Julia Walochnik
Michael Duchêne *Editors*

Molecular Parasitology

Protozoan Parasites and their Molecules

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Julia Walochnik
Institute of Specific Prophylaxis
and Tropical Medicine
Center for Pathophysiology, Infectiology
and Immunology
Medical University of Vienna
Vienna
Austria

Michael Duchêne
Institute of Specific Prophylaxis
and Tropical Medicine
Center for Pathophysiology, Infectiology
and Immunology
Medical University of Vienna
Vienna
Austria

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Contributors

Anton Aebischer Head of FG 16 Infections, Robert Koch-Institute, Berlin, Germany

Carsten Balczun Central Institute of the Bundeswehr, Medical Service Koblenz, Koblenz, Germany

Ulrike Boehme Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, UK

Christian Bogdan Mikrobiologisches Institut – Klinische Mikrobiologie, Immunologie und Hygiene, Friederich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen, Erlangen, Germany

Reto Brun Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

Christine Clayton Group Leader in the DKFZ-ZMBH Alliance, ZMBH, Heidelberg, Germany

Alan F. Cowman Head, Division of Infection and Immunity, The Walter and Eliza Hall institute of Medical Research, Parkville, VIC, Australia

Department of Medical Biology, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, VIC, Australia

Kathryn Crouch Wellcome Trust Centre for Molecular Parasitology, Glasgow, UK

Joel B. Dacks Department of Cell Biology, Canada Research Chair in Evolutionary Cell Biology, University of Alberta, Edmonton, Alberta, Canada

Daniele Dessì Department of Biomedical Sciences, University of Sassari, Sassari, Italy

Michael Duchêne Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Pier Luigi Fiori Department of Biomedical Sciences, University of Sassari, Sassari, Italy

Friedrich Frischknecht Parasitology - Department of Infectious Diseases, University of Heidelberg Medical School, Heidelberg, Germany

Tim Gilberger, PhD Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

Sven Gould Institute for Molecular Evolution, Heinrich-Heine-University, Düsseldorf, Germany

Vladimír Hampl Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Omar S. Harb Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

Julie Healer Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

Emily Herman Department of Cell Biology, Canada Research Chair in Evolutionary Cell Biology, University of Alberta, Edmonton, Alberta, Canada

Volker Heussler Institute of Cell Biology, University of Bern, Bern, Switzerland

Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Robert Hirt Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK

Ivan Hrdy Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Shanshan Hu Menzies Institute for Medical Research, University of Tasmania, Tasmania, Australia

Olukemi O. Ifeonu University of Maryland School of Medicine, Institute for Genome Sciences, Baltimore, MD, USA

Anna Karnkowska Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Christen Klinger Department of Cell Biology, Canada Research Chair in Evolutionary Cell Biology, University of Alberta, Edmonton, AL, Canada

Martina Köhsler Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Heinrich Körner Menzies Institute for Medical Research, Tasmania, Australia

Carsten G.K. Lüder Department of Medical Microbiology, Göttingen University Medical School, Göttingen, Germany

Pascal Mäser Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

Martin Mrva Department of Zoology, Comenius University in Bratislava, Faculty of Natural Sciences, Bratislava, Slovakia

Norbert Müller Institute for Parasitology, University of Berne, Bern, Switzerland

Joachim Müller Institute for Parasitology, Vetsuisse and Medical Faculty, University of Berne, Bern, Switzerland

Paola Rappelli Department of Biomedical Sciences, University of Sassari, Sassari, Italy

David S. Roos Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

Günter A. Schaub Zoologie/Parasitologie, Ruhr-Universität-Bochum, Bochum, Germany

Frank Seeber 2 Robert-Koch-Institut, Berlin, Germany

Joana C. Silva University of Maryland School of Medicine, Institute for Genome Sciences, Baltimore, MD, USA

Fatima Silva-Franco Institute of Integrative Biology, University of Liverpool, Liverpool, UK

Tobias Spielmann Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Staffan Svärd Uppsala University, Uppsala University, BMC, Uppsala, Sweden

Jan Tachezy Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Kyle Tretina University of Maryland School of Medicine, Institute for Genome Sciences, Baltimore, MD, USA

Patric Vogel Zoology/Parasitology Group, Ruhr-Universität, Bochum, Germany

Julia Walochnik Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Jonathan Wastling Executive Dean, Faculty of Natural Sciences, Keele University, Keele, UK

Gareth Weedall Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, UK

Iain B.H. Wilson Department for Chemistry, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

Dong Xia Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool, UK

Part I

The Molecules

Genomics

1

Omar S. Harb, Ulrike Boehme, Kathryn Crouch, Olukemi O. Ifeonu, David S. Roos, Joana C. Silva, Fatima Silva-Franco, Staffan Svärd, Kyle Tretina, and Gareth Weedall

Abstract

In the last decade, the rise of affordable high-throughput sequencing technologies has led to rapid advances across the biological sciences. At the time of writing,

O.S. Harb (✉) • D.S. Roos
Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA
e-mail: oharb@upenn.edu

U. Boehme
Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus,
Hinxton, Cambridgeshire CB10 1SA, UK
e-mail: ucb@sanger.ac.uk

K. Crouch
Wellcome Trust Centre for Molecular Parasitology,
B6-28 SGDB, 120 University Place, Glasgow G12 8TA, UK
e-mail: kathryn.crouch@glasgow.ac.uk

O.O. Ifeonu • J.C. Silva • K. Tretina
Institute for Genome Sciences, University of Maryland School of Medicine,
BioPark II, Room 645, 801W. Baltimore St., Baltimore MD 21201, USA
e-mail: KAbolude@som.umaryland.edu; jcsilva@som.umaryland.edu;
KTretina@som.umaryland.edu

F. Silva-Franco
Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK
e-mail: F.Silva-Franco@liverpool.ac.uk

S. Svärd
Department of Cell and Molecular Biology, Uppsala University, BMC,
Box 596, Uppsala SE-75123, Sweden
e-mail: staffan.svard@icm.uu.se

G. Weedall
Vector Biology Department, Liverpool School of Tropical Medicine,
Pembroke Place, Liverpool L3 5QA, UK
e-mail: gareth.weedall@lstmed.ac.uk

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annotated reference genomes are available for most clades of eukaryotic pathogens. Over 550 genomes, including unannotated sequences, are available in total. This has greatly facilitated studies in many areas of parasitology. In addition, the volume of functional genomics data, including analysis of differential transcription and DNA-protein interactions, has increased exponentially. With this unprecedented increase in publicly available data, tools to search and compare datasets are becoming ever more important. A number of database resources are available, and access to these has become fundamental for a majority of research groups. This chapter discusses the current state of genomics research for a number of eukaryotic parasites, addressing the genome and functional genomics resources available and highlighting functionally important or unique aspects of the genome for each group. Publicly accessible database resources pertaining to eukaryotic parasites are also discussed.

1.1 Introduction

Arguably the field of genomics began when Friedrich Miescher first isolated DNA in 1869 (Dahm 2005), paving the way for the work of many scientists in understanding the role of this material in heredity (Avery et al. 1944), discovering its double-helical structure (Watson and Crick 1953) and deciphering the genetic code (Nirenberg et al. 1965). However, the technological advance that the entire field of genomics rests on is sequencing (Gilbert and Maxam 1973; Sanger et al. 1977; Wu 1972). The ability to read the genetic code is relatively new, having only been developed in the last 50 years. Sanger sequencing, which relies on dideoxy chain termination, remained the method of choice for several decades; however, early implementations of dideoxy chain termination methods were not well parallelized, and analysis was initially a painstaking manual process. Later, data analysis was carried out computationally but limited by the processing capacity of computers of the era. These factors combined to limit early sequencing to individual genes, small genomic fragments, or the genomes of small viruses and organelles. The emergence of techniques such as fluorescence-based cycle sequencing and the polymerase chain reaction in addition to the increased use of computational power to automatically read and analyze results allowed larger-scale genome projects to be undertaken (Prober et al. 1987). Indeed within a few years of this marriage of techniques and fields, the first bacterial, protozoan, fungal, plant, and animal genomes were sequenced (Fleischmann et al. 1995; Fiers et al. 1976; Goffeau et al. 1996; Gardner et al. 2002; The Arabidopsis Genome Initiative 2000; The *C. elegans* Sequencing Consortium 1998). Despite these advances, sequencing of whole genomes remained relatively costly and time consuming. As an example, sequencing the human genome took roughly 10 years at a price tag of 3 billion US dollars (https://www.genome.gov/11006943) (Lander et al. 2001).

The first forays into high-throughput analysis of sequence data came in the form of microarrays. A microarray consists of a panel of oligonucleotide probes bonded to a solid surface such as a glass slide. Hybridization of nucleic acids from a specimen to individual probes is detected by the intensity of a fluorescent signal (Schena et al. 1995). This technique was the first to make querying of sequence polymorphisms,

transcript expression levels, and segmental duplications possible on a genomic level and cheap enough to be widely available. In addition, microarrays forced the development of computational tools and techniques to handle data on a genomic scale. However, an important limitation of microarrays is the requirement for prior knowledge of the genome and the coincident inability to make de novo discoveries (i.e., one can query the presence of known SNPs but not discover new SNPs). A large volume of functional genomics data has been obtained using microarray technologies, but with a small number of exceptions (such as diagnostics), microarrays have for the most part been superseded by next-generation sequencing technologies.

Two factors have been instrumental in enabling sequencing to be taken to the next level: continued growth of computer processing capacity following Moore's law (Moore 1998) and the development of "next-generation" sequencing (NGS) methods (also known as second-generation sequencing), which enable massively parallel sequencing of millions of fragments by synthesis (Margulies et al. 2005; Shendure et al. 2005). One of the major advantages of next-generation sequencing is that it can be applied to a wide variety of methodologies (*readers are directed to an excellent series of manuscripts*; http://www.nature.com/nrg/series/nextgeneration/index.html) and unlike microarrays, does not require any prior knowledge of the sample. Methodologies include:

- DNA sequencing: High-throughput technology makes sequencing for de novo assembly of new genomes ever more affordable. Comparison of re-sequenced isolates against a reference is a common technique for discovery of sequence polymorphisms, while analysis of coverage depth and mapping topology can reveal information about structural variations such as chromosomal translocations and segmental duplications.
- RNA sequencing: Sequencing of RNA can provide important information about gene structure such as the locations of UTRs and intron/exon boundaries and the presence of alternative or *trans*-splice variants. Analysis of RNA-seq coverage depth over a time course or under different experimental conditions reveals information about transcription of genes under differing conditions, and combination of this technique with ribosomal profiling enables identification of the translational status of the genome. Specialized sample preparation techniques enable the sequencing of noncoding RNA species such as those involved in the RNAi-mediated translational silencing.
- Epigenomics: Chromatin immunoprecipitation (ChIP) sequencing is a powerful technique that allows determination of the "footprint" of DNA-binding proteins. This can be used to examine promoter-binding sites; transcription, replication, and repair mechanisms; and factors such as histone modification that can affect transcription. Other techniques are available, such as bisulfite sequencing, which enables profiling of DNA methylation.
- Metagenomics: Sequencing of DNA extracted from samples that contain mixed populations of organisms can be used to survey populations in environmental samples (such as soil) or biological samples (such as gut microbiomes). Metagenomics techniques can be used to determine the makeup of populations and to survey how this changes over time or under different conditions. Metagenomics analysis is a fast-growing field in which the problems of analysis have not yet been solved.

It is not surprising that the dawn of large-scale sequencing projects necessitated an expansion in the field of bioinformatics and data management. As high-throughput sequencing has become cheaper, it has moved from being a specialist technique to a tool used daily in labs across the world. This has necessitated the development of user-friendly tools that can run on desktop machines and thrust the field of bioinformatics into the foreground. The expansion of massively parallel sequencing has also led to a revolution in the teaching of biology, with computational techniques for management and analysis of genomic-scale datasets now being taught in many undergraduate courses. Data warehousing is also becoming a priority, with data repositories such as the National Center for Biotechnology Information (NCBI) (Kodama et al. 2012) having to rethink both their submissions procedures and their approaches to storage.

1.2 Parasite Genomics

The field of parasite genomics has benefited tremendously from the sequencing revolution. While only a handful of parasite genomes were sequenced by 2005, the number has exploded to over 550 genomes (http://genomesonline.org) (Reddy et al. 2015) by 2015. This number reflects both annotated and unannotated genomes and will already be out of date by the time this chapter is in print. Besides the technological advances, this increase in sequences has been aided by a number of initiatives with parasitology components. These include projects supported by the Wellcome Trust Sanger Institute in the United Kingdom and a number of parasite-specific genome sequencing white papers supported by the National Institute of Allergy and Infectious Diseases (NIAID) Genomic Centers for Infectious Diseases (GCID) in the United States. Together these centers have generated sequence, assemblies, and annotation from many important human and veterinary parasites. All data from these projects are available via project-specific websites (i.e., GeneDB: http://genedb.org) (Logan-Klumpler et al. 2012) and/or through the International Nucleotide Sequence Databases (GenBank, EMBL Nucleotide Sequence Database, and the DNA Data Bank of Japan (Kulikova et al. 2006; Benson et al. 2015; Tateno et al. 1998)).

1.3 General Features of Protozoan Parasite Genomes

1.3.1 *Giardia*

Giardia duodenalis, also known as *Giardia intestinalis* or *Giardia lamblia*, is a unicellular protozoan parasite that infects the upper intestinal tract of humans and animals (Ankarklev et al. 2010). The disease, giardiasis, manifests in humans as an acute diarrhea that can develop to a chronic diarrhea, but the majority of infections remain asymptomatic (Ankarklev et al. 2010). Giardiasis has a global distribution with 280 million cases reported annually, with its impact being more pronounced in the developing world.

G. duodenalis is divided into eight morphologically identical genotypes or assemblages (A to H). Only assemblages A and B have been associated with human infections, and they are further divided into sub-assemblages: AI, AII, AIII, BIII, and BIV (Cacciò and Ryan 2008). Despite extensive efforts to associate specific assemblages to symptoms, conflicting results have been obtained, and there is to date no clear correlation between assemblage and symptoms.

Giardia, like the other diplomonads, has two nuclei and each nucleus is diploid, resulting in a tetraploid genome (Bernander et al. 2001). *G. duodenalis* has five different linear chromosomes with ribosomal DNA tandem repeats next to TAGGG telomeric repeats (Adam 2001). The study of the genome structure and architecture in *Giardia* using pulsed-field gel electrophoresis (PFGE) revealed differences in size of individual chromosomes within and between *G. duodenalis* isolates (Adam et al. 1988). The size differences were attributed to frequently recombining telomeric regions and differences in copy number of rDNA arrays (Adam 2001). Evidence of aneuploidy has been suggested in individual *Giardia* cells based on cytogenetic evidence (Tůmová et al. 2006), with the most common karyotype differing between different assemblage A and B isolates.

The genomes of six *G. duodenalis* isolates, representing three different assemblages (A, B, and E), are available to date (Adam et al. 2013; Jerlström-Hultqvist et al. 2010; Morrison et al. 2007; Ankarklev et al. 2015). The first genome to be sequenced was WB-C6 (assemblage A1), which has a haploid size of ~11.7 MB distributed over the five chromosomes (Morrison et al. 2007). The compact genome contains few introns, and promoters are short and AT rich. 6470 open reading frames (ORFs) were identified but only 4787 were later shown to be associated with transcription (Birkeland et al. 2010). Genes are placed on both DNA strands and sometimes even overlapping. Reduction of components in metabolic pathways, DNA replication, and transcription was also detected. Several genes had bacterial origin and are candidates of lateral gene transfer (Morrison et al. 2007). Variable surface proteins (VSPs) are involved in antigenic variation in *Giardia* and later analyses have shown that there are 186 unique VSP genes in the WB genome (Adam et al. 2013). Chromosome-wide maps have been established by optical mapping of the WB genome (Perry et al. 2011). The results resolved some misassemblies in the genome and indicated that the actual genome size of the WB isolate is 12.1 Mb, in close agreement with PFGE analyses. The major discrepancy was an underestimation of the size of chromosome 5, the largest of the *Giardia* chromosomes. Chromosome 5 contained an 819 kbp gap in the optical map, most likely rDNA repeats (Perry et al. 2011).

Shortly after publication of the WB genome, the genome of the GS isolate (assemblage B) was sequenced using 454 technology (Franzén et al. 2009). However, the genome was highly fragmented with 2931 contigs. 4470 ORFs were identified and the genomes show 78 % amino acid identity in protein-coding regions. The repertoire of *vsp* genes was very different compared to the WB isolate, but only 14 VSP genes were complete. The GS genome was later re-sequenced, resulting in 544 contigs and a much more complete repertoire of VSPs, totaling 275 genes (Morrison et al. 2007). Moreover, the GS genome had a much higher level of allelic sequence heterozygosity (ASH) compared to WB (0.5 % versus 0.01 %). ASH was

distributed differently into low and high ASH regions over the GS genomic contigs (Franzén et al. 2009).

The third genome represents the first isolate to be sequenced that was not obtained from a human host. The P15 isolate originates from a symptomatic pig (piglet no. 15) and belongs to assemblage E (Jerlström-Hultqvist et al. 2010). Assemblage E has been found to be more closely related to assemblage A than to assemblage B (Cacciò and Ryan 2008), and the identity of protein-coding sequences was 90 % between P15 and WB and 81 % between P15 and GS (Jerlström-Hultqvist et al. 2010), consistent with earlier results. Obtaining the sequence of three phylogenetically distinct *Giardia* groups (WB, P15, and GS) made it possible to assign lineage specificity to the genes identified in the three genomes. 91 % of the genes (~4500 protein-encoding genes) were found to be present in all three *Giardia* genomes (three-way orthologs). The rest of the genes (9 %) are variable, belonging mostly to four large gene families (the variant-specific surface proteins (VSP), NEK kinases, Protein 21.1, and high-cysteine membrane proteins (HCMPs)). The highest number of isolate-specific genes (38) was found in the P15 isolate, followed by GS (31) and WB (5). The P15 and GS isolates shared 20 proteins to the exclusion of WB, with 13 of these found in a cluster of 20 kbp in the P15 genome (Jerlström-Hultqvist et al. 2010). Interestingly the ORFs in this genomic cluster are not expressed in any of the conditions tested. The chromosomal architecture in *Giardia* shows core gene-rich stable regions with maintained gene order interspersed with non-syntenic regions harboring VSPs and other non-core genes. These regions often have a higher GC% and show nucleotide signatures that deviate from surrounding regions, in part due to the common occurrence of VSP and genes encoding high-cysteine membrane proteins (HCMPs) that are more GC rich than the genome on average. The level of ASH in the P15 isolate was lower than in the GS isolate, 0.0023 % (Jerlström-Hultqvist et al. 2010).

Three assemblage AII isolates have been sequenced (DH1, AS98, and AS175) (Adam et al. 2013; Ankarklev et al. 2015). The amount of genetic diversity was characterized in relation to the genome of WB, the assemblage A reference genome. The analyses showed that the divergence between AI and AII is approximately 1 %, represented by ~100,000 single nucleotide polymorphisms (SNP) distributed over the chromosomes with enrichment in the variable genomic regions containing VSPs and HCMPs (Ankarklev et al. 2015). The level of ASH in two of the AII isolates (AS98 and AS175) was found to be 0.25–0.35 %, which is 25–30-fold higher than in the WB isolate and tenfold higher than the assemblage AII isolate DH1 (0.037 %, (Ankarklev et al. 2015)).

There is a need for further genomic analyses of *Giardia* genomes. The assemblage A (WB) and B (GS) reference genomes can be improved, which will facilitate reference-based genome mapping of data from clinical and environmental isolates. More isolates from the A and B assemblages should be sequenced so that all the genetic differences between the human-infecting isolates can be identified. Genomic information from the remaining assemblages, C-D, F-H, can reveal species-specific genomic features. Sequence data from other *Giardia* species, like *Giardia muris*, will be important for further studies of the evolution of *Giardia* biology and virulence. In addition to the underlying genomic sequence and annotation, a number of functional datasets are available for the GiardiaDB.

1.3.2 Trypanosomatids

Trypanosomatids are a group of parasitic unicellular flagellate eukaryotes. Their range of hosts is diverse and includes humans as well as a wide variety of species from both the animal and plant kingdoms. Trypanosomatids belong to the Kinetoplastida, which is included in the phylum Euglenozoa, a branch that diverged early in the eukaryotic tree (Campbell et al. 2003; Simpson et al. 2006). While a number of Kinetoplastida are pathogenic parasites, most are free-living organisms found in soils and aquatic habitats. The name Kinetoplastida derives from the presence of large amounts of mitochondrial DNA, visible by light microscopy as a dense mass known as the kinetoplast with its contained DNA referred to as kDNA. Trypanosomatids are obligate parasites that can be monoxenous or dixenous (usually an insect vector and other animal or plant (Votýpka et al. 2015)).

1.3.2.1 Trypanosomatid Genomes

The nuclear genome of trypanosomatids has some unusual characteristics when compared with other eukaryotic genomes. Their genome is organized in polycistronic transcriptional units (PTUs) and the production of individual mRNAs from PTUs requires trans-splicing of a splice leader (SL) sequence (Martínez-Calvillo et al. 2010). PTUs are well conserved and exhibit a high degree of synteny between species. The kDNA has an unusual physical structure, being arranged in circles of DNA that are interlocked in a chain-mail-like network. These mitochondrial mRNAs require post-processing in the form of insertion and deletion of uridines before being translated into proteins, a process known as RNA editing (Aphasizhev and Aphasizheva 2014; Lukeš et al. 2002). Other peculiarities of trypanosomatid genomes include the almost complete lack of introns, kinetoplastid-specific histone modifications and histone variants, unique origins of replication in some genera, a special DNA base (Base J) (Maree and Patterton 2014), and the transcription of protein-coding genes by RNA pol I in African trypanosomes, a behavior unique among eukaryotes (Daniels et al. 2010). Although none of these unusual features seem to be exclusive of trypanosomatids and are also present, at least in some basic form, in other free-living kinetoplastids, they may be related to the development of parasitism in trypanosomatids (Simpson et al. 2006; Lukeš et al. 2014).

1.3.2.2 Regulation of Gene Expression in Polycistronic Transcriptional Units

One of the most striking characteristics of trypanosomatid genomes is the organization of their protein-coding genes into long polycistronic transcriptional units (PTUs) that contain tens to hundreds of genes in the same orientation. Individual mRNAs are produced from the precursor mRNA by the 5' trans-splicing of a capped mini-exon or splice leader (SL) sequence, followed by the polyadenylation of the 3' end. The 5' trans-splicing is linked to the polyadenylation of the upstream gene. Gene order within PTUs is highly conserved among trypanosomatids, and the main differences are usually in the regions between the PTUs and at the ends of the chromosomes (Martínez-Calvillo et al. 2010; Clayton 2014).

The genes included in a PTU are functionally unrelated and can be expressed at different times of the cell cycle or in different life stages. Nonetheless, each PTU is transcribed from a single transcriptional start site (TSS), severely limiting the amount of regulation that could be provided by the induction or repression of promoters. In some cases correlation between the location of a gene in a PTU and its expression level has been described. For example, in *T. brucei*, genes downregulated after heat shock tend to be closer to the transcriptions start site (TSS), while upregulated genes tend to be more distal. Also, the position of the genes along the PTUs correlates with gene regulation during the different cell cycle stages. However, most of the genes do not seem to be ordered depending on their transcriptional regulation (Campbell et al. 2003; Martínez-Calvillo et al. 2010; Kelly et al. 2012).

In most organisms, the start of transcription is a fundamental step in the regulation of gene expression. In trypanosomatids this layer is constrained, but a swift and specific regulation of gene expression is still needed. The medically relevant kinetoplastids are dixenous parasites with complex life cycles that require fast and extensive changes in morphology and metabolism. These changes depend, ultimately, on changes in gene expression. For example, the parasite has to quickly adapt to differences in temperature, energy sources, and host immune system (Daniels et al. 2010; Kelly et al. 2012). Besides the regulation at the start of transcription, it is possible to modulate other steps in the transcription and translation process. Additional levels of control include transcriptional elongation, mRNA processing (trans-splicing and polyadenylation), export from the nucleus, mRNA degradation (in the cytoplasm and nucleus), translation (start and elongation), and protein degradation (Martínez-Calvillo et al. 2010; Clayton 2014).

Both mRNA processing and the control of the mRNA stability are important regulatory steps in trypanosomatids. The stability of the mRNAs depends on elements present in the 3' UTRs, for instance, duplicated genes in tandem arrays can be differentially regulated due to differences in their 3' UTRs. In *T. brucei,* the range of half-lives of mature mRNAs is very diverse and is also determined by the life cycle stage. In addition, the half-life of a mRNA not only depends on the stability of the mature mRNA but also on the rates of destruction of the precursor mRNA. If a mRNA undergoes a late or delayed polyadenylation, it is more susceptible to being degraded, even before finishing maturation (Clayton 2014; Jackson 2015).

Trypanosomatids contain a large number of RNA-binding proteins (RBPs) that likely regulate expression levels by binding to regulatory elements in the 3' UTRs of the mRNAs. The amount of RBPs is high compared with the number of mRNAs. Consequently the current hypothesis proposes the binding of multiple RBPs to each 3' UTR, which would compete or cooperate dynamically with other RBPs. The mix of RBPs would determine the stability of the mRNA and could also modulate the translation process (Clayton 2014; Clayton 2013). The expression of protein-coding genes can also be regulated at the translational level. In ribosome profiling studies, it has been shown that there is a wide range in the density of ribosomes associated to mRNAs, with differences between life stages. In addition, trypanosome mRNAs can contain upstream open reading frames in their 5' UTRs, which decrease the translation of the main ORF (Clayton 2014; Vasquez et al. 2014; Jensen et al. 2014).

1.3.2.3 Multi-copy Families of Surface Proteins

Genome reduction is frequent in parasites with functions that are essential for a free-living organism becoming obsolete inside a host. Surprisingly, compared with other single-cell parasitic eukaryotes, trypanosomatid genomes do not appear to be specially reduced in size or function. On the contrary, in the evolution of parasitism in trypanosomatids, the gain of new competences seems to have been more important than the loss of functions (Jackson 2015). One example of this gain of functions is the presence of large multi-copy families that encode surface proteins. These families are specific to trypanosomatids and usually have a nonrandom distribution in the genome. A number of them have been implicated in pathogenesis and defense against the host immune system, such as the major surface protease (MSP) family of metalloproteases involved in pathogenesis and conserved in all trypanosomatids. Other well-known examples are the variant surface glycoprotein (VSG) and procyclin in *T. brucei*, delta-amastin and promastigote surface antigen (PSA) in *Leishmania*, and trans-sialidases in *T. cruzi* (Jackson 2015; Rogers et al. 2011; El-Sayed et al. 2005a).

1.3.2.4 Epigenetic Regulation

In eukaryotes, nuclear DNA is organized into a complex of DNA and proteins known as chromatin. The nucleosome is the basic unit of the chromatin, providing a sevenfold condensation. It comprises an octamer made of two copies of each of the core histones (H2A, H2B, H3, and H4) around which approximately 147 bp of DNA is wrapped. In addition, there is a histone (H1) in the DNA region between two nucleosomes that helps stabilize the chromatin. The chromatin is folded into a 30 nm chromatin fiber that can be further compacted, up to the level of the distinct chromosomes that can be visualized during the eukaryotic mitosis (Martínez-Calvillo et al. 2010; Maree and Patterton 2014). Although the nucleosomes are still the basic unit of chromatin in trypanosomatids, their histones are divergent from those found in yeast and vertebrates. DNA in trypanosomatids is not condensed into the 30 nm chromatin fiber nor do chromosomes condense during mitosis. However, some differences in the level of condensation between life cycle stages have been described (Martínez-Calvillo et al. 2010; Daniels et al. 2010).

Mechanisms that influence the structure of chromatin have been implicated in the regulation of gene expression. In trypanosomatids, as in other eukaryotes, specific modifications of the N-terminal tails of histones, or the presence of histone variants, correlate with regions of active or repressed transcription. As of yet, no conserved sequences have been identified in the transcription start sites (TSSs) of the PTUs. It has been proposed that TSSs could be determined by chromatin structure rather than the presence of conserved sequence motifs. Some of the histone modifications described in trypanosomatids are common in eukaryotes, but there are also some modifications and histone variations specific to trypanosomatids, such as H3V and H4V (probable markers of transcription termination sites) (Maree and Patterton 2014; Siegel et al. 2009).

1.3.2.5 Mitochondrial Genome: Architecture and RNA Editing

The kDNA is made up of circles of DNA that are interlocked in a chain-mail-like network and are of two types: maxicircles and minicircles. Maxicircles store information for classical mitochondrial genes and proteins, but their transcripts require RNA editing, the insertion or deletion of uridines, before being translated. Minicircles encode guide RNAs (gRNAs) that act as templates during the editing process. Unlike other eukaryotes, mitochondrial tRNAs are found in the nuclear genome and require specific target sequences to be transported into the mitochondria (Campbell et al. 2003; Lukeš et al. 2002). The mitochondrial genome contains a few dozens of maxicircles, with identical sequence and a size of 20–40 kb, and thousands of minicircles. Minicircles differ in sequence content, but their size is species specific and uniform, usually between 0.5 and 10 kb. Maxicircles are concatenated together and simultaneously interlinked with the minicircle network. The DNA network and associated proteins are organized in a dense disk visible by light microscopy. While all kinetoplastids contain maxi- and minicircles, the concatenated network is unique to trypanosomatids (Simpson et al. 2006; Marande et al. 2005). During the RNA editing, uridines are inserted or deleted from mitochondrial mRNAs fixing errors in the sequence and restoring a viable coding sequence. The sequences to be used as templates are stored in the gRNAs (50–60 nt). These encode only a small portion of the information needed to repair a mRNA; therefore, multiple gRNAs are required to edit each mRNA. RNA editing is catalyzed by the RNA editing core complex or editosome. Several modules can combine to build different versions of the editosome, each with different specificities (Aphasizhev and Aphasizheva 2014; Liu et al. 2005).

1.3.2.6 Base J

Base J, or the modification of thymine to beta-D-glucosyl-hydroxymethyluracil, is enriched at the ends of PTUs, at potential transcription terminal sites (TTSs) and in repetitive DNA elements, such as the telomeric repeats (Campbell et al. 2003; Maree and Patterton 2014).

1.3.2.7 Transposable Elements

The two main classes of transposable element are DNA transposons and RNA retrotransposons. DNA transposons move by "cut and paste" and depend on a DNA intermediate, while RNA retrotransposons use a "copy and paste" strategy, with a RNA intermediate. DNA transposons have not been found in trypanosomatid genomes, but RNA retrotransposons have been shown to be present. For example, several classes of potentially active retrotransposons have been identified in *T. brucei* and *T. cruzi*, some of which could be involved in the regulation of gene expression, such as SIDER2, which localizes to the 3'UTRs of mRNAs and affects its stability (Martínez-Calvillo et al. 2010; Bringaud et al. 2006; Bringaud et al. 2008).

1.3.2.8 Sequenced Genomes

The first trypanosomatids sequenced were *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*, the causative agents of sleeping sickness, Chagas disease, and one form of cutaneous leishmaniasis in humans (Berriman et al. 2005; Ivens

et al. 2005; El-Sayed et al. 2005b). Since then, the genomes of other medically relevant trypanosomes have been published. *Leishmania* species that have been sequenced include *L. donovani* (Downing et al. 2011), *L. infantum*, *L. braziliensis* (Peacock et al. 2007), *L. mexicana* (Rogers et al. 2011), *L. panamensis* (Llanes et al. 2015), *L. peruviana* (Valdivia et al. 2015), and *L. amazonensis* (Real et al. 2013). *Trypanosoma* species include *T. rangeli* (Stoco et al. 2014). Apart from the reference genomes, multiple strains and hundreds of isolates have been sequenced and are available in the databases [TriTrypDB, NCBI]. The range of published genomes has expanded to other dixenous species and includes parasites of reptiles (*Trypanosoma grayi* (Kelly et al. 2014) and *Leishmania tarentolae* (Raymond et al. 2012)), parasites of livestock (*T. evansi* (Global 2015)), or parasites of plants (*Phytomonas serpens*, *Phytomonas* spp. (Kořený et al. 2012; Porcel et al. 2014)). In addition, the genomes of a few monoxenous trypanosomatids have been published (*Leptomonas seymouri* (Kraeva et al. 2015) and *Lotmaria passim* (Runckel et al. 2014)). Some of these species harbor symbiotic bacteria and have been used as a model to study the evolution of organelles (*Crithidia acanthocephali*, *Herpetomonas muscarum*, *Strigomonas oncopelti*, *Strigomonas galati*, *Strigomonas culicis*, *Angomonas desouzai*, and *Angomonas deanei*) (Alves et al. 2013). Additional genomes are available prepublication in the genome databases (TriTrypDB, NCBI) and include *Endotrypanum monterogeii*, *Leptomonas pyrrhocoris*, and *Crithidia fasciculata*; the *Leishmanias L. aethiopica*, *L. tropica*, *L. gerbilli*, *L. enrietti*, and *L. turanica*, and the *Trypanosomas T. congolense* and *T. vivax*.

1.3.3 *Toxoplasma* and Related Organisms

Toxoplasma gondii is a member of the tissue cyst-forming coccidian parasites, which include *Neospora caninum*, *Hammondia hammondi*, and *Sarcocystis* spp., among others (Dubey and Ferguson 2014; Dubey and Lindsay 1996; Levine 1986; Dubey et al. 1998). Of these *T. gondii* appears to be the most widely distributed both geographically and by host diversity and is able to infect virtually any warm-blooded animal. While the diversity of *T. gondii* is restricted to three clonal lineages in Europe and North America, isolates from the southern hemisphere exhibit much wider genetic variability (Sibley and Ajioka 2008). Amazingly, while *T. gondii* can infect a wide variety of warm-blooded organisms, it can only undergo sexual recombination in Felidae. Cats shed infective sporozoites containing environmentally resistant cysts, which can be transmitted orally to other organisms such as rodents (Dubey et al. 1998). Following oral infection, sporozoites cross the small intestine and can infect a variety of cells where they undergo a developmental switch to fast-growing tachyzoites (Sibley and Ajioka 2008). Tachyzoites replicate through a process called endodyogeny where two daughter cells are formed within a mother cell by a combination of de novo building of cytoskeletal and secretory components, replication and segregation of mother cell components (i.e., nucleus, mitochondria, and apicoplast), and recycling of mother cell components (Francia and Striepen 2014; Ouologuem and Roos 2014). Pressure from the host immune system forces

tachyzoites to undergo another developmental change into bradyzoites (Miller et al. 2009). These semi-quiescent cells form clusters called tissue cysts that settle in brain and/or muscle tissue where they may remain for the life of the host, although reactivation of bradyzoites can occur in immunocompromised individuals. Bradyzoites also serve as a reservoir of transmission if an infected host is eaten by another animal. Interestingly, the tissue cyst tropism varies markedly between hosts. The fast replicating tachyzoite stage is often asymptomatic but can cause acute morbidity or mortality in immunocompromised individuals. Placental transmission is known to cause fetal mortality or serious congenital defects.

T. gondii contains a ~65 Mb nuclear genome comprising 14 chromosomes (Khan et al. 2005; Reid et al. 2012; Lorenzi et al. 2016), a 35 Kb apicoplast genome (Köhler et al. 1997), and a mitochondrial genome. *T. gondii* genomic-scale data such as expressed sequenced tags, sequenced BAC clones, and whole-genome shotgun sequencing were first made available through ToxoDB beginning in 2001 (Kissinger et al. 2003). Since then, additional genomic-scale data have been generated including genome sequence and transcriptomic data from a large-scale population sequencing project (Lorenzi et al. 2016). The genome of the closely related *H. hammondi* and *N. caninum* is ~65 Mb and ~62 Mb in size, respectively, and not surprisingly also comprises 14 chromosomes each (Table 1.1) (Reid et al. 2012; Lorenzi et al. 2016; Walzer et al. 2013). The genome of the more divergent *S. neurona* is almost twice the size of those previously described at ~130 Mb, while a GC content of roughly 53 % is common across this group (Table 1.1) (Blazejewski et al. 2015). A high degree of genomic synteny is observed between *T. gondii*, *H. hammondi*, and *N. caninum.* This level of synteny is not maintained with between this group and *S. neurona* (Reid et al. 2012; Lorenzi et al. 2016; Blazejewski et al. 2015).

Apicomplexan parasites in general have evolved secretory systems that transport effector molecules into their host cells. These have a range of functions, including modification of the intracellular environment, promotion of immune evasion, and modulation of host-cell transcription (Hakimi and Bougdour 2015). Most information about secretory effectors in coccidian parasites comes from *T. gondii* where numerous studies have defined dense granule (Mercier and Cesbron-Delauw 2015), rhoptry (Boothroyd and Dubremetz 2008), microneme (Carruthers and Tomley

Table 1.1 Basic genome statistics for *T. gondii* and related organisms

	Toxoplasma gondii[a]	*Hammondia hammondi* H.H.34	*Neospora caninum* Liverpool	*Sarcocystis neurona*[b]
Genome size (Mb)	63	65	62	128
No. of chromosomes	14	14	14	ND
No. of genes	8707	8176	7266	7140
% of genes with introns	76	76	77	81

ND not determined

[a]Average statistics from three strains: ME49, VEG, and GT1

[b]Average statistics from two strains: SN1 and SN3

2008), and SAG1-related sequence (SRS) proteins (Wasmuth et al. 2012). Comparative genomic analysis revealed that one of the primary features differentiating both different species of coccidian parasite and different strains of *T. gondii* is sequence diversity and copy number variation (CNV) at secretory effector loci (Reid et al. 2012; Lorenzi et al. 2016; Walzer et al. 2013). A comparison of 62 isolates of *T. gondii* and one isolate of *H. hammondi* showed that secretory effectors are often found in genomic regions exhibiting tandem amplification (Lorenzi et al. 2016). A comparison of reference isolates from the 16 major *Toxoplasma* haplogroups showed that all possess a repertoire of secretory effectors with most diversity occurring in rhoptry and SRS genes. Further comparison of secretory effectors between *T. gondii*, *H. hammondi*, and *N. caninum* revealed additional diversity and a *T. gondii*-specific family (*Tg*FAMs) of effectors, which may be important for host range and definitive host preferences (Lorenzi et al. 2016). Interestingly, a number of the *Tg*FAMs are clustered in telomeric regions and contain a variable region, which may implicate them in immune evasion (Lorenzi et al. 2016; Dalmasso et al. 2014), but they also may play a role during sexual development since many are expressed in the cat and in oocysts (Behnke et al. 2014).

1.3.4 *Cryptosporidium*

Cryptosporidium spp. are protozoan parasites with significant impact to the health of humans and livestock. They infect the intestinal and gastric epithelium of a variety of vertebrates, causing a disease known as cryptosporidiosis. Human cryptosporidiosis is responsible for diarrhea-induced death of young children in developing countries, and in immune-compromised adults, it constitutes an acute, usually self-limiting, diarrheal illness that results in significant morbidity and sometimes death. A recent study found *Cryptosporidium* to be the second leading cause of moderate-to-severe diarrhea in developing countries, with diarrheal diseases being the second principal cause of death among children under 5, globally (Kotloff et al. 2013).

There are no licensed vaccines against *Cryptosporidium*, and the only FDA-approved drug (nitazoxanide) is only effective in immunocompetent patients. Thus, the development of alternative therapeutic agents and vaccines against this disease is urgently required and remains a high public health priority. The lack of a practical and reproducible axenic in vitro culture system for *Cryptosporidium* is a major limitation to the development of specific anti-cryptosporidial vaccines (Arrowood 2002; Karanis and Aldeyarbi 2011). Advances in next-generation sequencing technologies and in genome assembly and annotation methodologies (Niedringhaus et al. 2011; Nagarajan and Pop 2013; Martin and Wang 2011; Yandell and Ence 2012) have facilitated the generation of -omics data for *Cryptosporidium*, with genomics resources now available for multiple *Cryptosporidium* species (Table 1.2, (Heiges et al. 2006)). These developments prompted a shift to in silico studies aiming to identify a wide pool of potential vaccine targets, to be further filtered according to properties common to antigens (Manque et al. 2011). This approach is similar to reverse vaccinology studies that have led to licensed vaccines in other organisms

Table 1.2 *Cryptosporidium* species with completed or draft genomes

Species	Number of draft genomes	Natural host range	Predilection site
C. hominis	8	Human, primates	Intestinal
C. parvum	8	Human, bovine	Intestinal
C. meleagridis	1	Various vertebrates	Intestinal
C. baileyi	1	Birds	Respiratory
C. muris	1	Rodents	Gastric
C. sp. chipmunk LX-2015	1	Rodents, human	Intestinal

(Donati and Rappuoli 2013; Kelly and Rappuoli 2005) and is particularly promising in organisms that, like *Cryptosporidium*, are difficult to cultivate continuously in the laboratory.

Apart from human, *Cryptosporidium* species infect other vertebrates including fish, birds, and rodents, and some species are capable of zoonotic transmission (Xiao and Herd 1994; Bouzid et al.). Some have a somewhat restricted host range, such as *Cryptosporidium hominis*, a human parasite that infects the small intestine; *Cryptosporidium muris*, a gastric parasite of rodents; and *Cryptosporidium baileyi*, an avian parasite. *Cryptosporidium parvum* and *Cryptosporidium meleagridis* have a wider host range and are known to infect both avian and mammalian species, including humans. *C. parvum* and *C. hominis* are considered class B agent of bioterrorism and are significant causes of gastrointestinal infections worldwide.

1.3.4.1 *Cryptosporidium* Genomic Resources

Cryptosporidium genomes are compact, with >75 % consisting of protein-coding sequences, and have an average size of approximately 8.5–9.5 mega base pairs (Mbp), and each encodes ~4000 genes (Table 1.3). *C. parvum* (isolate Iowa II) was the first species for which a genome was published (Abrahamsen et al. 2004). The genome was found to be 9.1 Mbp in length, assembled into 13 supercontigs. Pulsed-field gel electrophoresis studies had shown the nuclear-encoded genome to consist of eight chromosomes, and therefore the assembly includes five unresolved gaps. About 5 % of the 3807 predicted protein-coding genes in this assembly contained introns, and the average gene length was 1795 base pairs (bp). At about the same time, the genome of *C. hominis* (isolate TU502) was published (Xu et al. 2004). Since the two species were known to be closely related, with about 95–97 % DNA sequence identity between them, the *C. hominis* genome was sequenced to a much lower depth of coverage. The primary goal was to identify differences relative to *C. parvum*, rather than reconstruct a gold-standard genome assembly. Consequently, this assembly is much more fragmented, with the likely eight chromosomes split among 1413 contigs, which are grouped into ~240 scaffolds.

There were some fundamental differences between the annotated gene sets in the two species. The average gene length of *C. hominis* was 1360 bp, about 500 bp less than that of *C. parvum*, and about 5–20 % of the *C. hominis* genes were predicted to contain introns, compared to 5 % in *C. parvum* (Abrahamsen et al. 2004; Widmer

Table 1.3 Genome statistics for representative *Cryptosporidium* species

Species	Isolate	GenBank accession	Assembly length (Mb)	No. of contigs	Largest contig (bp)	No. of protein-coding genes	Average gene length (bp)	Percent coding (%)
C. hominis	TU502 (2004)	AAEL00000000	8.7	1413	90,444	3886	1360	60.4
C. hominis	TU502_new (2014)	SUB482083	9.1	120	1,270,815	3745	1845	75.8
C. parvum	Iowa	AAEE00000000	9.1	13	1,278,458	3807	1795	75.3
C. parvum[a]	Iowa	AAEE00000000	9.1	13	1,278,458	3865	1783	75.7
C. meleagridis	UKMEL1	SUB482042	9.0	57	732,862	4326	1861	89.7
C. baileyi	TAMU-09Q1	SUB482078	8.5	153	702,637	3700	1776	77.3
C. muris	RN66	AAZY02000000	9.2	84	1,324,930	3934	1780	79.2

[a]2015 re-annotation

et al. 2012). In addition, only 60 % of the *C. hominis* genome was estimated to be coding compared to 75 % for *C. parvum*. These differences are remarkable for such closely related taxa and were thought to be due to erroneous gene models in *C. hominis* related to the high degree of fragmentation of the genome assembly. To address these questions, the *C. hominis* genome has recently been re-sequenced, assembled, and annotated, improving the assembly from draft to "nearly finished" form, with preliminary data available in CryptoDB.org. This effort increased the average gene length by 500 bp, bringing it to 1845 bp, in line with gene length in *C. parvum* (Table 1.3). The improved genome assembly consists of only 120 contigs, a tenfold reduction in contig number relative to the original *C. hominis* assembly. The genome assembly is more comprehensive, with an additional 370 Kb sequence, also now comparable in length to that of *C. parvum*. Finally, there was a 25 % increase in the predicted fraction of the genome that encodes for proteins. The now marked similarities between the re-annotated *C. hominis* gene set and that of *C. parvum* provide encouraging evidence that the predicted genes are a significant improvement over the original annotation, but validation of gene structures awaits community effort. *C. parvum* Iowa II was also recently re-annotated, based on full-length cDNA clone sequences and RNA-seq data (Table 1.3, (Isaza et al. 2015)).

Both *C. hominis* and *C. parvum* are intestinal parasites. *C. muris* (isolate RN66), the third species sequenced, was chosen for two primary reasons: its evolutionary distance to *C. hominis* and *C. parvum* and the fact that it is a gastric species, which is rare among *Cryptosporidium* parasites. Currently, the field is rapidly expanding, with the genome sequence for several isolates of *C. parvum* and of *C. hominis* now available as well as the genomes of other species (Table 1.4).

The availability of multiple isolate genomes per species allows analyses that can shed light into species evolution, including age and population structure, and will facilitate studies that address key questions of great translational impact, including the amino acid sequence variations in current candidate vaccine antigens and the identification of genomic correlates of virulence whenever isolates with different pathogenic potential are available. In an effort to support research that addresses key questions in the evolution of the *Cryptosporidium* genus, and the discovery of parasite-encoded factors that control host specificity, *C. meleagridis* UKMEL1 was sequenced, a species which appears to lack host specificity and that is considerably more distantly related to *C. hominis* and *C. parvum* than they are to each other, but a closer relative to them, that is, *C. muris. C. baileyi* can complete its life cycle in embryonated chicken eggs, of critical importance for the establishment of an avian model system of cryptosporidiosis, and *C. baileyi* TAMU-09Q1 was sequenced to support the development of such a system. Determining the proportion of *Cryptosporidium* infections that are caused by human-specific parasites rather than by zoonotic infections remains a critical question in the field. Accordingly, the genome of a zoonotic infection by a *Cryptosporidium* species with origin in the chipmunk was sequenced with the goal of identifying genotyping markers that differentiate among *Cryptosporidium* subtypes (Guo et al. 2015).

A major challenge for the generation of *Cryptosporidium* whole-genome sequence data has been the need to propagate the parasites in vertebrate hosts, a step

Table 1.4 *Cryptosporidium* genomes available in CryptoDB

Species	Isolate	Year	Sequencing institution	GenBank accession	RNA-seq SRA accession	Assembly length (bp)	No. of contigs	Largest contig (bp)
C. hominis	TU502	2004 2013	VCU	AAEL01 AAEL02	– –	8,743,570 8,915,516	1422 358	90,444 282,140
C. hominis	TU502_new	2014	IGS/Tufts	SUB482083	SRS566230	9,110,085	120	1,270,815
C. hominis	37999	2014	CDC	JRXJ01	–	9,054,010	78	1,029,232
C. hominis	UKH1	2014	IGS/Tufts	SUB482088	SRS566214	9,141,398	156	542,781
C. hominis	UKH3	2015	PHW	LJRW01	–	9,136,308	34	1,295,005
C. hominis	UKH4	2015	PHW	LKHI01	–	9,158,280	18	1,295,931
C. hominis	UKH5	2015	PHW	LKHJ01	–	9,179,731	18	1,281,265
C. parvum	Iowa II	2004	Univ. Minnesota	AAEE01	–	9,087,724	18	1,278,458
C. parvum	UKP2	2015	PHW	LKHK01	–	9,126,082	18	1,285,807
C. parvum	UKP3	2015	PHW	LKHL01	–	9,085,686	18	1,258,884
C. parvum	UKP4	2015	PHW	LKHM01	–	9,001,535	18	1,283,549
C. parvum	UKP5	2015	PHW	LKHN01	–	9,283,240	18	1,284,088
C. parvum	UKP6	2015	PHW	LKCK01	–	9,112,937	18	1,296,567
C. parvum	UKP7	2015	PHW	LKCL01	–	9,221,024	18	1,295,191
C. parvum	UKP8	2015	PHW	LKCJ01	–	9,203,314	18	1,288,507
C. meleagridis	UKMEL1	2014	IGS//Tufts	SUB482042	–	8,973,224	57	732,862
C. baileyi	TAMU-09Q1	2014	IGS/ Texas A&M	SUB482078	SRS566232	8,502,994	153	702,637
C. muris	RN66	2008	TIGR/Tufts	AAZY02	SRS000463	9,238,736	97	1,182,920
C. sp.	chipmunk LX-2015	2015	CDC	JXRN01	–	9,509,783	853	478,353

Division of Foodborne, Waterborne, and Environmental Diseases, *CDC* Centers for Disease Control and Prevention, *IGS* Institute for Genome Sciences, *PHW* Public Health Wales (Microbiology), *TIGR* The Institute for Genome Research, *VCU* Virginia Commonwealth University

needed to generate DNA material in sufficient quantity and of the quality need for use in high-throughput sequencing applications. A novel method for preparing genomic *Cryptosporidium* DNA directly from human stool samples that satisfies the criteria these applications has now been developed (Hadfield et al. 2015). The authors used this approach to generate five assemblies each for *C. parvum* and *C. hominis*. Finally, a new *C. hominis* (isolate UdeA01) also isolated from human stool has been sequenced independently (Isaza et al. 2015).

All the genomics data described above are publicly available through CryptoDB (Heiges et al. 2006). This database also provides a platform to easily query the annotation and a variety of precomputed analysis data (including homology information across taxa). Multiple aspects of the data can be easily visualized, including synteny, polymorphism, and expression data. CryptoDB also contains *Cryptosporidi*um information other than genome sequences, including gene expression and proteomics data (Table 1.5).

1.3.5 Piroplasms

Piroplasms are a vast group of poorly characterized Haemosporidia that are named after their pyriform (pear-shaped) structure visible during intracellular stages in the host erythrocytes. They are found in numerous mammals, birds, and reptiles and are often transmitted by ixodid ticks after parasite replication in the tick gut (Manwell 1964). While little is known about the life cycle of most piroplasms, well-described species of *Theileria* commonly infect mammalian host leukocytes, followed by a tick-infective stage in red blood cells (RBCs), while *Babesia* do not have a leukocyte-infective stage (Cornillot et al. 2012; Gardner et al. 2005). Some *Babesia* species are known to infect humans (*B. microti, B. divergens, B. duncani, B. venatorum*), where they cause babesiosis, a malaria-like disease (Cornillot et al. 2012). The diseases caused by these parasites can lead to fevers and even death in equid and ruminant livestock species all around the world. Consequently, most of the genomics resources developed for piroplasm research to date have focused on species that infect bovids (Table 1.6). Most of these resources are available through PiroplasmaDB (http://PiroplasmaDB.com).

The first piroplasm genomes were published in 2005 and consisted of *Theileria* species of domestic cattle and wild buffalo. *T. parva* causes a tremendous economic impact in Eastern, Central, and Southern Africa (Gardner et al. 2005), while *T. annulata* is distributed throughout much of Southern Asia and Southeast Europe (Pain et al. 2005). Their genomes are small at ~8.3 Mbp in length, are AT rich, with GC content of 33 %, and contain ~4000 nuclear protein-coding genes. These properties are similar to the genomes of other Piroplasmida that have been sequenced since (Table 1.7). Several genomic features were uncovered that are typical of other sequenced piroplasm genomes, such as the presence of telomeric multigene families, and several incomplete or absent biosynthetic pathways, implying a critical dependence on salvaging resources from their hosts (Chaudhary and Roos 2005). These two piroplasms are unique, however, in their ability to transform host

Table 1.5 Other *Cryptosporidium* genomic resources available in CryptoDB

Data type	Description	Species	Reference
EST	EST library and predicted full-length cDNA	*C. parvum* HNJ-1	(Yamagishi et al. 2011)
EST	ESTs from database of Expressed Sequence Tags (dbEST)	*C. baileyi* TAMU-09Q1, *C. hominis* TU502, *C. meleagridis* UKMEL1, *C. muris* RN66, *C. parvum* Iowa II	(Boguski et al. 1993)
RT-PCR	Expression profiling of life cycle stages post-infection	*C. parvum* Iowa II	(Mauzy et al. 2012)
Microarray	Global gene expression in oocysts (environmental stage) and oocysts treated with UV	*C. parvum* Iowa II	(Zhang et al. 2012)
RNA-seq	Transcriptome of sporozoites and HTC-8 infection time course	*C. parvum* Iowa II	(Lippuner et al. Unpublished)
RNA-seq	Transcriptome in normal culture conditions	*Chromera velia* CCMP2878, *Vitrella brassicaformis* CCMP3155	(Woo et al. 2015)
Mass spectrometry	Enriched cytoskeletal and membrane fractions	*C. parvum* Iowa II	(Madrid-Aliste et al. 2009)
Mass spectrometry	Mitochondrial fraction proteomics	*C. parvum* Iowa II	(Putignani et al. Unpublished)
Mass spectrometry	Proteome of intact oocyst, oocyst wall, and sporozoites by linear ion trap MS	*C. parvum* Iowa II	(Truong and Ferrari 2006)
Mass spectrometry	Proteome during sporozoite excystation	*C. parvum* ISSC162	(Snelling et al. 2007)
Mass spectrometry	Sporozoite peptides from 2D gel LC-MS/MS analysis	*C. parvum* Iowa II	(Sanderson et al. 2008)
SNPs	SNPs determined by aligning high-throughput sequencing reads of *C. parvum* TU114 to the *C. parvum* reference genome	*C. parvum* TU114, *C. parvum* Iowa II	(Widmer et al. 2012)

leukocytes to have cancer-like phenotype. This phenotype correlates with the expansion of two multigene families: the subtelomere-encoded variable secreted protein (SVSP) gene family and the *T. annulata* schizont AT-hook/*T. parva* Host Nucleus (TashAT/TpHN) gene families (Hayashida et al. 2012b; Tretina et al. 2015). Two other *Theileria* species have been sequenced, *T. orientalis* (Hayashida et al. 2012b), an economically important pathogen of cattle in eastern Asia, and *T. equi* (Kappmeyer et al. 2012), which has a worldwide distribution and infects equids. These two genomes have many similar features, with the exception that the genome of *T. equi* is larger, mostly due to a significant increase in the number of species-specific genes,

Table 1.6 The first publication of available piroplasm whole-genome sequences and a few features of their genomes

Genus	Species	Strain(s)	Year published	Reference	Hosts	Assembly length (Mbp)	Genome %GC	# nuclear, protein-encoding genes
Babesia	*bigemina*	BOND[a], PR, BbiS3P, JG29	2014	Jackson et al. (2014)	Bovids	13.8	51	4457
Babesia	*bovis*	T2Bo	2007	Brayton et al. (2007a)	Bovids	8.2	41.8	3671
Babesia	*divergens*	1802A[a], Rouen1987	2014	Jackson et al. (2014)	Bovids	9.6	42	4134
Babesia	*microti*	RI	2012	Cornillot et al. (2012)	Rodents, humans	6.5	36	3513
Cytauxzoon	*felis*	Winnie	2013	Tarigo et al. (2013)	Felids	9.1	31.8	4323
Theileria	*annulata*	Ankara	2005	Pain et al. (2005)	Bovids	8.4	32.5	3792
Theileria	*equi*	WA	2012	Kappmeyer et al. (2012)	Equids	11.6	39.5	5330
Theileria	*parva*	Muguga	2005	Gardner et al. (2005)	Bovids	8.3	34.1	4035
Theileria	*orientalis*	Shintoku	2012	Hayashida et al. (2012a)	Bovids	9	41.6	3995

All of these are available at PiroplasmaDB, with the exception of *B. divergens*
%GC percentage GC nucleotide content for the whole genome

[a]Genomic statistics shown for this isolate

Table 1.7 Whole-genome data for several piroplasm species

Genus	Species	Strains	Year published	Data type	Reference
Babesia	*bovis*	C9.1	2014	WGS	Jackson et al. (2014)
Babesia	*divergens*	None	2014	WGS, draft assembly	Cuesta et al. (2014)
Babesia	*microti*	R1, Gray	2013	Complete genome assembly	Cornillot et al. (2013)
Babesia	*bovis*	T2Bo_Vir., T2Bo_Att., L17_Vir., L17_Att., T_Vir., T_Att.	2011	WGS	Lau et al. (2011)
Theileria	*parva*	Marikebuni, Uganda, MugugaMarikebuni, MugugaUganda	2012	WGS, draft assemblies	Henson et al. (2012)
Theileria	*parva*	ChitongoZ2, KateteB2, Kiambu Z464/C12, MandaliZ22H10, Entebbe, Nyakizu, Katumba, Buffalo LAWR, Buffalo Z5E5	2013	WGS	Hayashida et al. (2013)
Theileria	*parva*	Muguga, Kiambu5, Serengeti-transformed	2015	WGS	Norling et al. (2015)

These resources are not available at PiroplasmaDB but can be found associated with their respective references

including antigen-encoding families such as the equi merozoite antigen (EMA) family (Kappmeyer et al. 2012).

With a genome size of ~8.2Mpb, the *B. bovis* genome sequence revealed a genomic organization that is remarkably similar to *T. parva*, with extensive synteny and multiple, large multigene families potentially contributing to host immune evasion (Brayton et al. 2007b). However, the smallest apicomplexan genome sequenced to date is *B. microti*, the principal agent of human babesiosis and a common pathogen transmitted by blood transfusions (Cornillot et al. 2012; Cornillot et al. 2013). With a genome size of 6.5Mbp, *B. microti* represents the closest record of a naturally occurring apicomplexan "core genome," and comparative genomics with this reduced genome may yield insights into the most essential gene products of apicomplexans, which could make excellent chemotherapeutic targets. *B. microti* is also the only example of an apicomplexan with a circular mitochondrial genome (Cornillot et al. 2013).

One apicomplexan with somewhat unclear phylogenetic position is *Cytauxzoon felis*. While originally considered a separate genus, the existence of exoerythrocytic forms, particularly schizonts, in macrophages/monocytes indicates that this parasite might be more appropriately considered in the family Theileriidae. *C. felis* is an emerging pathogen of domestic cats (*Felis catus*) in the Southern United States, and, as such, its genome was sequenced in an effort to identify potential vaccine targets (Tarigo et al. 2013). With a 9.1 Mbp genome, it has more protein-coding

genes in common with *T. parva* than it does with *B. bovis* and was found to encode a gene that is syntenic with a block of genes around the *T. parva* antigen and vaccine candidate, p67 (Tarigo et al. 2013).

There is currently only one licensed vaccine against apicomplexans for use in humans, the RTS,S subunit malaria vaccine based on the circumsporozoite protein (CSP) (see Chap. 16). With a few notable exceptions, such as those against coccidiosis (*Eimeria*), toxoplasmosis (*Toxoplasma*), and East Coast fever (*Theileria parva*), very few vaccines against piroplasms have been used on a commercial scale, which may be due, in part, to antigenic diversity in these parasites (Cornelissen and Schetters 1996). Genomic resources have also recently started to become available for some piroplasms (Table 1.8). These data are critical for identifying potential virulence genes, mapping recombination hotspots, and estimating genome-wide variation among various isolates, including vaccine strains (Norling et al. 2015). One weakness of piroplasm whole-genome datasets is their reliance on ab initio gene predictors for the majority of their structural annotations (determining where exons start and end in the genome). Given the fact that these genomes are smaller, denser, and more AT rich than most eukaryotes sequenced to date, these gene predictors may not be optimal for gene prediction in these genomes, and experimental evidence should be rigorously incorporated into genome re-annotation efforts in order to take full advantage of the genome sequences that are available for these apicomplexans. Of particular usefulness in this context is RNA-seq data (Table 1.8). The coupling of whole-genome variation data with gene expression data is a powerful method to give insight into gene structure, variation, and function and will hopefully assist the design of better prophylaxis against piroplasm-mediated diseases.

1.3.6 *Plasmodium* Reference Genomes

To date several complete reference genomes of *Plasmodium*, the etiological agent of malaria, have been sequenced. Advances in technology have also led to the sequencing of many additional lab strains and clinical isolates with the aim to produce reference genomes. The first reference to be published in 2002 was *P. falciparum* 3D7 (Gardner et al. 2002), the species responsible for the majority of human morbidity. Additional genomes of species that infect humans have been sequenced (*P. vivax* (Carlton et al. 2008)) or are in the process of being sequenced and *analyzed* (*P. malariae* and *P. ovale*). The simian- and human-infecting *P. knowlesi* (Pain et al. 2008), the chimpanzee malaria *P. reichenowi* (Otto et al. 2014a), and the simian malaria parasite *P. cynomolgi* (Tachibana et al. 2012) are also part of the reference genome collection. Draft genomes of three rodent malaria parasites that are widely used as model systems, *P. yoelii yoelii* (Carlton et al. 2002), *P. chabaudi chabaudi* AS, and *P. berghei* ANKA, were initially sequenced and analyzed in 2005 (Hall et al. 2005). Due to the highly fragmented nature of these genomes, they were re-sequenced in 2014 (Otto et al. 2014b). Two avian malaria genomes, *P. relictum* and

Table 1.8 Gene expression data not found at PiroplasmaDB for several piroplasm species

Genus	Species	Strains	Year published	Reference	Data type
Babesia	*bovis*	T2Bo	2007	Lau et al. (2007)	Microarray
Babesia	*bovis*	T2Bo	2013	Pedroni et al. (2013)	RNA-seq
Babesia	*bovis*	T2Bo_Vir., T2Bo_Att., L17_Vir., L17_Att., T_Vir., T_Att.	2013	Pedroni et al. (2013)	Microarray, RNA-seq
Babesia	*bigemina*	PR	2014	Jackson et al. (2014)	LC-MS
Cytauxzoon	*felis*	Winnie	2013	Tarigo et al. (2013)	EST
Theileria	*annulata*	Ankara	2012	Durrani et al. (2012)	Microarray
Theileria	*annulata*	Ankara	2013	Kinnaird et al. (2013)	Microarray
Theileria	*annulata*	Ankara	2013	Witschi et al. (2013)	LC-MS/MS
Theileria	*parva*	Muguga	2005	Bishop et al. (2005)	MPSS

Most expression data, including more EST data, is found at PiroplasmaDB for piroplasms

P. gallinaceum, have been sequenced and are in the process of being analyzed. They will provide a valuable missing link to understand the evolutionary context of human malaria. The Broad Institute has published a white paper that proposes the sequencing of additional *Plasmodium* genomes. This includes *P. coatneyi*, *P. inui*, and *P. fragile*; all of them belong to the monkey malaria clade and the sequencing of additional rodent malaria genomes. Further details can be found at https://www.broadinstitute.org. All of the published genomes mentioned above can be searched in PlasmoDB (Harb and Roos 2015) and GeneDB (Logan-Klumpler et al. 2012).

The publication of *P. falciparum* 3D7 in 2002 was a major milestone (Gardner et al. 2002). It enabled the malaria community to systematically analyze the gene content and tailor their experiments based on genomic data. This is also shown by over 3000 citations of the genome paper since publication. After the initial publication, assembly and annotation of the *P. falciparum* 3D7 genome has been

continuously improved over time. In 2011 a new *P. falciparum* 3D7 assembly (version 3) was made publicly available. This new version includes the correction of major misassemblies. The current genome version has a size of 23.3 Mb and encodes 5429 genes (Table 1.9). It is highly AT rich with a GC content of only 19.3 %. The overall structure of *Plasmodium* genomes sequenced to date is very similar (Table 1.9). The nuclear genome consists of 14 chromosomes, and the size ranges from 19 to 26 Mb with a comparable number of genes. About three quarters of genes are conserved across all *Plasmodium* genomes, representing the core genome. *Plasmodium* genomes also exhibit a high degree of synteny. The majority of the variation between *Plasmodium* species is found in the subtelomeric regions at the end of the chromosomes. In these regions, each of the *Plasmodium* species has a unique set of gene families that are assumed to be involved in immune evasion and virulence. The most important gene family in *P. falciparum* 3D7 is the *var* gene family that encodes the erythrocyte membrane protein 1 (PfEMP1) and plays a role in antigenic variation. Of around 60 gene family members, only one protein is expressed on the surface of an infected red blood cell at a time. PfEMP1 can also bind to host endothelial receptors and therefore plays an important role in pathogenicity. The *Plasmodium* interspersed repeat (PIR) superfamily composed of subtelomeric rifins and stevors in *P. falciparum* has orthologs in all *Plasmodium* spp. and ranges in number from 70 to 980 copies (Table 1.8) (Otto et al. 2014a). While the function of stevors is unknown, rifins are expressed on the surface of infected red blood cells where they mediate microvascular binding of infected red blood cells (Goel et al. 2015).

Closely related to *P falciparum* is the chimpanzee malaria parasite *P. reichenowi*. A comparative genomics analysis only showed minor differences between these two genomes. There is an almost complete colinearity in the core areas of the genome. The organization of *var* genes and other virulence-associated genes is also conserved. Differences were found in the reticulocyte-binding proteins, a gene family involved in invasion. These genes encode ligands that are important for the recognition of host erythrocytes. Members of this gene family are located on chromosome 13, where two almost identical genes are present in *P. falciparum* (RH2a and RH2b). *P. reichenowi* lacks RH2a but encodes a new reticulocyte-binding protein, RH7. The most significant difference between *P. reichenowi* and *P. falciparum* was found in the rifin and stevor multigene families. There are currently 463 rifins and 66 stevors annotated in *P. reichenowi*, while *P. falciparum* only encodes 185 rifins and 42 stevors. The difference in this multigene family also explains the difference in the overall number of genes found in the nuclear genome (Table 1.9).

P. vivax is the major source of human malaria outside of Africa. In contrast to *P. falciparum,* this species has a dormant stage in the human liver and can stay inactive for years. The nuclear genome of the Salvador I strain *P. vivax* has a size of 26.8 Mb and encodes 5433 genes (Table 1.9). With a GC content of 42.3 %, *P. vivax* has the highest GC content found so far in *Plasmodium*. Unique to *P. vivax* is an isochore structure. Chromosomes have AT-rich subtelomeres and internal regions of high GC content.

Table 1.9 *Plasmodium* reference genomes

	P. falciparum 3D7 (v3)[c]	*P. reichenowi* CDC (v1)[c]	*P. vivax* Sal1[a]	*P. knowlesi* H (v2)[c]	*P. cynomolgi* B[b]	*P. berghei* ANKA (v3)[c]	*P. chabaudi* AS (v3)[c]	*P. yoelii yoelii* 17X (v3)[c]
Genome size (Mb)	23.2	24	26.8	24.4	26.2	18.8	18.9	22.7
No. of chromosomes	14	14	14	14	14	14	14	14
G+C content (%)	19.3	19.2	42.3	38.6	40.4	22	23.6	21.5
No. of unassigned contigs	0	237	2745	148	1649	5	0	138
No. of genes[d]	5429	5736	5433	5290	5722	5034	5183	5948
% of genes with introns	54.1	55.9	52.1	54	ND	52.4	53.5	59.8
No. of PIRs[e]	227	529	346	70	256	217	208	980
Manually curated	Yes	Yes	No	Yes	No	Yes	Yes	Yes

[a]Carlton et al. (Carlton et al. 2008)
[b]Tachibana et al. (Tachibana et al. 2012)
[c]Genome version from 1.10.2015
[d]Including pseudogenes and partial genes, excluding noncoding RNA genes
[e]Including pseudogenes and partial genes

Closely related to *P. vivax* is the malaria parasite *P. knowlesi. P. knowlesi* is primarily a simian-infecting malaria parasite but has also been reported to cause natural infections in humans mainly in Southeast Asia. The nuclear genome has a size of 24.4 Mb and a GC content of 38.6 %; the number of protein-coding genes is 5290 (Table 1.9). There are two novel features in the *P. knowlesi* genome. The major variant gene families that are usually located in subtelomeres are found in chromosome-internal regions dispersed on all 14 chromosomes. These regions are often also associated with intrachromosomal telomeric repeats. Another unusual feature unique to *P. knowlesi* is a phenomenon called molecular mimicry. KIR proteins that are part of the PIR superfamily contain stretches of sequences that are identical to the host proteins AHNAK and CD99, which has a critical immunoregulatory role in host T-cell function. It is speculated that these proteins might interfere with host recognition processes. Another important gene family is the SICAvar (schizont-infected cell agglutination) gene family. SICAvars are expressed on the surface of infected erythrocytes and are the largest family of variable surface antigens in *P. knowlesi*.

Phylogenetically related to *P. knowlesi* and *P. vivax* is the simian malaria parasite *P. cynomolgi. P. cynomolgi* is used as a model organism for human *P. vivax* infections. Both parasites share the ability to form a dormant liver stage. Strain B of *P. cynomolgi* has been sequenced and published in 2011 (Tachibana et al. 2012). The genome has a size of 26.2 Mb and encodes 5722 genes (Table 1.9). Of those, around 90 % have 1:1 orthologs to *P. vivax* and *P. knowlesi. P. cynomolgi* and *P. vivax* share a common isochore structure, while the presence of intrachromosomal telomeric repeats is common to *P. cynomolgi* and *P. knowlesi.* Comparative genome analysis found a number of copy number variants in multigene families, e.g., in reticulocyte-binding proteins.

Of particular interest are the rodent malaria parasites, *P. berghei*, *P. chabaudi chabaudi*, and *P. yoelii yoelii*. They are used as model organisms for experimental studies of human malaria. The genome size of the rodent malaria parasite genomes ranges from 18.8 to 22.7 Mb (Table 1.9). The GC content is around 22 %. *P. yoelii yoelii* has the highest number of genes in the nuclear genome, mostly due to a large expansion of PIR genes (980). Gene synteny is conserved along the 14 chromosomes of the rodent malaria parasites, with only one known synteny breakpoint. Analysis of gene families in the rodent-infective species reveals that the gene family is the PIR gene family (Otto et al. 2014b). The second largest gene family encodes fam-a proteins. Fam-a proteins are exported to the infected red blood cell and are expanded in the rodent malaria parasites. All other *Plasmodium* genomes sequenced to date have only one fam-a family gene. The number ranges from 161 in *P. yoelii yoelii* to 148 in *P. chabaudi chabaudi* and 74 in *P. berghei*.

1.3.7 Amoebae

The amoebae, single-celled eukaryotes that last shared a common ancestor with humans/animals (metazoans) after fungi, are from a sparsely sampled and little-studied domain of the tree of life. As with most protists, the best known are those that cause disease in humans, such as species of the genera *Entamoeba* and *Acanthamoeba*. *Entamoeba* spp. are intestinal parasites or commensals of a wide range of animals in addition to humans. *Acanthamoeba* spp. are free-living amoebae of interest to humans primarily as opportunistic pathogens. These two and the social amoebae, such as *Dictyostelium* species, are the best studied amoebae and those for which there exist sequenced genome assemblies (Clarke et al. 2013; Eichinger et al. 2005; Lorenzi et al. 2010, Loftus et al. 2005).

1.3.7.1 *Entamoeba*

The described species of *Entamoeba* are generally obligate parasites or commensals. They have simple life cycles consisting of a vegetative stage, the trophozoite, which lives in the host's large intestine and feeds upon bacteria and a transmissible stage, the cyst, which allows survival outside the host and transmission to a new host. Possible exceptions to these rules include two species (*Entamoeba moshkovskii* and *Entamoeba bangladeshi*) that can survive outside of the host and may be primarily free-living organisms and one species (*Entamoeba gingivalis*) that colonizes the mouth and may have lost the ability to form cysts, instead being transmitted directly in the trophozoite form.

The human pathogen *Entamoeba histolytica* is the most studied species of the genus. A draft genome assembly was first published in 2005, with subsequent updates, though it remains fragmented and chromosomes cannot be defined (Lorenzi et al. 2010; Loftus et al. 2005; Clark et al. 2007). Unusual features of the *E. histolytica* genome include an unusual organization of tRNA genes, which occur in arrays of sets of tRNA genes separated by repetitive intergenic DNA (Clark et al. 2006) and rRNA genes encoded on extrachromosomal circular DNA occurring in multiple copies per cell (Bhattacharya et al. 1989). Two features of Entamoebae associated with their anaerobic environments are the loss of the function and genome of the mitochondrion, which occurs as a relict organelle, the mitosome, and the related lateral transfer of genes, many involved in anaerobic metabolic processes and apparently derived from anaerobic bacteria (Rosenthal et al. 1997).

Genomic re-sequencing suggests little nucleotide diversity among *E. histolytica*, even among lineages derived from widely separated geographical locations (Weedall et al. 2012). In contrast, gene copy number variation appears to be extensive (Weedall et al. 2012), which may be associated with the genomic plasticity observed among *E. histolytica* lineages (Willhoeft and Tannich 1999). Studies using tRNA-associated

repetitive intergenic DNA or SNP markers also suggest very little linkage disequilibrium among markers, which suggests extensive outcrossing among parasite lineages (Weedall et al. 2012; Gilchrist et al. 2012; Zaki et al. 2003). Genetic diversity in other *Entamoeba* species is largely unknown, apart from studies of the 18S ribosomal RNA gene, which indicate that some "species" may in fact be species complexes (Stensvold et al. 2011).

Genomic data exist for four other species of *Entamoeba*: *E. nuttalli*, *E. dispar*, *E. moshkovskii*, and *E. invadens*. For the first three of these, the data are available but no reference publication yet exists. Most closely related to *E. histolytica*, *Entamoeba nuttalli* is a pathogen of macaques (Tachibana et al. 2007; Tachibana et al. 2013; Feng et al. 2013). A slightly more divergent species, *Entamoeba dispar*, infects humans and is of primary interest as a relative of *E. histolytica* (which, due to both species being morphologically indistinguishable, was only recently recognized as a separate species) that appears to be nonpathogenic (Diamond and Clark 1993). *Entamoeba moshkovskii* is of uncertain status as a parasite or a free-living organism and has recently been associated with disease in humans (Ali et al. 2003; Shimokawa et al. 2012). *Entamoeba invadens*, a pathogen of reptiles, is of primary interest as a model species for the process of encystation (which cannot be induced in axenic *E. histolytica* cultures). The genome of *E. invadens* is considerably larger than that of *E. histolytica* (Ehrenkaufer et al. 2013). Genomic data for a number of *E. histolytica* strains, from a range of geographical locations and associated with different disease manifestations, are available via AmoebaDB (http://AmoebaDB.org) (Aurrecoechea et al. 2011) (Table 1.10).

1.3.7.2 *Acanthamoeba*

The Acanthamoebae are of importance for human health as a cause of keratitis when they infect the eye, often via contaminated contact lenses (Lorenzo-Morales et al. 2013). More commonly, they are free-living, soil-dwelling protists preying on bacteria.

A draft genome assembly of *Acanthamoeba castellanii* was published in 2013 (Clarke et al. 2013). The genome encodes large families of genes involved in cell signaling and environmental sensing, such as protein kinases (Clarke et al. 2013). As in the Entamoebae, a proportion of genes appear to have been acquired by lateral gene transfer, though the number of such genes in *A. castellanii* is larger and a larger proportion appear to have been acquired from aerobic and free-living bacteria (Clarke et al. 2013). Interestingly, in contrast to *Entamoeba* genes, which contain few introns, *Acanthamoeba* genes are intron rich (Clarke et al. 2013). Thirteen additional *Acanthamoeba* genome assemblies, representing a geographically diverse range of species and strains, were recently made available via AmoebaDB (Dr. Andrew Jackson, University of Liverpool; Table 1.11).

Table 1.10 Genome datasets of amoebae available in AmoebaDB

Species	Strain	Dataset	Sequencing platform	Reference
Entamoeba histolytica	HM-1:IMSS	De novo genome assembly	Sanger	Lorenzi et al. (2010), Loftus et al. (2005)
Entamoeba histolytica	HM-1:IMSS-A	De novo genome assembly	454, Illumina	
Entamoeba histolytica	HM-1:IMSS-B	De novo genome assembly	454, Illumina	
Entamoeba histolytica	HM-1:CA	De novo genome assembly	454, Illumina	
Entamoeba histolytica	HM-3:IMSS	De novo genome assembly	454, Illumina	
Entamoeba histolytica	KU27	De novo genome assembly	454, Illumina	
Entamoeba histolytica	KU48	De novo genome assembly	454, Illumina	
Entamoeba histolytica	KU50	De novo genome assembly	454, Illumina	
Entamoeba histolytica	MS96-3382	De novo genome assembly	454, Illumina	
Entamoeba histolytica	DS4-868	De novo genome assembly	454, Illumina	
Entamoeba histolytica	Rahman	De novo genome assembly	454	
Entamoeba histolytica	HM-1:IMSS-A	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	HM-1:IMSS-B	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	Rahman	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	2592100	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	MS84-1373	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	MS27-5030	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	PVBM08B	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	PVBM08F	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba histolytica	HK-9	Re-sequencing	SOLiD	Weedall et al. (2012)

(continued)

Table 1.10 (continued)

Species	Strain	Dataset	Sequencing platform	Reference
Entamoeba histolytica	IULA:1092:1	Re-sequencing	SOLiD	Weedall et al. (2012)
Entamoeba nuttalli	P19	De novo genome assembly	Illumina	
Entamoeba dispar	SAW760	De novo genome assembly	Sanger	
Entamoeba moshkovskii	Laredo	De novo genome assembly	454	
Entamoeba invadens	IP1	De novo genome assembly	Sanger	Ehrenkaufer et al. (2013)
Acanthamoeba castellanii	Neff	De novo genome assembly	Sanger, 454, Illumina	Clarke et al. (2013)
Acanthamoeba castellanii	Ma	De novo genome assembly	Illumina	
Acanthamoeba mauritaniensis	1652	De novo genome assembly	Illumina	
Acanthamoeba quina	Vil3	De novo genome assembly	Illumina	
Acanthamoeba astronyxis		De novo genome assembly	Illumina	
Acanthamoeba palestinensis		De novo genome assembly	Illumina	
Acanthamoeba sp. (T4b-type)		De novo genome assembly	Illumina	
Acanthamoeba triangularis	SH621	De novo genome assembly	Illumina	
Acanthamoeba sp. Incertae sedis		De novo genome assembly	Illumina	
Acanthamoeba sp.	Galka	De novo genome assembly	Illumina	
Acanthamoeba lugdunensis	L3a	De novo genome assembly	Illumina	
Acanthamoeba culbertsoni	A1	De novo genome assembly	Illumina	
Acanthamoeba rhysodes	Singh	De novo genome assembly	Illumina	
Acanthamoeba lenticulata	PD2S	De novo genome assembly	Illumina	

Table 1.11 Online resources for genomic-scale data

Resource name	Acronym	Content and functionality	Web address (URL)
National Center for Biotechnology Information	NCBI	Data repository and search capability (International Nucleotide Sequence Database Collaboration)	http://www.ncbi.nlm.nih.gov
The European Bioinformatics Institute	EMBL-EBI	Data repository and search capability (International Nucleotide Sequence Database Collaboration)	http://www.ebi.ac.uk
DNA Data Bank of Japan	DDBJ	Data repository and search capability (International Nucleotide Sequence Database Collaboration)	http://www.ddbj.nig.ac.jp/
Ensembl Protists	Ensembl Protists	Part of the larger Ensembl Genomes which is a joint European Bioinformatics Institute and the Wellcome Trust Sanger Institute project providing Ensembl tools, data visualization, data mining, and comparative analysis	http://protists.ensembl.org/
GeneDB	GeneDB	Core part of the Sanger Institute's Pathogen Genomics initiative. Provides early access to the latest sequence data and annotation/curation. In addition, the site includes some basic search functionality and genome browsing	http://www.genedb.org/
The Eukaryotic Pathogen Databases	EuPathDB	One of four National Institute of Allergy and Infectious Diseases bioinformatic centers. Provides integrated search capabilities of genomes and functional data dedicated to eukaryotic pathogens (and related organisms). Includes AmoebaDB, FungiDB, GiardiaDB, MicrosporidiaDB, PiroplasmaDB, PlasmoDB, ToxoDB, TrichDB, TriTrypDB, OrthoMCL, and HostDB	http://EuPathDB.org http://amoebadb.org http://cryptodb.org http://fungidb.org http://microsporidiadb.org http://piroplasmadb.org http://plasmodb.org http://toxodb.org http://trichdb.org http://tritrypdb.org http://orthomcl.org http://hostdb.org

1.4 Data Integration and Accessibility

Several databases exist that provide online and free access to parasite genomes, annotation, and functional data (Table 1.11). The National Institute of Allergy and Infectious Diseases (NIAID) in the United States initiated established bioinformatics resource centers (BRCs) in 2004 whose goal is to provide the global pathogen research community with free and online tools to mine genomic and functional genomic data and additional data types essential for pathogen surveillance and control (Greene et al. 2007). The BRCs included one specifically tasked with providing support for the eukaryotic pathogen scientific community (EuPathDB, initially known as ApiDB) (Aurrecoechea et al. 2010). Now in its third 5-year funding cycle, EuPathDB incorporates data from over 240 parasitic and evolutionarily related organisms spanning multiple phyla such as the Amoebozoa, Apicomplexa, Euglenozoa, Metamonada, Sarcomastigophora, and numerous fungal phyla. Data includes genome sequence, structural and functional annotation, and functional data covering the omics landscape including transcriptomics, proteomics, and metabolomics. Most current database content can be accessed here: http://eupathdb.org/eupathdb/eupathGenome.jsp

Data within EuPathDB and its component sites are searchable via an intuitive graphical user interface that allows the development of complex in silico experiments to support hypothesis-driven experiments. Data types include the underlying genomic sequences and annotations (close to 250 genomes represented), transcript level data (SAGE-tag, EST, microarray, and RNA sequence data), protein expression data (including quantitative), epigenomic data (ChIP-chip and ChIP-seq), population-level (SNP) and isolate data, and host response data (antibody array). In addition, genomic analyses provide the ability to search for gene features, subcellular localization, motifs (InterPro and user defined), function (Enzyme Commission annotation and GO terms), and evolutionary relationships based on gene orthology. Detailed tutorials and usage instructions are available through publications and online tutorials and exercises (Harb and Roos 2015; Aurrecoechea et al. 2013). A number of YouTube tutorials are available: https://www.youtube.com/user/EuPathDB/. EuPathDB resources provide community annotation, and curation via user comments (including images, files, PubMed records, etc.) can be added to records in EuPathDB sites (comments become immediately visible and searchable). A graphical search system allows building complex searches in a stepwise manner that can be saved, modified, and shared. An example strategy can be seen in Fig. 1.1 and accessed online by following this link: http://plasmodb.org/plasmo/im.do?s=df42a71ae3acbb1e. Browsing capability is available through a genome browser integrating genomes, annotation, analyses, and functional data. Column and result analysis tools are also available to generate word cloud graphics, histograms, and GO term and pathway enrichment analyses.

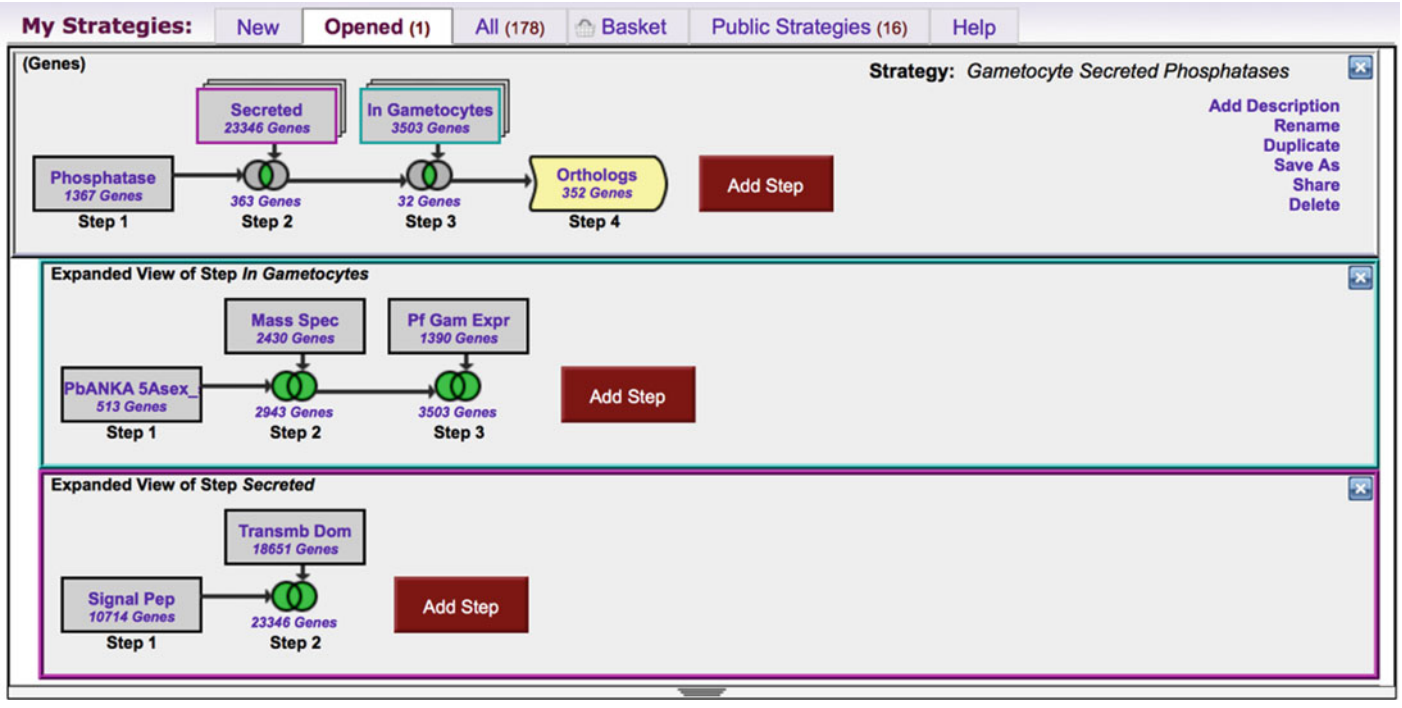

Fig. 1.1 Screenshot from PlasmoDB depicting a search strategy that identifies putative phosphatases that are secreted and expressed in gametocytes based on proteomics, RNA sequence, and microarray experiments. Search strategies are constructed by adding steps that query underlying data. *Step 1* identifies all putative phosphatases based on a text search. *Step 2* identifies any of the genes in Step 1 that also have a secretory signal peptide, at least one transmembrane domain, or both (see expanded view of "Secreted"). *Step 3* identifies any genes in Step 2 that have evidence of expression based on data from three experiments in *P. falciparum* (see expanded view "Gametocytes") (Otto et al. 2014b; Florens et al. 2002; Silvestrini et al. 2010). Step 4 transforms the results in Step 3 to all orthologs in PlasmoDB

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Proteomics

2

Jonathan Wastling and Dong Xia

Abstract

Systems biology integrates high-throughput molecular sciences such as genomics, proteomics, metabolomics and glycomics to advance our understanding on pathways, functional nodules and large-scale organisation (Oltvai and Barabasi, Science. 298(5594):763–764, 2002). Developed in the late 1990s, proteomics is now established as one of the pillars of 'systems biology'. Strategies to characterise the abundance, stability and modification status of proteins, the functional molecules of the cell, are of great interest to enhance our understanding of how cells function and communicate. Proteomic approaches have been exploited extensively by the protozoan parasitology field. In this chapter, we review the present state of proteomic research in protozoan parasites and discuss how various proteomic platforms combined with advanced bioinformatic tools have been used to enhance our understanding of specific biological questions in host-parasite interaction systems.

2.1 Major Proteomic Techniques and Applications

2.1.1 High-Throughput Protein Identification

Proteins are the end products of most genes; the identification of expressed proteins and their functions are central to the understanding of biological meaning of the genome. The term proteome was introduced by Marc Wilkins in 1994 at a

J. Wastling (✉)
Executive Dean, Faculty of Natural Sciences, Keele University, Keele, ST5 5BG, UK
e-mail: j.wastling@keele.ac.uk

D. Xia
Department of Infection Biology, Institute of Infection and Global Health,
University of Liverpool, Liverpool L69 7ZJ, UK
e-mail: d.xia@liverpool.ac.uk

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conference (Wilkins 1994) to describe the entire complement of proteins expressed by a genome, cell, tissue or organism. More specifically, it is the set of expressed proteins at a given time under defined conditions. Papers that began to use the term were published thereafter (Wasinger et al. 1995; Wilkins et al. 1996).

Before the availability of annotated parasite genomes, 'top-down' approaches in which intact proteins were analysed directly by mass spectrometry (MS) (along with non-MS-based approaches such as amino acid sequencing by Edman degradation) were the only practical way to obtain protein sequence information. Due to the instrument limitations, analysing intact protein samples is more difficult to achieve by MS, although progress has been made in recent years, which will be reviewed later in this chapter. The availability of annotated parasite genomes as described in Chap. 1 as well as the improvement of MS instrumentation has enabled so-called 'bottom-up' proteomics. There are many excellent reviews on proteomics in the literature (Aebersold and Mann 2003; Cox and Mann 2011; Mallick and Kuster 2010; Nesvizhskii 2014; Olsen and Mann 2013), and these and others should be referred to for a more thorough introduction to the general topic. However, before dealing specifically with proteomic studies in parasitology, we will briefly review some of the basic principles of this relatively new area of technology.

In 'bottom-up' proteomic approach, MS is used to analyse enzymatically digested or chemically produced peptides from protein samples. Due to the complexity of the proteome under investigation, sample separation at either the protein level or peptide level is almost always performed prior to MS analysis. Protein separation is typically achieved by techniques such as gel electrophoresis or other forms of chromatography followed by in-gel or in-solution digestion, while peptide separation is typically achieved by using reverse phase liquid chromatography. A typical workflow for high-throughput identification proteomics in parasite systems is shown in Fig. 2.1. The resulting MS spectra, usually contains information on mass/charge (m/z) and intensity, are then used to infer peptide sequence by matching to a computationally derived database from annotated genome sequence data provided by sequence databases such as UniProt (2015) and EuPathDB (Aurrecoechea et al. 2010), using search engines such as Mascot (Perkins et al. 1999), Sequest (Yates et al. 1995), X!Tandem (Craig et al. 2004), PEAKS DB (Zhang et al. 2012) and MS-GF+ (Kim and Pevzner 2014).

The field of protozoan parasitology has adopted emerging proteomic technologies very promptly. Early studies typically used in-gel digestion of protein spots separated by two-dimensional electrophoresis (2DE) followed by protein identification made using peptide mass fingerprinting (PMF) on a MALDI-TOF instrument such as the global proteomic study on *T. gondii* tachyzoite (Cohen et al. 2002). A similar approach employing MALDI-TOF has also been used to analyse the proteome of *Cryptosporidium parvum* oocysts (Magnuson et al. 2000) and intact *Giardia duodenalis* and *Giardia muris* cysts (Villegas et al. 2006). The advances in MS instrumentation enabled a more comprehensive analysis of parasite proteomes, including the more widespread use of CID (collisional-induced dissociation) based liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of peptides. This platform has allowed the high-throughput identification of multiple

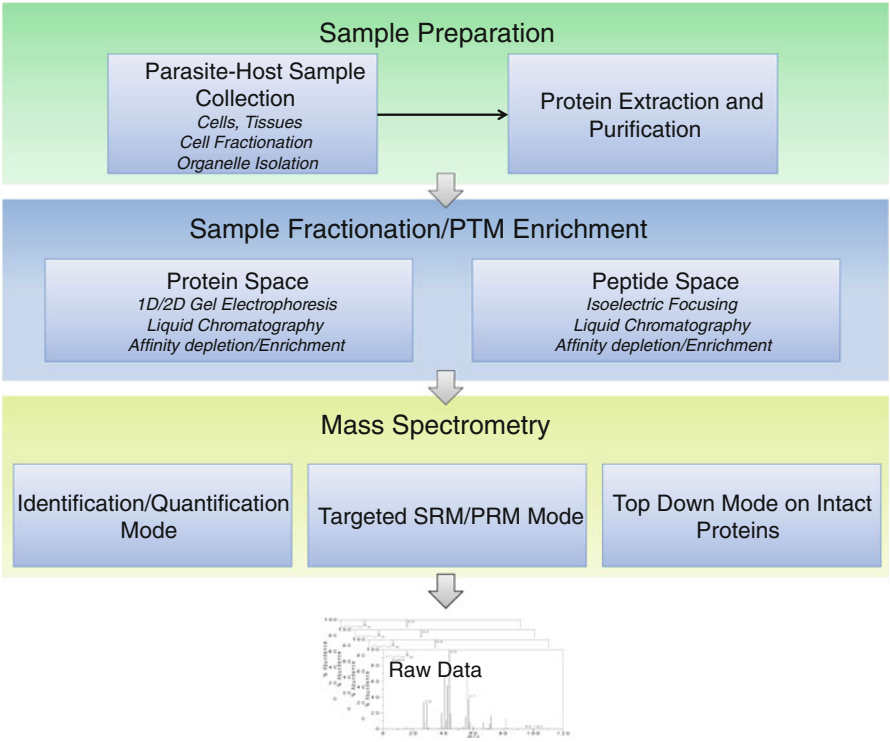

Fig. 2.1 A schematic diagram for a proteomics workflow. A typical workflow for a high-throughput identification and quantification proteomics experiment. The workflow consists of three major steps: sample preparation (collection, protein extraction and purification), sample fractionation (in either protein or peptide space) and PTM enrichment, followed by mass spectrometry analysis. The collected raw mass spectra data are then analysed by various bioinformatics pipelines illustrated in figure 2.

proteins present in 1DE or 2DE spots or in whole digests – fractionated or un-fractionated.

Two pioneering examples took advantage of high-throughput proteomics for the analysis of the life cycle of *Plasmodium falciparum* (Florens et al. 2002; Lasonder et al. 2002). These global strategies were used to analyse the changes that the parasite undergoes as it traverses its life cycle in multiple hosts. Both studies utilised a combination of pre-fraction strategies and online multidimensional protein identification technology (MudPIT) which used the complementary separation power of strong cation exchange and reverse phase chromatography and 1D gel electrophoresis combined with MS/MS analysis to identify both parasite and human proteins. Evidence for the translation of more than 1000 predicted 'hypothetical' proteins was confirmed by use of high-throughput proteomic techniques. Large-scale proteomic studies on protozoan parasites appeared soon after including a comprehensive proteomic survey on *Trypanosoma cruzi* (Atwood et al. 2005) and the proteomes of other Apicomplexa including *Cryptosporidium parvum* (Sanderson et al. 2008;

Snelling et al. 2007) and *Toxoplasma gondii* (Dybas et al. 2008; Xia et al. 2008). There are now over 20 protozoan species with ongoing proteomic projects, half of which have greater than 30% coverage and are headed by *Plasmodium* and *Toxoplasma,* each with around 70% coverage (Table 2.1).

In addition to global proteome profiling, specific sub-proteomes of protozoan parasites have also been investigated in detail. One key area of research is to understand the proteins expressed in specific cellular compartment or organelles. Bradley et al. pioneered the proteomic investigation of apicomplexan rhoptry organelles, identifying many novel components of the rhoptry and rhoptry neck of *T. gondii* (Bradley et al. 2005). Rhoptry-enriched fractions have been investigated in

Table 2.1 Current proteomics projects hosted on Eukaryotic Pathogen Database Resources (EuPathDB: http://eupathdb.org) (release 26)

Organism	Protein coding genes	No. protein IDs	Coverage (%)	Database
Plasmodium falciparum 3D7	5542	4117	74	PlasmoDB
Toxoplasma gondii ME49	8322	5748	69	ToxoDB
Trypanosoma brucei TREU927	11,567	6638	57	TriTrypDB
Toxoplasma gondii GT1	8460	4488	53	ToxoDB
Plasmodium berghei ANKA	5076	2532	50	PlasmoDB
Leishmania donovani BPK282A1	8083	3999	49	TriTrypDB
Leishmania major strain Friedlin	8400	3790	45	TriTrypDB
Giardia Assemblage A isolate WB	9667	3598	37	GiardiaDB
Trypanosoma cruzi CL Brener Esmeraldo-like	10,339	3687	36	TriTrypDB
Trypanosoma cruzi CL Brener non-Esmeraldo-like	10,831	3862	36	TriTrypDB
Cryptosporidium parvum Iowa II	3805	1320	35	CryptoDB
Theileria annulata strain Ankara	3796	1237	33	PiroplasmaDB
Entamoeba histolytica HM-1:IMSS	8306	2443	29	AmoebaDB
Plasmodium yoelii yoelii 17X	5978	1720	29	PlasmoDB
Leishmania mexicana MHOM/GT/2001/U1103	8250	1666	20	TriTrypDB
Trypanosoma cruzi strain CL Brener	2135	374	18	TriTrypDB
Plasmodium vivax Sal-1	5586	865	15	PlasmoDB
Leishmania infantum JPCM5	8239	1046	13	TriTrypDB
Eimeria tenella strain Houghton	8597	279	3	ToxoDB
Trichomonas vaginalis G3	97,475	1830	2	TrichDB
Leishmania braziliensis MHOM/BR/75/M2904	8357	68	1	TriTrypDB

There are over 20 protozoan species with ongoing proteomic projects, half of which have greater than 30% coverage. EuPathDB (Aurrecoechea et al. 2010) acts as one of the main portals for eukaryotic pathogen proteomics, where individual proteome projects listed in the table can be accessed in its dedicated daughter databases

Plasmodium merozoites (Sam-Yellowe et al. 2004). Cytoskeletal and sub-compartments in *T. gondii* (Hu et al. 2006; Lorestani et al. 2012) have also been examined. Ferella et al. coupled proteomics with immunofluorescence microscopy to identify novel proteins in various organelles from enriched subcellular fractions that were undetected by whole cell proteomic methods (Ferella et al. 2008).

Another important contribution of sub-proteome research comes from the identification of excretory-secretory products of the parasites that are essential for host-pathogen interaction. The biotin-streptavidin-fractionated surface protein of parasite-infected erythrocytes of *P. falciparum* was investigated using shotgun proteomics (Florens et al. 2004). Key proteins involved in host cell invasion by *T. gondii* tachyzoites have also been characterised on MIC2/M2AP complex (Zhou et al. 2004), excreted/secreted antigens (ESA) (Zhou et al. 2005) and N-glycosylated structures (Fauquenoy et al. 2008). Proteomic analysis of *Leishmania donovani* secretome revealed major discrepancies between observed and *in silico* prediction of secreted proteins, identifying the existence of nonclassical secretion pathways comparing to classic eukaryotic secretion signal peptides (Silverman et al. 2008).

Advances in MS instrumentation and protein separation technology will continue to increase the number of identifications that can be obtained from a single sample. Increasing resolution and accuracy have improved the reliability of these identifications, as has the use of more sophisticated bioinformatic tools to improve data processing and to ensure identifications are supported statistically. It is only a matter of time until relatively extensive proteomic coverage has been reported for most parasites of relevance to human and animal health. However, it is worth considering the extent to which a fully comprehensive proteome for some organisms is really achievable. Issues with sample preparation, incomplete tryptic digestion (Brownridge and Beynon 2011), dynamic range, bias against certain classes of proteins, imperfect genome annotation and concerns over peptide coverage, detectability and specificity ('proteotypic' peptides) (Beck et al. 2011) make full coverage of a proteome challenging. Extensive pre-fractionation of the protein or peptide samples can go some way to overcome some of these problems, but in doing so adds a great deal of experimental redundancy as well as greatly increasing instrument analysis time and cost. Finally, parasites are characterised by often complex life cycles, sometimes in multiple hosts or survival in the external environment. A truly comprehensive proteome for any parasite is therefore never expressed at any one moment; rather it is a dynamic and responsive facet of the host-parasite system for which a great range of temporal changes captured by both identification and quantification data is required before a full picture can be achieved.

2.1.2 Advances in Quantitative Proteomics in Parasite and Hosts

In addition to identify the proteins expressed, being able to measure the changes in the abundance of proteins from one condition to another or determine changes in the protein composition of protein complexes and organelles under different conditions

provides key information in understanding how the proteome responds in a dynamic host-parasite interaction. Quantitative proteomics is now one of the foremost topics in proteomic research, which can be categorised into two main approaches: label-based methods and label-free methods.

Common labelling techniques involve either stable isotope labelling through in vivo metabolic labelling, chemical modification or labelling by fluorescent dyes. In vivo labelling metabolically labels proteins by incorporation of stable isotope labels with amino acids in cell culture (SILAC) (Ong et al. 2002). Popular chemical labelling includes proteins labelled with isotope-coded affinity tags (ICAT) (Gygi et al. 1999), tandem mass tags (TMTs) (Thompson et al. 2003) and isobaric tag for relative and absolute quantitation (iTRAQ), which uses a multiplexed set of isobaric reagents that yield amine-derivatised peptides for relative and absolute quantitation (Ross et al. 2004). Fluorescent dye labelling is commonly used in 2DE fluorescence difference gel electrophoresis (2DE DIGE) (Unlu et al. 1997).

More recently, label-free quantification has gained popularity due to straightforward experimental implementation. Label-free techniques directly use raw data from parallel MS runs to compare relative or absolute protein abundances in different runs, which are typically achieved by spectral counting or intensity-based quantification. Spectral counting generally infers protein abundance using the number of peptide-spectrum matches (PSMs) in a given run. However, due to different ionisation efficiencies caused by biophysical properties of each peptide, the raw spectral counting has been proved as less reliable as a quantification indication. Several software packages have been developed to normalise spectral counting, such as APEX (Lu et al. 2007), NSAF (Zybailov et al. 2006) and emPAI (Ishihama et al. 2005). Intensity-based methods align precursor ion spectra of the same peptide from parallel runs according to their retention times (RT), and protein quantification is acquired by summing ion intensities that have been matched to all nonconflicting or top three peptides for a given protein (Grossmann et al. 2010; Schwanhausser et al. 2011). This approach has been implemented by several commercial software packages such as Progenesis QI (NonLinear Dynamics) and PEAKS Studio (Bioinformatics Solutions Inc.), as well as open source packages such as MaxQuant (Cox and Mann 2008), OpenMS (Sturm et al. 2008) and MSight (Palagi et al. 2005).

2.1.2.1 Label-Based Quantitative Proteomics in Host-Parasite Systems

In parasitology research, the quantitative approach used usually depends on the nature of the host-parasite system under investigation. If the parasite can be cultured, then in vivo metabolic labelling with a stable isotope can be achieved. SILAC has been extensively performed in cell culture, with virtually the entire proteomes of diploid and haploid yeast being compared using SILAC (de Godoy et al. 2008). It has also been employed in various protozoan parasitology studies. For example, quantitative proteome of the trophozoite stages of the malarial parasite *Plasmodium falciparum* following chloroquine and artemisinin treatment was examined using a stable isotope approach that used ^{14}N-isoleucine and $^{13}C_6$,$^{15}N_1$-isoleucine combined with a MudPIT peptide separation method (Prieto et al. 2008). Actively secreted

proteins in *Leishmania donovani* was quantitatively identified by comparing protein secreted into promastigote-conditioned medium in relation to the same protein that remained in cell-associated population (Silverman et al. 2008). The role of the antibiotic paromomycin on the global proteomes of susceptible and resistant strains of *Leishmania donovani* was investigated with SILAC (Chawla et al. 2011). In *Trypanosoma brucei*, SILAC has been used to compare protein expression profiles between bloodstream and procyclic life cycle stages (Butter et al. 2013; Urbaniak et al. 2012) as well as characterise the glycosome proteome where isotope ratios were used to discriminate glycosomal from mitochondrial and other contaminating proteins (Guther et al. 2014).

SILAC-based quantification benefits from high accuracy and the fact that the labelled 'heavy' proteome is essentially indistinguishable from the 'light' or normal proteome and can be combined early on in the procedure, at the cell level or just after lysis, meaning that less variation or inaccuracies will be introduced during sample preparation and pre-fractionation before MS analysis. It has also been popularly used to couple with post-translational modification (PTM) studies to provide quantitative information on certain modifications that will be reviewed in a later section.

Despite being an accurate and versatile approach, many parasites cannot easily be labelled in vivo, which makes SILAC an impractical approach. An alternative labelling technique is therefore to post-label by chemically modifying peptides or protein preparations from experiments using tagged stable isotope or fluorescent dyes. The most widespread of the stable isotope tag techniques are iTRAQ and TMT. Up to eight different samples can be labelled with iTRAQ (Choe et al. 2007; Pierce et al. 2008), whereas recent extension of TMT allows multiplexing to ten conditions (McAlister et al. 2012; Werner et al. 2012). Labelled peptides from different samples can be mixed and analysed in the same MS run. During MS, the tags are fragmented into reporter groups with a different mass for each tag. The MS intensity recorded for each tag can then be used to derive the relative abundance of corresponding peptides in the starting sample. Protein abundance changes in the malaria parasite *P. falciparum* following doxycycline treatment were measured using iTRAQ (Briolant et al. 2010) as was the differential protein expression over the life stages of *Trypanosoma congolense* (Eyford et al. 2011). The protein abundance differential measured using iTRAQ was used to distinguish putative mitosomal proteins from copurified contamination in *Giardia duodenalis* extracts (Jedelský et al. 2011). Combining quantitative iTRAQ proteomic profiling with transcriptomics showed that the expression of merozoite proteins in *P. falciparum* was regulated post-translationally during invasion pathway switching as an adaptation to variations of the host cell (Kuss et al. 2011). TMT labelling has assisted the discovery of promising markers for staging human African trypanosomiasis by quantitatively determining patient proteins overexpressed in the second stage of the disease (Tiberti et al. 2010).

Difference gel electrophoresis (DIGE) can be used to label up to three different samples with a fluorescent dye. The samples are then mixed and analysed by 2D electrophoresis. Differences in protein abundance between the samples can be

measured by excitation at different wavelengths, and gel images are matched and analysed to produce candidate protein spot for MS identification. This technique helps reduce the variability between samples run on separate 2DE gels. DIGE has been used to monitor the changes in the host cell proteome to invasion by *Toxoplasma gondii*, highlighting significant changes in key metabolic pathways and in post-translational protein modification (Nelson et al. 2008), in sulphadiazine-resistant and sulphadiazine-sensitive strains of *T. gondii* (Doliwa et al. 2013), measuring the key protein changes during *Neospora* differentiation (Marugán-Hernández et al. 2010) and identifying changes in the abundance of proteins involved with energy metabolism in the head proteome of the *Anopheles* mosquito after infection with *Plasmodium* (Lefevre et al. 2007). The plasma proteomes of several individuals infected with *Leishmania donovani* were compared to control individuals using DIGE (Rukmangadachar et al. 2011) identifying several putative biomarkers.

2.1.2.2 Label-Free Quantitative Proteomics in Parasite Systems

Another popular quantitative proteomic approach is the label-free technique. The principle behind this technique is that two samples can be compared without the need to modify or label protein preparations using the mass spectra alone. Commonly used label-free techniques can be divided into two categories, spectral counting and intensity-based approaches. Spectral counting is based on a straightforward principle of more spectra observed equals higher peptides abundance. It can provide semi-quantitative data on protein abundances in shotgun identification proteomics, which is usually automatically performed on peptide identification data sets (e.g. emPAI in the Mascot search engine). The straightforward nature of this approach has enabled pioneering study from Le Roch and co-workers to understand post-transcriptional controls regulating mRNA and protein abundance across the *P. falciparum* life cycle, where a moderately positive relationship was observed (Le Roch et al. 2004). Label-free quantitative proteomics of the early gametocyte phase of *P. falciparum* identified that proteins involved in erythrocyte remodelling were enriched (Silvestrini et al. 2010). Spectral counting approach has also been used to quantify global protein expressions in *Toxoplasma* oocysts (Fritz et al. 2012), *Giardia duodenalis* encystation (Faso et al. 2013) and *G. duodenalis* assemblage A (Emery et al. 2015). Spectral counting was also coupled with PTM studies to provide relative protein abundance estimations in the global analysis of protein palmitoylation in *T. brucei* (Emmer et al. 2011) and phosphoproteomes of *P. falciparum* and *T. gondii* (Treeck et al. 2011).

Intensity-based label-free approach aligns separate LC-MS/MS runs of peptide mixtures and calculates the differences in intensities of the same peptides detected in each run. This approach tends to be more accurate than spectral counting due to the cross sample alignment employed which was used to underpin several large-scale mammalian quantitative proteomic studies (Nagaraj et al. 2011; Schwanhausser et al. 2011). This technique has been used in combination with RNA-seq to characterise global gene expression profiles of three different development forms of *T. vivax* where significant differences in gene expression affecting metabolism in the fly and a suite of *T. vivax*-specific genes with predicted cell-surface expression were

observed comparing to other African trypanosomes (Jackson et al. 2015). This approach has also been used to provide quantitative evidence of differential expression profiles of different parasite-derived variant erythrocyte surface antigen (VESA) families in *Babesia bigemina* (Jackson et al. 2014).

To this point, high-throughput discovery proteomic approaches have been discussed which have made no *a priori* assumptions as to the proteins to be analysed. While this is highly valuable in generating assumption-free hypotheses, it has the disadvantage of pushing separation techniques and instrumentation to its limits due to the divergent physiochemical properties of protein species and the dynamic range of the targets. Moreover, it is sometimes unnecessary to employ an extensive and in-depth shotgun discovery experiment to answer a specific biological question. Targeted proteomic approaches are now being developed to enable the MS instrument measurements to be focused only on specific peptides from predetermined sets of selected proteins.

2.1.2.3 Targeted Quantitative Proteomics

Targeted proteomics is gaining popularity and was selected as the 2012 Method of the Year by Nature Methods (Marx 2013). It has the potential for greater accuracy since the instrumentation and bioinformatics can be tuned to a relatively small subset of protein targets, which allows a rapid and accurate quantitative profiling of a repeated set of proteins across samples from different conditions. The techniques are implemented both by triple quadrupole mass spectrometer (QqQ) in the form of selected/multiple reaction monitoring (SRM, MRM) (Bertsch et al. 2010; Lange et al. 2008) and by the widely used Thermo Q Exactive instrument in the form of parallel reaction monitoring (PRM) technology (Peterson et al. 2012). SRM/MRM is typically used to selectively record fragmentation events that are specific for the peptides of interest. A QqQ is used to achieve peptide targeting in SRM/MRM experiments. The first quadrupole is used to isolate precursor ions in a narrow mass range, and the selected ions are then fragmented in the second quadrupole. The third quadrupole is used to specifically detect a set of fragment ions that is characteristic for the target peptides. This sequential isolation of targeted ions enables a great reduction in background noise and makes this approach one of the most sensitive MS strategy available. PRM on the other hand takes advantage of Q Exactive quadrupole-Orbitrap MS which possesses geometry similar to QqQ except the substitution of the third quadrupole with a high resolution and accuracy Orbitrap. Being able to monitor all transitions on targeted peptides, PRM approach effectively minimises the effort required to develop and optimise the traditional SRM/MRM assay and offers experimental flexibility of both high-performance quantitative and high-throughput discovery proteomics (Peterson et al. 2012).

An application of targeted proteomics strategy has been used to obtain the absolute quantity of low abundance proteins in *P. falciparum* crude cell extracts (Southworth et al. 2011). It also helped to quantify four *P. falciparum* proteins in 48 individual patients where PfHPRT is proposed to be a promising biomarker to diagnose *P. falciparum* malaria infection (Thezenas et al. 2013). Using MRM, a rapid and simple assay to quantify a new antimalarial compound (TK900D) from small

volume (20 µl) whole blood was developed, which makes it readily suitable for the analysis of large sample batches (Abay et al. 2014). Along with the instrument advancement and the increasing availability of public databases for tandem MS spectra and validated SRM/MRM assays (Farrah et al. 2012; Perez-Riverol et al. 2015), targeted proteomics will inevitably gain more popularity in the proteomic field. Its advantages over discovery proteomics in terms of sensitivity, accuracy and reproducibility would be harnessed to enhance various hypothesis-driven tasks in parasitology research in the coming years.

2.1.2.4 Transcriptomics and Proteomics in Parasite Systems

The gene expression process can be simply summarised using the classical central dogma of gene-Transcription-Translation. However, many levels of transcriptional and translational control and regulation events during this process introduce variables to the system where a simple one-to-one expression is not achieved. Early comparisons between transcriptomic data and proteomic data have generally indicated a weak correlation (de Sousa Abreu et al. 2009; Maier et al. 2009). The same phenomenon has also been observed in protozoan parasites and has been summarised in reviews of model systems such as *Plasmodium* (Kooij et al. 2006) and other apicomplexan parasites (Wastling et al. 2009). These studies highlighted the discrepancies of mRNA and protein expression and the important involvement of the regulation of expression, which is likely to involve biological explanations such as selective protein degradation and variations in protein turnover rates (Doherty et al. 2009; Yen et al. 2008) as well as post-translational regulations such as mRNA decay (Shock et al. 2007) and translational repression (Hakimi and Deitsch 2007).

In addition to the biological reasons, technical factors have also contributed to the discrepancies observed between transcriptome and proteome. Firstly, the lack of simultaneously collected samples for both, proteomics and transcriptomics, analyses has contributed to the discrepancy observed. Studies carried out by Schwanhäusser et al. on mouse NIH3T3 cells (Schwanhausser et al. 2011) and Nagaraj et al. on human HeLa cells (Nagaraj et al. 2011) are among the first large-scale comparisons between RNA-seq data and intensity-based quantitative proteomic data from simultaneously collected samples. However, the reported correlation coefficient remains rather poor being between 0.41 and 0.6 (Spearman) (Nagaraj et al. 2011; Schwanhausser et al. 2011).

Secondly, the lack of advanced statistical models to combat the inherent errors in each high-throughput transcriptome and proteome platforms has also been highlighted by a recent review from Li et al. (2015). Various approaches have been proposed to rectify measurement errors incurred. For example, Li et al. used classic data from the literature to correct the nonlinear scaling error in protein abundance estimations, and a subset of the other errors in RNA-seq and protein abundance data was estimated from replica and other control data (Li et al. 2014). In a recent study on lipopolysaccharide-stimulated mouse dendritic cells, Jovanovic et al. used a Bayesian model to estimate the true rates of translation and protein degradation from noisy mass spectrometry data as well as estimates of stochastic mRNA-seq errors. The results revealed that newly activated cellular functions are mainly

regulated by transcriptional induction while the pre-existing proteome that performs basic cellular functions is remodelled by protein production or degradation (Jovanovic et al. 2015). This interesting finding on functional-based gene expression regulation would further advance our understanding of various host-pathogen interaction models in protozoan parasites.

2.1.3 Post-translational Modifications

Proteins can undergo a great range of modifications after translation. These post-translational modifications (PTMs) can determine the localisation, activity state, turnover, structure of a protein, as well as its interactions with other proteins, cells or organisms. Understanding the roles of these PTMs in parasite regulation, survival and pathogenesis as well as their contribution to the adaptation and evolution of the host require both highly sensitive and precise detection and reliable high-throughput methodologies to quantify protein changes in a complex mixture.

The low dynamic range, stability and sometimes transient changes of protein modifications combined with attempting to relate these modifications to biological events create a challenge to modern technologies. There are many proteomic approaches to studying PTMs, ranging from bottom-up and top-down mass spectrometry, gel and gel-free techniques to affinity-based methodologies. Nowadays, many PTMs such as phosphorylation, ubiquitination, glycosylation and acetylation can be efficiently identified and confidently localised in a high-throughput fashion using proteomic approach (Olsen and Mann 2013).

Classically, gel-based techniques paired with mass spectrometry have been used to highlight PTMs. Two-dimensional electrophoresis separates proteins by their charge and molecular weight. The resolving power of this technique can separate differentially expressed modified forms of a given protein. Further selectively in the detection of specific PTMs by using certain stains, metabolic labelling, antibodies or specific probes can aid in detection and identification of PTMs and have been used to identify phosphorylated proteins in erythrocytes infected by the human malaria parasite *P. falciparum* (Wu et al. 2009). Fluorescent or colorimetric stains for gels or Western blots (e.g. Pro-Q Diamond stain, Invitrogen) allow simple selective detection of phosphoproteins (Nunes et al. 2010). A more sensitive technique for phosphoprotein detection is the radiolabelling of proteins by ^{32}P incorporation (Leykauf et al. 2010) or immunoblotting (Wu et al. 2009). Glycosylation can also be detected using specific stains such as Pro-Q Emerald (Invitrogen)-conjugated lectins or differential glycosidase digestion (Rebello et al. 2011). Changes in the relative abundance and phosphorylation of protein components of the invasion motor complex during host cell invasion by the apicomplexan parasite *Toxoplasma* were also monitored by SILAC-based quantitative proteomics (Nebl et al. 2011).

The scarcity of many PTMs requires the enrichment of the subpopulation of select modified proteins. Affinity-based enrichment can be performed at the protein or peptide level and targets specific or groups of PTMs. Immobilised metal ion affinity chromatography (IMAC) utilises the affinity of chelated Fe(III) or Ga(III)

ions to the phosphate group of phosphopeptides. Crude protein mixtures from *Leishmania donovani* extracts were enriched for phosphoproteins using IMAC then digested with trypsin and analysed for life stage-specific phosphoprotein abundance (Hem et al. 2010). Metal oxide such as TiO_2 was popularly used to selectively isolate phosphoproteins. It was used to identify 491 phosphoproteins in bloodstream form *T. brucei*, among which the conserved phosphorylation sites detected in 44 eukaryotic protein kinases revealed that phosphorylation-based signalling is a general and fundamental regulatory process that extends to this highly divergent lower eukaryote (Nett et al. 2009). Coupled with SILAC-based quantitation, it was used to compare phosphoproteomes between *P. falciparum* and *T. gondii*, which revealed the prevalence of protein phosphorylation within and beyond parasite boundaries and unusual phosphorylation motifs possessed by *P. falciparum* (Treeck et al. 2011). The same enrichment strategy was also used to identify phosphoproteome of *Trichomonas vaginalis* (Yeh et al. 2013) and *Theileria annulata* (Wiens et al. 2014), where stage-specific phosphorylated proteins and cell cycle-dependent surface proteins were characterised, respectively. Specific antibodies can also act to isolate other PTMs, for example, the global analysis of acetylation, methylation and nitration of peptides. Carbohydrate binding proteins (lectins) are used to enrich glycoproteins and glycopeptides using affinity chromatography. Affinity resins that bind polyubiquitin protein conjugates are commercially available.

PTMs can also be specifically targeted by chemical derivatisation. Affinity tags can be introduced by beta elimination of phosphoric acid from pSER or pThr followed by the addition of affinity groups such as biotin to allow enrichment of phosphoproteins by chromatography. Solid-phase extraction of glycopeptides can be achieved by immobilisation of carbohydrates to a hydrazide-activated resin followed by release by PNGase F and analysis with LC-MS/MS. Hydrophilic interaction liquid chromatography (HILIC)-based methods can also be used to isolate glycopeptides. Recently hexapeptide libraries have been applied to large-scale glycomic analysis (Huhn et al. 2011), and arrays have been used to profile glycans (Lepenies and Seeberger 2010; Lonardi et al. 2010). In fact, glycomics, which studies glycoconjugates such as glycoproteins and glycolipids, is such a rapidly growing field in protozoan parasite research that is extensively reviewed in the next chapter.

Among over 200 known in vivo modifications and many other chemically induced modifications, only a handful of them, such as phosphorylation, ubiquitination, glycosylation and acetylation, are well studied and supported by established high-throughput workflows. There are many other important modifications that remain to be exploited. For example, covalent PTMs of cysteine are mediators of redox regulation and signalling, which influence protein structure and stability when involved in disulphide bonds. S-nitrosylation, s-glutathionylation, palmitoylation and prenylation are all PTMs of cysteine found in parasites (Jortzik et al. 2011) that warrant further characterisations. On the instrument side, top-down proteomics, although currently restricted by the throughput and detection of higher molecular weight proteins, has also shown promising potentials in recent years with its ability to measure differences in the expression of the constellation of unique proteoforms of intact proteins (Tran et al. 2011). With ever growing arsenal of separation and enrichment

protocols as well as statistically rigorous bioinformatic implementations on identification, site localisation and functional interpretation, the future of PTM research in the protozoan parasitology field would be much more prosperous.

2.1.4 Protein-Protein Interactions

Most cellular processes are governed by protein-protein interactions (PPIs). These can range from the interaction of two proteins to the formation of large macromolecular complexes consisting of many different proteins in differing ratios. Interactions can be strong or transient. In parasitology research, it can be used to understand binding partners between different parasite proteins that form a functional complex as well as to characterise interface of host-parasite interactions.

There are several methods for experimentally determining protein-protein interactions. The two most widely used are the yeast two-hybrid system and affinity purification followed by MS methods (AP/MS). The yeast two-hybrid system measures the interaction of a bait protein which is fused to the DNA-binding domain of the yeast protein Gal4 and the prey protein, which is fused to the transactivation domain of Gal4. When the bait and prey interact, a downstream reporter gene is activated (Uetz et al. 2000). AP/MS offers advantages over other methods under near-physiological conditions in identifying PPIs by identifying protein complexes instead of binary interactions. By performing AP of a bait protein followed by LC-MS/MS identification and quantification, the partner proteins that form complexes with the bait can be identified (Altelaar et al. 2013).

A variation of the yeast two-hybrid system has been used to investigate interaction networks in *P. falciparum* (LaCount et al. 2005), identifying 2846 unique interactions involving 1312 proteins and highlighting a group of interacting proteins involved with host cell invasion, including 19 uncharacterised proteins. Fleckenstein et al. prepared Glutathione Sepharose 4B beads loaded with glutathione S-transferase (GST)-Irga6 to find interactions between Irga6 and *T. gondii*-derived proteins and identified ROP5 proteins act as essential cofactors for virulence factor ROP18 by assisting its binding to the host immunity-related GTPases (IRG) proteins (Fleckenstein et al. 2012). Protein G magnetic beads immobilised with Fc-RON4-8His recombinant proteins were used to investigate host cellular proteins that interact with parasite rhoptry neck protein 4 (RON4) in *T. gondii* and *P. falciparum* and demonstrated a parasite-specific interaction between TgRON4 and host cellular β-tubulin (Takemae et al. 2013). To study the redox interactome of *T. cruzi*, Dynabeads® His-Tag Isolation and Pulldown (Invitrogen) was used to tag a mutated form of tryparedoxin 1 (TcTXN1), where 15 binding partners were identified that are involved in oxidative metabolism and protein synthesis and degradation (Pineyro et al. 2011).

Biological processes rely on the combined activities of proteins to form an interconnected network. Interactome studies characterising PPIs as well as interactions between protein and other molecules form the building blocks of our understanding in the complex biological system, where genome-scale molecular networks can be mapped and dynamic models can be generated.

2.1.5 Bioinformatic Resources for Protozoan Parasite Proteomics

There are two major components in any proteomic experiment. One is the utilisation of the MS instrumentation itself and second, but equally as crucial, is the successful bioinformatic processing of the large quantity of data generated. The involvement of various bioinformatic tasks in processing and interpreting proteomic data is summarised in Fig. 2.2. Software packages fulfilling the identification and quantification purposes have been reviewed in the previous sections. The scale and complexity of the data generated by such a workflow require the development of integrated database pipelines, which ensure that these data are stored and rendered accessible for easy use by the community. Subsequently, the focus is then on downstream interpretation in relation to protein function and localisation prediction, pathway and network analysis, since these are the aspects of bioinformatics which have the potential to turn an elegant data-gathering exercise into one which can reveal genuine insights into function. The following sections will briefly introduce examples of bioinformatic solutions for these downstream analyses.

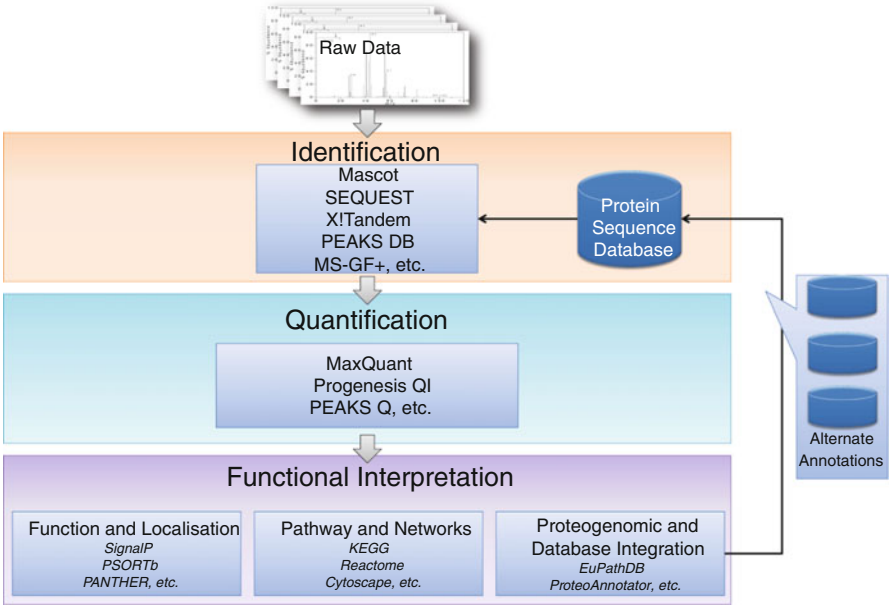

Fig. 2.2 **A bioinformatics pipeline for large-scale proteomics data processing, querying and interpretation**. The involvement of various bioinformatics tasks in processing and interpreting proteomics data is summarised. Raw mass spectra data collected from an experimental pipeline are subjected to proteomics identification packages. The results are then analysed by appropriate quantification packages where relative or absolute quantifications of the identified proteins are calculated. Protein function and localisation prediction and pathway and network analysis tools have been developed to infer the biological meaning of the identification and quantification data. Proteogenomic and database integration pipelines are available to facilitate data integration with online databases and improve genome annotation using alternate annotations.

2.1.5.1 Data Repositories for Parasite Proteomic Data

Several public repositories host proteomic data for the research communities, such as the proteomics identifications database (PRIDE) (Jones et al. 2006), the Global Proteome Machine Databases (GPMDB) (Craig et al. 2004) and PeptideAtlas (Desiere et al. 2006). More recently, ProteomeXchange consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al. 2013) has made considerable effort to provide a user-friendly interface and long-term stability to proteomic data repository.

While these databases are useful for storage and re-querying of proteomic data generated, the integration of proteomic data with organism-specific genomic and proteomic resources provides an essential technical step to data interpretation (Xia et al. 2008). For protozoan parasites, the most advanced example of this is the hosting of proteomic data in EuPathDB (Aurrecoechea et al. 2010). The easily accessible format on EuPathDB has opened-up proteomic identification and quantitation data on both host and parasite to the entire research community in a way that was difficult to envisage.

EuPathDB acts as a portal to eukaryotic pathogens (Aurrecoechea et al. 2010). It is an integrated genome database composed of a family of dedicated pathogen databases including PlasmoDB, ToxoDB (also serves *Neospora caninum*), CryptoDB, GiardiaDB, TrichDB, TriTrypDB, PiroplasmaDB, AmoebaDB, FungiDB, MicrosporidiaDB and an ortholog database, OrthoMCL. More detailed introductions to these databases have been published through online tutorials, individual websites and journal publications (Aslett et al. 2010; Aurrecoechea et al. 2009a, b, 2010; Gajria et al. 2008; Heiges et al. 2006).

Proteomic data of *T. gondii* and *C. parvum* pioneered the full integration of proteomic data into EuPathDB (Sanderson et al. 2008; Xia et al. 2008). The latest version of EuPathDB (Release 26) hosts identification and quantification proteomic data for 55,307 proteins from 21 parasite strains and species (Table 2.1). In addition to advance querying and downloading tools for proteomic data, visualisation and analysis tools such as Generic Genome Browser (GBrowse) (Stein et al. 2002) have been incorporated to facilitate the browsing, functional prediction and comparison of proteomic data with other types of genomic data.

2.1.5.2 Post-identification Bioinformatic Analysis

Once the protein identification and quantification has been determined, signature-based resources can be used to infer function and subcellular localisations where one or more protein signatures can be identified. Protein signatures are defined by either a regular expression method that shows patterns of conserved amino acid residues (Sigrist et al. 2002) or the Hidden Markov Model (HMM) method which provides a statistical profile based on probabilities of finding an amino acid at a given position in the sequence (Krogh et al. 1994). There are many publicly available signature databases of protein families and domains, including both sequence-based and structure-based databases. Sequence-based databases include PROSITE, an ExPASy database for protein domains, families and functional sites (Sigrist et al. 2002); Pfam, a collection of protein families (Finn et al. 2008); PRINTS, a compendium of protein fingerprints (a group of conserved motifs) (Attwood et al.

2003); and PANTHER, an online resource for comprehensive protein evolutionary and functional classification (Mi et al. 2007). Structure-based databases include websites such as SUPERFAMILY, a database of protein structural and functional annotations (Oates et al. 2015; Wilson et al. 2007), and Gene3D, a comprehensive database of protein domain assignments for sequences from the major sequence databases (Lees et al. 2012). Protein signatures can be used in combination to predict protein functions. For example, proteins with no significant sequence similarity but which have similar functions might be expected to share some common features like post-translational modifications, protein-sorting signals and similar subcellular localisations. In parasitology research, the identification of signal peptide and transmembrane domains is of special interests. The entry of virtually all proteins into the secretory pathway is controlled by signal peptides (Gierasch 1989; von Heijne 1990), and transmembrane proteins support essential biological functions acting as receptors, transporters or channels, which are essential in host-parasite interactions (Baxt et al. 2008; Dowse and Soldati 2005; O'Donnell et al. 2006).

Universal software packages were developed to predict certain protein features based on a set of trained rules, such as SignalP for signal peptide prediction (Bendtsen et al. 2004), TMHMM for transmembrane domain prediction (Krogh et al. 2001) and PSORTb for general subcellular localisation prediction (Yu et al. 2010). Additional organism-specific prediction tools and databases were also developed to predict important features in the organism under study, with more targeted training data, for example, PSEApred (Verma et al. 2008), PlasMit (Bender et al. 2003) and ApiLoc (http://apiloc.biochem.unimelb.edu.au/apiloc/apiloc) for apicomplexan parasites.

The analysis of individual protein products can be further integrated by the interface of pathways and networks. KEGG (Kanehisa and Goto 2000), MetaCyc (Caspi et al. 2014), Reactome (Croft et al. 2011), DAVID (Jiao et al. 2012) and Cytoscape (Shannon et al. 2003) are such tools developed to facilitate pathway browsing and data analysis.

2.1.5.3 Proteogenomic-Assisted Genome Annotation

While 'bottom-up' proteomic identification most commonly depends on the availability of annotated genomes, the large amount of experimental MS spectra generated along the process has fuelled an emerging area of application, proteogenomics, whereas proteomic data is used to validate and improve gene models. Due to the limitation of comparative genomic alignment and *ab initio* methods used, genome annotations on non-model organisms are often plagued with incorrect models and missing regions despite the availability of high-quality genomic sequence data. Even with iterations of high-quality curation effort made to genome models in *T. gondii*, several proteomic studies have highlighted genomic regions where official gene models remain inaccurate (Che et al. 2011; Dybas et al. 2008; Xia et al. 2008). A similar approach has also been applied to the study of *Leishmania major*, where novel genes and correct N-terminal positions of existing genes were identified (Pawar et al. 2014). Using ProteoAnnotator (Ghali et al. 2014), Krishna et al. has further refined the proteogenomic approach in *T. gondii* and *N. caninum* by

harmonising proteomic data sets with next-generation sequencing data, leading to the identification of over 400 potential loci that are absent from current official annotation (Krishna et al. 2015). These include cases such as wrong start site predicted, truncated gene models, alternative splicing events and novel protein-coding regions, which substantially improved gene models for these organisms (Krishna et al. 2015). The identification of alternative slicing events in the study has also released the potential to exploit proteoforms of parasite proteins that possess variable molecular forms in reflection of various functional requirements (Smith et al. 2013).

In light of the importance of incorporating experimental data from both proteomics and RNA-seq studies into routine genome annotation protocols, the tools and results developed in that study have been installed locally at the EuPathDB database to be used as part of a standard build pipeline for submitting proteomic data to different subsites (Krishna et al. 2015). On the other hand, with increasingly easy access to next-generation sequencing and proteomic platforms, a combination of proteogenomic pipeline and novel RNA-seq *de novo* assembly tools such as Trinity (Haas et al. 2013), STM (Surget-Groba and Montoya-Burgos 2010) and Trans-ABySS (Robertson et al. 2010) platforms would release proteomic analysis of novel parasite species from the usual waiting for the availability of high-quality genome annotations. In fact, a recently published software system based on Galaxy platform (Goecks et al. 2010) has made this proteomics informed by transcriptomics (PIT) approach into a user-friendly, single software pipeline (Fan et al. 2015).

Conclusion

Proteomic research is a rapidly evolving field; as the number and type of MS-based applications expands, new technology will be developed to meet the growing needs to further our understanding of ever-changing proteomes of host-parasite interaction systems. For the foreseeable future, peptide-based 'bottom-up' approaches will continue to dominate the diverse applications in proteomic research from basic high-throughput identification to accurate quantification of various proteoforms and PPIs. With the maturation of 'top-down' approaches, protein level characterisation that measures changes of the proteins themselves rather than using a select few of their constituent peptides would become more popular. Realising the full potential of any MS-based proteomic platforms relies on advance bioinformatic support. With the increasing amount of knowledge and resources accumulated by the research community, advanced statistical, prediction and integration algorithms are continuously developed to better utilising the data acquired from precise biological samples.

Just as mRNA analysis cannot explain everything about the function of genes, proteins too cannot be understood entirely in isolation. Biological events are the result of the concerted action of individual system components such as the genome, transcriptome, proteome and glycome. Supported by the increasing volume of data sets hosted on dedicated open access databases such as EuPathDB, the goal of realising system level modelling of pathways and networks in these

unique and fascinating host-parasite interaction systems in protozoan parasites will be the focus of future research in the coming years.

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Glycomics

3

Iain B.H. Wilson

Abstract

The carbohydrate-containing molecules of protozoan parasites have been studied for some 40 or so years, and the results of the many studies indicate that these glycoconjugates display a high degree of species specificity. Nevertheless, a number of major classes of glycoconjugate can be distinguished – whether the glycans be N, O or P linked to protein or are lipid linked. Although modern glycomic analyses have enabled advances in our knowledge, many questions remain regarding structure, biosynthesis and function. In many cases, roles in virulence can be proposed, and the potential for glycans to be important for diagnosis or therapy is significant.

3.1 Introductory Comments

The surfaces of all cells display a range of glycoconjugates, ranging from simple glycolipids, glycoproteins with N- and O-linked oligosaccharides and/or glycolipid anchors through highly heterogeneous polysaccharides (Varki 2011). Protozoan parasites are no exception; indeed, these organisms express a wide range of carbohydrates, many of which are unique to certain species or show divergence from those from more familiar organisms (Guha-Niyogi et al. 2001). Furthermore, interactions of parasites with hosts and vectors are dependent on glycans with either the parasite expressing a glycoconjugate recognised by the host or vector or vice versa. By comparison to bacterial pathogens and metazoan parasites, it is not unlikely that protozoan glycoconjugates are involved in immunoregulation or are potential

I.B.H. Wilson
Department of Chemistry, University of Natural Resources and Life Sciences (BOKU), Muthgasse 18, A-1190 Vienna, Austria
e-mail: iain.wilson@boku.ac.at

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vaccine candidates; however, the 'non-template-driven' nature of glycan biosynthesis and the branched nature of many oligosaccharides mean that they are challenging molecules to study in terms of their molecular structure and biological function.

3.2 Major Glycomic Techniques

The fact that a typical monosaccharide can be attached through any of four free hydroxyl groups to another monosaccharide means that glycan structures have an inherent structural complexity quite unlike that of proteins and nucleic acids; this means that glycan analysis is much more than the 'reading off' of series of constituent components of a linear polymer; not only is the 'connectivity' (which type of monosaccharide or other modifications is next in the row) to be considered but also the position of the modification around the sugar ring, the potential branching of the glycopolymer and the chirality of the linkages. This means a strategy analogous to Sanger or Edman sequencing as for DNA or peptides is not appropriate. Furthermore, a glycan is generally part of a glycoconjugate; very often the glycan part is analysed in isolation and thus must be released from the underlying lipid or protein by enzymatic or chemical means.

Over the years, a number of ways of determining glycan structures, with greater or lesser degrees of detail, have been introduced and applied (Geyer and Geyer 2006). A traditional approach was to radiolabel the glycoconjugates, either metabolically or post release from the underlying peptide or lipid, and then perform anionic exchange, gel filtration or paper chromatographic analyses before and after enzymatic or chemical treatments. However, the definition of the actual structure is difficult to discern; thus, in older studies, if sufficient quantities were available, gas chromatography in conjunction with mass spectrometry (GC-MS) and/or nuclear magnetic resonance (NMR) studies were performed, but these require special expertise in terms of carrying out the experiments and interpreting the results, especially when standards were not available for comparison.

In terms of chromatography, simple systems have been replaced by HPLC and, most recently, UPLC: in general, fluorescent labelling of one end of the glycan (the so-called reducing end) has superseded radioactive labelling, and then a variety of columns containing reversed-phase (RP), graphitised carbon, anion exchange, normal-phase (NP) or hydrophilic interaction (HILIC) resins can be used in combination with appropriate enzymatic or chemical treatments. An alternative, less used now, is to perform analyses of glycans at high pH; this results in deprotonation also of the hydroxyl side chains and facilitates the use of anion exchange in conjunction with amperometric detection (HPAEC-PAD). However, HPLC retention times alone cannot define a glycan structure and for 'unknown' structures; the lack of standards means conclusions from such analyses have to be considered with caution.

In the past couple of decades, advances in the types and the sensitivity of mass spectrometer have contributed very much to the analyses of glycans and glycoconjugates (Alley and Novotny 2013; Haslam et al. 2001). First, fast atom

bombardment mass spectrometers (FAB-MS) were used to analyse glycopolymers of various types and from various sources. The glycans, though, had to be chemically modified so that all hydroxyl groups were present in methylated or acetylated forms (hence, permethylated or peracetylated). Then matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry was developed (MALDI-TOF MS) in which either native or permethylated glycans could be analysed by 'firing' a laser at a sample dried with a matrix; also, various electrospray methods (ESI-MS, either with injection or in combination with liquid chromatography) with different types of detector (e.g. time of flight or ion trap) currently occupy an important place in glycomics. Particularly the ability to fragment glycans is an important aspect in MS-based analyses, but the preparation and separation methodologies often are key to obtaining the best results. Thus, LC-MS in various forms ('online' using electrospray-type techniques or 'offline' with MALDI-TOF-MS) is normally required, as glycans of low abundance or of poor ionising ability are otherwise suppressed if only examining a whole glycome; also, chemical and enzymatic treatments followed by reanalysis can maximise the ability to make clear conclusions from the obtained data. Therefore, in my own laboratory, a combination of enzymatic release, fluorescent labelling, HPLC, chemical and enzymatic digestions and MALDI-TOF-MS is used.

However, the glycan itself is not the whole molecule, and so glycoproteomics and glycolipidomics which consider both parts of the glycoconjugate are important, yet challenging, techniques. Here, again, mass spectrometry is the key modern technique (Thaysen-Andersen and Packer 2014). Thereby, not just the type of glycan structure but its attachment to the polypeptide or the nature of the lipid moiety can be examined. However, as glycoproteins and glycolipids tend to be complex mixtures of different molecular species, the range of glycoforms can be immense, even if the peptide or the lipid portion does not change. This so-called microheterogeneity is a result of the non-template-driven nature of glycan modifications, particularly those occurring during the passage of a glycoconjugate through the Golgi apparatus (Schachter 1986).

For simple screens, very often lectins and anti-carbohydrate antibodies can be used in blotting experiments (Iskratsch et al. 2009); however, the specificity of such reagents can sometimes be low and so misleading conclusions are often made. Nevertheless, the range of specificities of lectins and antisera is of interest, particularly as in animals, as both can be key to immune responses. Thus, the definition of lectin and antibody specificities is of continued interest, and new array-based methods can yield important clues as to which glycans are bound by which proteins (Cummings and Pierce 2014). Such arrays need, though, an appropriate set of host and parasite glycans in order to make biologically relevant conclusions; unfortunately, currently available arrays have a poor selection of parasite glycans, and so the synthesis or isolation of such carbohydrates is required. Another problem for parasite glycomics is that care must be taken to consider the medium or organism in which they were cultivated as nonparasite glycans (e.g. from foetal calf serum) may also be present in the sample to be analysed; otherwise misleading results can be obtained which are then remaining in the literature.

3.3 State of the Art

3.3.1 General Principles of Glycosylation in Eukaryotes

Complex carbohydrates come in a variety of forms; in general, they consist of a set of different monosaccharide units, linked together in linear or branched forms, often covalently attached to some other biological macromolecules such as a lipid or protein. In the latter case, the carbohydrate (oligosaccharide) chain is classified on the basis of the type of linkage between the oligosaccharide and the polypeptide: N-, O- and C-linked glycans are known (see Table 3.1), in which the linkage is via nitrogen (of asparagine), oxygen (of an hydroxyamino acid such as serine, threonine or hydroxyproline) or carbon (of tryptophan) atom (Spiro 2002). Further subclassifications of the N- and O-linked glycans have been defined, primarily based on glycan types known from mammals. Thus, N-glycans may be oligomannosidic, complex or hybrid; O-glycans vary depending on the connecting sugar (e.g. *N*-acetylgalactosamine for mucin-type glycans or xylose for chondroitin or heparin sulphates). The biosynthesis of these glycan types also differs: whereas N-glycans are formed from pre-synthesised oligosaccharide precursors transferred to newly synthesised proteins, O-glycans result from a stepwise addition of individual monosaccharide units. Another glyco-modification of proteins is represented by the glycosylphosphatidylinositol (GPI) anchors in which a glycan acts as a bridge between the C-terminus of the protein and a lipid and so enables the anchoring of a protein in the outer leaflet of the plasma membrane, acting instead of a typical transmembrane domain (Ferguson et al. 1994).

The classical N-glycosylation pathway, familiar from higher organisms (in general more complicated than that in protozoans), begins with the assembly of a heptasaccharide, consisting of two *N*-acetylglucosamines and five mannose residues ($Man_5GlcNAc_2$), linked to a long-chain polyisoprenol (dolichol) on the cytosolic face of the endoplasmic reticulum; this dolichol-linked glycan is then 'flipped' through the membrane to then present the glycan chain to the glycosyltransferases on the luminal face of the endoplasmic reticulum.

The final result of this process is the $Glc_3Man_9GlcNAc_2$ tetradecasaccharide (see Fig. 3.1). A set of asparagine-linked glycosylation (*alg*) gene products using either nucleotide sugar or lipid sugar donors is responsible for the formation of this glycan, which is then transferred by a so-called oligosaccharyltransferase (OST), to

Table 3.1 Overview of types of protein-linked glycans

N-glycans	Linkage of GlcNAc to the amide of asparagine (N) side chains
O-glycans	Linkage of various monosaccharides (e.g. GalNAc, GlcNAc, Fuc, Xyl) via hydroxyl groups of, e.g. serine or threonine
C-glycans	Linkage of Man to tryptophan
P-glycans	Linkage of glycans via phosphodiesters of hydroxyamino acids
GPI anchors	Linkage of glycosylphosphatidylinositols to protein C-termini

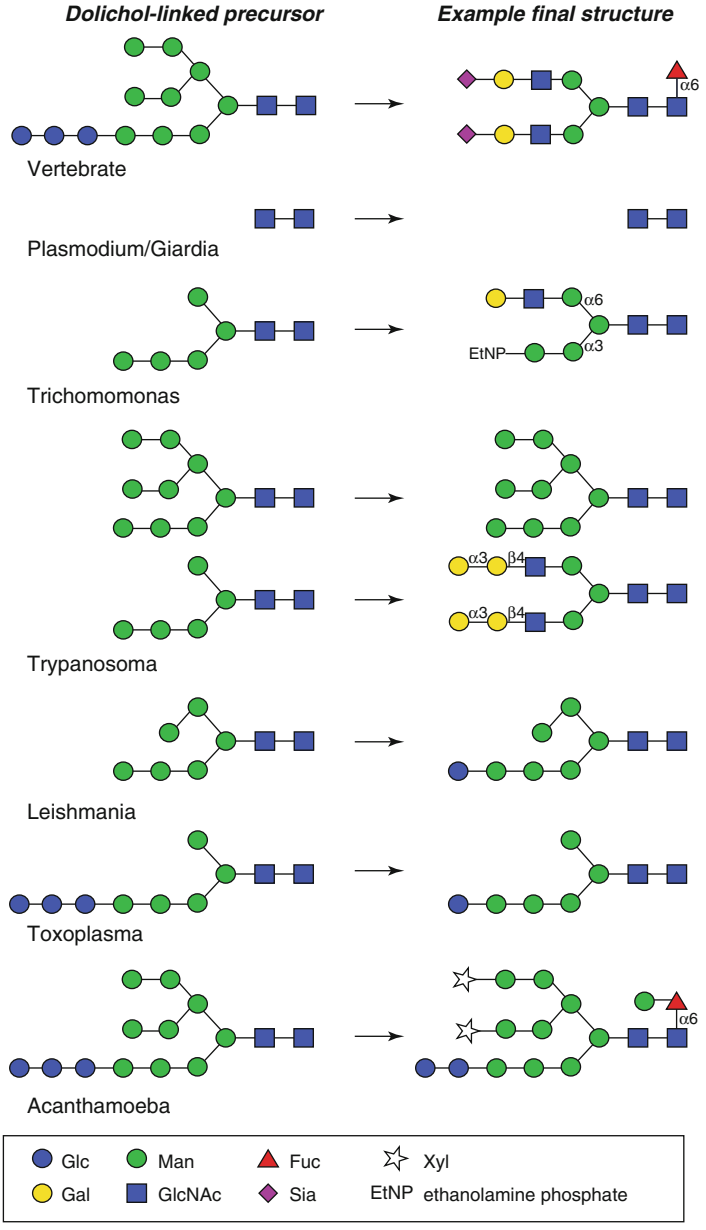

Fig. 3.1 Example N-glycan precursors and final structures in protists. On the *left*, the dolichol-linked precursors typical for the species and, on the *right*, one possible final protein-linked structure depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics (*circles* hexoses, *squares N*-acetylhexosamines, *triangles* deoxyhexoses, *diamonds* acidic monosaccharides, *stars* pentoses)

newly synthesised proteins inside the endoplasmic reticulum (Aebi 2013). However, rarely is $Glc_3Man_9GlcNAc_2$ found on isolated glycoproteins; a process of trimming down and building up of the N-glycan chain takes place on the glycoprotein's route from the endoplasmic reticulum and Golgi apparatus to the plasma membrane or extracellular space. This highly variable set of trimming and extension reactions is directed by the accessibility of the glycan on the glycoprotein surface to the enzymes along the secretory pathway. Indeed, glycosylation affects, and is affected by, protein folding.

Other factors in glycan processing are the levels of the relevant enzymes (glycosidases which trim and glycosyltransferases which add monosaccharide units), which are the result of varying rates of transcription, translation and trafficking (all of these being dependent on the species, cell type or stage of development). A further variable is the concentration within the Golgi lumen of the relevant activated nucleotide sugars. Therefore, the final 'shape' of the glycan is the result of a highly dynamic process, which is only partially determined by the genetic code. Indeed, a typical N-glycoprotein is a mixed population of individual 'glycoforms', each of which is a polypeptide 'decorated' by one specific glycan structure at a particular glycosylation site – thus, a high degree of heterogeneity of even 'pure' glycoproteins results. N-glycosylation to asparagine residues, in context of Asn-Xaa-Ser/Thr 'sequons', is a process not only found in almost all eukaryotes (including most protozoans) but also found in some bacteria, including *Campylobacter* (Abu-Qarn et al. 2008). A complication is, though, that parasitic protists tend not to have the full complement of *alg* genes and so have truncated dolichol-linked precursors as compared to multicellular organisms (Samuelson et al. 2005; Samuelson and Robbins 2014).

In the case of O-glycosylation, there are a wide variety of O-glycan-initiating enzymes, often operating in the lumen of the Golgi apparatus, including the mucin-type glycans with GalNAc, *O*-mannose on dystroglycan or the glycosaminoglycans with xylose as the linking sugar (Wopereis et al. 2006); there are also forms of O-glycosylation found in the cytoplasm, e.g. O-linked *N*-acetylglucosamine as found on serine and threonine residues as a modification of nuclear and cytoplasmic proteins (Hart et al. 2007) such as histones and RNA polymerase II.

3.3.2 Apicomplexan and Diplomonad Glycans

Amongst the simplest glycomes of any eukaryotes are those of apicomplexans (such as *Plasmodium* and *Toxoplasma*) and diplomonads (exemplified by *Giardia*). Indeed, in the case of *Plasmodium* and *Giardia*, there were contradictory reports as to the extent and the structures of N-glycans. However, the latest analytical and genomic data indicate that both these organisms are capable of transferring a precursor consisting of just one or two *N*-acetylglucosamine residues to certain asparagines of their proteins (Bushkin et al. 2010; Ratner et al. 2008); a report that *Giardia* possessed mammalian-type complex glycans was the result of analysis of residual foetal calf serum carried over into the parasite preparation (Robbins and Samuelson

2005). This highly vestigial N-glycosylation capacity is due to the lack of the vast majority (12 of 14) of the *alg* genes during the evolution of these parasites (Samuelson et al. 2005); even more extreme is *Theileria*, which completely lacks the capacity to N-glycosylate its proteins. In contrast, *Toxoplasma* is an organism which not only synthesises its own $Glc_3Man_5GlcNAc_2$ precursors for N-glycosylate but also 'steals' $Glc_3Man_9GlcNAc_2$ from the mammalian cells in which it resides (Garénaux et al. 2008). However, there is little in the way of further modification of these N-glycans, even in *Toxoplasma.* Regarding O-glycans, peptide-modifying *N*-acetylgalactosaminyltransferases and *N*-acetylglucosaminyltransferases have been described from *Toxoplasma*, *Cryptosporidium* and *Giardia,* but not from *Plasmodium* (Bhat et al. 2013; Stwora-Wojczyk et al. 2004; Banerjee et al. 2009); C-mannosylation and O-fucosylation, on the other hand, are respectively predicted to occur in *Toxoplasma* and *Plasmodium* (Buettner et al. 2013; Sanz et al. 2013).

Of other glycoconjugate types, various glycolipids are also known in apicomplexans. These include the GPI-related protein-free glycerophosphatidylinositols in *Babesia*; indeed this parasite lacks one of the standard GPI biosynthesis genes, and so its GPI consists only of inositol, glucosamine and two mannose residues (Rodriguez et al. 2010). In contrast, *Plasmodium* GPIs (whether free or as protein anchors; see Fig. 3.2) contain three or four mannose residues (Gowda et al. 1997); these are considered also to be 'toxic' and have pro-inflammatory activity, and a full set of GPI biosynthesis genes has been identified (Delorenzi et al. 2002). Over a decade ago, a chemically synthesised GPI was tested as a potential vaccine in a rodent model; however, despite amelioration of symptoms, the parasites were not eliminated (Schofield et al. 2002). On the other hand, immobilised synthetic GPIs may be suitable for serodiagnosis of *Plasmodium* and *Toxoplasma* (Kamena et al. 2008; Götze et al. 2014).

3.3.3 Trichomonad Glycans

Like *Toxoplasma*, the N-glycosylation potential of various trichomonads is based on synthesis by the parasite of a $Man_5GlcNAc_2$ precursor (Samuelson et al. 2005). This means that after flipping across the endoplasmic reticulum membrane, there is no further elongation of the precursor prior to transfer to protein. However, once present on the protein, this glycan can be modified by transferases in the secretory pathway. First, a glucose residue may be transferred, a process which may be associated with chaperone-dependent protein folding (Banerjee et al. 2007); then, as revealed by data from my laboratory, the $Man_5GlcNAc_2$ may be processed in *Trichomonas vaginalis* to contain further xylose, *N*-acetylglucosamine, galactose, phosphate or phosphoethanolamine groups in a strain-dependent manner (Paschinger et al. 2012). The ability of *T. vaginalis* to synthesise xylose and galactose has been proven by the activity of relevant recombinant UDP-sugar nucleotide synthases/epimerases (Rosenberger et al. 2012). Other modifications may be present in *Tritrichomonas foetus* and *Trichomonas gallinae* (unpublished data). A protein O-glycosylation activity has also been found (Grabinská et al. 2008).

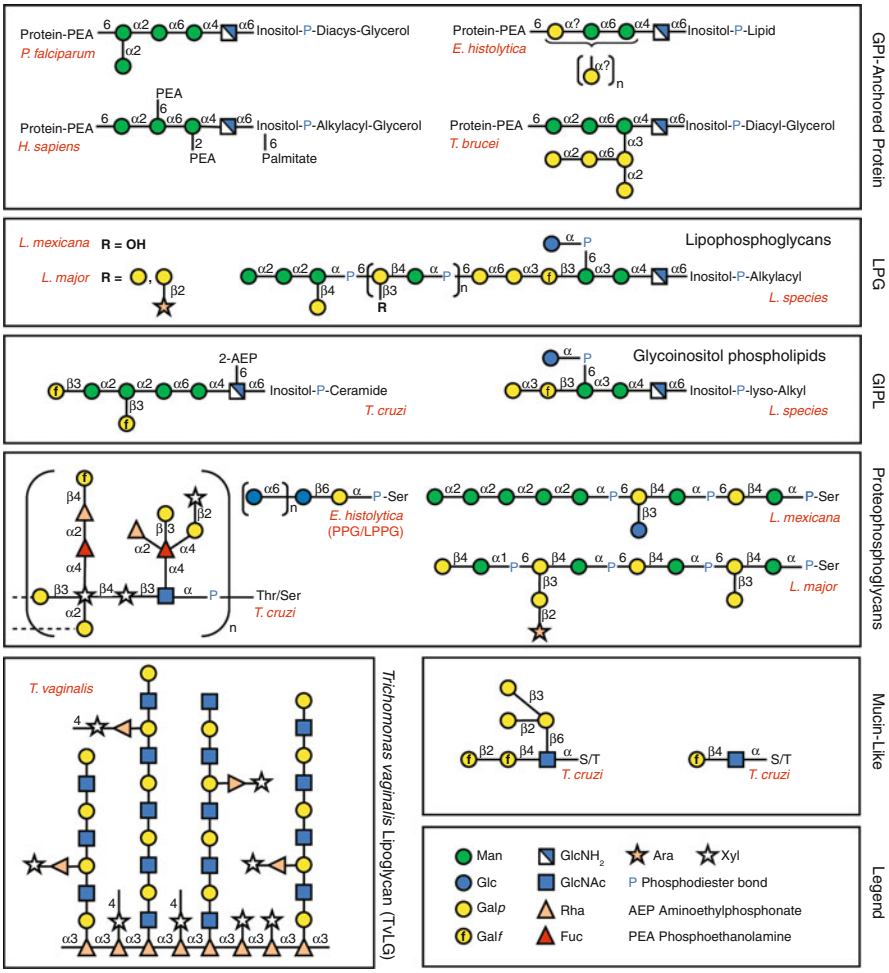

Fig. 3.2 Examples of lipid-linked glycans, phosphoglycans and O-linked oligosaccharides in parasitic protists. Several structures like glycosylphosphatidylinositol (*GPI*)-anchored protein, lipophosphoglycans (*LPG*), glycoinositolphospholipids (*GIPL*), proteophosphoglycans (*PPG*), mucin-like oligosaccharides and *Trichomonas vaginalis* lipoglycan (*TvLG*) are shown as illustrations of the huge diversity in glycosylation that can be found in parasitic protists. The full taxonomic names of parasitic protists use in this figure are *Entamoeba histolytica*, *Leishmania major* and *L. mexicana*, *Plasmodium falciparum*, *Trypanosoma brucei* and *T. cruzi* and *Trichomonas vaginalis*. Monosaccharide units are depicted according to the symbolic nomenclature of the Consortium for Functional Glycomics

One special feature of trichomonads appears to be their lipoglycans (see Fig. 3.2 and Table 3.2); only recently was it shown that these have a unusual structure in which a lipid is directly modified with chains consisting of polyrhamnose chains substituted with Galβ1, 4GlcNAc repeats as well as xylose residues in *T. vaginalis* (Ryan et al. 2011). In *T. foetus,* fucose residues may be also present on the

Table 3.2 Examples of different types of protozoan lipid-linked glycans

GIPL	Glycoinositolphospholipids (like a GPI without the protein)
GPI	Glycosylphosphatidylinositol (protein anchor)
LG	Lipoglycans (typical of *Trichomonas*; mistakenly earlier considered to be a type of LPG; lacks a GPI-like moiety)
LPG	Lipophosphoglycan (e.g. in *Leishmania*, molecule with phosphoglycan repeats on a GPI-like anchor) or lipophosphonoglycan (with unknown structure from *Acanthamoeba*)
LPPG	Lipopeptidophosphoglycan or lipophosphopeptidoglycan (e.g. phosphoglycan-modified protein with a GPI anchor from *Entamoeba* or old name for GIPLs of *T. cruzi*)
PPG	Proteophosphoglycan (protein modified with phosphodiester-linked glycans, e.g. in *Leishmania* or *Entamoeba*)

See Fig. 3.2 for some example structures; some 'historic' nomenclatures have changed as a result of more recent structural analyses

analogous molecule (Singh et al. 1994). These lipoglycans are unlike similar molecules (lipophosphoglycans, lipoproteophosphoglycans, etc.) found in other protists: trichomonad lipoglycan is not based on glycosylphosphatidylinositol (GPI). Indeed, trichomonads lack entirely the capacity to make any GPI-like molecules whatsoever. The lipoglycan appears to be important for adherence to the genital epithelium (Bastida-Corcuera et al. 2005).

3.3.4 Kinetoplastid Glycans

In terms of glycosylation, trypanosomatids are probably the most intensely studied protists. Indeed, the *Trypanosoma brucei* variant surface glycoproteins (VSGs), which are anchored to the plasma membrane of the parasite via a glycosylphosphatidylinositol anchor and carry various types of N-glycans, have been an important model for studying the structure of GPI anchors. The first structure of a GPI anchor was that derived from *T. brucei* (Ferguson et al. 1988), and its elucidation laid the foundations for studies of GPI anchors from other organisms. As expression of GPI anchors is essential for presentation of VSGs on the plasma membrane of the bloodstream form of the parasite, there has been some effort to investigate differences in GPI biosynthesis between trypanosomatids and other organisms, and a specific inhibitor toxic to *T. brucei* has been synthesised (Smith et al. 2004).

VSGs are also interesting as models for site-specific N-glycosylation, as the different VSG genes encode divergent proteins which carry either oligomannosidic or complex N-glycans dependent on the type of VSG (Zamze et al. 1990). Relevant to these different glycosylation patterns is the presence of three genes encoding the oligosaccharyltransferase in *T. brucei*, which transfer $Man_5GlcNAc_2$ or $Man_9GlcNAc_2$ to proteins (Izquierdo et al. 2009) (see also Fig. 3.1); in contrast to more typical eukaryotes, the trypanosomal oligosaccharyltransferases consist of a single catalytic STT3 subunit. There appears to be not just a glycan specificity in terms of which

oligosaccharyltransferase is transferred to the protein, but there is also a site specificity regarding which asparagine is modified by a particular STT3 isoform. Whether $Man_5GlcNAc_2$ or $Man_9GlcNAc_2$ has been transferred then dictates the final processing, with more 'complicated' forms resulting from the penta-mannosylated form (including then N-glycans which are very similar to mammalian complex N-glycans and contain terminal α- and β-galactose residues or poly-*N*-acetyllactosamine sequences) and oligomannosidic structures originating from the nona-mannosylated form (Manthri et al. 2008; Jones et al. 2005).

Leishmania also transfers truncated glycans to proteins, but the precursors only contain six mannose residues. These can also be post-transferred or glucosylated; thus, the gp63 protease from *L. mexicana* carries a mixture of $Glc_{0\text{-}1}Man_{4\text{-}6}GlcNAc_2$ glycans (Olafson et al. 1990). Earlier reports from other trypanosomatids suggest the presence of other sugar modifications such as galactofuranose and ribose (Mendelzon and Parodi 1986; Merello et al. 1994).

In terms of O-glycosylation, perhaps the best-studied trypanosomatid is *Trypanosoma cruzi*. The surface of this parasite carries mucin molecules which are GPI anchored (Serrano et al. 1995); the term 'mucin', though, should not be taken to indicate identity with animal mucins based on *N*-acetylgalactosamine which is secreted by various epithelia. Indeed, in *T. cruzi*, the O-glycans are linked through *N*-acetylglucosamine; they are also the acceptor for trans-sialylation in which a parasite enzyme removes sialic acid from mammalian host proteins and transfers this moiety to the mucins (Schenkman et al. 1993). Relevant *N*-acetylglucosaminyltransferases, which can initiate the modification of the mucin core polypeptide, have been identified (Heise et al. 2009). *T. cruzi* also features forms of phosphoglycosylation with a glycan containing fucose, rhamnose, xylose and other monosaccharides being present on gp72 (Allen et al. 2013) and a mannose-1-phosphate modification of the epimastigote NETNES protein (Macrae et al. 2005). The latter linkage is the same as that linking phosphoglycan repeats to form proteophosphoglycans in *Leishmania* (Ilg et al. 1998) (see also Fig. 3.2).

In trypanosomatids, there are also a variety of glycolipids based on GPI anchors with names such as glycosylinositol phospholipids (GIPLs), lipophosphoglycans (LPGs) and lipopeptidophosphoglycans (LPPGs) (McConville et al. 1990, 1995; de Lederkremer et al. 1991) (see Fig. 3.2 and Table 3.2). Defects in lipophosphoglycan biosynthesis may affect the ability of *Leishmania* to establish infections in their hosts (Späth et al. 2003).

3.3.5 Amoebal Glycans

Three amoebae have been subject to N-glycomic analyses: one obligate parasite (*Entamoeba histolytica*), one facultative parasite (*Acanthamoeba*) and one nonparasitic social amoeba (*Dictyostelium discoideum*). Despite all belonging to the amoebozoans, their N-glycans could not be more different. *Entamoeba*, like *Trichomonas*, lacks a number of the *alg* genes and only transfers $Man_5GlcNAc_2$ to proteins (Samuelson et al. 2005); after transfer, some modifications, particularly with α1,2-linked galactose residues or an α1,3-linked glucose residue, are possible (Magnelli

et al. 2008). *Acanthamoeba* and *Dictyostelium*, on the other hand, have full-length $Glc_3Man_9GlcNAc_2$ precursors. These then are modified in multiple ways. The *Acanthamoeba* N-glycome depends strongly on the strain, with a variety of modifications with xylose, methyl and α1,6-fucose, but there is (as yet) no discernible correlation with clinical relevance (Schiller et al. 2012). In contrast, *Dictyostelium* modifies its glycans with methylphosphate, sulphate, intersecting and bisecting *N*-acetylglucosamine and α1,3-fucose (Hykollari et al. 2013). Both *Acanthamoeba* and *Dictyostelium* extracts have been shown to have a special GlcNAc-phosphotransferase activity of the type also present in mammalian cells (Couso et al. 1986) and required for the formation of the Man-6-phosphate moiety required for lysosomal enzyme trafficking.

In parasitic amoebae, there are also molecules which have been named lipopeptidophosphoglycan, lipophosphoglycan or lipophosphonoglycan (see also Fig. 3.2 and Table 3.2), but these contrast with the LPGs from trypanosomatids. Indeed, the *Entamoeba* 'LPG-like molecule' is now known as a proteophosphoglycan (PPG) because it appears to be a GPI-linked protein decorated with poly-α1,6-linked glucose chains linked to the polypeptide core via Gal-1-phosphate (Moody-Haupt et al. 2000); the PPG is a potential drug target as antibodies recognising it are protective (Marinets et al. 1997). On the other hand, the LPG from *Acanthamoeba* probably does not contain protein, but is incompletely characterised (Dearborn et al. 1976; Karaś and Russa 2013). *Acanthamoeba* also produces cellulose during encystation (Potter and Weisman 1972), just as *Dictyostelium* does during fruiting body formation (Blanton et al. 2000).

3.4 Available Databases

Unlike DNA and protein databases, there is nothing like a 'GlycoBank' along the lines of Genbank or Swiss-Prot. There have been a number of attempts to initiate glycan databases, but financial or formatting problems have been recurrent: indeed, it is the chemical nature of carbohydrates (especially branching), which means that a standard linear code cannot summarise a glycan structure. However, attempts are continuing (e.g. UniCarbKB), and discussions regarding the formatting and minimal reporting requirements are ongoing (Campbell et al. 2014; York et al. 2014).

The biological meaning of glycans is to be found in their interactions with proteins, and there are examples of host-parasite interactions based on sugars. For instance, human galectin-1 binds *T. vaginalis* probably via its lipoglycan (Okumura et al. 2008), and *Acanthamoeba* mannose-binding protein may have a role in binding to the cornea (Garate et al. 2004). In terms of databases of lectin/antibody interactions, the results of array screening by the Consortium for Functional Glycomics (www.functionalglycomics.org) represent an interesting resource, but due to its mammalian-centred nature, the data are often of limited value when considering binding of host proteins to parasite glycans. On the other hand, there is high potential to study binding of parasite lectins to host glycans with the currently available glycan array tools, but also new 'shotgun glycomics' approaches should enable more natural ligands to be screened for interactions with host proteins (Arthur et al. 2014).

Similarly, still in its infancy is the process of cataloguing the enzymes required for the synthesis of protozoan glycans. The Carbohydrate-Active Enzyme (CAZy) database has homology-based listings of different families of glycosidases and glycosyltransferases (Lombard et al. 2014). The curations are most advanced for model organisms and bacteria, but for protists, a community effort will be necessary to identify potential 'CAZy' reading frames in these organisms. Other than very few exceptions, there is little biochemical data to back up any predictions of such bioinformatic studies on enzymes involved in protist glycan biosynthesis.

Conclusion

Glycomics is a challenging topic and the study of glycans in protists is no exception; however, as all cells are covered in 'sugar' (whether host or parasite), the so-called glycocalyx is normally the first point at which different cells come together during parasite adherence and invasion. Therefore, studying the glycome is as important to understanding host-parasite interactions and for development of new anti-parasite strategies as examinations of the proteome and genome. There is an immense variation between species, which makes generalisations difficult; there is even no correlation as regards glycomic complexity whether a parasite is intra- or extracellular, although obligate parasites may have some 'simplifications' as compared to facultative species. Nevertheless, it is certain that as technologies to characterise glycans and their interactions advance, new insights into these key molecules will continue to be made in the coming years.

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Part II

The Parasites (and Their Molecules)

Giardia

4

Norbert Müller and Joachim Müller

Abstract

The intestinal protozoan parasite *Giardia duodenalis* (syn. *Giardia lamblia*, *Giardia intestinalis*) causes diarrhoea in humans and animals worldwide. The life cycle of *G. duodenalis* consists of two stages, the flagellated trophozoite proliferating in the upper part of the small intestine and the nonproliferative cyst representing the infectious stage of the parasite. Both stages can be handled in vitro and in vivo. Trophozoites are pear-shaped, motile cells exhibiting a convex dorsal and a concave ventral side. The cell body is formed by a microtubule cytoskeleton. The whole genome contained in two diploid nuclei per trophozoite has been sequenced and characterised. It has some prokaryote-like features such as short promoter sequences. Moreover, some key enzymes of energy and intermediate metabolisms share common features with prokaryotic enzymes and may have been acquired by lateral transfer. *Giardia* does not contain mitochondria and peroxisomes, but mitosomes, most likely an evolutionarily reduced version of a mitochondrion. The energy metabolism is chemoheterotrophic and works under anaerobic or semiaerobic conditions with glucose as main energy and carbon source and arginine as another important energy source. The present book chapter selectively reviews current knowledge in *Giardia* research highlighting its basic genetic, physiological and, to a lower extent, its immunological properties. Furthermore, this chapter also shows that *G. duodenalis* is a suitable cellular model system for the investigation of fundamental biological principles.

N. Müller (✉)
Institute for Parasitology, Vetsuisse and Medical Faculty, University of Berne, Länggass-Strasse 122, Bern CH – 3012, Switzerland
e-mail: norbert.mueller@vetsuisse.unibe.ch

J. Müller
Institute for Parasitology, Vetsuisse and Medical Faculty, University of Berne, Länggass-Strasse 122, Bern CH – 3012, Switzerland

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4.1 Introductory Remarks

The intestinal protozoan parasite *Giardia duodenalis* (syn. *Giardia lamblia*, *Giardia intestinalis*) causes diarrhoea in humans and animals worldwide (Thompson et al. 2000; Müller and von Allmen 2005; Jerlström-Hultqvist et al. 2010). In giardiasis, infection occurs via peroral uptake of water or food contaminated with *G. duodenalis* cysts. *Giardia* is of particular relevance in dogs and cattle where infections associated with severe symptoms are frequently observed in young animals. In humans, infection with *G. duodenalis* often remains asymptomatic, but occasionally individuals may suffer from acute, or chronic, watery diarrhoea as a consequence of malabsorption. The infection may even be associated with long-term clinical manifestations such as postinfectious irritable bowel syndrome (Halliez and Buret 2013).

The life cycle of *G. duodenalis* consists of two major stages: the flagellated trophozoite proliferating in the upper part of the small intestine and the nonproliferative cyst representing the environmentally resistant, infectious stage of the parasite (Fig. 4.1). Upon ingestion of *Giardia* cysts by a host, excystation is induced through exposure of cysts to the acidic, proteolytic conditions existing in the gastric environment (Ankarklev et al. 2010). Excystation is a rapid process, and excyzoites released from ingested cysts immediately divide into trophozoites that subsequently colonise in the upper part of the small intestine. Adherence of the trophozoites to the intestinal mucosa essentially occurs through the sucking function of a disc on the ventral surface of the cell. Moreover, *G. duodenalis* has four pairs of flagella, allowing nonadherent trophozoites to swim and translocate to a different mucosal site. Both strong attachment and high motility are vital cellular functions enabling the parasite to resist immediate elimination by intestinal peristalsis.

The development of the symptoms is supposed to be influenced by the immunological status of the infected individual (Müller and von Allmen 2005; Cotton et al. 2011). However, physiological factors determined by the intestinal environment are also involved in the interaction between the host and the parasite. For example, as a semiaerobic microorganism, *G. duodenalis* has developed unique enzymatic and metabolic pathways, allowing the parasite to cope with varying concentrations of oxygen in the intestinal habitat and with varying nitrosative stress conditions, e.g. related to the differential diets of its carnivorous, herbivorous and omnivorous hosts (Brown et al. 1998). Finally, *G. duodenalis* has adopted a genetic regulatory system to perform continuous surface antigen variation involving the highly antigenic variant surface proteins (VSPs) and allowing the parasite's survival within the hostile immunological and physiological environment of its intestinal habitat (Jerlström-Hultqvist et al. 2010; Müller and von Allmen 2005).

The two major life cycle stages of *G. duodenalis* can easily be handled both in vitro and in vivo. Moreover, the complete genome sequence is available (GiardiaDB; www.giardiadb.org). Therefore, *G. duodenalis* is an ideal model organism for biological and biomedical research. The present book chapter selectively reviews current knowledge in *Giardia* research highlighting its basic genetic, physiological and, to a minor extent, immunological properties. Furthermore, this chapter also shows that *G. duodenalis* is a suitable cellular model system for the

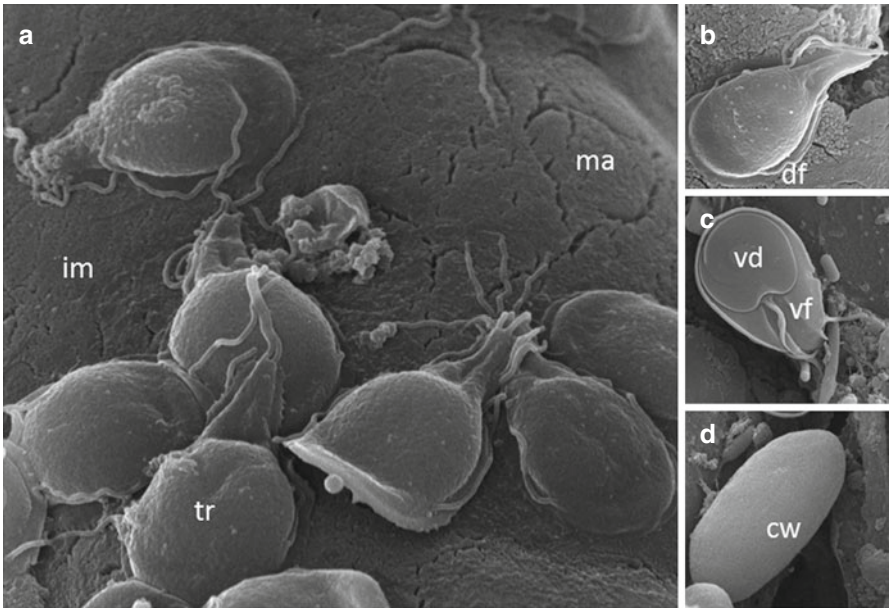

Fig. 4.1 Scanning electron micrography of *Giardia duodenalis* trophozoites (**a**–**c**) and a cyst (**d**) on murine intestinal mucosa. *im* intestinal mucosa, *ma* marks of attachment, *tr* trophozoite, *df* dorsal flagellum, *vf* ventral flagellum, *vd* ventral disc and *cw* cyst wall

investigation of fundamental biological principles such as intracellular protein transport (Faso and Hehl 2011). Accordingly, concise data on giardial intracellular protein trafficking associated with surface antigen coat and cyst wall formation will be presented.

4.2 Well-Established Facts

4.2.1 The Genome

4.2.1.1 Genome Organisation

Giardia trophozoites carry two diploid, supposedly genetically equivalent, and transcriptionally active nuclei that during binary fission of the trophozoites are equally segregated between the daughter cells. Encystation is associated with an incomplete mitosis leading to a cyst with four diploid daughter nuclei. Excystation involves two rounds of cytokinesis followed by one round of karyokinesis providing the transient excyzoite stage that harbours four tetraploid nuclei. Excystation is completed by two cellular divisions without DNA replication providing four trophozoites equipped with two diploid nuclei each. Although not definitely proven yet, genetic recombination particularly supposed to occur in cysts was suggested as a mechanism to reduce heterozygosity among the nuclei (Ankarklev et al. 2010).

Numerous genetic studies have led to the identification of eight genotypes referred to as assemblages A to H. Assemblages A and B are found both in animals and humans and therefore exhibit a zoonotic potential; others are only found in animals (Ryan and Cacciò 2013). The best studied isolate from assemblage A, WB C6, carries a diploid set of five major chromosomes, and the haploid size of the genome is approximately 11.7 Mb (Morrison et al. 2007; Franzén et al. 2009). A genome sequence comparison between isolates from assemblages A, B and E reveals significant variation in the gene content and composition, as well as in the chromosomal structure. This variation is exemplified by assemblage-specific differences in numbers, genetic characteristics and chromosomal organisation of *vsp* genes within isolates (Morrison et al. 2007; Franzén et al. 2009; Jerlström-Hultqvist et al. 2010). Conversely, many genes, e.g. involved in housekeeping and metabolism, are highly conserved. Furthermore, it seems that particularly some of those genes supporting the semiaerobic lifestyle of *G. duodenalis* have been evolutionary adopted from bacteria (Nixon et al. 2002, 2003; Feng et al. 2012).

4.2.1.2 Gene Expression

In a general view, giardial promoters cover less than 70 base pairs and are thus extremely short in comparison with promoters from higher eukaryotes (Elmendorf et al. 2001; Morrison et al. 2007; Jerlström-Hultqvist et al. 2010). mRNA maturation largely follows the features typical for eukaryotic cells including 5′ capping (Hausmann et al. 2004), mRNA polyadenylation (Adam 2000; Morrison et al. 2007) and, in very few cases, classical intron splicing mediated by spliceosomes (Morrison et al. 2007). Typically, mRNAs from *Giardia* contain short (0–14 nucleotides) 5′-terminal and somewhat longer (10–30 nucleotides) 3′-terminal untranslated regions (Morrison et al. 2007). Furthermore, the open-reading frames contain a 13-nucleotide downstream box that is recognised by a complementary sequence in the 16-s ribosomal RNA, thus initiating translation of the mRNAs into the corresponding proteins (Morrison et al. 2007).

4.2.2 Morphological and Cellular Features of Different Life Cycle Stages

4.2.2.1 Trophozoites

Trophozoites of *G. duodenalis* are pear-shaped, motile cells exhibiting a convex dorsal and a concave ventral side (Ankarklev et al. 2010). This polar cell body is essentially formed by a microtubule cytoskeleton. Trophozoites have two genetically equivalent and bilaterally symmetric nuclei. They contain four microtubule-rich structures including the ventral disc, three pairs of dorsal and one pair of ventral flagella, the median body and the funis. Like in any eukaryotic cell, giardial microtubules are formed by heterodimers consisting of alpha and beta tubulin. These structures have been identified as high-abundant components of the cytoskeleton contributing to the cellular shape and plasticity of giardial trophozoites (Dawson 2010). Moreover, microtubules participate in intracellular trafficking, encystation/

excystation, and they are involved in cell division by forming two mitotic spindles around the nuclear envelope mediating segregation of the chromosomes. In *G. duodenalis*, microtubule structures are associated with a variety of proteins, particularly with giardins representing a complex protein family unique for this organism. Conversely, other cytoskeletal components such as microfilaments basically built up by actin or intermediate filaments mainly consisting of lamins are low abundant or even absent, respectively.

The ventral disc of *G. duodenalis* has a concave profile and is primarily shaped by a spiral array of membrane-linked microtubules that are associated with trilaminar structures called microribbons. Upon attachment, the concave configuration of the disc increases and thus creates a vacuum that mediates mechanical adherence of the trophozoites. This adhesive process is supposed to be supported by the contractile function of a lateral crest, an as yet poorly characterised fibrillar structure surrounding the disc (Dawson and House 2010a, b).

Motility of *G. duodenalis* trophozoites is achieved by a coordinated flapping motion of four pairs of flagella (anterior, posterolateral, ventral and caudal) that originate from basal bodies. The basal bodies are located between the paired nuclei and dorsal to the ventral disc and represent formations of microtubule triplets, cross-bridged by electron-dense elements of unknown composition. The flagellar cytoskeleton is called axoneme consisting of a 9+2 microtubular structure (nine pairs of microtubules organised in a circle with two microtubules in the centre). These dynamic structures are associated with dyneins that act as energy-dependent microtubule motors responsible for the motile function of the flagella (Dawson and House 2010a, b).

The funis consists of sheets of microtubules that are arranged along the axonemes from the caudal flagella and extend to the posterior region of the trophozoite. Part of the funis is transversally covered by the median body, a dense microtubule structure of as yet unknown cellular function (Benchimol et al. 2004).

Giardia does not contain mitochondria and peroxisomes, but mitosomes, double-membrane-bounded cytoplasmic compartments, functionally involved in iron-sulphur protein maturation. The mitosome is supposed to be an evolutionarily reduced version of a mitochondrion. This organelle has lost its mitochondrial genome DNA as well as typical mitochondrial metabolic pathways such as the citric acid cycle and the respiratory chain. Furthermore, *G. duodenalis* contains an endoplasmic reticulum (ER) and a developmentally regulated, primitive Golgi apparatus that both are required, e.g. for secretion of cyst wall proteins (CWP). Conversely, endocytosis and digestion of exterior fluid material such as nutrients are carried out by the peripheral vesicles (PVs) located below the plasma membrane (Faso and Hehl 2011).

4.2.2.2 Cysts and Excyzoites

Giardia cysts are oval shaped and are covered by a robust wall that protects the parasite from mechanical and chemical stress (Ankarklev et al. 2010). The cyst wall is rather thick (0.3–0.5 μm) and is underlaid with a double membrane. The outer part of the cyst wall has a filamentous structure, which contains both carbohydrate and protein components.

Conversion of motile, proliferating trophozoites to immotile, non-proliferating, metabolically dormant cysts coincides with complex ultrastructural changes involving an as yet poorly understood reconfiguration of the cytoskeleton. These alterations are associated with internalisation of flagella and fragmentation of the ventral disc. During an infection event, internalised debris are "recycled", thus allowing rapid (re-)constitution of respective functional elements in excyzoites and in early trophozoite populations (Faso and Hehl 2011). This phenomenon may be important for the establishment of a *G. duodenalis* infection in a host because it may facilitate rapid intestinal colonisation of trophozoites emerging upon the excystation process.

Excyzoites appear immediately upon excystation; they possess eight flagella and four nuclei (Ankarklev et al. 2010) and are characterised by a distinct gene expression pattern (Birkeland et al. 2010). These cells represent a transient life cycle stage rapidly converting into proliferating trophozoites.

4.2.3 Enzymes

4.2.3.1 Pyruvate Oxidoreductase

Some key enzymes of giardial energy and intermediate metabolisms share common features with prokaryotic enzymes and may have even been acquired by lateral transfer (Nixon et al. 2002). These enzymes may constitute suitable targets for antigiardial drugs and are therefore investigated in detail by several groups. One of the enzymes that have gained major attention during the last 25 years (Smith et al. 1988) is pyruvate oxidoreductase or pyruvate ferredoxin oxidoreductase (PFOR). PFOR is a 138-kD homodimer (Townson et al. 1996) membrane-associated cytosolic enzyme (Emelyanov and Goldberg 2011) encoded by two homologous genes, namely, GL50803_114609 and GL50803_17063, the derived polypeptide sequences having 1,253 and 1,199 amino acids, respectively (GiardiaDB). PFOR decarboxylates pyruvate in the presence of coenzyme A-SH yielding acetyl-CoA, CO_2 and a pair of electrons that is transferred to ferredoxin. Thus, PFOR is different from the pyruvate dehydrogenase multienzyme complex found in mitochondria of higher eukaryotes. The electrons transferred to ferredoxin are believed to activate the antigiardial drug metronidazole. Downregulation of PFOR by a hammerhead ribozyme flanked with various lengths of antisense PFOR leads to an enhanced resistance to metronidazole and to oxygen (Dan et al. 2000). There are, however, metronidazole-resistant *G. duodenalis* lines with normally expressed (Müller et al. 2007) or even fully functional PFORs indicating that downregulation of PFOR is not the only way to metronidazole resistance (Leitsch et al. 2011; Leitsch 2015).

4.2.3.2 Alcohol Dehydrogenase

The *Giardia* genome contains four gene-encoding alcohol dehydrogenases. GL50803_93358 encodes a 97-kDa protein expressed in trophozoites and downregulated during encystation. GL50803_3861 (alcohol dehydrogenase 3 lateral transfer candidate) encodes a 45-kDa protein upregulated during encystation.

GL50803_13350, another alcohol dehydrogenase lateral transfer candidate, encodes a 48-kDa protein upregulated during encystation. An ortholog of this gene, GL50803_3593, encodes a 44-kDa protein with no distinct expression pattern (data from GiardiaDB).

The gene product of GL50803_93358 has been characterised in more detail. It is a bifunctional aldehyde/alcohol dehydrogenase enzyme (Sánchez 1998). Interestingly, strains with a hammerhead ribozyme knock-down of this enzyme have a relatively poorer growth than the wild type under anaerobic growth conditions and a relatively better growth than the wild type under aerobic conditions (Dan and Wang 2000). This may be explained by a better availability of NADH for oxygen consumption under semiaerobic growth conditions.

4.2.3.3 Enzymes Involved in Oxidative Stress Protection

Giardia trophozoites consume oxygen but lack the conventional mechanisms of oxidative stress management, including superoxide dismutase, catalase, peroxidase and glutathione cycling present in most eukaryotes (Mastronicola et al. 2015). In their place, *Giardia* contains H_2O-producing NADH oxidase, a membrane-associated NADH peroxidase, a broad-range prokaryotic thioredoxin reductase-like disulphide reductase and the low molecular weight thiols, cysteine, thioglycolate, sulphite and coenzyme A. NADH oxidase is a major component of the electron transport pathway of *Giardia* which, in conjunction with disulphide reductase, protects oxygen-labile proteins such as ferredoxin and pyruvate ferredoxin oxidoreductase against oxidative stress by maintaining a reduced intracellular environment. As a terminal oxidase, NADH oxidase provides a means of removing excess electrons and recycling NADH, thereby enabling continued pyruvate decarboxylation and the resultant production of acetate and adenosine triphosphate (ATP) (Brown et al. 1996a, b). The *Giardia* genome contains two gene-encoding NADH oxidase isoforms, namely, Gl50803_33769, a prokaryote-like enzyme, and Gl50803_9719, a membrane-associated enzyme.

Thioredoxin reductase (Gl50803_9827) is a dimeric flavoprotein containing 1-mol FAD per subunit with an apparent subunit molecular mass of 35 kDa. The purified enzyme reduces a variety of substrates, including cystine and oxidised glutathione suggesting that it is a broad-range disulphide reductase, probably accounting for the majority of thiol cycling activity (Brown et al. 1996a, b). This broad-range activity may also be involved in the mode of action of antigiardial drugs such as 5-nitroimidazoles and metronidazole (Leitsch et al. 2011). Interestingly, the antirheumatic drug auranofin inhibits thioredoxin oxidoreductase and is effective against *G. duodenalis* in the micromolar range (Tejman-Yarden et al. 2013).

4.2.3.4 Protein Disulphide Isomerases

Another process where oxidative stress protection is crucial is the formation and correct maintenance of disulphide bonds within and between polypeptide chains by protein disulphide isomerases (PDIs). The *Giardia* genome contains five gene-encoding protein disulphide isomerase isoforms, namely, PDI 1 (Gl50803_29487; ca. 26 kDa), PDI 2 (GL50803_9413; ca. 50 kDa), PDI 3 (Gl50803_14670; ca.

13 kDa), PDI 4 (GL50803_103713; ca. 39 kDa) and PDI 5 (GL50803_8064; ca. 15 kDa). All PDIs have signal peptides indicating a translocation to the ER. PDI 1 and PDI 3 have no KDEL retention signal indicating that they may be secreted. Unlike most other eukaryotic PDIs, all *Giardia* PDIs have only one active site. The active-site sequence motif in these five PDIs, namely, CGHC, is found only in PDIs, but not in other members of the thioredoxin superfamily that have one active site, such as thioredoxin and Dsb proteins from Gram-negative bacteria. All *Giardia* PDIs rearrange disulphide bonds in in vitro assays (Knodler et al. 1999; Müller et al. 2007). PDI 2 also displays oxidant and reductant activities. Surprisingly, the PDI 1, PDI 2 and PDI 3 also have calcium-dependent transglutaminase activities (Knodler et al. 1999). This transglutaminase may have a biological function since differentiating trophozoites contain epsilon-(gamma-glutamyl)-lysine-bonds, the product of transglutaminase activity that results in irreversible cross-linking of proteins in vivo. The highest isopeptide bond content is found in encysting cells. This is in good agreement with the observation that assays of encysting parasites revealed that PDIs 1–3 are located in encystation secretory vesicles. Moreover, PDI 2 is localised on the cell surface (Davids et al. 2004).

Phylogenetic analyses of eukaryotic PDIs support common ancestry from a thioredoxin ancestor and independent duplications of thioredoxin-like domains within PDIs throughout eukaryote evolution, e.g. in *Acanthamoeba*, *Dictyostelium* and mammals. In contrast, gene duplication, instead of domain duplication, produces PDI diversity in *G. duodenalis*. The five single-domain PDIs of *G. duodenalis* may reflect an ancestral mechanism of protein folding in the eukaryotic endoplasmic reticulum, a combination of PDIs being used as a redox chain analogous to that known for bacterial Dsb proteins (McArthur et al. 2001).

4.2.4 Metabolism

4.2.4.1 Energy Metabolism

G. duodenalis has a chemoheterotrophic metabolism working under anaerobic or semiaerobic conditions. Under in vitro conditions, the main carbon and energy source is glucose, which is catabolised by the Embden-Meyerhof-Parnas and hexose monophosphate pathways. These pathways seem to rely on pyrophosphate rather than adenosine triphosphate since they contain two typically bacterial glycolytic enzymes, which are pyrophosphate dependent. Pyruvate decarboxylation and subsequent electron transport to as yet unidentified anaerobic electron acceptors rely on a eubacterial-like pyruvate ferredoxin oxidoreductase and an archaebacterial/eubacterial-like ferredoxin. The presence of another 2-keto acid oxidoreductase (with a preference for alpha-ketobutyrate) and multiple ferredoxins in *Giardia* is also a trait shared with the anaerobic bacteria (Brown et al. 1998).

Due to the lack of mitochondria, energy is produced by substrate-level phosphorylation (Jarroll et al. 1989). The main catabolic end products are CO_2, ethanol, acetate and alanine (Edwards et al. 1989). Ethanol formation in *G. duodenalis* is a two-step process. First, acetyl-CoA is reduced to acetaldehyde + CoA-SH. Then,

acetaldehyde is reduced to ethanol. Both reactions need NADH as a cosubstrate and are catalysed by a bifunctional enzyme (Sánchez 1998). In contrast to most eukaryotes in which ethanol formation proceeds from pyruvate via acetaldehyde, the *Giardia* pathway departs from acetyl-CoA, a more distal product of extended glycolysis. Ethanol production declines with a decrease of glucose supply. *G. duodenalis* trophozoites continue, however, to grow and produce the same metabolites in a medium containing little or no glucose, which indicates that glucose is not the only metabolic fuel. Moreover, the presence of alanine as a catabolic end product suggests an intimate link between energy, carbon and nitrogen metabolism (Schofield et al. 1991). The pathway of CoA biosynthesis in *G. duodenalis* is conserved (Genschel 2004).

Another bacterial-like trait of energy metabolism in *Giardia* is the utilisation of arginine as an energy source. Supplementation of the trophozoite culture medium with arginine results in an accelerated trophozoite growth over the first 2 days even in the absence of glucose. The corresponding rapid utilisation of arginine is associated with the appearance in the growth medium of 1 mol of ornithine and 2 mol of NH_3 per mol of arginine utilised (Edwards et al. 1992). Further studies have shown that arginine is catabolised by the arginine dehydrogenase pathway to carbamoyl phosphate and ornithine, which is exported via an arginine-ornithine-antiport system. Carbamyl phosphate is further catabolised to CO_2 and NH_3, yielding 1 mol of ATP per mol of arginine. Thus, arginine is a major potential energy source independent of glucose (Knodler et al. 1995). Besides arginine, alanine is taken up and contributes to CO_2 formation as shown by labelling experiments (Edwards et al. 1993). More recent results reveal that arginine consumption by trophozoites may have another beneficial side effect for the parasite, namely, the reduction of intestinal cell proliferation (Stadelmann et al. 2012).

Redox equivalents accumulated during glycolysis may be transferred to acetyl-CoA, yielding ethanol as a major final product of fermentation. Under semiaerobic conditions, redox equivalents are transferred to oxygen via NADH oxidase (Brown et al. 1996a, b).

A scheme of energy metabolism is shown in Fig. 4.2.

4.2.4.2 Intermediate Metabolism

Gluconeogenesis

Studies on gluconeogenesis and related enzymes in *Giardia* are scarce. Gluconeogenesis may start with the ATP-dependent synthesis of acetyl-CoA by an acetyl-CoA synthetase. The *Giardia* genome contains one homologous gene (GL50803_13608) encoding an unusual ADP-forming acetyl-CoA synthetase. Another possibility to start gluconeogenesis is the decarboxylation of oxaloacetate via phosphoenolpyruvate carboxykinase. A gene encoding a putative GTP-specific phosphoenolpyruvate carboxykinase exists in the genome (GL50803_10623). The deduced amino acid sequence is related most closely to homologs from hyperthermophilic archaebacteria and only more distantly to homologs from eubacteria and metazoa (Suguri et al. 2001). Since mitochondria are absent, one probable source of

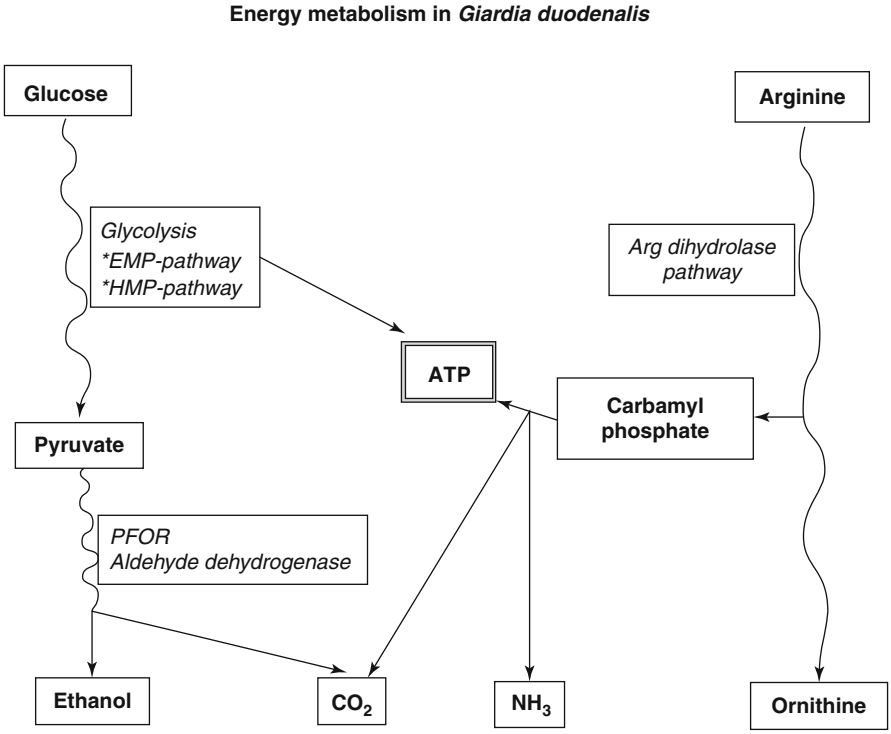

Fig. 4.2 Scheme of energy metabolism in *Giardia duodenalis*, depicting the main energy sources (glucose and arginine), intermediate compounds and end products as well as the main pathways (in italics). The references are given in the text

oxaloacetate is the deamination of alanine. Trophozoites produce alanine as an end product of fermentation (Paget et al. 1990) and excrete alanine during growth (Nygaard et al. 1994), probably via an antiport system relying on closely related amino acids (Schofield et al. 1995). Since alanine and other amino acids are also used as energy sources, their uptake, metabolism and excretion may be dependent on the energy and redox status of the cell (Mendis et al. 1992).

Purine and Pyrimidine Salvage

One striking feature of intermediate metabolism of *Giardia* is the entire dependence on exogenous purine and pyrimidine bases and deoxynucleosides (Baum et al. 1989).

Salvage of purines and their nucleosides is accomplished by adenine phosphoribosyltransferase (APRT), adenosine hydrolase, guanine phosphoribosyltransferase (GPRT) and guanine hydrolase. When GPRT is knocked down by a hammerhead ribozyme carrying GPRT antisense RNA, GPRT enzyme activity and replicative cell growth decrease to less than 10–20 % of wild-type levels. In parallel with growth reduction, the guanine ribonucleotide pools are depleted (Munagala and Wang 2002).

The absence of de novo pyrimidine synthesis is evidenced by the lack of incorporation of bicarbonate, orotate and aspartate into nucleotides and by the lack of detectable levels of the enzymes of de novo pyrimidine synthesis (Aldritt et al. 1985). Salvage appears to be accomplished by the action of uracil phosphoribosyltransferase, uridine hydrolase, uridine phosphotransferase, cytidine deaminase, cytidine hydrolase, cytosine phosphoribosyltransferase and thymidine phosphotransferase. Nucleotides of uracil may be converted to nucleotides of cytosine by cytidine triphosphate synthetase (Jiménez and O'Sullivan 1994), but thymidylate synthetase and dihydrofolate reductase activities are not detected. Uptake of pyrimidine nucleosides, and perhaps pyrimidines, appears to be accomplished by carrier-mediated transport, and the common site for uptake of uridine and cytidine is distinct from the site for thymidine. Thymine does not appear to be incorporated into nucleotide pools (Jarroll et al. 1989).

Lipid Metabolism

As in the case of purine and pyrimidine metabolism, *Giardia* trophozoites appear to rely on preformed lipids rather than synthesising them de novo (Jarroll et al. 1989). The lipids have to be taken up from the environment thus from the small intestine or from the culture medium. Interestingly, the presence of trophozoites in the small intestine of suckling rats does not alter their fatty acid digestion (Magne et al. 1994). A complete lipidomic analysis reveals that phosphatidylglycerols are the major phospholipids followed by phosphatidylcholines and phosphatidylethanolamines in non-encysting and encysting trophozoites, as well as in cysts. The fatty acids attached to these phospholipids consist mostly of palmitate, palmitoleate, oleate and linoleate. Phosphatidylglycerols and ethanolamines are not present in bovine bile and serum, the major sources of lipids of the culture medium. Therefore, they may be produced by a fatty acid and headgroup remodelling reactions, circumventing the synthesis of entirely new phospholipids via de novo pathways (Yichoy et al. 2009). In a more recent review, the authors give a detailed overview of investigated and presumptive pathways of giardial lipid metabolism (Yichoy et al. 2011).

4.2.5 Surface Structure and Protein Secretion

4.2.5.1 Variant Surface Proteins (VSPs) and Antigenic Variation

In the past, a large number of molecular, biochemical and immunological investigations were focused on the ability of *G. duodenalis* to undergo surface antigenic variation (Müller and Gottstein 1998; Müller and von Allmen 2005; Prucca and Lujan 2009; Rópolo and Touz 2010; Lujan 2011; Prucca et al. 2011). This phenomenon is supposed to be a strategy of the parasite to evade the immune response and thus to increase the survival time within its host. Alterations of the surface antigen properties are associated with a unique family of surface antigens, namely, the VSPs.

In *G. duodenalis*, antigenic variability is based on the availability of a large VSP repertoire encoded by approximately 200 distinct genes (Morrison et al. 2007). VSPs are cysteine-rich proteins containing repeated CXXC motifs and possessing a

relatively conserved membrane-spanning region followed by an invariant cytoplasmatic CRGKA tail at the carboxy terminus and vary from approximately 20 to 200 kDa in size (Nash and Mowatt 1992). Antigenic variability is mediated by the amino-terminal region of the proteins. The extracellular domains of the VSPs containing the antigenic epitopes are cleaved and released into the environment. This antigen stripping may reduce binding of cytotoxic antibodies to the surface of trophozoites and thus contribute the immunoevasive strategy of the parasite (Papanastasiou et al. 1996).

The process of antigenic variation of *G. duodenalis* has most extensively been studied using the murine infection model (Müller and Gottstein 1998; Müller and von Allmen 2005). During infections, initial VSP-type trophozoite populations are eliminated primarily by the intestinal antibody (IgA) response and are replaced by a mixture of new variant antigen types (Nash 1997). In immunocompetent mice, VSP variants are negatively selected most likely by a direct cytotoxic effect of the antibodies. In immunodeficient mice, both positive and negative selections occur (Singer et al. 2001). This process continues until the humoral and cellular anti-*Giardia* immune responses succeed in resolving the acute infection. In those rare cases where the infection enters a chronic phase, antigenic variation may contribute to persistence of the parasite inside the intestinal habitat. The importance of antigenic variation for survival of the parasite is experimentally demonstrated. Primary infection with trophozoites expressing many VSPs and immunisation with the entire repertoire of purified VSPs protect gerbils from subsequent infections (Rivero et al. 2010). This finding also suggests further investigations of highly complex VSP cocktails covering the entire repertoire from one or several *Giardia* genotypes regarding their suitability as vaccine against giardiasis in humans and various animal hosts.

Interestingly, a few of the VSPs characterised so far seem to possess a peptide conformation that resists exposure to intestinal proteases (Nash et al. 1991). Accordingly, a certain number of VSPs may protect trophozoites from intestinal enzymatic attack, thus favouring proliferation of the corresponding variant type within the intestinal environment. In combination, these observations indicate that the growth-selective processes modulate antigenic diversification of an intestinal trophozoite population. These processes are determined by the interaction of immunological and physiological factors, rather than by the immunological factors alone.

In the last few years, the genetic mechanisms controlling switching of the VSP expression within an individual trophozoite have been investigated in great detail. Depending on the parasite isolate investigated, VSP switching is a spontaneous event that leads to the substitution of a single species of VSP by another one on an individual trophozoite. Such events occur approximately every 6 to 13 generations (Nash et al. 1990). This antigenic switch may be affected by deimination of the final arginine residue from the conserved VSP carboxy terminus by arginine deiminase (Touz et al. 2008).

According to a current model, all *vsp* genes are transcribed simultaneously and constitutively, but only one mRNA encoding the VSP that is expressed on the cellular surface accumulates in the cytoplasm. Thus, VSP expression in *G. duodenalis*

is regulated post-transcriptionally by involving RNA interference (RNAi)-like mechanisms (Prucca and Lujan 2009; Lujan 2011; Prucca et al. 2011). RNAi eliminates the cytoplasmatic mRNAs from all other members of the existing VSP repertoire. Several enzymes typical for a eukaryotic RNAi machinery participate in silencing of *vsp* mRNA. Once the transcripts are released into the cytoplasm, complementary antisense transcripts are synthesised by an RNA-dependent RNA polymerase (RdRP). The double-stranded (ds) RNAs formed by template sense and newly synthesised complementary antisense RNA are cleaved by the dsRNA endonucleases Dicer and Argonaute (Prucca et al. 2008). Resulting micro (mi)RNA molecules approximately 26 nt in size are integrated into an RNA-induced silencing complex (RISC). Then, the diverse *vsp* mRNA molecules are degraded by RISCs containing the corresponding miRNAs as sensors for the mRNA target sites. Since the *vsp* transcripts contain many target sites, multiple miRISCs can operate in a concerted manner and thus completely repress corresponding VSP expression (Li et al. 2011, 2012; Saraiya et al. 2011).

This model of post-transcriptional regulation of *vsp* gene expression was verified in that transgenic RdRP and Dicer knock-down *G. duodenalis* strains exhibited a simultaneous expression of several VSPs (Rivero et al. 2010). However, this model cannot answer the two questions: (i) how the mRNA corresponding to the VSP that is expressed by the parasite is able to bypass the silencing process and (ii) by which regulatory mechanism VSP switching actually is initiated. Although rather speculative, the species of VSP transcripts constitutively synthesised at the highest concentration may be the one that bypasses the silencing process at a given time point. Provided that differential transcription of the *vsp* genes really occurs, this process may be modulated by an epigenetic mechanism. This mechanism is supposed to underlie the above-described post-transcriptional regulation of VSP gene expression in *G. duodenalis* (Kulakova et al. 2006).

As already described above, VSPs are plasma membrane-bound surface proteins that form a dense and variable antigen coat of *Giardia* trophozoites. This surface coat is a dynamic structure because stripping of the extracellular VSP domains into the environment (Papanastasiou et al. 1996) requires a constant secretion and turnover of VSPs on the membrane. Accordingly, intracellular transport of VSP occurs in a constitutive manner. Trafficking of VSP via the ER to the plasma membrane involves the functions of conserved sorting motifs including the membrane-anchoring domain and the cytoplasmic CRGKA domain at the extreme carboxy terminus of VSP (Marti et al. 2003; Touz et al. 2003; Hehl and Marti 2004; Faso and Hehl 2011). A similar secretory pathway has also been postulated for other surface proteins such as those belonging to the family of the invariant cysteine-rich proteins (Davids et al. 2006).

4.2.5.2 Cyst Wall Protein (CWP) Secretion and Cyst Formation

The *G. duodenalis* cyst wall is formed by aggregates composed of three cyst wall proteins (CWP1, CWP2 and CWP3) and a β-(1–3)-GalNAc homopolymer, which represents about 60 % of the cyst wall material (Jarroll et al. 1989; Gerwig et al. 2002; Chatterjee et al. 2010). Apart from CWP1–3, the cyst wall contains some minor proteins including the high cysteine non-variant cyst protein and several

EGF-like cyst proteins (Chiu et al. 2010). In contrast to membrane-anchored surface proteins, proteins constituting the cyst wall of *G. duodenalis* are secreted by a developmentally regulated secretory pathway (Luján et al. 1995; Marti and Hehl 2003; Faso and Hehl 2011). In vivo, detached trophozoites encyst during translocation from the duodenum to lower parts of the small intestine. In vitro, encystation is induced by a basic pH, increased levels of bile salt or decreased levels of cholesterol. Stage conversion is associated with activation of various genes that are structurally involved in cyst formation of the parasite (Faso and Hehl 2011). During encystation, the gene-encoding CWP1–3 (GL50803_5638, GL50803_5435, GL50803_2421) and the gene-encoding enzymes that participate in cyst wall carbohydrate synthesis-like ceramide glucosyltransferase (GL50803_11642) are substantially upregulated during encystation (see information in GiardiaDB). Respective gene regulation occurs on the transcriptional level and involves *cis*-acting elements located in the 5′ flanking region of the genes. Transcription of these genes is activated by a Myb2-like transcription factor that initiates transcription upon interaction with specific binding site in the promoter region (Huang et al. 2008). As particularly shown for CWP1, steady-state levels of cytoplasmatic mRNA are modulated by a cis-element in the 3′ untranslated region. This element seems to be essential for the processing of the transcripts in the late-stage encystation phase (Hehl et al. 2000). At the end of the encystation process, CWP expression and cyst wall formation are supposed to be downregulated by an epigenetic mechanism as discussed for the VSPs. Moreover, regulation may occur on the protein level by deamination of C-terminal arginine residues by arginine deiminase (Vranych et al. 2014).

Cellular synthesis and secretion of CWPs as well as integration of these proteins into the cyst wall have already been elucidated in great detail (Faso and Hehl 2011; Faso et al. 2013). CWP1–CWP3 are closely related proteins of different lengths (CWP1, 241 amino acids; CWP2, 362 amino acids; CWP3, 247 amino acids). They share a hydrophobic signal sequence as a secretory element at the amino terminus, a central region containing four to five tandemly arranged leucine-rich repeats and a cysteine-rich region towards the carboxy terminus. In contrast to CWP1 and CWP3, CWP2 additionally carries a 121 amino acid carboxy-terminal tail that is rich in basic amino acids (see GiardiaDB for details).

In the initial phase of encystation, CWPs are synthesised and are imported into the ER lumen. Simultaneously, early encysting trophozoites start to form encystation-specific vesicles (ESVs) at the ER exit sites (Faso et al. 2012). These vesicular membrane compartments are functionally related to the cisterna of a classical Golgi from a eukaryotic cell and act as carriers for secretion of the de novo synthesised CWPs. Inside the ESVs, accumulated CWPs separates into two fractions that are secreted in a consecutive manner: CWP3 and the proteolytically cleaved, carboxy-terminal part of CWP2 form two distinct condensates within the ESVs (Konrad et al. 2010). Conversely, CWP1 and the amino-terminal part of CWP2 remain in a fluid state. They are separated from the condensed material and accumulate in small compartments close to the cellular periphery. In a next step, the fluid material is secreted and rapidly forms a dense extracellular matrix. Finally, the condensed material retained in the ESVs is slowly secreted and probably constitutes the inner

layer of the cyst wall. This final maturation step stabilises the cyst wall and is essential for infectivity of the cysts.

4.2.5.3 Secreted Enzymes Involved in Host-Parasite Interaction

In the past, various in vitro and in vivo studies demonstrated that attachment of trophozoites to intestinal epithelial cells is fundamental for colonisation of the intestine and for damage of the intestinal mucosa. In these investigations it became evident that mechanical adhesion via the ventral disc plays the major role in attachment of the parasite to the intestinal epithelium. Since the surface of trophozoites contains unique N-acetyl-glucosamine polysaccharides (Ward et al. 1988; Midlej et al. 2013), cross-linking to host cell polysaccharides via specific lectins is also an option for attachment and has been demonstrated by various authors (Magne et al. 1991; Pegado and de Souza 1994; Sousa et al. 2001; Samuelson and Robbins 2011).

During the interaction between the parasite and the intestinal mucosa, several enzymes are secreted by the trophozoites. One example is secreted protease that seems to be involved in the pathogenesis causing damage of the host intestinal mucosa (de Carvalho et al. 2008).

Another example is enzyme that interferes in the production of epithelial nitric oxide (NO). This molecule is supposed to be released by the intestinal tissue of the host and inhibits proliferation of trophozoites as shown by in vitro studies (Eckmann 2003; Fernandes and Assreuy 1997). In intestinal epithelial cells, NO is essentially produced through the enzymatic activity of the inducible NO synthase that relies on arginine as a substrate for NO synthesis (Salzman et al. 1996). Trophozoites are able to counteract epithelial NO production by depletion of arginine via secretion of arginine-consuming enzymes, particularly arginine deiminase and ornithine carbamoyl transferase (Ringqvist et al. 2008; Stadelmann et al. 2012, 2013). This effect is enhanced by the capability of *G. duodenalis* to efficiently absorb, and metabolise, arginine, thus allowing the parasite to further lower the local concentration of this compound in the intestinal environment (Edwards et al. 1992). Interestingly, arginine deiminase secreted by trophozoites also affects the phenotype and cytokine production of dendritic cells (Banik et al. 2013) and reduces proliferation of T cells in vitro (Stadelmann et al. 2013). Both epithelial NO production and T-cell proliferation can be restored to normal levels by addition of arginine, or citrulline (a metabolite of arginine), to the culture medium (Stadelmann et al. 2013). Complementary to the mechanisms described above, protection of *Giardia* from NO attack is conferred by the intracellular activity of NO-detoxifying enzymes such as flavohemoglobin (Mastronicola et al. 2010; Stadelmann et al. 2013) or nitroreductases (Nillius et al. 2011; Müller et al. 2013, 2015).

4.3 Recent Advances and Open Questions

In the last few years, the *Giardia* genome-sequencing effort (Morrison et al. 2007) has generated suitable tools to study the cellular and molecular biology of the parasite. As a consequence, its importance as a model organism to investigate basic

cellular functions of a primitive eukaryote increases. Studies on protein trafficking, for instance, reveal simple but efficient mechanisms of this cellular pathway (Faso and Hehl 2011). Most of these investigations are based on reverse genetic approaches profiting from recent advances in plasmid vector development and transfection technology. These technical achievements allow (over)expression of recombinant proteins in trophozoites (Davis-Hayman and Nash 2002). Until now, recombinant expression can be achieved either constitutively, e.g. under control of the glutamate dehydrogenase gene promoter (Yee et al. 2000; Müller et al. 2009; Nillius et al. 2011) or under control of a CWP promoter that can be induced by initiating encystation in trophozoite cultures (Faso and Hehl 2011). Furthermore, alternative vector systems conferring inducible expression of recombinant proteins in non-encysting, proliferative trophozoites are currently in preparation. A disadvantage of *Giardia* as compared to model systems such as yeast is the lack of gene knockout systems based on homologous recombination. Recently, a technique involving transient expression of the Cre/loxP recombination/excision system has been developed (Wampfler et al. 2014). This tool will be further developed to allow insertional inactivation of all loci from any target gene on the binucleate and tetraploid genome of the parasite.

In future *Giardia* research, insertional inactivation of selected target genes and/or insertion of detectable markers within such genes will yield novel tools to explore if sexual recombination in *Giardia* effectively occurs, i. e. if the paired nuclei present in *G. duodenalis* trophozoites are able to exchange genetic material. If the individual nuclei can be targeted with different genetic markers, researchers will be able to assess if the two nuclei in fact are equivalent regarding their genetic contents and their transcriptional activities. In this respect, it will be especially interesting to learn more about the regulatory mechanisms that coordinate the transcription of the two nuclei. Perhaps, a combination of those approaches mentioned above will contribute to the elucidation of the biological significance of the binucleate characteristics of *G. duodenalis* trophozoites. Similar transgenic strategies may also be suited to examine the mechanisms responsible for RNAi-dependent VSP switching during antigenic variation.

During a *G. duodenalis* infection, the parasite undergoes cellular differentiation from an environmentally resistant but metabolically dormant cyst to a proliferating trophozoite that is able to persist within its hostile intestinal habitat. In order to sense, and subsequently adapt to, these changing living conditions, the parasite should have efficient receptor-signalling systems. Meanwhile, cAMP and calcium-dependent protein kinases have been identified (Abel et al. 2001; Reiner et al. 2003; Gibson et al. 2006; Cho et al. 2012). Unfortunately, a profound characterisation of respective surface receptor molecules as well as the molecular mechanisms initiating the corresponding signalling cascades is lacking and should be prioritized in future investigations. Here again, the knowledge of the entire *G. duodenalis* genome is a prerequisite for unravelling the complex sensing and signalling pathways of the parasite.

Finally, we do not know which parasite- and host-derived factors direct a *G. duodenalis* infection to either an asymptomatic, acute or chronic course (Müller and von Allmen 2005; Roxström-Lindquist et al. 2006; Solaymani-Mohammadi and Singer 2010). Various immunocompetent and immunocompromised murine

infection models help to understand the (patho)immunological processes associated with a *Giardia* infection. However, most animal models applied so far have strong experimental limitations particularly because they are unable to adequately simulate a symptomatic *Giardia* infection as it may take place in most natural hosts. As an experimental strategy complementary to studies in animal infection models, the molecular and cellular host-parasite interphase can also be explored in an in vitro cocultivation system where *G. duodenalis* trophozoites are grown in the presence of intestinal epithelial cells. Here, a cocultivation system separating host and parasite via a semipermeable microcultivation chamber is a promising tool for future studies. Immunocompetent cells may even be included in a third compartment (Bermudez-Brito et al. 2013). It is evident that compartmentalised cocultures reflect the complex situation existing within the intestinal environment from a *G. duodenalis*-infected host only to limited extent. However, such a coculture represents a relatively well-defined experimental set-up that is compatible with modern molecular or biochemical techniques such as reverse transcript microarray-based transcriptomics or mass spectrometry-based proteomics (Hutchins 2014). These methods are predestinated to monitor the gene/protein expression patterns underlying the overall characteristics of the host-parasite relationship. In combination with comprehensive data available from in vivo or ex vivo experiments, findings from such investigations may uncover essential molecular clues to antigiardial immunity and *Giardia*-induced immunopathology that both strongly influence the intensity and severity of a *G. duodenalis* infection.

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Trichomonas

5

Pier Luigi Fiori, Paola Rappelli, Daniele Dessì, Robert Hirt, Sven Gould, Jan Tachezy, and Ivan Hrdy

Abstract

Trichomonas vaginalis is a flagellated eukaryotic microorganism, which parasitizes the human urogenital mucosa. This pathogen is responsible for the most prevalent, nonviral, sexually transmitted infection worldwide; despite the high prevalence of the disease, relevant aspects of the pathophysiology of *T. vaginalis* are still obscure, and the impact of the infection on public health is greatly underestimated, especially in developing countries. Recently, trichomoniasis has been classified as a neglected infection. In order to establish the infection in humans, *T. vaginalis* has evolved a number of sophisticated and multifaceted colonization and virulence strategies, based both on production of toxic molecules and enzymes and on subversion of the host immune response. Even if trichomoniasis is primarily considered as a source of morbidity in the human reproductive tract, increasing interests of parasitologists and clinicians have recently focused on severe complications associated with *Trichomonas* infection, such as adverse pregnancy outcomes,

P.L. Fiori (✉) • P. Rappelli • D. Dessì
Department of Biomedical Sciences, University of Sassari,
Viale San Pietro 43/b, 07100 Sassari, Italy
e-mail: fioripl@uniss.it

R. Hirt
Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences,
Newcastle University, Newcastle upon Tyne, UK
e-mail: robert.hirt@newcastle.ac.uk

S. Gould
Institute for Molecular Evolution, Heinrich-Heine-University,
Universitätsstr. 1, Düsseldorf 40225, Germany
e-mail: gould@hhu.de

J. Tachezy • I. Hrdy
Department of Parasitology, Faculty of Science, Charles University in Prague,
Vinicna 7, Prague 2 128 44, Czech Republic
e-mail: jan.tachezy@natur.cuni.cz

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facilitation of HIV transmission, and association with cervical and prostate cancer. Nitroimidazole derivatives remain the sole treatment of trichomoniasis, and despite an ever increasing number of metronidazole-resistant isolates has been reported in the last years, effective alternative therapies are not yet available. All these aspects of pathobiology of *T. vaginalis* will be discussed in the present chapter, on the basis of the most recent results of molecular and "omics" investigations.

5.1 Introductory Remarks

Trichomonas vaginalis is an early diverging flagellated protist belonging to the Parabasalia, a group which comprises a monophyletic assemblage of 80 genera and 400 species. Even if most parabasalids inhabit the digestive tract of animals (including birds, termites, and cockroaches), several species are considered of medical importance.

T. vaginalis is the etiological agent of the most common nonviral sexually transmitted disease in humans. Despite having the highest prevalence of any nonviral sexually transmitted disease, with an estimated incidence of 248 million new cases per year, higher than syphilis, gonorrhea, and chlamydia combined (World Health Organization 2012), many fundamental aspects of *T. vaginalis* pathobiology remain poorly understood, and trichomoniasis can be considered a neglected disease.

Trichomoniasis has long been overlooked as simply a curable sexually transmitted infection, clinically characterized by vulvar inflammation, copious greenish malodorous vaginal discharge, and dyspareunia in females and urethral inflammation with discharge and dysuria in males. Almost all males and 50 % of women are asymptomatic, and infection can persist for a long time in the urogenital tract. In recent years, the infection has been associated with a number of important pathological conditions such as adverse pregnancy outcomes (Fichorova 2009), infertility, pelvic inflammation, up to two–fourfold promotion of HIV-1 infection acquisition (McClelland et al. 2007), and increased incidence of aggressive cervical and prostate cancer (Zhang and Begg 1994; Sutcliffe et al. 2006; Twu et al. 2014).

One of the most intriguing aspects of *T. vaginalis* is the complex relationship with two intracellular symbionts: a dsRNA virus (TVV) and the bacterium *Mycoplasma hominis*. Both microorganisms seem to influence the lifestyle of the protist, upregulating the inflammatory response of the human host, thus potentially affecting the clinical evolution of the protozoan infection. The endosymbiotic relationship between *T. vaginalis* and *M. hominis* is the first described involving two obligate human parasites that produce independent diseases in the same anatomical region.

Routine diagnosis of *T. vaginalis* infection is based on direct microscopic examination of wet mount preparations and on culture-based techniques, but both are limited by a low sensitivity (from 38 to 82 %), being strongly dependent on the number and on the viability of protozoa and expertise of the analyst. In recent years, more sensitive and specific molecular techniques have been proposed (Hobbs and Seña 2013).

The therapy against infection is still based on the use of a single class of antimicrobial molecules, the nitroimidazole derivatives (i.e., metronidazole or tinidazole),

but an increasing number of cases of allergic reactions and resistance to drug treatment have been recently reported.

Thanks to the complete genome sequencing, the recent proteomic and transcriptomic approaches, and the studies of genome-wide variations in different isolates, a better understanding on some fundamental biological features of this intriguing neglected protist has been achieved.

5.2 Well-Established Facts

5.2.1 The Genome and Its Transcription (Sven Gould and Robert Hirt)

5.2.1.1 A Surprising Large Genome and a Massive Potential Proteome

With an estimated size of ~160 Mbp, the parabasalian protist *T. vaginalis* (isolate G3) is currently the parasitic protist endowed with the largest sequenced genome (Table 5.1) (Carlton et al. 2007, 2010; Zubacova et al. 2008). This relatively large haploid genome is distributed over six chromosomes (Carlton et al. 2007). This was a rather unexpected discovery for a genome that was assumed to be ~10× smaller and flew against the general perception that parasites tend to reduce their genomes compared to their free-living relatives as they adapt to a host-dependent lifestyle (Carlton et al. 2007). It is in particular much larger than the extracellular mucosal parasites *Giardia lamblia* and *Entamoeba histolytica* (Table 5.1), with somehow similar mucosal anaerobic/micro-aero-tolerant lifestyles sharing a number of metabolic similarities (Clark et al. 2010), some acquired from bacterial sources through lateral gene transfers (LGTs) (Carlton et al. 2007; Alsmark et al. 2013) (Table 5.1). A survey of 11 genomes from nine different trichomonads species indicated that *T. vaginalis* is not unique in respect to its large genome with values ranging from 86 to 177 Mbp (Zubacova et al. 2008). Even more unexpected was that a remarkable 97,883 protein coding genes were initially annotated from the 17,290 scaffolds generated for this highly repetitive (65 % is repetitive) genome, explaining the difficulty in assembling the sequence reads and complicating gene annotations (Carlton et al. 2007; Conrad et al. 2013). Further scrutiny reduced this vast conceptual proteome to a still astonishing 59,681 genes considered to represent the core set of genes, currently the largest repertoire of annotated protein coding genes for a microbial eukaryote (Conrad et al. 2013). This number is a remarkable seven to nine times larger than for the extracellular mucosal parasites *E. histolytica* and *G. lamblia* (Table 5.1). The remaining 38,213 genes were deprecated as highly repetitive elements including ~14,000 genes related to transposable elements and viral-like genes and 31 unclassified repetitive families (Carlton et al. 2007; Conrad et al. 2013). Most of the 59,681 protein-coding genes are members of gene families, some rather large (the largest 31 families make up 24,981 proteins), highlighting numerous gene duplication events (Carlton et al. 2007; Conrad et al. 2013). This is in contrast to other microbial parasites with relatively large genomes such as the recently

Table 5.1 Comparison of the taxonomy, lifestyle, and genome of *T. vaginalis* with selected parasitic protists

Species (strains/isolate)	Taxonomy[a]	Parasitic lifestyle	Estimated genome size (Mbp)	#Annotated protein coding genes	Source
Trichomonas vaginalis (G3)	Excavata – Metamonada	Extracellular, mucosal – sexually transmitted	~160	59,671	Carlton et al. (2007)
Entamoeba histolytica (HM-1:IMSS)	Amoebozoa – Archamoebae	Extracellular, mucosal – oral-fecal route	20.8	8,201	Caler and Lorenzi (2010)
Giardia lamblia (WB)	Excavata – Metamonada	Extracellular, mucosal – oral-fecal route	11.7	6,470	Morrison et al. (2007)
Sarcocystis neurona (SN3)	SAR[b] – Alveolata	Intracellular mucosal/other tissues – oral-fecal and predatory-prey cycle	130	7,093	Blazejewski et al. (2015)
Toxoplasma gondii (ME49)	SAR[b] – Alveolata	Intracellular mucosal/other tissues – oral-fecal and predatory-prey cycle	65.7	8,322	EuPathDB (2013)
Trypanosoma cruzi (CL Brener Non-Esmeraldo-like)	Excavata – Discoba	Extra- and intracellular, insect transmitted parasite of mammals[c]	32.5	9,380	EuPathDB (2014)
Encephalitozoon intestinalis (ATCC 50506)	Opisthokonta – Nucletmycea (fungi)	Intracellular, mucosal – oral-fecal route	2.3	1,939	EupathDB (2014)

[a]Taxonomy according to Adl et al. (2012). Only the super-group and the following taxonomic level are indicated

[b]SAR stands for Stramenopile, Alveolata, and Rhizaria (Adl et al. 2012)

[c]Can infect mammalian hosts through skin injuries or mucosal surfaces from the digestive tract

sequenced *Sarcocystis neurona* (130 Mbp) that is characterized by similar protein complement (~7,000 genes) compared to other apicomplexans with smaller genomes such as *Toxoplasma gondii* (63 Mbp, ~8,000 genes). The larger genome of *S. neurona* is explained by an increase of repetitive elements and larger introns and intergenic regions (Blazejewski et al. 2015). Notably, only 65 *T. vaginalis* protein coding genes were identified to possess introns (~0.1 % among 46,000 genes) (Carlton et al. 2007), in mark contrast to other parasites such as *E. histolytica* and *T.*

gondii with 24 % and 74 % of their genes, respectively, possessing introns (Caler and Lorenzi 2010; Khan et al. 2007). In the case of *T. vaginalis*, gene duplications have contributed in dramatic ways to both the relatively large genome and its vast coding capacity (Carlton et al. 2007).

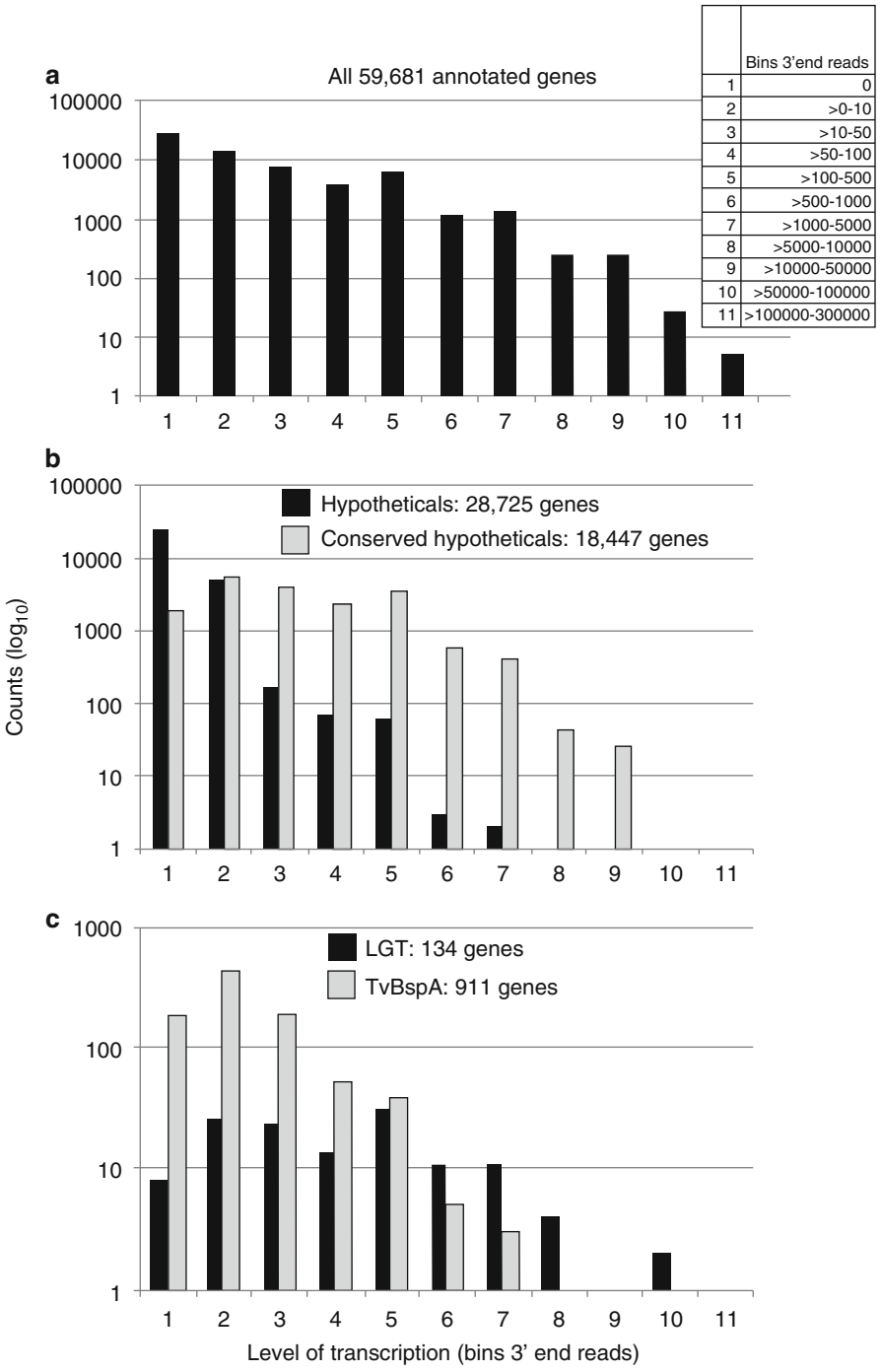
Identifying likely gene fragments in the *T. vaginalis* G3 annotation (annotated genes 100 % identical to candidate full-length genes) identified a core set of ~46,000 candidate complete genes (Smith and Johnson 2011). This is still a rather formidable coding capacity for a microbial eukaryote and a parasite in particular. Hence, it will be important to compare a number of *T. vaginalis* strains and related species to rationalize the origins of this vast protein coding capacity and contribute at deciphering their biological relevance (Conrad et al. 2013; Maritz et al. 2014).

5.2.1.2 A Transcriptomics and Proteomics View on the Vast Potential Proteome

With the potential to encode such a large repertoire of proteins, it is essential to establish what genes are expressed and functional across *T. vaginalis* isolates to be able to investigate more effectively the molecular cell biology of the parasite and its links with its pathobiology. A number of proteomics analyses have directly investigated expressed proteins including surface (411 proteins) (de Miguel et al. 2010a), secreted exosomal proteins (215 entries) (Twu et al. 2013), and hydrogenosomal proteins (~600 entries) (Schneider et al. 2011; Rada et al. 2011; Beltran et al. 2013a) and various other proteins including proteases (Quintas-Granados et al. 2013; Ramon-Luing et al. 2010; Huang et al. 2009). Proteomics surveys provide the most direct evidence for functional proteins and uncovered important new players in the host/parasite interactions and in the parasite biochemistry (Hirt et al. 2011); however these investigations identified so far only a small fraction of the annotated proteins (~1300 proteins versus 46,000, ~3 %).

More recently, deep sequencing of cDNA libraries for two distinct *T. vaginalis* isolates grown in different conditions (exposure to oxygen and human epithelial cells or glucose restriction) generated the first global investigations of the transcription profile for the parasite (Gould et al. 2013; Huang et al. 2014; Woehle et al. 2014), complementing data based on microarrays and ESTs (reviewed in (Conrad et al. 2013)). The RNA-seq data supported the expression of about half of the 59,681 annotated genes and a core set of ~23,000 genes that were found to be expressed in all 11 sequenced libraries by (Gould et al. 2013). While only about half of all the annotated genes are expressed, in almost all cases at least one member of a given gene family is transcribed (Gould et al. 2013). The proportion of expressed members from a gene family is neither associated with the size of the gene family nor with the overall expression level. Notably the largest set of non-transcribed genes corresponds to hypothetical genes, i.e., those with no hits in proteins databases during the initial annotation (Carlton et al. 2007) (Fig. 5.1).

The level of transcription of different gene sets was contrasted through histograms with 11 bins reflecting no read (bin 1, zero mean 3′-normalized reads across 11 conditions in Gould et al. 2013) to the highest level of transcription (bin 11, >100,000–300,000 mean reads). The inset in the panel A shows all bins with different levels of


a
All 59,681 annotated genes
100000
10000
1000
100
10
1
1 2 3 4 5 6 7 8 9 10 11
Bins 3'end reads
1 0
2 >0-10
3 >10-50
4 >50-100
5 >100-500
6 >500-1000
7 >1000-5000
8 >5000-10000
9 >10000-50000
10 >50000-100000
11 >100000-300000
b
Hypotheticals: 28,725 genes
Conserved hypotheticals: 18,447 genes
Counts (log10)
c
LGT: 134 genes
TvBspA: 911 genes
Level of transcription (bins 3' end reads)

transcription expressed as the mean of 3'-normalised reads across 11 tested growth conditions from Gould et al. (2013). (A) The variation in transcription level between all annotated protein coding genes from the *T. vaginalis* G3 genome sequence data (see main text, Carlton et al. 2007). (B) Comparison of protein-coding genes annotated as "hypotheticals" (black bars, with no BlastP hits in databases) versus "hypothetical conserved" (gray bars, with BlastP hits in databases) (Annotation in GenBank derived from Carlton et al. 2007). (C) Comparison of the level of transcription of all 134 LGT cases identified in Alsmark et al. (2013) (black bars) versus the 911 TvBspA genes identified by Noel et al. (2010) (*gray bars*). The LGT genes are notably skewed toward the right hand side of the histogram (higher level of transcription) compared to the TvBspA and "hypotheticals" genes. This suggests that the majority of LGTs are likely to be functionally integrated into the biology of the parasite whereas the great majority of "hypotheticals" are not and might represent pseudogenes or miss-annotations of spurious genes. A number of TvBspA are not transcribed or have low level of transcription whereas a number of gene have higher level of transcription. Counts in the different bins are shown as Log10 to facilitate comparisons between large and small counts. Modified from Hirt et al. (2015).

These non-transcribed hypothetical genes could correspond to novel genes (formed de novo from scratch (Chen et al. 2013)), highly divergent versions of genes with homologues in databases, homologues of currently un-sampled genes or spurious annotations. Comparison between different clinical strains and a broader range of growth conditions will be important to establish a more accurate estimation of the proportion of transcribed genes. Furthermore comparative genomics and transcriptomics with various trichomonads species will also play a role in identifying genuine functional genes, in particular the *Trichomonas* species isolated from birds that are closely related to *T. vaginalis* (Maritz et al. 2014).

Fig. 5.1 Transcription level of different gene categories. The level of transcription of different gene sets was contrasted through histograms with 11 bins reflecting no read (bin 1, zero mean 3'-normalized reads across 11 conditions in Gould et al. 2013) to the highest level of transcription (bin 11, >100,000 to 300,000 mean reads). The inset in the panel A shows all bins with different levels of transcription expressed as the mean of 3-'normalised reads across 11 tested growth conditions from Gould et al. (2013). (**a**) The variation in transcription level between all annotated protein coding genes from the T. vaginalis G3 genome sequence data (see main text, Carlton et al. 2007). (**b**) Comparison of protein-coding genes annotated as "hypotheticals" (*black bars*, with no BlastP hits in databases) versus "hypothetical conserved" (*grey bars*, with BlastP hits in databases) (Annotation in GenBank derived from Carlton et al. 2007). (**c**) Comparison of the level of transcription of all 134 LGT cases identified in Alsmark et al. (2013) (black bars) versus the 911 TvBspA genes identified by Noel et al. (2010) (*grey bars*). The LGT genes are notably skewed towards the right hand side of the histogram (higher level of transcription) compared to the TvBspA and "hypotheticals" genes. This suggests that the majority of LGTs are likely to be functionally integrated into the biology of the parasite whereas the great majority of "hypotheticals" are not and might represent pseudogenes or miss-annotations of spurious genes. A number ot TvBspA are not transcribed or have low level of transcription whereas a number of gene have higher level of transcription. Counts in the different bins are shown as Log10 to facilitate comparisons between large and small counts. Modified from Hirt et al. (2015)

5.2.1.3 Non-coding RNA (ncRNA)

It is increasingly recognized that an important and broad diversity of functions (in addition to tRNAs and rRNAs mediating translation), including regulation of gene expression and protection against foreign nucleic acids, is mediated by ncRNAs (Cech and Steitz 2014). Compared to mammals and other more intensively studied model systems, far less is known about ncRNA among parasitic protists such as *T. vaginalis* (Collins 2011). It was recently established that *T. vaginalis* expresses a large quantity of pseudogenes and long non-coding RNA (lncRNA) and it was hypothesized that these might represent an important gene pool for gene innovation (Woehle et al. 2014). In contrast to yeast, the lncRNAs from *T. vaginalis* were shown to be transcribed from their own promoters (Woehle et al. 2014). There is also accumulating evidence that the parasite expresses other ncRNAs including small ncRNAs (Collins 2011). Based on these initial data, *T. vaginalis* promises to represent an important model system to broaden our understanding of ncRNA functions and evolution (Woehle et al. 2014; Collins 2011). Investigating ncRNA in *T. vaginalis* might also identify novel important aspects of the molecular basis of its pathobiology and by doing so novel candidate targets may be identified to disrupt the workings of the parasite for therapeutic purposes (Collins 2011).

5.2.2 Cellular Architecture (Sven Gould and Paola Rappelli)

The general cell biology of *T. vaginalis* is that of a typical protist cell. *Trichomonas vaginalis* trophozoites can vary in size and shape, depending on environmental conditions such as temperature and pH. The shape of individual *T. vaginalis* cells is usually more uniform among in vitro laboratory cultures than those of freshly isolated protists. A nondividing *T. vaginalis* grown under axenic conditions is typically piriform (pear-shaped) and measures, on average, 10×7 μm. The protist has five flagella, four of which are located at its anterior end, plus a fifth recurrent flagellum that is anchored to an undulating membrane that runs toward the posterior end by unknown means. This fifth flagellum and the undulating membrane are responsible for the typical quivering motility of the protist. The majority of the rough endoplasmic reticulum is often observed to form an almost closed ring around the nucleus, but which is composed of a few individual sheets. The cells also have a rather pronounced Golgi apparatus located toward the apical end of the cell that is often seen in duplicate form, also in cells that do not appear to be dividing.

Binary fission is the predominant form of *Trichomonas* division, but cells with more than two, and up to eight nuclei, have been observed, from which individual daughter cells separate (Kusdian et al. 2013; Yusof and Kumar 2012). Cell division commences with the internalization of the flagella and division of the pelta, driven by the centrosome-like atractophore (Bricheux et al. 2007). In interphase the *T. vaginalis* trophozoite has a single nucleus located at the anterior portion of the parasite, surrounded by a porous nuclear envelope. The nucleus contains six chromosomes, together encoding a genome of approximately 160–175 Mbp (Carlton et al. 2010; Smith and Johnson 2011), making it the largest protist genome sequenced so far.

The parasite uses both the tubulin and the actin cytoskeleton for locomotion. The latter plays a major role during morphogenesis and when the cells turn amoeboid after being exposed to host tissue or fibronectin-coated slides, as shown through experiments focusing on alpha-actinin (Addis et al. 1998) and fimbrin (Kusdian et al. 2013). In fresh isolates the cells are more often observed to be amoeboid than under axenic in vitro culture. How severe trichomoniasis develops depends very much on the parasite's ability to undergo morphogenesis. Some, but not all, *T. vaginalis* strains have the ability to switch from a free-swimming flagellate to an adherent amoeboid form upon contact with host tissue (Lal et al. 2006). Only *Trichomonas* strains able to undergo morphogenesis are considered to be pathogenic. Amoeboid parasites can migrate across host tissue with up to 20 μm/min using actin its accessory proteins (Kusdian et al. 2013). This type of phenotypic plasticity is rather special in comparison to other parasites: it is much faster than in the heterolobsean amoeba *Naegleria* (in some virulent strains such as FMV1 it takes only minutes), it is life cycle independent, and the force that allows the parasite to migrate across host tissue is myosin independent. To coordinate morphogenesis the parasite encodes virtually all canonical actin- and tubulin-interacting proteins conserved among eukaryotes (Kusdian et al. 2013). Amoeboid parasites do not internalize their five flagella—this only occurs during mitosis—and the switch back to a free-swimming protist is hence also rapid. It is not known how the difference in the response to host tissue and subsequent morphogenesis is linked to the large genetic variety observed among hundreds of isolates (Conrad et al. 2012).

A few things, however, are special in *Trichomonas*. The parasite is a rare example of a eukaryote that appears to completely lack peroxisomes. *Trichomonas* also houses no canonical mitochondria, but, like all other parabasalian protists, anaerobic hydrogenosome that are reduced mitochondria. Hydrogenosomes are spherical organelles, measuring from 200 nm to 1 μm, and that can sometimes be associated with the axostyle and costa (paracostal and paraxostilar hydrogenosomes). Like mitochondria they have two closely apposed membranes that surround an electron-dense granular matrix. Hydrogenosomes lack a genome as shown by immunoelectron microscopy, coupled with in situ nick translation and hence also a translation machinery. They import all their several hundred proteins from the cytosol (Rada et al. 2011; Burstein et al. 2012; Schneider et al. 2011).

A slender noncontractile structure, the costa, is thought to support the undulating membrane from the cytosolic side against the sheering forces generated by the attached flagella. Together with the pelta-axostyle complex, the costa represents a characteristic cytoskeletal feature of the Trichomonadida lineage. The exact nature of the costa is unknown, but appears to be mainly composed of proteins ranging between 100 and 135 kDa (Viscogliosi and Brugerolle 1994). The pelta-axostyle complex is a rigid cytoskeletal structure composed of dozens of tightly in parallel arranged microtubules that are connected by microfibrillar bridges (de Andrade Rosa et al. 2013). No free cytoplasmic MTs have been found in this parasite. The pelta forms the flagellar canal and supports the anterior flagella. The anterior part of the axostyle is wider and forms the capitulum, into which the nucleus is partly

embedded. Posteriorly to the nucleus it turns upon itself, forming a tube known as the axostilar trunk that extends through the entire cell and protrudes through the posterior end of the parasite as a thin tip, still covered by the plasma membrane. Apart from the microscopic observation that the axostyle provides the cell with a stiff axis, other functions, if they exist, remain unknown.

5.2.3 Surface Structures (Robert Hirt)

Surface structures from both parasites and their host cells are key players in host/parasite interactions but we still know little about this fundamental aspect of *T. vaginalis* pathobiology with only a few parasite surface proteins and glycolipids functionally investigated (Hirt et al. 2011; Ryan et al. 2011a; Kusdian and Gould 2014). Notably, only one human receptor has been identified so far with galectin-1 being targeted by the parasites' lypoglycans (Ryan et al. 2011b; Okumura et al. 2008). Here we will focus on a selection of some recent data and direct the readers to complementary reviews for a broader perspective on the topic (Hirt et al. 2011; Ryan et al. 2011a; Kusdian and Gould 2014; Figueroa-Angulo et al. 2012a).

The availability of the genome sequence and its annotation have greatly benefited the study of surface proteins (Carlton et al. 2007; Hirt et al. 2011; Hirt et al. 2007). There is indeed now a large list of candidate surface proteins identified in silico of which molecular cell parasitologists have taken advantage to boost the characterization of the complex interactions taking place between *T. vaginalis* and the various landmarks of human mucosal surfaces from both male and female hosts. Of particular interest are a number of gene families likely to be important for the biology of the parasite that have been selectively expanded or retained after gene (and/or genome wide) duplication events (Hirt et al. 2011; Hirt et al. 2007). Functional characterization of these expanded protein family members will be required to test the hypothesis they are mediating important functions. Indeed, existing data on surface proteins differentially expressed by different strains and similarities between *T. vaginalis* proteins and proteins from other pathogens have highlighted a number of interesting features for different gene families (de Miguel et al. 2010a; Hirt et al. 2011).

The *Bacteroides forsythus* surface protein A (TvBspA) family represents such a potential candidate family as bacterial BspA-like proteins were shown to play various roles in the pathobiology of several bacterial pathogens (Noel et al. 2010). It is one of the largest gene families of *T. vaginalis* and 721 of the 911 annotated TvBspA genes (Noel et al. 2010) were transcribed in tested growth conditions (Gould et al. 2013) (Fig. 5.2). Nearly 200 TvBspA proteins possess an identifiable transmembrane domain (TMD) and some of these possess conserved C-terminal domains shared with other candidate surface proteins, which are characterized by sequence features that potentially recognized by the endocytic/phagosomal machinery (Hirt et al. 2011; Noel et al. 2010). The N-termini of the BspA proteins are characterized by leucine-rich repeats (LRRs) and are inferred to be exposed to the extracellular milieu and are highly diverse in both length and sequence, which makes them prime

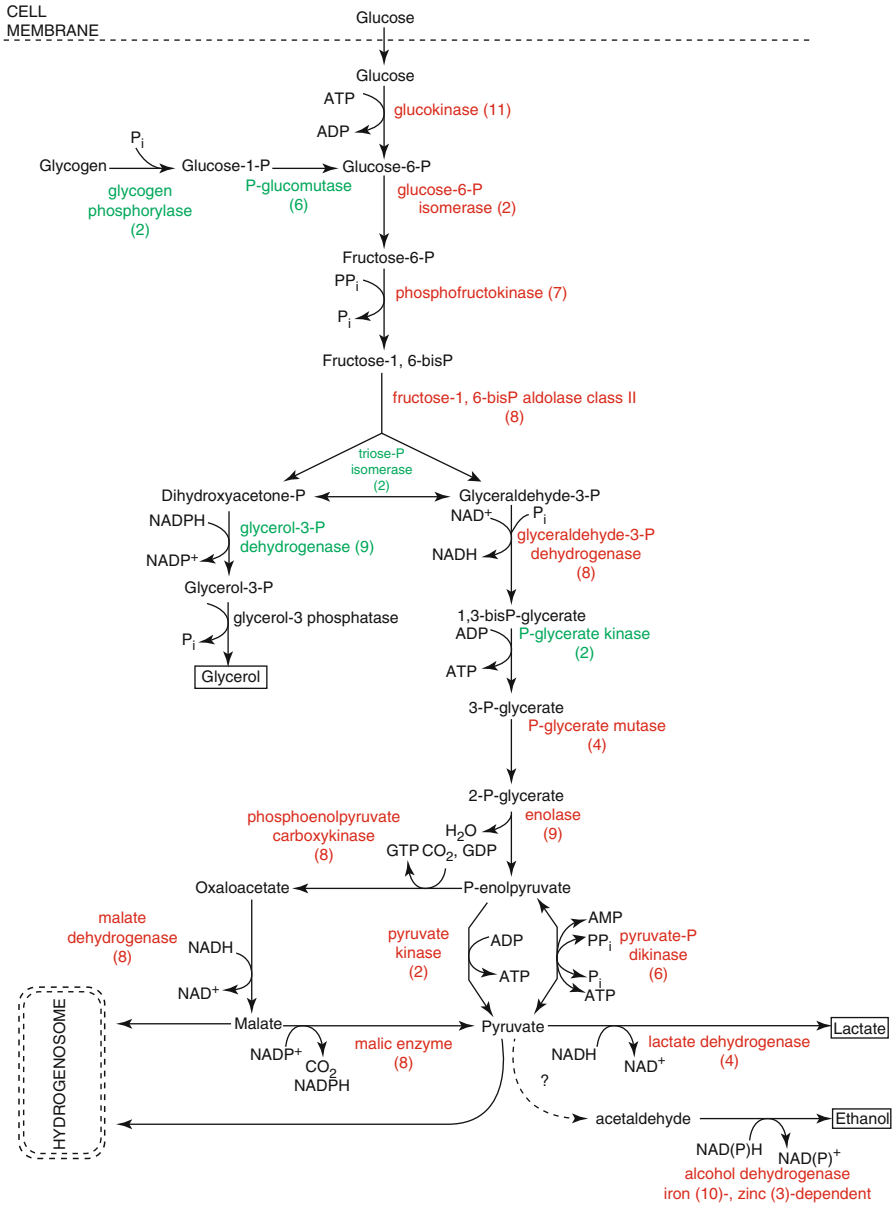


Fig. 5.2 The mosaic character of glycolytic pathway in *Trichomonas vaginalis*. Glycolysis proceeds via two pyrophosphate (PPi)-dependent enzymes: PPi-dependent phosphofructokinase and pyruvate phosphate dikinase. These enzymes allow the increased yield of glycolytic ATP due to the replacement of ATP with PPi as a phosphate donor. The main glycolytic end-products are lactate and glycerol, although small amount of ethanol is also formed. Malate and pyruvate serve as hydrogenosomal substrates. Phylogenetic analysis of glycolytic enzymes suggested that only five enzymes are of eukaryotic origin (*in green*), while most enzymes were probably gained by lateral gene transfer from various prokaryotes (*in red*). Numbers in brackets indicate the number of paralogs in *T. vaginalis* genome

candidates for "variant surface proteins" (Hirt et al. 2011; Noel et al. 2010). Their functions might include binding to extracellular targets such as members of the microbiota, host-secreted and cell surface proteins, evading the host adaptive immune response and involved in parasite-parasite interactions (Hirt et al. 2011). One TvBspA protein with a TMD (TVAG_073760) was expressed on the cell surface of the parasite grown in vitro and elicited an antibody response in *T. vaginalis*-infected patients (Noel et al. 2010). Another TvBspA protein (TVAG_240680) with a TMD was shown to be secreted via exosomes and could contribute at modulating the parasite binding properties to host cells after the TvBspA protein is inserted into the host plasma membrane following the fusion of the parasite exosomes to the host plasma membrane (Twu et al. 2013).

Another family of *T. vaginalis* membrane proteins of great interest which has been investigated in more detailed are the tetraspanins (TvTSPs), known in other systems to coordinate numerous intracellular and intercellular processes including signaling, adhesion, migration, and host-pathogen interactions via a network of protein-protein interactions thought to regulate cell and membrane compartmentalization (Charrin et al. 2014). There are at least 13 TvTSP members, with most entries expressed on the parasite cell surface and one (TvTSP1) that is also secreted via exosomes (Twu et al. 2013; Coceres et al. 2015; de Miguel et al. 2012). The expression of most TvTSP genes was also shown to be upregulated upon parasite binding to human VEC cells (Coceres et al. 2015). One tetraspanin (TvTSP8) was also shown to modulate parasite-parasite interactions potentially playing an important role during parasite swarming, a process influencing both parasite binding properties and cytotoxicity to human cells (Coceres et al. 2015; Lustig et al. 2013; Honigberg 1990).

5.2.4 Important Enzymes and Special Metabolic Features (Jan Tachezy and Ivan Hrdy)

5.2.4.1 Background Information

Trichomonas vaginalis is an anaerobic pathogen in the sense that (i) its energy metabolism is not dependent on molecular oxygen as a terminal electron acceptor, (ii) the parasite is insensitive to inhibitors of mitochondrial respiration, and (iii) the cells can grow well under fully anaerobic conditions. Cellular free-energy carrier ATP is exclusively synthesized by substrate-level phosphorylation including Embden-Meyerhof-Parnas (EMP) glycolytic pathway in the cytosol (Arese and Cappuccinelli 1974; Steinbüchel and Müller 1986a) and extended glycolysis in hydrogenosomes (Hrdy and Müller 2008; Lindmark et al. 1975), an anaerobic form of mitochondria (Müller 1997; Müller et al. 2012). In addition to carbohydrates, energy conservation is supported by amino acid metabolism, particularly by the arginine dihydrolase pathway (Yarlett 1988; Yarlett et al. 1996). However, *T. vaginalis* is not a strict anaerobe. The cells are well adapted to tolerate fluctuating levels of oxygen that is present in the environment of the vaginal mucosa. The cytosol possesses highly active oxygen-scavenging enzymes such as NADH and NADPH

oxidases (Leitsch et al. 2014; Linstead and Bradley 1988; Rasoloson et al. 2001; Tanabe 1979), thioredoxin system (Coombs et al. 2004; Iulek et al. 2006), and superoxide dismutase, respectively (Rasoloson et al. 2001; Ellis et al. 1992; Hwang et al. 2002; Viscogliosi et al. 1996). The hydrogenosomes represent the second cellular compartment that metabolizes oxygen. Consumption of oxygen by hydrogenosomes was described as respiration (Cerkasov et al. 1978; Müller and Lindmark 1978; Ohnishi et al. 1980); however, unlike mitochondria hydrogenosomes do not possess a respiratory chain and reduction of oxygen is catalyzed by soluble oxidoreductases such as the flavodiiron protein (Smutna et al. 2009). Therefore, it was proposed to consider *T. vaginalis* an anaerobic aerotolerant pathogen. Moreover, testing of in vitro *T. vaginalis* growth conditions revealed that parasites grow significantly faster under the atmosphere containing low (micromolar) level of oxygen and millimolar concentration of CO_2 in comparison with strict anaerobiosis (Paget and Lloyd 1990). These observations suggested that *T. vaginalis* is possibly not only aerotolerant but rather a microaerophilic organism.

5.2.4.2 Energy Metabolism

In most eukaryotes, cytosol and mitochondrion are the main sites of energy metabolism to which glycolysis and oxidative phosphorylation are compartmentalized, respectively. Glycolysis converts 1 mol of glucose into 2 moles of pyruvate with a net yield of 2 moles of ATP. Mitochondrial metabolism of pyruvate and glycolysis-derived-reducing equivalents allows synthesis of up to additional 30 moles of ATP per mole of glucose of which 23 moles are generated by oxidative phosphorylation. Anaerobes such as *T. vaginalis* are unable to utilize oxygen in energy-conserving reactions, thus the efficiency of their energy metabolism is dramatically reduced. In hydrogenosomes, oxidative phosphorylation is absent, and only a single mole of ATP per mole of pyruvate is synthesized by substrate-level phosphorylation. To increase glycolytic efficiency, *T. vaginalis* replaces the two glycolytic enzymes ATP-dependent phosphofructokinase (PFK) and ADP-dependent pyruvate kinase (PK) by pyrophosphate (PPi)-dependent PFK (Mertens et al. 1998) and pyruvate-phosphate dikinase (PPDK) (Slamovits and Keeling 2006). Utilization of PPi in these reactions can increase the ATP yield to 3–5 ATP molecules per glucose that might be crucial in absence of oxygen-dependent energy metabolism (Mertens 1993).

Free glycogen in vaginal fluid is believed to be the main carbohydrate substrate for *T. vaginalis* to conserve energy in form of ATP. The glycogen content increases in the vaginal epithelial cells upon stimulation of estrogen and undergo cyclic changes during the menstrual cycle in reproductive years, whereas content of glycogen is low during childhood and menopause (Farage and Maibach 2011). The changes in estrogen levels correspond to differences of female susceptibility to *T. vaginalis* infection during ontogenesis; trichomoniasis appears in individuals during reproductive years, rarely in newborn infants of infected mothers (al Salihi et al. 1974). It has been also observed that the amount of glycogen in vaginal liquids decreases during *T. vaginalis* infections (Simonetti et al. 1989). In addition, relationship between glycogen content stimulated by estradiol and susceptibility to *T. vaginalis* infection was suggested based on experimental infections in animals (Kulda and B M 1989). However,

mechanisms of external glycogen acquisition and degradation are not known. *T. vaginalis* synthesizes its own glycogen as carbohydrate storage, which constitutes up to 15 % of dry weight (Müller 1989). The glycogen reserve is utilized upon external carbohydrate depletion (Mack and Muller 1980). The initial step of glycogen break down is catalyzed by glycogen phosphorylase that liberates a molecule of glucose from glycogen in form of glucose-1-phosphate (Nielsen et al. 2012; Wu and Müller 2003). Subsequently, phosphoglucomutase converts glucose-1-phosphate to glucose 6-phosphate that is further used in glycolysis. These two enzymes were suggested to mediate utilization of cytosolic glycogen storage, whereas their role in utilization of external glycogen is unlikely. Presence of external glycogen debranching enzymes such as isoamylases has not been reported in *T. vaginalis*, thus far. However, *T. vaginalis* has capacity to efficiently hydrolase external disaccharide maltose. Maltose is utilized in most of the media for in vitro growth of *T. vaginalis* (Diamond 1957; Johnson and Trussell 1943; Kupferberg et al. 1948) as the yield of *T. vaginalis* grown on maltose is considerably higher than growth on glucose (ter Kuile 1994). Investigations of maltose and glucose import revealed that glucose is taken up through facilitated diffusion, whereas the maltose import system is absent in *T. vaginalis* (ter Kuile and Müller 1993). The mechanism of maltose utilization requires its extracellular hydrolysis to produce glucose at the cell surface by extracellular α-glucosidase activity and the liberated glucose is then transported into the cell (ter Kuile et al. 2000). Multiple genes for α-glucosidases are currently annotated in the *T. vaginalis* genome; however, their cellular localization and substrate specificity for maltose and possibly other glucose polymers remain to be determined.

5.2.4.3 Glycolysis

The EMP glycolytic pathway constitutes the core of *T. vaginalis* energy metabolism in the cytosol that converts glucose to glycerol and lactate as the main cytosolic end product. In addition, ethanol and alanine are formed as minor end products. Although the *T. vaginalis* EMP pathway consists of a usual sequence of metabolic steps, several reactions are catalyzed by enzymes which represent alternatives to the classical eukaryotic glycolytic machinery.

Initial phosphorylation of glucose is catalyzed by glucokinase that is a strictly substrate-specific type of hexokinase. Unlike other hexokinases, the *T. vaginalis* glucokinase does not seem allosterically regulated; no physiological inhibitor has been found. A related parasite in cattle, *Tritrichomonas foetus*, possesses also fructokinase activity; however, this activity has not been detected in *T. vaginalis* (Mertens and Müller 1990).

Phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate is catalyzed by PPi-dependent PFK (Mertens et al. 1998). In contrast to eukaryotic ATP-PFK, PPi-PFK is not a subject to allosteric regulation and the reaction is reversible. Interestingly, in addition to PPi-PFK, *T. vaginalis* also possesses genes coding for ATP-PFK that are structurally similar to bacterial PFK. Surprisingly, cell localization studies revealed that the trichomonad ATP-PFK resides in hydrogenosomes, although no other glycolytic partner enzymes are present in this compartment (Rada et al. 2011). What is a physiological function of ATP-PFK is currently unknown.

In most eukaryotes, the fourth glycolytic step, the cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, is catalyzed by fructose-bisphosphate aldolase (FBA) class I. In *T. vaginalis*, the same reaction is catalyzed by structurally unrelated enzyme FBA class II type B, which is present in several anaerobic eukaryotes and some bacteria (Sánchez et al. 2002).

Dihydroxyacetone phosphate is converted to glyceraldehyde 3-phosphate by conventional triosephosphate isomerase. Alternatively, *T. vaginalis* can use dihydroxyacetone phosphate for production of glycerol, which forms up to 16 % of the fermentation products (Steinbüchel and Müller 1986b). First, dihydroxyacetone phosphate is converted by NADP-dependent glycerol 3-phosphate dehydrogenase into glycerol 3-phosphate and the phosphate is subsequently liberated by activity of Mg^{2+}-dependent glycerol 3-phosphatase. Maintenance of redox balance seems to be the chief reason for production of glycerol during which NADPH generated by the cytosolic malic enzyme is reoxidized.

3-Phosphoglycerate is converted into 2-phosphoglycerate by phosphoglycerate mutase (PGM) activity that is activated by 2,3-bisphosphoglycerate. *T. vaginalis* catalyzes this step with alternative PGM, whose activity is 2,3-bisphosphoglycerate-independent. The trichomonad PGM is related more to bacterial orthologues than to eukaryotic enzymes (Liapounova et al. 2006).

T. vaginalis possesses three enzymes that can metabolize phosphoenolpyruvate (PEP). Conversion of PEP to pyruvate with concomitant synthesis of ATP is catalyzed either by standard ADP-dependent PK or PPDK that utilizes AMP and PPi (Slamovits and Keeling 2006; Mertens et al. 1992). The reaction catalyzed by the latter enzyme requires a small Gibbs free energy and thus it is reversible. Coexistence of both enzyme activities in the same compartment might be problematic. However, transcriptomic data suggest that they might be differentially expressed under various environmental conditions (Horváthová et al. 2012). Alternatively, PEP can be converted via oxaloacetate to malate by activities of GTP-dependent PEP carboxykinase (PEPCK) and malate dehydrogenase (MDH) (Drmota et al. 1997). Malate can be either imported to hydrogenosomes or oxidatively decarboxylated by cytosolic malic enzyme (Dolezal et al. 2004). Involvement of MDH and ME with specificity to NADH and NADPH, respectively, allows transfer of reducing equivalents (transhydrogenase) between these two cofactors and thus may provide NADPH for glycerol production.

Pyruvate is converted to lactate by NADH-dependent lactate dehydrogenase (LDH) (Wu et al. 1999) and/or enters to hydrogenosomes. *T. vaginalis* produces also a small amount of ethanol as well as secondary alcohols such as 2-propanol and 2-butanol. Indeed, zinc-dependent secondary alcohol dehydrogenase (S-ADH) that can utilize acetaldehyde for ethanol production as well as ketones for production of secondary alcohols has been identified and characterized in *T. vaginalis* (Leitsch et al. 2013; Sutak et al. 2012). *T. vaginalis* also possesses multiple genes for Fe-dependent ADH, though their contribution to the cytosolic ADH activity seems to be negligible (Sutak et al. 2012). Importantly, *T. vaginalis* lacks pyruvate decarboxylate that catalyzes conversion of pyruvate to acetaldehyde in *Tritrichomonas foetus* (Sutak et al. 2004a). Thus, a source of acetaldehyde and sources of ketones in

T. vaginalis remain unknown. Finally, some pyruvate can be converted in the cytosol to alanine by alanine aminotransferase (Coombs and Müller 1995; Huang et al. 2014). Unlike the other metabolic end products, alanine is not released to the culture media and accumulates within the cells (Lowe and Rowe 1986; ter Kuile 1996).

5.2.4.4 Energy Metabolism in Hydrogenosomes

Hydrogenosomes are modified forms of mitochondria that are adapted to function under anaerobic or oxygen-poor environment (Hrdy and Müller 2008; Embley and Martin 2006; Martin 2008; Muller et al. 2012; Yarlett and Hackstein 2005). Originally, hydrogenosomes were defined based on a single biochemical property: production of molecular hydrogen, a phenomenon that was first described in trichomonads (Lindmark et al. 1975; Lindmark and Müller 1973). However, further investigations revealed a number of various forms of hydrogen-producing mitochondria that are adapted in various extent to anaerobiosis (Barbera et al. 2010; Jerlstrom-Hultqvist et al. 2013; Leger et al. 2013; Nyvltova et al. 2015; Stairs et al. 2014; Stechmann et al. 2008; Yarlett et al. 1981; Yarlett et al. 1983; Yarlett et al. 1986) leading to a redefinition of hydrogenosomes. According to the current functional classification, hydrogenosomes are organelles of the mitochondrial family class four that (i) produce molecular hydrogen, (ii) synthesize ATP exclusively by substrate-level phosphorylation, (iii) lack of membrane-associated electron transport, and (iv) lack of genome (Müller et al. 2012).

Hydrogenosomal energy metabolism is based on oxidative breakdown of malate and/or pyruvate to carbon dioxide, molecular hydrogen, and acetate (Steinbüchel and Müller 1986a). Pyruvate is either imported from hydrogenosomes or it is a product of oxidative decarboxylation of malate. This bidirectional reaction is catalyzed by hydrogenosomal NAD-dependent malic enzyme (ME) that is one of the most abundant hydrogenosomal proteins (Hrdy and Müller 2008; Drmota et al. 1996; Hrdy and Müller 1995b). The hydrogenosomal ME is structurally distinct from NADP-dependent ME in the cytosol (Dolezal et al. 2004). Pyruvate is oxidatively decarboxylated by pyruvate: ferredoxin oxidoreductase (PFO) to acetyl-CoA with high energy thioester bound (Hrdy and Müller 1995a; Williams et al. 1987). The CoA moiety is then transferred to succinate by acetate: succinate CoA transferase (Lindmark 1976; van Grinsven et al. 2008) and acetate are released as metabolic end products. Succinyl-CoA is utilized for ATP synthesis by succinyl-CoA synthetase (SCS) and succinate is recycled (Jenkins et al. 1991). SCS is a hetero-tetrameric enzyme which consists of α and β subunits (Lahti et al. 1994; Lahti et al. 1992). It has been suggested that pyruvate might be alternatively metabolized by ferredoxin-independent 2-keto acid oxidoreductase (Brown et al. 1999). However, recent studies did not confirm presence of this activity in the hydrogenosomes (Zednikova et al. 2012). In some anaerobic protists, ATP is synthesized directly from acetyl-CoA by cytosolic or organellar acetyl-CoA synthetase (ACS) (Nyvltova et al. 2015; Tielens et al. 2010). Involvement of this enzyme in the energy metabolism of *T. vaginalis* was originally proposed, yet only the presence of SCS activity and not of ACS activity has been reported so far. Nevertheless, an activity of ACS in *T. vaginalis* hydrogenosomes cannot be excluded as a gene coding for ASC is annotated in

the *T. vaginalis* genome. Electrons that are generated during oxidation of malate and pyruvate are utilized for hydrogen synthesis by [FeFe]hydrogenase (Payne et al. 1993). [2Fe2S]ferredoxin is the key low redox potential electron transporter that transfer electrons directly from PFO to hydrogenase (Gorrell et al. 1984). During oxidative decarboxylation of malate, reducing equivalents are captured by NAD. Subsequently, NADH is reoxidized by activity of NADH dehydrogenase (NDH) that transfers electrons to ferredoxin. *T. vaginalis* NDH consists of two subunits, namely, Tvh47 and Tvh22 that are homologues of the 51-kDa and 24-kDa subunits of the NADH dehydrogenase module in the mitochondrial respiratory complex I (Hrdy et al. 2004).

Similarly to mitochondria, hydrogenosomes are able to generate ATP. However, low ATP yield in hydrogenosomes accounting for approximately 25 % of overall ATP production raises a question whether the ATP generated in hydrogenosomes is (i) exported from hydrogenosomes to be used in the cytosol and other cellular compartments and (ii) is able to cover ATP demands only in hydrogenosomes or (iii) the amount of ATP is not sufficient and additional ATP is imported from the cytosol into hydrogenosomes. Intrahydrogenosomal ATP is required for at least two processes: the import of hydrogenosomal proteins (Bradley et al. 1997) that are all synthesized in cytosol as hydrogenosomes lacks of its genome (Clemens and Johnson 2000) and for FeS clusters formation in apoproteins (Sutak et al. 2004b). These pathways are dependent on ATP hydrolysis associated with activity of HSP70. Similarly to mitochondria, hydrogenosomes possess ADP/ATP carriers (Rada et al. 2011; Tjaden et al. 2004) in inner hydrogenosomal membrane that allows exchange of adenine nucleotide phosphates; however, the direction of ATP/ADP exchanges in metabolically active hydrogenosomes has not been studied. Nevertheless, it is very likely that ATP is imported to the organelles, when hydrogenosomal energy metabolism is reduced or silenced. For example, key enzymatic activities of energy metabolism (ME, PFO, NDH, hydrogenase) are low or absent in strains with high resistance to metronidazole (Rasoloson et al. 2002) a drug of choice for treatment of trichomoniasis. Metronidazole and other 5-nitroimidazoles are in part activated within hydrogenosomes, where its nontoxic form is reduced to toxic nitroradicals (Chapman et al. 1985; Moreno et al. 1983); at the same time, they are in part activated by a cytosolic flavin-based mechanism (Leitsch et al. 2009). Because organisms with high resistance to metronidazole lack pyruvate/malate metabolism in hydrogenosomes, it is unlikely that ATP is generated within the hydrogenosomes to cover intrahydrogenosomal ATP demands.

Previous biochemical and more recent transciptomic and proteomic data revealed mutual regulation of cytosolic and hydrogenosomal pathways that allow compensation of impaired hydrogenosomal energy metabolism by increased rate of glycolysis. During in vitro induction of resistance to increasing concentrations of metronidazole (from 3 to 100 μg/ml), the hydrogenosomal energy metabolism gradually decreases, which is reflected by gradual decrease or loss of acetate and hydrogen production, respectively. In parallel, amounts of lactate, a glycolytic end product, increase to reach about three fold higher concentration in the most resistant strain in comparison to parent metronidazole-susceptible trichomonads (Rasoloson et al. 2002).

Interestingly, a similar effect has been observed in trichomonads that were cultivated under iron-restricted conditions (Vanacova et al. 2001). Iron is required for the activity of a number of hydrogenosomal proteins that possess FeS clusters in their active site such as PFO, ferredoxin, hydrogenase, and NDH as well as iron regulate expression of hydrogenosomal proteins (Horváthová et al. 2012). Proteomic analysis of hydrogenosomes revealed at least 58 proteins that are differentially regulated by iron (Beltran et al. 2013b). Iron deficiency led to the downregulations of proteins involved in hydrogenosomal energy metabolism, whereas proteins involved in FeS cluster assembly were upregulated. Decreased energy metabolism in hydrogenosomes was associated with increased expression of cytosolic ME and LDH upon iron-restricted conditions that allow conversion of malate via pyruvate to lactate. Conversely, iron-rich conditions strongly upregulate expression of cytosolic PEPCK, a component of the pathway leading to production of malate that is available for hydrogenosomal energy metabolism (Beltran et al. 2013b). Interestingly, most of glycolytic as well as hydrogenosomal enzymes are encoded by multiple gene copies that are differentially expressed upon effect of iron; thus only some copies are expressed whereas the expression of others was iron independent (Horváthová et al. 2012). Expression of other copies is affected by other conditions such as oxidative and cold stress, interaction with fibronectin, or vaginal epithelial cells (Gould et al. 2013; Huang et al. 2014; Fang et al. 2014; Huang et al. 2012). These findings indicate a stringent regulation of differentially expressed multiple gene copies in response to highly dynamic changes in the parasite environment or interaction with drugs.

Production of molecular hydrogen is a hallmark of hydrogenosomes. Hydrogen generated within these organelles passes through the cytosol and is released to the cell environment. *T. vaginalis* cells produce from 1.8 up to 171 nmol H_2 per min/mg protein (Steinbüchel and Müller 1986a; Rasoloson et al. 2002). Production of volatile hydrogen is an elegant mechanism to remove reducing equivalents generated during pyruvate/malate oxidations. However, is it possible that hydrogen may serve as a source of reducing equivalents for cytosolic metabolism when increased reducing power is required? It has been observed that *T. vaginalis* is able to efficiently reduce external acetone to 2-propanol (Sutak et al. 2012). This reaction is catalyzed by cytosolic S-ADH that requires reducing power in form of NADPH. Interestingly, increased production of 2-propanol was associated with increased production of acetate. Acetate is generated in equimolar amounts with hydrogen during pyruvate oxidation in hydrogenosomes; thus increased production of hydrogen could be expected as well. Instead, reduction of acetone to 2-propanol is accompanied with a considerably lower amount of hydrogen released outside the cells. The most plausible explanation of this observation is that hydrogen may be utilized in the cytosol to maintain cellular redox balance (Sutak et al. 2012).

5.2.4.5 Amino Acid Metabolism and Polyamine Formation

Several reviews provide detailed information about the importance of arginine metabolism and polyamines in *T. vaginalis* (Yarlett 1988; Yarlett and Bacchi 1991). Arginine is an alternative substrate, metabolism of which leads to ATP synthesis. *T. vaginalis* possesses an arginine dihydrolase (ADH) pathway that is present in few

anaerobic protists such as *Trichomonas* and *Giardia*, Gram-negative bacteria, and Mollicutes (Morada et al. 2011). The pathway converts arginine to ammonia and ornithine with the generation of ATP via three enzymes. In the initial step, nitrogen is liberated from arginine to form citrulline by activity of arginine deiminase (ADI). Intriguingly, ADI resides in hydrogenosomes, while other components of ADH pathway are localized in *T. vaginalis* parasite cytosol (Morada et al. 2011; Yarlett et al. 1994). Citrulline is metabolized by catabolic ornithine carbamoyltransferase (OCT) to ornithine and carbamoyl phosphate which is used by carbamoyl phosphate kinase for synthesis of ATP (Yarlett et al. 1994; Linstead and Cranshaw 1983). It has been shown that *T. vaginalis* cells incubated under glucose-restricted conditions upregulate expression of OCT and carbamoyl phosphate kinase, which is consistent with a significant role of ADH as alternative ATP-generating pathway (Huang et al. 2014).

Ornithine serves for synthesis of polyamines. Ornithine decarboxylase (ODC) splits ornithine to carbon dioxide and putrescine that form a large intracellular pool (Yarlett et al. 1993). Putrescine is eventually exported via an antiport with simultaneous import of spermine that is converted to spermidine (Yarlett et al. 1994; Yarlett et al. 2000). This conversion includes acetylation of spermine via a spermidine: spermine N^1-acetyl transferase to N^1-acetylspermine in the cytosol; then N^1-acetylspermine is oxidized to spermidine by polyamine oxidase, whose activity is predominantly associated with hydrogenosomes (Yarlett et al. 2000). Putrescine is released to the culture media in concentration up to 40 μM (Yarlett and Bacchi 1988). Importantly, a rather high concentration of arginine in vaginal fluids, over 200 μM in noninfected individuals (Chen et al. 1982), decreases to undetectable level upon *T. vaginalis* infection, whereas an amount of putrescine increases from undetectable level in noninfected individual to concentration up to 2420 μM in patients with trichomoniasis (Sanderson et al. 1983). Another support to underline the importance of ADH pathways has been provided by a recent proteomic study comparing low and highly virulent *T. vaginalis* strains to reveal higher expression of OCT in the strain with highly virulent phenotype (Cuervo et al. 2008). These observations strongly suggest that arginine metabolism may provide an important contribution to the energy metabolism in the vaginal environment. Moreover, ADH pathways may partially compensate the impaired hydrogenosomal metabolism in metronidazole-resistant strains. The amount of ATP produced via ADH pathway accounts for about 1 % of the ATP synthesis during glucose fermentation by in vitro-cultivated *T. vaginalis*. However, contribution of the ADH pathway to ATP formation in strains with in vitro-induced metronidazole resistance was about tenfold higher than in the parent strain, also reflected by increased synthesis of putrescine (Yarlett et al. 1996).

In addition to energy metabolism, putrescine appeared to be a highly active molecule in various processes mostly related to the parasite's virulence. Production of basic polyamines might contribute to the increase of pH in vaginal fluids during *T. vaginalis* infection and changes in vaginal biocenosis. Inhibition of ODC using putrescine analogue diamino-2-butane (DAB) causes inhibition of trichomonad growth, which was restored by addition of putrescine (Garcia et al. 2005).

DAB-treated parasites downregulate expression of cytosolic and surface cysteine proteases TvCP39 and TvCP65 that are likely involved in *T. vaginalis* cytotoxicity to the host cells (Alvarez-Sanchez et al. 2008; Carvajal-Gamez et al. 2014) and may be considered a translation initiation factor TvelF-5A (Carvajal-Gamez et al. 2011). Finally, in addition to putrescine, *T. vaginalis* can produce cadaverine via decarboxylation of lysine by ODC, although at a slower rate in comparison to decarboxylation of ornithine. Production of both polyamines is inhibited by ODC inhibitor DL-α-difluoromethylornithine (Yarlett 1988; Yarlett et al. 1993; Yarlett et al. 1992). Similarly to putrescine, cadaverine is exported from *T. vaginalis* to growth media (Yarlett and Bacchi 1988) and various levels of cadaverine (55–2020 μM) have been found in vaginal fluids of infected patients (Sanderson et al. 1983). Increased biosynthesis of cadaverine has been observed during the early stage of *Entamoeba invadens* encystation (Jeelani et al. 2012). Whether biosynthesis of cadaverine in *T. vaginalis* is somehow involved in formation of trichomonad pseudocysts or in some other cellular functions is not known.

Additional sources of energy may provide sulfur amino acid metabolism. *T. vaginalis* possesses the unique cytosolic enzyme methionine γ-lyase (MGL) converting sulfur-containing amino acids such as methionine, homocysteine, and cysteine to ammonia, thiols, and corresponding α-keto acid (Lockwood and Coombs 1991). Methionine is converted to 2-oxobutyrate and methanethiol, which has been detected among volatile metabolic end products (Sutak et al. 2012; Thong et al. 1987). MGL also catalyzes breakdown of homocysteine to 2-oxobutyrate, NH_3 and H_2S. It was suggested that following conversion of 2-oxobutyrate to propionate with concomitant ATP synthesis might contribute to energy metabolism (McKie et al. 1998). In *T. vaginalis*, cysteine is believed to be a central antioxidant comprising over 70 % of the total thiols in this parasite (Westrop et al. 2009). Cysteine is acquired either from *T. vaginalis* environment, or it is synthesized from phosphoserine and H_2S by cysteine synthase (Westrop et al. 2006). There are at least three enzymes including MGL that utilize cysteine as substrate: (i) cysteine desulfurase, a component of the hydrogenosomal iron-sulfur cluster assembly machinery, produces sulfur for formation of FeS clusters and alanine in hydrogenosomes (Sutak et al. 2004b); (ii) aspartate aminotransferase converts cysteine into 3-mercaptopyruvate and 3-mercaptopyruvate is further catabolized to pyruvate and H_2S by mercaptopyruvate sulfurtransferase (Westrop et al. 2009); and (iii) MGL converts cysteine to pyruvate, H_2S, and NH_3 (Lockwood and Coombs 1991). Pyruvate produced by the last two reactions can be utilized by hydrogenosomal energy metabolism.

5.2.5 Secreted Molecules (Daniele Dessì)

Given the peculiar obligate extracellular parasitic lifestyle of *T. vaginalis*, the role of secreted/excreted products in the crosstalk with the host mucosal environment is of paramount importance. In recent years, the secretome analysis of a number of parasitic protists has received much attention, helping to unravel many aspects of the biology and of host/parasite relationships of *Plasmodium falciparum*, *Leishmania*

donovani, and *Trypanosoma cruzi* among others (Bayer-Santos et al. 2013; Silverman et al. 2008; van Ooij et al. 2008). While a comprehensive proteomic profiling of *T. vaginalis* secretome is still lacking, in the last 20 years many researchers have focused their efforts to the characterization of several aspects of host/parasite relationships involving secreted proteins. The generally accepted mechanism by which *T. vaginalis* exerts its cytopathic effect implies a preliminary adhesion step, leading to a contact-dependent host cell lysis. Nevertheless, seminal studies conducted by Fiori and coworkers (Fiori et al. 1999) showed how acellular supernatants of *T. vaginalis* cultures are able to induce hemolysis in human red blood cells (RBCs) in vitro, with a mechanism that leads to the functional formation of pores on the target cell membranes. This contact-independent hemolytic activity is strongly dependent on environmental pH: indeed, an acidic pH triggers the release of the molecules mediating the hemolytic activity (pore-forming proteins) in the extracellular milieu, where they bind to and insert themselves into the phospholipid bilayer of the RBC plasma membrane. Despite the pore-forming activity of *T. vaginalis* is well characterized, the effectors involved in such activity have never been identified. The *T. vaginalis* genome-sequencing project (Carlton et al. 2007), however, allowed the identification of a family of 12 candidate pore-forming proteins belonging to the family of saposin-like proteins (TvSaplips). *Entamoeba histolytica* and *Naegleria fowleri* secrete pore-forming proteins called amoebapores and naegleriapores, respectively, both belonging to the saposin-like protein family (Leippe et al. 2005). Unfortunately, experimental evidences supporting a role for these proteins as pore-forming factors are still lacking, and the quest for the proteins mediating the *T. vaginalis*-induced host cell lysis is still open, albeit a number of secreted proteases are thought to contribute to this phenomenon (Cárdenas-Guerra et al. 2013), which then appears to be mediated by multiple factors that are not necessarily secreted molecules (Figueroa-Angulo et al. 2012b; Fiori et al. 1999). Secreted and surface proteases of *T. vaginalis* have been extensively studied and are involved in a number of mechanisms taking place at the host/parasite interface, thus playing a fundamental role in infection. Numerous proteinases and antibodies to trichomonad proteinases, in effect, have been found in vaginal washes of patients infected by *T. vaginalis* (Alderete et al. 1991). Secreted proteases, and cysteine proteases (CPs) in particular, may degrade the mucin layer and extracellular matrix proteins, in order to allow direct contact of trichomonad cells with the underlying epithelium (Hirt et al. 2007). An iron-regulated extracellular fraction named CP30, composed of four different CPs (CP2, CP3, CP4, CPT), was shown to induce apoptosis in cultured human vaginal epithelial cells (Kummer et al. 2008). Secreted molecules are also involved in a fundamental aspect of *Trichomonas* pathogenesis, which allows the establishment of a successful chronic infection: immune modulation and evasion from the host immune response. Several reports describe the immunomodulatory activity of *T. vaginalis*-secreted products on innate immune cells such as neutrophils and macrophages, inducing a proinflammatory response that may contribute to local tissue damage (Fiori et al. 2013; Han et al. 2009; Ryu et al. 2004). Secreted CPs play a role also in immune escape, by degrading human IgA, IgG, and IgM (Provenzano and Alderete 1995) and complement C3b (Lehker and Alderete 2000). A hypothetical mechanism by which *T. vaginalis* could

evade the adaptive immune response is the continuous secretion of highly immunogenic proteins, which may bind and neutralize secreted antibodies, thus short-circuiting specific anti-*T. vaginalis* immunity (Figueroa-Angulo et al. 2012b).

One of the major advances in the field of *T. vaginalis*-secreted molecules is represented by a recent work by Twu and colleagues (Twu et al. 2013), which showed that *Trichomonas* is able to produce exosomes. Exosomes are small (50–100 nm) membrane vesicles released by eukaryotic cells in the extracellular milieu, mediating a plethora of important biological functions, such as cell-cell communication and gene expression regulation (Silverman and Reiner 2010). *T. vaginalis* exosomes were shown to fuse to and deliver their content to host cells in vitro, modulating immune responses by eliciting IL-6 and downregulating IL-8 secretion in ectocervical cells. Importantly, exosomes appear to play a key role in parasite adhesion processes. Indeed, microvesicles purified from a highly adherent *T. vaginalis* strain are able to increase adherence of strains characterized by a low-adhesion phenotype. The small RNAs found in *T. vaginalis* exosomes, similarly to what happens in higher eukaryotes, may represent a new mean of cell-cell communication. Furthermore, these short RNAs might hypothetically affect host cells gene expression upon delivery.

The study of molecules secreted by *T. vaginalis* has received a growing attention over the last years, but the whole picture of the secretome of this important human parasite is far from being complete. The fundamental role of this class of molecules in host/parasite interactions is well established, and their potential use in clinical microbiology as new targets for innovative therapeutic interventions and as perspective biomarkers to develop new noninvasive, sensitive, and specific diagnostic tools is of paramount importance. Given these premises, there is a tremendous need of a comprehensive analysis, description, and characterization of the complete set of gene products released by *T. vaginalis*, with both conventional and unconventional secretion mechanisms.

5.2.6 Pathogenicity (Pier Luigi Fiori)

Humans are the only natural host of *T. vaginalis*, and the evidence for sexual transmission of trichomoniasis is indisputable. During infection, *T vaginalis* colonizes the genital mucosa of both female and male. Mammalian mucosal surfaces are exposed to potentially harmful pathogens and hence have developed a sophisticated first line of defense based on innate and adaptive immune mechanisms.

The *T. vaginalis* genome sequencing has led to an increase in knowledge on a number of new gene families involved in the host/parasite relationship, prompting new avenues of research to better understand the mechanisms of the parasite pathogenicity (Carlton et al. 2007).

Trophozoites attach to different mucosal surfaces of the lower urogenital tract (i.e., the vagina, cervix, urethra, prostate, and epididymis) and they are able to survive for long periods of time in the environment despite adverse conditions, such as acidic pH, and a robust immune response (Harp and Chowdhury 2011). In addition,

the vaginal microenvironment is continuously changing due to hormonal variations during the menstrual cycle. Likewise a number of other microbial pathogens, *T. vaginalis*, have evolved various mechanisms to adapt to such a hostile niche and to cope with a robust host immune response, by acquisition of virulence factors and strategies that allow a successful colonization and to cause disease in the human host.

After entering the human body, adherence to epithelial cells is a critical step for parasite survival within its host (Fiori et al. 1999), together with the establishment of a subtle balance between innate and adaptive immune response (Song et al. 2015). Therefore, the identification of adhesins is critical to our understanding of the colonization process. Immediately after binding to epithelial cells, the free-swimming protozoa transform from typical pear-shaped cells to amoeboid forms, dramatically rearranging their cytoskeleton (Edwards et al. 2014). This phase transition probably leads to an increase of cell-to-cell contact surface, forming cytoplasmic projections that interdigitate with target cells. Over the last 30 years a number of papers from a single group have identified several *T. vaginalis* adhesins. However, all these molecules have been characterized as hydrogenosomal enzymes (Alderete et al. 1995; Engbring et al. 1996). The authors explain the double function and localization by proposing the inclusion of *T. vaginalis* adhesins in the group of the so-called "moonlighting molecules": the double function is explained by the claim that *T. vaginalis* needs to diversify the functions of a small set of proteins as it possesses a small genome, similar to some viruses or bacteria (Alderete et al. 2001). The recent sequencing of *T. vaginalis* genome has shown that this protist has a very large genome encoding a massive proteome. Data from the *T. vaginalis* genome project showed that this protist possesses a number of candidate surface proteins, including some putative adhesins. Based on these recent data, the role of hydrogenosomal enzymes as adhesins is highly debatable (Addis et al. 2000). A complete list of open questions regarding the putative role of hydrogenosome enzymes as adhesins is discussed in a recent review article (Hirt et al. 2007).

The annotation of the *T. vaginalis* genome has identified a plethora of candidate surface protein families putatively involved in mediating interaction with host cells and tissues (Hirt et al. 2011; Hirt et al. 2007; Cui et al. 2010). Notably, a number of these molecules express domains shared with surface proteins implicated in adhesion and virulence in other mucosal pathogenic microorganisms (Hirt et al. 2007; Hirt et al. 2002). Proteomic studies have been used to identify the massive number of surface proteins of *T. vaginalis* (de Miguel et al. 2010b). The largest gene family encoding potential surface proteins is known as BspA-like proteins. These proteins show a specific type of leucine-rich repeat (TpLRR), a domain expressed in a number of mucosal prokaryotic microorganisms in which it is involved in binding to host cells and/or extracellular matrix (Kobe and Kajava 2001) (Kedzierski et al. 2004). These proteins are also expressed in mucosal eukaryotic microorganisms, in particular in *Entamoeba histolytica* and *E. dispar*. An exhaustive description of TvBspA-like proteins is reported in the section entitled "Surface Molecules" of the present chapter.

The list of putative surface proteins includes not only TvBspA proteins but also GP63-like metallopeptidases (a metalloprotease implicated in *Leishmania*

virulence), subtilisin-like serine peptidases (involved in egress in apicomplexan), calpain-like cysteine peptidases, P270 surface immunogen, chlamydial polymorphic membrane proteins (Pmp), immunodominant variable surface antigens, legume-like lectin receptors, and giardial variant surface protein VSP-like proteins (Carlton et al. 2007; Hirt et al. 2007; de Miguel et al. 2010b). Recently a novel domain named M60-like/PF13402 has been identified: orthologues of this domain were detected among both prokaryotic and eukaryotic microbes associated with host mucosae. The domain is characterized by a zinc-metallopeptidase-like motif and is able to degrade mammalian mucin; in a number of microorganisms, these peptidases are involved in colonization processes. A total of 25 TvM60-like/PF13402-containing proteins were identified in *T. vaginalis* (Nakjang et al. 2012). Additional novel surface protein families include at least three tetraspanins (de Miguel et al. 2012), a rhomboid-like serine peptidase (Freeman 2008), and a nicastrin precursor. Finally, 37 % of the 411 proteins identified in the surface proteome were annotated as hypothetical.

Even if membrane proteins play important roles in *T. vaginalis* adhesion to host cells, several evidences support the involvement of surface carbohydrates in attachment and colonization processes. In particular, *T. vaginalis* lipoglycan (LG) is a large molecule expressed on the surface of the protest which is considered a major candidate in host/parasite interactions during colonization. LG, with a total of $2–3 \times 10^6$ molecules per cell, is the most abundant molecule of the surface glycocalyx. This molecule plays a primary role in attachment and colonization of the parasite, as demonstrated by experiments with *T. vaginalis* mutants expressing truncated LG. Mutated protozoa were significantly less adherent and as a consequence less cytotoxic to human ectocervical cells in vitro, as compared to wild-type protozoa (Bastida-Corcuera et al. 2005). The same group has also identified human galactose-binding protein Gal-1 as a ligand for *T. vaginalis*, demonstrating that human Gal-1 (but not galectin-7) specifically binds to *T. vaginalis* in a carbohydrate-dependent manner. In addition, silencing of Gal-1 in ectocervical cells leads to a reduction of parasite binding. Thus, Gal-1 is the only host cell receptor identified for *T. vaginalis* so far (Okumura et al. 2008). LG could also play a role in modulation of host immune response during infection (Fichorova et al. 2006; Singh et al. 2009) by upregulating the production of pro-inflammatory cytokines. LG contributes to the immunopathogenesis of *T. vaginalis*, probably due to its ability to bind toll-like receptor 4 (TLR-4) and to activate the innate immune response. Recent studies demonstrated that LG is able to stimulate host soluble immune response, eliciting production of antibodies both in sera and in vaginal secretion of infected women; notably, during normal pregnancies infected women had significantly higher vaginal anti-LG antibody titer than those with adverse outcomes of pregnancy (Bastida-Corcuera et al. 2013).

All these results suggest that a high number of adhesion factors, from surface proteins to surface glycolipids, are involved in colonization during the first stage of infection and in host/parasite interaction (Harp and Chowdhury 2011; de Miguel et al. 2010b).

The second stage of infection is characterized by the production of toxic molecules and the subsequent host tissue damage (Fiori et al. 1999). Recently Lustig

et al. (Lustig et al. 2013) demonstrated that even if adhesion to epithelial cells is fundamental to induce cytolysis, higher levels of attachment do not induce a massive cytolysis. The authors suggest that adhesion to target cells might trigger a contact-independent mechanism for killing, probably based on release or activation of secreted/excreted molecules. The secretion of a number of molecules with cytotoxic activity, such as proteases, cell-detaching factors, and putative pore-forming proteins, is thought to play a central role in *T. vaginalis*-mediated epithelial damage, as discussed above. Very recently a secretion mechanism based on production of exosomes has been reported (Twu et al. 2013); these authors also demonstrated a pivotal role of secreted vesicles in immune response modulation and host/parasite relationships, by packaging virulence molecules and delivering their content (soluble proteins and RNA) through fusion with host cell membrane. Proteomic analysis of secreted molecules suggests a role for proteases in colonization and infection. The protozoan releases cysteine proteinases into the extracellular space, resulting in disruption of the vaginal and cervical epithelium (Hernandez et al. 2014). A wide range of hydrolases (with molecular mass ranging from 20 to 110 kDa) have been identified in *T. vaginalis* (De Jesus et al. 2009). Studies in the past decade have demonstrated the role of 30, 62, 65, and 39 kDa cysteine proteinases in colonization and infection (Hernandez et al. 2014). Some enzymes show a trypsin-like activity and are able to function as cell-detaching factors by degrading laminin, fibronectin, and other components of the extracellular matrix (ECM). Four different cysteine proteinase genes of *T. vaginalis* have 45 % homology to cysteine proteinase genes of *Dictyostelium discoideum* and are L-cathepsin and H-papain-type proteinases (León-Sicairos et al. 2004). A role for these enzymes in degrading the protective mucus barrier has been suggested. Between the molecules involved in virulence, there also are several phospholipases: Lubick et al. suggest that phospholipase A2 is involved in host cells destruction (Lubick and Burgess 2004).

On the basis of indirect observations, it has been hypothesized that the cytotoxic effect was mediated by functional pore insertion into the host cell membrane, by means of pore-forming proteins (trichopores) production (Fiori et al. 1996; Fiori et al. 1993). An exhaustive description of trichopores is reported in the section "Secreted molecules" of the present chapter.

An additional cytolytic mechanism has been described. During in vitro infection the protist dramatically disrupts the subcortical membrane cytoskeleton of red blood cells in an extremely fast and effective way by degrading spectrin (or fodrin, the spectrin-like protein in mammalian nucleate cells) (Fiori et al. 1997). Demolition of the subcortical cytoskeleton has fundamental effects on host cell architecture, since it arranges into a complex network responsible for maintenance of cell shape and membrane integrity. The complete disappearance of the subcortical cytoskeleton affects the connection with the cytoplasmic actin network, leading to an enhanced sensitivity to osmotic lysis mediated by functional pore insertion. Interestingly, the ability to enzymatically degrade fodrin in host cells leading to actin cytoskeleton disruption has been reported for other microbial pathogens, such as the enteroaggregative *E. coli* (Villaseca et al. 2000), indicating common virulence strategies among different microbial pathogens.

All these data depict a fundamental role for a number of secreted/excreted molecules in mechanisms of pathogenicity; nevertheless, production of toxins or other virulence molecules as the sole strategy for pathogenicity is a rather unusual event, and microbial virulence cannot merely be ascribed to the release of toxic effectors into the environment. As a matter of fact, the evolution of infections is strongly influenced by innate and adaptive responses of the hosts: it is well known that a number of microbial pathogens can indirectly damage tissues by stimulating host inflammatory response, contributing to cell death directly caused by pathogens. Inflammatory response can be considered as a double-edged sword: if immune response is excessive, high levels of pro-inflammatory mediators can be dangerous not only for microorganisms but also for the host surrounding tissues. Likewise a number of pathogens, *T. vaginalis* can temperate and limit the spread of inflammation, inducing a chronic infection: chronicization represents a good compromise between pathogens and their hosts (Malla et al. 2014). *T. vaginalis* relationship with immune system is further on complicated by concomitant microbial flora (van de Wijgert et al. 2014). Even if normal vaginal flora does not stimulate local inflammatory response, coinfecting pathogens can induce a strong inflammatory response and influence the evolution of *Trichomonas* infection. Our research group has demonstrated that *M. hominis* stably infects *T. vaginalis*, establishing a symbiotic relationship (the only one described between two human pathogens) (Dessi et al. 2006). *M. hominis* is able to survive and multiply within *T. vaginalis* cells (Dessi et al. 2005; Morada et al. 2010). Mycoplasmal cell surface structures are well-known ligands for TLRs and consequently induce inflammatory response; interestingly, infected strains induce a synergistic effect on pro-inflammatory cytokine secretion, upregulating basal inflammatory activation (Fiori et al. 2013). Very recently, Fettweis et al. demonstrated a new species of uncultivated vaginal mycoplasma strictly and specifically associated with *T. vaginalis* infection and proposed the name of "*Candidatus* Mycoplasma girerdii" for this new bacterium (Fettweis et al. 2014). The symbiosis with intracellular *M. hominis* and the upregulation of inflammatory response may affect the evolution of the protozoan infection and might have important implications on severe diseases associated with trichomoniasis, such as HIV infection and cervical and prostate cancer. Finally, a role in modulation of host immune response has been reported for endosymbiont dsRNA virus TVV: the viruses are sensed by human epithelial cells by activation of TLR-3, upregulating secretion of pro-inflammatory cytokines, thus influencing preterm birth delivery and HIV infection susceptibility (Fichorova et al. 2012).

5.2.7 Recent Advances (Pier Luigi Fiori)

Recently, based on epidemiological or serological approaches, a number of papers focused on the potential association of *T. vaginalis* infection with aggressive cervical and prostate cancers. An increasing number of pathogens (including viruses, bacteria, and parasites) have been reported as strictly involved in tumor promotion, and it has been well established that cancer can be exacerbated by inflammatory response to microbial infections. Mediators of inflammation released during microbial infections

can induce genetic and epigenetic changes in host cells, leading to the development and progression of tumors, and chronic inflammation maintains a tumor-supporting microenvironment; recent data demonstrate that infection and chronic inflammation contribute to about 1 in 4 of all tumors (Elinav et al. 2013; Porta et al. 2011).

T. vaginalis infection has been implicated as a risk factor for cervical cancer (Tao et al. 2014), and a relation between *T. vaginalis* infection and benign prostate hyperplasia (Mitteregger et al. 2012), as well as with aggressive prostate cancer, has been reported by several authors (Sutcliffe et al. 2006) on the basis of serological data based on large case-control studies (Stark et al. 2009). The magnitude of the association between *T. vaginalis* seropositivity and overall prostate cancer risk is between 1.23 and 1.43. Despite these indirect data suggest an association between of *T. vaginalis* infection and prostate and cervical cancers, a direct molecular mechanism explaining a role in transformation and/or progression of tumors was only hypothesized in recent years.

A possible involvement of *M. hominis* infection in prostate cancer has been reported by Barykova and colleagues (Barykova et al. 2011), who demonstrated a significant association of *Mycoplasma* infections with malignant transformation and genomic instability (Feng et al. 1999; Namiki et al. 2009). Interestingly, a high number of *T. vaginalis* isolates are parasitized by *M. hominis* (Dessi et al. 2006; Dessi et al. 2005; Feng et al. 1999; Namiki et al. 2009): the bacteria synergistically upregulate the host inflammatory response (Fiori et al. 2013), thus potentially contributing to induce cancer-promoting conditions.

Only recently, a direct molecular mechanism of the potential *T. vaginalis* involvement in prostate tumor transformation and progression has been proposed (Twu et al. 2014). Reported data demonstrate that *T. vaginalis* secretes a homologue of human macrophage migration inhibitory factor (MIF), a well-known pleiotropic cytokine that mediates inflammation as upstream regulator of pro-inflammatory response. MIF exhibits a number of unusual properties including two distinct enzymatic activities: oxidoreductase and tautomerase. MIF binds to a specific receptor, CD74, inducing its phosphorylation and the recruitment of CD44 which in turn activates tyrosine kinases (Syk and PI3K/Akt). An increasing number of evidences indicate that human MIF is responsible for promotion of tumorigenesis by inhibiting p53 accumulation and cellular proliferation through activation of the MAPK family, in association with antiapoptotic, angiogenic, and metastatic activities. MIF is hyper-expressed in all stages in a large number of tumors, and its expression levels have been found to correlate with disease severity and metastasis. In the past years, several authors demonstrated that human MIF is a key molecule in prostate cancer (del Vecchio et al. 2000). Interestingly, the MIF molecule secreted by *T. vaginalis* (TvMIF) show all features of its human homologue (i.e., enzymatic activities, ability to inhibit macrophage migration, binding to CD74, ERK1/2, and Akt/BAD phosphorylation, production of IL-6 and IL-8) including proliferation and invasiveness of normal and tumor-derived prostate cells. In addition, a high number of sera from patients infected with *T. vaginalis* are reactive to TvMIF, especially in males, demonstrating the secretion of TvMIF during protozoan infection.

All these data indicate that secreted TvMIF in combination with chronic inflammation during trichomoniasis, and especially in case of asymptomatic infection, may have a role in increased risk of prostate cancer (and probably also cervical cancer), thus leading to consider *T. vaginalis* as a potential target for secondary chemoprevention.

5.2.8 Perspectives and Open Questions

Despite the high prevalence of trichomoniasis and the social impact of infection and complications, several important aspects of the causative agent, *T. vaginalis*, remain poorly understood. Future studies on basic and molecular biology of this parasite, together with a new knowledge on immune response strategies of the host, will certainly contribute to the understanding of the complex series of events taking place at the host/parasite interface. Even though complex sets of data from complete genome sequencing and from "omics" technologies have elucidated important aspects of the pathophysiology of this parasite, gaps in our knowledge still remain.

A list of still open questions includes:

- Can sexual recombination among different isolates occur? The presence of a complete set of meiosis-specific conserved genes indicates that *T. vaginalis* might be capable of genetic recombination. The hypothetic ability of protists to undergo genetic exchange has significant implications for the understanding of trichomoniasis biology.
  Recent observations based on microsatellite studies indicate the existence of two genetically different *T. vaginalis* "types": type 1 and type 2 have different geographic distributions and show highly significant difference in the frequency of TVV infection; in addition, type 2 isolates show a higher resistance to metronidazole (Conrad et al. 2012).
- What is the role of adhesins and trichopores in pathogenicity? The detailed mechanisms of adhesion and cytopathogenicity should be elucidated, in order to characterize molecules directly involved in virulence, such as adhesins and secreted toxins. The pathogen attaches to different mucosal surfaces of male and female urogenital tracts: *T. vaginalis* is able to colonize different ecological niches, modulating virulence mechanisms and adapting to different environmental conditions. It is still obscure if protozoa undergo a global transcriptional reprogramming upon contact with different epithelial surfaces (vaginal, urethral, prostatic). At the same time, the strategies of host immune response should be elucidated, especially during chronic infections. The explanation of the intricate relationships between the pathogen and the human host is fundamental to set up strategies to produce effective vaccines.
- Is it possible to identify *T. vaginalis* during chronic asymptomatic infections? It is important to set up highly sensitive and specific diagnostic techniques capable of identifying not only acute but also subclinical infections, in order to prevent disease transmission from asymptomatic donors and to prevent complications related to long-lasting disease.

- Is it possible to design alternative and effective drugs? Metronidazole is considered the only effective therapy for trichomoniasis. It is estimated that 2.5–5 % of all cases of infections are resistant to treatment with this drug, and the number of resistant isolates is steadily increasing. Even if the drug resistance can sometimes be overcome with higher metronidazole doses, and alternative 5-nitroimidazole derivatives such as tinidazole have been widely used, mechanisms of drug resistance should be analyzed in depth in order to design new effective pharmacological therapies.
- What are the interactions with intracellular symbionts and their influence on protozoa biology? A very exciting and still uncovered issue is the study of the complex and intriguing interactions of *T. vaginalis* with the vaginal microbiome, including commensal microorganisms and other pathogens able to colonize the human urogenital tract. The influence of the two intracellular symbionts *T. vaginalis* dsRNA virus (TVV) and *M. hominis* on metabolism and virulence deserves to be studied carefully, as well as the role of the protozoon as a "Trojan horse" in transmission of concomitant infections.
- Can *T. vaginalis* be considered as a cause of transformation or maintenance of tumours? Following the elucidation of the potential functions of TvMIF in prostate tumorigenesis, the role of *T. vaginalis* in malignant transformation and maintenance of a tumor-supporting environment, including characterization of pro-oncogenic molecules produced during genital infection, should be studied in depth and carefully taken into account. Potentially, controlling *T. vaginalis* infections might mean controlling a possible cofactor in prostate and cervical cancer.

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Trypanosoma

6

Christine Clayton

Abstract

This chapter describes the unique features of kinetoplastid molecular and cellular biology. Various adaptations, including antigenic variation and intracellular invasion, allow the parasites to survive in different environments within mammalian and arthropod hosts. Some molecular features are so unique that they allow kinetoplastids to serve as biological model systems.

6.1 Introduction

Trypanosomes are unicellular flagellates which belong to the class Kinetoplastea and the order Trypanosomatida, which also includes the leishmanias. Organisms of the class Kinetoplastea are parasitic and have a single nucleus, mitochondrion and flagellum. They show a variety of forms varying from long, thin, motile spindle shapes to spherical organisms in which the flagellum is reduced to a tiny stub. One of the original defining characteristics was the assemblage of mitochondrial DNA, which is called the kinetoplast and, after appropriate staining, is visible by light microscopy. In addition to their medical and economic impact, the trypanosomes exhibit many remarkable biological features and have become model systems for various aspects of eukaryotic molecular cell biology. Kinetoplastids probably evolved as parasites of insects, with infection of secondary hosts as a later development. There are numerous trypanosome species; only those of medical or veterinary importance will be considered here.

C. Clayton
Group Leader in the DKFZ-ZMBH Alliance, ZMBH, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany
e-mail: cclayton@zmbh.uni-heidelberg.de

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The salivarian trypanosomes replicate extracellularly in the blood and tissue fluids of mammals and in the digestive system of tsetse flies. Various *Glossina* species are involved in transmission, which occurs when the fly feeds on a permissive mammalian host. The tsetse-transmitted diseases of medical and veterinary relevance, which are restricted to sub-Saharan Africa, include nagana in cattle and sleeping sickness in humans. *Trypanosoma congolense* and *Trypanosoma vivax* are the major pathogens of cattle and goats, and *Trypanosoma simiae* infects pigs. All of the aforementioned parasites are killed by human serum. *Trypanosoma brucei* falls into several subspecies. While *Trypanosoma brucei brucei* is a minor parasite of cattle, the subspecies *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* have acquired minor changes that enable them to survive in human serum: these are the causative agents of sleeping sickness. (From now on, these will be referred to as *T. brucei*, *T. rhodesiense* and *T. gambiense*.) Due to the ease with which it can be maintained in the laboratory, and the wide spectrum of available tools for reverse genetics, *T. brucei* has become the organism of choice for investigating kinetoplastid molecular and cellular biology.

T. brucei subspecies that have defective kinetoplast DNA are unable to multiply in tsetse; the resulting parasites, which are called *Trypanosoma equiperdum* or *Trypanosoma evansi*, can be transmitted either venereally, or mechanically by biting flies. These trypanosomes are distributed across Asia and in South America.

Stercorarian trypanosomes multiply extracellularly in the digestive system of various *Rhodnius*, *Triatoma* and *Panstrongylus* species – reduviid bugs. Infection of mammalian hosts occurs when infected bug faeces are rubbed into the bite site, and in this host, the parasites multiply intracellularly. *T. cruzi* can enter mucosal membranes or be transmitted congenitally or orally.

Trypanosoma cruzi causes Chagas disease in humans; *Trypanosoma rangeli*, which appears superficially similar, is non-pathogenic for humans but pathogenic for the bug. Although *T. cruzi* and *T. rangeli* are currently restricted to the Americas, related trypanosomes are found worldwide in mammals and reptiles, including bats and crocodiles. *T. cruzi* can be subdivided into several different groups based on differences in genomic sequence; the groups vary in geographical distribution and virulence.

There are numerous other trypanosomes, but these have been little studied at the molecular level, so they will not be considered further here.

6.2 Cell Structure

Figure 6.1 illustrates the most important components of trypomastigotes. Light microscopy of live cells reveals elongated, wriggling organisms of varying sizes; for example, bloodstream-form *T. brucei* are about 20 μm long and 2 μm wide. Stains that reveal DNA, such as Giemsa or intercalating dyes, reveal the nucleus (with an approximately diploid chromosome content) and the kinetoplast, which is composed of multiple DNA circles. The flagellar basal body is linked to the kinetoplast by structural proteins, which form links across the mitochondrial inner and outer

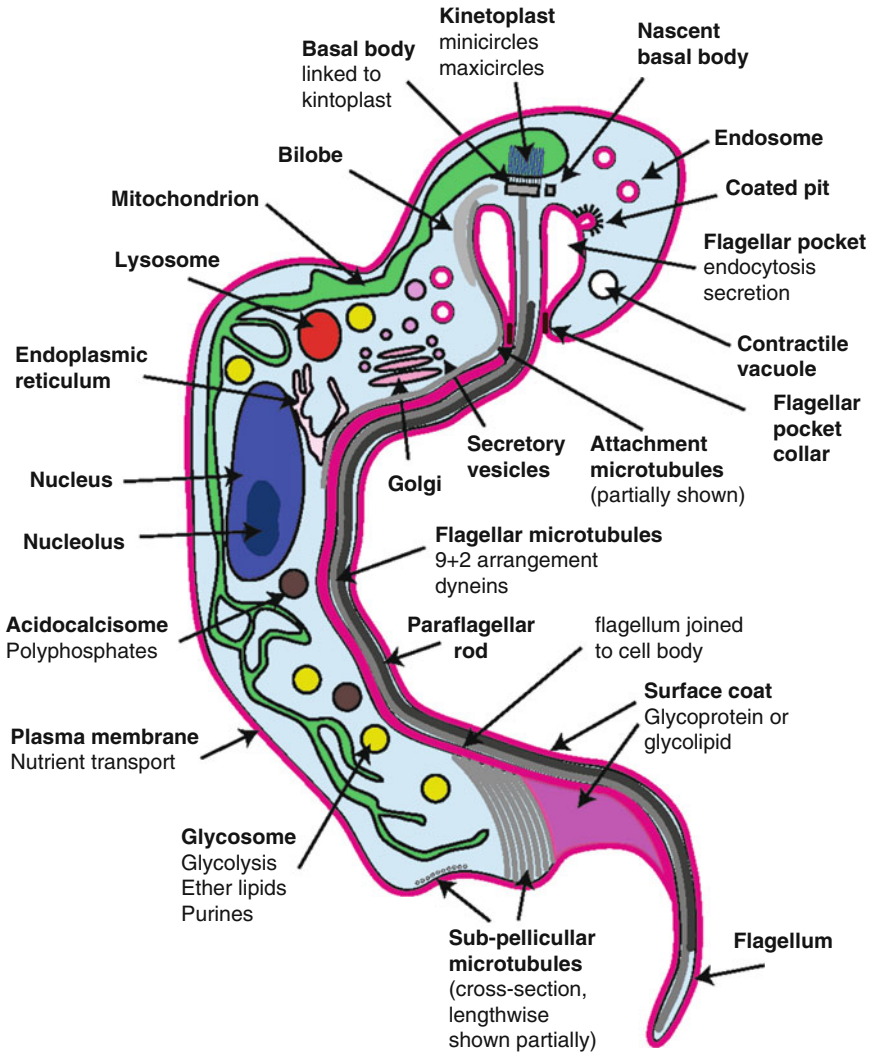

Fig. 6.1 Trypanosome structure

membranes. The flagellum emerges from the flagellar pocket, which is at the posterior end of the cell relative to the direction of swimming. The shape of the parasite is maintained by a corset of microtubules, which spiral around the parasite; this is shown only partially in Fig. 6.1. As cells grow, more microtubules are added between the existing ones; the "plus" ends are towards the posterior of the parasite. The flagellum is joined to the parasite body along most of its length and also spirals around the cell body. (For simplicity, it has been straightened out in Fig. 6.1). Four specialised microtubules, which are linked to the basal body by the bilobe, run down the body of the parasite immediately underneath the flagellum. The bilobe is not

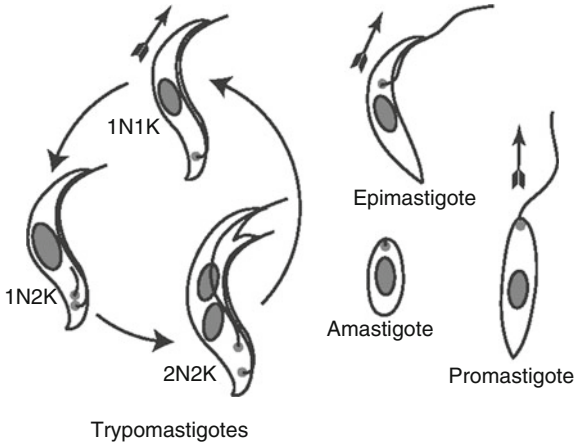

Fig. 6.2 Variations in trypanosome form. The *arrow* indicates the direction of swimming. "1N1K" indicates that the cell has one nucleus and one kinetoplast; the diagram shows that kinetoplast division precedes nuclear division

visible by either light of electron microscopy, but can be detected with antibodies to its constituent proteins.

The flagellum contains, in addition to microtubules in the classical 9 + 2 configuration, a protein assemblage called the paraflagellar rod which is essential for flagellar shape and motility. During cell multiplication, synthesis of the kDNA slightly precedes nuclear S phase. After basal body duplication, a new flagellar pocket is formed, and the new flagellum starts to grow out, and the kinetoplast divides. Nuclear division is then followed by cytokinesis (Fig. 6.2). Other developmental stages differ in shape, flagellar length and attachment, and the position of the kinetoplast/basal body assemblage relative to the nucleus (Fig. 6.2). In the amastigote stage, the flagellum is present only as a stub that does not protrude beyond the flagellar pocket entrance.

Kinetoplastid protists use specialised surface macromolecules to protect themselves from the external environment. The salivarian trypanosomes have glycoprotein surface coats, which vary according to species and life cycle stage. As in all eukaryotes, the surface glycoproteins are synthesised in the rough endoplasmic reticulum (ER), and the glycan chains are modified in the Golgi. Each kinetoplastid possesses only one or two Golgi, which have to be replicated once per cell division; the single organelle in procyclic forms makes trypanosomes a very convenient model system for the study of Golgi replication. Glycoproteins and glycolipids are secreted into the flagellar pocket, which is the only part of the organism that lacks the microtubule corset. The flagellar pocket is also the only part of the parasite that is available for uptake of nutrients and recycling of cell surface proteins via endocytosis. Its integrity is ensured by specialised structural proteins that join the neck of the pocket to the flagellum, in a structure called the "collar". Endocytosed nutrients are transported in vesicles to the single lysosome. The lysosome is also the site for recycling of cellular contents – including organelles – by autophagy; this is probably particularly important for cellular remodelling as the parasite progresses from one life cycle stage to the next. *T. cruzi* has specialised late endosomes called reservosomes. There are several present in each cell, and they probably have a variety of functions.

Three organelles are important in cellular metabolism. The mitochondrion shows varying degrees of elaboration. When the cells are dependent on amino acids and require active oxidative phosphorylation, the mitochondrion forms an extensive network; under conditions of abundant glucose, the mitochondrion regresses. The glycosomes are microbodies: that is, they fall into the same family as peroxisomes. They contain most enzymes of glycolysis and parts of several additional pathways. Acidocalcisomes – as implied by their name – are an acidic compartment that stores polyphosphates and calcium.

6.3 Variations During the Life Cycle

6.3.1 Salivarian trypanosomes

Salivarian trypanosomes multiply in the blood and tissue fluids as long slender trypomastigotes. They depend primarily on glucose for energy generation and the mitochondrion is rather rudimentary. Long slender bloodstream-form trypomastigotes are protected from humoral immunity by a coat of Variant Surface Glycoprotein (VSG). A minority of parasites arrest in the G_0 phase of the *T. brucei* cell cycle and preadapt for survival in tsetse: they become shorter and up-regulate the expression of some mitochondrial proteins. After uptake into tsetse via the proboscis, the parasites develop into procyclic trypomastigotes, in which the kinetoplast is a little nearer to the nucleus. Procyclics have an elaborate mitochondrial network with considerable metabolic capabilities, adapted to energy generation via oxidative phosphorylation with amino acids – particularly proline – as the energy source. The parasite surface is now covered with a glycoprotein called procyclin.

After 1–2 weeks in the tsetse midgut the parasites migrate to the proventriculus, there is an asymmetric division involving a short, and an extremely elongated form; this is likely to be the point at which epimastigotes are formed (Fig. 6.2). The latter then migrate to the salivary glands, where many adhere to the salivary gland epithelium and some undergo meiosis and mating. Unusual forms that have apparently haploid DNA content, and in which the kinetoplast is next to the nucleus, have been suggested as the gametes, but this is controversial. Finally, the epimastigotes convert into metacyclic trypomastigotes, which have surface VSG and are – unlike procyclic forms – able to withstand human complement.

The cycle of *T. congolense* is similar to that of *T. brucei*, although rather fewer shape variations are seen; the surface proteins are described below. *T. vivax* procyclic forms are restricted to the foregut and the proventriculus. Stumpy bloodstream forms have not been observed for either species, but their cycles have been much less studied than those of *T. brucei*.

Differentiation and cell cycle control require the trypanosome to regulate gene expression in response to changes in the extracellular environment. The only developmental switch that has been studied in any detail is the transition of bloodstream-form *T. brucei* to the procyclic form. *T. brucei* long slender bloodstream forms (which grow at 37 °C) are able to measure their own cell density (a process called

"quorum sensing"); they secrete a mysterious signal molecule of under 500 Da called "stumpy induction factor" (SIF). When the SIF concentration surpasses a threshold, stumpy formation is initiated, with a rise in the intracellular AMP concentration. (Several papers implicated cAMP, but it turns out that the only cAMP analogues that work are those that can be metabolised to AMP.) This argues against a classical cAMP-mediated signalling mechanism. In addition to some mitochondrial enzymes, stumpy forms express cis-aconitate transporters called PAD1 and PAD2. Further differentiation to procyclic forms is initiated by a change in the medium – especially, by addition of cis-aconitate – and a decrease in temperature to 27 °C. The transition can be further enhanced by incubating the cells at 20 °C, which increases the amount of PAD2 on the cell surface. PAD1 or PAD2 must be present for perception of the cis-aconitate signal. The signalling is effected by several kinases and phosphatases, but the connection between those and gene expression is not yet understood.

6.3.2 Stercorarian Trypanosomes

Nearly all work on stercorarian molecular biology has focussed on *T. cruzi*. The parasites multiply inside the gut of reduviid bugs as epimastigotes (Fig. 6.2), transition to metacyclic trypomastigotes in the insect hindgut, and are passed in the faeces as the insect takes a blood meal. Upon entering the mammalian host, the trypanosomes spread in the blood and are taken up by a variety of cell types. They escape the initial phagolysosome to replicate freely in the cytosol as amastigotes. Differentiation into a non-replicating trypomastigote form precedes host cell lysis, whereupon emergent trypomastigotes circulate and can either reinvade another mammalian cell or infect a reduviid bug. *T. cruzi* pathology in the chronic stage reflects its preferential replication in smooth and cardiac muscle cells.

T. cruzi has several different mechanisms of invasion, which depend partly on the cell type. Various host cell molecules have been implicated in initial attachment; they include the LDL receptor, cell surface glycosaminoglycans and sialylated glycans.

Both trypomastigotes and epimastigotes are efficiently internalised by macrophages, but epimastigotes are destroyed within the lysosomes by NO and other reactive oxygen species. Invasion of macrophages may involve two different mechanisms, one of which involves active invasion by the parasite, while the other requires host actin polymerisation. There is evidence for involvement of flotillins and caveolae.

T. cruzi trypomastigote invasion of non-phagocytic host cells mimics a plasma membrane injury and repair process. It involves calcium-dependent lysosome exocytosis at the parasite attachment site, release of lysosomal acid sphingomyelinase and the localised production of ceramide in the exoplasmic leaflet of the plasma membrane. This prompts membrane invagination and parasite entry. Some of the vacuoles are initially devoid of lysosomal markers, but contain phosphoinositides; these acquire early endosomal markers, EEA1 and Rab 5, then late endosome and

lysosomal markers, in a process that resembles endosome maturation. The remainder of parasite vacuoles fuses immediately with host lysosomes and/or autolysosomes. It is currently unclear whether the acid sphingomyelinase-dependent, ceramide-enriched vacuoles are also enriched for phosphoinositides or whether there are two separate entry pathways for *T. cruzi*. Regardless, the fusion of parasitophorous vacuoles with lysosomes causes internal acidification, which allows *T. cruzi* to escape to the cytosol.

6.4 The Cell Surface

6.4.1 Surface Molecules: Salivarian Trypanosomes

Salivarian trypanosome bloodstream forms are covered by Variant Surface Glycoprotein (VSG) dimers comprising 5–10 % of the total cell protein (about ten million molecules per cell). The VSG molecules have molecular weights varying between 50 and 65 kDa and completely conceal most underlying essential membrane proteins such as receptors and transporters. Thus, although membrane proteins will definitely be presented as antigens after trypanosome lysis, in most cases antibodies cannot bind to them on live cells. Each VSG carries a glycosyl phosphatidylinositol anchor with two myristate chains (Fig. 6.3). The mechanism of antigenic variation will be discussed below. Diagnostic screening for *T. gambiense* infection relies on the presence of antibodies to two highly conserved VSGs.

The bloodstream forms of *T. brucei* and *T. congolense* also have a transferrin receptor; this is built of two proteins called ESAG6 and ESAG7 which are related to VSG (Fig. 6.3). Only ESAG6 carries an anchor: since the myristate chains are only 14 carbon units long, attachment of the receptor is somewhat unstable, and it can be released into the flagellar pocket.

The family of "Invariant Surface Glycoproteins" (ISG65 and ISG75) is expressed in bloodstream-form *T. vivax*, *T. congolense* and *T. brucei*; these may be of some interest for diagnosis of these three infections. It has been suggested that ISG75 might be the receptor for the anti-trypanosomal drug suramin. These are about 1,000 times less abundant than VSG. The haptoglobin-haemoglobin receptor of *T. congolense* (and presumably also *T. brucei*) has an elongated structure, which is expected to protrude above the VSG coat.

Salivarian trypanosomes have several genes encoding surface proteases (MSPA – MSPD) that are related to *Leishmania* GP63 and are expressed to various levels in the different stages. Most, but not all, have signals for GPI anchor addition. *T. brucei* MSPB is known to assist in cleavage of VSG from the surface during differentiation from the bloodstream to the procyclic form; the other enzyme involved, a glycosyl phosphatidylinositol phospholipase C, removes the GPI anchor.

Trypanosomatids that multiply in the gut of arthropods are limited by various components of the host innate immune system. *T. brucei* subspecies switch to expression of procyclins upon arrival in the midgut: these are "procyclins" with central EP or GPEET repeats, which probably form an extended structure; the

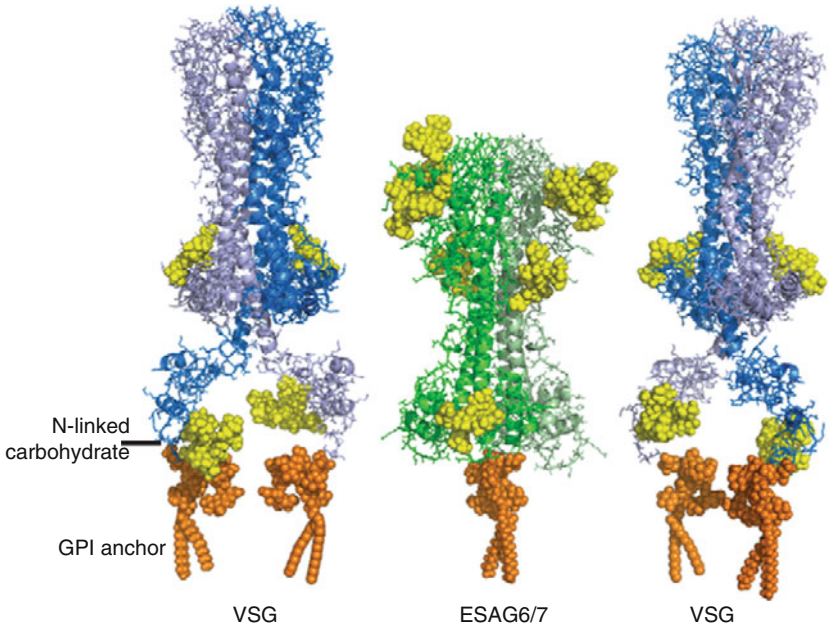

Fig. 6.3 Approximate structures of VSG and the transferrin receptor (Adapted from Mehlert et al. 2012)

protein is fixed into the membrane by inositol-acylated monoacyl phosphatidylinositol with 16- or 18-carbon chains. The inositol acylation prevents cleavage by GPI-PLC. The choice between GPEET and EP procyclin is influenced by carbon source availability and the activities of mitochondrial pathways, GPEET being turned off by hypoxia. Rather surprisingly, the procyclins are not required for transmission of trypanosomes through tsetse. Epimastigotes express BARP (brucei alanine-rich proteins). Bioinformatic analysis revealed the existence of several more GPI-anchored proteins, but their functions are unknown.

T. congolense insect stages express at least four different GPI-anchored proteins. Insect-stage surface antigen (TcCISSA) is >60 % identical to *T. brucei* procyclin: it has EPGENGT repeats. There are also *T. congolense* epimastigote-specific protein, CESP, and glutamic acid- and alanine-rich protein (GARP) and protease-resistant surface molecule (PRS). The carbohydrate on PRS is rich in galactose and sialic acid residues; the latter are presumably transferred by the parasite trans-sialidase. The anchor on PRS is composed of inositol-acylated diacyl-phosphatidylinositols containing myristate: there are thus three lipid chains involved in anchoring.

6.4.2 Surface Molecules in Stercorarian Trypanosomes

T. cruzi has two major surface protein families: the trans-sialidases and the mucins. Mucins consist of 40 % protein and 60 % carbohydrate. Mucin genes comprise 1 % of the parasite genome. There are extensive variations between

strains both in the protein core and the carbohydrates. The protein core is 50–200 amino acids long, with many serines and threonines available for O-glycosylation. The carbohydrates have a core of beta-galactopyranose or galactofuranose linked to n-acetyl glucosamine. The core is decorated with sialic acid, transferred from host carbohydrates to the mucins by the trans-sialidase. This is thought to protect the parasite from antibodies to galactopyranose.

T. cruzi has many trans-sialidase genes in repetitive arrays. In one strain, over 200 genes were detected, although only 15 were predicted to encode enzymatically active proteins. Other strains may have more. Trans-sialidase has an N-terminal catalytic domain, which sometimes has a mutation which abolishes enzyme activity without affecting the ability to bind sialic acid. Next there is a lectin-like globular domain. This can bind to the nerve growth factor receptor. In epimastigotes, this dual structure is anchored to the plasma membrane via a transmembrane peptide. In trypomastigotes, there is five times more trans-sialidase, and it is more complex. There is an additional domain of 12-amino acid "shed acute-phase antigen" (SAPA) repeats, and the whole is attached to the surface via a GPI anchor. Hydrolysis of the anchor can result in shedding of trans-sialidase into the blood and tissue fluids.

The precise role of trans-sialidase in both hosts is not known. Since there are multiple genes, and RNAi is absent from *T. cruzi*, it is not possible to investigate the properties of *T. cruzi* that lack trans-sialidase. It is thought that in the vector, trans-sialidase mainly transfers sialic acid to the surface mucins. In the mammalian host, there are additional functions. Binding of the globular domain to the nerve growth factor receptors is thought to facilitate invasion of neural, glial and epithelial cells. Antibodies to trans-sialidase can block invasion and inhibit the enzyme activity, but some antibodies are thought to be non-inhibitory. There are also effects on cellular immunity, which prevent parasite elimination.

T. cruzi also has several hundred genes encoding proteins related to the salivarian MSPs, but the roles of these proteins are not known.

6.5 Genomes and Their Replication

6.5.1 The Nuclear Genome

Salivarian trypanosome genomes are diploid. With the exception of some *VSG* genes, the protein-coding genes of *T. brucei* are distributed between 11 diploid chromosomes; a single set constitutes at least 30 Mb of DNA. The smallest chromosome is about 1 Mb. In addition, the salivarian trypanosomes have a diverse collection of smaller chromosomes called "mini" chromosomes, which act as a repository of inactive *VSG* genes. The chromosomes of stercorarian trypanosomes have considerable synteny with those of the Salivaria, but there have been extensive rearrangements, and the parasites are not strictly diploid. The telomeres are composed of TTAGGG repeats, as in vertebrates.

Trypanosomes possess the classical core histones – H2A, H2B, H3 and H4 – plus some variants and a rather divergent version of histone H1. In all kinetoplastids analysed so far, some thymine bases are modified to glycosylated hydroxymethyluracil ("base J"), but DNA methylation has not been observed.

The most remarkable aspect is the arrangement of open reading frames in polycistronic transcription units, which can include 100 or more open reading frames (discussed in more detail below). There are thus relatively few places where polymerase II transcription appears to initiate and terminate. Most polymerase II initiation sites are at places where the direction of transcription changes (divergent strand-switch regions), and most termination is at convergent strand-switch regions. However, polymerase II initiation and termination can also occur without a strand switch; obviously, also, it must occur at the beginning and end of regions which are transcribed by a different polymerase, such as rRNA or tRNA loci. Polymerase II initiation sites are "marked" by histone variants H2A.Z and H2B.V and termination sites by H3.V and H4.V, in addition to acetylation and methylation of specific residues in the classical core histones. Base J was first found in *T. brucei* telomeres but is also present in polymerase II initiation and termination sites.

6.5.2 Replication of Trypanosome Nuclear DNA and Chromosome Segregation

In Opisthokonts, origins of replication are recognised by an origin recognition complex composed of "ORC" proteins, and more factors are required for recruitment of the replicative helicase, composed of the MCM (minichromosome maintenance complex) and additional proteins. Once the DNA has been melted, the remaining components, including DNA polymerase, are recruited, and replication can be initiated. This machinery is generally conserved in trypanosomes, with the exception of proteins required for origin recognition, which are highly diverged.

Origins of replication were found by identifying sequences associated with ORC1 and by looking at genomic sequence coverage in synchronised cells in early S phase. Within the polymerase-II-transcribed "cores" of the large chromosomes, all of the origins corresponded with sites of RNA polymerase II transcription initiation or termination. This, combined with the absence of any consensus sequences, leads to the suspicion that both are specified by chromatin structure. Only a fifth of these ORC binding sites become active replication origins. The remaining ORC binding sites are in non-transcribed subtelomeric regions and are not used for replication. Methylation of histone H3 is involved in the regulation of replication.

Once the DNA has been replicated, chromosomes must be segregated accurately. In both *T. cruzi* and *T. brucei*, each of the large (megabase-length) chromosomes has a centromere-like region. One way that these can be recognised is by their ability to confer mitotic stability on small DNA fragments or circles. Another method relies on the fact that topoisomerase accumulates at centromeres during prometaphase, where it is needed to resolve accumulated catenations. Treatment with etoposide results in cleavage without religation, so cleavages are concentrated in centromeric

regions. In both *T. cruzi* and *T. brucei*, the centromeres are located between transcription units, and both contain degenerate retroposons or other transposable elements. In *T. cruzi*, the regions are GC-rich, whereas *T. brucei* centromeres include 20–120 kb of 147 bp AT-rich tandem repeats. The details of how spindle microtubules are attached to the centromeres are not understood. Salivarian trypanosome minichromosomes lack centromeres and so are probably distributed at random.

Kinetochores are highly complex protein complexes that form on centromeres and connect them to the mitotic spindle. Until recently, it was supposed that the eukaryotic kinetochore was highly conserved, but this is not true. Kinetoplastids do not have homologues of any of the components, including the histone H3 variant CENP-A, which marks centromeres in all other eukaryotic groups analysed so far. Instead, they have a kinetochore that is composed of 19 kinetoplastid-specific proteins.

The trypanosome cell cycle is governed, as in other eukaryotes, by cyclins and cyclin-dependent kinases, but trypanosomes lack some of "checkpoints" – points at which the cell division cycle can be arrested if progress is not satisfactory. For example, in yeast, a failure of cell division after nuclear division will halt further DNA replication, but in *T. brucei* bloodstream forms, DNA replication and nuclear division can carry on for several cycles, resulting in multinucleate "monsters". Conversely, if the kDNA divides, but the nucleus does not, the cells can still divide, to give one cell with both kDNA and a nucleus, and a "zoid" with kDNA but no nucleus.

The basic machineries for recombination and DNA repair are similar to those from Opisthokonts. In *T. brucei*, recombination and repair are vital for antigenic variation as well as for accurate DNA replication. DNA repair pathways include nucleotide excision repair, mismatch repair and double-strand break repair. All of these are active in trypanosomes. Though defects in a pathway tend not to affect growth of the parasites in culture, at least in the short term, they result in increased susceptibility to treatments that damage DNA. Nucleotide excision repair in *T. brucei* is predominantly coupled to the recognition of lesions that block transcription. UV lesions are not repaired very efficiently: in the wild, of course, the parasites are unlikely to be exposed to UV irradiation since they are always inside the host. Mismatch repair is required to correct errors in DNA replication, and trypanosomes that lack it show instabilities in the lengths of loci containing repeats and increased susceptibility to hydrogen peroxide. In other organisms, double-strand breaks can be repaired either by joining of non-homologous ends, or by recombination between ends with varying degrees of homology (homologous regions or regions of microhomology). In *T. brucei*, the non-homologous pathway seems to be absent. An important consequence for the researcher is that homologous recombination is very efficient, which greatly facilitates targeted integration of transfected DNA.

Genetic exchange occurs in *T. brucei*, within the tsetse fly. Meiosis-specific genes are conserved and are expressed in the salivary glands. Using populations of parasites tagged with two different cytosolic fluorescent proteins, and microscopic DNA quantitation, gametes have been detected in the salivary glands. As judged by morphology, and mixing of the fluorescent proteins, mating also occurs there,

starting with interactions between the two flagella. Mixing of cytosolic proteins, however, does not necessarily mean that nuclear fusion will occur, and so far, the latter has not been directly observed. Mating also occurs in *T. cruzi* and clear genetic hybrids are found.

6.5.3 Kinetoplast DNA and Its Replication

The kinetoplast DNA is visible by light microscopy when suitably stained. It consists of hundreds of minicircles of fairly uniform size – 1 kb in the Salivaria – concatenated together in an ordered, disc-like assemblage. Threaded within them are about 20 maxicircles (around 30 kb), which are the main mitochondrial genome. The maxicircles contain the genes for mitochondrial rRNA and a small number of mitochondrial proteins; the minicircles assist in production of mature mitochondrial mRNAs in a process called RNA editing (described later). Faithful replication and segregation of both are therefore required in order to preserve mitochondrial gene expression.

Both minicircles and maxicircles are replicated from a single origin, resulting in theta structures. In order to segregate the copies, however, the entire structure has to be decatenated (Fig. 6.4). The replication process therefore involves numerous different DNA polymerases, ligases and topoisomerases (not named individually in the figure), whose positions can be determined by immunofluorescence. Meanwhile, DNA replication itself can be visualised by incorporation of bromo-deoxyuridine or by adding a fluorescent nucleotide to nicks and gaps using terminal

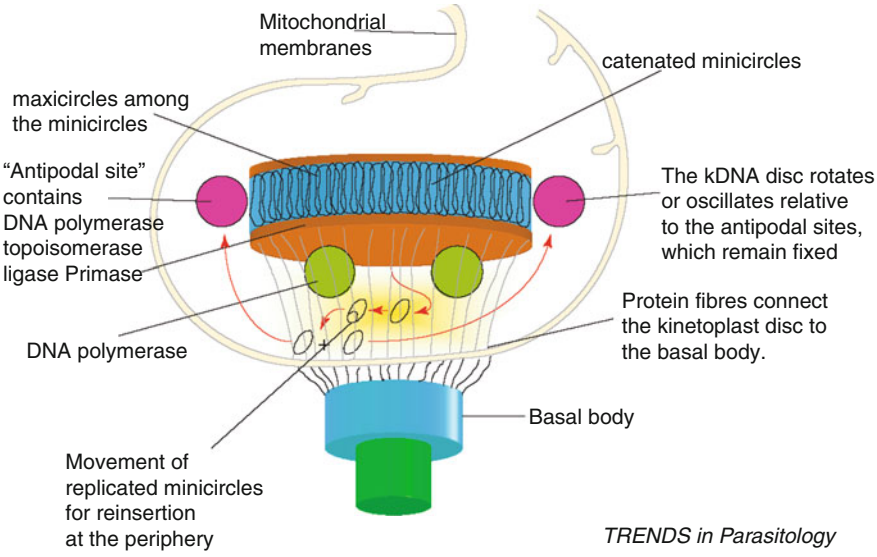

Fig. 6.4 Kinetoplast structure and replication (Adapted from *Trends in Parasitology*, vol. 21 No. 8, August 2005)

deoxynucleotidyl transferase. The kDNA disc is connected to the basal bodies via filaments that link across the mitochondrial membranes. Once the basal body has duplicated, it can pull the two kDNA discs apart to ensure accurate distribution of one to each daughter cell. A decrease in one of the fibre proteins correspondingly resulted in defective kDNA segregation.

Not surprisingly, the process of kDNA replication is highly susceptible to inhibition by intercalating dyes. Most salivarian trypanosomes require expression of at least one kDNA-encoded protein in order to grow. Cultivation with intercalating drugs leads to formation of non-viable cells that lack the kDNA. Indeed, the drug homidium, which is a derivative of ethidium bromide, is still used for cattle trypanosomiasis. Prolonged cultivation with such drugs can lead to permanent loss of the kDNA, which is compensated by nuclear mutations. (This is explained later.)

6.6 Gene Expression

6.6.1 Nuclear Gene Arrangement and Transcription

Kinetoplastid polycistronic transcription units consist of from 2 to over 100 open reading frames that are all in the same orientation on the DNA. The genes in a unit are usually not related, either with regard to regulation of their expression or to the function of the encoded protein. Tandem repeats of identical genes are an exception to this: very abundant, constitutively expressed proteins such as cytoskeletal proteins and histones are often encoded by such repeated genes.

As already noted above, RNA polymerase II transcription initiates in ORF-free regions of a few kilobases which are associated with variant histones and histones bearing characteristic post-translational modifications – acetylation and methylation of specific residues (Fig. 6.5, purple arrows). Initiation happens in a region, rather than at one specific nucleotide, and is usually (though not always) bidirectional. Evidence so far indicates that the transcription of protein-coding genes is not under specific control: all transcription units are transcribed equally, and initiation may depend mainly on the presence of relatively open chromatin. Correspondingly,

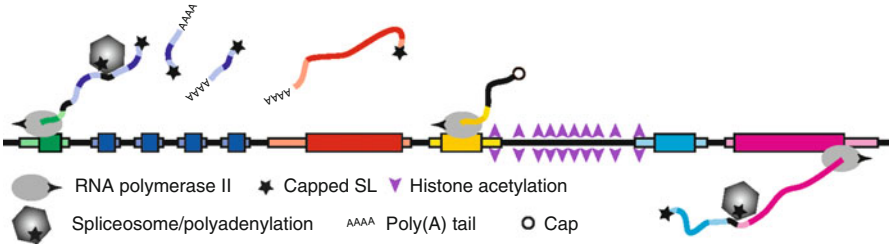

Fig. 6.5 Kinetoplastid gene expression. The *black line* is DNA that will not be represented as mature mRNA. *Thick boxes* are coding regions; *thinner boxes* are the 5′- and 3′-untranslated regions. The *dark-blue* genes on the left are a tandem repeat. The figure shows the beginning of two oppositely oriented polymerase II transcription units

there is no evidence for regulatory polymerase II transcription factors. There are, however times when transcription in general is decreased; this occurs when cell growth has stopped, such as in stumpy trypomastigotes of *T. brucei*. Termination sites are also epigenetically marked, with histone variants and modifications as well as base J. Base J is essential in *Leishmania*, where it is required for accurate polymerase II termination, and probably also in *T. cruzi*, where reduced J caused global changes in gene expression; but it is not essential in *T. brucei*.

To make mRNAs that contain only a single open reading frame, the precursor must be processed. This is achieved by *trans* splicing of a 39 nt "spliced leader" (*SL*) upstream of the start codon, and polyadenylation at the 3′-end. The *SL* comes from a precursor of about 140 nt called the *SLRNA*. The hundred or so *SLRNA* genes are arranged as a repeat, and each has an RNA polymerase II promoter that is recognised by specific transcription factors and has a unique transcription start site. As in other eukaryotes, *SLRNA* transcription initiation is accompanied by capping – addition of a 5′–5′ linked 7-methylguanosine residue to the 5′ end of the primary transcript. In addition, the first four nt of the *SL* are methylated. Since the mRNAs from up to 10,000 protein-coding genes have to be *trans* spliced, a substantial and continuous supply of *SLRNA* is required.

As in all other eukaryotes, the ribosomal RNA (rRNA) genes are transcribed by RNA polymerase I. The transcription complex includes both conserved and trypanosome-specific subunits, and its function has been studied extensively in an in vitro system. In the Salivaria, the genes encoding *VSG* and the procyclins are also transcribed by RNA polymerase I. This is unique to trypanosomes and can only happen because the 5′ end of the mRNA is capped post-transcriptionally by *trans* splicing. The *rRNA*, *VSG* and *EP*/*GPEET* promoters have distinct sequences but seem so far to rely mostly on the same transcription factors. RNAi experiments showed that histone H1 is required for regulated suppression of polymerase I transcription.

Genes that encode structural RNAs are interspersed between the polycistronic units. They include the rRNA genes, which are transcribed by RNA polymerase I, and tRNA genes, which are transcribed by RNA polymerase III and are often found at the points where polymerase II transcription units converge. The spliceosomal U RNAs, the U3 RNA (which is involved in rRNA processing) and the 7SL RNA (a component of the signal recognition particle) are transcribed by RNA polymerase III, with upstream tRNAs serving as promoters. In contrast, the small nucleolar RNAs (snoRNAs), which are involved in rRNA processing and modification, are processed from polycistronic polymerase II transcripts.

6.6.2 Processing of RNAs in the Nucleus

The trypanosome *trans*-splicing machinery is similar to that for *cis* splicing in other eukaryotes, except that the *SL* snRNP, which provides the 5′ trans-splice site, takes over the function that would normally be played by U1. A functional *SL* snRNP can only form with capped, methylated *SLRNA*. The *trans*-splicing acceptor

site – equivalent to the 3′ splice site in *cis* splicing – is usually an AG dinucleotide, situated about 25 nt downstream of a ~18 nt U-rich polypyrimidine tract. (All distances given are for *T. brucei*.) The spacing and polypyrimidine tract lengths vary considerably, and alternative acceptor sites are very often used. This can lead to the production of alternative proteins with different N-termini. The polypyrimidine tract is recognised by splicing factors, which recruit the U2, U4, U5 and U6 snRNPs. Only two *T. brucei* genes have been shown to be (rather inefficiently) *cis* spliced. *Trans* splicing probably occurs 1–3 min after the splice acceptor site has been made by the polymerase (Fig. 6.5).

There are no consensus polyadenylation signals in the mRNA. Instead, polyadenylation happens at several sites, located 100–140 nt upstream of the next polypyrimidine tract. All evidence so far indicates that splicing and polyadenylation are interdependent: maybe they occur in a single complex. The median 3′-untranslated region length in *T. brucei* is 400 nt, but lengths of over 6 kb are not uncommon. Since there are often several polypyrimidine tracts between the end of one open reading frame and the beginning of the next, the average *T. brucei* gene uses about ten poly(A) sites, arranged in clusters upstream of the different polypyrimidine tracts. This has implications for subsequent regulatory steps.

Kinetoplastid rRNAs also undergo quite unusual processing. The most obvious symptom of this is the fact that the large subunit rRNA is cut into two pieces. Thus, when kinetoplastid total RNA is run on agarose gels and stained with ethidium bromide, there are three prominent bands instead of the usual two. The rRNAs are made from a precursor of about 12 kb. A series of cleavage and steps produces the precursors of the small subunit rRNA, the 5.8S rRNA, the two main large subunit rRNAs and four other large subunit fragments. These are then trimmed by 5′ and 3′ exoribonucleases. The enzymes responsible for the 5′ trimming have not been identified, but 3′ processing is – as in Opisthokonts – effected by the exosome. Modification of the rRNA is guided by a bevy of snoRNAs. The processing and modification of tRNAs have been relatively little studied.

6.6.3 Control of Cytosolic mRNA Abundance

Since most protein-coding genes are constitutively transcribed, gene expression must be controlled at subsequent points. Some mRNAs are spliced faster than others, which will in turn affect the efficiency of upstream polyadenylation. In the nucleus, mRNA processing competes with degradation, so mRNAs that are spliced or polyadenylated slowly are in danger of being destroyed without ever reaching the cytosol. Moreover, the longer an mRNA is, the less efficiently it seems to be made; this could also be a consequence of co-transcriptional destruction. Alternative splicing can be developmentally regulated, but the mechanisms are not yet known.

Once the mRNA is mature, it is exported through the nuclear pores to the cytosol. This process appears to be quite similar to that in other eukaryotes. Subsequently, the mRNA can be translated, but is also exposed to the mRNA degradation machinery. The median mRNA half-life is 12 min in bloodstream-form trypanosomes, and

20 min in procyclics, which are bigger and grow a bit slower (division times in culture of 6–8 h and 12 h, respectively). However, some mRNAs have half-lives exceeding 4 h. Work with bloodstream forms has shown that very unstable mRNAs (half-lives under 10 min) are preferentially attacked from the 5′ end, by decapping and digestion by a 5′–3′ exoribonuclease. This will immediately stop translation initiation, which depends on the 5′ cap. Most mRNAs, however, are subject to gradual removal of the poly(A) tail, which does not prevent translation; this is done mainly by the CAF1/NOT complex. The poly(A) tail is bound by poly(A)-binding protein (PABP). Once the poly(A) tail is too short for PABP to bind, the mRNA becomes exposed to 3′–5′ degradation by the exosome; decapping and 5′–3′ degradation are also facilitated.

The rate of mRNA degradation strongly influences the steady-state mRNA level, and can be a major determinant of developmental regulation. The rate of degradation is decided by the mRNA sequence, usually (but not always) in the 3′-untranslated region (3′-UTR). Several regulatory sequences have been identified using transgenic reporter gene assays, and in a few cases, the proteins that bind to the regulatory sequences are also known. Trypanosome genomes encode over 150 proteins with known RNA-binding domains, and a high-throughput screen identified nearly 300 trypanosome proteins that are capable of affecting mRNA fate. Only a small fraction of these proteins have been studied in detail. The mechanisms of action of the RNA-binding proteins are likely to involve interactions with the degradation or translation machineries, but so far none has been documented.

6.6.4 Cytosolic Translation

Classical eukaryotic mRNA translation starts with binding of eIF4E to the 5′-cap. eIF4E recruits eIF4G, which in turn recruits the helicase eIF4A. eIF4G can also interact with poly(A)-binding protein: this results in circularization of the mRNA and its protection against both deadenylases and the decapping enzyme. The next steps are binding of a 43S complex of activated eIF2, a charged methionyl tRNA and the small ribosomal subunit. The resulting 46S complex scans in the 3′ direction until it encounters an initiation codon (ATG). At this point, various translation factors are exchanged, the 60S subunit joins and translation can start. General translation inhibition can occur through modification of the eIF2-alpha translation factor. Trypanosomes have three potential eIF2-alpha kinases, one of which is localised to the flagellar pocket membrane. More specific inhibition can be – for example – by binding of a protein to a secondary structure in the 5′-UTR, or by binding of regulatory proteins to eIF4E, hindering recruitment of eIF4G.

The kinetoplastid translation machinery has one major unusual twist: the presence of multiple proteins with strong sequence identities with either eIF4E or eIF4G. In *T. brucei*, there are six eIF4Es and five eIF4Gs. The eIF4Es show varied polysome association and abilities to bind to the methylated cap and eIF4Gs. eIF4E3 and eIF4E4 seem to be the main eIF4Es involved in translation initiation, interacting mainly with eIF4G4 and eIF4G3, respectively. eIF4E1 can bind the cap, but – in

bloodstream-form trypanosomes at least – seems to be a negative regulator, along with its partner protein 4E-IP.

6.6.5 RNA Interference

RNA interference was discovered in trypanosomes in the same year as for metazoa, when the Ullu lab observed the appearance of "fat" cells after expression of a double-stranded RNA from the beta tubulin gene. Its natural role – as in other organisms – is the suppression of expression of mRNAs from retrotransposons and thus of genome instability, but there are also siRNAs from satellite repeats and transcription convergence regions. RNAi in trypanosomes is most effective if the dsRNAs are several hundred nucleotides long. As in other eukaryotes, the dsRNA is cut into pieces of about 21 nt by Dicer enzymes – there are two, DCL1 in the cytoplasm and DCL2 in the nucleus. DCL2 is required for RNAi targeting nuclear RNAs such as the snoRNAs. In the cytoplasm, siRNA is assembled into a complex that includes Argonaute protein AGO1 and two kinetoplastid-specific proteins: RIF4 is a 3′–5′ exonuclease that converts the ds siRNA into a single strand, and RIF5 is a DCL1 cofactor.

The genes required for RNA interference are absent in *T. cruzi* and several *Leishmania* species, including *L. major*. It has been suggested that conservation of RNAi is correlated with the presence of active transposons.

6.6.6 Genetic Manipulation

Ease of genetic manipulation has made African trypanosomes into a workhorse for kinetoplastid research. DNA can be put into trypanosomes by electroporation. Nearly all work is done using permanent cell lines, in which the DNA of interest has been integrated into the genome by homologous recombination. For this, one has to transfect a linear DNA that contains a selectable marker, flanked on each side by at least 70 bp of sequence that is identical to the genomic region to be targeted; longer homologous regions increase the efficiency. For high-throughput screens of transgenic trypanosome libraries, the efficiency of integration – and therefore of selection of transfected parasites – can be enhanced by two orders of magnitude by introducing a double-strand break into the genomic locus to be targeted. This is done by inserting a target site for a site-specific endonuclease, and expressing the nuclease at the time of transfection.

The absence of non-homologous recombination greatly facilitates generation of classical gene knockouts: for this, one simply has to transfect a selectable marker with homologies on either side. Similarly, it is possible to target DNA such that an endogenous gene has an added sequence encoding an epitope tag at either the N- or the C-terminus of the protein. This is particularly useful for estimating protein localization without over-expression and to monitor the efficiency of RNAi. For transgene expression, one needs a splice acceptor signal and untranslated region at

the 5′ end, and a full intergenic region (3′-untranslated region and downstream splice site) at the 3′-end. If such a transgene is inserted into a polycistronic polymerase II transcription unit, it will be expressed at a fairly normal level (dictated by the 3′-untranslated region). To increase expression, a polymerase I promoter is added; in this case, to ensure correct expression, the construct has to be inserted into a silent region of the genome, such as the space between rRNA loci. Expression can be made inducible if the host trypanosomes express a bacterial repressor protein such as the *tet* repressor, and there are two matching operators (e.g. tet operator) immediately downstream of the polymerase I promoter. To obtain even higher expression, one can use trypanosomes that also express T7 polymerase and use a T7 promoter instead of a promoter for RNA polymerase I.

There are three alternative methods to shut off expression of an endogenous gene. Two of them involve RNA interference. In one, the sequence to be targeted is cloned twice, in opposite orientations, and transcription is driven by a tetracycline-inducible polymerase I promoter. In the other, the sequence is cloned once between two opposing tetracycline-inducible T7 promoters. The second method tends to be a little more "leaky" (i.e. RNAi is seen even without tetracycline). The third option is to take a cell line that has an inducible copy of the gene in question, and knock out both of the endogenous copies. This last method is much more laborious than RNAi, but tends to give the most effective regulation.

The availability of high-efficiency transformation made it possible to screen all genes in the genome by RNAi. This "inducible RNAi library" approach yielded a catalogue of genes whose expression is required for survival in the bloodstream and procyclic form and during differentiation. It was also used to identify genes required for the action of various anti-trypanosomal drugs, in particular revealing proteins required for drug uptake. Conversely, libraries that inducibly over-express proteins can be used to select for specific phenotypes. For example, over-expression of a drug target can result in drug resistance. Such approaches can also be used to answer more complicated questions.

All of the inducible expression methods have the disadvantage that some of the targeted protein remains in the cells. As little as 10 % of the normal protein amount may be sufficient for fully normal function, and the amount of RNA may not predict the amount of protein. Another problem with all genetic manipulation in trypanosomes is their flexibility. Any cell line which has a selective disadvantage – even from leaky expression from an inducible promoter – can result in rapid loss of the desired expression. In bloodstream trypanosomes, this "escape" is usually after about 5 days of culture in the presence of inducer, but it can also even happen when parasites are thawed after storage in liquid nitrogen.

Although five selectable markers are in routine use, sometimes these are not sufficient. Transfected DNA can be eliminated from the genome if the sequence is flanked with target sites for Cre recombinase. The recombinase itself can then be expressed inducibly or from a transiently transfected circular plasmid. This method can also be used for sudden deletion of a gene encoding a protein under investigation. This would, transiently, give a cell population in which the majority have a knockout phenotype.

Most of these methods, including inducible expression, are available for *T. cruzi*. Reducing gene expression can however be more difficult – there is no RNAi, and many genes are present in more than two copies, which makes generation of knockouts more complicated.

6.6.7 RNA Editing in the Mitochondrion

Most of the protein-coding genes in the mitochondrial maxicircle are incomplete. Comparison with the corresponding mRNAs reveals that numerous U residues have been inserted into the mRNAs, but some have also been deleted, in a process called RNA editing. The minicircles are required to provide sequence templates, so both they and the maxicircles are essential.

The alteration of the primary sequence of an mRNA after transcription is known as RNA editing, and was discovered for the first time in trypanosome mitochondria. Kinetoplastid editing is effected by editosome complexes, which vary depending on the type of editing involved and contain not only the core enzymes but also proteins with RNA binding and various other activities. The sequence to be edited is recognised by a guide RNA. Each guide RNA consists of the 5′ "anchor" sequence, which hybridises to the mRNA; a further segment that is complementary to the correctly edited mRNA; a 3′ portion that can also hybridise to the region downstream; and then a poly(U) tail. After the first guide RNA has hybridised with the unedited mRNA, the latter is cleaved by an endonuclease at the point of mismatch (Fig. 6.6). During insertion editing, Us are then added by a terminal uridylyl transferase (TUTase) to match A residues in the guide RNA. The mRNA substrate is then religated. During excision editing, surplus Us are removed instead (Fig. 6.6).

6.6.8 Protein Degradation

Kinetoplastids need to degrade damaged proteins and to remove unwanted proteins during differentiation and the cell cycle; they also have to recycle or destroy endocytosed material. Cytosolic degradation is effected by the proteasome and perhaps other proteases such as the metacaspases. Degradation also occurs in the mitochondrion and the lysosome (discussed later), and release of lysosomal proteases is probably involved in some types of stress-induced cell death. A variety of protease inhibitors kills the parasites, but these may have more than one target.

Trypanosomes have classical proteasomes. The 20S proteasome is composed of seven alpha-type and seven beta-type subunits, and the 19S regulator complex is composed of 11 proteins; together they form a 26S complex. The *T. brucei* proteasome is active in VSG quality control and is required for cell cycle progression. Proteins are targeted to the proteasome by ubiquitination, which is effected by ubiquitin ligases. E3 ligases determine the specificity of ubiquitination; in yeast, these are the anaphase-promoting complex (APC) or cyclosome, and the Skp1-Cdc53/cullin-F-box protein complex (SCF). *T. brucei* has a ten-subunit APC, which is required for

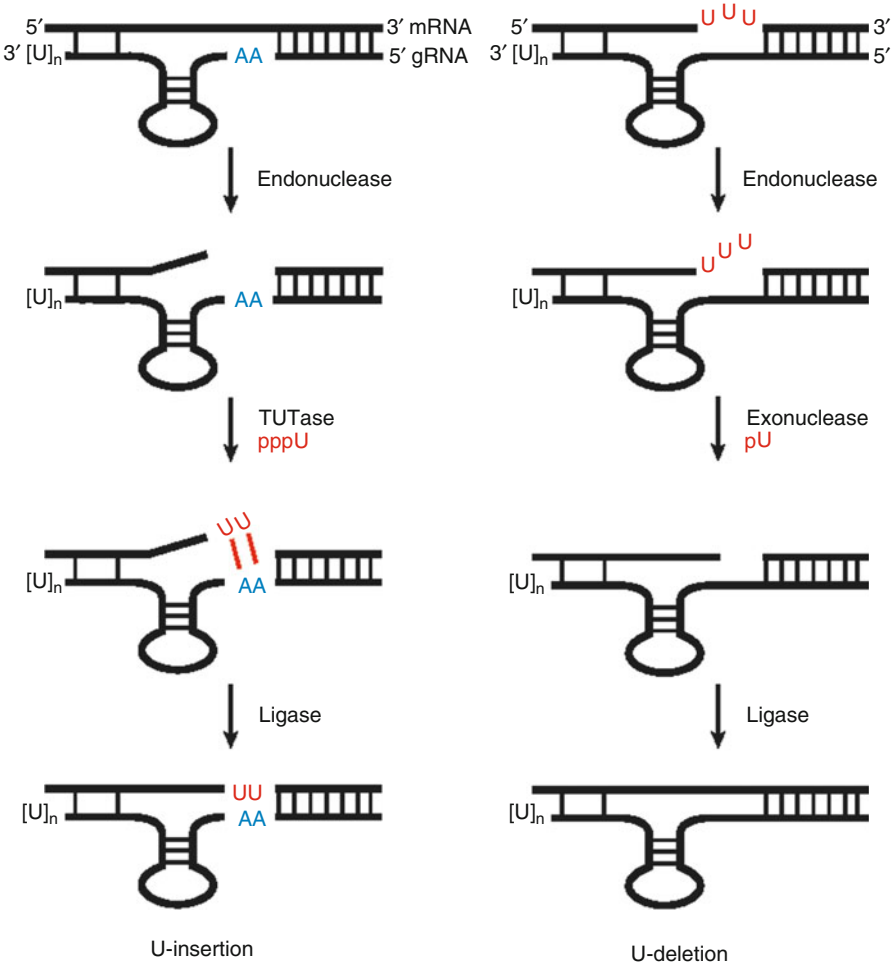

Fig. 6.6 RNA editing (From Goringer 2012)

degradation of cyclin B. The genome has one annotated *SKP1* gene, and encodes nine cullin-domain proteins and several proteins with cyclin F-box domains. E1 subunits activate ubiquitin, while E2 ligases transfer the ubiquitin to the target protein – either directly or as part of an elongating ubiquitin chain. There are at least six annotated E1s and ten E2s; their individual functions have not been disentangled.

Metacaspases are cysteine proteases that are found in plants, fungi and protists but not in mammals. The *T. brucei* genome encodes 5 metacaspases, of which three (MCAs 2, 3 and 5) have intact active sites. They are associated with endosomes. There is no evidence that trypanosome metacaspases are involved in programmed cell death. RNAi targeting all active MCAs caused cell cycle arrest. However, all three genes could be sequentially deleted, suggesting that during selection of the deletion mutants, other proteins could take over MCA function.

6.7 Mechanisms of Antigenic Variation

Every salivarian trypanosome has hundreds of different VSG genes, but only one is expressed at a time. The expressed *VSG* gene is positioned at a telomere, at the end of a polycistronic unit that is transcribed by RNA polymerase I. Upstream are some 70 bp repeats. In each bloodstream-form expression site, there is a selection of 12 different additional genes called "Expression Site-Associated Genes" (ESAGs). Metacyclic expression sites are activated in metacyclic trypanosomes in the tsetse salivary glands; these have a slightly different promoter sequence and lack ESAGs. Transcription of these persists for the first few days after the parasites enter a mammal. Sequencing of telomeres of one trypanosome line yielded 14 different bloodstream-form expression sites and five metacyclic expression sites, suggesting that all but four of the 22 megabase chromosome telomeres host expression sites.

ESAG6 and *ESAG7* encode a dimeric transferrin receptor, which is anchored somewhat weakly to the flagellar pocket membrane by a GPI anchor attached to ESAG6. Of the remaining *ESAG* products, ESAG4 is thought to be adenylate cyclase and perhaps a transmembrane receptor; ESAG10 is a possible biopterin transporter. The functions of most other ESAGs are unknown, though ESAGs 3, 5 and 11 are thought to be membrane-targeted proteins. ESAG9 mRNA is increased in stumpy forms, and the protein is N-glycosylated and released from the parasites. All of the functional bloodstream-form expression sites had ESAGs 5, 6 and 7; and most included ESAGs 1–8 plus ESAG11. Some of the ESAGs have numerous paralogues, both in the subtelomeres and more chromosome-internal locations. They include some genes downstream of, and co-transcribed with, the procyclins but are also scattered in other locations. This has impeded analysis of ESAG function considerably. Nevertheless, the use of a dominant-negative strategy revealed that ESAG4 family proteins are important for suppression of host innate immunity: phagocytosed cells release ESAG4, which increases phagocyte cytosolic cAMP. This in turn reduces secretion of TNF-alpha.

In addition to the VSGs at the end of expression sites, there are hundreds of genes and pseudogenes that are "stored" in subtelomeric regions of the megabase chromosomes or on the telomeres of minichromosomes. For one strain, exhaustive sequencing and assembly revealed nearly 2,500 genes encoding at least 250 amino acids of a VSG, of which about 300 were functionally complete (including both the N-terminal signal sequence and the C-terminal signal for GPI anchor addition). Interestingly, the minichromosomes had a higher share of intact VSG genes: 50 out of 67. Comparing lines over time reveals gene loss and duplication. Many *VSG* genes have at least one copy of the 70 bp repeat upstream, and in the studied line, about 150 had a conserved 14 bp sequence downstream; the latter ends up in the 3′-untranslated region of the mRNA.

To understand antigenic variation, one needs to know how transcription is controlled, such that only one expression site is active? And how does variation occur? Control of expression site transcription is the knottier problem to solve. It is well established that active expression sites have more open chromatin than inactive ones. Expression site promoters are suppressed in procyclic forms, although there is

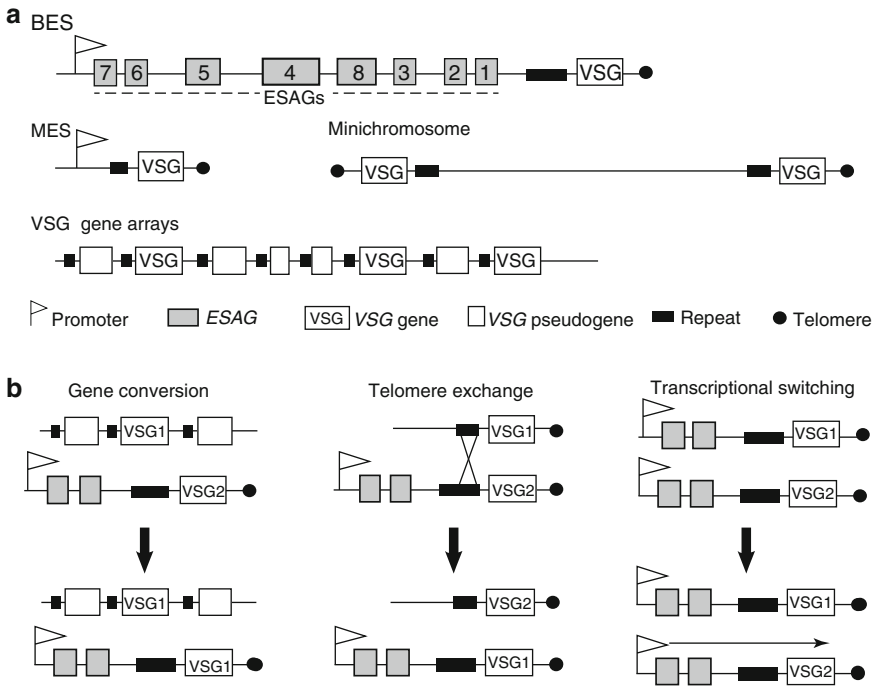

Fig. 6.7 VSG expression site and mechanisms of VSG switching (From Schwede and Carrington 2010)

a low level of transcription near the start site. A VSG promoter that has been transplanted to a different (non-telomeric) part of the genome is active. What controls this chromatin is however not yet fully understood. In bloodstream forms, the active VSG expression site is located in the "expression site body" (ESB), which contains RNA polymerase I and is outside the nucleolus.

Several gene products have been shown to influence the control of expression site transcription. Classical telomeric silencing seems not to be important, although the telomere complex component RAP1 has a role in suppression in procyclic forms. Transcription from silent expression site promoters is increased by depletion of histone H1 or histone H3, various proteins involved in chromatin remodelling and a protein involved in histone H3 trimethylation. At the time of writing, several groups were trying to find other components of the ESB. It is assumed that this single location contains factors that are essential for expression site transcription and are absent from the rest of the nucleus.

The question about variation is rather easier to answer (Fig. 6.7):

(a) The VSG can switch via transcriptional control. One expression site is switched off, and another is switched on.
(b) The VSG can be changed via genomic rearrangements. These include exchange between telomeres, either from other expression sites or the minichromosomes;

and either double-crossover homologous recombination or gene conversion with chromosome-internal VSGs. Gene conversion and recombination can generate novel VSGs and probably occur continuously between silent VSGs and pseudogenes, resulting in continuous evolution of the repertoire. Variation through genetic rearrangement involves both microhomology-mediated and homology-mediated end joining and is stimulated by the presence of a double-stranded break in the active expression site. Such breaks probably occur because of natural telomere instability.

Inheritance of VSG expression requires that the expression site retains its epigenetic marks and remains associated with the ESB during cell division. The switch frequency is increased by RNAi targeting the origin recognition complex or cohesin (which holds chromatids together). This has led to the idea that premature separation of the chromatids during cell division can result in a failure of ESB inheritance.

6.8 Protein Localisation, Transport and Degradation

6.8.1 Vesicular Transport

The vesicular transport machinery of trypanosomes (Fig. 6.8a) is interesting because it is highly polarised (trafficking exclusively to and from the flagellar pocket), the presence of only one or two Golgi, and shows exceptionally fast secretion and recycling kinetics. The kinetoplastid signal recognition particle (SRP) contains two RNAs – the 7SL RNA and a tRNA-like RNA. Many membrane proteins contain classical, cleaved N-terminal signal sequences, while others are polytopic membrane proteins lacking signal sequences. Interestingly, in procyclic trypanosomes, down-regulation of the SRP preferentially affected transport of polytopic membrane proteins. In contrast, proteins with signal sequences might be imported into the ER by either SRP-independent or SRP-dependent pathways. In bloodstream forms, VSG is associated with the rough ER, suggesting that it is co-translationally imported; and import depends as expected on the SEC61 pore.

N-glycosylation and GPI anchor addition occur by mechanisms similar to those in other "model" eukaryotes; the mechanism of GPI anchor biosynthesis was first described for bloodstream-form trypanosomes. The heavy dependence of *T. brucei* on GPI anchors, and their rather unique use of myristoyl groups, makes N-myristoyltransferase an attractive drug target.

Transport from the Golgi to the ER, and from ER to Golgi, is also quite well conserved. As in Opisthokonts, various small GTPases are specifically associated with the various vesicles involved in vesicular transport, and these are used as markers for protein localisation studies (Fig. 6.8a). Endocytosis is exclusively clathrin mediated, and depletion of clathrin or other proteins involved in endocytosis results in dramatic enlargement of the flagellar pocket. Normal bloodstream-form trypanosomes were calculated to recycle their VSG at a remarkable rate of 5×10^5 molecules/min (one

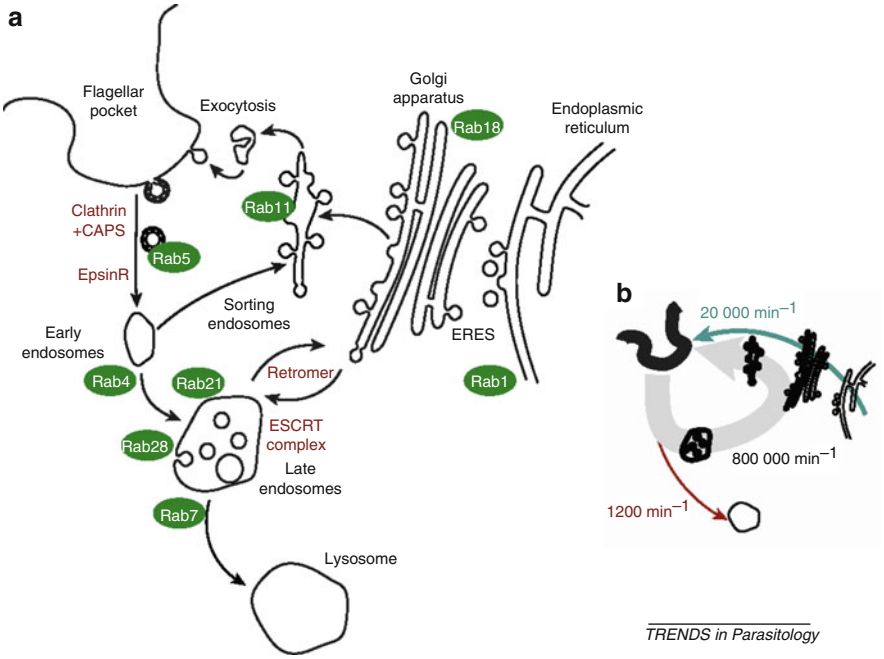

Fig. 6.8 Vesicular transport in trypanosomes. (**a**) Summary of the system showing useful markers (**b**) Synthesis and recycling of VSG, numbers are in molecules per minute (Taken from Manna et al. 2014)

example of measurements is in Fig. 6.8b). This would mean that the entire coat (about 5×10^6 dimers, 90 % of the plasma membrane protein) is recycled in about 30 min. Other calculations suggest recycling of the entire plasma membrane in 12 min. When antibodies bind, they behave like a "sail", and antibody-bearing VSGs are swept into the flagellar pocket by the fluid that flows past the moving trypanosome. (For this it is important to remember that the pocket is at the posterior of moving trypanosomes.) It is not clear, though, whether this "sail" phenomenon is important in vivo since a loss of forward motion does not affect pathogenesis. The rate of recycling in procyclic forms is much lower, and the levels of the proteins involved are much decreased. It has been estimated that about 80,000 VSG molecules have been made per minute, although only a third of them are correctly folded, processed and reach the cell surface. This VSG reaches the surface in about 15 min. This means that when the expressed VSG gene is switched, it will take about 6 h to replace 10^7 VSG molecules. However, if division is continuing, more VSG would be needed so replacement would take longer.

During differentiation of the bloodstream to the procyclic form when VSG synthesis is turned off and procyclin synthesis has begun, the VSG is removed by a combination of proteolysis (using a surface-associated protease related to *Leishmania* GP63) and cleavage of the GPI anchor by a GPI-specific phospholipase C.

ER-targeted proteins are destined for a variety of locations apart from the plasma membrane, and even within that, some proteins are restricted to the flagellar pocket

or the flagellum. Targeting of a large cysteine-rich transmembrane protein to the flagellar pocket required cytosolic tail at the C-terminus. Targeting of GPI-PLC to the flagellum requires the presence of three cysteines, which are acylated, and a proline residue, and depends on the presence of the flagellar pocket collar (see Fig. 6.1). Restriction of the transferrin receptor to the pocket is somewhat paradoxical since it has no transmembrane domain; the restriction might either be due to its ready loss into the pocket matrix, since the dimer has only two myristates.

Opisthokonts respond to defects in the folding or processing of ER proteins by initiation of an unfolded protein response, which operates by signalling from the ER to a transcription factor. Genes required for this response are absent from kinetoplastids. Instead, they have a unique mechanism that has been called SL silencing. ER stress induced by *SEC61* or *SEC63* silencing, low pH or DTT treatment results in the translocation of protein kinase 3 to the nucleus, where it phosphorylates the transcription factor TRF4 (the trypanosome homologue of TATA-box binding protein). This results in a shut-off of *SLRNA* transcription, and therefore in mRNA processing. As a consequence, no new mRNA is made. However, some mRNAs that may be required for survival increase in relative abundance. Ultimately, however, the cells die. This seems to be a kinetoplastid-specific form of programmed cell death. It is not known whether the pathway confers an advantage to cells that are subject to transient stresses.

The secretory pathway is also required for delivery of proteins to some other organelles. Proteins that are trafficked via the ER include some integral membrane proteins of the glycosome, and all proteins of the lysosome and acidocalcisome. Lysosomal targeting also seems likely to be conserved (e.g. lysosomal membrane proteins have cytosolic dileucine motifs).

The acidocalcisome is a specialised lysosome which is important in storage of high-energy phosphate and calcium and for osmoregulation. A short-chain polyphosphate kinase is responsible for creation of polyphosphate chains. During osmotic stress, levels of polyphosphate change in order to regulate the amount of water taken up into the organelle. A proton pyrophosphatase maintains the acidic pH, and an inositol 1,4,5-trisphosphate receptor mediates calcium release in response to inositol triphosphate. The acidocalcisome is therefore important in intracellular calcium signalling. *T. cruzi* also has a contractile vacuole that is involved in osmoregulation.

6.8.2 Receptor-Mediated Endocytosis and the Lysosome

The flagellar pocket is important not just for recycling of membrane proteins, but also for receptor-mediated endocytosis. The best characterised pathway is that for iron uptake. Iron is essential for the function of various mitochondrial proteins, including the cytochromes and the alternative oxidase; cytosolic iron-dependent proteins include iron-superoxide dismutases and enzymes for base J synthesis. In bloodstream-form trypanosomes, iron is taken up bound to transferrin. The ESAG6/ESAG7 transferrin receptor binds transferrin in the flagellar pocket and delivers it to the lysosome by endocytosis. Structural analysis shows that the receptor is

accessible to transferrin, but not antibodies; it is therefore not a protective immunogen. The ESAG6/7 pairs encoded by different expression sites show varying specificities for the various mammalian host transferrins, but it is not known whether this has any physiological implications, since the serum transferrin concentration is usually considerably higher than any of the dissociation constants. Within the lysosome, both the transferrin and the receptor are degraded, and the iron is delivered to the cytoplasm via a protein orthologous to the mammalian endolysosomal cation channel mucolipin 1. The iron in the lysosome has the potential to cause considerable oxidative damage, with peroxidation of membrane proteins. This is prevented by cytosolic peroxidases.

Neither intracellular *T. cruzi* nor the parasites in the arthropod vector have access to transferrin. Culture medium for the procyclic forms and epimastigotes usually contains haem as an iron source, but this may be inappropriate: procyclic *T. brucei* actually takes up iron from ferric complexes by a reductive mechanism. The machinery for creation of iron-sulphur complexes is broadly similar to that in other eukaryotes.

Relatively few other endocytic receptors have been identified. The receptor for the anti-trypanosomal drug suramin is a glycoprotein called ISG75 (Invariant Surface Glycoprotein 75kD). Suramin is used for stage I *T. rhodesiense* infection – early infection when the parasites have not crossed the blood-brain barrier. Bloodstream-form trypanosomes take up low-density lipoprotein, but their receptor is unknown.

The lysosomes are also important during differentiation. Ubiquitinated proteins can be disposed of by uptake into the multivesicular body – a specialised late endosome that delivers its content to the lysosome. In addition, the lysosome is required for destruction of organelles by autophagy. For example, the content of glycosomes (see below) changes considerably during differentiation of the bloodstream form to the procyclic form, and the old organelles are found in autophagosomes.

The lysosomes of African trypanosomes have received much attention because they are important for host specificity. *T. brucei* are killed by apolipoprotein particles in human serum. There are two types of trypanosome lytic factor particle, TLF1 and TLF2. Two components of TLF1 are important for the effect. The complex of haptoglobin-related protein with haemoglobin is important for uptake of TLF1: it is bound by a GPI-anchored haptoglobin-haemoglobin receptor in the flagellar pocket (Fig. 6.9). This leads to endocytosis and transfer of the entire complex to the lysosome. Acidification releases the lethal component, apolipoprotein L1, which inserts into the lysosomal membrane and creates pores, thus increasing ion permeability. The lysosome swells and bursts, and the cells die. Apolipoprotein L1 is also the toxic ligand in TLF2, but uptake of the latter is not well understood – some, at least, must get in by fluid-phase endocytosis. Loss of the haptoglobin-haemoglobin receptor reduces susceptibility of *T. brucei* to human serum 100-fold and to TLF1 by 10,000-fold.

T. rhodesiense are resistant to TLF. The only difference between *T. rhodesiense* and *T. brucei* is that the former express a serum resistance-associated (SRA) protein from within a VSG expression site. SRA interacts with ApoLI and inhibits pore formation. Interestingly, some West African sera can kill *T. rhodesiense*, because they have a mutation in ApoL1 that reduces its binding to SRA. This mutation is

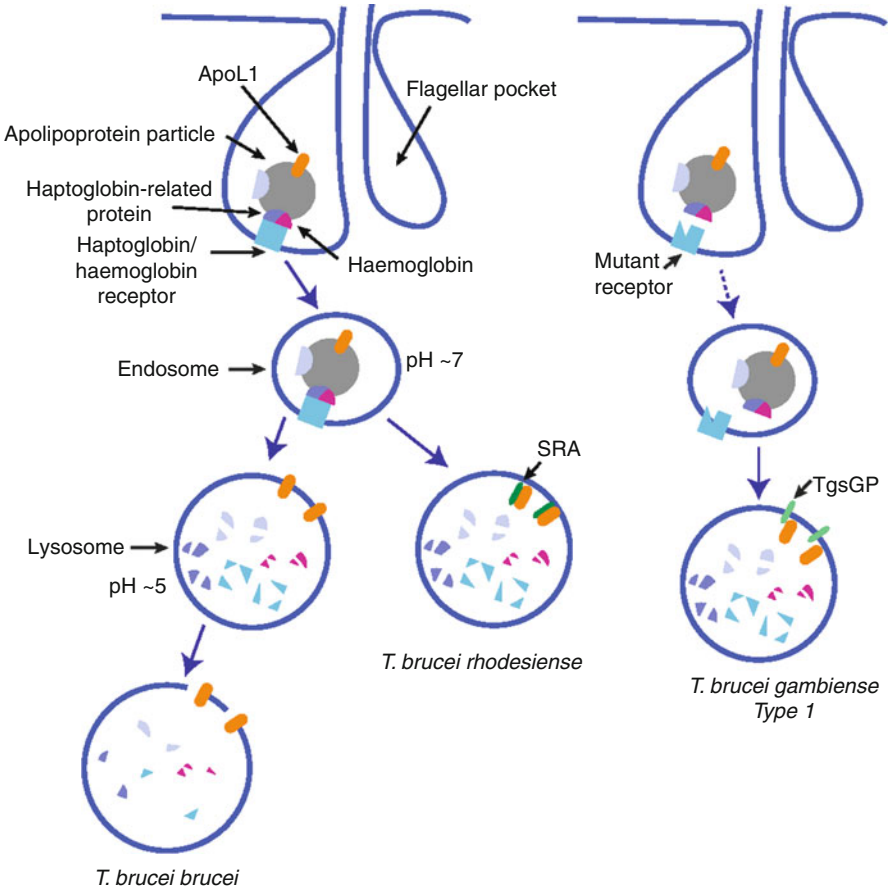

Fig. 6.9 African trypanosomes and the effect of human serum

unfortunately also linked to kidney disease. It is not clear how selection for the mutant version could have occurred, since *T. rhodesiense* is not found in West Africa; perhaps the altered ApoL1 has a selective advantage for other infections. An interesting side effect of the genomic location of the *SRA* gene is that while in humans, *T. rhodesiense* can only use one expression site.

T. b. gambiense falls into two clear evolutionary groups, type 1 and type 2. Resistance of type 1 *T. gambiense* to human serum is now partially understood. Firstly, the trypanosomes have a mutation in the haptoglobin-haemoglobin receptor that reduces TLF uptake. (The mutation also compromises uptake of haem, though this is not completely eliminated.) Secondly, a glycoprotein called TgsGP1 prevents the action of endocytosed ApoL1, perhaps by stiffening the membrane in some way. Deletion of the *T. gambiense* gene encoding the glycoprotein TgsGP makes *T. gambiense* susceptible to human serum and ApoL1, but expression of the protein in *T. brucei* is not sufficient to give resistance.

ApoL1 is found in old world (but not new world) primates, and baboon ApoL1 can kill not only *T. rhodesiense* but also other salivarian trypanosomes. This has prompted attempts to make transgenic cattle expressing baboon ApoL1. If sufficient ApoL1 were produced, these might be resistant to all economically important African trypanosomes.

6.8.3 Organellar and Nuclear Trafficking of Proteins and RNAs

Only a very small proportion of mitochondrial proteins is encoded by the mitochondrial DNA: the rest are imported from the cytosol. Some proteins that are imported into the matrix have N-terminal targeting signals that are very similar to those of Opisthokonts, which are cleaved after import by an evolutionarily conserved mitochondrial processing peptidase. Other proteins have signals that are extremely short – down to about eight residues – which means that standard programmes to predict protein subcellular location do not work for trypanosomes. However, several proteomic analyses have yielded extensive lists of possible mitochondrial proteins.

The mitochondrial import apparatus is very different from that of Opisthokonts. The most diverged component is the outer membrane translocase, "archaic translocase of the outer membrane" (ATOM), which is remotely similar to both the canonical outer membrane translocase Tom40 and a bacterial outer membrane protein, Omp85. ATOM36 is an integral membrane protein which interacts with ATOM, but not SAM50. It is essential throughout the life cycle and needed for import of some, but not all, matrix proteins.

The inner membrane translocase consists of a single TIM protein with homology to yeast Tim17, 22 and 23. Import is driven, as in other eukaryotes, by PAM18, TIM44 and mitochondrial HSP70. Membrane proteins are delivered by the "tiny TIM" proteins (TIM9–TIM10 hexamer and a protein resembling TIM8 and TIM13). An inner membrane protease is responsible for cleaving some inner membrane and inter-membrane space proteins; there is one of these in trypanosome IMP2. The OXA complex assists assembly of some inner membrane proteins, while outer membrane proteins are delivered to the SAM complex.

Trypanosome mitochondria also need to import all of their tRNAs. This import, which may require the TIM complex, is abolished by RNAi targeting either TIM17 or the mitochondrial HSP70. For most tRNAs, there is no specificity – a fraction of the cytosolic pool is imported. The same applies to most aminoacyl tRNA synthetases. However, in a few cases, there are two genes – one for the mitochondrial and one for the cytosolic protein – and in one case, proteins with and without mitochondrial targeting signals are generated by alternative *trans* splicing.

Kinetoplastid glycosomes are evolutionarily related to the microbodies of other eukaryotes. The glycosomal membrane originates from the ER and is dominated by proteins of the Pex11 family (PEX11, GIM5A and GIM5B). Import of glycosomal matrix proteins relies on the same system as for other microbodies. Most proteins have a C-terminal tripeptide "PTS1" signal (related to SKL) and are recognised by PEX5; a few have an N-terminal PTS2 and are recognised by PEX7. A third group

has no signal at all; these are probably imported in a complex with a protein that does have a PTS. That is possible because glycosomal matrix proteins are imported in a fully folded state. PEX5 or PEX7 deliver their bound cargo to the peroxisomal import machinery, which includes the membrane proteins PEX13 and PEX14. PEX5 and PEX7 are thought to enter with their cargo, with subsequent recycling to the cytosol.

Nuclear import and export also appear to be quite conserved. A proteomic study showed that the composition of the trypanosome nuclear pore, and the structure of the subunits, is broadly similar to those in other studied eukaryotes. Trypanosomes lack nuclear lamins, but instead have a lamin-like protein called NUP1, which is required to maintain nuclear shape. Proteins to be imported are bound by importin alpha and beta, and there are also an exportin, putative RNA GTPases and Ran-binding proteins. There is a putative tRNA exportin, and export of mRNAs is mediated by MEX67. Spliced mRNAs in mammals are recognised by a complex of Y14 and Mago; this is conserved in trypanosomes, but interaction with the *trans*-splicing site on mRNAs has not been demonstrated.

6.9 The Flagellum

The microtubules of the flagellum show a classical 9+2 arrangement, with the nine adjacent doublets connected by a nexin-dynein complex. The proteome of the flagellum has been catalogued. Next to the axoneme is the paraflagellar rod, which has three layers. Depletion of rod proteins affects trypanosome motility, and some of the associated proteins may be involved in calcium signalling. In trypomastigotes, the flagellum adheres to the cell body via the flagellar attachment zone. Underlying this are four specialised ER-associated microtubules. Various proteins link the flagellum through to the cell body cytoskeleton, and their depletion can liberate the flagellum, which impacts motility. The trypomastigote body spirals as the cell moves, and the cellular velocity is influenced by the presence of barriers in the environment.

Assembly of the new flagellum starts quite early in the cell cycle. New proteins are transported towards the tip by the intraflagellar transport machinery, and the position of the attached flagellum is guided by the pathway taken by the old one. RNAi that results in severe shortening of the flagellar attachment zone filament results in loss of viability, suggesting that a certain length is required for cell division.

All components that are essential for motility are also required for bloodstream-form (but not procyclic-form) *T. brucei* survival, although a mutant which had flagellar beating, but no forward motion, had unimpaired virulence. The wave of flagellar beating starts at the flagellar tip and moves towards the flagellar pocket; if it reverses, the cells tumble, and depletion of one protein even caused the cells to go backwards.

Procyclic cells growing on soft agarose plates show social motility: groups of parasites move together, and groups seem to sense and move towards each other. The signals for this are unknown. However, various proteins connected with

signalling – including calcium-binding proteins and proteins resembling adenylate cyclases – are associated with the flagellum. Loss of GPI-PLC, calflagins and metacaspase C – all of them flagellar proteins – reduces trypanosome virulence in mice.

Epimastigotes adhere closely to the salivary gland epithelium via adhesion complexes that have been seen by electron microscopy, but not characterised. This may facilitate colonisation of the salivary gland. Flagellar interactions might also be involved in mating.

6.10 Metabolism and Anti-trypanosomal Drugs

This section will cover only a few very well-characterised aspects of trypanosome metabolism. The mechanisms of action of anti-trypanosomal compounds that are in current use as drugs, or in clinical trials, will also be mentioned. Numerous enzymes have been proposed as potential targets for anti-trypanosomal drugs, on the basis of being essential for parasite survival and either being absent from the host or significantly different from the host enzyme.

At the time of writing, most of the compounds that are under development as anti-trypanosomal drugs were found by phenotypic screens: parasite cultures in microtitre plates were treated with hundreds of thousands of different compounds, and compounds that killed the parasites without affecting human cells were selected. The targets of these compounds are not yet known.

6.10.1 Energy Metabolism

The energy metabolism of trypanosomes is developmentally regulated, allowing survival using available carbon sources. Bloodstream-form *T. brucei* are heavily reliant on glycolysis (Fig. 6.10). Results of metabolic modelling and inhibitor studies suggest that the rate of ATP generation is limited mainly by the import of glucose by the glucose transporter. The glucose enters the glycosome, where it is metabolised to 3-phosphoglycerate. This exits the glycosome; the only reaction that generates ATP in the cytosol is catalysed by pyruvate kinase, which generates pyruvate as the main end product. Correspondingly, pyruvate kinase is the only enzyme that is subject to control by the cytosolic ATP/ADP ratio.

A lack of permeability of the glycosomal membrane to ATP, ADP, NAD+ and NADH has major implications, since the levels of each have to be kept in balance. One consequence of this is that the initial phosphorylation reactions are not subject to control by the ATP/ADP ratio. The second consequence is more complicated. Fructose-bisphosphate aldolase makes glyceraldehyde 3-phosphate (GA-3-P) and dihydroxyacetone phosphate (DHAP). If the latter is converted to glyceraldehyde phosphate, the overall yield of ATP from the glucose equals the ATP consumed in the first two phosphorylation steps, so glycosomal ATP and ADP balance is maintained. Also, for every glucose, two molecules of 3-phosphoglycerate (3-PGA) exit the glycosome, yielding two ATP in the cytosol. However, direct conversion of dihydroxyacetone phosphate would result in a redox imbalance, since NAD+ is reduced by

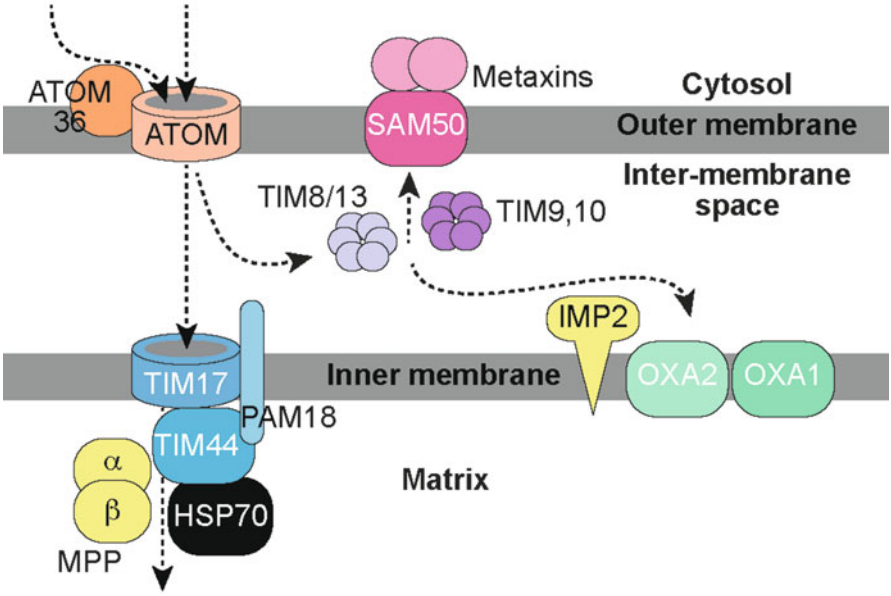

Fig. 6.10 Mitochondrial protein import. The pathway of proteins is shown by the *dotted arrows*

glyceraldehyde phosphate dehydrogenase. Instead, the NADH is reoxidised with concomitant reduction of dihydroxyacetone phosphate to glycerol phosphate. The glycerol phosphate exits the glycosome, to be reoxidised on the mitochondrial inner membrane by a glycerol phosphate dehydrogenase coupled to a ubiquinone-dependent alternative oxidase (Fig. 6.10). The resulting dihydroxyacetone phosphate can then re-enter the glycosome for conversion to glyceraldehyde phosphate by triosephosphate isomerase. If the alternative oxidase is inhibited, or the parasites are maintained under anaerobic conditions, the parasites instead convert the glycerol phosphate to glycerol, generating ATP. This maintains the intra-glycosomal ATP/ADP and NAD+/NADH balances, but yields only one ATP per glucose. That is not enough, so the cells start to die after a few hours. Triosephosphate isomerase (TPI) is essential for the same reason. The need to maintain the ATP/ADP ratio is illustrated by the fact that loss of cytosolic phosphoglycerate kinase (cPGK) is lethal in bloodstream forms. The alternative oxidase – which is found in plants, but absent from mammals – is therefore a possible target for chemotherapy; parasites are killed by the experimental drug candidate ascofuranone, which inhibits the alternative oxidase. The relative simplicity of trypanosome glucose and glycerol metabolism has made it an ideal model for computerised metabolic modelling: this has not only shown the exquisite dependence on glucose transport, but can also predict the consequences of various manipulations on metabolite levels. The pentose phosphate pathway is present, but flux through the pathway is minor compared with glycolysis. It has been suggested that glycolysis is an important target of suramin, but the drug probably has multiple molecular targets.

The energy metabolism of procyclic *T. brucei* is much more complex than that of bloodstream forms (Fig. 6.11). Glucose from the blood will not persist long enough

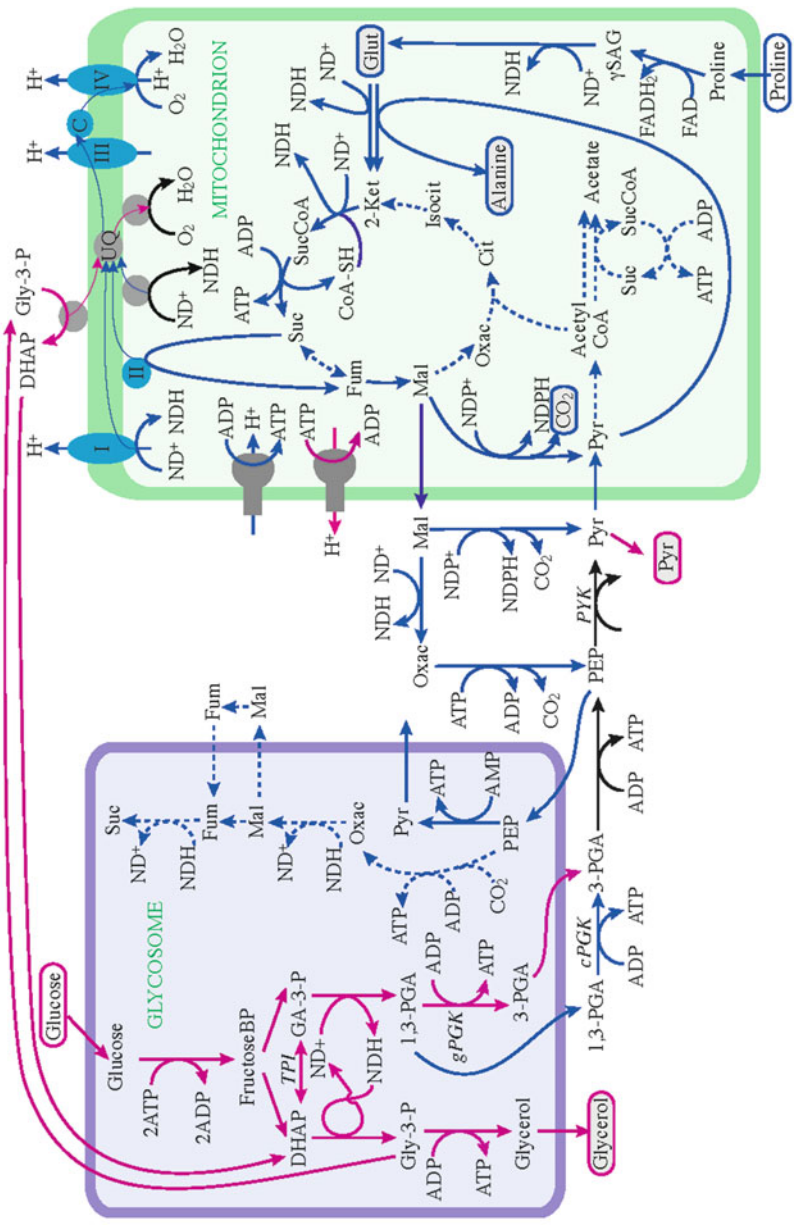

Fig. 6.11 Energy metabolism in *T. brucei*. Pathways that dominate in the bloodstream form are in *magenta*; reactions that are up-regulated in the procyclic form are in *blue*. *ND+*, *NDH* NAD+, *NDH* NADH, *FructoseBP* fructose bisphosphate, *DHAP* dihydroxyacetone phosphate, *GA-3-P* glyceraldehyde 3-phosphate, *Gly-3-P* glycerol 3-phophate, *PGA* phosphoglycerate, *suc* succinate, *Fum* fumarate, *Mal* malate, *Oxac* oxaloacetate, *PEP* phosphoenol pyruvate, *Pyr* pyruvate, *SucCoA* succinyl CoA, *Cit* citrate, *Isocit* isocitrate, *Glut* glutamate, *γSAG* glutamate γ-semialdehyde, *TPI* triosephosphate isomerase, {*GK* phosphoglycerate kinase (g-glycosomal, c-cytosolic), *UQ* ubiquinone, *I*, *II*, *III*, *IV* respiratory complexes. Substrates are *circled* and products are *circled with a grey background*

for the trypanosome infection to mature. Based on the content of the tsetse haemolymph, it is thought that the main carbon source is likely to be proline. This is metabolised in the mitochondria, major products being alanine, glutamate and carbon dioxide. Procyclic forms absolutely rely on the respiratory chain, so dyskinetoplastic parasites cannot colonise tsetse. These dyskinetoplastic forms can, however, be transmitted by other types of body fluid transfer such as blood (biting flies) or, for *T. evansi*, venereally.

The *T. brucei* mitochondrial genome encodes a few components of respiratory complexes and the "a" subunit of F_0. The fact that bloodstream-form trypanosomes do not require oxidative phosphorylation therefore suggests that they should not need kDNA. Indeed, *Trypanosoma evansi* and *Trypanosoma equiperdum* are variants of *T. brucei* that lack some, or all, of the kDNA. On the other hand, two classes of anti-trypanosomal drugs, the diamidines (e.g. diminazene aceturate, pentamidine) and the phenanthridines (e.g. isometamidium and ethidium bromide), are known to accumulate in the mitochondrion, and the phenanthridines were thought to act by intercalating into mitochondrial DNA. Moreover, bloodstream forms are killed by depletion of proteins required for RNA editing, which would make the latter a promising potential drug target. If *T. brucei* is grown in acridine orange for many months, dyskinetoplastic forms may eventually emerge, but this is clearly a rare event. The explanation for this conundrum lies within the F_1F_0 ATPase. This is used to make ATP in the procyclic form, but in the bloodstream form, it works in reverse, hydrolysing ATP in order to maintain the proton gradient. The proton gradient is required for import of proteins into the mitochondrion – including the alternative oxidase. It turns out that dyskinetoplastic trypanosomes have a mutation in the nuclear-encoded F_1F_0-ATPase subunit-γ which allows the ATPase to function in the absence of the mitochondrially encoded "a" subunit. (Similar mutations are seen in petite yeast.) When this mutant subunit is expressed in *T. brucei*, the cells develop 8-fold resistance to the diamidines and 100-fold resistance to phenanthridines.

No *T. cruzi* life cycle stage shows the minimalistic energy metabolism seen in *T. brucei* bloodstream forms. The pentose phosphate pathway is active throughout the life cycle, and the mitochondrion is probably also required in all stages. The energy metabolism of *T. cruzi* epimastigotes is likely to be proline dependent, similar to that of *T. brucei* procyclics. The metabolism of amastigotes was studied in an ingenious screen: host cell gene expression was down-regulated by siRNA prior to infection, and the effects on parasite intracellular growth were assessed. Growth of the cells was impaired by siRNAs that inhibited host cell beta-oxidation of long-chain fatty acids, but increased in cells in which pyruvate dehydrogenase activity or conversion of pyruvate to lactate was decreased. Indeed, infection itself is known to cause an increase in beta-oxidation and pyruvate dehydrogenase down-regulation. Interpretation is complicated, but the results do show that amastigotes depend on products of fatty acid oxidation. This result may explain the ability of *T. cruzi* to replicate in cardiac and smooth muscle and in adipose tissues, where the pathway is active.

Fatty acids are, of course, needed for purposes other than energy, such as membrane assembly and production of GPI anchors. Some relevant information is available, but it is beyond the scope of this chapter. Bloodstream-form

trypanosomes scavenge cholesterol from the host, but *T. cruzi* amastigotes, like *Leishmania*, use ergosterol instead of cholesterol. Experimental *T. cruzi* infections can be treated with antifungal drugs that inhibit sterol 14α-demethylase, but so far, such drugs have not reached the clinic.

6.10.2 Nucleotide and Cofactor Metabolism

Nucleotides are needed for many purposes including synthesis of nucleic acids, as a source of high-energy bonds (ATP, GTP) and reducing power (NAD, NADP), and synthesis of glycans. Trypanosomes are purine auxotrophs, but are able to synthesise pyrimidines.

T. brucei has several purine transporters, with a range of specificities. The transporter AT1 has gained notoriety, since it contributes to the accumulation of two classes of drug. The arsenical drug melarsoprol is an extremely toxic compound whose use is now restricted mainly to stage II *T. rhodesiense* infection, when the parasites have entered the brain. Melarsoprol may act by reacting with thiol groups. A major reason that it shows any specificity for trypanosomes at all is probably the fact that drug is accumulated within trypanosomes by AT1 and also a second carrier, AQP2 (an aquaglyceroporin). Loss of either transporter can reduce sensitivity, but loss of AT1 and AQP2 is needed for high-level resistance. The second groups of AT1 substrates are the diamidines. These include pentamidine, which is used for stage I *T. gambiense* infection, and diminazene aceturate, which is used to treat trypanosomiasis in cattle. Pentamidine also enters via AQP2 and at least one other transporter. The existence of alternative purine transporters has meant that the *AT1* gene can be mutated, or even lost, without any effect on cell viability or virulence. This results in drug resistance, and the mutation has become quite widespread in parts of Africa where the drugs are used. Interestingly, *T. congolense* lacks the AT1 gene, although it is susceptible to the diamidine diminazene aceturate. Instead, diminazene appears to be taken up by a *T. congolense* pteridine transporter.

Trypanosomes are able to make pyrimidines de novo, from orotate, aspartate or glutamate, but evidence suggests that salvage is likely to contribute. *T. cruzi* amastigotes show reduced survival in cells with reduced pyrimidines. Bloodstream-form *T. brucei* express a high-affinity uracil transporter and can survive – albeit with reduced virulence – by pyrimidine salvage alone.

6.10.3 Redox Metabolism

All organisms need to maintain a reducing environment in the cytosol, since many enzyme active sites rely on cysteine residues and all must protect themselves from damage by reactive oxygen species. Infectious microorganisms may be exposed to additional oxidative stress from the activated immune system. Trypanosomes lack several of the systems present in mammalian cells, but they nevertheless have

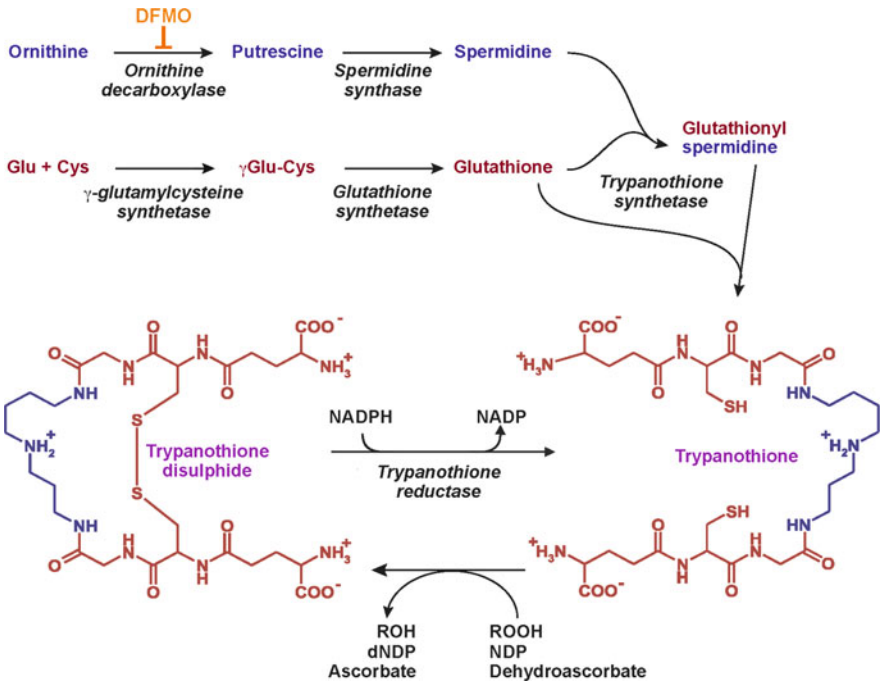

Fig. 6.12 Trypanothione metabolism. The two glutathiones are in red and the spermidine is in blue. Enzymes are in *italics*. Examples of reactions that depend on trypanothione are indicated at the *bottom*: ROOH (hydroperoxides) are reduced to the corresponding alcohols by trypanothione peroxidases. Ribonucleotides (NDP) are reduced to deoxyribonucleotides by ribonucleotide reductase, and dehydroascorbate is reduced chemically (Adapted from Schmidt and Krauth-Siegel, *Curr Top Med Chem* 2:1237–1256)

efficient redox control. The most notable difference is that instead of glutathione, the cofactor that is the basis of mammalian redox control, they have trypanothione (Fig. 6.12).

Trypanothione is a conjugate of two glutathiones with spermidine. The presence of two SH groups on one molecule makes reduction a unimolecular reaction, which is further potentiated by the basic nature of the spermidine bridge, which makes the thiol groups very reactive at neutral pH. This makes trypanothione a much stronger reducing agent than glutathione. Oxidised trypanothione is reduced by NADPH-dependent trypanothione reductase. Trypanothione is required by all trypanosome reactions that depend on glutathione in mammals. It reduces dehydroascorbate and disulphides in proteins such as thioredoxin and tryparedoxin. Tryparedoxin in turn is required to reduce ribonucleotide reductase, which is needed to make deoxyribonucleotides. Trypanothione is the cofactor for peroxidases which detoxify hydroperoxides; this compensates for the fact that they lack catalase. Numerous other trypanothione-dependent enzymes are found. For example, cytosolic glutathione peroxidase-type enzymes protect the cells against iron-mediated damage by

repairing lipid peroxidation. Trypanothione-dependent enzymes are even in the mitochondrion, although there is no trypanothione reductase; it is not clear, therefore, how trypanothione could be recycled in the organelle.

The presence of numerous enzymes that depend on a cofactor that is absent from mammalian cells has raised reasonable expectations that it would be possible to develop some of the relevant enzymes as drug targets: trypanothione binding pockets are larger than those that accommodate two glutathiones, since they must also include the basic spermidine moiety. A drug would, however, have to compete for binding with intracellular trypanothione, which is in the high micromolar range. This means that highly potent – and probably irreversible – inhibitors are required. So far, such molecules have not been found. The large size of the trypanothione binding pocket may be partly responsible for the difficulty in finding inhibitors with the desired properties.

Trypanothione metabolism is nevertheless central to the mode of action of one drug that is in clinical use. Trypanothione synthesis relies on steady supplies of spermidine and glutathione. Putrescine is synthesised from ornithine by ornithine decarboxylase (ODC), which is irreversibly inhibited by dihydrofluoroornithine (DFMO). DFMO is the first-line drug for treatment of stage II *T. gambiense* trypanosomiasis. In fact, it also inhibits ODC in mammalian cells and *T. rhodesiense*. DFMO is selective for *T. gambiense* because *T. gambiense* ODC has a long half-life. Mammalian ODC – and, unfortunately, *T. rhodesiense* ODC – turns over much more rapidly, so that irreversibly inhibited ODC is rapidly replaced by new, active enzyme. DFMO is taken up into trypanosomes by an amino acid transporter called AAT6. Cells with RNAi targeting *AAT6* are therefore resistant to the drug, but fortunately this has not yet been seen in patients. *T. cruzi* cannot be treated with DFMO because it scavenges polyamines from the host.

Trypanothione can also form conjugates with heavy-metal-containing drugs such as those containing arsenic or antimony. In the bloodstream, melarsoprol is converted to melarsen oxide. Once this has been accumulated within the parasite, it reacts non-specifically with all thiols including those of trypanothione conjugates. The trypanothione conjugates are, in turn, substrates of a multidrug resistance transporter called MRPA. There is no evidence that MRPA is involved in arsenical resistance in patients, but over-expression of the same transporter is implicated in clinical resistance to antimonial drugs in *Leishmania*, and resistance is made worse if enzymes required for trypanothione synthesis are also over-expressed (Fig. 6.13).

Benznidazole is the first-line drug for *T. cruzi* infection; nifurtimox is also effective against *T. cruzi* and is currently used in combination with DFMO against stage II *T. gambiense* infection. Both are prodrugs. They have to be nitro reduced in order to become active, probably by an unusual nitroreductase called NTR1. Mammalian cells are unable to activate the drug, explaining its selectivity. Metabolomic analysis of benznidazole-treated *T. cruzi* revealed – in addition to various reduced derivatives – conjugates between the imidazole ring and various small thiols. Similar work with nifurtimox revealed that it actually slightly antagonises DFMO action; however, use of the combination may at least delay the

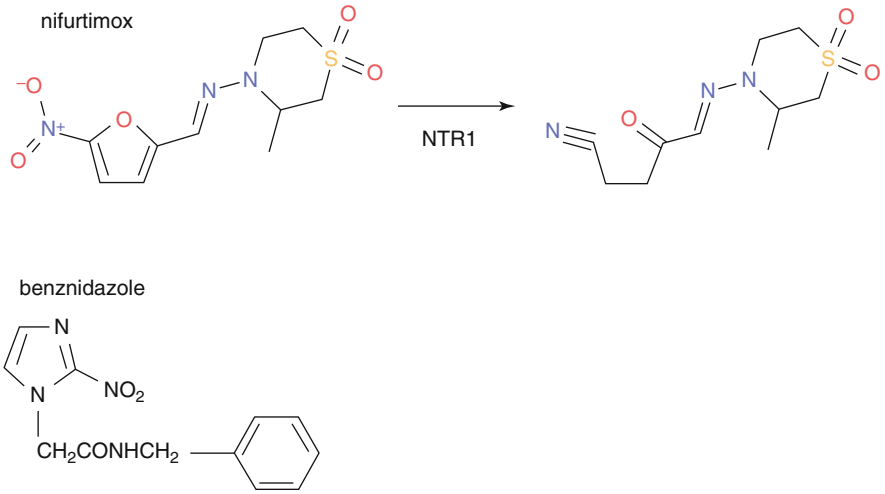

Fig. 6.13 Structures of nifurtimox and benznidazole

appearance of resistance. Another nitroheterocycle, fexinidazole, which can be given to patients orally, is in advanced clinical trials for stage 2 HAT. Fexinidazole is also active against *Leishmania* and *T. cruzi*.

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Leishmania

7

Anton Aebischer and Martin Mrva

Abstract

It is more than 125 years since Piotr Fokich Borovsky reported in 1898 on *Leishmania* parasites as the etiologic agent of oriental cutaneous leishmaniasis. Publication of the first *Leishmania* genome in 2005 propelled research on this organism into a new dimension. Genome information together with reverse genetics, -omics methodologies, and bioinformatics have become the modern point of departure to study also these pathogens. In the following chapter, selected aspects of the biology of *Leishmania* spp. are introduced with a non-familiar reader in mind. The aim was to integrate genome information into the presentation of cell morphology, sub-cellular organization, molecular biology, and metabolic adaptation to different habitats as these unicellular protozoans revolve through their lifecycle.

7.1 Introductory Remarks

It is more than 125 years since 1898 when Piotr Fokich Borovsky, a Russian military physician serving in Tashkent (today: Capital of Uzbekistan), reported on *Leishmania* parasites as the etiologic agent of oriental sore, i.e., cutaneous leishmaniasis. However, the parasite genus was named after Sir William Boog Leishman. He detected similar organisms a few years after Borovsky when performing necropsy on a patient who died in India of what we now know as

A. Aebischer (✉)
Head of FG 16 Infections, Robert Koch-Institute, Postfach: 650261,
D-13302 Berlin, Germany
e-mail: AebischerA@rki.de

M. Mrva
Department of Zoology, Comenius University in Bratislava, Faculty of Natural Sciences,
Mlynská dolina Ilkovičova 6, 842 15 Bratislava, Slovakia
e-mail: mrva@fns.uniba.sk

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visceral leishmaniasis. At the time, microscopy and novel staining techniques allowed the detection of organisms in cells of infected tissue and highlighted a distinctive structural feature, the kinetoplast, linking this pathogen to its kin, the kinetoplastea. All human pathogenic *Leishmania* have digenetic life cycles that involve intracellular replication in phagocytes of mammalian hosts and extracellular proliferation in their blood-feeding insect vectors of the *Lutzomyia* and *Phlebotomus* genera, responsible for the transmission of *Leishmania* species, in the New and Old World, respectively.

Since Borovsky's discovery, more than 20 human pathogenic *Leishmania* species alone have been identified. They are agents of a spectrum of diseases ranging from self-healing cutaneous to fatal visceral infections. The public health burden is considerable with more than 350 million people in 98 countries at risk, and, based on mortality due to parasitic infection, visceral leishmaniasis is thought to be second only to malaria in importance. Drug-based therapies are available but often too costly, and the mechanistic understanding of the action of current drugs is limited, preventing rational improvements.

From their discovery until the end of the twentieth century, research on these parasites has been driven by the quest for effective, affordable therapies and the promise of a vaccine, but progress has been incremental at best. Publication of the first full sequence of a *Leishmania* genome in 2005 has propelled this pace of discovery into a new dimension. This resource – together with the application of reverse genetics, -omics methodologies, and bioinformatics allowing highly parallelized analyses – has become the modern point of departure to study these organisms and will also be the point of reference for this chapter.

7.2 Well-Established Facts

7.2.1 Genome

The first haploid nuclear *Leishmania* genome deciphered was that of a *L. major* isolate, with genomes of *L. infantum*, *L. donovani*, *L. mexicana*, and *L. braziliensis* isolates following. These constitute a valuable though limited population genome sampling of the major *Leishmania* species responsible for human disease. Additional genome data sets have since been generated by different groups, and the compilation of this and additional information into a "single stop" database for eukaryotic pathogens has been a visionary initiative of David Roos and colleagues who provide a truly enabling resource to the interested communities through EuPathDB (cf. http://eupathdb.org/eupathdb/).

According to this data source, the size of a haploid *Leishmania* genome typically is 32–35 MB. It is physically organized on 34–36 chromosomes and these range from ~2–3 MB to 0.2 MB in length. The differences in the haploid chromosome number between species known so far arose from fusion events between members of the set of 36 chromosomes. The predicted gene number also varies

between isolates but is in the order of 8000–9500 which is similar in complexity to, e.g., yeast model organisms (c.f. http://www.yeastgenome.org/). Many leishmania protein-encoding genes belong to families and these are often organized in tandem gene arrays. In different species, the synteny of orthologous arrays is relatively conserved, but regions often differ in the number of family members they contain. Almost all protein-coding genes are intronless, separated from each other by short intergenic sequences. On the chromosomes, genes are organized into directional transcriptional clusters which in the *L. major* genome number 133. These clusters can contain very few but also up to hundreds of genes encoded on the same strand of the double helix. Clusters are separated by short (1–14 kB) strand switching regions that are often characterized by tRNA, rRNA, or snRNA sequences, and the directionality of the sense strand of two adjacent clusters can be divergent or convergent (c.f. (Ivens et al. 2005)). A number of genes, particularly enzymes, appear to have been introduced by lateral gene transfer. Their origin may be beta-proteobacteria which are related to symbionts present in extant species of monoxenic – that is, insect host only parasitizing – relatives of the *Leishmanias* and *Trypanosomas*. As is true for many other organisms, the function of the majority of identifiable genes remains unknown; hence, they are annotated as encoding hypothetical proteins.

Sequences of the initial genomes were derived from long-term cultured laboratory isolates and suggested that *Leishmania* are essentially diploid organisms. However, the genomes of field isolates studied now show that this is rather the exception and that *Leishmania* spp. have to be considered as aneuploid organisms. Isolates can contain few to many so-called supernumerary chromosomes, and the identity of the chromosomes affected differs between isolates. Also single parasites within an isolate may differ in chromosomal content. It is thought that this allows for genetic variation in an organism that was long believed not to undergo sexual recombination. However, the latter has recently been shown to be possible in the insect vector (Akopyants et al. 2009; Romano et al. 2014). How often sexual recombination between distinct isolates occurs in nature and how this may drive population genetics of these parasites is unclear and a very current topic in the field with a first report demonstrating genetic exchange even between species in 2014 (Romano et al. 2014).

Leishmania also contain a mitochondrial genome that is distributed over two major classes of circular elements, maxi- and minicircles that are 20–40 kB and 0.2–2.5 kB in length. The single mitochondrion contains 20–50 maxicircles and in the order of 10^4 minicircles that are intertwined into a complex network (Flegontov et al. 2009; Simpson 1986; Yatawara et al. 2008). This assembly is called the kinetoplast – a distinctive feature when cells are processed for microscopy with nucleic acid-detecting stains – that gave the higher taxonomic unit its name. Both classes of circles show sequence heterogeneity. Maxicircles encode mitochondrial genes, but open reading frames are often faulty and require mending at the transcript level by RNA editing which depends on guide RNAs derived from minicircle sequences (Simpson et al. 2010; Simpson and Shaw 1989).

7.2.1.1 Genome Replication

Nuclear and mitochondrial genome replication as parasites proliferate is intricate, and, although involving more or less of the canonical molecular mechanisms described for other eukaryotes, the processes are not fully understood but better studied in trypanosomes and are therefore discussed in more detail in the respective chapter of this book on trypanosomes.

7.2.1.2 Gene Expression

As other kinetoplastida, *Leishmania* spp. regulate gene expression mostly posttranscriptionally at the level of mRNA stability and translation efficiency and not by the activity of promoters (see again chapter on trypanosomes for more details and Clayton (2013), Michaeli (2011)). The directional gene clusters are transcribed polycistronically by RNA polymerase II. The long transcripts are subsequently processed. Processing involves two steps, trans-splicing of a small leader RNA (mini-exon encoded in numerous copies elsewhere in the genome) onto splice acceptor sites present in the intergenic region 5′ of the individual open reading frames and polyadenylation of the preceding cistron. More conventional cis-splicing can occur but this only concerns transcript processing of a hand full of genes. The fact that gene expression is regulated posttranscriptionally is thought to explain the comparatively high number of RNA-binding proteins encoded in the genomes of these organisms. Moreover, a clear bias in the use of codons in ORFs encoding highly abundant proteins has been noted. Thus, on a genome scale, codon usage is correlated with protein abundance in these organisms.

Differential gene expression in the different life cycle stages seems to be regulated by sensing and integrating two major environmental clues: temperature and pH. This results when free-living promastigotes are transferred by the insect during a blood meal; the parasites become exposed to a higher temperature (at least when transferred to a warm-blooded host) and eventually become located in the acidic environment of a phagolysosomal compartment.

A hallmark of transcription of mitochondrial genes in these organisms is RNA editing. Maxicircles encode mitochondrial genes, but open reading frames are often faulty and require mending at the transcript level by an editing process that depends on guide RNAs derived from minicircle sequences. Again this process is described in instructive detail for trypanosomes elsewhere in this volume (see also Simpson et al. 2010).

7.2.1.3 Genetic Manipulation

As mentioned before, sexual recombination is a rare event and has only recently been discovered in *Leishmania* spp. Consequently, the analytical power of classical genetics to assign and study gene functions cannot yet be harnessed for these diploid organisms. In contrast, after pioneering efforts of Diane Wirth's and Steve Beverley's laboratories in the late 1980s, the tool box for genetic manipulation of *Leishmania* parasites has been constantly expanding (Beverley 2003; Dean et al. 2015) and allows generation of transgenic *Leishmania* expressing additional specific genes of interest as well as the targeted ablation of individual genes or gene clusters by homologous recombination events. The power of RNAi approaches has so far not been harnessed for application in *Leishmania*. The reason for this is mainly that the

required enzymatic machinery has been lost from the genomes of Leishmanias species other than the Viannia species group. This New World Leishmanias contains species such as *L. braziliensis*, *L. guyanensis*, and *L. panamensis* (Atayde et al. 2013). Adapting the bacterial CRISPR-Cas9 system for use in *Leishmania* is likely to eventually enable full genome screens by reverse genetics in the near future.

7.2.1.4 Cell Morphology

The genome encodes almost all information that is necessary to form a living unicellular organism that for most species oscillates between a free living, flagellated, so-called promastigote form thriving in the intestinal tract of the insect vector and aflagellated amastigotes that replicate within an endolysosomal compartment of phagocytic vertebrate host cells. Promastigotes are 10–15 μm in lengths and 2–4 μm in width, motile with a flagellum emanating from cellular invagination, the flagellar pocket. The flagellum propels the cells in its direction and can reach twice the lengths of the cell body in the so-called metacyclic promastigotes that differentiate from replicating parasites within the female insect vector to form parasites ready for transmission to the vertebrate host. Amastigotes are much smaller (~4 x 3 μm) ovoid shaped, and the flagellum is reduced to a rudimentary structure not extending from the flagellar pocket. From what we know currently, the single-celled organisms possess the canonical subcellular compartments and structures expected from a eukaryotic cell with certain peculiarities (Fig. 7.1; c.f also (Besteiro et al. 2007; McConville et al. 2002)):

- Cell shape is dictated by a dense set of sub-pellicular microtubules running with a helical twist as parallel rods below the plasma membrane along the entire length of the cell.
- This microtubular corset is thought to limit exo- and endocytic processes to the flagellar pocket membrane.
- The promastigote surface is dominated by a dense coat of extended lipophosphoglycans (LPG) and few mostly glycoinositol-phospholipid (GPI)-anchored protein species, of which a metalloprotease, gp63, is the dominating species with $>10^6$ molecules per cell.
- The amastigote surface, in contrast, is characterized by the absence of the dominating molecular species of the promastigotes (LPG and gp63), a general paucity of proteins, and a coat composed of glycoinositolphospholipids (GIPLs).
- A single mitochondrion with the kinetoplast, the mitochondrial DNA located beneath the flagellar origin to which it seems linked in a unit also encompassing the basal body.
- A lysosomal compartment that takes the shape of a long multivesicular tubule in promastigotes and of a large vesicular body (megasome) in amastigotes.
- Glycosomes and acidocalcisomes as major vesicle classes, besides multivesicular bodies, autophagosomes, and a number of currently still poorly defined endo- and exocytic vesicles that ensure receptor-mediated endocytosis.
- An endoplasmic reticulum that is structured in several functional subdomains such as the nuclear envelope, rough and smooth domains as in other eukaryotes, and a special transitional zone in form of a cysternal extension that lays opposite of the Golgi.

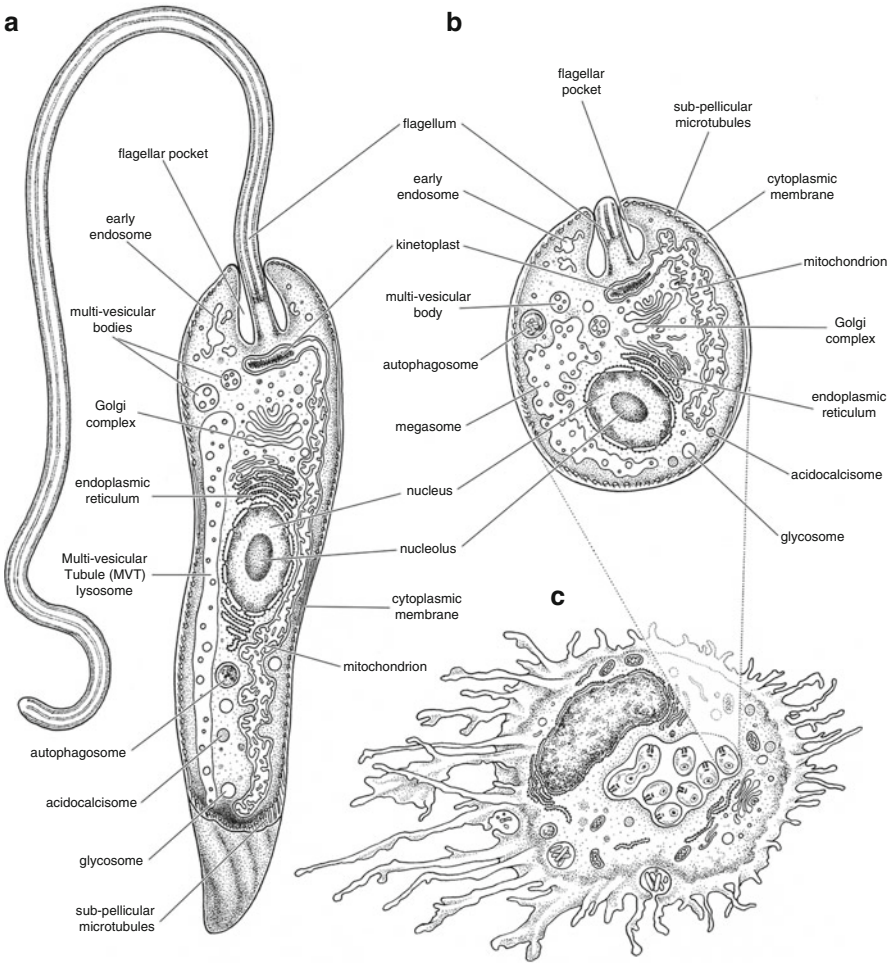


Fig. 7.1 Schematic representation of the cellular and subcellular architecture of a *Leishmania*. Promastigote (**a**) and intracellular amastigote (**b**). Amastigotes dwell in a parasitophorous vacuole (PV) inside mammalian phagocytes (**c**). Old World *Leishmania* species reside in individual PVs, while New World forms thrive in a communal PV (**c**)

A single, discernable Golgi apparatus of 3–10 stacks which in contrast to other eukaryotes is not breaking down during cell cycle but instead is distributed onto daughter cells be medial fission. These structures provide the context for the living cell, its replication and metabolic compartmentalization.

7.2.1.5 Cellular Replication

Leishmania parasites divide by mitosis and binary fission, but very few studies have analyzed this process in detail and only in promastigotes also because chromosomal condensation does either not occur or is not observable. Only recently has there

been a more thorough analysis of this process at least in *L. mexicana* (Ambit et al. 2011; da Silva et al. 2013; Wheeler et al. 2011). The generation time of logarithmically growing cells in rich media is 6–8 h. Replication proceeds through a series of steps that start with cell growth which may be equivalent to a G1 phase and lasts in between 2.5 and 3 h, followed by the S phase of DNA replication of the nuclear and kinetoplast DNA which is estimated to take another 3 h. The end of S phase coincides with the emergence of a new flagellum besides the remaining older one, and then nuclear mitotic division follows and finally segregation of duplicated kinetoplast. The sequence of the latter two events may not be strictly conserved in all *Leishmania* species (Ambit et al. 2011; da Silva et al. 2013; Wheeler et al. 2011). This was indeed a surprising finding since it is also opposite to what is known from *Trypanosoma brucei* or *Crithidia fasciculata* that also duplicate firstly the flagellum but then the kinetoplast segregates before nuclear mitosis. Finally, *Leishmania* undergoes cytokinesis through which daughter cells separate along their longitudinal axis starting from the flagellar end. One daughter cell inherits the old flagellum which can be distinguished from the newly formed one on the basis of being longer. The sub-pellicular microtubular corset on the other hand seems to be inherited in a semiconservative and symmetric way. All the post-S phase events require roughly an hour or less than 20 % of the cell cycle (Fig. 7.2).

The replication (see Chap. 2.3) and segregation machineries acting on the nuclear genome are only slowly becoming elucidated through the use of transgenic parasites, proteome, and genome analyses (Akiyoshi and Gull 2014).

Through the life cycle of these parasites, one broadly distinguishes three main forms: procyclic promastigotes replicating within the midgut of their insect vectors;

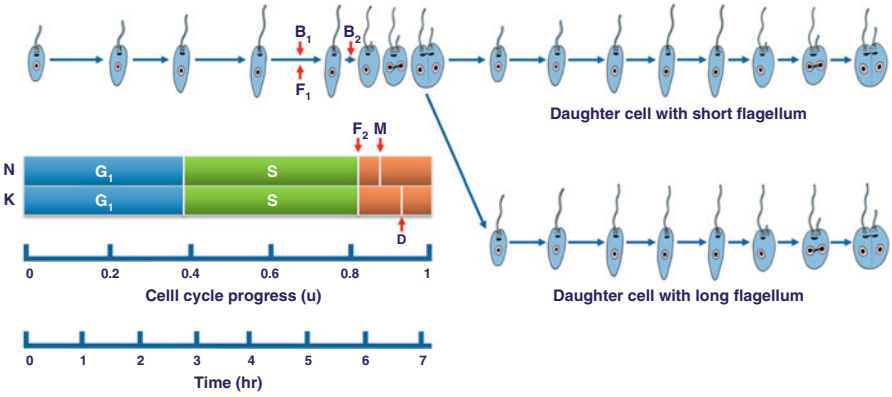


Fig. 7.2 *Leishmania* replication cycle. Promastigotes increase in cell volume and length as they progress through S phase of the cell cycle, toward the end of the S phase, new basal body (*B1*) and axoneme (*F1*) formation starts, and repositioning of these newly formed structures is initiated (*B2*) before nucleus (*N*) and kinetoplast (*K*) divide. *F2* denotes the relative time of daughter flagellum emergence from the flagellar pocket. The relative timing of nuclear mitosis (*M*) and kinetoplast division (*D*) is noted as observed for *L. mexicana* may differ between species (Adapted from Wheeler et al. (2011))

metacyclic promastigotes that do no longer divide and constitute a differentiated form of the promastigote ready to be transmitted (these locate to a parasite-derived glycan-rich plug in the stomodeal valve of the insect's sucking apparatus): and finally, the mentioned amastigotes into which metacyclic promastigotes transform once phagocytosed by host cells and confronted with a higher temperature and acidic pH in the phagosome.

7.2.1.6 Subcellular Vesicular Compartments and Associated Metabolic Features

The annotated genome of *Leishmania* spp. is a powerful resource of information on the metabolic capacities and subcellular distribution of enzymes and proteins of these parasites (Doyle et al. 2009; Opperdoes and Coombs 2007). This resource complements and expands the many experimental studies that had been performed over decades in the pre-genomic era to elucidate the metabolism and cellular architecture of these organisms. The information gained through the genome sequences also instructs and facilitates functional analyses by reverse genetics.

7.2.1.7 Glycosomes

In kinetoplastids, peroxisome-like compartments were described that lacked catalase, the hallmark enzyme of peroxisomes, but showed a high content of enzymes involved in glycolysis (Opperdoes 1990). They were thus called glycosomes and in *Leishmania* are crucial for fueling the energy metabolism of extracellular promastigotes (see also Fig. 7.1 and 7.4) through glycolysis.

In the post-genomic era, proteomic studies and genome-based bioinformatic prediction of glycosomal proteins confirmed that all enzymes required for glycolysis supposedly localize to this compartment but also revealed additional features. The sequence-based prediction of glycosome-located proteins exploits the relation of this compartment to ER-derived peroxisomes to which proteins containing peroxisomal targeting sequences (PTS) are transported via the help of carrier proteins, peroxins, that are shuttled from the cytosol to the glycosome lumen and back by a dedicated microbody membrane-associated machinery. Searching the genome for PTS containing open reading frames identified already nearly 200 proteins simply by analyzing the coding sequences predicted from the *L. major* genome (Opperdoes and Szikora 2006). Many of these proteins represented enzymes. The analysis of their function confirmed known and revealed additional metabolic pathways located to this compartment. These pathways include glycolysis and gluconeogenesis. Data from *T. brucei* show that compartmentalization of the enzymes hexokinase and phosphofructokinase in glycosomes seems essential since activity of these enzymes is not regulated by product inhibition and mis-localization in the cytosol results in cell death. Enzymes of other metabolic pathways are also localized to the glycosomes; this includes the hexose-monophosphate pathway, purine-salvage and pyrimidine synthesis, steps in ether lipid synthesis and sterol synthesis, and the remodeling of long-chain fatty acids. In addition, certain paralogues of genes encoding enzymes involved in polyamine and trypanothione metabolism, the key

molecule in trypanosomatids to keep the REDOX balance and to detoxify reactive oxygen species (c.f. chapter on trypanosomes for information on trypanothione synthesis), display PTS sequences. It is suspected that the respective proteins at least partly localize to the glycosomes. Many of these bioinformatically identified proteins were also found when analyzing the proteome of glycosomes isolated from extracellular promastigotes (Colasante et al. 2013). However, the dependence of the energy metabolism of the parasites on glycolysis varies with the life cycle stage. While it is very pronounced in the extracellular promastigote stage, amastigotes rather rely on the degradation of fatty acids and amino acids (Opperdoes and Coombs 2007). Whether this correlates also with a change in the enzymatic content of the glycosomes in the intracellular parasite is not yet known.

7.2.1.8 Acidocalcisomes

These organelles are present in eukaryotes as well as certain bacteria and were originally defined in trypanosomatids as acidic, calcium-storing compartments. In addition, they contain inorganic and polyphosphates and other divalent cations such as Zn. The generation of these vesicular compartments is thought to be linked to lysosomes, and their function is critically dependent on the recruitment of proton ATPases required for acidification. They are assumed to have a role in several forms of stress responses. Shown so far for *L. major*, their number is higher in intracellular amastigotes forms than in promastigotes. Mutants of this parasite with defects in acidocalcisome function or formation (*L. major* with targeted genetic lesions in a gene identified by searching the parasite genome for mammalian target of rapamycin (TOR)-like kinase genes (LmjF34.3940) (Madeira da Silva and Beverley 2010) or *L. major* lacking a functional adaptor protein 3 complex and, in consequence, lacking proton pump ATPases in acidocalcisome membranes (Besteiro et al. 2008)) are stress susceptible and cannot induce disease anymore, indicating that intracellular survival depends on functional acidocalcisomes.

7.2.1.9 Lysosomes/Multivesicular Tubes/Megasomes

Leishmania possess a lysosomal compartment that is the final point of the endocytic pathway (McConville et al. 2002) and play a key role in cell nutrition. However, this compartment displays changing properties depending on life cycle stage. In replicating promastigotes, it takes on the shape of a single long tubular structure that contains luminal vesicles called the multivesicular tube (MVT) that is not very acidic. In so-called metacyclic promastigotes, i.e., the insect-vector-borne forms that differentiate from proliferating parasites into a non-replicating form ready for transmission, the MVT is restructured into numerous electrodense vacuoles that become more degradative and richer in proteohydrolases. In amastigotes of the New World Leishmanias, the lysosomes take the shape of one or several large vesicles, called megasomes. The size and number of this compartment can vary between species, but this acidic compartment can take up up to 15 % of the total parasite cell volume and is very rich in cysteine proteases.

7.2.1.10 Autophagosomes

Starting from the trypanosomatid genomes, searches for genes encoding protein involved in autophagy in other organisms such as yeast have revealed more than 40 candidate orthologues (Besteiro et al. 2007). Macroautophagy does occur in *Leishmania* spp., and experimental evidence suggests that autophagosomes in these parasites can contain cytosolic proteins, multivesicular bodies, acidocalcisomes, and, in analogy to pexophagy – that is, the autophagy of peroxisomes – in yeast, glycosomes. Autophagosomes are eventually delivered to the MVT and megasomes and become degraded there. That glycosomes which are less numerous in amastigotes are destined for degradation via autophagosome formation as whole organelles is thought to highlight the importance of autophagy in metabolic adaptation from the extracellular lifestyle to the intracellular habitat that engenders a switch off of glycolysis (see below).

7.2.1.11 Flagellar Pocket

The flagellar pocket (FP) is an invagination of the cell membrane at the base of the flagellum and represents only few percent of the total cell membrane. The FP shows several subdomains; there is a collar-like structure at the site where the flagellum exits the pocket. The FP is precisely oriented with respect to the flagellum and the Golgi, and this orientation defines an asymmetry of the pocket membranes in relation to other cellular elements (McConville et al. 2002). This cellular compartment is much better studied in trypanosomes (Field and Carrington 2009), but many aspects may also be true for *Leishmania* spp. As mentioned FP is the site wherein trypanosomatid parasites' exo- and endocytosis occur.

Vesicles involved in transporting cargo from the Golgi to the FP are most likely located between these two compartments, and morphologically distinct populations can be observed in this space. Gp63 the major GPI-anchored surface protein of *Leishmania* has been located in larger, translucent vesicles in this area.

7.2.1.12 Protein Targeting and Subcellular Localization

With genome information available, a very common strategy to predict function and subcellular localization of proteins is the analysis for the occurrence of a particular amino acid sequence that is known from other organisms' proteins to be indicative of a particular function or to be important for protein routing. This concept has been validated experimentally by expression of say a mitochondrial protein of a plant species in yeast and observing that the protein is correctly targeted to the yeast mitochondrion. Such homology-based strategies generate quickly overall useful and testable parts lists of the protein and enzymatic make up of subcellular compartments. Available software tools for these predictions are constantly being refined taking into consideration that searches are more likely to predict protein localization correctly if the algorithms take the evolutionary context of an organism under study into consideration (Bohnsack and Schleiff 2010).

Current concept of phylogeny relates eukaryote organisms to six supergroups. Kinetoplastids belong to the so-called Excavata supergroup (Burki 2014). In contrast, most deeply investigated model organisms such as yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, or *Mus musculus* all belong to the Opisthokont

supergroup. Since the presumed common ancestor of these supergroups is thought to have had already a system of endomembranes, mitochondria, and peroxisomes, protein sequence-dependent signals that governed localization to these subcellular compartments are expected to maintain a degree of conservation through evolution to extant species.

Such homology-based search concepts exploiting the *Leishmania* genome information have been iteratively improved to identify specific protein localization signals and also import machineries operating in mitochondria and glycosomes based on sequences of predicted genes. These data sets are often complemented (in fact need to be complemented) by analyses of proteomes of purified subcellular compartments. The mitochondrial protein import machinery known so far for kinetoplastids was discussed in the chapter on trypanosomes and is essentially also operative in *Leishmania* as shown for *L. tarentolae* (Eckers et al. 2012). The major molecular difference in the kinetoplastid system as compared to the core components of the canonical import system known from Opisthokont model organisms is that the protein translocase in the outer membrane, ATOM, has homology to a family of bacterial translocases of the Omp85/TpsB protein superfamily but not to the functional equivalent in Opisthokonts, TOM40. Nonetheless, import signals are identified in an apparently functionally conserved way by the TOM40 and ATOM machineries since mitochondrial proteins of Opisthokont model organisms are imported by *Leishmania* mitochondria and vice versa.

Homology-based searches were also used to predict proteins localized to glycosomes by searching the genome for coding sequences with the information for peroxisomal targeting sequences (Opperdoes and Szikora 2006). This approach identified a list of 259 candidate glycosomal proteins. Proteomic analyses of glycosomal preparations from *L. tarentolae* add a complementary data set of more than 400 putative glycosomal proteins (Colasante et al. 2013). The data suggest that *Leishmania* glycosomes are equipped with a comparatively higher capacity to metabolize different sugars than the glycosomes of African trypanosomes.

Other primary sequence-encoded signals that influence protein localization in kinetoplastids are similar to those in eukaryotes in general and can be used to suggest protein localization based on genome data. The results of the predicting algorithms have different degrees of uncertainty and are prone to yield false-positives, as well as false-negative assignments. This caveat needs always to be kept in mind, but this uncertainty is balanced by the fact that the predictions are more than valuable means to exploit more of what the genome information can teach us. The clues in the sequence predictive of protein localization include signal peptide sequences, amino acid compatible with those known to form transmembrane domains, and signals predicting posttranslational modifications linked to protein localization such as glycoinositolphospholipid anchor addition sites or lipid-addition regions.

Signal peptides were predicted for 1409–1541 proteins depending on the species of which the genome is analyzed (try, e.g., the http://tritrypdb.org search for Genes, click protein features on the drop down menu, and select predicted signal peptides). A similar search strategy for ORFs with predicted TM yields 1409–1544 different ORFs per genome encoding at least one TM. There are a number of additional

protein sequence motifs that have been experimentally shown to target proteins in *Leishmania* to specific subcellular compartments. For example, the C-terminal tetrapeptide motif MDD results in ER retention of proteins; C-terminal KKXX motifs have also been shown to result in ER enrichment. Sequence motifs in the N-terminal cytoplasmic tails direct different glucose transporters in *Leishmania* to either the flagellum or the cell body.

Published analyses of genomes for proteins encoding ORFs that feature GPI anchor addition site sequences identified 82 ORFs in *L. major* encoding proteins that feature both a signal peptide and a GPI anchor addition sequence. Since GPI-anchored proteins dominate the surface of *Leishmania* promastigotes, this set of proteins contains many proteins known to be surface located such as the already mentioned GP-63 metalloprotease. The GPI anchor is added in the lumen of the ER to the C-terminal GPI attachment sites. How efficiently such proteins can then be transported from this compartment to the cell membrane has been illustrated elsewhere for the VSG proteins of trypanosomes, and the process for the major surface protein GP63 may be similar. Acylation -dependent localization of proteins has been demonstrated for a number of proteins in *Leishmania* sp. and in trypanosomes (Goldston et al. 2014). The current data are consistent with the idea that N-myristoylation alone routes proteins to compartments such as the Golgi and the pellicular membrane, while additional palmitoylation results to further trafficking, e.g., to the flagellum. The amino acid motives for N-myristoylation is a 9-amino-acid-long sequence that contains a glycine right after the starting methionine (see Fig. 7.3). A total of 72 ORFs in the *L. major* genome were predicted to contain such a N-myristoylation site.

There is only one N-myristoyltransferase (NMT) encoded in trypanosomatid genomes, and deletion of this gene or full inhibition leads to parasite death, not only in *Leishmania* but also in trypanosomes. Thus, drugs inhibiting NMT were anticipated to open a new avenue to target these organisms. Indeed, inhibitors of trypanosome NMT have been identified, and although these show species specific activity, screening for additional NMT inhibitors active also against *Leishmania* NMT has commenced.

Molecular trafficking including protein trafficking depends not only on signals such as those discussed above but, on trafficking by moving compartments such as vesicles with the help of molecular machines that are multi-subunit complexes actually mediating sorting, transport, and compartment fusion/fission. Homology searches mining the genome information and taking into account the evolutionary context of the trypanosomatids have again been used to identify likely components of such machines. These searches (Field and Carrington 2004; Murungi et al. 2014; Wideman et al. 2014) suggest that these organisms retained protein homologues belonging to the SNARE, retromer, vesicular coat proteins, and ancestral RAB and ARF proteins that orchestrate these processes (as discussed for trypanosomes in the respective chapter). However, the precise function of the individual proteins has yet to be established.

7.2.1.13 Metabolic Adaptation to Life Cycle Habitats

The genome information has also enabled in silico reconstruction of the metabolic network of the parasites (Chavali et al. 2008; Doyle et al. 2009). Together with

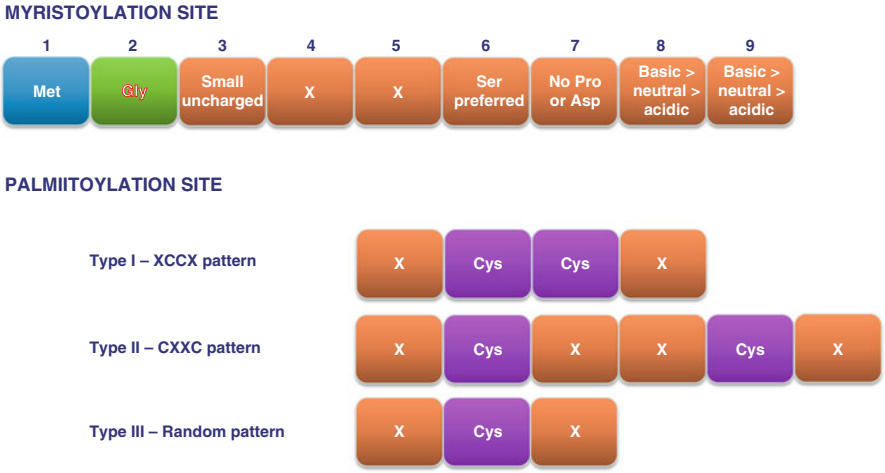
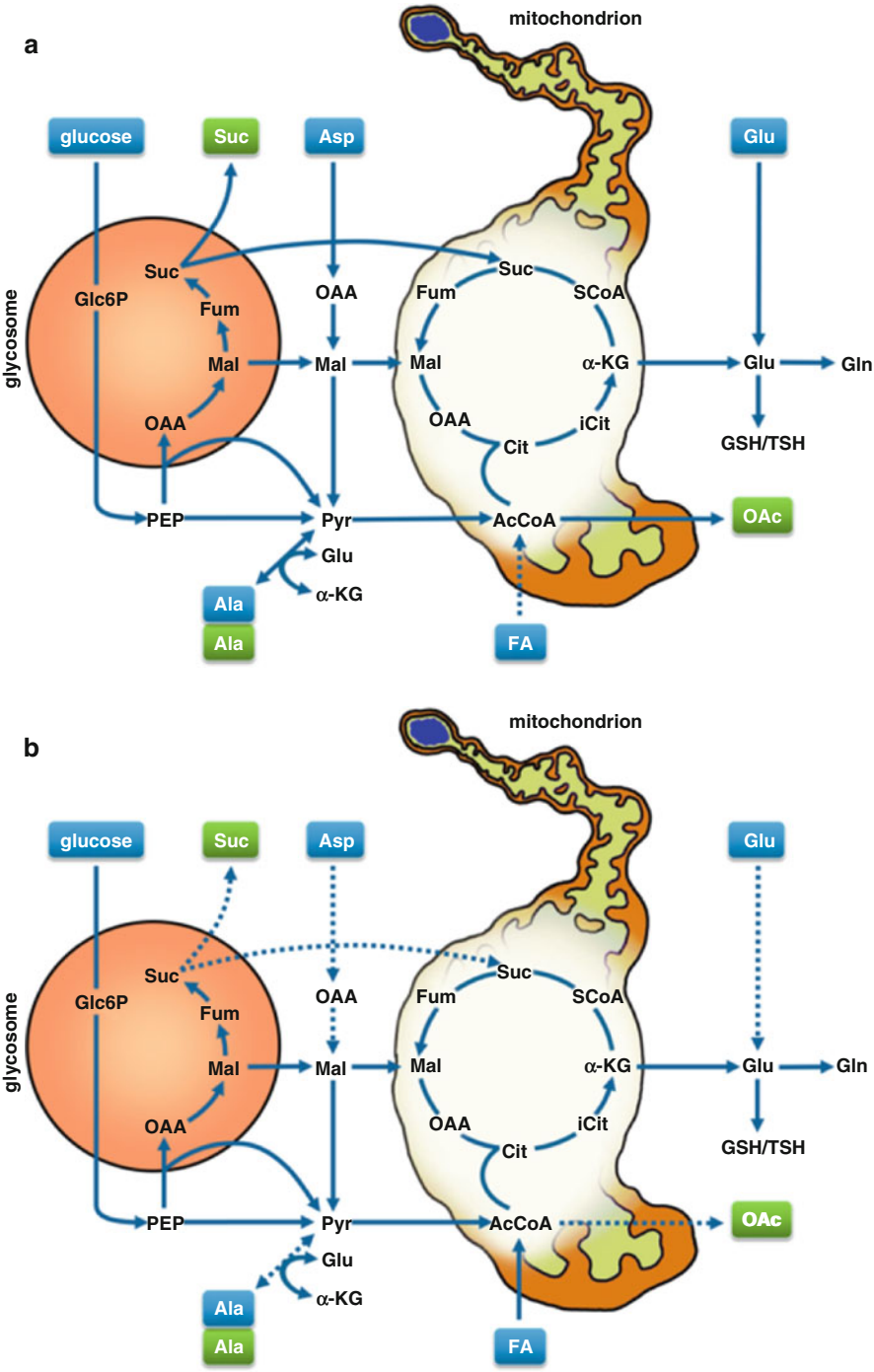


Fig. 7.3 Protein sequence motifs leading to myristoylation and palmitoylation. Sequence motifs in proteins enabling myristoylation (*top*) that occurs at a glycine at position to 2 from the N-terminus; motifs linked to palmitoylation (*bottom*). *X* denotes any amino acid, for palmitoylation neutral amino acids are preferred over basic and these over acidic ones (Adapted from Goldston et al. *TiParasitol*. 30:350–360)

proteome (Paape and Aebischer 2011) and increasingly metabolome (Saunders et al. 2015) data sets, the substantial amount of knowledge that was generated with classical biochemical investigations of the metabolic capacity of the parasites is rapidly growing. With reverse genetics possible and the availability of a chemically defined growth medium (at least for promastigotes (Merlen et al. 1999)), generating hypotheses about functional metabolic pathways and testing their relevance under the different conditions of the life cycle have become very informative. The major habitats encountered through the life cycle are those of free-living promastigotes in the midgut of a sand fly vector that provides a mainly plant-derived sugar-rich diet, such as honeydew, versus the environment in which amastigotes thrive, i.e., a phagolysosomal, intracellular compartment that is thought to be relatively rich in amino acids and lipids but less so in saccharides.

Early analyses (c.f. Opperdoes and Coombs (2007) for references) of major biochemical pathways operating at the different life cycle stages had already highlighted that the energy metabolism of promastigotes was primarily dependent on glycolysis and that of amastigotes on β-oxidation of fatty acids. Both life cycle stages under in vitro conditions produced CO_2, succinate, and alanine and glycine.

In contrast, freshly isolated amastigotes were mainly fueling their metabolism by degrading fatty acids. The technological advancement of metabolomics that enables the simultaneous analysis of many metabolites and, again exploiting genome information, reverse genetics confirmed and extended these findings considerably. It has become clear that when parasites differentiate into amastigotes, they not only switch to the use of fatty acids to cover their energy metabolism but also depend on this pathway providing building blocks for anabolic reactions via the tricarboxylic acid cycle (TCA) (see Fig. 7.4; Saunders et al. 2014).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AcCoA
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Accordingly, fatty acid degradation is thought to feed the TCA leading to the formation of a ketoglutarate and glutamine. Thus, in stark contrast to what is known about the TCA cycle in extracellular *T. brucei*, the full cycle is operative in both life forms of *Leishmania* spp. In addition, amastigotes require essential amino acids, at least low levels of hexoses from the host and a functional gluconeogenic pathway to grow and proliferate (McConville et al. 2007). This metabolic adaptation seems optimized for intracellular growth and the release of the metabolic end products mentioned above is much reduced when compared to promastigotes.

Information from the annotated genome also allows for predictions, refinements, and analysis of the metabolic potential of the parasites. In silico reconstruction of the predictable metabolic network has been performed by several groups (Chavali et al. 2008; Doyle et al. 2009), and a kind of a tutorial on how this can be achieved is available (see further reading (Saunders et al. 2012)). The EuPathDB/TriTrypDB database (http://tritrypdb.org/tritrypdb/) has incorporated and integrated metabolic pathway information with other data sets for online use. The online resources and tools are rapidly evolving, and information accessibility will hopefully become permanent.

One use of this information is the prediction of minimal requirements for parasite growth and the generation of hypotheses which of the metabolic pathways may be essential for growth. When pairing these predictions with information on the mode of action and kind of targets of existing and approved drugs, new treatment regimens can be predicted based on a so-reasoned approach (Chavali et al. 2012). The rational and workflow of a recent example of such an analysis are depicted in Fig. 7.5. The algorithm identified 15 enzymes that may be potential targets of 254 drugs approved for human use but most of them never tested in the context of leishmaniasis. However, within this set of 254, nine of the 13 drugs that are currently used to treat the disease were present. The major known pathways targeted by current therapies are the ergosterol synthesis by azoles and amphotericin B and the trypanothione-dependent redox balance system by penta- and trivalent antimony. Like in trypanosomes, the redox balance in *Leishmania* depends on the synthesis and level of trypanothione (see respective book section on trypanosomes for the synthesis of this molecule).

7.2.1.14 Surface Dominating Molecules

As mentioned before, GIPLs and GPI-anchored glycans and similarly anchored glycoproteins are dominating features of the parasite surface (Ilg 2000b; Ilgoutz and McConville 2001). The structure and biosynthetic pathway, in particular of the GPI-anchored glycans, has been a major research focus in the decade before the genome information became available and many steps were identified by first applications of reverse and forward genetics with tools developed largely by the Beverley group.

Fig. 7.4 Remodeling of central carbon metabolism depending on life cycle stage. The main pathways of carbon source and metabolism are depicted for promastigotes (*left*) and amastigotes (*right*) with the main subcellular compartments involved. *Dotted lines* indicate pathways downregulated relative to the other life cycle stage. *Abbreviations*: *αKG* α ketoglutarate, *AcCoA* acetyl-CoA; amino acids are indicated by their 3-letter code, *Cit* citrate, *Fum* fumarate, *FA* fatty acids, *G-6P* glucose 6-phosphate, *G3P* glyceraldehyde 3-phosphate, *GSH/TSH* glutathione/trypanothione, *Mal* malate, *OAA* oxaloacetate, *OAc* acetate, *PEP* phosphoenolpyruvate, *Pyr* pyruvate, *S-CoA* succinyl-CoA, *Suc* succinate (Adapted from Saunders et al. (2014))

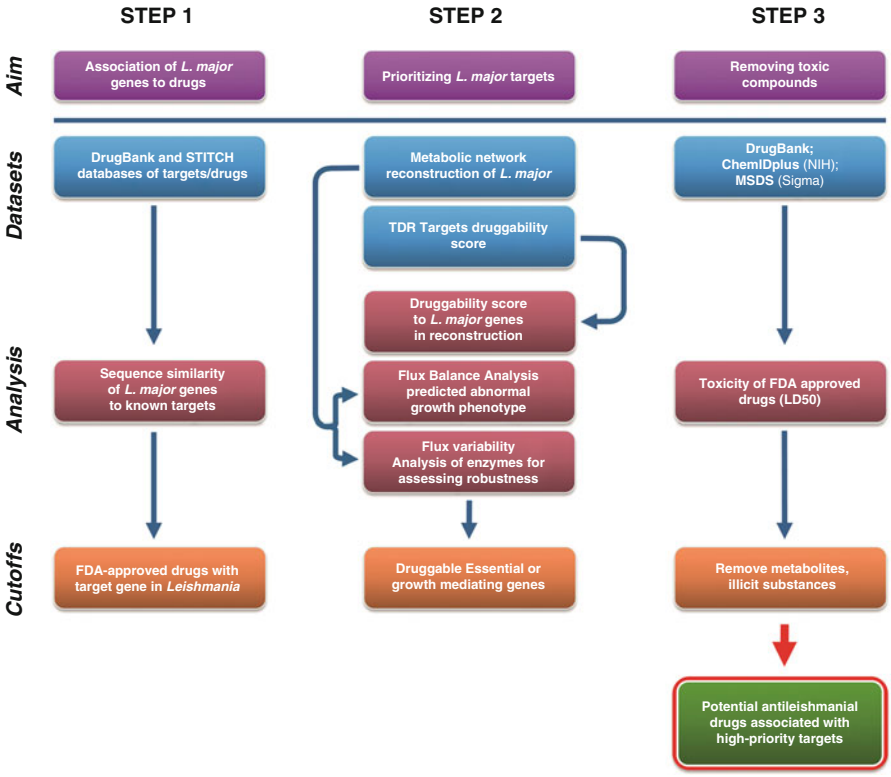

Fig. 7.5 Metabolic network guided Drug selection pipeline concept. The flowchart illustrates the conceptual framework (MetDP) for the prioritization of drug and drug targets against *L. major*. The first step deals with the association of *L. major* genes and drugs. The second step includes the prioritization of *L. major* targets and drugs using constraints such as druggability, gene essentiality, and flux variability. The final step allows for the removal of toxic compounds from the prioritized list of drugs to yield a set of low-toxic candidate antileishmanials (Adapted from Chavali et al. (2012))

The glycan parts of these molecule classes are related and were first elucidated for LPG (Fig. 7.6). They are composed of a repeated backbone of 6-galactoseβ1-4 mannoseα1 disaccharides linked by phosphodiesters. These extended phosphosaccharide repeats are capped by mannose-rich and substituted by side chains of several additional oligosaccharides. The lengths of composition of the caps and sidechains are variable. This variation exists between promastigote stages and parasite species and to a degree also between isolates of the same species. Apart from LPG, glycoproteins of a family of proteins with Ser-rich repeat domains that exist as GPI-anchored but also as secreted and/or surface-adsorbed constitute an abundant and specific feature of *Leishmania*. The proteins are heavily modified by a type of O-glycosylation unique to the parasites, namely, phosphogycosylation of Ser residues of the type R-Manα1-PO_4-Ser with R representing glycans related to the LPG building blocks. The basic structure of these glycans and how they are molecularly related are discussed in detail in (Ilg 2000b) and are summarized in Fig. 7.6. Collectively these proteins are called proteophophoglycans or PPGs.

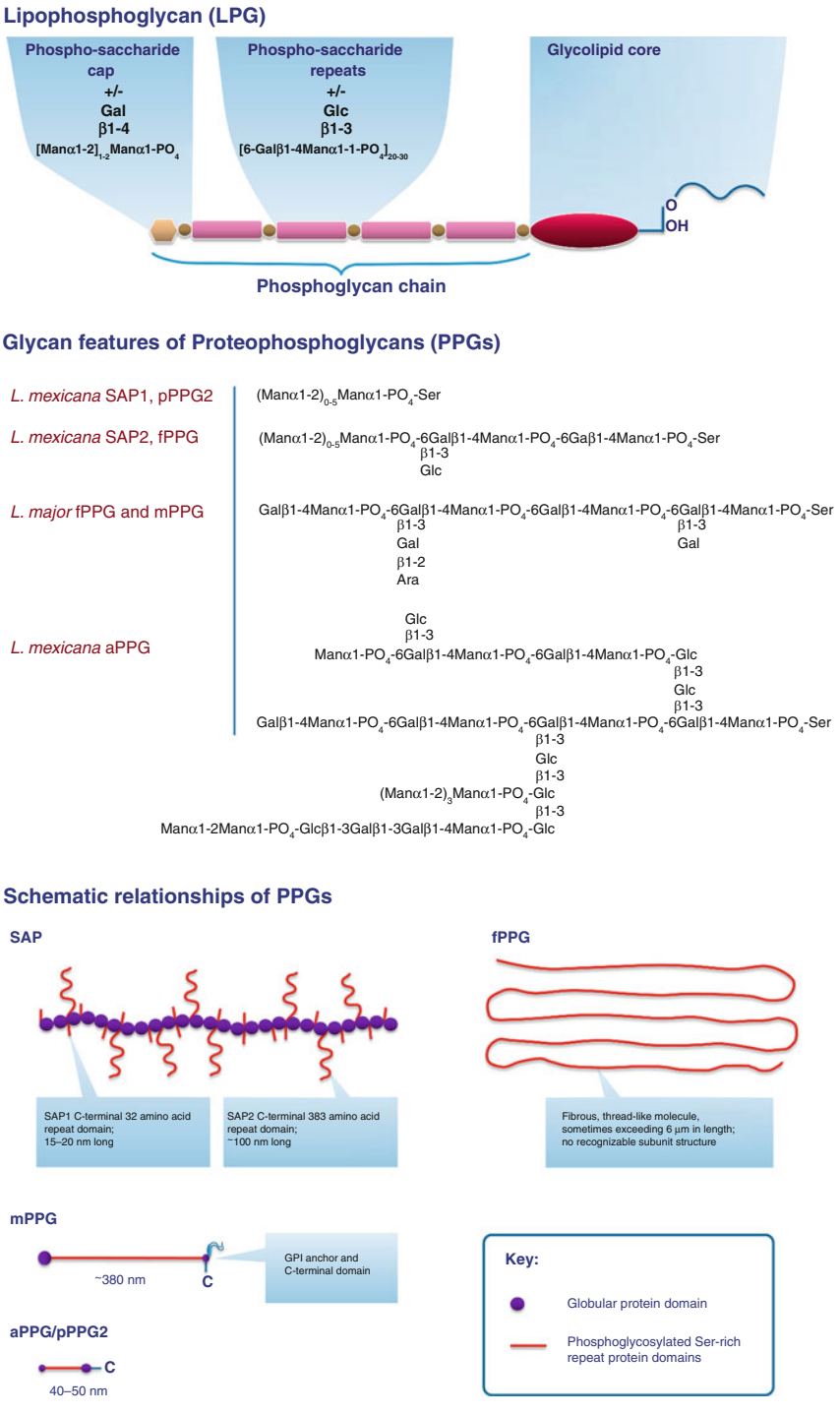

Fig. 7.6 Schematic of structural features of phospho- and proteophosphoglycans of *Leishmania* (Adapted from Ilg (2000a, b))

Astonishingly, while PPGs and LPG and GPI-anchored proteins such as GP63 dominate the promastigote surface, their relevance for the parasites remains incompletely understood.

Deletion mutants of at least one species, *L. mexicana*, that lack PPGs, LPG (Ilg 2000; Garami) (Garami et al. 2001; Ilg 2000a), or GPI-anchored proteins (Hilley 2000) (Hilley et al. 2000) retain infectivity to mammalian cells and can cause disease in murine models of cutaneous disease. These molecules may play a relevant role in the insect vectors, but, to date, these aspects have not attracted the same attention and remain unclear (Dostalova and Volf 2012) However, categorically speaking from the perspective of a mammalian host, parasites can lack all dominating surface features of promastigotes and remain pathogenic. This agrees well with the long known fact that the surface of the intracellular amastigotes is devoid of all these molecules and is instead dominated by small GIPLs.

7.2.2 Surface Ligands for Engagement of Receptors on Host Cells

To date *Leishmania* spp. have not been shown to invade cells; instead they are actively taken up by host cells, in particular by phagocytic cells. Phagocytosis is thought of as a receptor-mediated process, and complement receptors, lectin-type receptors, scavenger receptors, Ig-binding receptors, and others have been shown to mediate this type of endocytosis. Since *Leishmania* is transmitted in the promastigote form by female sand flies during their attempts to obtain a blood meal, dominant promastigote surface molecules on the parasite side and on the host side, complement components and their receptors, and other blood components have been studied for their relevance for parasite uptake (Fig. 7.7). Many of these aspects have been investigated in the pre-genomic information era and also before reverse genetics tools became available (for recent review, see Ueno and Wilson 2012). The findings, however, agree well with what we learned about the non-requirement of most of the abundant promastigote surface molecules since the latter approaches became available (see above) and show that ligands and receptors show functional redundancy which may be more or less pronounced depending on the parasite species and host cell or host species combination studied. However, no single interaction is decisive and this holds true for the uptake of both pro- and amastigotes.

The different receptor-ligand interactions involved in parasite phagocytosis are known also from other systems to link to and modulate downstream effects in the mammalian host cells such as signaling and immune response generation. For example, immunoglobulin-Fc receptor-mediated uptake has been shown to lead to disease aggravation in mice infected with tegumentary leishmaniasis causing species (Peters et al. 1997). Thus, the redundancy with respect to uptake is less evident when assessing other parameters of infection.

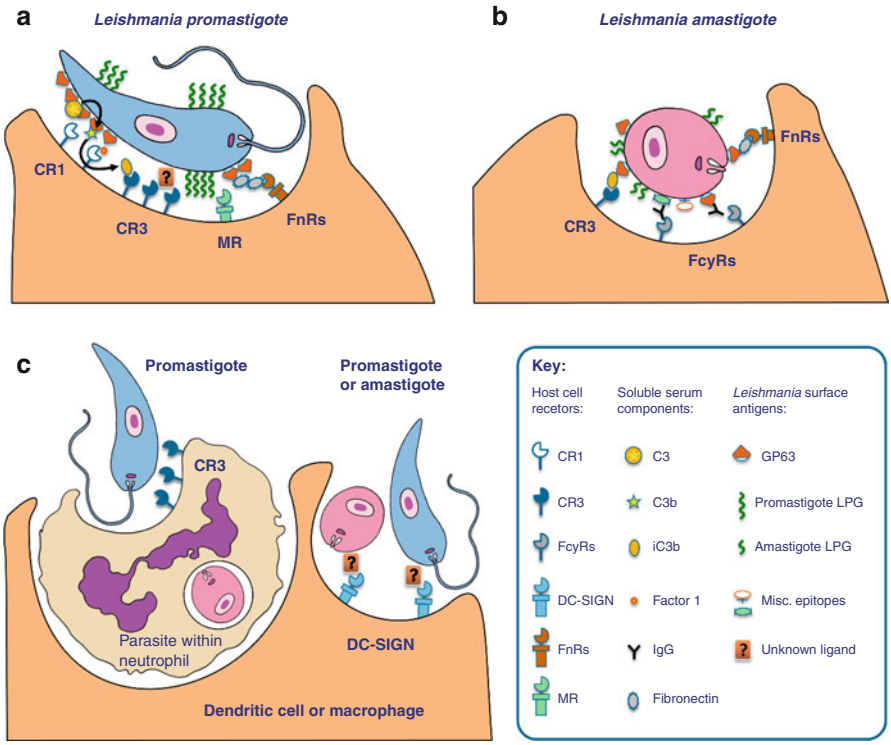

Fig. 7.7 Receptors for *Leishmania* entry into host phagocytes. *Leishmania* employ receptor-mediated entry pathways into host phagocytes (macrophages, neutrophils, or dendritic cells). Promastigotes are depicted in blue, amastigotes in magenta, and host cells in orange (**a**) GP63, which is highly expressed in promastigotes and converts C3 (third complement protein) opsonins into C3b, the natural ligand for CR1 (complement receptor 1). CR1, with factor I, cleaves C3b into iC3b (inactivated C3b), facilitating binding to CR3. CR3 may also mediate direct binding to promastigotes via a yet unknown surface epitope on promastigotes. The terminal sugar residues on LPG may be recognized by mannose receptor (MR); this awaits proof. GP63 also binds fibronectin, which can bridge parasites to fibronectin receptors (FnRs). (**b**) LPG expression on amastigotes is absent or downregulated; amastigotes may also express low levels of GP63 that could act as a ligand for complement components as in (a). The relevance of both ligands for amastigote binding remains unclear. In contrast, antibody and fibronectin on amastigotes lead to ligation of Fc gamma receptors (FcγRs) and FnRs, respectively. (**c**) Immediately following inoculation by the sand fly, promastigotes become phagocytosed predominantly by neutrophils. Promastigotes are taken up by these cells via CR3. Some of these parasite-containing neutrophils cells thought to be engulfed by macrophages or DCs much like dying cells. Promastigotes and amastigotes may also directly enter DCs via DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), but the ligand on the parasite surface has yet to be identified (Adapted from Ueno and Wilson (2012))

7.3 Perspectives and Open Questions

As mentioned above, in this post-genomic era, the potential to query the biology of any organism but, specifically that kinetoplastids has been tremendously boosted. Yet, the full potential of this information has yet to be harnessed. What we are still lacking are high-throughput methods to exploit fully the power of genetics but these seem to appear on the horizon. Genomics has already revealed that the concept of *Leishmania* being a diploid organism has to be reconsidered. Rather, these parasites seem to be largely aneuploid when recently sampled from the natural population (Rogers et al. 2011, 2014). Thus, the plasticity of the species genome is much greater than anticipated. It will be tremendously interesting to investigate whether and how this impacts and, specifically, what aspects of the biology of the parasite it affects as this protist revolves through the life cycle.

It is also expected that high-throughput methods for reverse and forward genetic analyses (or in fact the exploitation of classical genetics once its modality is better understood (Romano et al. 2014)) will eventually enable screening approaches to decipher the exact mechanism and molecular networks that rule differential gene expression or processing of environmental clues that lead to differentiation.

High-throughput methods and genome-wide analyses will hopefully also enable addressing the reasons for the diverse clinical manifestations of the leishmaniases. These can be mostly explained as being the result of infections with different *Leishmania* species. However, we do not really have a clue as to what is the molecular reason behind this. Increased thermotolerance or general stress tolerance is one hypothesis that may explain why, e.g., *L. donovani* can cause visceral infection while *L. mexicana* infections localize to arguably cooler parts of the body such as the ears. However, explanations at molecular resolution are not yet available. Of course the study of this aspect is complicated as host factors are also involved.

Finally, and probably most relevant, the integration of genome information into every aspect of teaching particular parasitology is a challenge that deserved to be taken on.

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Toxoplasma

8

Carsten G.K. Lüder and Frank Seeber

Abstract

Toxoplasma gondii is the most widespread intracellular parasite of warm-blooded vertebrates including humans. Infections are mostly asymptomatic or benign in immunocompetent hosts but can be life threatening in immunocompromised individuals and in fetuses after vertical transmission. *T. gondii* has become a model organism for intracellular parasitism as well as for other Apicomplexa. Genome and transcriptome data for different *T. gondii* strains are publically available, and powerful forward and reverse genetic tools have been developed. Here we summarize molecular and cellular features of *T. gondii* that are critical for the biology of the parasite and its interaction with the host. This includes characteristics of the three parasite genomes and how gene expression may be controlled. Examples for *T. gondii*-specific metabolic features including metabolic pathways of the apicoplast are highlighted. Being an intracellular parasite, the mechanism of host cell invasion is also of major interest. It involves adhesins of the SAG-related sequence protein family, sequential secretion of a large number of proteins from three characteristic secretory organelles (i.e., micronemes, rhoptries, and dense granules), and a unique form of motility accomplished by the "glideosome" multi-protein complex. Polymorphic excretory-secretory proteins from the rhoptries and the dense granules injected into the host cell also extensively modify host responses and determine parasite virulence. Finally, conversion from the proliferative tachyzoite to the dormant bradyzoite stage is critical for the establishment of a chronic infection and allows host (and parasite) survival and transmission to new hosts.

C.G.K. Lüder (✉)
Department of Medical Microbiology, Göttingen University Medical School, Kreuzbergring 57, D-37075 Göttingen, Germany
e-mail: clueder@gwdg.de

F. Seeber
Robert Koch-Institut, FG16, Nordufer 20, D-13353 Berlin, Germany

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8.1 Introductory Remarks

Toxoplasma gondii is ubiquitously distributed, and with an estimated prevalence of 30 % worldwide, it is one of the most prevalent parasites. It belongs to the phylum Apicomplexa and hence possesses two characteristic organelles, namely, the apical complex, which is crucial for host cell invasion, and the apicoplast, i.e., a non-photosynthetic plastid. As most other members of the phylum, it is obligatory intracellular. *T. gondii* is also characterized by a complex life cycle that includes reproduction by asexual schizogony, sexual gamogony, and asexual sporogony.

A main feature of *T. gondii*'s biology is its broad host and host cell range. Any warm-blooded vertebrate, i.e., birds and mammals including humans, support asexual reproduction and can serve as intermediate hosts. Within these hosts all nucleated cell types sustain the intracellular replication of the parasite. In contrast, only felids can serve as definitive hosts and allow sexual reproduction of *T. gondii* in their intestinal epithelium. Oocysts are shed with the cat feces and, after sporulation, can be infective for intermediate hosts by their ingestion via contaminated food or water or by uptake from the environment. After traversal of the intestinal epithelium, sporozoites transform to fast replicating tachyzoites that disseminate within the host using predominantly monocytes and dendritic cells as vehicles. After several rounds of intracellular replication and within an estimated 1–2 weeks of infection, tachyzoites transform to slowly replicating and metabolically largely inactive bradyzoites, which reside in intracellular tissue cysts. These cysts are able to persist for extended periods of time predominantly within neural and muscular tissues. Consequently, hosts infected with *T. gondii* will remain latently infected for the host's life span. Uptake of tissue cysts via ingestion of latently infected prey (e.g., cats hunting mice) or raw or undercooked meat products from latently infected livestock (e.g., humans eating pork) may transmit the parasite to new definitive and intermediate hosts, respectively.

T. gondii is an important pathogen of humans and livestock. Although most infections of immunocompetent hosts remain asymptomatic or benign, *T. gondii* can lead to severe illnesses including lymphadenopathy and recurrent posterior uveitis (an inflammatory eye disease) in some patients. In addition, immunosuppression (e.g., in AIDS patients or in those under immunosuppressive therapy) is a common predisposing factor which enables persisting bradyzoites to reactivate and convert to fast replicating and tissue-damaging tachyzoites. This may lead to life-threatening toxoplasmosis if not treated with available parasiticidal drugs. Finally, primary infection of pregnant women can lead to transplacental infection of the fetus, eventually leading to stillbirth, to severe congenital toxoplasmosis of the fetus, or to sequelae during childhood.

8.2 Molecular Biology of *Toxoplasma gondii*

8.2.1 Cellular Architecture

Tachyzoites are the most extensively studied stage of *T. gondii* because they can be easily propagated in various mammalian cells in vitro. They are crescent-shaped cells of approximately 2×7 μm (Fig. 8.1) (Dubey et al. 1998) surrounded by a

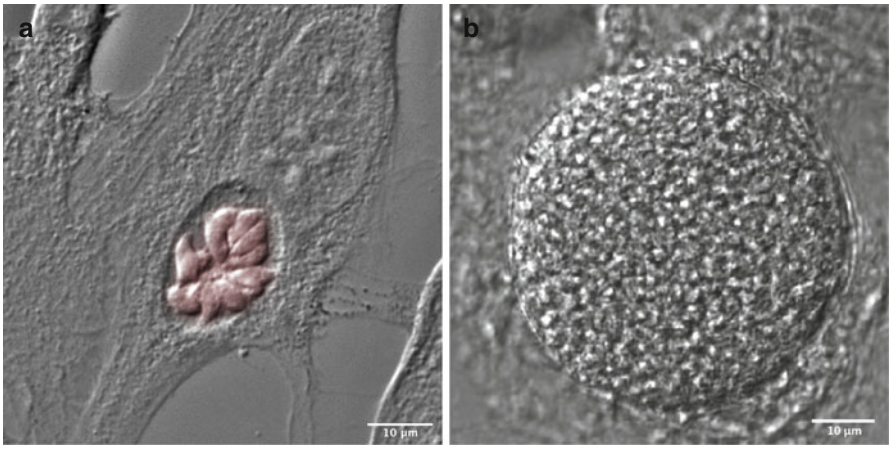

Fig. 8.1 Tachyzoites and bradyzoites of *T. gondii*. (**a**) Tachyzoites residing within a parasitophorous vacuole after infection of a human foreskin fibroblast in vitro. For illustration, the parasites have been colorized *red*. (**b**) Tissue cyst containing hundreds of individual bradyzoites that has been isolated from the brain of an infected mouse. Scale bars 10 µm

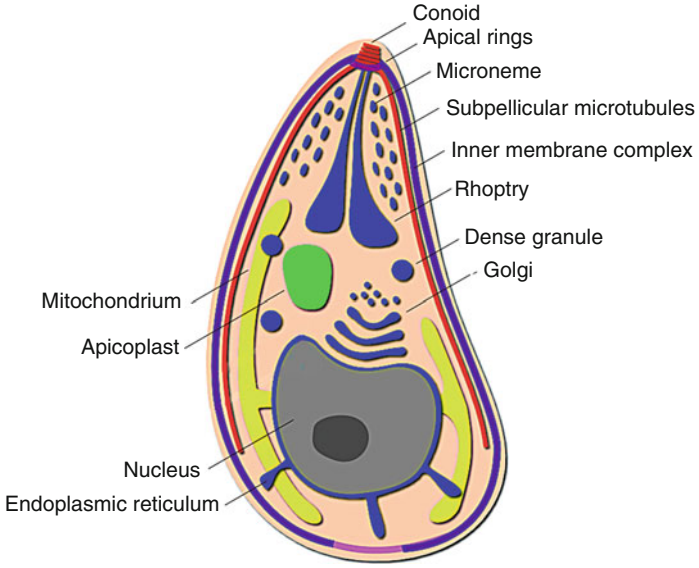

Fig. 8.2 Schematic representation of the ultrastructure of a *T. gondii* tachyzoite. See text for further details (©Striepen B, Jordan CN, Reiff S, van Dooren GG (2007) Building the perfect parasite: cell division in Apicomplexa. PLoS Pathog 3(6): e78. doi: 10.1271/journal.ppat.0030078. http://www.plospathogens.org/article/info:doi/10.1371/journal.ppat.0030078 (modified))

complex membranous structure called the pellicle. The pellicle consists of an outer plasma membrane and an inner membrane complex (IMC) composed of a layer of flattened and partially fused vesicles (Fig. 8.2). The cytosolic face of the IMC

interacts with subpellicular microtubules which provide mechanical strength to the *T. gondii* cell. The outer membrane of the IMC and the plasma membrane are connected to each other by multi-protein complexes which comprise F-actin and myosin A (MyoA) motor proteins, "glideosome"-associated anchor proteins (GAPs), myosin light chain (MLC1), aldolase, and the transmembrane adhesin MIC2 (Lebrun et al. 2014) (see below). Together, these components form the glideosome motor complex that enables *T. gondii* to move on a substrate by a process called gliding motility, to invade host cells, and in some cases to egress from host cells (Frenal et al. 2010).

The *T. gondii* cell is highly polarized, with several distinctive structures particularly at the apical pole of the cell (Fig. 8.2). These include four cytoskeletal ring structures and the conoid, which is a 250 nm long channel composed of tubulin filaments. In addition, two types of excretory-secretory organelles, namely, the club-shaped elongated rhoptries and the small rod-shaped micronemes, are located at the apical pole of the *T. gondii* cell. Before and during host cell invasion, the contents from micronemes and rhoptries are sequentially secreted and are essential for the invasion process (see below). The conoid may mechanically facilitate the penetration of the host cell. A third group of excretory-secretory organelles are the dense granules which are mostly located in the posterior part of the cell. They are discharged into the parasitophorous vacuole (i.e., a membrane-bound compartment *T. gondii* resides in; PV) after host cell invasion and may transform the PV into a suitable environment for parasite development.

Although the apicoplast had been described on an ultrastructural level already in the 1960s, it was only in the 1990s when it was identified as a non-photosynthetic plastid (Kohler et al. 1997). It originated from the engulfment of probably red algae by a process called secondary endosymbiosis, i.e., uptake of a plastid-bearing eukaryote by the ancestor of the apicomplexans. Despite the small size of the apicoplast (~300 nm in diameter), which hampers analysis by electron microscopy, it is now widely accepted that the apicoplast of *T. gondii* is bound by four membranes. Their origins are two of the endosymbiotic plastid, one of the algal secondary endosymbiont and one from the apicomplexan ancestor which engulfed the algae.

Besides the distinctive structures described above, *T. gondii* comprises also universal organelles including endoplasmic reticulum, ribosomes, Golgi apparatus, nucleus, and a single fused and elongated mitochondrium (Fig. 8.2). Although the general morphology is similar in tachyzoites, bradyzoites, and sporozoites, there are also stage-specific differences (Dubey et al. 1998). For example, the nucleus is located in the central part of tachyzoites, whereas it is more posterior in bradyzoites and sporozoites. Furthermore, bradyzoites and sporozoites but not tachyzoites contain numerous polysaccharide granules. They also differ in the numbers or the appearance of their excretory-secretory organelles.

Important differences between the three cellular forms relate to the structural entities in which they reside. Tachyzoites and bradyzoites are obligatory intracellular and reside within a PV or a tissue cyst, respectively (Fig. 8.1). The PV is bound by a single lipid membrane (PVM), which is extensively modified by parasite

proteins, particularly of the rhoptry (hence called ROPs) and dense granule (GRAs) families. In addition, within the lumen of the PV, a tubulo-vesicular network (TVN) develops shortly after invasion that contains several GRA proteins. The functions of the PVM-modifying proteins as well as the TVN are not fully resolved yet, but they may be critical for nutrient acquisition, exchange of cofactors, and interaction of the parasite with its host cell. One of the well-established features of the *T. gondii* PV is its fusion incompetence with the endosomal-lysosomal compartment of the host cell (Jones and Hirsch 1972) and the absence of its acidification (Sibley et al. 1985), probably because of the exclusion of host cell transmembrane proteins from the PVM during host cell invasion. The tissue cyst in which the bradyzoites reside is also a membrane-bound intracellular compartment; however, it is modified by formation of a prominent ~0.5 μm thick cyst wall. It likely confers physical strength to tissue cysts, which may contain up to several hundreds of individual bradyzoites and can measure up to 100 μm in diameter (Dubey et al. 1998). It consists of the PVM and membrane-bound vesicles embedded in a layer of granular material beneath the membrane. The cyst wall is highly glycosylated, and lectins are regularly used to mark the cyst wall of *T. gondii* tissue cysts. A cyst wall protein named CST1, which is recognized by a specific lectin, has been recently cloned and characterized (Tomita et al. 2013). It is a 250 kDa protein of the SRS (SAG1-related sequence) family and bears a mucin-like domain which is responsible for lectin binding. Interestingly, the mucin domain of CST1 appears to be important for formation of a normal cyst wall and for wall integrity under stress conditions (Tomita et al. 2013).

8.2.2 Genome and Transcriptome

T. gondii is largely present in North America as four and in Europe as three clonal lineages but shows greater evidence of sexual recombination in South America, the reasons for which are currently unclear. Extensive genotyping of several hundred globally sampled diverse *T. gondii* isolates revealed a population structure of a small number of haplogroups comprising six major clades (Su et al. 2012). A subsequent study used whole-genome single nucleotide-polymorphism analysis of 26 representative *T. gondii* strains and concluded that most sequenced strains did not fit into the previously proposed haplogroups (Minot et al. 2012). Further analyses are required to come to a consensus, but evidently there seems to be more genetic recombination going on in nature than previously anticipated. Since the products of polymorphic genes are known virulence factors (see Sect. 8.2.7), the knowledge of *T. gondii* genotypes and their natural distribution is of great importance to also understand host-parasite relationships.

T. gondii contains three genomes: one nuclear of 14 chromosomes (haploid in all asexual stages), one mitochondrial containing only three genes (the smallest mitochondrial genome known), and one circular apicoplast-localized genome (Khan et al. 2007; Kissinger and DeBarry 2011; Seeber et al. 2014). Due to the model character of *T. gondii* for other apicomplexans, there is already an impressive

Table 8.1 Genome size and number of putative genes and proteins of different *T. gondii* strains and related Apicomplexa

Organism	Mbp	Genes	Proteins
Toxoplasma gondii GT1	63.95	8,637	8,460
Toxoplasma gondii ME49	65.67	8,920	8,322
Toxoplasma gondii VEG	64.52	8,563	8,410
Neospora caninum Liverpool	59.10	7,266	7,122
Plasmodium falciparum 3D7	23.33	5,777	5,542
Plasmodium berghei ANKA	18.78	5,254	5,076

Taken from EuPathDB Release 28 (June 2016)
Mbp mega base pairs

number of genomic and transcriptomic data available from different strains. Most of them are accessible through the Web-based *T. gondii* database (ToxoDB; www.toxodb.org).

The numbers in Table 8.1 are still changing with every new ToxoDB release, e.g., due to re-sequencing of different genomic regions, and new data from next-generation sequencing projects of RNA, which allow to build better gene models or to verify existing ones. It is apparent from Table 8.1 that *T. gondii* has a considerably larger genome and consequently also a higher number of putative genes than, e.g., *Plasmodium* sp. This most likely reflects the fact that *T. gondii* has a much broader host range, both in terms of species and host cell types, than *Plasmodium*, which is restricted to a few cell types and a single intermediate host. Therefore, *T. gondii* can be assumed to be equipped with a larger repertoire of proteins involved in, for example, cell attachment, invasion, immune evasion, or metabolic enzymes that allow flexible adjustments to different growth environments (see Sect. 8.2.4). In this respect it is interesting that genome comparisons between *T. gondii* and the closely related *Neospora caninum*, which is more host restricted and has the dog as definite host, revealed only a very limited number (<250 genes) of sequences unique to either parasite. The encoded proteins are therefore good candidates for being involved in organism-specific differences (Reid et al. 2012). However, both species differ to a larger extend in surface protein families, with *N. caninum* having a larger repertoire but with *T. gondii* having seemingly more of those genes expressed at a given time (Reid et al. 2012).

Compared to the nuclear genomes, those of the mitochondrion differ only little within the Apicomplexa with regard to gene content: only three mRNAs encoding apo-cytochrome b (cob), cytochrome c oxidase I (cox1), and cytochrome c oxidase III (cox3) are present on the 6 kb linear genome, surrounded by sequences similar to highly conserved regions of rRNAs, and some internal repeats (Seeber et al. 2014). To date, its localization in the mitochondrial organelle has not been formally proven but is considered highly likely.

The apicoplast genome and its encoding proteins are much better characterized (Seeber et al. 2014; van Dooren and Striepen 2013). It consists of a 35 kb circle present in several copies (5–25) and is thus one of the smallest known plastid

genomes. Most proteins that make up the organelle are nuclear encoded (see below). However, it encodes a full set of proteins and RNAs required for ribosome assembly, i.e., small and large subunit rRNAs, ribosomal proteins as well as all tRNAs, the translation elongation factor Tu, and most subunits of an eubacterial-like RNA polymerase. Only two other open reading frames coding for proteins with known functions are present: one encoding a protein involved in iron-sulfur cluster biosynthesis (SUFB) and one potential orthologue of CLPC (a chaperone component of the "*T*ranslocon at the *i*nner envelope membrane of *c*hloroplasts," Tic complex). Division of the apicoplast is coordinated with the cell cycle of the organism, thereby ensuring that each daughter cell receives a single apicoplast.

How gene expression is regulated in *T. gondii* is constantly emerging. One of the most important discoveries was the definition of a large family of plant-related AP2 transcription factors (Balaji et al. 2005) that are now known to be involved in the regulation of core promoter activity, in particular during parasite development (see Sect. 8.2.5). In addition, chromatin remodeling via a diverse set of histone modifications also contributes to gene regulation in this context.

The regulation of mRNA abundance through the cell cycle is another topic of interest. About 40 % of mRNAs are expressed in such a coordinated way that proteins are apparently produced "just in time" for delivery to the daughter cell (Behnke et al. 2010). It is thought that in addition to translational control, transcript processing via splicing, mRNA capping and polyadenylation, and degradation or stabilization of mRNA play important roles in this regulation rather than AP2 transcription factors (Suvorova and White 2014).

Powerful forward and reverse genetic tools have been developed for *T. gondii* (Jacot et al. 2014), like inducible expression of genes and reporters, conditional gene knockins/knockouts, chemical and insertional mutagenesis combined with genome sequencing, and the possibility to conduct genetic crosses in the cat. Together with the available high-quality genome sequences for several strains, this toolbox will ensure that *T. gondii* remains the model organism for other genetically less amenable Apicomplexa.

8.2.3 Important Enzymes

Since most anti-infectives are inhibitors of enzymes of the pathogen, it is not surprising that most enzymes that are well described in *T. gondii* belong to the "class" of drug targets and by definition are also important for parasite survival. In this paragraph only a few examples will be given for enzymes that are targets of drugs already in use or where the enzymes have been shown to be essential.

The combination of pyrimethamine (PYR) together with sulfadiazine (SDZ) or sulfadoxine (SDX) for treatment of acute toxoplasmosis or uncomplicated malaria, respectively, has been in use for decades and relies on the inhibition of the enzymes dihydrofolate reductase (DHFR) by PYR and dihydropteroate synthetase (DHPS) by SDZ/SDX (Müller and Hyde 2013). Both enzymes are involved in the generation of folates, which are required for the conversion of deoxyuridine 5′-monophosphate

(dUMP) to deoxythymidine 5′-monophosphate (dTMP) and thus ultimately for RNA and DNA synthesis (for details, see Müller and Hyde 2013). Since the parasites cannot salvage thymidine from the host and lack a thymidine kinase that could convert deoxythymidine to dTMP, inhibition of dTMP synthesis by antifolates is highly efficient in killing them. While the excessive use of PYR/SDX as antimalarial in the last decades has led to its widespread worldwide resistance in *Plasmodium* spp., PYR/SDZ for treatment of toxoplasmosis is still effective.

The second class of drugs in use for both, *T. gondii* and *Plasmodium* spp., are compounds that inhibit organellar transcription, translation, or replication and which are of (cyano)bacterial origin (Wiesner and Seeber 2005). Whereas tetracyclines and macrolides, like clindamycin, target ribosomal proteins, rifampicin blocks the activity of bacterial RNA polymerase, and the fluoroquinolone ciprofloxacin inhibits DNA gyrase. To what extent the mitochondrial or apicoplast machinery (or both) are the actual targets for those drugs is not entirely clear yet.

Since the apicoplast is known to be essential for parasite survival and harbors a number of plant-like enzymatic reactions not found in the mammalian hosts, most recent studies on new drug targets have focused on this organelle (Botte et al. 2012). Most of the estimated 500–600 proteins constituting the proteome of the apicoplast are nuclear encoded and can be detected in the genome with some confidence by bioinformatic means due to the presence of N-terminal sequence extensions required for protein import into the organelle. From such analyses, it has become apparent that synthesis of heme (only partly), fatty acids (bacterial FAS II pathway), and isoprenoids (so-called *m*ethyl*e*rythritol *p*hosphate or MEP pathway) takes place in the apicoplast and follows routes that differ substantially from those of the host (Seeber and Soldati-Favre 2010). The essential nature of FAS II in *T. gondii* has been shown by genetic deletion and pharmacologic experiments (Crawford et al. 2006; Mazumdar et al. 2006), whereas in *Plasmodium* sp., contrary to expectation, FAS II gene deletions turned out to be deleterious only in liver but not in blood stages (Tarun et al. 2009). It seems that only in infected red blood cells fatty acids derived from the host are capable of supplementing the FAS II deficiency in transgenic malaria parasites, whereas this is not possible (or not sufficient) in those parasite stages that reside in nucleated cells, like the liver stages of *Plasmodium* and *T. gondii*.

The latter highlights an important general aspect of host-parasite relationship, i.e., the accessibility to and transport of nutrients (metabolites) and/or drugs (anti-metabolites) within the infected cell. This is very well illustrated for an inhibitor of another essential apicoplast enzyme called 1-*deo*xy-D-*x*ylulose-5-*p*hosphate (or DOXP) reductoisomerase (DXR). Starting from D-glyceraldehyde-3-phosphate and pyruvate for the synthesis of the isoprenoid precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), this protein catalyzes the second step in this pathway (Seeber and Soldati-Favre 2010). The inhibitors fosmidomycin and its derivative FR900098 are structurally similar compounds to the DXR substrate DOXP (Fig. 8.3) and bind with high affinity in the catalytic center of the enzyme. Due to their highly charged nature, the inhibitors cannot easily pass through cellular membranes and in the case of cells infected with *T. gondii* are thus not accumulating to a concentration that would allow parasite

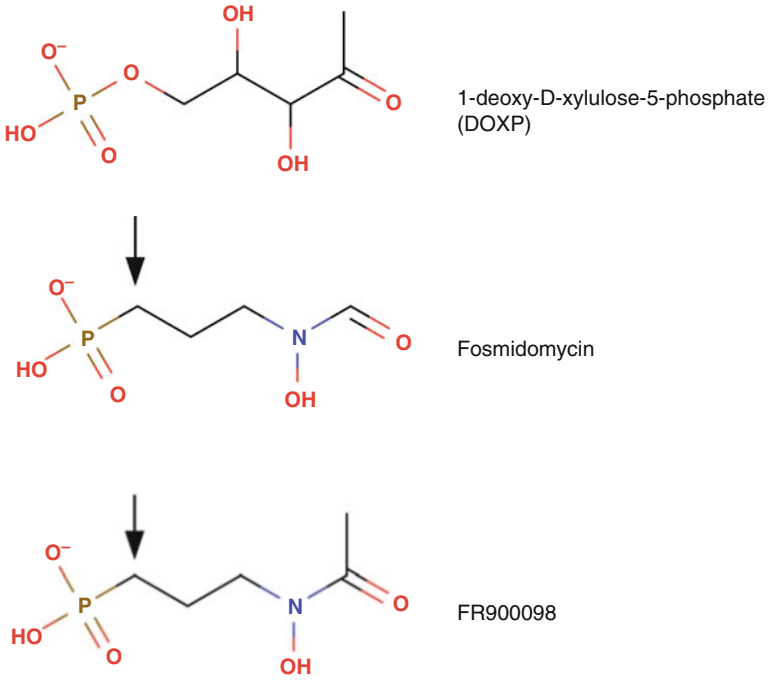

Fig. 8.3 Substrate (*top*) and inhibitors of DOXP reductoisomerase, an enzyme involved in apicoplast isoprenoid biosynthesis. The *arrow* indicates the replacement of a carbon atom in the inhibitors, compared to the oxygen in the phosphate group in DOXP, resulting in a non-hydrolysable (more stable) molecule by cellular phosphatases

killing, although the drugs readily inhibit the enzyme (Baumeister et al. 2011; Nair et al. 2011). In contrast, red blood cells infected with *P. falciparum* change the permeability of the host cell membrane through an ill-defined mechanism called "new permeability pathway," which enables fosmidomycin and FR900098 to enter the infected cell, bind to and inhibit DXR, and thus kill *Plasmodium* (Baumeister et al. 2011). This example shows that all aspects of enzyme-substrate (or inhibitor) interactions need to be considered before a compound can be regarded as a potential drug.

8.2.4 Special Metabolic Features

A hallmark of cellular parasitism is the battle for nutrients between pathogen and host. If the host cell is a constant bountiful dining table, the parasite can afford to lose whole metabolic pathways that its free-living ancestors still had to rely on. Loss of entire synthesis routes is connected on one hand to the dependence on the host but on the other hand also means much less metabolic (energetic) burden for the parasite. For instance, to generate one molecule of the C_{16}-fatty acid palmitic acid, 7 molecules of ATP and 14 molecules of NADPH are required, whereas scavenging it from the host is almost free.

T. gondii and other Apicomplexa have to different extents lost many pathways, making them now auxotrophic for a number of metabolites they have to scavenge entirely from the surrounding host cell (Seeber et al. 2008). These include purines (required for nucleic acid synthesis); arginine and all essential amino acids; polyamines, cholesterol, and choline (important for membrane integrity and signaling); and vitamins like biotin (required for some enzymes). Other compounds like pyrimidines, isoprenoids, fatty acids, and lipids can to some extent be synthesized by the parasite but are also scavenged from the host cell for optimal growth. This way *T. gondii* preserves quite some metabolic flexibility, which is certainly one prerequisite for its large host range and also a reason for its larger genome size compared to other Apicomplexa (Seeber et al. 2008).

A unique situation in nature is the apparent auxotrophy of *T. gondii* for the cofactor lipoic acid (LA) (required for activity by three enzymes in the mitochondrion and one in the apicoplast), despite its synthesis in the apicoplast (Crawford et al. 2006). It seems that LA from the plastid organelle is not able to reach the parasite's mitochondrion in sufficient amounts (or at all) to compensate for the lack of LA synthesis there. Interfering pharmacologically with LA scavenging from the host leads to parasite death, indicating the essential nature of host-derived LA for *T. gondii* growth (Crawford et al. 2006).

A metabolic paradox has been the exclusive apicoplast localization of the pyruvate dehydrogenase (PDH) complex and its absence in the mitochondrion in *T. gondii* and other Apicomplexa. This implied that, contrary to almost all other eukaryotes, Apicomplexa would be unable to convert glycolysis-derived pyruvate into acetyl-CoA in their mitochondrion. Without acetyl-CoA fueling the mitochondrial TCA cycle, the functional significance of the latter would be questionable. Alternatively, some other enzymatic entity could fulfill the task of PDH. Previously it was hypothesized that the mitochondrial *b*ranched-*c*hain *k*etoacid *deh*ydrogenase (BCKDH) complex (involved in the degradation of the three amino acids leucine, isoleucine, and valine) might have taken over the missing PDH function (Seeber et al. 2008). Both complexes share a number of structural and enzymatic properties: they are both made of three subunits with significant sequence homologies; they share the 4th subunit, lipoamide dehydrogenase; and they catalyze similar reactions in central carbon metabolism (decarboxylation of α-ketoacids) using similar substrates (Fig. 8.4). Recently, it has been experimentally proven that indeed the BCKDH complex fulfills PDH's function in the mitochondrion of *T. gondii* while still maintaining some of its BCKDH activity (Oppenheim et al. 2014). This is a further example of microbial reductive genome evolution whereby the function of two enzymatic entities has been combined into a single complex with extended capabilities.

The recent discovery of a photosynthetic free-living close relative to Apicomplexa (*Chromera velia*) (Moore et al. 2008) will allow to compare its whole predicted metabolism with that of *T. gondii* to define more precisely at the biochemical level the evolution from free-living to parasitic Apicomplexa. One potential outcome of these studies might be the definition of an axenic culture medium that would allow growing *T. gondii* in the absence of host cells, something that is currently not possible.

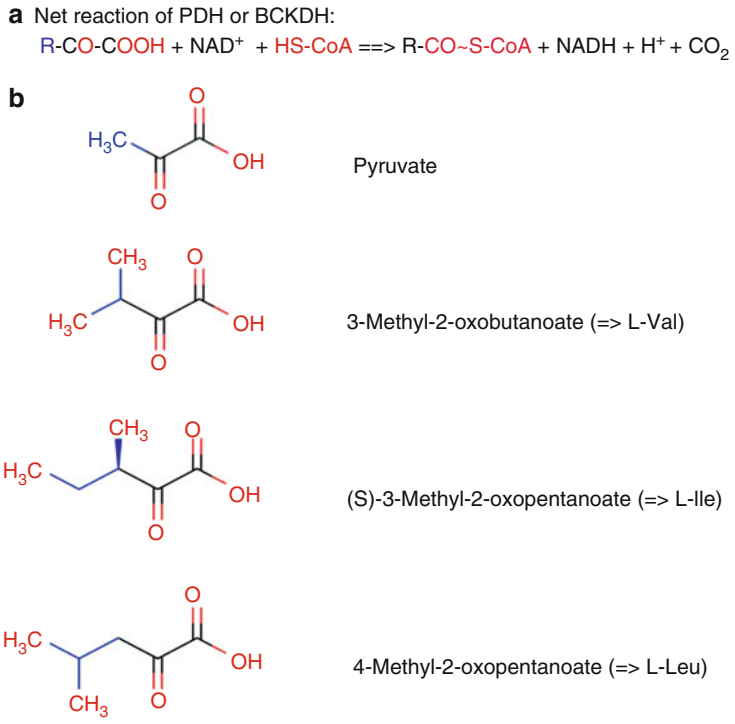

Fig. 8.4 General reaction of α-ketoacids dehydrogenases PDH and BCKDH (**a**) and their substrates (**b**). HS-CoA, coenzyme A; R-CO ~ S-CoA, substrate CoA-thioester. Pyruvate is the single substrate for PDH, whereas BCKDH from *T. gondii* in addition to pyruvate can also accept the other three metabolites (resulting from the initial deamination by the enzyme branched-chain amino acid transferase, BCAT) of the respective branched-chain L-amino acids mentioned in parentheses

8.2.5 Stage Conversion

The life cycle of *T. gondii* includes developmental switches between different parasite stages, i.e., sporozoites, tachyzoites, and bradyzoites. The differentiation of fast replicating tachyzoites to metabolically inactive bradyzoites is critical for the transition from an acute infection to chronic parasite persistence. It also enables natural parasite transmission from one to another new intermediate host or to the definite host via bradyzoite-containing tissue cysts. Finally, bradyzoites can reconvert to tachyzoites in immunosuppressed hosts, thereby leading to overt disease (referred to as reactivated toxoplasmosis) and possibly host death. The interconversion of bradyzoites to tachyzoites and vice versa is commonly referred to as stage conversion.

Tachyzoites and bradyzoites differ in morphology (see above), gene expression, metabolic turnover, and cell cycle progression. Stage conversion is associated with up- and downregulation of numerous stage-specific genes. For example, parasites of

the type I RH strain upregulate 177 and downregulate 84 genes at least twofold in response to bradyzoite-inducing conditions in vitro (Naguleswaran et al. 2010). The number of differentially expressed genes during stage conversion might even be higher in type II and type III strains which are generally more prone to tissue cyst formation. Proteins differentially expressed in tachyzoites and bradyzoites include various surface proteins, heat shock proteins, enzymes including some of the glucose metabolism, excretory-secretory proteins, cyst wall proteins, transcription factors, and others. Analyses of *T. gondii* mutants that are defective in bradyzoite formation have revealed a hierarchy of developmentally regulated genes, with a core set of genes whose upregulation appears crucial for bradyzoite formation (Singh et al. 2002). Recently, several transcription factors with AP2 DNA-binding domains have been identified in *T. gondii*, some of which are upregulated during bradyzoite development (Radke et al. 2013; Walker et al. 2013). Among them, TgAP2IX-9 and TgAP2XI-4 are able to regulate expression of bradyzoite-specific genes in a negative or positive manner, respectively. This indicates that both transcriptional repressors and activators are required for regulating the complex developmental program from tachyzoites via intermediate early bradyzoites toward mature bradyzoites. Promoter elements of *T. gondii* bradyzoite-specific genes which bind AP2 transcription factors have been identified (Behnke et al. 2008; Radke et al. 2013). Finally, the transcriptional reprogramming during the tachyzoite-to-bradyzoite transition is also governed by extensive chromatin remodeling (Bougdour et al. 2009; Hakimi and Deitsch 2007), and factors which regulate the chromatin state in *T. gondii* have been identified (Bougdour et al. 2010). In addition to changes in gene transcription, bradyzoite formation is associated with phosphorylation of eIF2α (eukaryotic initiation factor-2) which inhibits global de novo protein synthesis but favors translation of mRNAs encoding stress response and bradyzoite-specific proteins (Narasimhan et al. 2008; Sullivan et al. 2004). It is conceivable that the extensive morphological, transcriptional, and biochemical remodeling that is observed during the differentiation process of *T. gondii* imposes significant pressure on the parasite, which requires an adequate stress response in order to survive the transition process.

Importantly, bradyzoite formation is preceded by a slower growth rate and a prolonged parasite cell cycle. Particularly the appearance of a population of near-diploid parasites with a DNA content of 1.8–2 N, i.e., parasites in a G2 phase of the cell cycle, appears to be required for bradyzoite development (Radke et al. 2003). Thus, the transit through a G2-related cell cycle checkpoint may be crucial in regulating stage differentiation. Since bradyzoites in mature tissue cysts are uniformly haploid (1 N), it is assumed that parasites in G2 are intermediate stages which undergo mitosis and cytokinesis before entering a cell cycle-arrested G0 phase (Radke et al. 2003). The cell cycle arrest in mature bradyzoites explains why during chronic toxoplasmosis parasites remain almost quiescent within tissue cysts and can probably persist for years without harming the host.

Tachyzoites, which emerge from infections with bradyzoites or sporozoites, start to differentiate spontaneously to bradyzoites following ~20 cell divisions (Radke et al. 2003). This suggests that a developmental clock operates triggering stage

conversion. Bradyzoite formation can also be induced or accelerated in vitro by external stressors, e.g., alkaline pH, heat stress, chemotherapeutics, or deprivation of nutrients. In vivo, it commences with development of a strong inflammatory immune response, and immunosuppression predisposes for reactivation of chronic toxoplasmosis. Host immunity against the parasite is thus required at least to prevent reactivation of chronic stage bradyzoites; whether it is also instrumental to induce the initial differentiation of tachyzoites toward bradyzoites is unknown, however. In vivo, tissue cysts are predominantly located in brain and muscle tissues. Mature neurons and muscle cells are terminally differentiated and long-living cells and thus appear well suited as host cells for parasite persistence. Remarkably, *T. gondii* readily transforms from tachyzoites to bradyzoites in cell cycle-arrested polynucleated skeletal muscle cells but not in mononuclear and proliferating myoblasts (Swierzy and Luder 2015). Furthermore, knockdown of the negative cell cycle regulator Tspyl-2 in mouse skeletal muscle cells and overexpression of the human orthologue CDA1 in human fibroblasts abrogates or induces *T. gondii* bradyzoite formation, respectively (Radke et al. 2006; Swierzy and Luder 2015). This suggests that *T. gondii* might sense a distinct environmental factor which is predominantly present in mature muscle cells and neurons and which is then transduced by the parasite into the cellular pathway that initiates formation of a G2 subpopulation and bradyzoite-specific gene expression. This could explain the preferred tissue localization of *T. gondii* during chronic toxoplasmosis.

8.2.6 Surface Structure

The *T. gondii* surface is decorated with members of a large protein family, i.e., the SRS (SAG1-related sequence) family. Since the description of the gene encoding SAG1 (*s*urface *a*nti*g*en 1; now called SRS29B) in 1988, this family has grown continuously. It now comprises 122 different protein-coding genes (and additional 60 pseudogenes, i.e., sequences containing SRS domains but not located within a predicted gene) in two major subfamilies (Wasmuth et al. 2012). Comparison of the three archetypal lineages ME49, GT1, and VEG (see above) revealed a core set of 69 common SRS genes present in all three *T. gondii* strains. This indicates a substantial heterogeneity in the presence or absence of distinct SRS genes between different parasite lineages. In addition, strain-specific polymorphisms in the core gene set further increase interstrain diversity (Wasmuth et al. 2012). It is assumed that such diversity of *T. gondii* surface proteins facilitates colonization of different niches (e.g., host species, tissues) and/or contributes to strain-specific differences in virulence (see also below).

Importantly, sets of SRS genes are expressed in a mostly parasite stage-specific manner. For instance, the immunodominant surface antigens of *T. gondii*, SRS29B (SAG1) and SRS34A (SAG2A), are specific to tachyzoites and are downregulated in the bradyzoite stage. Stage-specific expression of immunodominant SRS genes contributes to the strong humoral and cellular immune response as observed toward the tachyzoite but not the bradyzoite stage. It has been hypothesized that stable

expression of immunodominant SRS genes by tachyzoites is required to limit propagation of this potentially deadly acute infection stage, whereas the persistent and transmissible bradyzoite stage remains largely unrecognized and might even be favored by anti-tachyzoite immune responses (Lekutis et al. 2001). SRS35 (SAG4) and SRS16C (BSR4) are specific to bradyzoites, and SRS28 (SporoSAG) is the most abundant surface protein of sporozoites.

SRS proteins enter the excretory pathway by N-terminal signal peptides and are linked to the cell surface membrane by *g*lycosyl*p*hosphatidyl*i*nositol (GPI) anchors. Six different GPIs have been isolated from *T. gondii*, which are recognized by human macrophages via pattern-recognition receptors to produce pro-inflammatory TNF-α (Debierre-Grockiego et al. 2003). SRS proteins typically contain one or two SRS domains with four to six cysteine residues per domain. They have ~25 % up to 99 % protein sequence identity. The crystal structure of immunodominant SRS29B revealed a homodimeric conformation in which the D2 domains of both monomers are GPI-linked to the plasma membrane and the D1 domains form a deep positively charged groove (He et al. 2002). Since the closely related SRS57 (SAG3) binds to negatively charged heparan sulfate proteoglycans (HSPGs) in vitro (Jacquet et al. 2001), it is possible that also SRS29B can bind such ligands, and recent studies confirmed this assumption (Azzouz et al. 2013). Importantly, crystals of SRS16C (i.e., BSR4) and SRS28 (SporoSAG) revealed substantial structural differences as compared to SRS29B, including a monomeric conformation as well as putative ligand binding grooves of distinct structure and chemistry (Crawford et al. 2010).

Consistent with the structural heterogeneity, SRS proteins also appear to fulfill different functions during infection. SRS57 (SAG3) serves as an adhesion molecule by binding to host cells in an HSPG-dependent manner. Importantly, SRS57-deficient mutants are also attenuated in infectivity and virulence in vivo (Dzierszinski et al. 2000). Using blocking antibodies, adhesive properties have also been attributed to SRS29B (SAG1); however, this was not confirmed after infections with SRS29B-deficient mutants (Lekutis et al. 2001). SRS29B rather mediates acute virulence at least in mice due to its strong immunogenicity and induction of local and systemic immunopathology. In contrast to SRS29B, SRS29C dampens acute *Toxoplasma* virulence. It is upregulated in genetically attenuated *T. gondii* strains, and overexpression of SRS29C reduces mortality substantially in infected mice (Wasmuth et al. 2012). Thus, distinct SRS surface proteins of *T. gondii* can both positively and negatively regulate virulence at least in the primary host. Finally, SRS16B (formerly SRS9) is one of the most abundant SRS proteins specific to the bradyzoite stage. Mice infected with SRS9-deficient parasites have substantially lower cyst burdens in the brain during chronic infection, indicating that this protein is required for establishing and/or maintaining chronic infection (Kim et al. 2007). Whether this is due to reduced adhesion of bradyzoites to host cells suitable for parasite persistence or to other functions important for bradyzoite biology is unknown.

Besides SRS proteins, a second family of surface proteins has been identified (Pollard et al. 2008). Their members are also GPI anchored, but they are distinct from SAGs and have therefore been named SAG-unrelated surface antigens (SUSA).

Thirty-one putative members have been identified in the *T. gondii* genome, and two of them have been confirmed to be surface located. SUSA proteins are highly polymorphic, and at least SUSA1 is specifically expressed in the bradyzoite stage (Pollard et al. 2008).

8.2.7 Secreted Molecules

Proteins secreted by *T. gondii* are key molecules for three essential processes of the parasite's biology, i.e., attachment to and invasion of host cells as well as establishment of intracellular infection. They are released in an ordered fashion during and after host cell entry from one of the three excretory-secretory organelles of *T. gondii*, namely, the micronemes, rhoptries, and dense granules (see Sect. 8.2.1).

Host cell invasion by *T. gondii* is a parasite-driven active process that is completed within ~20 s and which differs fundamentally from phagocytosis or induced uptake of a pathogen by a host cell. It commences with the discharge of microneme proteins which is triggered by a rise in cytosolic calcium levels in the parasite. More than 20 microneme proteins (MICs) have been characterized to date. They include both transmembrane and soluble proteins (Santos and Soldati-Favre 2011). Most MICs form multimeric complexes that are secreted from the micronemes onto the parasite surface where the transmembrane MIC anchors the complex in the parasite plasma membrane (Table 8.2). TgMIC2 appears to be the major adhesin of *T. gondii* since MIC2-deficient parasites show severe defects in attachment to host cells in vitro (and consequently in host cell invasion) as well as in virulence in mice (Huynh and Carruthers 2006). The broad array of the adhesive MICs may explain the parasite's ability to invade a broad range of different host cells from diverse warm-blooded vertebrates.

Apical membrane antigen 1 (TgAMA1) is also very important although its exact role in attachment/invasion is a matter of debate (Bargieri et al. 2014). It localizes to an electron dense ringlike structure at the interface of the parasite and the host cell surface, called the moving junction (MJ), where it binds to the rhoptry neck protein 2 (TgRON2) on the host cell surface. TgRON2 functions as a receptor by being inserted into the host cell together with other RONs. The N-terminal extracellular domains of most MICs contain conserved adhesion motifs present in higher eukaryotes including thrombospondin type I repeats, integrin-like A, and epidermal growth factor-like domains (Huynh and Carruthers 2007). Consequently, they bind to protein or carbohydrate motifs of host cell-expressed or parasite-translocated receptors and attach the parasite to the host cell. The C-terminal cytoplasmic part of transmembrane MICs can connect the MIC complex to the glideosome by binding to aldolase, thus establishing a molecular bridge between the host cell surface and the parasite motility machinery which is essential for host cell invasion. During invasion, the MIC-host cell receptor-holocomplexes are translocated toward the posterior end of the parasite by the force generated by the actin-myosin motor of the glideosome, thereby propelling the parasite into the host cell. However, this model has recently been challenged since tachyzoites with a deficient actin-myosin motor

Table 8.2 Excretory-secretory molecules discharged from micronemes, rhoptries, and dense granules

Micronemes	Complex partner(s)	Function
TgMIC2	MIC2-associated protein (TgM2AP)	Adhesion, gliding motility, invasion
TgMIC6	TgMIC1, TgMIC4	Targeting of adhesion complex
TgMIC8	TgMIC3	Rhoptry secretion, invasion
TgAMA1	Rhoptry neck proteins (TgRON2, 4, 5, 8)	Adhesion, invasion, MJ integrity (?)

Rhoptries	Complex partner(s)/host cell target(s)	Function
TgRON2, 4, 5, 8	TgAMA1	Formation of MJ
TgROP18	TgROP5, host IRGs, host ATF6β	Ablation of PVM disruption by IRGs
TgROP16	STAT3/STAT6 pathways	Modulation of host immunity, e.g., IL-12 downregulation
TgROP5	TgROP18, host IRGs	Binding IRGs, activation of ROP18
TgROP38	Unknown	Suppression of host gene expression
PP2C-hn	Unknown (host nuclear targets)	Phosphatase, facilitates parasite growth in vitro

Dense granules	Complex partner(s)/Host cell target(s)	Function
TgGRA2, 6	TgGRA4, 6 or TgGRA4, 2, respectively	Formation of TVN
TgGRA7	PVM, PVM tubules	Nutrient acquisition via endolysosome-mediated uptake from host to PV
TgNTPase-I, TgNTPase-II	–	Apyrase activity, egress from host cell
TgPI-1, TgPI-2	–	Serine protease inhibitor
TgGRA15	NF-kB pathway	Regulation of host gene expression, e.g., induction of IL-12
TgGRA16	HAUSP, PP2a-B55	P53 regulation
TgGRA24	MAPK p38α	Induction of IL-12 and MCP-1

could be generated that are still able to invade, although with low efficiency (Bargieri et al. 2014).

Distinct MIC proteins, including TgMIC8, also provide signals to induce protein discharge from the rhoptries. More than 40 proteins have been proven or are supposed to be discharged this way (Lebrun et al. 2014). Two major types of proteins are distinguished based on their localization within the rhoptries: rhoptry neck proteins (RONs) and rhoptry bulb proteins (ROPs). Rhoptries also store lipids, the roles of which are poorly defined, though. RONs and ROPs not only differ in their localization within the rhoptries but also in their function (Table 8.2). Four of the known RONs constitute the MJ at the site of parasite entry into the host cell (Besteiro et al.

2011). Whereas TgRON2 is predicted to localize to the host cell membrane and functions as a receptor for TgAMA1 (see above), RON4, 5 and 8 are soluble proteins which form a complex with RON2 and may have structural or signaling functions. ROPs are much more diverse and include kinases, pseudokinases (i.e., catalytically inactive kinases), phosphatases, proteases, and others. They are discharged early during invasion, but unlike RONs, they are discharged into the PV, locate to the PVM, or are even injected by the parasite into the host cell cytoplasm.

In the past years, kinases and pseudokinases of the ROP2 family have been intensively studied. Several members, including ROP18, ROP16, ROP5, and ROP38, modify extensively many host cell functions and partially serve as critical virulence factors of *T. gondii* (Table 8.2) (Saeij et al. 2007; Taylor et al. 2006). For example, ROP18 together with ROP5 can abrogate the activity of *i*mmunity-*r*elated *G*TPases (IRGs) and hence avoid disruption of the PVM and killing of *T. gondii*. Furthermore, the kinase ROP16 can prolong activation of STAT3/6, thereby altering host gene expression, including downregulation of the pro-inflammatory cytokine IL-12 (Hunter and Sibley 2012). These examples highlight that ROP2 family members comprise several parasite effectors that dampen immune responses to the parasite and thus facilitate parasite survival. Interestingly, genes encoding these effector molecules are highly polymorphic between different *T. gondii* lineages. This may have enabled the parasite to expand its host range during evolution. Besides kinases and pseudokinases, *T. gondii* also injects the protein phosphatase 2C into the host cell and which rapidly translocates into the host cell nucleus (hence designated TgPP2C-hn) via a conventional mammalian nuclear localization signal. The host molecules that are targeted by TgPP2c-hn are unknown, but it is interesting to note that *T. gondii* may modify regulatory relays like protein phosphorylation in the host nucleus for its own benefit using a parasite-derived protein phosphatase.

The dense granules are the third type of excretory-secretory organelles of Apicomplexa. They contain more than 20 different proteins (the actual number being probably much higher) which are discharged into the nascent and growing PV. The majority of *T. gondii* GRA proteins localize to the PVM, to the prominent membranous tubulo-vesicular network (TVN) present in the PV, or to the lumen of the PV (Table 8.2). Since functional characterization of GRA proteins is hampered due to the lack of characteristic domains or homologies to proteins of known function, only some of them have been functionally characterized. Both TgGRA2 and 6 form complexes together with TgGRA4, and TgGRA2 and TgGRA6 single or double knockout mutants revealed that they contribute to the organization of the TVN network (Lebrun et al. 2014). TgGRA7 is crucial for an interesting novel pathway of nutrient acquisition by which *T. gondii* sequesters material from the host endosomal-lysosomal compartment to the vacuolar space (Coppens et al. 2006). GRA7 deficiency indeed slows parasite growth under conditions of nutrient deprivation. Soluble proteins that are released from the dense granules into the PV lumen include two NTPase isoforms which are highly abundant (at least 2 % of total parasite protein) and potently cleave ATP to ADP and partially also ADP to AMP. Their functional role in the infected cell is unknown, but it has been proposed that they might provide the energy from ATP hydrolysis that is required for parasite egress

from the host cell (Lebrun et al. 2014). This view has been recently challenged by findings that NTPase I and II single or double knockouts did not show growth defects in vitro or a reduced virulence in mice (Olias and Sibley 2016). During the past few years, GRA proteins have been discovered which reach the host cell and which contribute to modulation of host responses (Table 8.2). For example, TgGRA15, 16, and 24 are transported into the host cell and can modulate NF-kB, p53, and mitogen-activated protein kinase (MAPK) p38α pathways, respectively (Bougdour et al. 2014; Hunter and Sibley 2012). Consequently, they alter host gene expression including activation of pro-inflammatory molecules like IL-12. Like several ROPs, they are highly polymorphic, which partially explains the different host immune responses seen after infection with different *T. gondii* strains.

8.3 Perspectives and Open Questions

Our toolbox to investigate the biology of *T. gondii* has tremendously expanded during the last 10–15 years. Different animal models are well established, and because tachyzoites are haploid and can be easily propagated in vitro, powerful forward and reverse genetics could be developed that are unsurpassed in all other Apicomplexa. Large-scale data sets on genomes, transcriptomes, proteomes, phosphoproteomes, and epigenomes are now available; at least genomes and transcriptomes are even available for different archetypal strains and for different life cycle stages, i.e., tachyzoites, bradyzoites, and sporozoites. Whole genome sequences of ~60 further strains of the different haplotypes and geographic locations have been characterized recently (Lorenzi et al. 2016). The wealth of "omics" data and methods has enabled the "*Toxoplasma* community" to increase our knowledge on various aspects of the parasite biology and the parasite-host interplay exponentially. Among many others, highlights that have been uncovered during recent years are certainly the identification of polymorphic ROPs and GRAs that regulate parasite virulence by manipulating host cell signaling cascades. Likewise, the discovery that the population structure of *T. gondii* is much more diverse than previously anticipated and may affect the outcome of disease is a major breakthrough. Finally, we now have a detailed picture of the unique molecular machineries that enable the parasite to glide and to invade host cells. Many more recent achievements could be added to this list.

There are, however, also still a lot of open questions that need to be resolved. For example, what are (if any) the environmental signals that induce the parasite to differentiate from the tachyzoite to the bradyzoite stage and how are these signals transduced to initiate the differentiation program? And how are the other developmental switches in the life cycle of *T. gondii* regulated, e.g., the sporozoite-to-tachyzoite transition or the bradyzoite-to-tachyzoite transition within the gut after oral infection? The molecular biology of the parasite stages, which develop within the cat intestine, i.e., merozoites, gametes, and oocysts, is also a largely unexplored field, mainly due to experimental limitations when working with intestinal stages in mice and cats. New stem-cell-based cultivation methods might provide an option in this respect (Klotz et al. 2012). A question of major medical

relevance is how the different parasite haplotypes and atypical *T. gondii* strains affect the course of infection in humans. It is very likely that the list of important polymorphic virulence factors is not yet complete and that new fascinating parasite-host interactions govern the course of infection. Of major interest is also to elucidate the molecular basis of the parasite's apparent ability to manipulate host behavior in rodents and whether this applies to humans as well. It can be expected that efforts undertaken by the scientific community will solve at least some of these important questions in the near future, probably even at higher speed than in the past.

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Plasmodium

9

Volker Heussler, Tobias Spielmann, Friedrich Frischknecht, and Tim Gilberger

Abstract

Malaria is the most important parasitic disease worldwide in terms of numbers of affected people and mortality. It is caused by parasites of the genus *Plasmodium*, which have a complex life cycle including insect vectors that are in the case of human malaria exclusively *Anopheles* mosquitoes. To date the genomes of several *Plasmodium* species have been sequenced. The overall genome organization is rather conserved, but highly divers species-specific gene families have been identified as well. The different life cycle stages exhibit a very variable morphology reflecting their respective needs. The change in cell shape during development is genetically inherited, but epigenetic factors also appear to play an important role. In the vertebrate host cell invasion and egress are crucial steps for the survival of the parasite and have evolved to highly orchestrated events, and some molecular details have been deciphered to date. Invasion occurs by invagination of the host cell membrane, and the parasite finally resides in a

V. Heussler (✉)
Institute of Cell Biology, University of Bern, Hochschulstrasse 4, CH-3012 Bern, Switzerland
e-mail: volker.heussler@izb.unibe.ch

Bernhard Nocht Institute for Tropical Medicine,
Bernhard-Nocht-Strasse 74, D-20359 Hamburg, Germany

T. Spielmann
Bernhard Nocht Institute for Tropical Medicine,
Bernhard-Nocht-Strasse 74, D-20359 Hamburg, Germany

F. Frischknecht
Parasitology-Department of Infectious Diseases, University of Heidelberg Medical School,
69120 Heidelberg, Germany

T. Gilberger, PhD
Pathology and Molecular Medicine, McMaster University,
1200 Main Street West, MDCL 2306, Hamilton, ON L8N 3Z5, USA

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parasitophorous vacuole. From there it controls the behavior of its host cell by secretion of proteins into the host cell cytoplasm and to its surface. Exposed parasite proteins at the surface of an infected red blood cell allow cytoadherence and are responsible for the pathogenicity of a *Plasmodium* infection. Egress is a two-step process initiated by the rupture of the parasitophorous vacuole membrane and followed by disintegration of the host cell membrane that involves the activation of proteases, kinases, and membrane lytic enzymes. Recent discoveries revealed completely new parasite strategies to switch from asexual to sexual development during the blood stage and to avoid elimination by cytosolic immune responses of host cells during infection of hepatocytes.

9.1 Introductory Remarks

Malaria is a prevalent life-threatening disease with more than 250 million infections per year worldwide and up to 3 billion people living in areas with malaria transmission. Malaria is caused by parasites of the *Plasmodium* genus, of which five species, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, are pathogenic in humans. The two species with the greatest infection prevalence and severity are *P. falciparum*, mainly found in sub-Saharan Africa, and *P. vivax*, which is endemic in large parts of Asia and South America. *P. falciparum* and *P. vivax* infections cause a severe febrile disease, which can result in death of the infected individual if not treated adequately and in time. If nonlethal, the infection causes severe symptoms similar to a heavy influenza infection, hence resulting in major suffering and economic losses in the endemic regions due to reduced work output by the affected individuals. In Africa, *P. falciparum* infections in children result in a high mortality rate, and it is now acknowledged that *P. vivax* infections can also be fatal (Mueller et al. 2009). With up to 300 million infections every year and up to 2.5 billion people at risk, *P. vivax* is a significant threat for developing countries in the tropical and sub-tropical regions. Further, *P. vivax* parasites form dormant stages in the liver, termed hypnozoites, which can hibernate for many months up to several years before being reactivated, triggering the pathogenic blood stage. In this way the parasite can survive in climates that do not allow constant transmission by *Anopheles* mosquitoes. Dormant forms of the parasite in the liver are not cleared by standard malaria chemotherapies, and malaria eradication cannot be envisioned without solving the problem of relapses caused by reactivated hypnozoites (Amino et al. 2011).

The majority of malaria research concentrates on the blood stage of *P. falciparum* because (1) it causes the pathology of the disease and (2) it is relatively easy to cultivate in vitro. However, model systems such as rodent-infecting *Plasmodium* species like *P. berghei* and *P. yoelii* make it possible to also more easily investigate preerythrocytic life cycle stages. Also, genetic manipulation of some rodent *Plasmodium* species is much more efficient than in *P. falciparum*. Despite the fact that rodent-infecting *Plasmodium* species are becoming a focus of malaria research,

including studies in the blood stages, one has to be aware of their limitations especially when studying aspects of immunity (Craig et al. 2012). In this chapter we provide an overview of what is known about the biology of the malaria parasite at the molecular and cellular level. However, it will also show the myriad of gaps in our knowledge of this medically relevant parasite and indicate that many opportunities for more discoveries lie in the future.

9.1.1 The *Plasmodium* Life Cycle

The life cycle of *Plasmodium* parasites is complex and alternates between the final host; the insect vector, where sexual reproduction takes place; and the secondary vertebrate host (Fig. 9.1). The latter can be humans, monkeys, rodents, and bats, but it is actually in birds and reptiles that most *Plasmodium* species reside. These little studied species show fascinating differences even in basic life cycle progression with some replicating in the skin and others replicating in the blood. Therefore, these *Plasmodium* species provide a rich source for understanding evolution and basic facts about the medically important human parasites. Nevertheless, in this chapter we will only discuss species infecting mammals, as not many molecular details are known of the other species.

During a blood meal, infected female *Anopheles* mosquitoes inject sporozoites into the skin of the host (Sidjanski and Vanderberg 1997). From the site of deposition, motile sporozoites disperse through the skin by moving within the extracellular matrix but also by passing through cells, a process called transmigration (Amino et al. 2008). They finally enter blood vessels, from where they are transported by blood flow to liver sinusoids. However, not all inoculated sporozoites find their way to the blood vessels (Amino et al. 2006). Around 50 % of them remain in the skin, where they are often taken up and eliminated by phagocytes. About 15 % of the inoculated parasites invade lymphatic vessels, most of them becoming trapped in lymph nodes and being phagocytized by dendritic cells (Amino et al. 2006). Sporozoites that have successfully invaded blood vessels (about 35 %) rapidly reach liver sinusoids where they bind to highly sulfated heparan sulfate proteoglycans (HSPGs) presented by hepatocytes through small channels in the endothelial cells, called fenestrae (Menard et al. 2013) (Fig. 9.2). Binding to the HSPGs triggers a signaling cascade in the parasite, involving calcium-dependent protein kinase 6 (CDPK-6) and other kinases. Following this, the circumsporozoite protein (CSP) is cleaved to expose a C-terminal cell-adhesive thrombospondin repeat (TSR) that allows the attachment to the endothelium (Coppi et al. 2011). After attachment, the sporozoites must cross the sinusoidal endothelium to reach the underlying hepatocytes. They primarily traverse Kupffer cells, which are liver-resident macrophages, but can also directly cross the endothelial barrier (Tavares et al. 2013). Sporozoites appear to invaginate the cell plasma membrane at the point of entry and cause cell fusion events at the site of exit (Pradel and Frevert 2001).

After traversing the space of Disse, which separates the endothelial cells from the hepatocytes, the parasites continue to migrate through several hepatocytes. It is

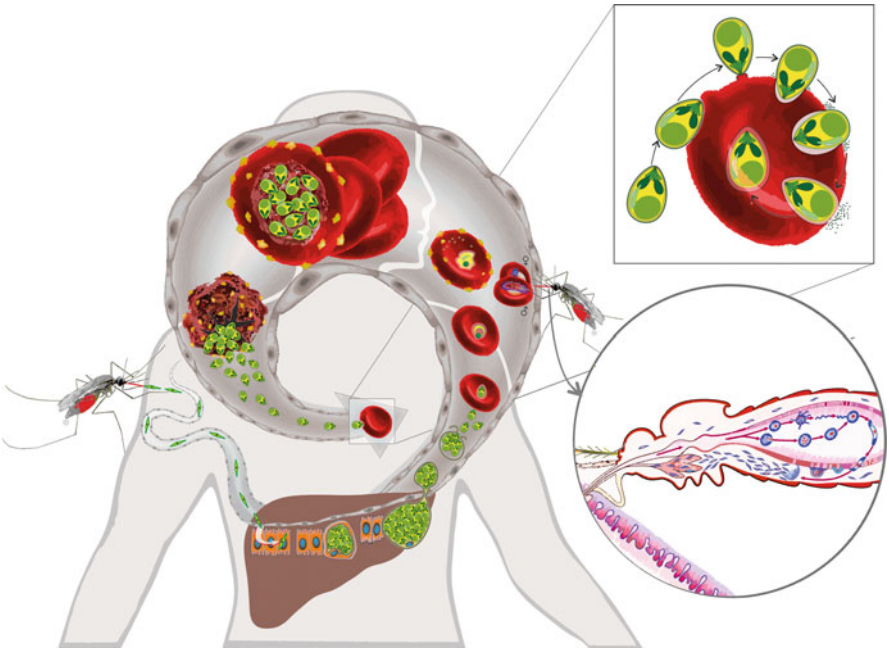
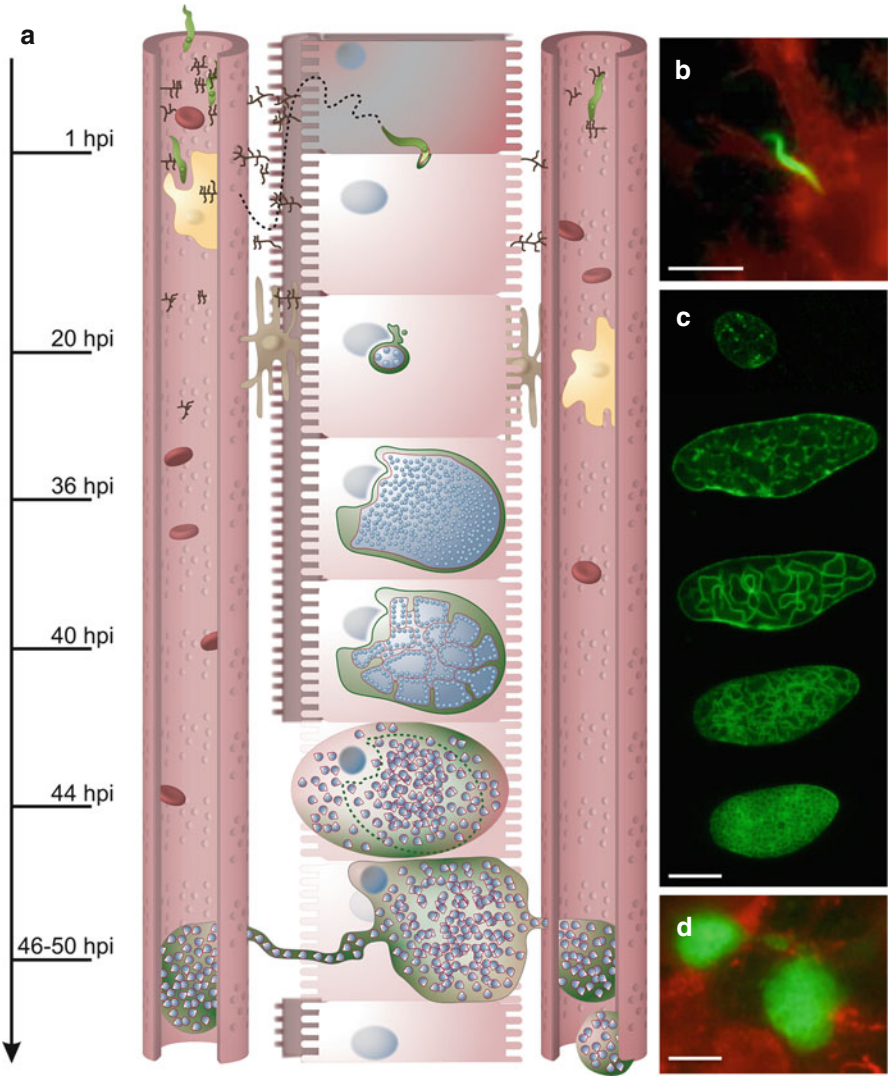


Fig. 9.1 *Plasmodium* life cycle: Once salivary gland sporozoites are inoculated into the host skin, a portion of them (about a third) actively invade blood vessels and passively reach the liver with the bloodstream. Details about liver stage invasion and development are provided in Fig. 9.2. Briefly, during liver stage development, the parasites multiply in a process called schizogony, which result in the formation of merozoites. Merozoites leave the liver inside a merosome, a membranous structure that protects the merozoites until their release. Free merozoites invade erythrocytes where they develop and multiply until the formation of new merozoites that invade new RBCs upon rupture of the infected RBC. Some of the merozoites can differentiate into gametocytes. These sexual forms are taken up while a mosquito feeds. Inside the midgut of the vector, male and female gametes fuse and form a zygote from which the motile ookinete emerges. Upon traversing the midgut, the ookinete develops to an oocyst between the basal membrane of the midgut and the basal lamina. Sporozoites develop inside the oocyst and are released into the hemocoel of the mosquito to eventually reach and penetrate the salivary glands of the mosquito

Fig. 9.2 *Plasmodium* liver stage development: (**a**) In the liver sinusoids, the blood flow is slow, and the sporozoites can attach to the endothelium by interacting with HSPGs presented by hepatocytes, through small channels (fenestrae) in endothelial cells. Upon crossing the endothelium, sporozoites transmigrate through several hepatocytes before settling in one and residing inside a PV. There it develops to a multinucleated schizont and by membrane invagination to the cytomere stage that finally forms thousands of merozoites. These are liberated from the PV into the host cell cytoplasm by PVM disruption. This event induces host cell death and the formation of vesicles, which are continuously filled with merozoites and reach into the blood vessel. Finally these vesicles (merosomes) bud off and are carried away by the bloodstream to reach the lung, where they



release merozoites to infect RBCs. (**b**) Sporozoite entering hepatocyte. Live imaging of fluorescent *P. berghei* sporozoite (*green*) enters red fluorescent hepatocyte. Note the flexibility of the sporozoite. (**c**) Series of the same transgenic parasite expressing a GFP-tagged parasite plasma membrane marker protein. (**d**) Intravital imaging of merosome formation. A mouse was infected with GFP-expressing *P. berghei* sporozoites, and the liver was examined by intravital microscopy at 45 hpi. Blood vessels are labeled with red fluorescent BSA. Note that the forming merosome (left vesicle) still receives merozoites (Images in **c** courtesy of Marco Schaffner). *HPSG* heperan sulfate proteoglycans; *PV* parasitophorous vacuole; *PVM* parasitophorous vacuole membrane; *RBCs* red blood cells; *GFP* green fluorescent protein; *BSA* bovine serum albumin

thought that this process breaches the plasma membrane of the hepatocyte and thus results in cell wounding (Mota et al. 2001). The exact molecular events and also the reasons for the transmigration are still a matter of debate. Like transmigration, the final invasion event is dependent on the active motility of the parasite and several interactions between the parasite and the host cell. The host cell plasma membrane invaginates, forming the parasitophorous vacuole membrane (PVM) (Bano et al. 2007). The known molecular details of invasion are discussed in detail in a separate section below.

After invasion, the parasite often localizes close to the host cell nucleus. How the parasite, which resides in a vacuole, senses this environment is not yet known. Close to the host cell nucleus, the parasitophorous vacuole (PV) is associated with the endoplasmic reticulum (ER) and the mitochondria of the host cell (Bano et al. 2007; Deschermeier et al. 2012). During the first hours of infection, the parasite transforms from its elongated form to a round trophozoite (Jayabalasingham et al. 2014). Approximately 20 h after invasion, the parasite nucleus starts to divide repeatedly and rapidly to form a multinucleated schizont. The replication rate of *Plasmodium* in the liver is one of the fastest known for animal cells, and it is not yet completely understood how the parasite obtains the nutrients and lipids required for its growth. In the infected erythrocyte, the parasite takes up hemoglobin and digests this in its food vacuole. However, to date, no similar organelle has been observed in the liver stage of the parasite. It is known that *Plasmodium* transforms its PV into a highly permeable compartment by creating channels within the PVM (Bano et al. 2007). These open channels permit the trafficking of a wide range of small metabolites from the host cytosol to the PV and might be used for nutrient uptake.

The fast replication of parasite nuclei is followed by subsequent invagination of the parasite membrane (Fig. 9.2). Invaginations first form spheres in which nuclei are peripherally located (cytomere stage), and the process progresses until each forming daughter parasite possesses its own membrane (Sturm et al. 2009). At the end of the clinically silent liver stage, the PVM disintegrates, and the generated merozoites are released in the host cell cytoplasm. Throughout liver stage development, the parasite blocks host cell apoptosis to allow for its development (van de Sand et al. 2005). However, during this final stage of development, the parasite initiates an atypical host cell death cascade that shares some features of apoptosis such as cytochrome c release and nuclear condensation. However, most apoptotic effector mechanisms are missing. There is no activation of caspases and no DNA fragmentation, and the phosphatidylserine asymmetry of the outer membrane remains intact, which protects the infected cell from elimination by phagocytes. During the atypical host cell death, merozoite-filled vesicles, termed as merosomes, bud off the infected hepatocyte and are released into the blood by an unknown mechanism (Sturm et al. 2006). Via the bloodstream, these merosomes travel to the lung capillaries where they rupture and release the infectious merozoites to continue the life cycle (Baer et al. 2007).

The duration of the liver stage from the hepatocyte invasion to the release of merozoites differs depending on the *Plasmodium* species, where it ranges from 2 days for rodent *Plasmodium* species to 15 days for *P. malariae*. In *P. vivax* and possibly *P. ovale* (Richter et al. 2010), hypnozoites might even rest for years. Interestingly, nothing is known about the molecular players that cause these differences in development.

When merozoites are released into the blood, they immediately start to attach to and subsequently infect erythrocytes, reticulocytes, or both depending on the *Plasmodium* species. The initial attachment most likely occurs by random collision. Subsequent adhesion is then mediated by specific proteins, such as members of the merozoite surface protein (MSP) family (Cowman and Crabb 2006). The parasite reorients itself until its apical end is facing toward the erythrocyte membrane. As in the case for hepatocyte invasion, the parasites invade red blood cells (RBCs) by forming a PVM (Spielmann et al. 2012). Since invasion is such an elementary process in the *Plasmodium* life cycle, a more detailed description of this well-documented process is provided in a separate section below.

Inside the RBC and surrounded by the PVM, the parasite first develops into a ring, then into a mononuclear trophozoite (from the Greek word "tropho" for "nourishment"), and subsequently into a multinucleated schizont (so called because of the curious way of nuclear division termed schizogony, from the Greek word for "dividing"). During this development, the parasite obtains nutrients, e.g., by taking up hemoglobin, which is then digested in the acidic food vacuole of the parasite. The breakdown of hemoglobin releases heme, which catalyzes the production of reactive oxygen species and hence is toxic to the parasite. The *Plasmodium* parasite therefore detoxifies heme by converting it into insoluble, crystalline hemozoin (Francis et al. 1997). The hemozoin is microscopically visible as dark brown deposit in stained and unstained parasites and is known as malaria pigment. The antimalarial drug chloroquine prevents this crystallization process, which leads to parasite death.

The final nuclear division initiates, as in the liver stage, invagination of the parasite plasma membrane, and up to 36 merozoites are formed, which are liberated via PVM rupture and cell lysis to infect new RBCs (Salmon et al. 2001). The time passing between invasion and egress of daughter merozoites depends on the *Plasmodium* species and takes between 24 and 72 h. To avoid clearance by the spleen, different *Plasmodium* parasites employ alternative strategies, but all appear to remodel their host RBCs to a certain extent. Erythrocyte remodeling by *P. falciparum* allows protein transport from the parasite to the host cell surface that leads to cytoadherence of the infected cell in the peripheral blood vessels of the infected individual. This way the parasite avoids passage through and elimination by the spleen, which is the central control organ for blood infections. The molecular details of cytoadherence of *Plasmodium*-infected RBCs are discussed separately in a section below.

After invasion of RBCs, some parasites undergo asexual reproduction in RBCs, while others differentiate into male and female forms called microgametocytes and macrogametocytes, respectively. These are cells under cell cycle arrest that await transmission back into the mosquito host. The commitment to differentiate into gametocytes is not only regulated at a genetic level but also by epigenetic factors as well as at the level of transcriptional control and will be discussed in more detail in a later section. When taken up by an *Anopheles* mosquito during blood feeding, only gametocytes survive in the midgut and give rise to the beginning of the mosquito stage of the life cycle. In the midgut macrogametocytes differentiate into the macrogametes, and each microgametocyte matures to eight flagellated microgametes, which are released from the RBC in a spectacular process called exflagellation (Sinden et al. 2010). These microgametes rapidly swim through the blood bolus

in the midgut to find a macrogamete. These reproductive forms then fuse to form a zygote, which develops into a motile and invasive ookinete (a motile egg). The ookinete penetrates the midgut epithelium (Vinetz 2005). Between the basal membrane of the midgut and the basal lamina, the ookinete develops into an oocyst, in which sporozoites are formed via asexual reproduction. Again the morphological events of parasite formation are similar to those in the liver stage including invagination of the parasite membrane. When the oocyst bursts, the sporozoites are released into the hemolymph of the mosquito from which they subsequently invade the salivary glands. When the mosquito takes the next blood meal, sporozoites are inoculated into the skin of the vertebrate, starting a new infection. The timing of the development from gametocytes to infectious sporozoites depends on the *Plasmodium* strain and the ambient temperature and takes between 8 and 18 days.

9.2 Well-Established Facts

9.2.1 Genome Organization

The genome organization is rather uniform in the various *Plasmodium* species infecting mammals. They have 23–27 megabase genomes across 14 chromosomes and about 5,500 genes organized in a highly syntenic manner (i.e., the vast majority of genes are kept at the same relative position on the chromosomes) (Kooij et al. 2005). In addition *Plasmodium* parasites have nonnuclear genomes in two organelles: a 35 kb circular genome in the apicoplast, a chloroplast-like organelle, and a 6 kb genome in the mitochondrion. About 10 % of the nuclear genome encodes for proteins that are targeted to the apicoplast (Ralph et al. 2004). More information about organelle formation and function is provided in the next section.

Importantly, almost 80 % of the genes of human and rodent *Plasmodium* species are orthologous (Carlton et al. 2008). Interestingly, the majority of the *Plasmodium* genes encode for proteins with unknown function. Another important feature found in the different genomes is that coding sequences of proteins that are exported to the surface of host RBCs have evolved much faster than those that remain in the cytoplasm. The accelerated evolution of exported proteins eventually resulted in the generation of gene families in *Plasmodium* parasites. Obviously the host immune system had a strong impact on this process. Along these lines, it is not surprising that genes coding for essential metabolic functions are highly conserved, whereas multi-copy gene families that are most likely involved in immune evasion show a low level of conservation or are even unique in the different *Plasmodium* species (Duffy and Tham 2007). Parasite invasion, including the recognition and binding of receptors on the surface of potential host cells, is certainly a strong factor and will be discussed later in a separate section. It is not surprising that *Plasmodium* species infecting different hosts bind to different receptors on RBCs, but *Plasmodium* species infecting the same host like the human parasites *P. falciparum* and *P. vivax* also vary quite considerably in their repertoire of invasion-relevant genes. The merozoite surface proteins (MSPs) in particular have evolved rather differently in human *Plasmodium* species. However, motor proteins thought to be involved in migration and

invasion are highly conserved, indicating that upon binding the various possible receptors, the merozoite invasion process is rather similar among the different *Plasmodium* species.

The spleen puts a very strong selective pressure on all blood-borne diseases including malaria as its function is to filter the blood and eliminate pathogens as well as infected RBCs. However, there are very different ways how *Plasmodium* parasites avoid elimination by the spleen, and this is reflected in the genome of the different species. *P. falciparum* avoids spleen passage by targeting adhesive PfEMP1 molecules, gene products of the variable "*var*" gene family, to the surface of the infected cell (Smith 2014). In contrast *P. vivax* avoids elimination by maintaining the flexibility of the infected RBCs as inflexible RBCs would be eliminated during passage through the spleen.

Because the expression of parasite molecules at the RBC surface provokes strong host immune responses, the *P. falciparum* genome contains a whole battery of more than 60 *var* genes with only one being expressed at any given time. The parasite has an interesting strategy to maintain a high recombination frequency of the genes involved in immune evasion. These genes are positioned in the periphery of the nucleus where recombination favors the generation of new variants. The generation of new variants could also be the reason for the high A+T content in the *Plasmodium* genome (80% in *P. falciparum*). Even in the relatively balanced *P. vivax* genome (60% A+T content), gene families are predominantly located in A+T rich regions, suggesting a higher potential for mutations and in consequence a high variability of the corresponding genes. To position the polymorphic genes at the nuclear periphery, it appears to be favorable that they are located mainly in the subtelomeric region of the chromosomes. This subtelomeric region does not maintain as high a level of synteny as all other chromosome regions do. Many other gene families involved in immune evasion are found in the subtelomeric species-specific region (Duffy and Tham 2007).

9.2.2 The Structure of Malaria Parasites

9.2.2.1 Organelles of Malaria Parasites: Same, More, and Different

Like most eukaryotic cells, malaria parasites contain a nucleus, an endomembraneous transport network (e.g., ER, Golgi apparatus), and a mitochondrion. In addition, they also contain an apicoplast: a remnant chloroplast derived from an ancient secondary endosymbiotic event when a progenitor parasite engulfed a red alga (van Dooren and Striepen 2013) (Fig. 9.3). This organelle is the target of a number of new drugs (including antibiotics such as doxycycline) that can be used to treat or prevent a malaria infection. The extracellular and invasive stages also contain secretory vesicles (named micronemes, rhoptries, and dense granules), which contain numerous proteins that are important for parasite migration and cell invasion (Tomavo et al. 2013).

The parasite's extracellular stages are not all alike (Fig. 9.4): merozoites, which infect RBCs, are teardrop-shaped tiny cells measuring only 1–2 μm in length. Ookinetes, which form from a zygote within the mosquito gut, are comparatively large, being 15 μm in length and about 3 microns in width. Sporozoites, the parasite forms that are injected by the mosquito into the vertebrate host, are slender cells

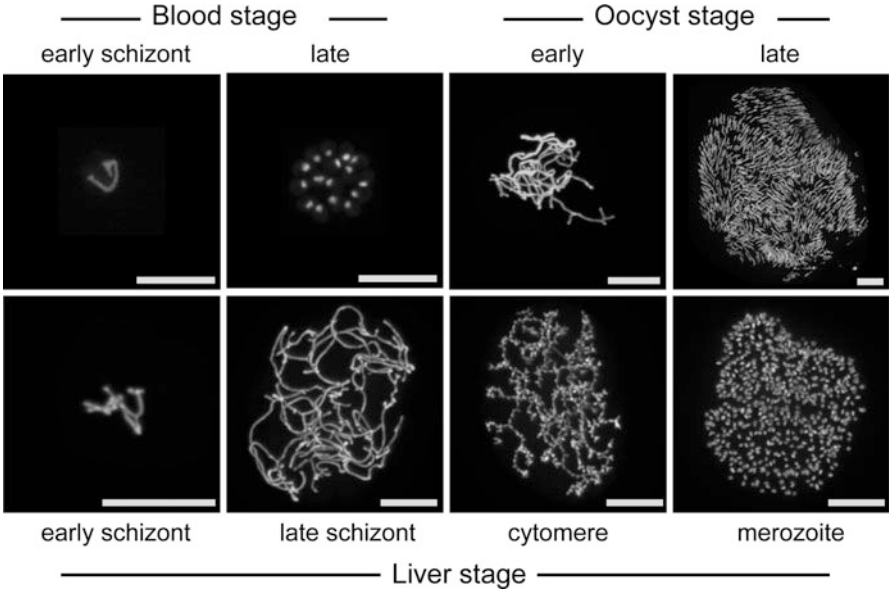


Fig. 9.3 Apicoplast development during asexual multiplication in the vertebrate and in the mosquito vector. Parasites expressing a GFP-tagged N-terminal part of the apicoplast marker protein APC were analyzed by live imaging in all main life cycle stages. In all cases, the apicoplast branches and is finally distributed to each daughter parasite. Organelle branching is most extensive during the oocyst development in the mosquito and during liver stage schizogony. During the cytomere stage in liver stage development, the apicoplast is directed toward the forming merozoites. Each merozoite finally contains a single apicoplast

measuring around 10 microns in length but just 1 micron in width. The shape differences of the zoites reflect a necessary adaptation to their different tasks: ookinetes and sporozoites need to cross epithelial and endothelial cell layers, respectively. They are thus highly motile and can migrate for tens and hundreds of microns, respectively. In contrast, merozoites float in the serum and then attach to and invade RBCs. Ookinetes leave the hostile environment in the midgut lumen by traversing cells but do not invade them. Thus, they lack the rhoptries, organelles that contain proteins necessary for host cell invasion and formation of the PVM. Interestingly, the ookinete contains a large and enigmatic organelle-like crystalloid body (Lemgruber and Lupetti 2012) that is absent from merozoites and sporozoites (Fig. 9.4).

Malaria parasites undergo many differentiation processes during their life cycle each of which is accompanied by a change in morphology. During intracellular replication, parasites multiply by schizogony. This produces many different parasitic shapes within the RBCs from ringlike parasites to grape-bunch structures (Fig. 9.1). During schizogony, the nuclear envelope is not disrupted and thus mitosis occurs, similarly to yeast, in a closed form. During closed mitosis the spindle pole body is integrated into the nuclear membrane, and mitotic microtubules emerge from it to target the chromosomes. While nuclear division occurs in the parasite

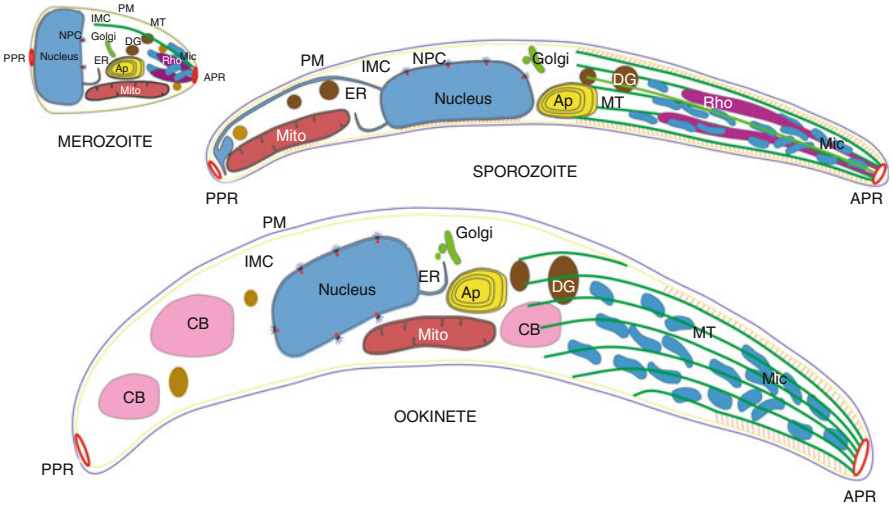


Fig. 9.4 Different structures of the invasive forms of *Plasmodium*. The different forms are roughly drawn to scale with the major organelles and features highlighted such as the nucleus (*blue*) with its associated nuclear pore complexes (*NPC*). Note that the NPCs are arranged on just one side of the nucleus in the merozoite and sporozoite. Likely this polarity is not present in the ookinete. *PM* denotes the plasma membrane, which is subtended by the inner membrane complex (IMC, *yellow*). The apical and proximal polar rings (*APR*, *PPR*) are shown in *red*. Mitochondrion (*Mito*) and apicoplast (*Ap*) are close to each other in the crammed cytoplasm of the merozoite, while they can be apart in the sporozoite, where the mitochondrion is located proximal to the nucleus. In the sporozoite different organelles and microtubules (*MT*, *green*) are linked to the IMC with short linkers (*red bars*). It is not clear if that is also the case in merozoites and ookinetes. While there are just few (3–4) microtubules in the merozoite, there are four times more in the sporozoite. In sporozoites there exists a curious polarity with one microtubule being on the dorsal side and all of the others on the ventral site as indicated with the light green microtubule. While in merozoites and sporozoites microtubules are straight, they are curved in ookinetes probably directing the helical movement of this stage. Secretory organelles/vesicles are indicated as Mic for micronemes (*cyan*); Rho for rhoptries (*magenta*), which are absent in ookinetes; and DG for dense granules (*brown*). Other vesicles exist too such as mononemes. In ookinetes the enigmatic crystalloid body (*CB*) is shown in pink. Golgi and ER are present in all stages with the Golgi being composed of just a single stack

schizont, the other major organelles are also multiplied. Both the mitochondrion and apicoplast grow into elaborate networks, which are then cleaved to yield dozens to hundreds to thousands of new organelles, depending on whether the parasite grows in the RBC, the oocyst, or the hepatocyte (Fig. 9.3) (Stanway et al. 2009, 2011). These new organelles must then be sorted at the plasma membrane of the schizont in a way that each daughter parasite receives one of each. During liver stage development, the apicoplast divides first followed later by the mitochondrion. In this manner, each merozoite receives the organelles it needs for subsequent survival, motility, and invasion (Stanway et al. 2011). This sorting involves proteins related to those responsible for proper positioning of flagella in motile algae (Francia et al. 2012). Organelle sorting is very efficient, yielding only few progeny parasites with the wrong number of organelles. Many molecular details regarding how

parasites are formed remain unsolved including what role the microtubules play, e.g., whether they also transport vesicles or organelles or just serve to stabilize the so-called pellicle. This structure envelops the parasite and consists of the plasma membrane and a set of underlying organelles. The pellicle is generated during the formation of progeny parasites. Subtending the plasma membrane at a distance of 25–30 nm lies the inner membrane complex (IMC), a flattened vesicular structure (Harding and Meissner 2014). In distantly related organisms such as *Paramecium*, this organelle serves among other functions as a calcium store important for cell motility. It is not clear, however, if the IMC has the same function in *Plasmodia*. Underneath the IMC a network of filamentous proteins make up the subpellicular complex, which is involved in generating the shape of the parasite. At the front (apex) and rear (posterior) of the parasite, a set of ringlike structures called the polar rings appear to hold the pellicle together. At the front several rings occur, one within another. From the outermost ring, the subpellicular microtubules emerge that seem to be essential during parasite formation (Figs. 9.4 and 9.5). The microtubules appear to be anchored to the IMC or the subpellicular network by long linker proteins that are arranged with a 32 nm periodicity along the microtubules, other organelles, and the IMC. Two proteins, subpellicular microtubule 1 (SPM1) and SPM2, most likely associate to the outside of microtubules and might also contribute to microtubule stability since one of them has this function in the related parasite *Toxoplasma gondii* (Tran et al. 2012). SPM1 was also shown to recruit other proteins, named TrxL1 and TrxL2 (for thioredoxin-like protein) to the microtubules (Liu et al. 2013). Thus, there appears to be a number of proteins contributing to the overall cellular shape in a manner that is currently not well understood.

9.2.2.2 The Network Underneath the IMC: How Parasites Get Their Shape

The subpellicular network is probably built up from dozens of proteins (Kono et al. 2012), some of which are named the alveolins (after the alveolates, a group of unicellular eukaryotes containing the apicomplexans as well as dinoflagellates) (Gould et al. 2008). In *Plasmodium*, several proteins that make up the subpellicular network have been investigated by either gene deletion or tagging the protein with a genetically encoded fluorescent marker. Along with expression profiling, this has shown stage-specific roles for some of them. For example, the protein IMC1a is required for the sporozoite to obtain its crescent shape (Khater et al. 2004). An antibody raised against IMC1a was shown to localize in the cytosol of sporozoites that have matured within the oocysts while it localized at the periphery in sporozoites isolated from the salivary gland. This suggests that it contributes to the building up of the subpellicular network during sporozoite maturation. Indeed the subpellicular network could only be detected in salivary gland-derived sporozoites but not in those from the oocysts (Kudryashev et al. 2012). And most importantly, deletion of IMC1a led to malformed sporozoites, which were unable to invade the salivary glands. Thus, it appears that IMC1a is an important component of the subpellicular network and that it plays a role in generating the shape of sporozoites. IMC1a-depleted sporozoites were also less able to withstand osmotic pressure further

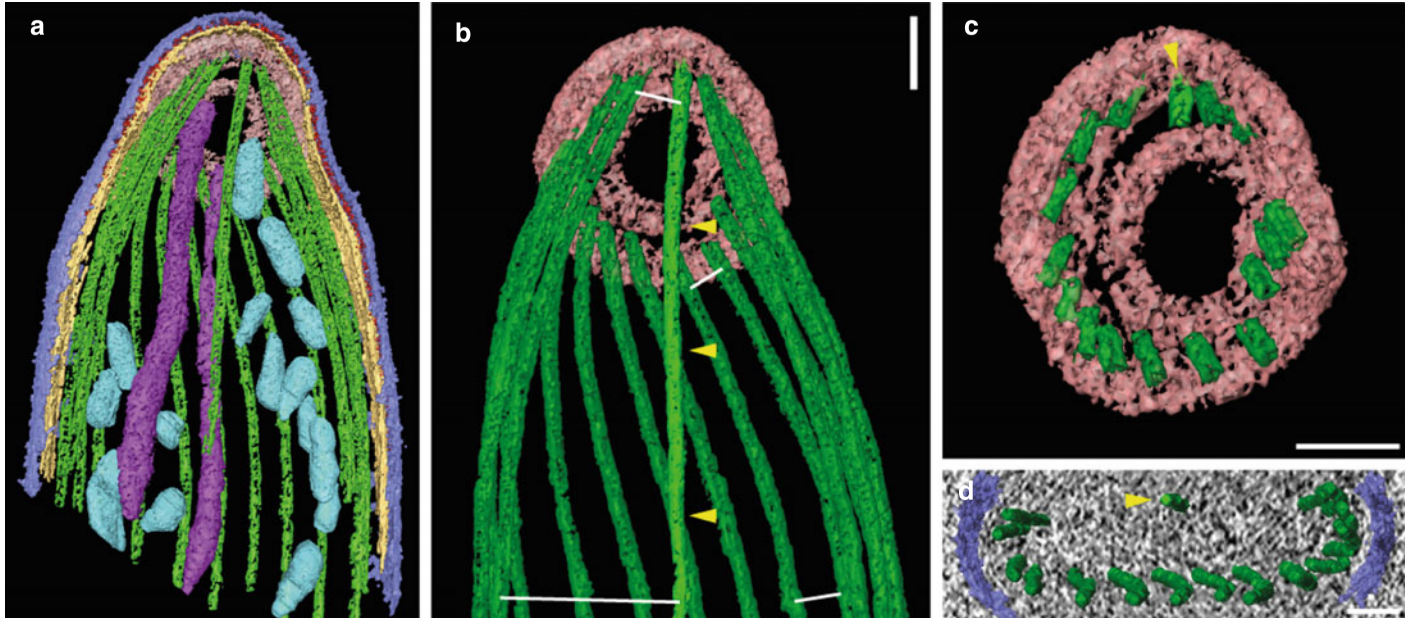


Fig. 9.5 Microtubular polarity in sporozoites. (**a**) Three-dimensional model derived from tomographic recordings of rapidly frozen *P. berghei* sporozoites. The shown structures are plasma membrane (*blue*), inner membrane complex (*yellow*), microtubules (*green*), apical polar rings (*pink*), micronemes (*cyan*), and rhoptries (*magenta*). (**b**) The same image as in **a** but now only microtubules and polar rings are shown. The single microtubule is shown in light green and indicated by *yellow arrowheads. White bars* show that the distance between the microtubules at the polar ring is the same, while further away they are larger for the distance between the single microtubule and the others. (**c**) Only polar rings and the first nanometers of the microtubules are shown. The yellow arrowhead points to the "single microtubule." (**d**) Distal ends of microtubules showing the large distance between the "single" microtubule and the others. Scale bars are 100 nanometers (Modified from Kudryashev et al. 2012)

suggesting that the subpellicular network is important for giving physical rigidity to the parasite. This physical rigidity is likely important during invasion of salivary glands and migration of the parasites in the skin, invasion of blood vessels, and extravasation from the blood into the liver parenchyma. Other proteins also clearly play a role. For example, the related IMC1h has a role in both ookinete- and sporozoite-shaped generation (Tremp and Dessens 2011; Volkmann et al. 2012). In contrast IMC1b has a major role in ookinetes (Tremp et al. 2008). While the IMC proteins appear to be located at the subpellicular network all around the parasite, other proteins, the so-called IMC sub-compartment proteins, localize to the subpellicular network mostly at the front end (Poulin et al. 2013). How these proteins interact with each other to generate the subpellicular network, to link the IMC to the microtubules, and to generate different parasite shapes in different stages remains largely unexplored.

Taken together, as is typical for other eukaryotic cells, the different elements of the cytoskeleton appear to be linked and form a dense network contributing to the shape of the parasite. In contrast to other cells, extracellular *Plasmodium* parasites have additional structural elements that give them the required rigidity to migrate and invade.

9.2.2.3 Sex and Cell Shape

While the parasite undergoes asexual replication in the blood, the oocysts, and the hepatocytes, it also has a sexual phase during its life cycle that spans the transmission from host to vector and causes a number of mind-boggling changes in cellular shapes (Fig. 9.1). The first differentiation happens within the RBCs, when parasites deviate from asexual replication to form gametocytes, the precursors of male and female gametes. Gametocytes of *P. falciparum* (but not most other *Plasmodia*) are of a crescent (falciform) shape that became the parasites namesake. This change is possibly driven by the polymerization of microtubules and the formation of an IMC during gametocyte development (Dixon et al. 2012). Microtubules are formed at one side of the parasite and elongate its shape. As the gametocyte grows, it stretches the membrane of the RBC. It is believed that the resistance of the RBC membrane then bends the parasite. This could have implications on the efficiency with which the parasite is sequestered in the spleen and bone marrow, as only the very late stages of gametocytes can again be detected in the peripheral blood. The few known molecular processes that regulate adhesion of gametocyte-infected RBCs are discussed below in the section about parasite-induced host cell modification.

Upon a blood feeding, gametocytes quickly differentiate in response to the drop of temperature, rise in pH, and the presence of xanthurenic acid within the mosquito gut (Billker et al. 1998). The male gametocyte undergoes the phenotypically most incredible change by generating several flagellated gametes, probably the smallest flagellated cells that exist. These are the only forms of the malaria parasite that actively swim using a flagellum (Wilson et al. 2013). Male gametes swim through the blood meal within the mosquito midgut at high speed and bind to female gametes using specific surface proteins termed Pfs48/45 (van Dijk et al. 2001). Interestingly, these proteins belong to the same family of proteins, 6-Cys (for 6

cysteines), as some of the proteins important in liver stage PVM formation. Fusion of a male and female gamete leads to the formation of a diploid zygote, from which the elongated ookinete emerges. The requirement of Pfs48/45 and other specific proteins on the surface of gametes and the fact that antibodies against these proteins can block zygote formation make them possible candidates for transmission-blocking vaccines (Saxena et al. 2007).

9.2.2.4 The Motor Underneath the Plasma Membrane: How Parasites Move

Cell movement is generally accomplished by an interaction of myosin and actin filaments, and this appears also to be the case for motile *Plasmodium* parasites (Fig. 9.6). Myosins localize to the plasma membrane-facing side of the IMC. These myosin motors, anchored in the IMC through associated proteins, are collectively referred to as "the glideosome" (Soldati and Meissner 2004). Glideosome-associated proteins (GAPs) are associated with myosin and other associated proteins like

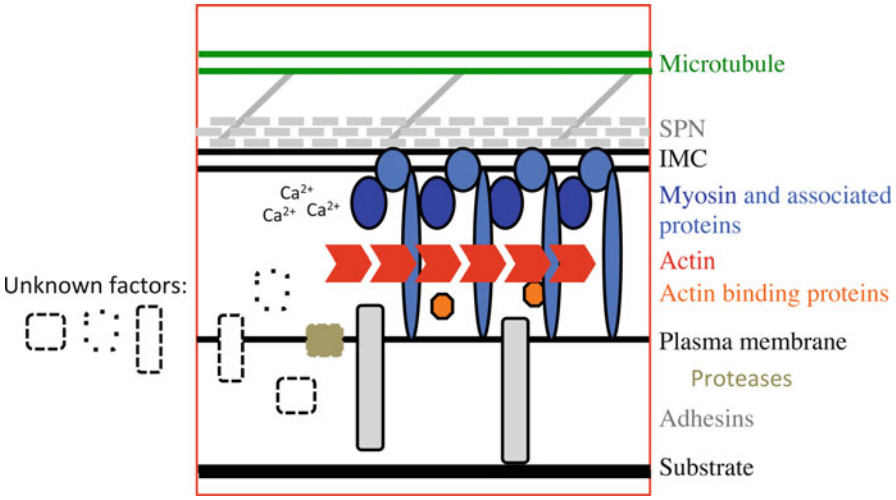


Fig. 9.6 A simplified model for actomyosin-based parasite motility. The parasite binds to the substrate with adhesins, proteins that span the plasma membrane. These proteins, e.g., from the TRAP family of adhesins can be cleaved by membrane-resident proteases of the rhomboid family. Intracellularly these adhesins link somehow to the actin filaments (*red*), which are likely short and turn over very fast. Actin filaments are regulated by actin-nucleating proteins of the formin family (not shown) and other small actin-binding proteins (*orange*). Myosin (*dark blue*) is anchored to the inner membrane complex (*IMC*) by a number of myosin-associated proteins, which also span that gap between the plasma membrane and the IMC. Some of these proteins might also span the IMC to link myosin stably to the subpellicular network (*SPN*) of intermediate filament like (and other) proteins that likely determine the rigidity of the parasite. Microtubules (*green*) are in turn linked to either the SPN or the IMC with long linker proteins. Calcium ions (Ca^{2+}) are essential for motility and mediate so far unknown signals. The *dotted shapes* indicate unknown factors that are necessary to complete the picture. Likely these include proteins on the outside, within the plasma membrane, and within the supra-alveolar space, as the space between plasma membrane and IMC is known

"myosin light chain 1" (MLC1) and myosin A tail-interacting protein (MTIP). They are presumably also linked to transmembrane domain proteins (GAPMs) of the IMC that might function to anchor the complex stably within the cell, possibly linking the motor to the subpellicular network. The myosin motor is thought to translocate short actin filaments from the front to the back of the parasite. The actin filaments themselves are thought to be linked to transmembrane proteins in the plasma membrane, which in turn anchor the parasite to the substrate (Baum et al. 2008). Thus, the myosins provide the force for forward locomotion of the parasite building the molecular basis of parasite gliding motility.

To make a connection to the external environment, sporozoites and ookinetes connect the actomyosin motor to transmembrane proteins. An important transmembrane protein for directional gliding is the transmembrane thrombospondin-related anonymous protein (TRAP) (Sultan et al. 1997). Careful analysis of sporozoites lacking TRAP showed that they were not able to move forward but instead moved back and forth over a single adhesion site and were thus completely noninfectious (Munter et al. 2009). In *T. gondii*, where much of the research has been done leading to the linear TRAP-actomyosin motor model, it was shown that parasites lacking proteins deemed essential for motility could be deleted from the genome without completely abrogating motility (Andenmatten et al. 2013). This suggests that alternative mechanisms and/or unknown redundancies play a role in motility. On its way from the insect hemocoel to the salivary glands and then from the skin to the liver and finally to the host hepatocytes, the sporozoite expresses a number of other TRAP-related proteins. These appear to have different functions that are crucial for cell adhesion and motility (Hegge et al. 2010). Once the gliding sporozoites have successfully entered hepatocytes, unknown processes cause the IMC to be dismantled, and the parasites round up to further differentiate and multiply.

9.2.3 Invasion of RBCs and Hepatocytes

9.2.3.1 RBC Invasion

Another significant step in the complex malaria life cycle is the invasion of mammalian erythrocytes – a step that is mandatory for massive parasite multiplication in the body (Fig. 9.1). This mass proliferation requires a substantial modification of RBCs and culminates in their destruction. It is responsible for all clinical symptoms associated with malaria. In a heavily infected person, over 20 % of the RBCs can be infected corresponding to a mass of nearly half a kilogram of parasites!

The parasites' choice of using erythrocytes as host cells for their exponential growth phase takes full advantage of this highly specialized cell type: their abundance (the human body has about 25 trillion RBCs), composition (more than 95% of its content is a single macromolecule, hemoglobin), architecture (highly flexible cell type with no endomembrane system), and the fact that they are immune privileged (no major histocompatibility complex capable of inducing immune activation). The flip side to this seemingly perfect home is that the malaria parasite cannot hijack or use any endocytosis-based mechanism for erythrocyte invasion from its host. To overcome this drawback, the parasites developed a highly

efficient molecular invasion machinery that encompasses several hundreds of proteins. These proteins are involved in multiple cellular processes such as adhesion, signaling, and motility that allow for invasion and subversion of its host – the erythrocyte.

Fast and Complex Process of Host Cell Entry

The invasion of erythrocytes by the parasite, at this stage termed merozoite, is a rapid and complex process that relies on an orchestrated cascade of interactions between invading parasite and host cell. After initial attachment of the parasite to the surface of the target cell, the intruder establishes a physical interaction (termed "tight junction") between its apical end and the host cell membrane. This tight junction progressively moves toward the posterior of the invading parasite as it enters the RBC (Fig. 9.1, enlarged box). The process is largely independent of the host cell and is driven by a parasite-specific, intracellular motor involving transmembrane proteins and myosin motor modules as already described in the section above (Cowman et al. 2012). The invasion process is terminated by the sealing of the erythrocyte membrane at the initial entry point. While the erythrocyte invasion itself takes less than 30 s, minimizing the exposure of the parasite to the human immune system, the whole process including erythrocyte membrane sealing takes about 3 min (Gilson and Crabb 2009).

The Perpetrator: The Merozoite Stage of the Parasite

Merozoites that are released into the bloodstream are custom-tailored for one thing: the invasion of new erythrocytes. Their surface is covered by antigenically diverse surface proteins such as the merozoite surface protein 1 (MSP1). These proteins may play a role in the reorientation of the parasite on the surface of the erythrocyte to bring the apical area into position for invasion and could be instrumental in providing a "smoke screen" against the human immune response. Several laboratories are trying to formulate a vaccine against malaria based on MSP1 (Fowkes et al. 2010). Additional cellular features guarantee efficient host cell invasion. First, the IMC also serves as a robust cellular scaffold for the invasion process (see above). Second, secretory organelles at the apical pole of the merozoite store and discharge several dozens of proteins that mediate host cell entry (Fig. 9.4).

The Challenging Gateway: The RBC Surface

Erythrocytes are much smaller than most other human cells and highly specialized. They are biconcave, disk-shaped cells with a diameter of 6–8 μm and a thickness of 2 μm. During erythropoiesis (the maturation process of erythrocytes), RBCs remove their nucleus, other organelles, and the machinery for protein biosynthesis and transport. Mature erythrocytes have a life span of about 120 days, and their aging is accompanied by dramatic changes in surface composition and rigidity.

This surface, which defines blood groups, is not only of particular interest for transfusion medicine but also provides a molecular docking station for the parasite. Glycophorins are by far the most abundant surface molecules of the erythrocyte. These glycophorins are conjugated with sialic acid, forming membrane-spanning

sialoglycoproteins. The sialic acid chains are responsible for the negatively charged surface of the erythrocytes which is critical for their circulation, preventing cell-cell aggregation. Coincidently, sialoglycophorins are also well-established receptors for the malaria parasites. Additional erythrocyte surface molecules that are known to be recognized and utilized as a gateway by the parasite are the transmembrane protein basigin and – in the case of *P. vivax* – the Duffy antigen. While the former one belongs to immunoglobulin superfamily, the latter represents a chemokine receptor (Wright and Rayner 2014). Interestingly, while *P. falciparum* is promiscuous and adaptive, invading most human erythrocytes independent of their maturation stage, age, or particular antigen composition, *P. vivax* only targets immature erythrocytes (reticulocytes), preferentially from Duffy-positive blood groups (Mercereau-Puijalon and Menard 2010).

The evolutionary pressure of malaria parasites on the human population has been so large during history that a number of polymorphisms have been selected for, the most famous of these being the sickle cell trait, which protects heterozygous carriers from the severe symptoms of a *P. falciparum* infection (Taylor et al. 2013). This is likely mediated by the increased rate of heme-oxidation in sickle cells, which inhibit transport of adhesins to the red cell surface and hence decrease the stickiness of the infected RBC (Cyrklaff et al. 2012). More impressively even is the polymorphism that does not allow *P. vivax* to enter a RBC, due to a mutation in the Duffy antigen. This polymorphism has spread widely through Africa and has protected these populations from *P. vivax* infection.

Lock-and-Key Principle: Receptor-Ligand Interactions

The establishment of solid cell-to-cell contact between invading parasite and erythrocyte is a prerequisite for successful invasion. During the invasion process, the parasite displays adhesive proteins on its surface that are able to bind with high affinity to surface molecules of the erythrocyte mediating host cell invasion. Two parasite protein families, the erythrocyte-binding antigens (EBA) and the reticulocyte-binding-like (RBL) proteins, play a major role in this process (Gunalan et al. 2013). As their name implies, members from these families bind with their extracellular, adhesive domains to specific structures of erythrocyte surface molecules. For instance, in *P. falciparum* EBA175 binds to glycophorin A, EBA140 binds to glycophorin C, and reticulocyte-binding-like protein homologue 5 (PfRh5) binds to basigin (Tham et al. 2012). Experimental evidence suggests that the roles of many of these receptor-ligand interactions are redundant and have most likely evolved as an adaptation to erythrocyte heterogeneity (Miller et al. 1994). Concurrently, the extracellular domain of the EBA or RBL proteins reveals a high degree of polymorphisms which presumably evolved under high immune system pressure of the host in order to circumvent interference with the receptor-ligand interactions.

While the establishment and use of redundant receptor-ligand interactions increase the erythrocyte invasion efficiency of *P. falciparum*, two parasite proteins and the respective binding partner seem to have a preeminent role in invasion: the interaction of the RBL protein PfRh5 with the erythrocyte receptor basigin (Crosnier et al. 2011) and apical membrane antigen 1 (AMA1) with the rhoptry neck protein

2 (RON2) (Richard et al. 2010). The first one is a very unusual RBL protein that does not possess a transmembrane domain and works in conjunction with other parasite proteins. The second one, AMA1, does not bind to a surface structure of the erythrocyte, but to a parasite transmembrane protein complex that is inserted into the membrane of the targeted cell during the invasion process. Both proteins are in the focus of experimental vaccine design given that antibody-mediated interference with their receptor binding can block invasion (Douglas et al. 2014; Dutta et al. 2013).

The Underlying Machinery That Powers Invasion

Receptor-ligand interactions and the linkage to the glideosome (for details see also section above) are embedded in a large functional protein network of several hundreds of proteins that mediates and controls host cell invasion (Hu et al. 2010). This network is composed of a diverse array of proteins including enzymes (proteases, transferases) and cytoskeletal proteins, as well as a substantial number of proteins without a known exact function. All these proteins are of interest in drug and vaccine development as the interference with this crucial biological process may disrupt the parasite life cycle. This notion is supported by two key observations. First, antibodies directed against known invasion-related proteins inhibit parasite invasion. Second, inhibitors targeting enzymes such as proteases or kinases involved in invasion block this process. A systematic functional assessment of this protein network will therefore help to identify rate-limiting steps, thus prioritizing candidate proteins for further therapeutic exploration.

9.2.3.2 The Same but Different: Sporozoite Invasion of Hepatocytes

In contrast to merozoites, which are in close proximity to their next host cell when they are liberated from an infected RBC, sporozoites require active gliding to reach their final destination, the hepatocytes. Sporozoites cross the skin with a speed of over 1 μm per second. This fast speed is curiously only needed for migration in the skin (Hellmann et al. 2011; Montagna et al. 2012). Once sporozoites have reached the liver sinusoids, they move at a slower speed to cross the endothelium, transmigrating through several hepatocytes and finally invading a hepatocyte (Coppi et al. 2011; Mota et al. 2002). The switch from motility to invasion appears to be a progressive event mediated by proteolytic processing of the circumsporozoite protein (CSP), the major sporozoite protein anchored on the parasite surface with a glycophosphatidylinositol (GPI) anchor. This results in the exposure of adhesive domains. It might be that invasion only happens if a sufficient majority of CSP is processed.

The next step for a sporozoite to reach its final host hepatocyte is the same as for a merozoite attaching to a RBC: the need to invade and establish in its respective host cells within a PVM. The major breakthrough in the analysis of sporozoite invasion was the establishment of an in vitro cell culture infection system for rodent *Plasmodium* species (Hollingdale et al. 1981). It turned out that *P. berghei* can infect in vitro, apart from primary hepatocytes, a wide range of cells including hepatoma cell lines of mouse and human origin, but also cell lines of non-hepatocyte background like HeLa cells and fibroblasts. It is well known that the *Plasmodium*-related parasite *T. gondii* has a broad spectrum of hosts, and within these hosts can infect a

wide range of differentiated cells. To achieve infection of so many different cell types, *T. gondii* injects rhoptry neck (RON) proteins such as RON2 into the host cell which then serve as receptors on the host cell plasma membrane for parasite surface proteins like AMA1 (Besteiro et al. 2011). Both types of proteins are well conserved among apicomplexan parasites, and it has been suggested that they have a similar function also in *Plasmodium* zoites (Ejigiri and Sinnis 2009). Although this concept is indeed very attractive and also occurs in some bacteria (Lai et al. 2013), it is still unknown as to how the sporozoites establish the initial contact with their final host cells. It has been shown that *P. yoelii* and *P. falciparum* sporozoite invasion depend to a certain extent on the expression of CD81 and SRBI (scavenger receptor, class B, type I) receptors on host cells (Rodrigues et al. 2008; Silvie et al. 2003; Yalaoui et al. 2008). However, it appears that other receptors might be involved as well since blocking the known receptors reduces but does not abolish sporozoites invasion. For example, the rodent-infecting *P. berghei* seems to not rely on CD81 and only partly on SRBI receptors.

For a long time, it was assumed that the sole driving force behind invasion is the gliding motility of the sporozoite, but it is now accepted that host cell actin also plays a role in sporozoite invasion (Gonzalez et al. 2009). Furthermore, sustained actin polymerization around the developing parasite appears to contribute to parasite elimination (Gomes-Santos et al. 2012). Interestingly, the same is true for the autophagy marker protein LC3, another host cell protein that is incorporated into the PVM briefly after sporozoite invasion (Grutzke et al. 2014). Obviously, the host cell recognizes the invading parasite and tries to label it incorporating LC3 into the PVM. LC3 labeling of membranes is followed by lysosome fusion, where recently it has been suggested that in *P. berghei*-infected hepatocytes, lysosomes indeed fuse with the PVM and contribute to nutrient and membrane supply for the growing parasite (Lopes da Silva et al. 2012).

It also remains to be solved how different parasite proteins are secreted in this highly ordered process. RON proteins are among the first proteins to be discharged, but other vesicles like micronemes or exonemes might be involved as well. It would also be important to determine how transmigrating sporozoites differ from invading sporozoites in terms of protein secretion. The content of the discharged vesicles most likely includes proteases; in *P. berghei* it has been shown that protease inhibitors added externally can block invasion (Coppi et al. 2005; Kumar et al. 1994). On the other hand, protease activity needs to be tightly controlled, and indeed sporozoites express a protease inhibitor termed *Pb*ICP (for *P. berghei* inhibitor of cysteine proteases) that is partly stored in micronemes and is constitutively secreted by the parasite (Rennenberg et al. 2010). Interestingly, neutralization of *Pb*ICP inhibition by a specific anti-*Pb*ICP antiserum resulted in a significant reduction of sporozoite invasion, and the generation of *Pb*ICP knockout parasites confirmed this (Lehmann et al. 2014). *Pb*ICP knockout parasites are completely unable to invade hepatocytes, and they process CSP and TRAP prematurely. However, the protease regulated by *Pb*ICP might have many other targets resulting in this drastic phenotype.

Sporozoite invasion of hepatocytes is accompanied by membrane invagination and the formation of a PVM. Although the PVM is of host cell origin, it is generally

accepted that the majority of host cell proteins are excluded during the invasion process similar to what is known for RBC invasion by merozoites, while parasite proteins are incorporated into the membrane (Spielmann et al. 2012; Dowse et al. 2008; Gratzer and Dluzewski 1993). Two members of a protein family called the 6-Cys, P36 and P52, are essential for the formation of the PVM. Parasites lacking both proteins seem to invade normally but do not form a proper PVM and hence are mostly unable to successfully complete liver stage development (Annoura et al. 2012; Ploemen et al. 2012). Curiously, it has been shown that rodent *Plasmodium* species can occasionally survive freely in the host cell cytoplasm without any protective PVM (Annoura et al. 2014), suggesting that the parasite does not strictly depend on this membrane.

Other essential PVM-resident proteins are the parasite-derived protein exported protein-1 (EXP1), upregulated in infectious sporozoites (UIS)3 and UIS4 (Mikolajczak et al. 2007; Mueller et al. 2005). UIS4 is thought to play a role in the uptake of host lipids for the biogenesis of parasite membranes to support the extensive growth of the liver schizont (Ramakrishnan et al. 2012). UIS4 interacts with the host cell apolipoprotein A1 (ApoA1), which can also be found inside the PVM and thus might be important for the synthesis of membrane material (Prudencio et al. 2006).

9.2.4 Host Cell Modifications and Consequences for Parasite Survival and Virulence

During blood stage development, malaria parasites face a unique environment inside a highly specialized host cell. In order to survive in this cell, the parasite has to install a plethora of modifications that depend on the export of a large number of parasite proteins into the host cell. Although it is well accepted that sporozoites-infecting hepatocytes also modify their host cell, much less molecular details are known in this respect. In contrast to RBCs, hepatocytes display the broad array of standard cellular functions. Thus, the parasite can benefit from the existing machinery the host hepatocyte provides rather than remodeling it to a great extent.

9.2.4.1 Host Cell Surface Changes Lead to Cytoadherence and Pathology

In the most virulent form of human malaria, caused by *P. falciparum*, only RBCs infected with young ring stage parasites are circulating in the peripheral blood of infected individuals. Older stages (trophozoites and schizonts) sequester in host organs such as the brain or lungs due to adhesion of the infected cells to the vascular endothelium (Miller 1969; Spitz 1946). This sequestration is a major cause of clinical complications in infected patients and hence is an important factor of parasite virulence (Miller et al. 2002). It is also crucial for the survival of *P. falciparum* parasites, as RBCs infected with older stage parasites that fail to adhere are removed from the peripheral blood circulation by the spleen. Conversely, in a splenectomized patient, where this selection is absent, all developmental stages could be detected in the peripheral blood (Bachmann et al. 2009), suggesting that

cytoadherence might come at additional costs. If the selective pressure for cytoadherence is removed, parasites lacking the underlying export machinery emerge in the blood circulation.

For *P. falciparum*-infected erythrocytes, adhesion to the endothelium is mediated by a parasite virulence factor termed *Pf*EMP1 displayed on the host cell surface (Scherf et al. 2008). *Pf*EMP1 is a single pass integral membrane protein concentrated and anchored in host cell modifications termed knobs (Fig. 9.7b). Knobs form characteristic protrusions on the host cell surface and are formed by parasite proteins accumulating in focal areas underneath the erythrocyte membrane that are connected to the host cell cytoskeleton (Oh et al. 2000). The large extracellular

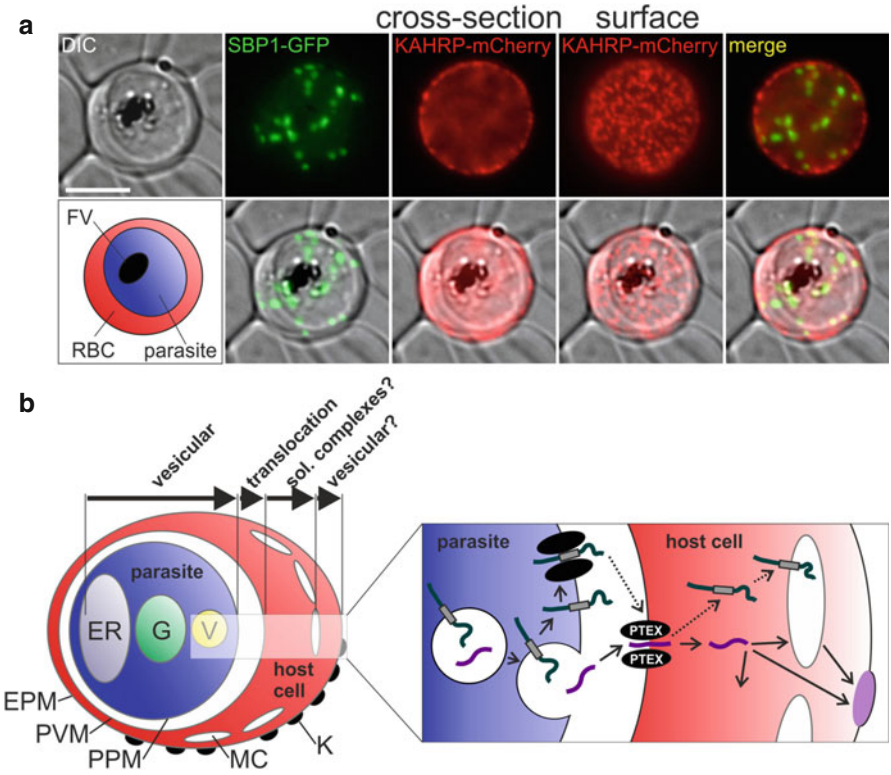


Fig. 9.7 (**a**) Examples of exported proteins in a red blood cell (*red*, in schematic) infected with a trophozoite (*blue*, in schematic). *Green* fluorescence shows a GFP-tagged version of SBP1; red fluorescence shows an mCherry-tagged version of KAHRP. "Cross section" and "surface" show two focus areas to highlight the focal KAHRP staining on the host cell surface. *DIC* differential interference contrast, *FV* food vacuole containing hemozoin (digested hemoglobin). (**b**) Top, schematic of an infected red blood cell (modified from Marti and Spielmann 2013), showing the different phases in export. *EPM* erythrocyte plasma membrane, *PVM* parasitophorous vacuolar membrane, *PPM* parasite plasma membrane, *MC* Maurer's cleft, *K* knob, *ER* endoplasmic reticulum, *G* Golgi, *V* vesicle delivering cargo from the Golgi to the PPM. *Bottom*, enlargement of the box in the schematic showing the pathway of soluble (*purple*) or transmembrane proteins (*green*)

variable part of *Pf*EMP1 protrudes from the knob to bind endothelial receptors such as CD36, ICAM, or chondroitin sulfate (CSA) (Cabrera et al. 2014). The more conserved internal part of *Pf*EMP1 binds the major structural component of the knob, the knob-associated histidine-rich protein (KAHRP) (Cooke et al. 2001), and a parasite protein of the PHISTb family (Oberli et al. 2014). Loss of one of these interaction partners reduces *Pf*EMP1 binding to the endothelium. KAHRP also binds the host cell cytoskeleton (Voigt et al. 2000), thereby connecting *Pf*EMP1 to the knob and the host cell cytoskeleton. Thus, knobs represent host cell cytoskeleton anchored platforms that concentrate *Pf*EMP1 in elevated areas on the red blood cell surface for optimal binding to the endothelium.

9.2.4.2 Antigenic Variation Leads to Altered Surface Changes

Each *P. falciparum* parasite genome contains ~60 *Pf*EMP1-encoding genes termed *var* genes of which only one is expressed at a given time (Voss et al. 2006). *Pf*EMP1 is a major target of the host's immune response (Chan et al. 2012). The mutually exclusive expression of *var* genes ensures that the parasite exposes only a single *Pf*EMP1 molecule to the immune system. The particular *Pf*EMP1 expressed determines the antigenic properties and defines the receptor binding-specificities of the infected cell. A switch in expression of the *Pf*EMP1 changes these properties and is thought to lead to evasion of the host's immune response and persistence of the parasites in the host. The changed receptor specificities can also influence the organ tropism. An example for this is the binding of infected RBCs to chondroitin sulfate A (CSA), leading to sequestration in the placenta of pregnant women (Salanti et al. 2003).

The *var* genes among different isolates display a high level of divergence (Gardner et al. 2002) and are constantly changing through recombination (Freitas-Junior et al. 2000). Hence, although each parasite genome contains only 60 *var* genes, the repertoire of different *Pf*EMP1 variants in the total population of *P. falciparum* parasites is formidable and is likely an important factor for the continued infection of the human population in endemic areas (Chen et al. 2011). After multiple infections, people in endemic areas acquire a semi-immunity characterized by reduced pathology or asymptomatic infections (Snow and Marsh 1998). This may be primarily attributable to recognition of *Pf*EMP1s (Chan et al. 2012).

9.2.4.3 Maurer's Clefts, J-Dots, and Protein Sorting in the Host Cell

Besides knobs, *P. falciparum* parasites cause a second kind of well-defined and prominent host cell modification referred to as Maurer's clefts (Mundwiler-Pachlatko and Beck 2013) (Fig. 9.7a, b). These vesicular structures are in the cytosol of infected RBCs and resemble large flattened disks that appear as long slender vesicles in electron microscopy sections (Tilley et al. 2008). Similar structures are also found in RBCs infected by many other malaria species (Aikawa et al. 1975). The origin of Maurer's clefts is unknown. They are first detectable within the first 2 h after erythrocyte invasion, after which no new clefts appear (Gruring et al. 2011). In early asexual stage parasites, Maurer's clefts are mobile. However, the movement stops during the transition from the ring to the trophozoite stage. This coincides

with the approximate life cycle stage in which *Pf*EMP1 appears on the host cell surface and hence the cytoadherence of infected RBCs (Kriek et al. 2003), a prerequisite for following rapid parasite growth. Maurer's cleft arrest may therefore signify completion of the host cell modifications required for trafficking of virulence factors, making the transition to the trophozoite stage possible. Parasite-induced structures termed tethers as well as host actin have been observed to connect the Maurer's clefts to the host cell membrane, but it is unclear whether they directly cause Maurer's clefts to cease moving (Cyrklaff et al. 2011; Pachlatko et al. 2010).

Maurer's clefts have a function in protein sorting in the host cell (Mundwiler-Pachlatko and Beck 2013). *Pf*EMP1, as well as the knob component KAHRP, is seen at the Maurer's clefts, indicating that these molecules pass through Maurer's clefts on their way to the host cell membrane. The trafficking function of Maurer's clefts is further supported by the fact that knockouts of resident Maurer's cleft proteins such as SBP1, MAHRP1, REX1, and several other proteins (Maier et al. 2008) prevent display of *Pf*EMP1 on the host cell surface. However, trafficking of other proteins to the surface of infected RBCs is not affected, indicating that this function seems to be rather specific for trafficking of cytoadherence factors. How the Maurer's cleft proteins orchestrate *Pf*EMP1 transport is so far unclear.

Other parasite-induced structures in the host cell also implicated in protein trafficking are the J-dots. These mobile focal structures contain exported parasite heat shock proteins (HSP) as well as *Pf*EMP1 and may represent protein transport aggregates, for instance, for trafficking to the Maurer's clefts or the host cell surface (Kulzer et al. 2012).

9.2.4.4 Exported Parasite Proteins Alter Host Cell Rigidity and Permeability to Nutrients

Less visually obvious host cell modifications are changes to the RBC cytoskeleton that often lead to a deformed morphology of the infected cell (Cranston et al. 1984). The ensuing increased rigidity is believed to be a reason for the splenic clearance of RBCs harboring later stages of *P. falciparum*.

Although KAHRP is a major contributor to host cell rigidity, many other parasite proteins also bind the host cell cytoskeleton with differing impact on cell deformability. These include *Pf*EMP2, *Pf*EMP3, *Pf*332, RESA, and several proteins identified in a large-scale knockout study (Maier et al. 2008). RESA also interacts with the host cell cytoskeleton, but it does not affect the deformability of the RBC (Pei et al. 2007). Instead it protects the infected cell from heat stress (Silva et al. 2005). It contains a PHISTb domain, a feature defining an entire group of host cell cytoskeleton-binding proteins, which may interlink different structures in the infected cell (Oberli et al. 2014).

To obtain nutrients, the parasite changes the permeability of the host cell plasma membrane to nutrients such as carbohydrates, nucleosides, or the amino acid isoleucine (Staines et al. 2000). The parasite protein CLAG3 has been linked to the genesis of these so-called new permeation pathways (Nguitragool et al. 2011). CLAG3 is a rhoptry protein that may be inserted into the host cell membrane upon invasion. However, additional parasite proteins exported during the intracellular development of the parasite are necessary for the activity of the new permeation pathways (Beck et al. 2014).

9.2.4.5 Protein Transport into the Host Cell

Protein export is most prominent and best studied during the development of *P. falciparum* parasites in RBCs, where hundreds of different proteins are exported (Hiller et al. 2004; Marti et al. 2004). As RBCs lack the capacity for protein sorting by a functional ER and Golgi, *Plasmodium* parasites not only require protein export to generate the host cell modifications described in the previous section but also need to install a protein trafficking system for the distribution of proteins in the infected cell. This all occurs beyond the parasite boundary and beyond the PVM, representing a formidable cell biological problem.

9.2.4.6 Malaria Parasites Contain a Typical Eukaryotic Secretory Pathway

Plasmodium parasites possess a classical intracellular secretory pathway with a perinuclear ER and a likely unstacked Golgi, both of which are detectable as a single structure in the mononuclear stage and divide shortly before or concomitant with nuclear division (Struck et al. 2005). The typical components of the anterograde and retrograde vesicle trafficking are encoded in the parasite's genome and, where analyzed, show localizations congruent with that in other eukaryotes (Deponte et al. 2012). For instance, the COPII coat components Sec13p and Sec24a are found at distinct sites of the ER, likely representing transitional ER (tER), and these sites were in close proximity to the cis-Golgi marker GRASP (Struck et al. 2008). The trans-Golgi, marked by Rab6, was found partially overlapping on the distal side of the cis-Golgi.

9.2.4.7 Export into the Host Cell Involves Parasite-Specific Trafficking Steps

Exported proteins use conventional secretory trafficking through the ER and Golgi before they reach the parasite boundary (Fig. 9.7b). Like in other eukaryotes, a signal peptide is sufficient for a protein to enter the ER in malaria parasites. In the absence of further signals (for instance, to retain it in the secretory pathway or to deliver it to other intracellular compartments), the protein will end up in the PV per default (Waller et al. 2000). This is equivalent to secreted proteins in other organisms. Exported proteins therefore require additional malaria parasite-specific signals to cross the PVM to reach the host cell and for trafficking in the host cell. The next sections will give an overview of the signals and pathways involved in protein export in malaria parasites (see also Fig. 9.7b).

9.2.4.8 The Majority of Exported Proteins Contain a Particular Export Motif

Many exported proteins contain a 5 amino acid sequence with the consensus RxLxE/Q/D (where x is an uncharged amino acid) located approximately 20 amino acid positions downstream of a signal peptide that is frequently recessed (Boddey and Cowman 2013). This motif is termed *Plasmodium* export element (PEXEL) or host targeting (HT) motif (Marti et al. 2004; Hiller et al. 2004). The PEXEL/HT is cleaved in the ER by the protease Plasmepsin V (Boddey et al. 2010). The resulting

protein with the newly exposed N-terminus is then N-acetylated and exported. Cleavage occurs after the third position of the motif (L), and mutations of this or the first position (R) abolish cleavage and export. The last conserved position (E/Q/D) is not important for processing but, together with other so far poorly defined sequences in the new N-terminus, drives post-ER export (Boddey and Cowman 2013). The PEXEL protease Plasmepsin V cleaving within the PEXEL motif is a promising drug target, as its inhibition leads to parasite death (Sleebs et al. 2014).

P. falciparum parasites encode more than 400 proteins with a predicted PEXEL motif, including a number of families such as the RIFINS, STEVOR, MC2TM, FIKK kinases, and PHIST domain proteins (Boddey and Cowman 2013). The knob component KAHRP (see Fig. 9.7a) and the J-dot component HSP40 are further PEXEL/HT proteins. *Pf*EMP1 on the other hand contains a PEXEL-like sequence with the consensus KxLxD/E that is not cleaved by Plasmepsin V but nevertheless is important for export (Marti et al. 2004). Based on this *Pf*EMP1s were classified as non-PEXEL proteins (Boddey et al. 2013).

PEXEL proteins are also exported in other *Plasmodium* species, but fewer of these proteins are predicted to exist in rodent and other human species (Marti and Spielmann 2013). This may reflect a genuinely reduced number of exported proteins, for instance, due to the lack of *Pf*EMP1 trafficking that is absent in these species. Alternatively it may either indicate a higher proportion of exported proteins without a PEXEL/HT motif (Siau et al. 2014) or an adapted consensus of the PEXEL motif in these species (Pick et al. 2011).

9.2.4.9 There Is a (Export) World Beyond PEXEL

Exported proteins without a PEXEL motif are referred to as PEXEL-negative exported proteins (PNEPs) (Spielmann and Gilberger 2010). In *P. falciparum*, PNEPs are comprised of a much smaller group compared to the PEXEL family (Heiber et al. 2013). However, it is likely that more PNEPs exist, as no signature motif is known to reliably predict these proteins.

Although PNEPs lack a defined export motif, their 10–20 N-terminal amino acids are capable of mediating export. This region is exchangeable with that of the new N-terminus of PEXEL proteins exposed after Plasmepsin V cleavage in the ER. It is therefore possible that these two groups of proteins share a similar and potentially universal export domain that mediates post-ER trafficking (Gruring et al. 2012). Examples of PNEPs include the Maurer's cleft proteins SBP1 (see Fig. 9.7a), MAHRP1, and REX1, all implicated in PfEMP1 trafficking, as well as a HSP70 protein found in the J-dots (Marti and Spielmann 2013). PNEPs are also present in *Plasmodium* species infecting rodents where they might represent a larger proportion of all exported proteins (Siau et al. 2014; Pasini et al. 2013).

9.2.4.10 Green Light for Trafficking Proteins

After release into the PV, PEXEL proteins and PNEPs are then transported across the PVM by components of the *Plasmodium* translocon of exported proteins (PTEX) complex (Elsworth et al. 2014). PTEX is a protein translocation machine

found on the luminal side of the PVM and consists of the parasite proteins EXP2, PTEX150, HSP101, PTEX88, and TRX2 (de Koning-Ward et al. 2009). HSP101 is an ATPase and thought to unfold translocation substrates that are then fed through a pore formed by EXP2. HSP101 and PTEX 150 are both essential for protein export, and their inactivation reversibly arrests parasite development from the ring to the trophozoite stage (Elsworth et al. 2014; Beck et al. 2014). PTEX88 can be knocked out but this leads to a pronounced reduction of parasite growth, whereas TRX2 knockout parasites show only a mild growth phenotype, indicating that these components are not strictly essential for PTEX function. No data on the function of the proposed pore component EXP2 are so far available. However, the proposition that PTEX is a protein translocator is further supported by the fact that the export of PEXEL proteins as well as PNEPs requires unfolding of the protein for export (Marti and Spielmann 2013).

9.2.4.11 General Model for the Trafficking Pathway of Exported Proteins

Combining the different phases in export into a general model results in a pathway where soluble exported proteins pass the parasite-internal secretory system by vesicular trafficking. These exported proteins are then released into the PV after which they are translocated across the PVM into the host cell (Fig. 9.7b, enlarged box). Once in the host cell, these proteins either remain soluble and attach directly to their destination site (e.g., the Maurer's clefts or the host cell cytoskeleton) or are further distributed via the Maurer's clefts such as in the case of KAHRP (Wickham et al. 2001).

The model is more complex for transmembrane proteins (Fig. 9.7b, enlarged box). Vesicle flow will deliver these proteins to the parasite plasma membrane, where they are subjected to a first translocation step. The steps thereafter have not been resolved, but PTEX components are involved which likely indicates PVM translocation. This would suggest a lack of vesicular trafficking at the PVM, which is congruent with time-lapse data on the export of transmembrane proteins and with the detection of soluble full-length transmembrane proteins in the host cell. The details of the trafficking of transmembrane proteins in the host cell are still obscure, but J-dots may play a role in post-PVM transport. Further trafficking from the Maurer's clefts to the host cell surface may again involve vesicular structures and a rearranged host cell cytoskeleton (Marti and Spielmann 2013).

9.2.4.12 Protein Export in Other Life Cycle Stages

Besides asexual blood stages, the sexual precursor parasites, termed gametocytes, also export proteins. While exported proteins also affect the rigidity of the infected cell, maturing gametocytes do not express *Pf*EMP1, and these stages preferentially sequester in the bone marrow in a cytoadherence-independent manner (Silvestrini et al. 2010). Less is known in liver stages where evidence for protein export is only slowly emerging (Montagna et al. 2014). However, for both of these stages, there is evidence for the presence of PTEX, although in gametocytes this only is the case in the younger stages, whereas later-stage gametocytes may not be capable to export proteins.

9.2.4.13 Controlling Host Hepatocyte Signaling: A Difficult Task for an Intracellular Parasite

It is well accepted that the presence of parasites in hepatocytes does substantially modify host cell signaling pathways, and therefore a protein export machinery of liver stage parasites can be predicted. It has been shown that the parasite inhibits host cell apoptosis to prevent elimination (van de Sand et al. 2005), and it has been suggested that CSP outcompetes NF-kB signaling to interfere with inflammatory host cell responses (Singh et al. 2007).

A straightforward concept of blocking host cell apoptosis is the secretion of inhibitors by the parasite. It has already been mentioned that *P. berghei* parasites express the highly potent inhibitor of cysteine proteases PbICP, a homologue of *Pf*ICP or falstatin (Pandey et al. 2006). In contrast to ICPs of other organisms, all currently identified *Plasmodium* ICPs contain a long N-terminus that might contain export signals for the transport into the host cell to block activation of cysteine proteases involved in the induction of apoptosis. In fact it was shown that PbICP expression in hepatoma cells protects them from apoptosis, and lack of PbICP by gene deletion had a profound effect on liver stage development suggesting an important role during this phase. However, as mentioned above, the main effect of deleting the *pbicp* gene was on sporozoite migration and invasion.

A parasite protein that is secreted into the host cell cytoplasm is liver-specific protein 2 (LISP2), but its function remains obscure (Orito et al. 2013). Although *P. berghei* LISP2 contains a signal sequence and atypical PEXEL sites, it appears not to show a typical PEXEL secretion pattern. Instead, it is transported in vesicles budding from the PVM. Upon release from these vesicles, LISP2 is partially translocated to the host cell nucleus. It has been speculated that LISP2-dependent modulation of host cell transcription might support parasite development. However, the main observed phenotype when LISP2 is knocked out is an impaired merozoite development within the host hepatocyte. *P. falciparum* LISP2 has not been analyzed experimentally but it also appeared to contain a putative signal sequence and no PEXEL sequence. This makes a similar transport as described for *P. berghei* more likely. Although LISP2 is so far the only known parasite protein that is exported during liver stage development into the host cell cytoplasm and even into the host cell nucleus, it is safe to assume that several other parasite proteins are exported in a similar way. The described vesicle transport obviously works independent of PEXEL sequences and might be accessible to other parasite proteins containing a signal sequence.

9.2.5 Getting Out: A Matter of Membrane Rupture

After their development in hepatocytes and RBCs, the parasites need to leave the vacuole they reside in and finally the host cell. In infected RBCs the PVM and the host cell membrane rupture almost simultaneously (Abkarian et al. 2011; Blackman 2008). In the liver stage of the parasite, these events are separated in time (Graewe et al. 2012). After disintegration of the PVM, parasite-filled vesicles bud from the infected host cell into the blood vessels. Within these vesicles, termed merosomes,

parasites are protected from the host immune system and can safely travel by blood flow until merosomes rupture and the pathogenic blood phase is initiated. This difference in parasite release makes perfect sense, as hepatocyte-derived merozoites need first to be transported to the blood vessel, whereas RBC-derived merozoites can immediately attach to and infect other RBCs.

Despite these obvious differences in the kinetics of egress, many similarities can be found at a molecular level between blood and liver stage egress, pointing to a common and conserved mechanism. In both stages, egress from the PV is blocked by the general cysteine protease inhibitor E64, and members of the serine repeat antigen (SERA) family are cleaved shortly before the release of the parasites (Schmidt-Christensen et al. 2008; Yeoh et al. 2007). In the blood stage, the processing proteases have already been identified as subtilisin-like protease 1 (SUB1) and dipeptidyl peptidase 3 (DPAP3). It was shown that *Pb*SUB1 also plays a crucial role in egress at the end of liver stage development (Suarez et al. 2013). Apart from proteases, kinases were also identified to play a key role during egress. Inhibition of the *P. falciparum* cGMP-dependent protein kinase (*Pf*PKG) prevented merozoite egress by blocking the discharge of the serine protease *Pf*SUB1 into the PV (Collins et al. 2013). Moreover, stimulation of the *Pf*PKG activity by blocking the parasite phosphodiesterase induced premature *Pf*SUB1 discharge and egress of immature noninfectious merozoites (Millholland et al. 2013).

While the importance of a proteolytic cascade for egress is well established, it is not clear how the PVM is disintegrated at a molecular level. Proteases could destabilize the PVM by cleavage of integral membrane proteins, but it is unlikely that protease activation results in the complete PVM disassembly that has been observed. A class of enzymes that could be important for PVM rupture are pore-forming proteins. Two perforin-like proteins are expressed in the blood stage, one of which localizes to the PVM in mature schizonts following its calcium-dependent secretion (Garg et al. 2013). Again, pore-forming proteins would not result in a complete PVM disassembly. This is rather the function of another class of enzymes: lipases. The genome of *Plasmodium* parasites contains several genes coding for putative lipases, and the role of *P. berghei* phospholipase (PbPL) in parasite egress from infected hepatocytes has recently been confirmed (Burda et al. 2015).

Another protein that has been identified to be involved in PVM disruption at the end of liver stage development is LISP1 in *P. berghei* (Ishino et al. 2009). It localizes to the PVM, and its deletion blocks parasite escape from the PV. LISP1 itself, however, has no recognizable functional domain and is therefore suspected to be either a membrane receptor for other PVM rupturing proteins like phospholipases or to be involved in the processing of proteases for activation.

Like for egress of merozoites from RBCs and from hepatocytes, proteases and perforin-like molecules play an important role in egress of gametocytes from its host RBC (Wirth et al. 2014). Interestingly, gametocyte egress is a two-step mechanism that appears as an intermediate between egress of merozoites from RBCs and from hepatocytes (see above). During gametocyte egress, the time between the rupture of the two membranes is in the range of minutes but can still clearly be distinguished. A gametocyte-specific event during egress is the discharge of osmiophilic bodies that are particularly abundant in female gametocytes into the PV. A number

of proteins have been associated with osmiophilic bodies including the gametocyte-specific protein *Pf*g377, gamete egress and sporozoite traversal (GEST), and male development 1 (MDV-1). Deletion of any of the corresponding genes resulted in an impaired egress of gametocytes indicating that they have a pivotal role in this process although the exact mechanism of how they rupture membranes is not known. Together it appears that the osmiophilic bodies have a similar function as exonemes in the egress of blood stage merozoites.

Another general principle during egress of *Plasmodium* parasites appears to be Ca^{2+} release from either host cell stores (in infected hepatocytes) or from parasite stores (asexual and sexual blood stage parasites). In gametocyte egress, Ca^{2+} release results in the activation of the calcium-dependent protein kinase CDPK-4 and the formation of male gametocytes (Billker et al. 2004). Curiously very little is known about how sporozoites leave the oocyst. A protease has been shown to be involved as a parasite lacking this specific protease does not egress and likely CSP processing is also involved (Aly and Matuschewski 2005).

9.3 Recent Advances and Open Questions

Malaria research has advanced our knowledge considerably about parasite biology in recent years. Several new concepts have been suggested which might revolutionize our thinking about the parasite, parasite-host interactions, and the resulting disease.

9.3.1 The Fight Inside the Host Cell: Intracellular Immune Responses

Once sporozoites have made their way to the final hepatocyte where they reside in a PV, they are by no means safe. It has become increasingly clear that the host cell is well equipped to eliminate invaders by processes considered to be cytosolic immune responses (Huang and Brumell 2014). The overall goal of the host cell in this regard is rather straightforward: isolate the pathogen in a compartment, label this compartment, recruit destructive vesicles such as lysosomes to the compartment, fuse them with the compartment membrane to acidify the content, and thus destroy the pathogen. The molecular mechanisms behind the defense strategy differ depending on the membrane surrounding the pathogen and involve many well-orchestrated events. In the case of *Plasmodium* parasites, the membrane that the host cell needs to recognize and label is the PVM. It is not clear yet how the host cell recognizes the PVM, but it has been shown that it is labeled by the autophagy marker LC3 (Grutzke et al. 2014; Prado et al. 2015). As LC3-I is constantly expressed as a soluble protein in the cytoplasm, it can thus immediately be recruited. This recruitment involves processing and lipidation steps, and finally the conjugated molecule is incorporated as LC3-II into the destination membrane, which, in the case of *Plasmodium*, is the PVM. So far it is not clear how the parasites escape

further destruction or whether it even makes use of this host cell defense system to actively attract LC3 to the PVM for the rapid PVM expansion during the extreme growth of the parasite during liver stage development.

9.3.2 How Intracellular Parasites Communicate: Microvesicles and Gametocytogenesis

Recent data indicates that malaria blood stage parasites induce the release of microvesicles budding from the host cell membrane. This process is involved in promoting conversion of parasites to sexual precursor stages (gametocytes) necessary for transmission to the mosquito host (Mantel et al. 2013; Regev-Rudzki et al. 2013). The microvesicles were shown to transport parasite antigens as well as parasite RNA and even DNA in the form of exogenously transfected plasmids. Studies analyzing microvesicles in hosts infected with *P. falciparum*, *P. vivax*, and *P. berghei* suggest that microvesicle numbers are elevated in malaria infections and immunomodulatory effects were found in *P. falciparum* culture-derived microvesicles (Mantel and Marti 2014). Interestingly, the microvesicles are released before rupture of the host cell, suggestive of an active release mechanism, and the exported parasite protein PTP2 has been implicated in this process. It will be exciting to unravel the exact mechanism for vesicle formation, release, and uptake as well as to how this affects commitment to gametocytogenesis. While immunomodulatory functions are clearly beneficial for the parasite, it remains to be determined why cell-cell communication and gametocytogenesis are so strongly linked and what the physiological advantage is for the parasite in vivo.

The fact that exogenous signals can influence the rate of gametocytogenesis is not only indicated by the work on microvesicles but by long-standing evidence whereby continuously passaged parasites tend to display reduction of gametocytogenesis (Alano and Carter 1990). This raises the question of how this process is regulated at the level of gene expression. Recent work revealed that a specific member of the ApiAP-2 transcription factors (Balaji et al. 2005), termed ApiAP-2-G, governs expression of gametocyte genes and controls commitment to sexual development (Sinha et al. 2014). ApiAP-2-G expression is in turn controlled by regulators of epigenetic gene silencing such as heterochromatin protein 1 (HP1) and the histone deacetylase 2 (HDA2), indicating that the actual switch to sexual development is controlled on the epigenetic level (Brancucci et al. 2014; Coleman et al. 2014). It will now be exciting to establish how microvesicle signaling fits into the picture. One possibility would be that microvesicles deliver cargo that acts by displacing factors like HP1 from the *apiap-2-g* locus to induce gametocytogenesis, an idea already speculatively put forward (Brancucci et al. 2014). Unraveling these aspects of parasite biology will be well worthwhile, as they present excellent targets for chemotherapeutic intervention. It is also interesting to note that HP1 and HDA2 are also responsible for the silencing of virulence genes and thereby maintain the mutually exclusive expression of a single *var* gene. Interventions interfering with these processes may therefore affect the parasite on

multiple levels, eventually leading to parasites that can be efficiently targeted by the immune system.

9.3.3 Sleepy Hollow: Dormant Liver Stages

In the last years, it became clear that the exoerythrocytic stage of the parasite, although clinically silent, will be crucial in the attempts to eradicate the disease (Amino et al. 2011). A major hurdle is the dormant hypnozoite form of the *P. vivax* and *P. ovale*, which can reside in the liver for several years. Although the dormant stages of *P. vivax* parasites are the major limiting factor for malaria eradication, there is currently only one drug available, primaquine, that radically eliminates the liver stage. Primaquine usage is restricted to tourists and military because a daily treatment for 14 days is necessary. Furthermore, primaquine causes severe hemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals and hence cannot be administered to this group as well as to pregnant women as the fetus could be G6PD deficient. This is particularly problematic as G6PD deficiency was evolutionary favored in malaria-endemic regions since it protects against severe malaria symptoms. Once again, this makes very clear that we need to understand the biology of the parasite much better before we can envisage eradication. So far we know very little about the dormant liver stage, largely because all attempts to cultivate the blood stage of *P. vivax* or other *Plasmodium* species forming hynozoites have failed. Without a continuous in vitro cultivation, researchers rely solely on blood from human volunteers or on primate infection models. Still some recent advances by successfully infecting humanized mice, in which mouse hepatocytes have been replaced by human hepatocytes, with *P. vivax* and by successful cultivation of *P. vivax* in vitro using hematopoietic stem cell-derived reticulocytes (Dembele et al. 2014; Noulin et al. 2014) raise the hope that this stage can be experimentally better approached in the future.

Our understanding of the *Plasmodium* biology at a molecular level has increased tremendously over the last two decades, but much more effort is needed to identify additional Achilles heels of the parasite and target them to develop strategies for malaria eradication. Clearly, commitment and investment in basic research are needed as, despite all efforts and substantial financial support, no commercial vaccine that confers sterile protection is available yet (Heppner 2013) and resistant parasites rapidly emerge to new drugs (Straimer et al. 2014; Mok et al. 2014; Ashley et al. 2014; Ariey et al. 2014). However, malaria control can be envisaged by a multidisciplinary approach exploring the recent advances in immunological, molecular biological, and microscopical techniques as well as in vector control measurements and in research on the dormant parasite stages and, of course, by the hard and inspiring work of future generations of malaria researches in the laboratory, public health workers in the field and committed politicians.

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Acanthamoeba

10

Martina Köhsler, Martin Mrva, and Julia Walochnik

Abstract

Acanthamoebae are ubiquitous free-living amoebae that occur abundantly in water and soil worldwide, being among the most versatile protozoan organisms. They generally do not need a host, but when they accidentally have contact to the human eye, lung or skin, they can cause severe disease. They are the causative agents of *Acanthamoeba* keratitis (AK), on the one hand, and of several disseminating infections in the immunocompromised host eventually leading to granulomatous amoebic encephalitis (GAE), on the other hand. The infective and invasive form of *Acanthamoeba* is the trophozoite; nevertheless, the cyst plays an important role in the distribution of the amoebae as well as in the course of disease. Acanthamoebae can form cysts within the host tissue, and these cysts are resistant against treatment and also often lead to reinfections.

Altogether, around 25 different species divided into three morphological groups have been described; however, the validity of many species has been challenged by molecular analyses. Currently, the genus is divided into 20 genotypes based on 18S rDNA sequencing, but the number of genotypes is growing constantly. Genotype T4 seems to be the most abundant one in most habitats and also the most common genotype in human infections; however, a classification into virulent and non-virulent genotypes is not possible. Acanthamoebae pathogenicity depends on cell-cell contact, the cytolytic event being triggered by an

M. Köhsler • J. Walochnik (✉)
Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna,
Kinderspitalgasse 15, Vienna A-1090, Austria
e-mail: martina.koehsler@meduniwien.ac.at; julia.walochnik@meduniwien.ac.at

M. Mrva
Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava,
Mlynská dolina Ilkovičova 6 842 15, Bratislava, Slovakia
e-mail: mrva@fns.uniba.sk

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intimate contact between the amoebae with the target cells, established primarily via lectin-like amoebic adherence molecules. The ability of acanthamoebae to lyse cells is mainly based on lysosomal hydrolases and phospholipases.

In 2013, a first genome has become available revealing a significant number of genes presumably acquired by lateral gene transfer and a rather complex cell communication repertoire. However, the genetics of *Acanthamoeba* spp. is far from being fully elucidated. Fact is that acanthamoebae have unusually broad metabolic and biosynthetic capabilities, being able to synthesise most amino acids (even multiple steps), co-factors and vitamins and nucleotides de novo. Moreover, they can digest a wide range of nutrients, they are among the few protozoa with a cellulase, and they are the only protozoans known to date with an alginate lyase. Although acanthamoebae generally divide by binary fission and there is no convincing evidence for genetic recombination, even their asexual nature has been challenged recently.

Abbreviations

AA	Arachidonic acid (AA)
AcAtg	*Acanthamoeba* autophagy-related proteins
AIF	Apoptosis-inducing factor
AK	*Acanthamoeba* keratitis
AOX	Alternative oxidase
aPA	*Acanthamoeba* plasminogen activator
cAMP	Cyclic adenosine monophosphate
CPE	Cytopathic effect
CRD	Carbohydrate recognition domains
CSCP	Cyst-specific cysteine protease
CXCL2	Chemokine (C-X-C motif) ligand 2
cPLA	Cytosolic phospholipase A
DAG	Diacylglycerol
DH	Dehydrogenase
ECM	Extracellular matrix
ERMES	ER-mitochondria encounter structure
EST	Expressed sequence tag
ETC	Electron transport chain
FFA	Free fatty acid
GAE	Granulomatous amoebic encephalitis
GMP	Guanosine monophosphate
GPCR	G protein-coupled receptors
HBMEC	Human brain microvascular endothelial cells
HCE	Human corneal epithelial cells
HNE	4-Hydroxy-2-nonenal
ICL	Isocitrate lyase
LBP	Laminin-binding protein

LGT	Lateral gene transfer
MalS	Malate synthase
MAPK	Mitogen-activated protein kinase
MBP	Mannose-binding protein
MIP	Mannose-induced protein
MMP	Matrix metalloprotease
ORF	Open reading frame
PAR	Protease-activated receptor
PDH	Pyruvate dehydrogenase
PHB	Polyhydroxybutyrate
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C-like gene
PMN	Polymorphonuclear leukocyte
PN	Purine nucleotide
pTyr	Phosphotyrosine
ROS	Reactive oxygen species
SAPLIP	Saposin-like protein
TCA	Tricarboxylic acid
TLR	Toll-like receptors (TLR)
UCP	Uncoupling protein

10.1 Introductory Remarks

Acanthamoebae are ubiquitous free-living amoebae; they occur worldwide in natural habitats such as soil, fresh water or marine water but also in man-made habitats such as swimming pools and airconditioning systems. With their low nutritional requirements and their highly resistant cysts, acanthamoebae can exist almost anywhere and are thus among the most frequently isolated amoebal species worldwide. The life cycle of *Acanthamoeba* spp. consists of two stages, a vegetative trophozoite stage, in which the organism feeds and multiplies, and a metabolically inactive cyst stage, which enables the amoeba not only to survive without nutrients and withstand desiccation and heat but also to resist disinfection and treatment. Generally, acanthamoebae feed on bacteria, algae and yeasts; they are, however, among the most versatile protozoans being able to feed on almost anything and to occur under aerobic and anaerobic conditions and also under extreme conditions concerning pH, salinity and temperature. They do not need a host, but they can cause serious disease upon contact, being the causative agents of very different disease entities, including *Acanthamoeba* keratitis (AK), a sight-threatening infection of the eye, and several disseminating infections, such as *Acanthamoeba* skin lesions and granulomatous amoebic encephalitis (GAE) (Khan 2006; Visvesvara 2010). While AK, particularly in the industrialised countries, occurs mainly in contact lens wearers, *Acanthamoeba* GAE seems to be restricted to immunodeficient patients. All *Acanthamoeba* infections are rare, but they usually show a severe progression, which is mainly due to the

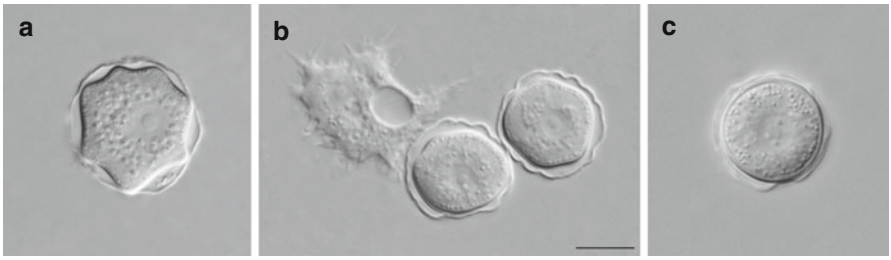

Fig. 10.1 *Acanthamoeba*. Morphological group I, cyst (**a**); morphological group II, trophozoite and cysts (**b**); morphological group III, cyst (**c**). Group I shows typical large trophozoites and cysts of more than 18 µm in diameter. Cysts have a rounded outer wall clearly separated from the inner wall, to which it is joined by radiations, forming a star-shaped structure. The opercula are not immersed into the outer cyst wall. Group II cysts are smaller with a variable endocyst shape, mostly smaller than 18 µm. Ecto- and endocyst may be separated or not. The ectocysts are thick or thin but usually wrinkled. The endocyst may be polygonal, triangular, or even round or oval. Normally no clear radiations are visible. The operculum is usually immersed into the ectocyst. In group III, the cysts show less than 18 µm in diameter and have poorly separated walls. The ectocyst is rather thin and very often ripped. The endocyst is usually round but sometimes slightly triangular or rectangular. Scale bar: 10 µm. Orig

unavailability of effective and easily manageable treatment. The dormant cysts pose a particular problem, residing within the tissue and leading to reinfection after termination of treatment, often even after keratoplasty.

Acanthamoeba is one of the most common and most robust genera of free-living amoebae, functioning also as host, vehicle and training ground for bacteria. Several bacteria, including important human pathogens such as legionellae, have been shown to be 'primed' for virulence by passage through amoebae. Indeed, acanthamoebae are phagocytic cells and behave similarly to the phagocytic cells of the mammalian immune system in many aspects (Siddiqui and Khan 2012).

Acanthamoebae were discovered in 1930 in a culture of *Cryptococcus pararoseus* (Castellani 1930) and placed into the genus *Acanthamoeba* one year later by Volkonsky (Volkonsky 1931). The genus *Acanthamoeba* comprises three morphological groups (Pussard and Pons 1977) based on the rather distinct, polygonal morphologies of the cysts (Fig. 10.1), the number of opercula considered to display species variation. But cysts, although deriving from one clone, may have varying morphologies, and intraspecific polymorphism generally is rather common in *Acanthamoeba*. Around 25 species have been described; however, the validity of most species has been challenged by molecular analyses (Moura et al. 1992; Nerad et al. 1995; Page 1987, 1991). Today, isolates can be assigned to genotypes based on their 18S rDNA sequences (Gast et al. 1996; Stothard et al. 1998). When this chapter was written, 20 *Acanthamoeba* genotypes (T1-T20) had been established. The most common genotype in human *Acanthamoeba* infections is T4; however, this generally seems to be the most abundant genotype. Other genotypes rather frequently isolated are T3 and T11 from AK patients, and T1, T10 and T12 from GAE, but a classification into virulent and non-virulent genotypes is not possible (Booton et al. 2005; Fuerst et al. 2015; Walochnik et al. 2015).

The first cases of AK were recorded in the 1970s in patients who had contact to contaminated water after experiencing a minor trauma in the eye (Jones et al. 1975; Nagington et al. 1974). In Central Europe, the first case of AK was reported in 1984 from Germany and was one of the very first cases of contact lens-associated AK (Witschel et al. 1984). Soon, the association between *Acanthamoeba* keratitis and contact lens wear was established (Moore et al. 1985). Today, the annual incidence of AK is estimated to lie between 1 and 10 cases per million inhabitants in the industrialised countries, the most important risk factor being low contact lens hygiene (Page and Mathers 2013; Walochnik et al. 2015). One of the first human cases of GAE was described in the USA in an AIDS patient (Wiley 1987). In Europe, the first GAE case occurred in an AIDS patient in Italy (Di Gregorio et al. 1992). Altogether, around 300 cases of GAE have been described around the world.

The infective and invasive form of *Acanthamoeba* is the trophozoite; nevertheless, the cysts are spread easily through the air and can transform into trophozoites rapidly under appropriate conditions. Moreover, the cysts play a significant role for the progression of disease, as they may survive treatment within the tissue and lead to reinfection. Importantly, the vast majority of healthy individuals do not develop disease in spite of regular contact to *Acanthamoeba*. Besides mechanical prerequisites, such as microlesions in the cornea and the skin, respectively, also immunologic features seem to play an important role for the establishment of disease.

A very important virulence factor is the ability of the acanthamoebae to adhere to surfaces including the corneal epithelium with the help of their acanthopodia. Trophozoites of *Acanthamoeba* do not only adhere to, but they also can hardly be removed by physical means from contact lenses. Moreover, the amoeboid locomotion enables the amoebae to pass through spaces as narrow as 2 μm (Bamforth 1985), which is of importance for the penetration of host tissue. It has been shown that not all species of free-living amoebae are equally adapted to tissue migration (Thong and Ferrante 1986). *Acanthamoeba* pathogenicity seems to depend on intimate cell-cell contact established primarily via a lectin-like amoebic adherence molecule. Also type IV collagen, laminin and fibronectin have been reported to function as binding sites (Gordon et al. 1993). The ability of amoebae to lyse cells is based on an enzymatic action induced by lysosomal hydrolases and phospholipases. Acanthamoebae were also shown to have an amoebapore protein being released into the intercellular space following cell-cell contact. This protein inserts into the cell membrane and forms ion channels through it. As a result, the target cell probably becomes permeable and lyses (Michalek et al. 2013). Table 10.1 gives an overview of important molecules involved in *Acanthamoeba* pathogenicity.

In 2013, a first genome has become available; several further genome projects are currently on the way (see Chap. 1, Tab. 2.0). The genome has a significant number of genes presumably acquired by lateral gene transfer, and it has a rather complex cell communication repertoire; however, the genetics of *Acanthamoeba* spp. is far from being fully elucidated. Acanthamoebae generally multiply by binary fission, and there still is no convincing evidence for genetic recombination, but *Acanthamoeba* has several genes implicated in meiosis in eukaryotes with sexual reproduction (Khan and Siddiqui 2015; Speijer et al. 2015).

Table 10.1 *Acanthamoeba* molecules involved in pathogenesis.

Process	Type	Characteristics	Known function	Citation
Attachment	Adhesins and lectins (carbohydrate/ glycan-binding proteins)	Mannose-binding protein (*MBP*, 400 kDa; composed of 130 kDa subunits)	Cell surface receptor binding to mannose glycoproteins, mediates adhesion and induces cytopathic effect	Yang et al. (1997)
		Laminin-binding proteins (28.2, 54, 55 kDa)	Adhesion	Gordon et al. (1993), Wang et al. (1994), Hong et al. (2004), Rocha-Azevedo et al. (2009, 2010)
		Surface glycoproteins (8 with mannose and 8 with N-acetyl glucosamine residues)	Adhesion	Soto-Arredondo et al. (2014)
		Acanthamoeba adhesin (207 kDa)	Adhesion	Kennett et al. (1999)
	Glycans	Oligomannosidic, paucimannosidic, hybrid/ complex type	Adhesion	Schiller et al. (2012)
	ecto-ATPases	62, 100, 218, 272, >300 kDa	Hydrolysing extracellular ATP, involved in adhesion	Mattana et al. (2002), Sissons et al. (2004)
Cell lysis	Serine proteases	*MIP-133* (133 kDa); 33, 35, 130, 133 kDa etc.	Hydrolysis of proteins (contact independent)	e.g. Hadas and Mazur (1993), Kong et al. (2000), Sissons et al. (2006)
		Elastases (70–130 kDa)	Degradation of connective tissue (degrade elastin, fibrinogen, collagen, proteoglycans)	Ferreira et al. (2009)
		Acanthamoeba plasminogen activator (aPA)	Facilitates penetration through membranes	Alizadeh et al. (2007), Mitra et al. (1995)
	Metalloproteases	P3; 80 kDa, 150 kDa	Contact dependent, degrade basement membranes and components of extracellular matrix (type I and type II collagens, fibronectins, laminin)	Sissons et al. (2006)

	Cysteine proteases	43 kDa, 65 kDa, 130 kDa etc.	Protein degradation	Hadas and Mazur (1993)
	Phospholipases	A1, A2, B, C, D	Hydrolysis of phospholipids, membrane disruption	Matin and Jung (2011)
	Neuraminidases	Membrane associated and released; optimal activity in acidic range	Degradation of sialylated glycoconjugates (colonisation of the sialic acid-rich corneal epithelium)	Pellegrin et al. (1991)
	Pore-forming proteins	Acanthaporin (probably amphipathic)	Permeabilises membranes	Michalek et al. (2013)
Interaction with host defence	Superoxide dismutases (SODs)	Iron SOD (~50 kDa) and copper-zinc SOD (~38 kDa)	Anti-oxidant and anti-inflammatory	Choi et al. (2000)
Encystment	G protein-coupled receptors (GPCRs)		Quorum sensing, induction of encystment (activation of Ras, activation of adenylate cyclase)	Aqeel et al. (2015), Fouque et al. (2012), Krishna Murti and Shukla (1984)
	Glycosidases and glycogen phosphorylase		Glycogen breakdown	Lorenzo-Morales et al. (2008)
	Cellulose synthase		Cyst wall formation	Moon et al. (2014)
	Xylose isomerase		Cyst wall formation	Aqeel et al. (2015)
	Rho kinase		Regulation of actin polymerisation	Dudley et al. (2009)
	Gelation factor		Actin cross-linking	Dudley et al. (2009)
	Proteases	Serine proteases (33 kDa)	Autolysis	Moon et al. (2008)
		Cysteine proteases, including a metacaspase type I (478 amino acids)	Autolysis	Leitsch et al. (2010), Moon et al. (2012), Trzyna et al. (2008)
	Autophagy-related proteins (AcAtgs)	AcAtg3, AcAtg8, AcAtg16	Autolysis	Moon et al. (2009; 2011)

10.2 Well-Established Facts

10.2.1 Genome

The genus *Acanthamoeba* is currently placed into the taxon Discosea within the phylum Amoebozoa. The Amoebozoa form a valid clade with the Obazoa, including the animals and fungi (see Chap. 12).

Although all available data support the monophyly of the Amoebozoa, the genomic divergence between, e.g. *Dictyostelium* and *Entamoeba* is higher than the one between animals and fungi. Amoebozoan genomes completed so far have a high (A + T) content and a relatively high percentage of horizontal gene transfer and transposal elements. *Dictyostelium* as well as *Acanthamoeba* have essential tRNAs in their mitochondrial genomes, which is not yet known from other organisms. Most studies on the genome of *Acanthamoeba* have been undertaken with the Neff strain of *Acanthamoeba castellanii*, genotype T4, the strain also used in the *Acanthamoeba* genome project. This strain has the advantage that it is very well characterised concerning also its cell biology; however, this strain is a non-pathogenic environmental isolate that has been grown under laboratory conditions since 1957 and, moreover, seems to be rather exceptional, as similar strains have rarely been isolated. Early studies estimated a total cellular DNA content of uni-nucleate acanthamoebae during log phase of 1–2 pg (Byers 1986) and a haploid genome size of 40–50 Mb (Bohnert and Herrmann 1974; Jantzen et al. 1988). The genome project revealed a 45 Mb genome encoding 15,455 compact genes with an average of 6.2 introns per gene, which is among the highest known in eukaryotes (Clarke et al. 2013). The ploidy level and also the number of chromosomes of *Acanthamoeba* spp. are still uncertain. Most probably, acanthamoebae are at least diploid, and the number of chromosomes, previously assumed to be in the range of several dozens, has more recently been estimated to lie between 9 and 21 (Byers 1986; Matsunaga et al. 1998).

10.2.1.1 Nuclear Genome

A first detailed sequence survey of parts of the genome of *A. castellanii* performed by Anderson et al. (Anderson et al. 2005) gave insights into the extensive metabolic capacities and environmental adaptabilities of this organism. In contrast to the parasitic entamoebae, *Acanthamoeba* has very broad biosynthetic potentials; it can synthesise most amino acids (even multiple steps), synthesise co-factors and vitamins and synthesise nucleotides (RNA & DNA) de novo. The biosynthetic machinery of *Acanthamoeba* includes a chorismate synthesis pathway for the synthesis of aromatic amino acids and folate as well as complete purine and pyrimidine biosynthetic pathways and thymidylate synthase and ribonucleotide reductase for the synthesis of ribonucleotides and deoxyribonucleotides.

Moreover, *Acanthamoeba* can utilise complex organic nutrients; as one of very few eukaryotes, it can utilise the bacterial storage compound polyhydroxybutyrate (PHB) as an energy source. It putatively possesses an enzyme for depolymerising PHB, which in bacteria is degraded to acetyl-CoA for the TCA cycle for energy production. *Acanthamoeba* also has a cellulase; it thus can also use ‘plant’ material as food, and unlike most eukaryonts, it also has a β-glucosidase, so it can degrade

cellulose to cellobiose and then to glucose. It can also feed on fungi and also has a putative chitinase. Further, *Acanthamoeba* is the only protozoan known to date with an alginate lyase. Alginate lyases are usually only found in marine invertebrates and bacteria. *Acanthamoeba* probably utilises alginate as a food source, breaking down biofilms and feeding on the enclosed bacteria.

Important enzymes involved in *Acanthamoeba* phagocytosis are a superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and a lysozyme to depolymerise bacterial peptidoglycan. Proteases have been shown to be essential for various functions, including pathogenicity, digestion and encystment. Five cysteine proteases of the papain family, three serine proteases of the subtilisin family and a metalloprotease were detected in the genome.

In contrast to several obligate parasites, *Acanthamoeba* has all standard asparagine-linked glycosylation (alg) genes in its genome and is capable of synthesising the complete lipid-linked oligosaccharide precursor dolichol-PP-$Glc_3Man_9GlcNAc_2$ (Schiller et al. 2012).

For signal transduction, several serine/threonine protein kinases, including putative receptor kinases as well as tyrosine kinases, belonging to the animal tyrosine kinase family, were found (Clarke et al. 2013). The identification of four proteins with predicted SH2 domains and several tyrosine phosphatases gave first evidence for the existence of primitive phosphotyrosine (pTyr) signalling. A group of 21 histidine kinases and 11 response regulator receiver domains were identified, potentially allowing the amoebae to sense and respond to environmental changes and stress conditions. The detection of a trehalose-6-phosphate synthase homolog indicates that one component of *A. castellanii*'s stress tolerance system appears to be the production of the disaccharide trehalose, which is involved in protection from desiccation, osmotic stress and extremes of temperature, suggesting that trehalose plays a role in stress adaptation and might be associated with the induction of encystment (Anderson et al. 2005).

10.2.1.2 Non-coding RNA (ncRNA)

The ribosomal gene repeat unit of *Acanthamoeba* is a typical eukaryotic one and includes one set of 18S, 5.8S and 28S rDNA plus spacer regions located between the genes and between neighbouring sets. As in other lower eukaryotes, the 5S rRNA gene is an integral part of the ribosomal repeat unit. The complete repeat unit is around 12 kb long and probably of a single kind as there is no evidence for sequence or size heterogeneity. The number of rRNA repeats in *Acanthamoeba* was estimated to be 24 per haploid genome; however, because of suspected common polyploidy, each cell might contain up to 600 rRNA genes (Yang et al. 1994). The 18S rRNA gene coding region is exceptionally long, namely, around 2,300 bp in most genotypes and even around 3,000 bp in genotypes having an intron (Stothard et al. 1998). Several species of *Acanthamoeba*, as, e.g. genotype T5, are known to have group I introns in the nuclear 18S rDNA (Gast et al. 1994; Schroeder-Diedrich et al. 1998). The 5.8S ribosomal RNA of *A. castellanii* is approximately 162 bp long and the 5S RNA gene is 119 bp long, and there may be up to 480 genes encoding the 5S RNA in each *A. castellanii* cell. The 5S rRNA genes are dispersed, which is highly unusual, since the majority of eukaryotic organisms contain 5S genes

clustered in tandem repeats (Zwick et al. 1991). The 28S coding sequence is subdivided into two segments of 2,400 and 2,000 bp separated by an internal transcribed spacer of about 200 bp (Byers et al. 1990). The intergenic spacer is 2,330 bp long and contains repeated sequence elements, which exhibit characteristics similar to polymerase I enhancers found in higher eukaryotes (Yang et al. 1994).

10.2.1.3 Mitochondrial Genome

The mitochondrial genome of *Acanthamoeba* spp. consists of circular molecules and is around 41 kb long and thus in the midrange of sizes for other protozoans (Burger et al. 1995; Fučíková and Lahr 2016). It encodes two rRNAs (small subunit and large subunit), 16 tRNAs and 41 proteins (Burger et al. 1995). The interstrain mtDNA sequence diversity in *Acanthamoeba* seems to be rather high (Byers et al. 1990). The mitochondrial genome encodes fewer than the minimal number of rRNA species required to support mitochondrial protein synthesis, suggesting that additional tRNAs are imported from the cytosol into the mitochondria (Burger et al. 1995). Another prominent feature of the mitochondrial genome of *Acanthamoeba* is the number of overlapping reading frames (Burger et al. 1995). Moreover, *Acanthamoeba*, similar to *Dictyostelium*, carries essential tRNAs in its mitochondrial genome, which is not known from any other organism so far.

Interestingly, the translation system in *A. castellanii* mitochondria does not use the standard genetic code (Gawryluk et al. 2014). The 41 proteins specified by the mitochondrial genome are all encoded on the 'plus' strand and are tightly packed with only 6.8 % of the total DNA sequence not having an evident coding function (Burger et al. 1995). There are only 40 predicted open reading frames (ORF), since subunits 1 and 2 of the cytochrome C oxidase (COX1 and COX2) are specified by a single continuous ORF (Lonergan and Gray 1996). Most proteins have assigned functions associated with respiration and translation. Additionally, there are three intron-encoded ORFs, predicted to specify LAGLIDADG homing endonucleases, and three appear to be unique to *A. castellanii* (Gawryluk et al. 2014). The encoded proteins associated with respiration include NADH dehydrogenase, apocytochrome b, cytochrome oxidase and the ATP synthase complex. Interestingly, compared to other (animal or fungal) mtDNAs, the *A. castellanii* mtDNA encodes extra NADH dehydrogenase and ATP synthase genes. The *A. castellanii* genome encodes an unusually high number of mitochondrion-targeted pentatricopeptide repeat proteins (PPR), organellar RNA metabolism factors, thought to be involved in RNA editing, intron splicing, transcript stabilisation and translational control. Altogether, the mitochondrial proteome of *Acanthamoeba* is highly complex in composition and function, not unlike that of multicellular eukaryotes (Gawryluk et al. 2014).

10.2.2 Lateral Gene Transfer

Lateral gene transfer (LGT) is considered a very important constituent of genome evolution. In *Acanthamoeba,* LGT is suggested to reflect trophic strategies driven by the selective pressure of new ecological niches. As mainly bacteriovorus

organisms, which tend to harbour endosymbionts, acanthamoebae are prone to pick up foreign DNA and hence typical candidates for LGT.

Clarke et al. (2013) performed a phylogenomic analysis determining cases of predicted interdomain LGT in the *Acanthamoeba* genome and identified 450 genes in the genome of *A. castellanii* which may have arisen through LGT. Being a host of various and phylogenetically different endocytobionts, *Acanthamoeba* might as well facilitate genetic exchange between phylogenetically disparate organisms, since miniature transposable elements occurring both, in cyanobacteria and DNA viruses, have been detected.

Acanthamoeba encodes 35 G protein-coupled receptors (GPCRs), sensors for extracellular stimuli that intracellularly activate signal transduction pathways and eventually cellular responses (see Sect. 10.2.4.1). In this context, three fungal-associated glucose-sensing Git3 GPCRs and an expansion in the number of frizzled/smoothened GPCRs have been identified. Moreover, seven G protein alpha subunits and a single putative target, phospholipase C, for GPCR-mediated signalling were detected. The exact function of the GPCRs in *Acanthamoeba* is not yet established; however, a role in detecting molecules secreted by their bacterial food source was suggested and an involvement in the induction of encystment seems possible. Additionally, 48 sensor histidine kinases of which 17 may function as receptors were found, which might also be involved in environmental sensing processes of *Acanthamoeba*. Two rhodopsins with homology to sensory rhodopsins in green algae might represent possible candidates for light sensors in *Acanthamoeba*.

Altogether, 377 protein kinases were identified in the genome of *Acanthamoeba*. Protein kinases are involved in the modulation of cellular responses to environmental stimuli, and this is the largest number of protein kinases predicted to date for any amoebozoan. Moreover, two homologues of mitogen-activated protein kinases have been identified.

Phosphotyrosine (pTyr) signalling mediated through tyrosine kinases for transmitting cellular regulatory information has been associated with intercellular communication and was considered absent from the amoebozoan lineage. pTyr signalling is mediated by a triad of signalling molecules; tyrosine kinase (writers) (PTKs), tyrosine phosphatase (erasers) (PTPs) and Src homology 2 (SH2) domains (readers). In *Acanthamoeba*, 22 PTKS, 12 PTPs and 48 SH2 domain-containing proteins were identified, representing a primitive, but already functional pTyr system (Clarke et al. 2013).

A considerable amount of exogenous DNA might derive from the numerous obligate and facultative endosymbionts or endocytobionts of *Acanthamoeba.* Acanthamoebae are known to (partially permanently) harbour viruses, bacteria and also fungi. Obligate bacterial endosymbionts are found from different lineages within the Proteobacteria, the Bacteroidetes and the Chlamydiae, e.g. *Parachlamydia acanthamoebae* (Horn and Wagner 2004). One of the most prominent viral examples is the Mimivirus, a giant virus with 1,181,404 bp linear dsDNA, more than 1,000 genes, many proteins having tandem IP22 repeats (as in *Dictyostelium*) and interestingly showing genome reduction after intra-amoebal culture (Boyer et al. 2011).

10.2.3 Cellular Architecture

10.2.3.1 Trophozoites

The trophozoites (Fig. 10.2a) are 15–45 μm in size and represent typical amoeboid cells, with a granuloplasma containing the cell organelles and a hyaloplasma producing the (sub)-pseudopodia. When the trophozoites attach to surfaces, they are rounded and flat, but they also have a so-called floating form, which has a more spatial shape with radiate pseudopodia. Acanthamoebae are typical eukaryotes, with a Golgi complex, smooth and rough endoplasmic reticula, free ribosomes, digestive vacuoles (performing pinocytosis or phagocytosis), lysosomes, mitochondria, microtubules and a nucleus with a large central nucleolus.

Generally, acanthamoebae are uni-nucleate; although grown under laboratory conditions, multinucleate cells are common (James and Byers 1967). The nucleus has approximately one-sixth the size of the trophozoite and is enclosed by a nuclear envelope, consisting of two membranes with nuclear pores for flow-through of RNA and proteins (Bowers and Korn 1969). The nuclear chromatin is finely granular, and acanthamoebae are characterised by a large, dense, centrally located nucleolus. Usually two Golgi complexes are found on opposite sides of the nucleus in one plane. Acanthamoebae normally reveal numerous oval- or round-shaped mitochondria with typically tubular cristae and intra-crystal inclusions, and they possess a full complement of 'ER-mitochondria encounter structure' (ERMES) proteins, initially thought to be restricted to fungi and so far only known from a few other amoebozoans and some excavates. The ERMES fixes the ER membrane to the mitochondrial outer membrane and thus allows the integration of mitochondria with non-endosymbiotically derived cell organelles (Wideman et al. 2013). The cytoplasm contains lipid droplets and polysaccharide reserves (Bowers and Korn 1969). *Acanthamoeba* trophozoites are to a large extent (approximately 30 %) composed of carbohydrates, of which most is glucose, most likely stored as glycogen. The main phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphoinositide and diphosphatidylglycerol. Oleic acids and longer polyunsaturated fatty acids are the predominant fatty acids (Ulsamer et al. 1971). Interestingly, only growing trophozoites are covered with a carbohydrate coat, while trophozoites in the stationary phase are not (Schiller et al. 2012). The thickness of the plasma membrane is approximately 100 Å, and it consists of proteins (33 %), phospholipids (25 %), sterols (13 %) and lipophosphonoglycan (29 %) (Dearborn and Korn 1974). Acanthamoebae exhibit microtubule-associated movement of mitochondria and small particles (Baumann and Murphy 1995). Microtubules originate from the Golgi complex and are found throughout the cytoplasm (Bowers and Korn 1969; Preston 1985). Another characteristic of *Acanthamoeba* is the prominent contractile vacuole that functions in osmoregulation of the cell. Water expulsion of the vacuole has been shown to be associated with alkaline phosphatase activity in the membrane (Bowers and Korn 1973), myosin-IC (Baines et al. 1992) and metacaspase activity (Saheb et al. 2013).

Acanthamoeba's movement is sluggish and involves the formation of usually a single pseudopodium (lobopodium) with hyaline sub-pseudopodia (acanthopodia)

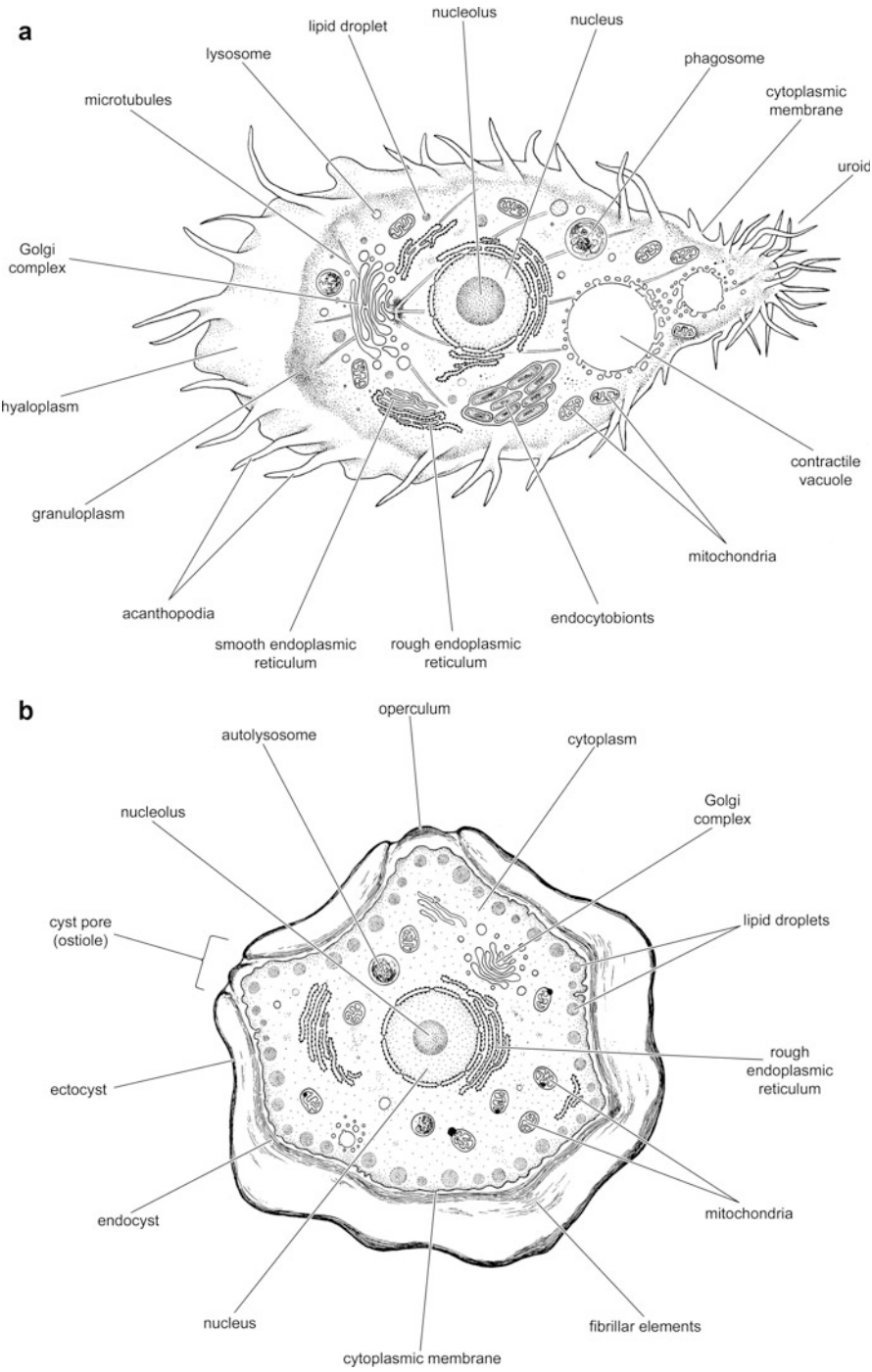

Fig. 10.2 Sketch of the ultrastructure of *Acanthamoeba* trophozoite (**a**) and cyst (**b**). Simplified and not entirely true to scale. Orig

on the entire surface of the cell. Acanthamoebae move with approximately 0.8 µm per second, accomplished by actin polymerisation anteriorly and accordingly a breakdown posteriorly in the advancing cell (Preston and King 1984). Actin, which constitutes about 20 % of the total protein, and myosin, together with more than 20 cytoskeletal proteins, are responsible for movement, intracellular transport and cell division (Gordon et al. 1976). The most important actin-binding proteins in *Acanthamoeba* are two classes of myosin, myosin-I and myosin-II (Pollard and Korn 1973a, b), two actin monomer-binding profilins (Reichstein and Korn 1979), actophorin (Cooper et al. 1986), an actin filament capping protein (Isenberg et al. 1980) and actin filament cross-linking proteins, the so-called gelation proteins (Pollard 1984). A typical feature of the genus are the characteristic spiny surface projections, the so-called acanthopodia, which are built up by actin fibres and networks (González-Robles et al. 2008) and which were shown to be particularly important in the infection process, since they allow the trophozoite to interact with the cell surfaces of the host (Omaña-Molina et al. 2004). Actin fibres and networks are also found in diverse endocytic structures that sometimes extend far away from the cell body (González-Robles et al. 2008).

Ingestion is linked very tightly to locomotion and represents a typical phagocytosis after cell-cell contact and consecutive invagination of the cell membrane. The trophozoite will phagocytose any particle of adequate size that it encounters, including nonorganic particles (Weisman and Korn 1967). Phagocytosed particles reach the phagosome, which subsequently fuses with a lysosome. Excretion is accomplished by the contractile vacuole, which empties and reappears in an actively growing culture within 1–2 min.

Trophozoites multiply rapidly in a favourable environment, growth rate depending on species. Under optimal conditions, they multiply every 6–8 h. Most species prefer temperatures of around 30 °C; however, many isolates can grow also at elevated temperatures, up to even 45 °C. Acanthamoebae divide by conventional mitosis, in which the nucleolus and the nuclear membrane disappear during cell division. For exponentially growing cells, cell division is largely occupied with the G2 phase (up to 90 %) and negligible with the G1 phase, 2–3 % M phase (mitosis) and 2–3 % S phase (synthesis) (Band and Mohrlok 1973; Byers et al. 1990, 1991). Mitosis is characterised by an early breakdown of the nuclear envelope as well as disintegration of the nucleolus and differs from the typical eukaryotic pattern in that the centrioles seem to be anomalous, although spindle fibres do terminate in centriolar equivalents (Bowers and Korn 1968).

10.2.3.2 Cysts

Under adverse environmental conditions, the amoebae form highly resistant cysts, whose morphologies were used for classification before molecular genotyping became standard.

Acanthamoeba cysts (Fig. 10.2b) are 12–32 µm in size. They have a double-layered cyst wall, an outer ectocyst consisting of proteins and polysaccharides (Neff and Neff 1969) and an inner endocyst, mostly composed of cellulose, which is only found in the cysts, not in the trophozoites (Neff and Benton 1962). Interestingly, besides glucose, galactose was found to be a major constituent of *Acanthamoeba* cyst walls (Dudley et al. 2009). The ectocyst and endocyst are separated by an

intercystic space and join only at the ostioles, pores covered by opercula, from which the amoebae emerge in the course of excystation (Chávez-Munguía et al. 2005). The ectocyst usually has an irregular surface, while the endocyst is rather smooth. Moreover, the ectocyst is more than twice as thick as the endocyst (650 nm versus 290 nm) and has numerous vesicles (67–167 nm) particularly towards the outer surface. The space between the two cyst walls has an average thickness of 301 nm and appears to be filled with 11-nm-thick filaments connecting the two walls (Lemgruber et al. 2010). The endocyst is primarily composed of cellulose, and it is assumed that cellulose is secreted through vesicles in the periphery region of the encysted amoeba (Lemgruber et al. 2010).

Acanthamoeba cysts maintain viability in the natural environment for at least 25 years (Mazur et al. 1995).

10.2.4 Life Cycle

10.2.4.1 Encystment

The encystment process of *Acanthamoeba* (Fig. 10.3) has been of specific interest since the pathogenic potential of these organisms became more apparent, not only because cyst formation is a typical reaction to treatment, with cysts being highly resistant against a broad variety of agents, but also due to the fact that pathogenic bacteria can persist in the cysts, well-protected from adverse environmental conditions. Encystment is accompanied by morphological changes, termination of cell growth and biochemical modifications. Initially, the amoebae become rounded, followed by a phase, in which the two cyst walls are synthesised. The first wall that is formed gives rise to the ectocyst. Subsequently, after the appearance of a well-defined layer, the endocyst is synthesised (Weisman 1976). Cyst wall synthesis is usually accompanied by a decrease of intracellular macromolecules such as proteins, glycogen and RNA and, particularly, a decrease in cytoplasmic mass by approximately 80 % through gradual dehydration, thereby causing retraction of the protoplast from the cyst wall (Bowers and Korn 1969).

In order to initiate the complex encystment ‘machinery’, acanthamoebae have to sense and react to adverse environmental conditions. In general, natural triggers for encystment are starvation and osmotic stress, while under laboratory conditions, catecholamines (epinephrine, norepinephrine), magnesium and taurine can efficiently induce encystment (Köhsler et al. 2008; Verma et al. 1974). Since high cell densities correlate with higher encystment rates, also quorum-sensing molecules have been suggested to be involved (Fouque et al. 2012). Similar to quorum-sensing molecules, an encystment-enhancing activity (EEA) secreted by amoebae and able to stimulate encystment in a density-dependent manner was reported by Akins and Byers (1980). The genome of *Acanthamoeba* encodes 35 G protein-coupled receptors (GPCRs), which act as sensors for extracellular stimuli and hence constitute potential candidates for the first step in signal transduction at the beginning of encystment (Clarke et al. 2013). This is consistent with the fact that biogenic amines as ligands for GPCRs sufficiently induce encystment and an involvement of GPCRs, in particular β-adrenergic receptors for catecholamines, in the encystment of *Acanthamoeba* has been demonstrated (Krishna Murti and

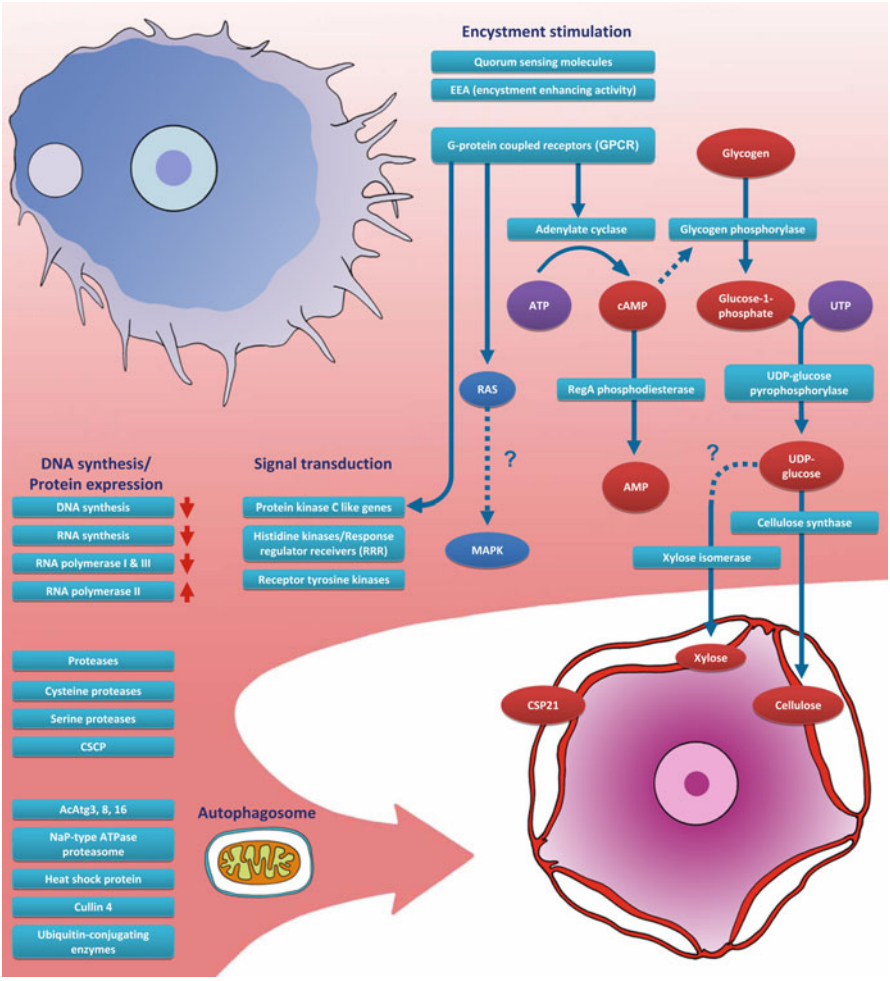

Fig. 10.3 Encystment pathway. Quorum-sensing molecules, EEA, GPCRs, histidine kinases/RRR and receptor tyrosine kinases are suggested to be involved in stimulation and signal transduction of encystment. GPCRs are supposed to activate protein kinase C-like genes, Ras and adenylate cyclase. Adenylate cyclase catalyses the rise of cAMP, leading to activation of glycogen phosphorylase breaking down glycogen for the synthesis of cellulose and xylose. Changes on the DNA/RNA level are indicated by upwards/downwards arrows. Highly expressed proteins involved in the encystment process are shown. *AcAtg Acanthamoeba* autophagy-related protein, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *CSCP* cyst-specific cysteine protease, *CSP21* cyst-specific protein 21, *RRR* response regulator receivers, *UDP* uridine diphosphate, *UTP* uridine triphosphate. Orig

Shukla 1984), and inhibition of β-adrenergic receptors reduces encystment in *Acanthamoeba* (Aqeel et al. 2015). GPCRs are involved in the activation of Ras, a small GTPase, which also has been shown to be involved in encystment, since an inhibitor of farnesylation of Ras leads to decreased encystment. In general, Ras activates the mitogen-activated protein kinase (MAPK) pathway, which, however could not yet be demonstrated during encystment (Dudley et al. 2009). Also activation of adenylate cyclase depends on GPCR-mediated activation of G proteins.

Adenylate cyclase catalyses the conversion from adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Adenylate cyclase activity of *Acanthamoeba* during exponential growth phase is generally low but rises two- to fourfold during the stationary phase to a peak, roughly at the time when cysts are detectable in the cultures (Achar and Weisman 1980). Correspondingly, cAMP levels rise two- to threefold within the first 8 h of encystment (Achar and Weisman 1980). The role of cAMP in encystation is thought to convert glycogen phosphorylase to the active form for glycogen degradation in order to synthesise cellulose for cyst wall formation (Siddiqui et al. 2012). cAMP degradation into AMP on the other hand is catalysed by the enzyme phosphodiesterase, which has been shown to play a crucial role during encystment as well, since inhibition of the phosphodiesterase RegA strongly stimulates encystation and causes a tenfold increase in cellular cAMP levels (Du et al. 2014).

The exact mechanisms for the regulation of signal transduction are far from being fully understood; however, the detection and response to a number of stress conditions are likely to be accomplished with a large set of sensor histidine kinases, with potential receptor function (Clarke et al. 2013). Additionally, response regulator receiver domains usually corresponding to membrane-bound histidine kinases have been detected (Anderson et al. 2005). Also tyrosine kinase-mediated signalling was suggested to be involved in encystment (Dudley et al. 2009) which is corroborated by the detection of potential receptor tyrosine kinases in the genome (Clarke et al. 2013).

Other signalling molecules, which have been suggested to participate in signal transduction pathways during encystation, are protein kinase C-like genes (PKC), which are activated through GPCRs and known to participate in signal transduction pathways that regulate cellular proliferation and differentiation (Hug and Sarre 1993). A great number of PKCs are highly expressed during the early stage of encystment with some of them localised in the cell membrane or nuclear membrane (Moon et al. 2012). The PKCs in *Acanthamoeba* have been identified as atypical PKCs, lacking a Ca^{2+} binding domain and a diacylglycerol (DAG) binding domain, indicating a more primitive composition. Moreover, signalling molecules interacting with PKCs have been detected in expressed sequence tags (ESTs) of *Acanthamoeba* cysts, such as G protein alpha subunit, G protein beta subunit, DAG kinase, tyrosine kinase, tyrosine phosphatase receptor, MAP kinase and calcium binding mitochondrial carrier, corroborating the involvement of PKCs in signal transduction during encystment (Moon et al. 2011).

Glycogen is a storage polysaccharide and is the most rapidly degraded molecule during encystment, since it is used as a precursor of the glucosyl residues of cellulose for cell wall synthesis (Bowers and Korn 1969). Cellular levels of glycogen decrease to between one-half and one-third within the first 4 h of encystment (Weisman et al. 1970). The glycogen breakdown during early encystment has been shown to be at least partly accomplished by the action of glycogen phosphorylase, which catalyses the cleavage of $\alpha(1 \rightarrow 4)$ glycosidic linkages of glycogen, releasing glucose-1-phosphate. The *Acanthamoeba* genome also includes a gene for a predicted glycogen debranching enzyme helping in glycogen breakdown. Interestingly, silencing of glycogen phosphorylase results in incomplete cyst formation, in particular, assembly of the cellulose-containing inner cyst wall could not be completed (Lorenzo-Morales et al. 2008). The expression of glycogen phosphorylase is

restricted to early encystment and appears to be repressed in growing cells. UDP (uridine diphosphate) glucose is synthesised from glucose-1-phosphate and uridine triphosphate catalysed by UDP-glucose pyrophosphorylase. UDP glucose levels increase fivefold within the first 4 h after induction of encystment. Subsequently, UDP glucose is incorporated into the cyst wall as $\beta(1\rightarrow4)$-glycans through the action of cellulose synthase (Aqeel et al. 2013; Moon et al. 2014; Weisman 1976).

Additionally, xylose isomerase appears to be crucial for wall formation (Aqeel et al. 2013). Analysis of cyst walls of *A. castellanii* has revealed that in addition to $\beta(1\rightarrow4)$-glucan-containing cellulose, an important constituent is xylose (Dudley et al. 2009). Obviously, both, cellulose synthase and xylose isomerase, contribute significantly to cyst wall formation (Aqeel et al. 2013; Moon et al. 2014).

Naturally, changes also occur on the DNA and RNA level. Nuclear activity is clearly required during encystment (Roti and Stevens 1975), but normal DNA synthesis is continued only at a significantly reduced rate (Byers et al. 1991). RNA levels decrease by 50 % during encystation and rRNA synthesis is completely shut down after the first 7 h of encystment (Weisman 1976). This is due to a covalent modification of RNA polymerase I, which prevents contact with the DNA-bound transcription factor TF-IB, and a loss of transcription factor TF-IIIA (Matthews et al. 1995). Activities of RNA polymerases I and III decrease during early encystment, while transcription by RNA polymerase II increases significantly (Orfeo and Bateman 1998). It has been shown that the transcription rates of many protein-coding genes are not substantially reduced during the first 16 h of encystment and mRNA pools persist even into the mature cyst stage. However, the increase of polymerase II indicates that additional genes, which might be actively repressed in trophozoites, are transcribed during encystment. Chromatin remodelling was suggested as a mechanism for repression and has been demonstrated to be involved in encystment to a certain extent (Köhsler et al. 2009). Hirukawa et al. (1998) reported a protein expressed exclusively in cysts, the cyst-specific protein CSP21, which is produced during early stages of encystment and associated with the cyst walls. This protein has been shown to be actively repressed in trophozoites; however, since chromatin remodelling was ruled out as repressing mechanism for CSP21, the exact mode of action remains to be established (Chen et al. 2004).

Cytoskeleton rearrangements are crucial for encystment. Rho kinase, which is involved in regulation of actin polymerisation, and the gelation factor, involved in actin cross-linking, have both been demonstrated to play an important role in the course of differentiation (Bouyer et al. 2009; Dudley et al. 2009).

Likewise, several proteases play an important role during the encystment process. A subtilisin-like serine protease, demonstrated in encysting cells, was suggested to be involved in promoting autolysis, since it associates with autophagosomes (Moon et al. 2008). Leitsch et al. (2010) demonstrated the involvement of cysteine proteases and suggested a promoting role for serine proteases, which could serve as mediators for the release of cysteine proteases by promoting the maturation of autophagosomes. Additionally, a specific role for cyst-specific cysteine proteases (CSCPs) in mitochondrial autolysis has been demonstrated. Since cysts require significantly fewer mitochondria, large numbers are degraded in the course of encystment mediated by CSCPs, probably providing macromolecules for building up the cyst walls (Moon et al. 2012).

Other factors related to differentiation of *Acanthamoeba* are P-type ATPases, proteasome and heat shock proteins, cullin 4, and ubiquitin-conjugating enzymes (Moon et al. 2008). Additionally, specific *Acanthamoeba* autophagy-related proteins (AcAtg) which are essential components of the autophagic machinery during encystment have been detected. AcAtg8 is involved in the formation of the autophagosomal membrane, while AcAtg3 plays an important role in AcAtg8 lipidation (Moon et al. 2009). AcAtg16 was found to be associated with small or large vesicular structures that partially co-localise with autophagolysosomes (Song et al. 2012).

Although encystment has been a focus of research in *Acanthamoeba* and many factors contributing to this complex process have been established lately, the interplay of all molecules involved is still far from being fully understood and clearly an important subject for future studies since the encystment process constitutes a promising target for specific therapeutic measures in *Acanthamoeba* infections.

10.2.4.2 Excystment

For the excystment process of *Acanthamoeba*, only very limited information is available. Excystment occurs under favourable conditions through the ostioles after removal of the operculum, leaving behind the outer shell. While the actual hatching from the cysts only lasts a couple of minutes, the entire excystment process, excystment takes around 12 h (Mattar and Byers 1971). The evidence of genes in the *Acanthamoeba* genome encoding cellulase and cellobiosidase corroborates the hypothesis that the amoebae break down cellulose from their own cyst walls and utilise it as a food source in the course of excystment (Anderson et al. 2005). This may, however, depend on general food supply, as under lab conditions with an oversupply of nutrients, intact empty cyst walls can be observed frequently.

10.2.5 Metabolism

Acanthamoeba has a heterotrophic metabolism and generally requires organic substrates for its growth and development. However, the metabolic and biosynthetic capabilities of acanthamoebae are very broad and they can indeed thrive on almost anything.

Generally, acanthamoebae produce energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation in the mitochondria with a functional tricarboxylic acid (TCA) cycle and electron transport chain (ETC) similar to those found in other aerobic eukaryotes (Fig. 10.4). In short, under aerobic conditions, pyruvate is decarboxylated via the pyruvate dehydrogenase (PDH) multi-enzyme complex and passed as acetyl-CoA into the TCA cycle, where reducing equivalents in the form of NADH (nicotinamide adenine dinucleotide) are generated and subsequently oxidised by the electron transport chain, resulting in an electrochemical gradient, which is utilised by the ATP synthase for the synthesis of ATP. In *A. castellanii*, the presence of all components of PDH, all subunits associated with the enzymatic activities of the TCA cycle and an ETC comprising all five complexes for oxidative phosphorylation has been confirmed (Gawryluk et al. 2012, 2014).

In addition, also a functional glyoxylate cycle, an anabolic variant of the TCA cycle for the synthesis of carbohydrates from acetyl-CoA by β-oxidation of fatty

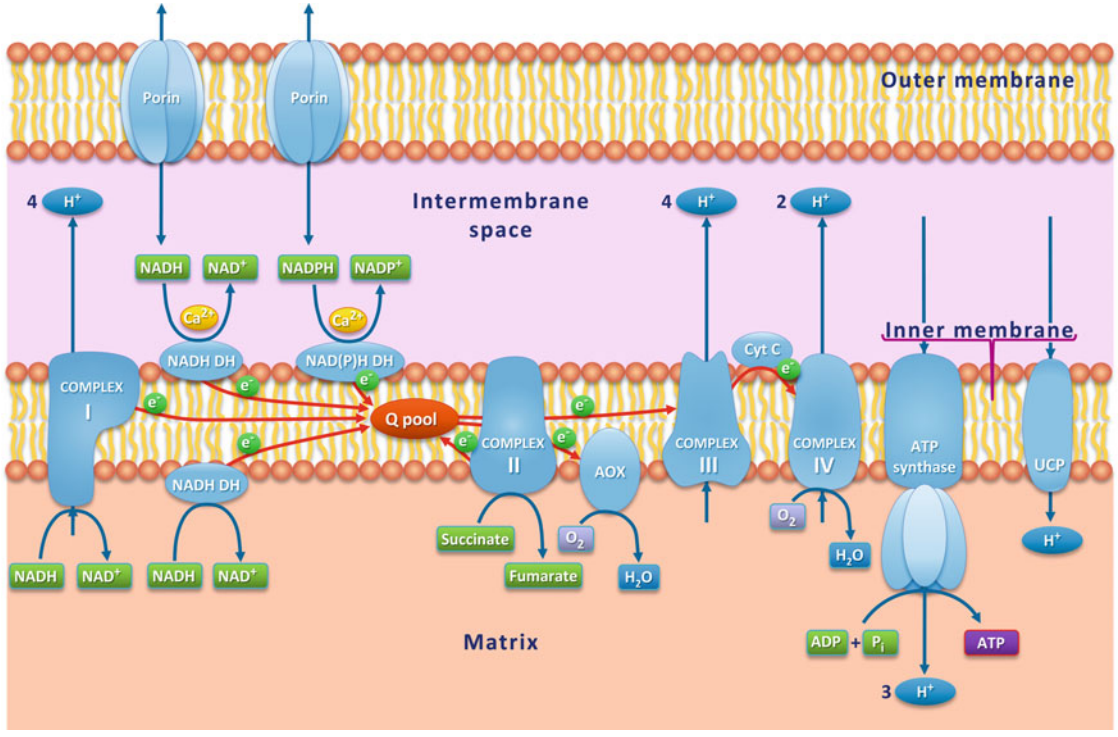

Fig. 10.4 ETC and ATP synthesis of *Acanthamoeba*. *Acanthamoeba* possess a plant-type respiratory chain. In addition to the four standard protein complexes (I–IV), the ETC contains additional enzymes for electron transport – an internal and external NADH DH and an external NAD(P)H DH. Alternative NADH DHs and NAD(P)H DH and complex I and II reduce ubiquinone (Q). AOX can bypass complexes III and IV and act as terminal oxidase, while UCP can bypass ATP synthase, both resulting in a lower ATP yield. *AOX* alternative oxidase, *DH* dehydrogenase, *NADH* nicotinamide adenine dinucleotide, *NADPH* nicotinamide adenine dinucleotide phosphate, *Q* ubiquinone (coenzyme Q), *UCP* uncoupling protein. Orig

acids has been demonstrated. The two key enzymes, isocitrate lyase (ICL) and malate synthase (MalS), have been detected and suggested to be fused in the mature protein (Gawryluk et al. 2012; Tomlinson 1967). ICL and MalS have also been suggested to be involved in the encystment process by providing glucose from lipid sources (Mehdi and Garg 1987). Interestingly, while in other organisms the glyoxylate cycle takes place in specialised peroxisomes, the glyoxysomes, in *Acanthamoeba* the site of the glyoxylate cycle appears to be the mitochondria. The presence of MalS and ICL as well as other glyoxylate cycle enzymes, such as citrate synthase, aconitate hydratase and malate dehydrogenase, has been demonstrated in the mitochondria (Gawryluk et al. 2012, 2014).

The mitochondria of *A. castellanii* possess a plant-type respiratory chain, which is highly branched and has additional electron transport complexes, bypassing the conventional complexes of oxidative phosphorylation, thereby dissipating redox energy instead of building up an electrochemical proton gradient. Complex I, the rotenone-sensitive NADH:ubiquinone oxidoreductase, situated in the inner mitochondrial membrane, can be bypassed by alternative NADH and NAD(P)H dehydrogenases (DH), namely, a rotenone-insensitive internal NADH DH (Hryniewiecka 1986), a rotenone-insensitive external NADH DH, located on the outer surface of the inner mitochondrial membrane (Hryniewiecka et al. 1980; Lloyd and Griffiths 1968), and a rotenone-insensitive, calcium-dependent external NAD(P)H DH (Antos-Krzeminska and Jarmuszkiewicz 2014). These alternative NADH and NAD(P)H DHs transport electrons but do not contribute to proton pumping. By oxidising cytosolic and matrix NADH and NAD(P)H, alternative NADH and NAD(P)H DHs together with complex I and II reduce a common ubiquinone pool. The exact physiological role of these alternative enzymes in *Acanthamoeba* has not been established yet; however, it has been suggested that these enzymes might enable the cell to adapt to changes in the surrounding environment more rapidly and make the mitochondrial respiratory chain more flexible by regulating the redox state of cytoplasmic and mitochondrial matrix NAD(P)H pools (Antos-Krzeminska and Jarmuszkiewicz 2014; Rasmusson et al. 2004). Possible functions were also assumed to be thermogenesis, oxidation of excess carbohydrates, oxidation of excess reductant for continuation of metabolic pathways, cold resistance and avoidance of reactive oxygen species (ROS) formation (Rasmusson et al. 2004).

In addition to alternative DHs, a cyanide-insensitive alternative oxidase (AOX) constitutes a bypass for complexes III and IV (Edwards and Lloyd 1978), and an alternative proton transporter, the uncoupling protein (UCP), allows proton flow from the intermembrane space to the matrix without ATP synthesis, thereby bypassing ATP synthase (Jarmuszkiewicz et al. 1999). Both systems lead to a decrease in ATP synthesis yield and are supposed to control the cellular energy balance as well as prevent the cell against the production of ROS.

An alternative respiratory pathway in *Acanthamoeba* spp. was first reported in 1978 (Edwards and Lloyd 1978), and AOX was first described in 1997 (Jarmuszkiewicz et al. 1997). In general, the AOX pathway branches from the main respiratory chain after ubiquinone, where AOX can potentially act as terminal oxidase. However, in response to low temperatures, it has been shown that AOX is upregulated without disturbing the conventional cytochrome pathway of complexes

III and IV (Jarmuszkiewicz et al. 2001). AOX was shown to be stimulated by purine nucleoside 5′-monophosphates, with guanosine monophosphate (GMP) being the most efficient. In addition to the binding of GMP, its activity is determined by the redox state of ubiquinone (coenzyme Q) as well as the matrix pH (Jarmuszkiewicz et al. 2002a, b). A possible function of AOX in *Acanthamoeba* is thought to be an alternative route for respiration during periods of oxidative stress (Czarna and Jarmuszkiewicz 2005; Jarmuszkiewicz et al. 2001).

Also, the *Acanthamoeba* UCPs are well studied and were first demonstrated in 1999 (Jarmuszkiewicz et al. 1999). UCPs are members of the mitochondrial anion carrier protein (MACP) family and are present in the mitochondrial inner membrane, where they mediate the free fatty acid (FFA)-activated, PN (purine nucleotide)-inhibited H+ leak that can divert energy from oxidative phosphorylation, thereby modulating the coupling of mitochondrial respiration and ATP synthesis (Jarmuszkiewicz et al. 2005). Additionally, UCP is activated by 4-hydroxy-2-nonenal (HNE), a reactive unsaturated aldehyde, which was shown to induce PN-sensitive uncoupling in *A. castellanii* (Woyda-Ploszczyca and Jarmuszkiewicz 2012). HNE is the most abundant end product of membrane lipid peroxidation, for which increased ROS production is a prerequisite. It has been shown that the redox state of membranous coenzyme Q is particularly important for the inhibition by PNs of FFA- and HNE-activated UCP in phosphorylating and non-phosphorylating mitochondria of *A. castellanii*, with a high coenzyme Q reduction level being essential for UCPs' activity, while the inhibitory effect of PNs can only be observed when coenzyme Q is sufficiently oxidised (Jarmuszkiewicz et al. 2005; Swida et al. 2008; Woyda-Ploszczyca and Jarmuszkiewicz 2013).

Through its redox state, coenzyme Q appears to be an ideal regulator for both AOX and UCP activity since it is able to sense the ATP demand of the cell. At a high ATP demand and low substrate availability, a low reduction level of coenzyme Q leads to inactivation of UCP and AOX and efficient ATP synthesis, while at low ATP demand and high substrate availability through a high reduction state of coenzyme Q, UCP and AOX are activated and might work as a safety valve to avoid overload in reducing power and ROS formation (Jarmuszkiewicz et al. 2010). In contrast to other organisms, where UCP and AOX do not occur simultaneously or are co-regulated by FFAs with an inhibitory effect on AOX (plants, fungi), in *Acanthamoeba* UCP and AOX appear to work in concert with a cumulative effect on the efficiency of oxidative phosphorylation. In particular, the protection against mitochondrial ROS production appears to be the physiological role of UCP and AOX, which might act as antioxidant systems to prevent damage to the cell at the level of energy production, but at the expense of oxidative phosphorylation (Czarna and Jarmuszkiewicz 2005; Czarna et al. 2007).

In addition to conventional aerobic respiration the presence of a complete hydrogenosome-like ATP generation pathway with a complete set of enzymes has been detected, probably functioning under anaerobic conditions (Leger et al. 2013). This anaerobic ATP generation pathway is similar to that found in *T. vaginalis* hydrogenosomes and includes the following enzymes: pyruvate ferredoxin

oxidoreductase (PFO); FeFe-hydrogenase; maturases HydE, HydG and HydF; and succinate CoA transferase. Interestingly, this hydrogenase exists in the presence of aerobic mitochondria and might thus, in contrast to the ones found, e.g. in *Trichomonas* or *Entamoeba*, constitute an oxygen-resistant hydrogenase, meaning that it rather stably could produce hydrogen even in the presence of air.

10.2.6 Pathogenesis

Different *Acanthamoeba* strains do not seem to be equally pathogenic, but it has not yet been really clarified, whether pathogenicity is a distinct character of specific strains or whether all strains are potentially pathogenic. Pathogenicity of *Acanthamoeba* isolates is usually assessed by animal inoculation, either into the eye (pig or hamster) or intranasally into the brain (mouse), or by evaluating cytopathic effects on various cell lines. Also, virulence usually correlates to high growth rate and temperature tolerance (Griffin 1972; Walochnik et al. 2000), but all these characters have shown to be rather epigenetically regulated than genetically defined (Köhsler et al. 2009; Pumidonming et al. 2010).

Generally, pathogenesis in *Acanthamoeba* is characterised by adhesion to the host cells, contact-mediated cytolysis and immunoreaction of the host. A scheme of the current understanding of this process is given in Fig. 10.5. Most studies on *Acanthamoeba* pathogenesis have focused on *Acanthamoeba* keratitis; however, the general steps can be assumed to be comparable in *Acanthamoeba* GAE. In AK, the amoebae particularly accumulate around the corneal nerves, which leads to radial neuritis and is the reason for the violent pain.

10.2.6.1 Adhesion

The acanthopodia allow the trophozoite to interact with other cells, the adhesins in the membrane anchoring to the cytoskeleton network (González-Robles et al. 2008). *Acanthamoeba* has a complex repertoire of surface proteins and glycoproteins that can potentially act as receptor adhesins to the host cells.

In *Acanthamoeba* keratitis, the first critical step in the pathogenesis is the adhesion of the amoebae to the surface of the cornea, which has been shown to be primarily mediated by a lectin, the mannose-binding protein (MBP), recognising mannosylated glycoproteins in the membrane of other cells and adhering to them (Garate et al. 2004, 2005; Yang et al. 1997). This *Acanthamoeba* lectin binds methyl-α-D-mannopyranoside and α – 1-3-D-mannobiose with highest affinity, and this adhesion is necessary for amoeba-induced cytolysis of target cells (Cao et al. 1998). The mannosylated glycoproteins on the cornea have been shown to be increasingly expressed in the course of corneal abrasion, a leading risk factor for the development of AK (Jaison et al. 1998). MPB is a 400 kDa protein that is composed of multiple 130 kDa subunits. It consists of a large N-terminal extracellular domain, a transmembrane domain and a short C-terminal cytoplasmic domain and has characteristics of a typical cell surface receptor (Garate et al. 2004). The cytoplasmic domain contains a number of phosphorylation sites and

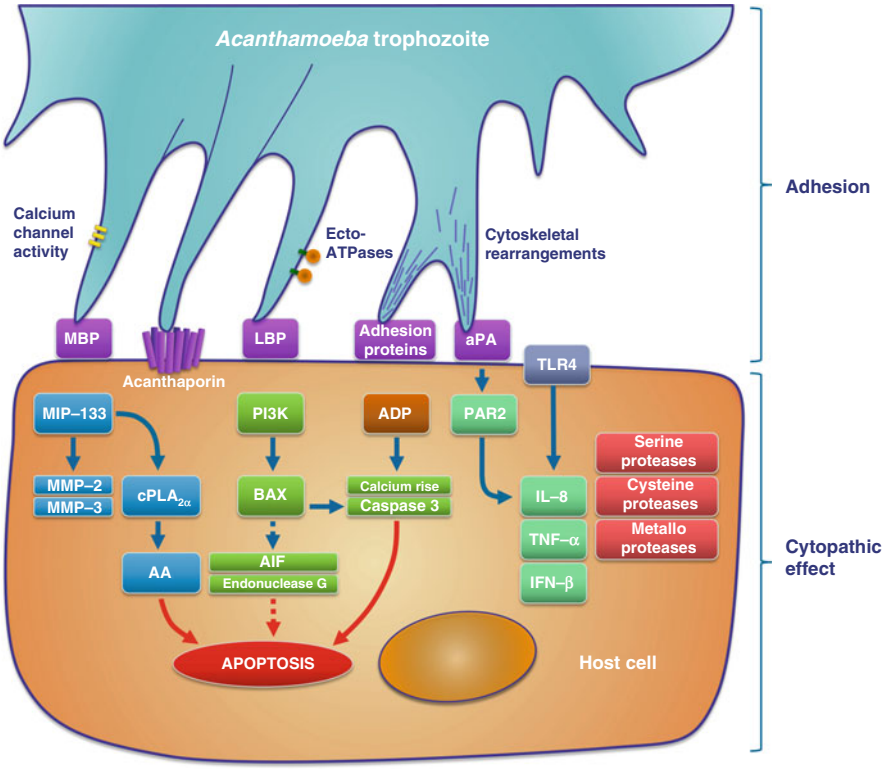

Fig. 10.5 Pathogenesis of *Acanthamoeba*. Adhesion to host cells is accompanied by calcium channel activity and cytoskeletal rearrangements and accomplished by MBP, LBP, ecto-ATPases and other adhesion proteins. Additionally, host cells can be permeabilised by the pore-forming protein acanthaporin. The secretion of MIP-133 leads to activation of MMPs and cPLA2α, the latter leading to the release of AA stimulating apoptosis. Alternatively, apoptosis might be induced by PI3K signalling pathways and ADP release. Host cell receptors involved are PAR2, activated by aPA resulting in IL-8 production and TLR4, inducing the secretion of IL-8 and TNF-α or IFN-β. Additionally, several proteases contribute to the cytopathic effect of *Acanthamoeba*. *AA* arachidonic acid, *ADP* adenosine diphosphate, *AIF* apoptosis-inducing factor, *aPA Acanthamoeba* plasminogen activator, *cPLA* cytosolic phospholipase A, *IFN* interferon, *IL* interleukin, *LBP* laminin-binding protein, *MBP* mannose-binding protein, *MMP* matrix metalloprotease, *PAR* protease-activated receptor, *PI3K* phosphatidylinositol 3-kinase, *TLR* toll-like receptor, *TNF* tumour necrosis factor. Orig

an NPLF motif, known for its ability to participate in cell signalling events leading to cell spreading and shape change. Size, number and location of the carbohydrate recognition domains (CRDs) of the MBP of *Acanthamoeba* remain to be determined; however, it appears that MBP contains at least one novel CRD, which lacks sequence similarity to well-characterised lectin CRDs (Panjwani 2010). For initiation of AK, amoebae adhere to the surface of the cornea via the CRD of the MBP, which leads to signal transduction events via the cytoplasmic domain, eventually resulting in the expression of components inducing the cytopathic effect (CPE).

However, since the adherence to host cells is a complex process, numerous other molecules have been suggested to be involved as well. For example, a 207 kDa adhesion molecule that does not bind to mannose has been detected by monoclonal antibodies blocking the adhesion of trophozoites to human corneal epithelial cells (HCEs) (Kennett et al. 1999). Furthermore, 16 potentially involved surface proteins, eight mannose glycoproteins and eight glycoproteins with N-acetyl glucosamine residues, were detected in *Acanthamoeba* (Soto-Arredondo et al. 2014). Since HCEs express proteins with Glc*N*Ac and Man residues on their surface (Panjwani et al. 1995), these proteins might represent potential receptor adhesins, corroborated by the fact that most of these proteins have been demonstrated to interact with neuronal and epithelial cells (Soto-Arredondo et al. 2014). Interestingly, *Acanthamoeba* seems to possess all ER glycosyltransferases involved in N-glycosylation as well as unusual fucosyl- and pentosyltransferases in the Golgi (Schiller et al. 2012).

Additionally, the interplay of amoebae and components of the host extracellular matrix (ECM) may be essential for adhesion and subsequent invasion. It has been reported that *A. polyphaga* binds to three different elements of the ECM: the basement membrane components laminin and collagen IV and the adhesive glycoprotein fibronectin (Gordon et al. 1993). In that context, a 28.2 kDa laminin-binding protein (LBP), which shows a definite binding specificity to mammalian laminin and higher expression levels in pathogenic strains (Hong et al. 2004), and a 54 kDa LBP protein, in that study only expressed in a pathogenic *A. culbertsoni* strain, have been described (Rocha-Azevedo et al. 2010). However, the actual involvement of other proteins than the MBP in the course of infection remains to be determined.

In addition to binding molecules, an important prerequisite for the establishment of a CPE are calcium channel activity and cytoskeletal rearrangements involving Rho-associated pathways (Taylor et al. 1995). Cytoskeletal participation is not only obvious due to formation of membrane structures like acanthopodia but also based on the fact that adhesins are strongly anchored to the cytoskeleton and disintegration of cytoskeletal elements leads to physiological changes for adhesins (Soto-Arredondo et al. 2014).

10.2.6.2 Cytopathic Effect

The next step in an AK is the release of a 133-kDa serine protease termed mannose-induced protein (MIP) 133, which is produced upon exposure to mannose and interacts with mannose receptors on the cell membrane of *Acanthamoeba* (Leher et al. 1998a). The expression of MIP-133 and the ability to cause disease have been shown to be directly related, since MIP-133 is expressed in clinical but not in soil isolates (Hurt et al. 2003a). The generation of MIP-133 seems to be a crucial component of the pathogenic cascade of *Acanthamoeba* keratitis, and several mechanisms have been described how the interaction of MIP-133 and host cells leads to cytolysis and desquamation. MIP-133 activates matrix metalloproteases (MMP), which have been shown to be expressed by corneal cells in response to pathogenic microorganisms (Fini et al. 1992). They are secreted as inactive enzymes and activated extracellularly via pathway-dependent proteases and participate in tissue degradation and remodelling under physiological and pathological conditions. In

HCEs the interaction with MIP-133 has been shown to lead to a two- to fourfold increase of the expression of MMP-2 and MMP-3, potentially facilitating the invasion of trophozoites (Alizadeh et al. 2008). Furthermore, MIP-133 can degrade human collagen types I and IV and is able to induce apoptosis via a caspase-3-dependent pathway (Hurt et al. 2003a). The mechanism to induce apoptosis through MIP-133 is supposed to be an interaction with phospholipids on the plasma membrane of corneal cells in order to activate cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) (Tripathi et al. 2012). cPLAs are involved in cell signalling processes, such as inflammatory responses, and lead to the release of arachidonic acid (AA), which stimulates apoptosis through activation of the mitochondrial pathway and is associated with loss of cell viability, caspase activation and DNA fragmentation (Taketo and Sonoshita 2002). $cPLA_{2\alpha}$ is regulated via phosphorylation by MAPKs at amino acid residue serine-505 or by Ca^{2+} to become its soluble form (p-$cPLA_{2\alpha}$), which translocates to the plasma membrane (Leslie 1997). In *Acanthamoeba* it still has to be determined, whether phosphorylation by MAPKs or interaction of MIP-133 with phosphatidylserine, a phospholipid exposed on the outer cell membrane of HCEs during apoptosis, induce $cPLA_{2\alpha}$. Furthermore, $cPLA_{2\alpha}$ appears to be involved in the production of chemokine (C-X-C motif) ligand 2 (CXCL2), which has a chemotactic effect on neutrophils (Tripathi et al. 2013). Collectively, activation of $cPLA_{2\alpha}$ induced by MIP-133 was shown to initiate a rapid immune response in corneal epithelial cells by inducing cytolysis and stimulating the accumulation of polymorphonuclear leukocytes (PMNs), accompanied by the production of IL-8, IL-6, IFN-γ and IL-1β (Tripathi et al. 2012). However, in human brain microvascular endothelial cells (HBMEC) activation of phosphatidylinositol 3-kinase (PI3K) was shown to be a prerequisite for apoptosis, since direct inhibition of PI3K significantly reduced *Acanthamoeba*-mediated HBMEC death (Sissons et al. 2005). In myeloma cells, it was demonstrated that downstream effectors of PI3K-mediated apoptosis involve activation of the pro-apoptotic proteins Bak and Bax (Thyrell et al. 2004).

Generally, activation of Bax (and other pro-apoptotic proteins) results in increased permeability of the outer mitochondrial membrane, which gives rise to the release of cytochrome c and other pro-apoptotic factors, leading to activation of caspases. In fact, in neuroblastoma cells, evidence for an involvement of the protein Bax in apoptosis via the mitochondrial pathway induced by *Acanthamoeba* has been demonstrated (Chusattayanond et al. 2010). In addition to the caspase-dependent pathway, also a caspase-independent pathway via upregulation of Bax was proposed, possibly involving apoptosis-inducing factor (AIF) and endonuclease G, which are released upon membrane permeabilisation induced by Bax activity and lead to apoptosis in a caspase-independent manner. They translocate to the nucleus and induce chromatin condensation and/or large-scale DNA fragmentation (Bröker et al. 2005).

An alternative way on how acanthamoebae accomplish to induce host cell death, which must be distinguished from protein-induced apoptosis and may reflect an additional strategy to overcome the host defence, is by release of adenosine diphosphate (ADP), which in vitro affects human epithelial cells by a

process that begins with a rise of cytosolic free-calcium concentrations and culminates in apoptosis (Mattana et al. 2001). In human monocytes, the effect of ADP is mediated by specific $P2y_2$ purinergic receptors (purinoceptors) expressed on the monocyte cell membrane and leads to a substantial rise of calcium and caspase-3 activation. Furthermore, ADP together with other excreted not yet identified soluble compounds has been shown not only to induce apoptosis but also to stimulate the secretion of pro-inflammatory cytokines (TNF-α and IL-1β) and IL-6 in human monocytes (Mattana et al. 2001, 2002). In this context, a potential role of ecto-ATPases in *Acanthamoeba* pathogenesis was suggested as well, since pathogenic and non-pathogenic *Acanthamoeba* strains exhibit distinct ecto-ATPases. In pathogenic strains, inhibition of these leads to decreased host cell cytotoxicity. Ecto-ATPases are glycoproteins in the plasma membrane with their active sites facing outward and hydrolysing extracellular ATP to ADP and phosphate. Since ecto-ATPases are supposed to be involved in cellular adhesion, also an association of ecto-ATPases and the MBP has been suggested, since mannose increases ecto-ATPase activities in pathogenic strains (Sissons et al. 2004).

Another important enzyme involved in the pathogenesis of AK is a 40 kDa *Acanthamoeba* plasminogen activator (aPA), a serine protease expressed from pathogenic, but not from non-pathogenic strains, activating plasminogen from several mammalian species including humans (Mitra et al. 1995). It has been demonstrated that the pathogenic potential of *Acanthamoeba* species closely correlates with aPA secretion, which facilitates the penetration of trophozoites through the basement membrane (Alizadeh et al. 2007). Four protease-activated receptors (PARs) have been identified so far (PAR1-4). Among these, functional PAR1 and PAR2 were shown to be expressed by human corneal epithelial cells (Lang et al. 2003). PARs are cleaved by proteases at an activation site, which eventually leads to activation and signalling. For *Acanthamoeba*, a specific activation of the PAR2 pathway by aPA has been demonstrated, resulting in the expression and production of IL-8 (Tripathi et al. 2014).

Also suggested to participate in the pathogenic cascade of *Acanthamoeba* infections is a newly identified pore-forming protein of pathogenic acanthamoebae, the so-called acanthaporin (Michalek et al. 2013). Pore-forming proteins are well-known virulence factors and are considered essential for host tissue destruction. In other pathogenic 'amoebae', including *Entamoeba histolytica* (amoebapore A) and *Naegleria fowleri* (naegleriapores), the existence and function of amoebapores have been described years ago (Leippe et al. 1994; Young and Lowrey 1989). Structurally, these proteins are members of the saposin-like protein (SAPLIP) family (Kolter et al. 2005). Interestingly, acanthaporin does not resemble previously described SAPLIP proteins, but its structure represents an entirely new protein fold, with which membranes can be permeabilised. Acanthaporins seem to be amphipathic; thus, a switch from the soluble form to a membrane-inserted state might be facilitated. Additionally, a very distinct activation mechanism for pore formation has been proposed. While in amoebapore A, at low pH, a protonated single histidine residue triggers the formation of an active dimer from inactive monomers to form a

hexameric pore (Leippe et al. 2005), acanthaporin becomes activated after protonation of histidine residues within the C-terminal helix by the transition from an inactive dimer to the active monomer resulting in the formation of oligomeric pores. Acanthaporin has been shown to be cytotoxic for human neuronal cells and a variety of bacterial strains by permeabilising their membranes, and its proposed primary function could be the combat against the growth of engulfed bacteria inside phagosomes. However, based on the contact-dependent cytotoxic reactions observed upon interaction of acanthaporin and HCEs, acanthaporin might as well take an effect extracellularly and contribute to the pathogenic cascade ultimately resulting in host cell death.

Several proteases have been described in *Acanthamoeba* that might play a role in phagocytosis, but might as well contribute to the penetration and dissolution of host tissue since proteolytic activities have been demonstrated for most of them. Some of these proteases seem to be specifically excreted by clinical isolates, indicating their potential as virulence factors. Upon contact to host cells, a 97 kDa serine protease is markedly upregulated as well as a cytotoxic 80 kDa metalloprotease, which, however, depends on mannose-mediated adhesion (Cao et al. 1998). A 65 kDa cysteine protease only expressed in pathogenic strains has also been suggested to be involved in pathogenicity, as well as a 150 kDa metalloprotease from an *Acanthamoeba* strain isolated from a GAE patient (Alsam et al. 2005; Hadas and Mazur 1993).

10.2.6.3 Immunobiology

Disseminating *Acanthamoeba* infections are clearly linked to immunodeficiency. And despite the ubiquitous nature of the acanthamoebae and many million contact lens wearers worldwide, also AK is very rare, suggesting that besides an impaired corneal surface, also factors of the host's immune system might influence the incidence and severity of this infection (Niederkorn 2002). Acanthamoebae generally are a source of constant antigenic stimulation. It has been shown in numerous studies that the vast majority of humans, including AK patients, have specific antibodies against these amoebae.

The most abundant immunoglobulin in mammalian tears is IgA, and IgA provides an immunological barrier for microorganisms, blocking their adherence to epithelial cells. AK patients seem to have lowered IgA levels, and this might constitute a supplementary risk factor for generating AK. If trophozoites achieve to bind to epithelial cells, other IgA-dependent elements of the immune system may be activated, including the complement system or inflammatory cells. The innate immune system, particularly macrophages, neutrophils and the complement system, is assumed to play an important role in the resistance against infections with *Acanthamoeba*. The presence of IgA together with the activation of the alternative pathway might serve as a first-line defence (Cursons et al. 1980; Niederkorn et al. 2002; Walochnik et al. 2001).

Macrophages may provide protection against AK, particularly in the very early stage of the infection. Macrophages are chemotactically attracted to *Acanthamoeba* trophozoites and have been shown to kill them in vitro, which is

even enhanced in the presence of specific antibodies (Marciano-Cabral and Toney 1998). Inflammatory infiltrates in *Acanthamoeba* infections are mainly composed of neutrophils, indicating that also neutrophils play an important role in the immunity to acanthamoebae. Also, neutrophils are more efficient than macrophages in killing *Acanthamoeba* cysts (Hurt et al. 2003b). Further, although the main localisations of *Acanthamoeba* in the human body, the eye and the brain, are immune-privileged sites, also complement components are continuously active at low levels at these sites (Bora et al. 2008; Woodruff et al. 2010). Thus, strains causing infections have to circumvent the action of activated complement proteins to invade and multiply. In vitro, acanthamoebae are killed by a complement-dependent process activated via the alternative pathway (Pumidonming et al. 2011). A concerted action of C5, C6, C7, C8 and C9 leads to membrane damage and in the following to lysis of the amoeba cell (Ferrante and Rowan-Kelly 1983). This is consistent with findings that membranes of acanthamoebae lack sialic acid (Ulsamer et al. 1971). Sialic acid on plasma membranes prevents the activation of the alternative pathway; thus, organisms with sialic acid are spared with this natural host defence mechanism. The uncontrolled activation of complement is also at least partially responsible for oedema and damage to blood vessel walls that are characteristic in *Acanthamoeba* infections.

Specific antibodies increase the amoebolytic capability of the immune system, inhibit host cell adherence and phagocytosis and seem to block the action of amoebal enzymes, thus neutralising their cytopathogenic effects (Ferrante and Abell 1986; Marciano-Cabral and Toney 1998; Stewart et al. 1994). They are most likely even transferred placentally as anti-*Acanthamoeba* antibodies can also be found in human cord blood (Cursons et al. 1980). However, immunological memory is not established in AK. In GAE, generally, immunoglobulin levels below the normal range are typical (Im and Kim 1998).

Host cell receptors that have been shown to be involved in *Acanthamoeba* recognition are Toll-like receptors (TLR), in particular TLR4 (Ren et al. 2010). TLRs recognise specific pathogen-associated molecular patterns leading to the activation of an inflammatory signalling cascade producing pro-inflammatory cytokines and chemokines. TLR4 is a unique member of the TLR family in that it signals through two distinct signalling pathways, the MyD88-dependent and TRIF-dependent pathway (the TIR domain-containing adapter inducing IFN-β). On ligand binding, most TLRs recruit the adapter molecule myeloid differentiation protein 88 (MyD88), which eventually results in induction of cytokines such as IL-6, IL-8 and TNF-α and chemokines (MyD88-dependent pathway). The TRIF-dependent pathway (or MyD88-independent pathway) uses TRIF to induce the activation of IFN-β and interferon-induced genes (Medzhitov et al. 1998; Yamamoto et al. 2002). Upon interaction of HCEs with *Acanthamoeba* trophozoites, the TLR4–MyD88 pathway is activated early and induces the secretion of IL-8 and TNF-α, while the TLR4–ERK1/2 pathway is activated later and induces the production of IFN-β (Ren et al. 2010; Ren and Wu 2011). Additionally, it was shown that clinical but not soil isolates induce an upregulation of TLR4 with production of IL-8 and CXCL2 gene

expression, which is of particular interest since IL-8 and CXCL2 are chemoattractants known to attract PMN to a site of infection, which is critical to disease severity in AK (Alizadeh et al. 2014).

Immunisation of mice with sonicated *Acanthamoeba* antigen induces a significant and moreover highly specific protection against a lethal challenge with this amoeba (Rowan-Kelly and Ferrante 1984). Successful immunisation against *Acanthamoeba* keratitis has also been demonstrated in a pig model (Alizadeh et al. 1995) and in Chinese hamsters (Leher et al. 1998b). However, recrudescence in AK suggests that corneal infection does not induce protective immunity. Moreover, it has been demonstrated that pathogenic acanthamoebae can evade the antibody-dependent amoebicidal activity of macrophages, are more resistant to the lytic activity of complement and can degrade human IgG and IgA antibodies (Kong et al. 2000; Marciano-Cabral and Toney 1998; Toney and Marciano-Cabral 1998).

In summary, one can assume that amoebae invading the body via skin lesions or lung tissue are opsonised by antibodies and complement. Antibodies may control the invasiveness of the amoebae by preventing amoebic adherence to tissue cells, neutralising cytolytic molecules and inhibiting amoebic phagocytosis. Subsequently phagocytic cells such as neutrophils are attracted to the infection site. A decreased antibody production, lowered complement levels, inhibited leukocyte function or an impaired lymphokine production result in a reduced resistance. A persistent non-ocular *Acanthamoeba* infection is the consequence of an immunodeficiency. In AK, immunobiology certainly also plays role; however, the details remain to be fully established.

10.3 Perspectives and Open Questions

Acanthamoeba is a particularly easy-to-culture eukaryote with very low requirements but a complex and highly evolved cellular repertoire. It thus represents an excellent model organism for cell biological studies in general, and with its similarity to phagocytotic cells of the mammalian immune system, it is also an interesting model for investigating infection processes at the cellular level. The completion of the first genome project and further genome projects expected to be finished soon gave this field of research a significant push forward, and it will certainly further expand in the future.

Also the surface structures of *Acanthamoeba* spp. are highly interesting and partially even unique. As there is evidence for differences in the glycomes between clinical and non-clinical isolates and as cell-cell attachment is the crucial step in the infection process, the elucidation of these structures will significantly advance our understanding of *Acanthamoeba* pathogenicity. Fact is, they are capable of synthesising a large number of highly uncommon N-glycans, including several entirely novel structures still waiting to be explored. Apart from their importance for attachment, these molecules may also represent interesting targets for specific treatment, which is still lacking.

Further, the role of *Acanthamoeba* spp. as permanent and transient host cells and vehicles for microorganisms, including not only bacteria of various clades but also fungi and viruses, has received increasing attention in the recent past. The

dimension and the importance of microbial interactions have long been seriously underestimated and are just now beginning to be understood. This field will certainly bring novel and fascinating findings in the next years.

And finally, the Amoebozoa, being phylogenetically relatively close to the animals and an extremely diverse group, are also highly interesting with respect to evolution, particularly to the emergence of multicellularity and sex. The complex cell communication machinery of *Acanthamoeba* possibly representing a precursor of multicellularity and the availability and significance of meiosis in amoebozoan taxa remain to be elucidated.

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Entamoeba

11

Michael Duchêne

Abstract

Entamoeba histolytica is an obligate human intestinal parasite causing amoebic dysentery and liver abscess. *Entamoeba dispar* is its close largely nonpathogenic relative. Both species are well adapted to the human host. The motile amoebae called trophozoites dwell in the lumen of the large intestine; in its distal part, they differentiate to cysts, which are excreted and can survive in the environment and infect new hosts. Although metronidazole and other nitroimidazoles represent an efficient treatment, there are more than 55,000 deaths worldwide every year.

The *E. histolytica* genome project led to a better understanding of the molecular equipment; two surprises were the significant number of genes acquired from bacteria by lateral gene transfer and a very high number of protein kinases and phosphatases underscoring the complex signal transduction pathways in this parasite.

The parasite attacks human cells by first attaching to them via a galactose and N-acetylgalactose (Gal/GalNAc)-specific lectin. The host cell membrane is pierced by a pore-forming protein called amoebapore, and cysteine proteinases destroy cellular components, antibodies and complement.

The human host mounts a massive humoral and cellular immune response against *E. histolytica* with macrophages and NKT cells being the most efficient defenders. The antibody response is limited in its impact through the ability of the amoebae to cap and shed antibodies bound to their surface. In addition, *E. histolytica* has developed a number of mechanisms to cope with immune cells.

M. Duchêne
Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna,
Kinderspitalgasse 15, Vienna A-1090, Austria
e-mail: michael.duchene@meduniwien.ac.at

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E. histolytica belongs to the microaerophilic microorganisms devoid of classical mitochondria and devoid of oxidative phosphorylation. So the main pathway to extract energy is by glycolysis, and when not needed, the energy is stored in the form of glycogen. The biosynthetic abilities are quite crippled; most useful molecules, sugars, amino acids, lipids and nucleotides are acquired from the host or from phagocytosed bacteria.

11.1 Introduction

Entamoeba histolytica is an obligate human intestinal parasite causing amoebic dysentery and liver abscess. Unlike *Acanthamoeba* spp. it lacks the ability to proliferate in the environment with the exception of artificial laboratory conditions. *E. histolytica* infects humans by the faecal-oral route, and it is prevalent in regions of this world with a lack of clean water supply and food. There is an old and much-cited study (Walsh 1986) which estimated about 36 to 50 million cases of amoebiasis each year and between 40,000 and 110,000 deaths. In some countries such as Vietnam or Sri Lanka, the prevalence has declined during the previous years. Nevertheless, in the Global Burden of Disease Study based on recent data from 2010, the global annual cost of *E. histolytica* infections was estimated at as much as 55,500 deaths and 2.24 million DALYs (years of life lost from premature death or disability) (Turkeltaub et al. 2015).

The parasite was discovered in 1875 by Fedor A. Lösch in St. Petersburg in the stool of a worker afflicted with amoebiasis. Lösch tried to use this stool to infect dogs with the parasite. As the dogs displayed either mild disease or none at all, the cause for the human disease remained unclear. Finally, Fritz Schaudinn was able in 1903 to unambiguously identify the agent causing amoebic dysentery and named it *Entamoeba histolytica*. Tragically, he died only 3 years later of amoebic liver abscess.

In the following years, it became clear that many more individuals carried amoebae in their gut than had a manifest disease. As a consequence, there had to be pathogenic and nonpathogenic amoebae. So the famous French parasitologist Émile Brumpt working in Paris suggested in 1925 that there could be two separate species, the pathogenic species *Entamoeba histolytica* and the nonpathogenic species *Entamoeba dispar*. For many years, this could not be confirmed as it was impossible to discriminate the two by microscopy. Only after several decades, Peter Sargeaunt in London was able to distinguish *Entamoeba* isolates by isoenzyme electrophoresis, a complex method in which lysates from cultured amoebae were separated on starch gels and stained with specific enzyme-specific reagents. By means of this method, hexokinase, phosphoglucomutase, glucose phosphate isomerase or malic enzyme could be visualised as bands in the starch gels. These band patterns, called zymodemes, were found to be stable, and it became clear that they could be used to identify pathogenic and nonpathogenic amoebae (Sargeaunt et al. 1982). In the following years, controversial reports were published about zymodeme conversions,

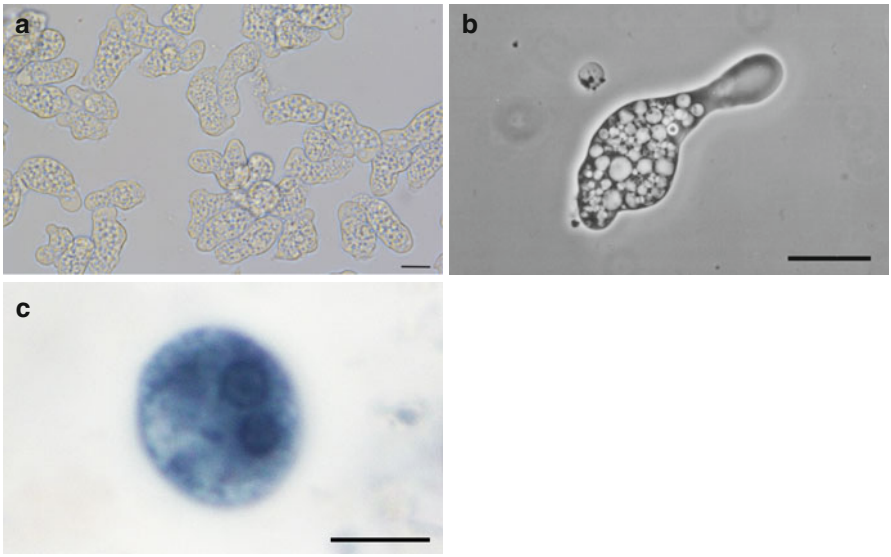

Fig. 11.1 Images of *E. histolytica*. (**a**) Trophozoites in culture (scale bar = 20 μm); (**b**) single trophozoite (scale bar = 20 μm); (**c**) cyst from stool sample (scale bar = 10 μm)

but finally the application of molecular biology settled the matter. The first DNA probe for the distinction of pathogenic and nonpathogenic amoebae was found in Hamburg (Tannich et al. 1989), and many more genetic differences were reported, until finally the pathogenic and nonpathogenic amoebae were redescribed (Diamond and Clark 1993) as *Entamoeba histolytica* and *Entamoeba dispar* using the old name originally proposed by Brumpt. Finally, the discriminating enzymes from Sargeaunt's zymodemes, hexokinase and phosphoglucomutase, were cloned and expressed in *Escherichia coli* (Ortner et al. 1997a, b). The genes from *E. histolytica* and *E. dispar* were clearly different, and the recombinant proteins exactly reproduced the patterns of the natural enzymes in starch-gel isoenzyme electrophoresis (e.g. Farri et al. 1980).

The life cycle of *E. histolytica* and *E. dispar* is simple as compared to other protozoan parasites (Stanley 2003). The infective form of the entamoebae is round cysts with four nuclei measuring about 10–15 μM in diameter. Cysts are able to survive in the environment for a limited time, especially in the presence of moisture, preferentially at cool temperatures. When the cysts are ingested, they pass through the stomach, surviving its acidic conditions, and through the small intestine. In the terminal ileum and the proximal colon, the suitable environment for excystation and proliferation is reached, and the nuclei divide and finally eight amoebae called trophozoites ("feeding stage") are released from each cyst. In contrast to the cysts, the trophozoites with a diameter of roughly between 20 and 50 μM (Fig. 11.1) are highly motile. Their forms constantly change (the term amoeba is derived from the Greek word amoibè which means change) which enables them to engulf and phagocytose bacteria or food particles in the content of the colon. The extracted energy

allows them to proliferate. The trophozoites also attach to the mucus and enterocytes on the inner face of the colon. As the trophozoites travel along the colon, the environment changes, probably getting less rich in freely available nutrients, which could be the signal for the trophozoites to encyst. For *E. histolytica* and *E. dispar*, the molecular signals for encystation are poorly understood, and so there are no reliable methods so far to reproduce this process in vitro. In contrast, the reptilian parasite *Entamoeba invadens* can be induced reliably to encyst, and therefore a number of studies addressing encystment and excystment were performed on this species. Finally the cysts are excreted with the faeces and have to wait passively to be taken up by a human host to start a new life cycle. Trophozoites are also released, but they cannot proliferate or even survive for a long time in the environment.

In the majority of cases, both *E. histolytica* and *E. dispar* trophozoites remain in the lumen of the colon. *E. histolytica* trophozoites attached to the colonic wall can digest the protective mucus barrier and then continue to invade the epithelial cell layer and then tissues beneath forming flask-shaped ulcers. The tissue-lysing properties led to the name "*histolytica*". When the trophozoites invade further, they can penetrate into mesenteric blood vessels, from where they are carried to the liver via the portal vein. In the liver they can establish serious abscesses (Stanley 2003). Further invasion such as into the peritoneum or to the brain is possible but rare. In contrast, until a few years ago, *E. dispar* was believed to be unable to invade the tissues, recent reports challenge this view (Dolabella et al. 2012; Oliveira et al. 2015).

Why *E. histolytica* remains asymptomatic and noninvasive or invades is not known. The outcome could be due to genetic host factors, to the fitness of the host's immune system, to the nutrients present in the environment, to the gut microbiome or to the properties of the *E. histolytica* strain. In the past, it was almost impossible to answer this question, but the new omic approaches could help in the search for molecular causes. A similar complex question is why some cases of invasive *E. histolytica* infection remain limited to the colon and others include an attack on the liver often even sparing the colon.

The symptoms of invasive amoebiasis depend on the site of attack (Stanley 2003). Amoebic colitis starts gradually. Patients develop abdominal pain and tenderness; they produce stools which may appear slimy and are sometimes but not always bloody. Fever is observed in less than 40 % of the patients. In some cases fulminant colitis develops with strong abdominal pain, fever, profuse bloody diarrhoea, and the danger of paralytic ileus and intestinal perforation. Other patients develop amoebomas, localised masses of inflammatory cells containing amoebae, which are able to mimic colon carcinomas.

Invasion of the mesenteric vessels commonly leads to migration of the amoebae to the liver and the establishment of abscesses (Stanley 2003). The patients suffer from pain in the upper right quadrant and hepatic tenderness. Modest fever is common as well as substantial weight loss. In contrast to viral hepatitis, jaundice is not common. Patients with amoebic liver abscess often do not suffer from the symptoms of amoebic colitis and vice versa. The amoebae are able to persist in the body for a long time, and liver abscesses sometimes develop up to more than 5 years after a journey to an endemic area with no symptoms during these years. It is not known why *E. histolytica* is able to persist for such a long time and where it is hidden.

Whereas amoebic dysentery afflicts both female and male individuals at a similar frequency, amoebic liver abscess is found predominantly in males.

In very fresh stool samples from patients with amoebic dysentery, motile trophozoites can be found; after a few hours, only cysts remain. The distinction of *E. histolytica* and *E. dispar* can be performed by an antigen ELISA (enzyme-linked immunosorbent assay) (Petri et al. 1990) or by PCR (polymerase chain reaction) (Blessmann et al. 2002). The serum is examined for *E. histolytica*-specific antibodies which are typical for both dysentery and liver abscess. To diagnose a liver abscess, the stool examination may be negative, but imaging methods such as ultrasonography can reveal the abscess.

Although invasive amoebiasis is a potentially fatal disease, it progresses slower than *P. falciparum* malaria, and it can usually be cured by treatment with metronidazole against the parasites that have invaded the tissues, if necessary combined with a luminal amoebicide to eliminate remaining trophozoites and cysts in the intestine (see below).

The following chapter will address the molecules of *E. histolytica*. A recent book edited by Tomoyoshi Nozaki and Alok Bhattacharya provides a much wider perspective on the biology and pathogenesis (Nozaki and Bhattacharya 2015).

11.2 Molecular Biology

11.2.1 Evolutionary Aspects of *E. histolytica*

In this book in the chapter on the phylogeny and evolutionary cell biology of parasites by Klinger et al. many aspects of the phylogeny of *E. histolytica* are discussed. The evolutionary history of *E. histolytica* is a field which has evolved rapidly in the recent years. The redescription of the closely related *E. histolytica* and *E. dispar* has been described above. In the larger perspective, the old hypothesis that *E. histolytica* is an archaic organism lacking mitochondria had to be dropped. The mitosomes (see below) are clearly derived from mitochondria. Although the Amoebozoa including parasitic and free-living amoebae are highly diverse, they are a clearly monophyletic taxon (Cavalier-Smith et al. 2015).

During the recent years, the universe of *Entamoeba* spp. has expanded significantly (Jacob et al. 2016). *E. nuttalli* infects macaques and is more closely related to *E. histolytica* than *E. dispar*, and many of its genes have been annotated. *E. bangladeshi* is a new species isolated from humans in Bangladesh which is more distant to *E. histolytica* than *E. dispar*.

11.2.2 The *Entamoeba histolytica* Genome

The *E. histolytica* genome sequence was determined in a collaboration between TIGR (The Institute for Genomic Research), now the J. Craig Venter Institute, in Rockville (MD), and the Sanger Institute in Cambridge (UK) (Loftus et al. 2005) and updated 5 years later after reassembly and reannotation (Lorenzi et al. 2010). The genome size

is 20.8 Mb; its overall G/C content is only 24 %. Within the genome, 49.7 % are protein-coding sequences; their G/C content is 28 %. The total number of genes predicted now stands at 8,201. For only 46 % of these proteins, putative functions could be assigned. The RNA-Seq (RNA sequencing, also called whole transcriptome shotgun sequencing) was applied to the HM-1:IMSS transcriptome (Hon et al. 2013). This powerful second-generation sequencing method allowed to verify 7,312 bona fide gene models; this means that most of the genes are transcribed. This work also allowed to define splicing and polyadenylation sites. More recently, genomic data from *E. dispar* have been obtained, but the analysis has only just begun (Wilson et al. 2012).

The determined *E. histolytica* sequence is organised into 1496 scaffolds. So far it has not been possible to assign these scaffolds to a defined set of chromosomes. Before the genome project, the karyotypes of the three *E. histolytica* strains HM-1:IMSS, 200:NIH and HK-9 were studied by separating their chromosomes by pulsed-field gel electrophoresis and Southern blotting (Willhoeft and Tannich 1999). Depending on the strains used, between 31 and 35 chromosomes of 0.3 to 2.2 Mb were identified. Probing these separated chromosomes with 68 independent cDNA probes led to an estimate of 14 linkage groups and a haploid genome size of $\leq$ 20 Mb which comes very close to the current estimation. As cDNAs of single-copy genes bound to as many as four chromosome-sized bands, the functional ploidy of *E. histolytica* was estimated to be at least four.

The sequences encoding tRNAs and rRNAs are present in many copies in *E. histolytica*, but they are organised in a fundamentally different way. Whereas the tRNA genes are repeated within the arrays that are part of the chromosomes, the genes encoding rRNA are found exclusively extrachromosomally on large plasmids present in about 200 copies per haploid genome equivalent of the trophozoite.

11.2.3 The tRNA Genes

The tRNA arrays comprise units ranging from 490 bp to 1775 bp. These units contain between one and five tRNA genes, nine arrays with a single tRNA gene, eleven with two different genes, three arrays with three tRNA genes each with four or five tRNA genes. In three cases, an additional 5S RNA gene was found within the arrays. The units are repeated many times, resulting in an estimated total of 4,500 tRNA genes (Clark et al. 2006). About 10 % of all reads of the genome project (Loftus et al. 2005) contained tRNA genes. If there are 61 anticodons of tRNAs carrying amino acids, this would correspond to 61 tRNA genes. So far, however, only tRNA genes for 45 anticodons have been identified by Clark et al. (2006) or annotated in the NCBI and AmoebaDB databases; 16 anticodons are apparently not accounted for (Fig. 11.2). All possible combinations of the first two bases are found; however, so it is likely that several of the tRNAs have to read more than one codon according to the revised Wobble Hypothesis as proposed by Guthrie and Abelson (1982) for the case of *Saccharomyces cerevisiae*. So taken together, it is likely that all *E. histolytica* tRNA genes have been found. There was no correlation between the relative abundance of a tRNA gene and the relative usage of the corresponding codon in protein-coding sequences of the genome (Fig. 11.2).

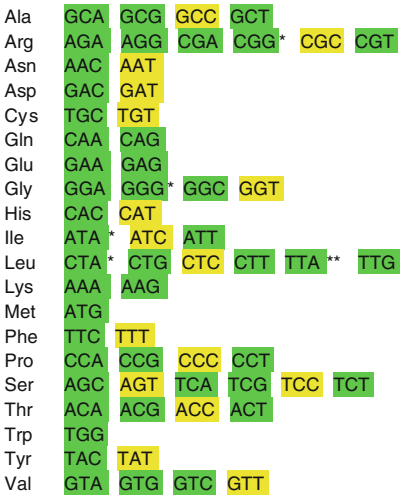

Fig. 11.2 The codons, for which tRNAs with the corresponding anticodons have been identified, are marked in *green*. Almost all these genes were found in tRNA arrays, the tRNA genes marked (*) were found as single copies and one gene marked (**) was found as a single copy as well as in an array (Clark et al. 2006). The codons, for which the tRNAs are lacking are marked in *yellow*

No telomere-like repeats are found in the genome, and very surprisingly, there is no putative telomerase gene in the *Entamoeba* genomes sequenced so far. This is unusual but not unique, e.g. the *Trichomonas vaginalis* genome also lacks an annotated telomerase gene, whereas the protozoan parasites *Acanthamoeba castellanii*, *Giardia duodenalis*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania donovani* all possess putative telomerases. Based on Southern blot data, it was hypothesised that tRNA arrays could be found at the end of chromosomes (Clark et al. 2006).

In axenic culture, the cell cycle of *E. histolytica* is unusual in that there is no strict order for DNA replication, mitosis and cell division. This leads to the presence of cells with multiple nuclei and nuclei with heterogeneous genome contents (Lohia et al. 2007). Flow cytometric analysis by DAPI fluorescence did not show discrete populations with 1x or 2x genome contents, but a broad distribution from 1x to 6x. Endo-reduplication of the genome appears to be common due to the lack of some crucial regulators not encoded in the *E. histolytica* genome.

11.2.4 The rRNA-encoding Episomes

In *E. histolytica*, the genes encoding the 18S, 28S and 5.8S ribosomal RNAs are located on episomal circular DNA elements (Bhattacharya et al. 1998). In the strain HM-1:IMSS, there are two repeats within a circle of 24.5 kb, similar to the strain 200:NIH. Other strains such as HK-9, Rahman or Laredo have only one set of rRNA-encoding genes per circle. The rRNA of nonpathogenic *E. dispar* is also encoded in circles. The high copy number of these elements is useful for PCR diagnosis (Blessmann et al. 2002). When *E. histolytica* resumes growth after a period of stress, the replication of the rDNA episome is initiated at dispersed sites around the molecule. During periods of stronger growth, when there is a concomitant rRNA transcription, replication starts from a site within the external transcribed spacer

(ETS) (Ghosh et al. 2003). The transcription of pre-rRNA by RNA polymerase I starts from either of two defined start sites in the ETS upstream of the 18S rRNA. Under normal conditions, the pre-rRNA is processed quickly, whereas during serum starvation, there is more transcription from the downstream start site, and the untranslated ETS RNA spontaneously forms circular RNAs of 766 and 912 nt. These RNA species appear to inhibit pre-rRNA processing (Gupta et al. 2012). The rDNA episomes are found in the periphery of the nuclei, not in conventional nucleolar structures (see below).

11.2.5 Repetitive Retroposon Sequences

More than 10 % of the *E. histolytica* genome consists of repetitive elements which are called LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements). These elements are generally found in intergenic regions of the genome and represent non-LTR (long terminal repeat) retroposons. One of the two open reading frames of the 4.8 kb LINE (ORF2) encodes the reverse transcriptase and endonuclease. The SINEs (0.54–0.65 kb) are nonautonomous partners of the LINEs. Under normal conditions, ORF2 is not transcribed. To study retroposition, an *E. histolytica* cell line was produced expressing ORF2 of LINE1 in a tetracycline-inducible manner, which can retrotranspose a marked SINE copy which was provided on a separate plasmid. In >20 % of the resulting clones, the integrated SINE was neither identical to the marked SINE nor to resident SINEs. This explains the high rate of mosaic formation of the SINEs (Yadav et al. 2012).

11.2.6 Histones

E. histolytica chromatin possesses nucleosome-like particles with a diameter of 10 nm. These are less regularly spaced than in typical mammalian chromatin, and some regions of the chromatin appear to be devoid of nucleosomes. Digestion of the chromatin with micrococcal nuclease therefore does not produce a regular banding pattern (Torres-Guerrero et al. 1991). So far, the histones have not been characterised on the biochemical level, but genes for all classical histones can be found in the *E. histolytica* genome. The first histone H3 and H4 cDNAs characterised (e.g. Födinger et al. 1993) had unusually divergent sequences.

In *E. histolytica* histone modification plays a significant role in the control of gene expression. *E. histolytica* possesses three divergent genes coding for histone acetyltransferases and one histone deacetylase gene. The histone deacetylase inhibitor trichostatin A (TSA) affected the expression of 163 genes (Ehrenkaufer et al. 2007). Interestingly, there was a significant overlap of the induced genes with genes regulated during the development from trophozoites to cysts, but the chitin synthase itself was not induced by TSA. In *E. invadens*, TSA inhibited cyst formation

(Byers et al. 2005), whereas in *Acanthamoeba castellanii*, the treatment with TSA and 5′-azacytidine reactivated the capacity of cyst formation which had been lost after long-term cultivation of the amoebae in the laboratory (Koehsler et al. 2009). Taken together, histone acetylation plays an important role in the differentiation from trophozoites to cysts, most likely in the early phases rather than in the actual production of the cyst wall (Ehrenkaufer et al. 2007).

In addition to the acetyl modification of histones, a dimethylation of Lys27 in histone H3 has been identified as a repressive mark (Foda and Singh 2015) observed during RNAi-mediated gene silencing, which is currently the most advanced technology for downregulation of genes in *E. histolytica* (see below).

11.2.7 mRNA Splicing

Based on the data of the genome project (Lorenzi et al. 2010), 24.4 % of the *E. histolytica* genes were predicted to contain introns. The RNA-Seq technology (Hon et al. 2013) allowed to provide a test for these predictions, and 81 % of the predicted junctions were confirmed. In addition, further junctions were found which are spliced rarely. Although there is clearly alternative splicing, it is not known if the resulting protein variants will differ in their function.

So far, there have been only limited biochemical studies on the splicing machinery of *E. histolytica*. In a proteomics approach, the recombinant snRNP protein component U1A with a haemagglutinin tag was used to immunoprecipitate early and late splicing complexes, which were analysed by tandem mass spectrometry (Valdés et al. 2014). In total, 36 spliceosomal components and 50 more hypothetical proteins were identified. One of the accessory components of the splicing machinery is the lariat debranching enzyme Dbr1 which opens the 2′,5′-branched splicing product to allow its degradation or sometimes the generation of miRNAs. The *E. histolytica* Dbr1 (EHI_062730) was the first such enzyme for which the structure could be solved (Montemayor et al. 2014).

11.2.8 Genetic Manipulation for Overexpression

Real ease of genetic manipulation of *E. histolytica* remains a future goal, although significant progress has been made in the recent years. As with other protozoan parasites, DNA is generally introduced into the amoebae by electroporation. Lipofection is an alternative more recent method (Baxt et al. 2010). The DNA vehicles were constructed on the basis of *E. coli* cloning vectors, in which the gene to be expressed was engineered. For successful transient expression, an *E. histolytica* promoter region upstream as well as a 3′-untranslated region of an abundantly expressed gene, e.g. an actin gene downstream region, is needed. In one study in Hamburg, the chloramphenicol acetyltransferase (CAT) gene flanked by actin 5′ and 3′ regions was expressed transiently (Nickel and Tannich 1994). In the other study in Charlottesville, the firefly luciferase was expressed with a construct

with the 5′ and 3′ regions of the gene encoding the Gal/GalNAc lectin heavy chain (Purdy et al. 1994).

So far it is not possible to integrate foreign genes into the *E. histolytica* genome by recombination; therefore, to overexpress genes more permanently, the shuttle vectors were equipped with a selectable marker gene such as the *neo* (neomycin phosphotransferase) gene or the *hyg* (hygromycin phosphotransferase) gene, conferring resistance to G418 or to hygromycin. A further step was to add a tetracycline-responsive element which allowed upregulation of the transfected gene (Hamann et al. 1997; Ramakrishnan et al. 1997). The upregulation of gene expression is limited, however, by a low but significant toxicity of tetracycline against *E. histolytica*. The Gateway™ system of vectors facilitates the investigation of a larger number of cDNAs once an entry vector has been constructed (Abhyankar et al. 2009). In an application of this technology (King et al. 2012), an *E. histolytica* cDNA library was engineered into such a vector. Trophozoites transfected with this library were then exposed to toxin-loaded erythrocytes, thus selecting those in which cDNAs were overexpressed that inhibited phagocytosis. These cDNAs could be retrieved from the surviving amoebae, as an example, two known proteins identified were profilin, an actin-binding protein, and LimA, a component of lipid rafts.

In some cases, the overexpression of a mutated version of a gene can have a dominant-negative effect. When a mutated gene coding for the alcohol dehydrogenase ADH2 (see below), with the active-site histidine residue codon replaced by a stop codon, was transfected into the trophozoites, the ADH2 activity was eliminated completely (Chen et al. 2004).

11.2.9 Downregulation of Genes by RNA Interference

For many open questions in *E. histolytica* research, clean methods would be needed to shut off precisely the expression of selected genes, which is possible in workhorse organisms such as *Saccharomyces cerevisiae* and largely in trypanosomes (see chapter on Trypanosoma). *E. histolytica* has several properties which make gene knockdown difficult to achieve: the recombination methods successful in many other fields do not appear to work in *E. histolytica*, the high functional ploidy of the genome requires silencing of several gene copies, and finally many proteins are encoded by large gene families of varying sequence similarities. In addition, it is not known, how long proteins can survive in *E. histolytica* and if a small remaining percentage could still have an activity even though transcription has terminated. Nevertheless and quite surprisingly, significant progress has been made in the recent years. All the methods were designed to mediate some kind of RNA interference, but some procedures led, sometimes unexpectedly, to a long-term shut-off of genes caused by epigenetic changes.

The classical method to decrease gene expression in *E. histolytica* was by plasmid-driven expression of antisense mRNA. The first targets were candidate factors of pathogenicity, so the production of antisense RNA to the *cp5* gene encoding the major cysteine proteinase decreased the *cp5* mRNA and reduced phagocytosis, however, not the cytopathic effect of the amoebae (Ankri et al. 1998).

A surprising outcome occurred when *E. histolytica* was transfected with the *ap-a* gene coding for the pore-forming peptide amoebapore A flanked by its 5′- and 3′-flanking regions (Bracha et al. 2003), both the amoebapore A mRNA and protein disappeared, and this effect persisted after the amoebae were cured from the transfecting plasmid by stopping the selective pressure. The same effect was observed, when only the 470 bp *ap-a* 5′-flanking segment was transfected. Significantly, this region contained a truncated short interspersed nuclear element (SINE) which needs to be transcribed for silencing to occur. Retransfection of *E. histolytica* with the *ap-a* gene with ribosomal protein flanking sequence failed to restore the amoebapore A mRNA or protein synthesis. The plasmid-less strain G3 was apathogenic and turned out to be a general tool for gene silencing (Bracha et al. 2006). Silencing could be achieved in many but not all cases by transfecting the G3 strain with a construct of the *ap-a* 5′-flanking region with the gene to be disrupted. One disadvantage of the strain is that its apathogenic nature hinders its use for the study of pathogenesis factors.

A significant advance in the understanding of gene silencing in the G3 strain was the discovery that small antisense 5′-polyphosphate RNAs (sRNAs) mediate the silencing of the *ap-a* gene and the silenced gene that had been fused to the *ap-a* region (Zhang et al. 2011). The length of the sRNAs is around 27 nt. They are found in the nucleus where they interact with the Argonaute protein (AGO2-2) and with the target gene leading to its silencing. The process leaves a repressive mark on histone H3 in the chromatin (Foda and Singh 2015) (see above). When the data from microarrays and from sRNA libraries were combined, the authors discovered that *E. histolytica* genes with a high level of sRNAs are transcribed at a very low level (Morf et al. 2013). When suitable portions of these genes are fused to other genes such as a luciferase gene or the *myb* gene and transfected into the trophozoites, the silencing gene triggers also the stable shut-off of transcription of the fused gene. The resulting technology is currently the most robust way to turn off genes in *E. histolytica* as well as in *E. invadens*.

11.3 Cell Biology and Pathophysiology

In this chapter, the architecture of the *E. histolytica* trophozoites and cysts will be presented. Then various activities of the trophozoites will be described at the molecular level. These activities, motility, adhesion, attack against host cells, phagocytosis, recognition of targets and signalling are all involved in one or the other way in the pathophysiology of amoebiasis. All these activities are sensed and countered by the human immune response which is described further below. A separate world of interactions is the interaction of the trophozoites with the gut microbiome, which certainly has a major influence on the virulence of the amoebae. At the same time, all parties are involved in a molecular small-talk exchanging nutrients, waste products, signalling molecules or toxins. Most of this complex world is unexplored, and it will be an art to identify and to focus on the most relevant interactions.

11.3.1 The Inner Architecture of Trophozoites

E. histolytica trophozoites are cultured in glass tubes or in closed plastic cell culture flasks, where they can be observed from below in an inverted microscope (Fig. 11.1). They come in various sizes and shapes; some round ones measure only 10 μm in diameter, others measure up to 50 μm, and elongated ones can reach 20×60 μm and sometimes stretch even more. The inside is filled with vesicles and granules of all sizes; the nucleus cannot be discriminated without staining. Occasionally, very small objects with motility can be observed in the culture flasks. As these are too small to contain a regular nucleus, they are probably the product of an aberrant cytokinesis. When the trophozoites move, they develop a pseudopod, the content of which appears clear for a short while, after which the granular part of the cellular inside follows, sometimes in a burst-like fashion. It is also possible that several pseudopods develop simultaneously, requiring some decision about the direction of the net movement. Most but not all of the amoebae are in contact with other amoebae, only few move in a solitary way. As long as the cell density allows, most amoebae remain at the bottom of the flask; when there is further growth, the amoebae form aggregates above the layer of the others. Setting the culture flask on ice or strong shaking will detach the amoebae.

Observing the unstained trophozoites by light microscopy does not reveal their organelles; also with the exception of the nuclei, isolation of defined other organelles by gradient centrifugation has been frustrating for a long time. So there is only limited knowledge about the structure of the various organelles.

There are some components which are definitively missing from *E. histolytica* trophozoites. Flagella are absent, there are no mitochondria carrying out oxidative phosphorylation, and there are no peroxisomes in this microaerophilic organism. The nuclei measuring variably between 3 and 8 μm are surrounded by a regular nuclear membrane. The nucleolus is the compartment carrying out the biosynthesis of rRNA. In *E. histolytica*, the rRNA encoding episomes, RNA polymerase I and fibrillarin, a methyltransferase associated with active RNA polymerase I, are all found localised around the periphery of the nuclei (Jhingan et al. 2009). In *E. invadens* trophozoites, the nucleolus is also spread around the periphery of the nucleus; however, this structure dissolves partially when the trophozoites are induced to form cysts (Jhingan et al. 2009).

In *E. histolytica*, many processes associated with the chromosomes in the nucleus appear less ordered than in other organisms. As described above, it is much harder than in the yeast *S. cerevisiae* to separate defined chromosomes. In addition, single amoebae also may contain several nuclei, and mitosis and cytokinesis are not well coordinated as in mammalian cells. This also results in highly heterogeneous sizes of the amoebae. In the nuclei, a variety of unusual microtubular assemblies can be observed such as multipolar mitotic spindles (Mukherjee et al. 2009). Sorted euploid cells can multiply to form polyploid cells and vice versa. Cytokinesis often is not symmetrical, and the final separation of the daughter cell requires the severing of a thin cytoplasmic bridge. It has been observed in several laboratories that this final separation is helped by other amoebae attracted to the site of cell division, which were humorously called “midwives” (Biron et al. 2001).

In human cells, the so-called chromosomal passenger complex (CPC) appears to play a major role for the coordination of mitosis and cytokinesis (Carmena et al. 2012). This complex contains four major components: the Aurora B kinase interacts with Incenp (inner centromere protein), borealin (also called cell division cycle-associated protein 8, CDCA8) and survivin, a small protein initially described as having anti-apoptotic activity. The search for similar *E. histolytica* sequences in AmoebaDB gave a clear result. There is a protein with significant full-length similarity to the Aurora B kinase, but all of the three other factors are absent. So this allows to hypothesise that the absence of a functional complete CPC could be a contributing cause for the disorder in cell division in *E. histolytica*.

The presence of numerous vesicles inside *E. histolytica* delayed the identification of the endoplasmic reticulum and Golgi apparatus until the start of the new century (Chávez-Munguía et al. 2000). In the genome data, the genes for a number of the associated factors were identified (Clark et al. 2007) pointing to a fully developed and complex trafficking machinery. The proteomic analysis of a total endomembrane fraction identified more than 1,500 proteins, roughly 20 % of the proteome of *E. histolytica* (Perdomo et al. 2015). Of these, 481 proteins with attributable functions included 152 proteins in the traffic category, components of the endoplasmic reticulum, the Golgi apparatus, the COPI and COPII (coat protein complex I and II) vesicles, exosomes and multivesicular bodies. In addition, there were 131 Rab, Ras, Rho and Gef/Gap GTPases, 81 cargo binders and 117 enzymes. An Alix-like protein was discovered (EHI_167710). Only the golgins, which are structural proteins of the Golgi apparatus, were missing. Interestingly, more than 400 of the identified components are not annotated underscoring the unexplored richness of the system.

The lack of oxidative phosphorylation and typical mitochondria in *E. histolytica* contributed to the view that this organism was an archezoan that had survived without an event of endosymbiosis. This view was first challenged by the discovery of the genes coding for the chaperonin Cpn60 and the pyridine nucleotide transhydrogenase. These proteins are localised in the mitochondria in other eukaryotes, and the *E. histolytica* Cpn60 sequence is related to the mitochondrial lineage (Clark and Roger 1995). So it appeared first that there had been mitochondria, which were lost secondarily, but then it was discovered that they were not lost altogether, but had developed into residual organelles called “mitosomes” (Tovar et al. 1999) or alternatively “cryptons” (Mai et al. 1999). In *E. histolytica*, the assembly of iron-sulphur (Fe-S) clusters under anaerobic conditions is carried out by NifS and NifU proteins (Ali et al. 2004), for which the genes were probably acquired by lateral gene transfer from bacteria. These proteins were not only found in the cytoplasm of the trophozoites but also significantly enriched in the mitosomes (Maralikova et al. 2010). In addition, the mitosomes were also enriched for rubrerythrin, a protein with Fe-S centres which is able to reduce hydrogen peroxide.

The successful isolation of mitosomes by ultracentrifugation through consecutive Percoll gradients allowed the identification of more components by mass spectrometry, and enzymes for sulphate activation were found almost exclusively in the mitosomes (Mi-ichi et al. 2009). ATP sulfurylase catalyses the synthesis of

adenosine-5′-phosphosulphate (APS) from ATP and sulphate, and APS kinase generates 3′-phosphoadenosine-5′-phosphosulphate (PAPS) from APS and ATP. This was very surprising as these enzymes are located in the cytoplasm in most eukaryotes, with a possible exception of *Mastigamoeba balamuthi*. Both sulphate activation and the action of Cpn60 require ATP, which is delivered into the mitosomes by an ATP/ADP transporter. The diameter of immunolabelled mitosomes was estimated at 150–400 nm with more than 6,000 mitosomes per trophozoite. *E. histolytica* is able to produce cholesteryl sulphate from cholesterol acquired from the host and from PAPS generated in the mitosomes (Mi-ichi et al. 2015). The cytoplasmic sulfotransferase SULT6 (EHI_146990) was identified as having this activity. Interestingly the free-living *M. balamuthi* has the organellar sulphate activation but does not produce cholesteryl sulphate. Cholesteryl sulphate appears to be very important for *Entamoeba* cyst formation. Patients with amoebic dysentery more than liver abscess patients develop a marked hypocholesterolaemia (Bansal et al. 2005). This condition is also observed in patients with sepsis and other severe infections and could be a consequence of the host inflammatory response; definitively the biomass of the *E. histolytica* cysts is many magnitudes too small to account for the missing cholesterol.

Whereas a number of infectious agents are able to attack *Acanthamoeba* spp., very little information is available on *E. histolytica* trophozoites being infected by other microorganisms. Around 40 years ago, a series of studies (e.g. Mattern et al. 1977) described polyhedral, filamentous or beaded DNA viruses in *E. histolytica*. These particles were able to pass through 0.2-μM filters. Some of these viruses were lytic for the trophozoites. There are no new studies, however, and no sequence information is available.

11.3.2 The Architecture of Cysts

The mature cysts of *E. histolytica* (Fig. 11.1c) are the infectious form which can survive in the environment. Their diameter is about 12–15 μm. Both *E. histolytica* and *E. dispar* cysts in their mature form contain four nuclei which go through another division before excystation, so that each cyst theoretically releases eight trophozoites. In the *Entamoeba* spp., the cyst wall is made up of chitin, while cysts from *Acanthamoeba* spp. contain cellulose. Chitin is a polymer of β-1,4-linked subunits of N-acetyl-glucosamine, whereas cellulose is a polymer of β-1,4-linked subunits of glucose. Chitin in insects and fungi and cellulose in plants are major glycans. The *Entamoeba* cyst wall is not as stiff as the insect exoskeleton as the latter is hardened by the protein sclerotin.

Unfortunately, the differentiation of *E. histolytica* trophozoites into cysts cannot be induced reliably in axenic culture in the laboratory, in contrast to the reptile parasite *E. invadens* which can be encysted readily in diluted glucose-free medium. This allowed to investigate the cyst wall architecture in more detail (Chatterjee et al. 2009). The chitin fibrils are formed in secretory vesicles and deposited onto Jacob lectin and chitinase on the cell surface. Finally, the Jessie3 lectin provides a kind of

mortar rendering the structure impermeable. The authors coined the term "wattle and daub" model as it resembles this historic building technology. As *E. histolytica* has all the homologues to these proteins, it is likely that the cyst structure of the human parasite is similar.

The chitin chain is synthesised by adding N-acetyl-glucosamine units from UDP-N-acetyl-glucosamine. There are two chitin synthases (EHI_170480 and EHI_044840) in *E. histolytica*, which are quite divergent is size and sequence (Campos-Góngora et al. 2004). *E. invadens* also possesses these two chitin synthases. Under normal culture conditions, trophozoites do not express the mRNAs, but 4–8 h after transfer to encystment medium, both mRNAs are upregulated, reach a peak and disappear by 48 h when most of the trophozoites have differentiated into cysts.

11.3.3 Cytoskeleton and Motility

Whereas the *E. histolytica* cysts are immotile and can only wait passively to be transported, the trophozoites display active amoeboid motility. Cultured trophozoites can travel up to about 24 μm/min (Zaki et al. 2006); interestingly, they move twice as fast in conditioned media compared to fresh media. The spent media, including ethanol, the product of amoebic metabolism, elicit a negative chemotactic response. In contrast, the amoebae migrate towards fibronectin, a component of the extracellular matrix, and even better towards fibronectin fragments (Franco et al. 1997), as well as towards erythrocytes and bacteria and also towards the pro-inflammatory cytokine tumour necrosis factor TNF (Blazquez et al. 2008).

As the trophozoites do not possess flagella, their movement is based on actin and myosin. The actin gene was the first *E. histolytica* gene to be cloned (Edman et al. 1987). The genome project revealed a large family of more than twenty actin and putative actin genes. In contrast, there is only one myosin II heavy chain gene (EHI_110180) and one gene encoding an unconventional myosin IB (EHI_110810), which is involved in phagocytosis (see below). In the search for a myosin light chain gene, a sequence with similarity to *Dictyostelium discoideum* myosin light chain and also to calmodulin was discovered which is currently annotated as calcium-binding family protein (EHI_177620). All *E. histolytica* strains possess a group of five genes either annotated as myosin light chain kinases or as Ser/Thr kinases, which are highly conserved between the strains. In smooth muscle cells, myosin light chain kinases are involved in calcium signalling.

When moving, the amoebae display a polarity. A clear bleb develops in the direction of locomotion (Fig. 11.1), which forms by the detachment of the plasma membrane from the actin cytoskeleton (Dufour et al. 2015). The major player in generating the necessary force is the myosin heavy chain located together with actin around the posterior end of the amoebae, the uroid. Actin staining shows that bundles of actin filaments (F-actin) are located towards the uroid, whereas the G-actin monomers are found more towards the pseudopod. Actin filaments are disassembled at the uroid and move towards the front end. The amoebae are able to transport

surface-bound components such as host antibodies towards the uroid where they can form caps and can be shed into the environment (Calderón et al. 1980). This remarkable ability to escape the host immune system also depends on the activity of the actomyosin filaments (Arhets et al. 1995).

The importance of myosin II is shown by a knockout experiment: wild-type *E. histolytica* is able to crawl over the surface of a monolayer of the intestinal Caco-2 cell line and to penetrate this monolayer within 25 min. A dominant-negative strain for myosin II moved only very slowly on a glass surface; on Caco-2 cells it did neither crawl nor penetrate the cell layer (Coudrier et al. 2005). This demonstrated that the myosin II-mediated motility contributes significantly to the pathogenicity of *E. histolytica*.

E. histolytica genes encoding a number of proteins interacting with actin can be found in the databases, such as profilin, a 120-kDa actin-binding protein, spectrin and gelsolin repeat proteins, actophorin, villidin, actinin, filamin, synapsin, caldesmon and a troponin family protein. Only some of these factors have been studied at the biochemical level. Trophozoites of increased virulence harvested from hamster livers express a higher level of the transcription factor EhPC4 (positive coactivator-4). Comparing the proteomes of wild-type and EhCP4-overexpressing trophozoites (de la Cruz et al. 2014) showed an increased amount of several proteins associated with cell migration such as a 16-kDa actin-binding protein EhABP16. Overexpression of this protein made the amoebae move faster and made them slightly more virulent against colon carcinoma cells, whereas knockdown of EhABP16 diminished motility.

11.3.4 Surface Molecules and Adhesion

The lifestyle of *E. histolytica* requires an extremely well-balanced system of motility and adhesion. On the intestinal surface, adhesion is important in order not to be transported along the colon too quickly. For invasion, motility as well as membrane attack and phagocytic capabilities are needed, as well as the ability to survive the attack from the human immune system.

The galactose/N-acetylgalactosamine (Gal/GalNAc)-specific lectin is the major adhesive factor on the surface of *E. histolytica* (Petri et al. 1987, 2002). It is able to bind to Gal and GalNAc residues which are found on the intestinal mucins and on the surface of enterocytes. This binding is inhibited by the free sugars. As long as the trophozoites adhere to the mucin, they behave rather like commensals. Binding to the enterocytes reveals their pathogenic character. Biochemically, the lectin consists of a large 170-kDa heavy subunit with a transmembrane domain and the largest cysteine-rich part containing the sugar-binding domain exposed to the environment. This part of the molecule also contains a domain mimicking the CD59 antigen of human cells. The function of this protein, e.g. on the surface of erythrocytes, is to prevent complement lysis by inhibiting the C5-C9 membrane attack complex. So the similar *E. histolytica* structure inhibits complement attack on the amoebae (Braga et al. 1992). The 35-kDa light subunit is inserted into the plasma membrane

via a glycosylphosphatidylinositol (GPI) anchor. In total, the five lectin heavy chain genes and six lectin light chain genes identified by classical molecular biology (Ramakrishnan et al. 1996) are also the complete set in the genome databases. In addition to the heavy and light chains, a lectin intermediate subunit was discovered, which does not possess a glycan-binding domain but appears to interact with the other chains (Cheng et al. 2001).

E. histolytica trophozoites like many other eukaryotic cells contain lipid rafts, cholesterol and sphingolipid-rich surface microdomains. Lipid rafts play an important role in signalling and adhesion, so it is not surprising that the Gal/GalNAc-specific lectin also localises to the rafts (Laughlin et al. 2004). Besides Gal- and GalNAc-containing glycans, the lectin also binds to collagen.

The cytoplasmic part of the lectin heavy chain resembles the cytoplasmic part of β2 integrins. When this part is overexpressed in the cytoplasm as a fusion protein, the consequence is a dominant-negative effect and adhesion is decreased (Vines et al. 1998); a similar construct with a mutated β2 integrin part does not produce the same effect. This means that adhesion of the trophozoites can be regulated from inside out via the lectin cytoplasmic part. Comparison of the wild-type and dominant-negative lectin strains also showed that whereas myosin II is important for the penetration through intestinal cells (see above), the lectin plays the larger role for the displacement within the liver (Coudrier et al. 2005).

The second abundant and complex surface molecule of *E. histolytica* is an O-glycan molecule variably described as LPPG (lipopeptidophosphoglycan), LPG (lipophosphoglycan) or PPG (proteophosphoglycan) and for simplicity will only be called LPPG here. The molecule possesses a novel GPI anchor with oligo-αGal ($n = 1–20$) side chains which anchors a putative protein in the plasma membrane. This putative core protein possesses Ser residues to which glycan side chains are linked. The first residue is galactose, and it is followed by a linear chain of α-1,6-linked glucose residues ($n = 4–25$) (Moody-Haupt et al. 2000). Similar dextran side chains are found in biofilm-forming bacteria such as *Leuconostoc* spp. The LPPG is expressed more in *E. histolytica* than in *E. dispar* (Marinets et al. 1997); also there is less LPPG in the presence of bacteria than in axenic culture (Bhattacharya et al. 1992).

Both the Gal/GalNAc-specific lectin and the LPG are major players in the immune response against *E. histolytica* which will be described below.

In addition to these major components, *E. histolytica* also possesses N-glycosylated surface proteins, which could be enriched for mass spectrometry analysis by ConA (concanavalin A) affinity chromatography (Carpentieri et al. 2010). This study showed that the Gal/GalNAc-specific lectin heavy and intermediate chains are also N-glycosylated and identified further 22 N-glycosylated surface proteins. The Asn-linked glycosylation begins with the assembly of the glycan chains on a dolichol pyrophosphate precursor by means of a set of specific glycosyltransferases. *E. histolytica* lacks several of the glycosyltransferases present in other organisms; as a consequence, it can make only a short dolichol-PP-$GlcNAc_2Man_5$ precursor as predicted from the available glycosyltransferases (Samuelson et al. 2005).

The genome annotation provided many more candidates for surface proteins, such as a large number of transporters for the uptake of nutrients as well as for the protection against toxic molecules and also many transmembrane kinases involved in sensing the environment (Clark et al. 2007). The P-glycoprotein Pgp5 is able to pump out drugs such as emetine from the trophozoites, and its overexpression results in resistance to this drug (López-Camarillo et al. 2003); however, it does not protect the amoebae from metronidazole.

11.3.5 Pore-Forming Peptides

E. histolytica produces a pore-forming peptide also named amoebapore. The major isoform amoebapore A is a polypeptide of 77 amino-acid residues produced from a precursor of 98 residues (EHI_159480). This is a membranolytic polypeptide with significant sequence and functional similarity to NK-lysin from natural killer cells (Leippe 1995). It is able to associate and insert itself into the membrane of target cells or bacteria forming a hydrophilic pore which allows the free traffic of ions with the consequence of a breakdown of the membrane potential. Trophozoites, in which the amoebapore A gene was disrupted, had lower cytolytic activity against mammalian nucleated cells and erythrocytes and were less able to inhibit the growth of bacteria (Bracha et al. 1999, 2003). In addition, the capability of liver abscess formation in hamsters was impaired. So amoebapore A is clearly a pathogenicity factor.

The structure of amoebapore A in solution was determined by nuclear magnetic resonance (NMR) (Hecht et al. 2004). The polypeptide comprises five α-helical regions which are linked by three disulphide bonds with a high similarity to the structures of NK-lysin and granulysin. Under acidic (pH 3.5) and basic (pH 8) conditions, amoebapore A exists as a monomer, whereas at pH 5.2 the molecule dimerises. This corresponds to the maximum pore-forming activity at pH 5.2 and a loss of activity at pH 6.5 or higher. With a pH of about 5.7 in the lumen of the caecum, this environment definitively allows amoebapore A activity against bacteria and enterocytes. In *E. histolytica*, two more pore-forming protein cDNAs are known, coding for amoebapore B (EHI_194540) and amoebapore C (EHI_118270); these might work optimally at another pH. *E. dispar* also possesses pore-forming peptides; they are expressed at a lower level and display lower activity.

11.3.6 Proteases

The cysteine proteases are a major family of pathogenicity factors of *E. histolytica*. They are neutral proteases related to papain or the mammalian cathepsins possessing a Cys residue in the active site. Over the years they have been shown to degrade a wide range of host molecules, including serum albumin, haemoglobin, collagen types I, IV and V, fibronectin, colonic mucin, IgA, IgG, and complement components as well as cytokines and chemokines. The first cysteine protease, now called

CP1, was cloned about 25 years ago (Tannich et al. 1991; Reed et al. 1993), and several more followed. Finally, the genome data allowed a full survey of the whole proteolytic inventory of *E. histolytica* (Tillack et al. 2007) listing 86 genes, including 50 cysteine peptidases, 22 metallopeptidases, 10 serine peptidases and 4 aspartic peptidases. The large number is not unusual among protozoan parasites; *P. falciparum*, as an example, had a list of 110 peptidases in 2007. The reannotation of *E. histolytica* (Lorenzi et al. 2010) deleted the genes for four cysteine peptidases, four metallopeptidases, three serine peptidases and one aspartic peptidase from this list, leaving 74 genes. Only a small part of this large repertoire which includes pathogenesis factors and housekeeping enzymes has been studied at the biochemical level, and only 21 out of 79 proteinases analysed are expressed in axenic culture (Tillack et al. 2007). The three proteinases with the highest expression are the best-characterised CP1, CP2 and CP5 (Bruchhaus et al. 1996). About 90 % of the transcripts in the trophozoites correspond to these CPs and virtually all of the protease activity. In contrast, *E. dispar* expresses only CP2 and CP3 with a much lower total activity. *E. dispar* does not have a functional CP5 gene; however, a positionally conserved DNA sequence was discovered with similarity to the *E. histolytica* CP5 sequence, but disrupted by numerous stop codons (Willhoeft et al. 1999). So this shows that *E. dispar* lost CP5 but can exist very well without it. In *E. histolytica*, CP5 is found on the surface. It contains a hydrophobic patch which enables it to stick to the plasma membrane (Jacobs et al. 1998). CP5 can be expressed in *E. coli* as a proenzyme which auto-activates under suitable conditions (Hellberg et al. 2002). When CP5 mRNA is overexpressed in the trophozoites, the activity of CP5 is increased but also the activities of CP1 and CP2, which shows that CP5 is also able to activate other cysteine proteases. The ability to destroy cell monolayers and to cause liver abscess was increased most in the CP5-overexpressing strains (Tillack et al. 2007).

A further cysteine protease CP112 and the adherence protein ADH112, which are encoded by two adjacent genes, together constitute the 112-kDa adhesin (García-Rivera et al. 1999). The complex is also associated with the trophozoite surface and is translocated to phagocytic vacuoles during phagocytosis. Downregulation of CP112 by siRNA technology decreases cell monolayer destruction and erythrophagocytosis.

One of the serine proteases, the rhomboid protease ROM1 (EHI_197460), is an intramembrane protease, which also relocalises to internal vesicles during erythrophagocytosis and to the base of the cap during surface receptor capping (Baxt et al. 2010). Downregulation of ROM1 expression using the epigenetic silencing mechanism of the G3 strain (see above) had no obvious effect on cap formation, but decreased the adhesion to cells and phagocytosis of healthy as well as apoptotic cells and rice starch.

During the invasion of *E. histolytica* into the intestinal wall, not all protease activity is derived from the parasite. Human colon explants exposed to trophozoites produce several matrix metalloproteases (Thibeaux et al. 2014). *E. histolytica* CP5 activates human pro-MMP-3 which in turn activates pro-MMP-1. Inhibition of MMP-1 and MMP-3 prevented degradation of the extracellular matrix and

amoebic invasion. Interestingly, in a parallel mechanism, pro-mature CP5 interacts with colonic cells to induce NFkB starting the inflammatory processes (please see Immunology Sec. 11.4.3).

11.3.7 Phagocytosis of Bacteria

Phagocytosis of bacteria appears to be a natural and straightforward ability of free-living and parasitic amoebae. In the intestinal environment, *E. histolytica* coexists with bacteria as is the case for the older xenic cultures of the parasite. The axenic culture of *E. histolytica* represented an important progress, as the parasite could for the first time be examined on its own. The axenic medium TYI-S-33 (Diamond et al. 1978) accompanied many decades of research. In axenic culture, the amoebae are nevertheless filled with vesicles. The ER, Golgi apparatus and mitosomes have been described above. A large part of the vesicles is represented by endosomes resulting from pinocytosis. The uptake of exogenous solutes was investigated by fluorescein isothiocyanate (FITC)-labelled dextran and horseradish peroxidase added to the medium (Aley et al. 1984). At 37 °C, not at 4 °C, both were taken up into large (>2 μm) vesicles in a linear fashion for 1 h and reached a maximum after 2 h. There was also a continuous recycling to the medium. Significantly, the pH within the vesicles remained close to the pH in the medium in the range between pH 6.8 and pH 8. In contrast, staining the amoebae with acridine orange showed a large number of small (<2 μm) vesicles with a pH of 5.2 typical for lysosomes. When the amoebae were incubated for 3 days with FITC-dextran and then for 10 h in medium without FITC-labelled dextran, the FITC-labelled dextran was also found in these lysosomes. So the amoebae quickly take up and recycle exogenous solute but take much longer to transfer materials into their lysosomes.

Phagocytosis of bacteria always starts with some kind of adhesion, mostly of glycan structures. Various species of bacteria interact differently with *E. histolytica* (Bracha et al. 1982). Whereas *E. coli* and *Serratia marcescens* can bind to mannose structures on *E. histolytica*, some isolates of *Staphylococcus aureus* or *Shigella flexneri* do not interact in this way. They can be opsonised, however, by ConA or human antibodies for phagocytosis by the amoebae. The phagocytic vesicles often contain several bacteria. *E. histolytica* is then able to recruit amoebapores and cysteine proteases to the phagosomes for the killing and digestion of the bacteria. In comparison to *E. histolytica*, *E. dispar* trophozoites have a much lower appetite for bacteria, their phagocytic vacuoles contain only one bacterium, and occasionally, bacteria may escape to the cytoplasm (Pimenta et al. 2002). Recently, an important receptor for bacteria was studied (Brewer et al. 2013). Metazoan phagocytes possess G-protein-coupled receptors (GPCRs) which initiate the phagocytosis of bacteria. *E. histolytica* also possesses such a receptor (GPCR-1) which is expressed at a much higher level than in *E. dispar*. This receptor was tested in a heterologous expression system in *S. cerevisiae* where it was activated specifically by bacterial lipopolysaccharide (LPS). Anti-LPS antibodies prevented this activation. There were also eight more members of the GPCR family in *E. histolytica*, but only GPCR-1 was expressed under normal conditions.

Several studies addressed the question if phagocytosed bacteria can increase the virulence of *E. histolytica* with variable results. As an example *E. histolytica* or *E. dispar* was fed with a commensal strain of *E. coli*, enteropathogenic *E. coli* (EPEC) or *S. dysenteriae*, and then the cytopathic effect on epithelial cell monolayers was determined (Galván-Moroyoqui et al. 2008). While *E. dispar* fed or unfed showed no cytopathogenicity, *E. histolytica* destroyed the cells. Feeding commensal *E. coli* only led to a slight increase, whereas feeding EPEC or *S. dysenteriae* led to a significant increase. This was accompanied by an increase in the Gal/GalNAc lectin and cysteine proteinases. One novel side observation was that *S. dysenteriae* survived for a significant time within *E. histolytica*.

11.3.8 Phagocytosis of Host Cells

Erythrocytes are the classical prey for *E. histolytica*. When an erythrocyte binds to the amoebal surface at 37 °C, a phagocytic cup forms rapidly within few seconds, and the erythrocyte can be engulfed after 10 s to 30 s (Bailey et al. 1985). The rapid process is accompanied by the accumulation of polymerised actin in the cup surrounding the erythrocyte. The phagocytic cup exerts significant force, as the red blood cell can be squeezed into elongated shape and even severed. Latex beads are also engulfed, but without significant actin accumulation. Erythrocytes inhibit uptake of latex beads, but not *vice versa*.

The comparison of eight *E. histolytica* isolates from dysentery patients and asymptomatic carriers showed that all were able to phagocytose erythrocytes, but the pathogenic strains were clearly more active than the nonpathogenic ones (Trissl et al. 1978). Recently, a similar study showed that *E. dispar* is also able to phagocytose red blood cells, but significantly fewer and at a significantly lower rate (Talamás-Lara et al. 2014); also in the process, adhesion of erythrocytes to *E. dispar* is much weaker. In an older study (Orozco et al. 1983), feeding *E. histolytica* with BrdU (5-bromo-2′-deoxyuridine) labelled bacteria and exposing the amoebae to UV radiation allowed to select an *E. histolytica* clone with low phagocytic activity. This clone had also lost its virulence in a hamster liver abscess model. After four passages through hamster livers, four out of seven subclones regained virulence in hamsters and also regained the ability to phagocytose red blood cells; three subclones had lost both abilities showing the close association between virulence and phagocytosis.

The molecular mechanisms of phagocytosis in *E. histolytica* are as complex as in higher organisms and have been reviewed recently (Christy and Petri 2011). As described above for the endomembrane system, several studies addressed the phagosome proteome in order to get a large view of the components involved. First, early phagosomes containing negatively charged latex beads were isolated from trophozoites by sucrose gradient centrifugation, and all phagosome-associated components were identified by tryptic digestion and mass spectrometry (Okada et al. 2005). The heavy, intermediate and light chains of the Gal/GalNAc lectin, numerous members of the Rac and Rab GTPase families, effectors of intracellular

signalling and actin remodelling, several transmembrane kinases, cysteine and serine proteinases, phospholipase A2, a calcium-transporting ATPase and many other components were identified. Later, the same authors showed that during the maturation of phagosomes the levels of the components change in a very dynamic way.

In an independent study, human serum-coated magnetic beads were offered to the trophozoites, and again proteins associated with the early phagosome were identified by mass spectrometry (Marion et al. 2005). This study also made use of a strain overexpressing the unconventional myosin IB. This molecule binds to and stiffens actin filaments, and in the overexpressing strain, phagocytosis is slowed down and rather better synchronised than in the wild-type trophozoites. Many of the phagosome proteins were similar to the work described above, but this study also identified a number of novel and well-known actin-rich cytoskeleton-associated proteins such as profilin, adenylyl cyclase-associated protein (CAP), actophorin, Arp2/3 complex subunits, actobindin, coactosin, cortexilins I and II, talin, a filamin homologue, ABP120, myosin II heavy chain, alpha-actinin and two diaphanous-related proteins. This sheds some light on the complexity of the machinery involved.

Calcium signalling is a further major aspect of phagocytosis in *E. histolytica*. The calcium-binding protein CaBP1 interacts with the C2-domain-containing protein kinase (C2PK) in the initiation of phagocytosis (Somlata et al. 2011). C2PK binds to phosphatidylserine, e.g. on the surface of erythrocytes, in the presence of calcium ions, and this attracts CaBP1 and actin to the membrane. Although both C2PK and CaBP1 bind to Ca^{2+} ions, their interaction is Ca^{2+}-independent. The kinase activity of C2PK is not involved in the interaction with CaBP1, but it is necessary for the progression of erythrophagocytosis. The trans-autophosphorylation of C2PK at the residue Ser428 is an important control mechanism. Both C2PK and CaBP1 are no longer necessary, however, at later stages and are absent from the mature phagosomes. When the phagocytic cup is closed and the phagosome forms, a second calmodulin-like Ca^{2+}-binding protein, CaBP3, enters the scene (Aslam et al. 2012). CaBP3 binds to G-actin and myosin IB in a Ca^{2+}-dependent way and recruits myosin IB to the site of cap closure. Antisense-mediated down-regulation of CaBP3 decreases the rate of erythrophagocytosis, and upregulation increases the rate.

From another phagosome proteome screen, a surface transmembrane kinase TMK96 (also called PATMK or phagocytosis-associated transmembrane kinase) was identified (Boettner et al. 2008). This putative kinase also accumulates at the site of contact between the trophozoites and human erythrocytes. Disruption of the gene by sh (short hairpin) RNA technology led to a decrease of erythrophagocytosis and reduced the ability of the amoebae to establish an intestinal infection in a mouse model while retaining their ability to cause liver abscesses.

E. histolytica killing of host enterocytes or liver cells elicits apoptosis in the target cells. When trophozoites are inoculated into the liver of severe combined immunodeficient (SCID) mice, numerous changes in mRNA expression in the liver tissue can be observed (Pelosof et al. 2006). Inflammatory, apoptotic, and regenerative pathways are induced. These include the mRNAs encoding the pro-apoptosis receptor Fas and factors acting downstream of Fas. A review lists a number of further

processes observed in cells killed by *E. histolytica* (Ralston and Petri 2011): lectin adherence to the cells is the first step; soon after, there is a strong Ca^{2+} influx increasing the intracellular Ca^{2+} levels about 30-fold. This is accompanied by a dephosphorylation of Tyr residues in cellular proteins, which may be triggered in part by the elevated Ca^{2+} concentrations. Caspase 3 plays an important role in the process of apoptosis, as it is inhibited in caspase 3-knockout mice in an intestinal model or by a caspase inhibitor in a liver abscess model. At 4 °C, cell killing does not occur; also extracts from *E. histolytica* do not kill host cells.

So far significant gaps remain in our understanding of the attack of amoebae on cells. A recent live microscopy study provided new insights at the macroscopic level (Ralston et al. 2014). *E. histolytica* trophozoites attack Jurkat cells or Caco-2 by biting off and ingesting pieces of the cells. This process was termed trogocytosis (from Greek *trogo* – nibble). The pieces remain surrounded by the plasma membrane of the target cell and mostly contain cytoplasm and sometimes mitochondria. One bite is not enough to kill a target cell, but it causes influx of Ca^{2+} and represents a significant damage. After several bites, the target cells die, but then the trophozoite detaches and stops interacting with the corpse. Trogocytosis requires physiological temperatures and involves signalling via the Gal/GalNAc lectin, C2PK (see above) and phosphatidylinositol 3-kinase (PI3K).

The loss of potassium ions from cells attacked by *E. histolytica* appears to play a major role, as was recently revealed by a whole-genome RNAi screen (Marie et al. 2015). The inhibition of several K^+ channels increased the survival of the cells. The efflux of K^+, which is stimulated by increasing intracellular Ca^{2+} concentration, results in the activation of caspase 3 and triggers apoptotic cell death in enterocytes. In macrophages, the decrease in intracellular K+ concentration causes the activation of caspase 1, inflammasome activation, secretion of IL-1β and pyroptotic cell death.

When *E. histolytica* trophozoites are starved for glucose, their virulence increases unexpectedly (Tovy et al. 2011), the rate of erythrophagocytosis and cell monolayer destruction rises, the amoebae adhere stronger to cells, and their motility increases. When they are transferred back to glucose-containing medium, their virulence returns to a normal level. Interestingly, the virulence was also increased in a strain disrupted for amoebapore A and CP5. So the recent years brought more insights, but also new, unexpected questions about the pathophysiology of this fascinating parasite.

11.4 Immunology

11.4.1 Antigenic Structures and the Prospects of Vaccine Development

Because *E. histolytica* is a rather large parasite, it is not able to hide within cells as *Leishmania* or *T. cruzi*. Therefore, it is necessary for survival to develop strategies against the action of antibodies and the complement system. The advantage of this lifestyle is of course the free mobility within the host. As long as the entamoebae

stay within the intestinal lumen and refrain from invasion, no antibody response is generated. Therefore, individuals carrying the largely nonpathogenic *E. dispar* mostly do not have anti-*Entamoeba* antibodies in their serum. The situation immediately changes, if *E. histolytica* invades the mucus and the intestinal wall. Normally some of the amoebae will be killed by macrophages, and all their components can be presented to the immune system. As a consequence, various antibodies, IgM, IgG and IgA, are generated, recognising surface as well as cytoplasmic components. Elevated antibody levels are therefore found in almost all patients with invasive amoebiasis, suffering from dysentery or from liver abscess.

To cope with the host antibodies, *E. histolytica* has developed several strategies. On the one hand, IgA and IgG antibodies can be inactivated by degradation through cysteine proteinases (Kelsall and Ravdin 1993; Tran et al. 1998); on the other hand, the parasite is able to transport surface-bound antibodies to the uroid, its posterior end, from where they are shed into the environment (Calderón et al. 1980). The transport is mediated by actin, and the shedding needs myosin II in addition in a process resembling cell division (Espinosa-Cantellano and Martínez-Palomo 1994).

The ability of *E. histolytica* to cope with antibodies through its high motility also obviates the need for fast antigenic variation. This strategy is very different from the systematic variation of the variable surface glycoproteins (VSGs) in *Trypanosoma* spp. Knowing this, there have been only a few attempts to compare the sequences of surface antigens in various isolates of *E. histolytica*. There is, however, clear evidence for gene conversion within the Gal/GalNAc gene complex (Weedall et al. 2011) which would be a mechanism also found in *P. falciparum* for generating diversity and possibly escaping the host immune response.

If there are several escape mechanisms, then are antibodies protective at all against human amoebiasis? Some answers are provided by several studies in a slum area of Dhaka, Bangladesh, where there is a high infection rate in preschool children. The children with a higher level of anti-*E. histolytica* IgA antibodies in their stool, specifically directed against the carbohydrate recognition domain (CRD) of the Gal/GalNAc lectin, were significantly less likely to acquire an infection with *E. histolytica* and to develop amoebic disease (Haque et al. 2006). Paradoxically, a previous study (Haque et al. 2002) showed that when the children had elevated serum IgG against trophozoite antigens, they were 37 % more likely to be reinfected by *E. histolytica* at 2 years of follow-up. The presence of any anti-amoebic antibodies cannot prevent, however, amoebiasis for a long time; it has been observed in travellers that it is possible to acquire invasive amoebiasis several times in spite of being cured between the episodes. So it is unlikely that a vaccine can be developed by current technology, conferring life-long protection as some of the antiviral vaccines.

A major obstacle to vaccine development is the lack of a truly representative small animal model. Rodents when fed *E. histolytica* cysts do not develop amoebic dysentery or liver abscess. Moreover, as in vitro methods for the differentiation of *E. histolytica* trophozoites to cysts are lacking, the cysts for a challenge would have to be isolated from patients' faeces.

Most of the vaccine studies reviewed recently (Quach et al. 2014) included a challenge, where the trophozoites were inoculated directly into the livers of

Mongolian gerbils (*Meriones unguiculatus*), golden hamsters (*Mesocricetus auratus*) or severe combined immunodeficient (SCID) mice; alternatively, in a mouse intestinal model, the trophozoites were injected into the caecum. The first choice for a vaccine component is the 170-kDa heavy chain of the Gal/GalNAc lectin. The molecule purified from trophozoites elicited 100 % protection in a gerbil liver abscess model when administered in combination with *E. histolytica* DNA (Ivory et al. 2008) and up to 100 % protection in a mouse model of intestinal infection (Houpt et al. 2004). Trials with recombinant parts of the lectin heavy chain gave only partial protection (Quach et al. 2014). In one study (Lotter et al. 1997), the cysteine-rich carbohydrate recognition domain (CRD) was best as a vaccine, while immunisation with the amino-terminal cysteine-poor portion resulted not only in no protection but even larger liver abscesses were observed.

The Gal/GalNAc lectin is the only antigen, which has been studied in a baboon model (Abd Alla et al. 2012). The baboons were immunised intranasally with a construct of four different peptides from the lectin heavy chain coupled to polylysine. The vaccinated and control animals were challenged by release of xenically grown trophozoites from an endoscope to the lumen of the caecum. Whereas four out of six control animals developed an inflammatory ulcerative colitis and five animals from this group remained infected after 9 weeks, all six immunised animals had cleared the amoebae by the 5th week, and none developed colitis. This protection was associated with high levels of anti-peptide and anti-lectin intestinal IgA.

Other surface antigens such as the serine-rich surface protein (SREHP), peroxiredoxin (initially called cysteine-rich surface antigen), CP112 and a maltose-binding protein also gave only partial protection as shown in several small animal trials (Quach et al. 2014); similarly the surface metalloprotease MSP-1 was studied more recently. Immunisation with a construct of four MLIF (monocyte locomotion inhibitory factor) peptides, (see below) bound to a lysine core, was more successful; it protected 100 % of gerbils against amoebic liver abscess, but not against liver abscess caused by *Listeria monocytogenes* (Giménez-Scherer et al. 2004). A construct with scrambled peptides had no activity.

Finally, two more antigens could be considered in the search for a vaccine, the LPPG elicits a strong immune response, and an IgG1 monoclonal antibody was able to passively protect 90 % of SCID mice from *E. histolytica* liver abscess (Marinets et al. 1997). The LPPG is a very complex molecule, however, and it will be challenging to produce a vaccine. The second molecule is the lectin intermediate chain Igl-1 which has been tested very recently in a hamster liver abscess model. In this study the carboxy-terminal part of the molecule protected 96 % of the hamsters, most likely via a Th2-type immune response (Min et al. 2016).

11.4.2 Unusual Products of the Amoebae Interacting with the Host Immune System

The inflammatory response of the human host represents a serious threat to the amoebae. An anti-inflammatory factor, initially described as monocyte locomotion

inhibitory factor (MLIF), is secreted by axenically grown *E. histolytica*. This factor, isolated from culture supernatants, is a heat-stable pentapeptide with the sequence Met-Gln-Cys-Asn-Ser (Kretschmer et al. 2001). Its activity can be mimicked by the synthetic peptide with the correct sequence, not with a scrambled sequence. As described above, a peptide vaccine containing MLIF is highly protective. Although MLIF is a rather simple molecule, many questions remain open both about its biosynthesis and about its molecular mode of action.

Prostaglandin E2 (PGE_2) is a lipid mediator with multiple activities in many organs. It inhibits the production of IL-2 and interferon-γ (IFN-γ) in T-lymphocytes and the release of interferon-1β and tumour necrosis factor (TNF) in macrophages. Various pathogenic bacteria are able to induce the synthesis of PGE_2 weakening the host immune response. This ability is also found in *E. histolytica*, but surprisingly and in addition, the amoebae are also able to synthesise their own PGE_2 (Dey et al. 2003). PGE_2 is made by a cyclooxygenase-like enzyme (EHI_164440) from arachidonic acid taken up from the host.

11.4.3 Immunology: Interaction of *E. histolytica* with the Human Defence Systems

The life of *E. histolytica* as a human parasite depends on the ability to survive in very divergent environments, outside the host as a cyst and also in the host in the gut lumen in competition with the intestinal flora, in the presence of mucus, epithelial cells, neutrophils, dendritic cells and macrophages around the surface of the intestinal wall and during invasion, in the short period in the mesenteric blood vessels on the way to the liver and finally in the presence of neutrophils, dendritic cells, migrating macrophages and resident Kupffer cells as well as NKT cells (natural killer T-cells) in the liver.

In the lumen of the colon, the amoebae are exposed to IgA in the form of sIgA (secretory IgA) as the first-line host defence. The IgA is synthesised by plasma cells in the gut-associated lymphoid tissue in an oligomeric form consisting of mostly two molecules of IgA linked via a joining (J) region. This molecule is received by the polymeric Ig receptor (pIgR) on the basolateral side of the enterocytes, where it gains a secretory component, a part derived from pIgR, and is secreted as the complete sIgA. Such antibodies specific for the Gal/GalNAc lectin inhibit the binding of *E. histolytica* trophozoites to mucus, enterocytes and immune cells and thus help to prevent intestinal invasion (Mortimer and Chadee 2010). As described above, this protective effect was also observed in children of an endemic region (Haque et al. 2006).

The mucus on the inner surface of the colon not only serves the amoebae to attach via the Gal/GalNAc lectin but it also hinders them to reach the epithelial cells. *E. histolytica* cysteine proteinases are able to cleave the mucin glycoprotein MUC2 in its carboxy-terminal region in which the core protein is exposed due to a lower level of glycosylation (Lidell et al. 2006); thus MUC2 loses its protective function. The downregulation of the mRNA for CP5 by antisense RNA (Moncada

et al. 2006) decreased the mucus degradation activity and led to trophozoites which were no longer able to disrupt monolayers of mucus-producing cell lines. The trophozoites retained, however, the ability to disrupt cells without mucus. A recent review (Cornick et al. 2015) shows the importance of the gut mucus not only as a passive barrier but also as an important and dynamic regulator of gut homeostasis.

Trophozoites crossing the mucus barrier encounter the enterocytes; the ability of the amoebae to bind to and destroy them has been described above. The enterocytes react by secreting interleukin-8 (IL-8) and other mediators which can attract neutrophils to the site. This was demonstrated in a number of cell lines (Eckmann et al. 1995) and then tested in vivo in a SCID mouse-human intestinal xenograft (SCID-HU-INT) model of intestinal disease (Seydel et al. 1997), with the result that the enterocytes themselves produced the IL-8. Four hours after inoculation with the trophozoites, lesions develop at some spots and grow, and after 24 h, numerous immune cells, predominantly neutrophils, are attracted to the sites of inflammation. A further step to study intestinal amoebiasis in the lab is provided by the ex vivo model of colon tissue explants from patients undergoing tumour surgery. At the borders of the tumour, some healthy tissue has to be removed for safety, and this explant is infected with *E. histolytica* (Bansal et al. 2009). Within 2 h, the trophozoites cross the mucus layer and soon after detach and kill enterocytes as measured by the release of lactate dehydrogenase (LDH). The amoebae attack preferentially in the interglandular region between the crypts of Lieberkühn and then invade the *lamina propria* along the basal side of the crypts. At 4 h after infection, a number of pro-inflammatory mediators such as IL-1β, IL-6, IL-8, IFN-γ and TNF are produced by the cells in the colon explants. *E. histolytica* trophozoites are not deterred by these mediators at all; in contrast, they are clearly attracted by TNF (Blazquez et al. 2008). *E. dispar* trophozoites, in contrast, do neither invade nor cause an inflammatory response. *E. histolytica* strains disrupted for the Gal/GalNAc lectin light and heavy chains or amoebapore A were still able to invade, whereas a strain disrupted for the cysteine proteinase CP5 and amoebapore A could only penetrate the mucus but then failed to invade and cause inflammation (Bansal et al. 2009). This shows the importance of CP5 in the process. Interestingly, CP5 may induce inflammation by a mechanism unrelated to its protease activity. Pro-mature CP5 (PCP5), which is abundantly secreted from the trophozoites, binds to the $\alpha_V\beta_3$ integrin (vitronectin receptor) on the surface of Caco-2 colonic cells, via an RGD motif of PCP5, and stimulates a pro-inflammatory response via the transcription factor NFkB (Hou et al. 2010). The other factor influencing inflammation is the trophozoite-derived PGE_2 described above (Dey et al. 2003).

Pathogenic strains of *E. histolytica* are well equipped for the fight against neutrophils; in vitro they may even survive an attack at a ratio of 1,000 neutrophils per amoeba (Guerrant et al. 1981), whereas nonpathogenic strains are killed by the neutrophils. Taken together, many pieces of evidence suggest that pathogenic amoebae promote inflammation and can cope well with the situation. It also appears that the host inflammatory response contributes strongly to the resulting tissue damage (Seydel et al. 1998). So can we disregard the protective role of neutrophils? A genetic study on host susceptibility leads to a different conclusion. Individuals

carrying the mutation Q223R in the leptin receptor are more susceptible to intestinal amoebiasis. Leptin expression is increased during infection and it also serves to attract neutrophils to the gut. Leptin signalling in the individuals carrying the mutation is impaired; as a consequence there are fewer neutrophils – this points to a significant role of these immune cells (Naylor et al. 2014).

The main role in the defence against tissue-invading *E. histolytica* trophozoites is played by the macrophages. An early study showed that virulent amoebae can be killed by human monocyte-derived macrophages if these are activated by ConA-induced lymphokines. During the fight, the amoebae diminish the viability of the macrophages. The non-virulent Laredo strain is killed without loss of viability (Salata et al. 1985). In hamsters, peritoneal macrophages protect from amoebic liver abscess. When the hamsters are treated intraperitoneally with silica particles, they develop more liver abscesses with more metastases to other organs (Ghadirian and Meerovitch 1982). A major weapon of macrophages is nitrogen oxide (NO) produced from arginine catalysed by nitric oxide synthase (NOS). TNF and IFN-γ cooperate to augment the expression of NOS mRNA in macrophages elevating their cytotoxicity against the amoebae (Lin et al. 1994). The production of TNF by the macrophages can be induced by IFN-γ in combination with bacterial lipopolysaccharide (LPS), but TNF production in macrophages is also enhanced by contact with the *E. histolytica* Gal/GalNAc lectin heavy chain, more specifically with its cysteine-rich domain (Séguin et al. 1995). As a possible strategy to counter poisoning by NO, the amoebae produce an L-arginase (EHI_152330) which prevents NO synthesis by diverting its precursor and thus protects the trophozoites from activated macrophages (Elnekave et al. 2003).

In the liver as in the gut, neutrophils are attracted quickly to the site of invasion; later they make way for the attack of macrophages, the resident Kupffer cells as well as migrating inflammatory macrophages (Helk et al. 2013). In the investigated immunocompetent mouse model, the liver tissue damage was attributed to the defending resident and migrating macrophages as well as the neutrophils rather than to the amoebae, with TNF playing the major role as mediator of inflammation. The complex interaction between *E. histolytica* trophozoites and immune cells has been reviewed recently (Begum et al. 2015).

In the liver, the natural killer T-cells (NKT cells) were found to play an important role (Lotter et al. 2006). Livers of immunocompetent C57BL/6 mice were infected with *E. histolytica* trophozoites. Female mice were able to clear the infection after 3 days, whereas in males, the parasites persisted for at least 2 weeks. In female mice, spleen cells produced predominantly IFN-γ, in male mice IL-4. In mice lacking NKT cells, large liver abscesses developed; similarly a monoclonal antibody against IFN-γ rendered female mice susceptible to liver abscess. Taken together, IFN-γ-producing NKT cells could help females to prevent an amoebic liver abscess. A later mouse study showed that these phenomena were directly related to the hormonal status; testosterone could increase liver abscesses in females, while orchidectomy led to decreased abscess sizes in males.

An important link between the LPPG surface antigen and NKT cells was discovered in a further mouse study (Lotter et al. 2009). The glycolipid α-galactosylceramide

was found to decrease the size of liver abscess lesions via the interaction with NKT cells; such an effect is also obtained by treatment with the LPPG antigen isolated from trophozoites. Moreover, the phosphatidylinositol (PI) part of the LPPG alone stimulates the production of protective IFN-γ in NKT cells. The chemical analysis reveals two unusual PI structures containing very long fatty acid chains of 28 or 30 C atoms. In a review from the laboratory of the discoverer of LPPG (Wong-Baeza et al. 2010), the immunology and biochemistry of this unusual molecule are described which is acting as a pathogen-associated molecular pattern (PAMP) molecule as well as an antigen, sometimes carrying out a beneficial role in the interaction with the host.

Finally, the role of lymphocytes in the defence against *E. histolytica* must not be neglected. As mentioned above, they are the source of protective IgA. Some older mouse data shed some doubt on the importance of lymphocytes, for example, SCID mice lacking B- and T-cells are able to clear an intraperitoneal amoebic infection. Splenectomy renders mice extremely resistant against intracaecal infection with polyxenic trophozoites, whereas non-splenectomised genetically susceptible mice develop numerous intestinal ulcers (Ghadirian and Kongshavn 1985).

Activated cytotoxic T-cells are able, however, to kill amoebae, just like activated macrophages and neutrophils. This was shown for human T-lymphocytes activated by phytohaemagglutinin (PHA). The activity is contact-dependent and does not require antibodies (Salata et al. 1987). A recent study focused on the protection via lymphocytes (Guo et al. 2011). When mice are immunised subcutaneously with the LecA domain of the Gal/GalNAc lectin, they develop a specific CD4+ and CD8+ T-cell response. Protection can be transferred to naïve mice both with the CD4+ T-helper cells and with the CD8+ cytotoxic T-cells from the vaccinated mice. The CD4+ cells produce mainly IFN-γ, and, unexpectedly, the CD8+ cells make little IFN-γ but a higher amount of IL-17. Both cell populations are amoebicidal, but the CD8+ cells are more potent.

Finally, whereas an inflammatory response is necessary, it also needs to be balanced. A population of regulatory T-cells CD4(+)CD25(+)FoxP3(+) additionally positive for the chemokine receptor CCR9 allows mice to clear an intestinal infection within 1 day postinfection. In contrast the regulatory T-cells from CCR9 (–/–) mice are recruited in a delayed way to the epithelium, resulting in a prolonged inflammation and the presence of amoebic trophozoites for at least 7 days (Rojas-López et al. 2012).

11.5 Chemical Biology and Drug Treatment

This section will cover some better-characterised areas of *E. histolytica* metabolism. Due to its exclusively parasitic lifestyle, the organism is able to acquire a number of metabolites from its host or from other gut microorganisms. Therefore, it is not surprising that its biosynthetic abilities are quite limited. The other major theme is the microaerophilic character of *E. histolytica*, which means that the amoebae lack mitochondria carrying out ATP synthesis through oxidative

phosphorylation. So glycolysis is the main source of energy. On the other hand, *E. histolytica* can survive and even make positive use of a limited amount of oxygen.

11.5.1 Energy Metabolism

E. histolytica trophozoites gain the major part of their energy by glycolysis (Reeves 1984). In addition, a minor part of the energy is derived from the catabolism of amino acids. Unlike the trypanosomes, for which glycolysis also plays a major role, *E. histolytica* does not possess glycosomes as a separate compartment, but the glycolytic enzymes are located in the cytoplasm. All the major intermediates of glycolysis are the same as found in the majority of organisms (Fig. 11.3); however, some unusual features of the enzymes or unusual cofactors have been observed by biochemical (Reeves 1984) or genome (Clark et al. 2007) analysis. In the new annotation of the genome data (Lorenzi et al. 2010), a number of genes were deleted giving a clearer picture.

E. histolytica possesses a specific glucose transport with a K_m of 1.6 mM. This system also transports other sugars, however, less efficiently (Serrano and Reeves 1974). Pinocytosis appears to play a lesser role for the uptake of glucose than in other amoebae. Although the activity of the glucose transporter has been described a long time ago, the gene has not yet been annotated. Glucose is phosphorylated by either of two hexokinases Hxk1 (EHI_098290) or Hxk2 (EHI_098560) (Ortner et al. 1997a). Whereas Hxk1 also acts on mannose, Hxk2 is mainly a glucokinase. Neither of them phosphorylates fructose (Kroschewski et al. 2000). Traditionally, the migration of the two hexokinases in starch gels was used as one marker to distinguish *E. histolytica* from *E. dispar* (Farri et al. 1980). In the culture medium for *E. histolytica*, glucose can be replaced by fructose without any apparent consequence for growth rate or viability. The genome project revealed a specific bacterial-type fructokinase gene (EHI_054510), which was shown to produce fructose 6-phosphate (Matt and Duchêne 2015). In individuals with fructose malabsorption, the intestinal fructose levels could be increased, and the amoebae could profit directly from this situation. Analysis of the metabolic control of glycolysis shows that the steps of glucose transport and phosphorylation exert a major influence on the total flux (Pineda et al. 2015).

In the main flow of glycolysis, glucose 6-phosphate is converted to fructose 6-phosphate by glucose-6-phosphate isomerase (EHI_047730). This enzyme was also used for differentiating *E. histolytica* strains by starch-gel isoenzyme electrophoresis, and several polymorphisms have been discovered (Razmjou et al. 2006).

For the next step of glycolysis, there is a 60-kDa bacterial-type phosphofructokinase (EHI_000730) (Deng et al. 1998), which has probably been acquired by lateral gene transfer and uses pyrophosphate PPi instead of ATP for the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate (Fig. 11.3). There is also a very divergent 48-kDa minor phosphofructokinase (EHI_103590) of tenfold lower activity (Chi et al. 2001) which uses ATP as the substrate. Interestingly, the substrate

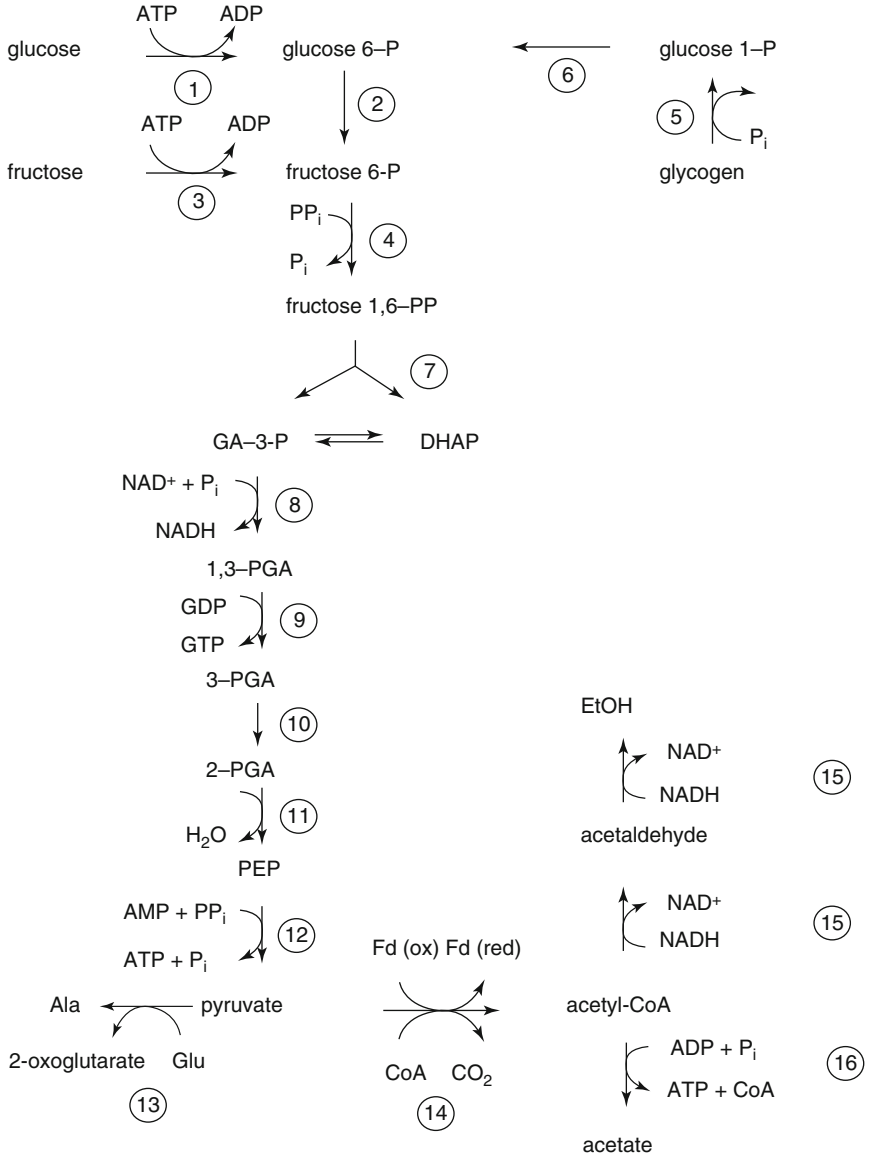

Fig. 11.3 Core energy metabolism of *E. histolytica*. Metabolites: GA-3-P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-PGA, 1,3-diphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate, Fd, ferredoxin; CoA, coenzyme-A. Enzymes: *1* hexokinase; *2* glucose-6-phosphate isomerase; *3* fructokinase; *4* phosphofructokinase; *5* glycogen phosphorylase; *6* phosphoglucomutase; *7* fructose-1,6-bisphosphate aldolase; *8* glyceraldehyde-3-phosphate dehydrogenase; *9* phosphoglycerate kinase; *10* phosphoglycerate mutase; *11* enolase; *12* pyruvate orthophosphate dikinase; *13* alanine transaminase; *14* pyruvate:ferredoxin oxidoreductase (PFOR); *15* alcohol aldehyde dehydrogenase ADH2; *16* acetyl-CoA synthase

specificity of the major 60-kDa phosphofructokinase can be switched from PPi to ATP by changing a single amino-acid residue.

In the next step, fructose-1,6-bisphosphate aldolase cleaves fructose 1,6-bisphosphate to the 3-carbon compounds glyceraldehyde 3-phosphate and dihydroxyacetone 3-phosphate. The enzyme, a class II aldolase (EHI_098570), is similar to eubacterial aldolases (Sánchez et al. 2002). A class I aldolase as found in the human host is absent in *E. histolytica*. This could make its aldolase a possible target for chemotherapy. This approach could be extended to other protists such as *T. vaginalis* and *G. duodenalis* which also have class II aldolases.

Triosephosphate isomerase converts dihydroxyacetone 3-phosphate into glyceraldehyde 3-phosphate. This dimer-forming enzyme has also been considered as a target for chemotherapy, and it is the first *E. histolytica* enzyme, for which a structure has been solved by X-ray crystallography (Rodríguez-Romero et al. 2002). The next enzyme in the pathway, another potential target of chemotherapy, is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which oxidises and phosphorylates glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (Reeves 1984). Phosphoglycerate kinase (EHI_188180) is an enzyme with an unusual substrate specificity. Whereas in most organisms, the high-energy phosphate is transferred to ADP, the *E. histolytica* enzyme transfers it to GDP generating a molecule of GTP. This has been known for a long time (Reeves 1984), but a few years ago, the molecular basis of the preference of GDP over ADP as an acceptor has been explained (Encalada et al. 2009). The amoebae could use GTP directly for protein synthesis and for signal transduction or convert it to ATP using a nucleoside-diphosphate kinase (EHI_104360).

Phosphoglycerate mutase (Reeves 1984) isomerises 3-phosphoglycerate to 2-phosphoglycerate. The current annotation lists five genes. There is a putative 2,3-bisphosphate-independent enzyme (EHI_050940) with significant similarity to bacterial phosphoglycerate mutases. Another entry is annotated as phosphoglycerate mutase family protein (EHI_129820); this entry is found on the list of lateral gene transfer candidates (Loftus et al. 2005) and could also be of bacterial origin. In addition, the databases list three more divergent genes. In contrast to the phosphoglycerate mutases, the enolase (2-phosphoglycerate dehydratase) is encoded by a single gene (EHI_130700), and it is related to eukaryotic enolases. Enolase dehydrates 2-phosphoglycerate to phosphoenolpyruvate and sets the stage for the generation of ATP. In many organisms, non-glycolytic functions have been described for enolases. As an example, in *E. histolytica* the enolase inhibits Dnmt2, the cytosine 5-methyltransferase-2 (Tovy et al. 2010).

The generation of ATP in the further step can proceed in two ways (Fig. 11.3). First, there is a pyruvate orthophosphate dikinase (EHI_009530) which converts AMP, PPi and phosphoenolpyruvate reversibly to pyruvate, ATP and Pi. This enzyme is normally found in bacteria and plants; as an example, in C4 plants this enzyme works in the direction of gluconeogenesis. In addition, the *E. histolytica* genome possesses a putative pyruvate kinase (EHI_098420), which transfers the phosphate directly from phosphoenolpyruvate to ADP in an exergonic reaction.

In the past, the presence of a glycolysis with important PPi-dependent steps led to the definition of a specific pyrophosphate-dependent type of glycolysis with *E.*

histolytica as the prime example (Mertens 1993). There are, however, also the conventional enzymes that do not use PPi. In addition, it was hypothesised that there should be no pyrophosphatase destroying the valuable PPi. In contrast, such an enzyme activity, albeit with a relatively high K_m of around 1 mM, had been discovered very early (McLaughlin et al. 1978), and there is a putative gene in the database (EHI_124880).

In the human host, pyruvate from glycolysis is oxidatively carboxylated to acetyl-CoA and CO_2 by pyruvate dehydrogenase, a large enzyme complex with thiamine pyrophosphate, lipoamide and FAD as catalytic and NAD^+ and CoA as stoichiometric cofactors. In *E. histolytica* in contrast, pyruvate is catabolised to the same products by a pyruvate:ferredoxin oxidoreductase (PFOR) (Reeves et al. 1977; Reeves 1984), for which there is one gene in the databases (EHI_051060). This gene is also a candidate for lateral gene transfer from bacteria. The electrons are transferred to ferredoxin and from the reduced ferredoxin to $NAD(P)^+$ and alternatively to H^+ ions. By structure modelling to the *Desulfovibrio africanus* PFOR structure, the *E. histolytica* PFOR is predicted to contain three [4Fe-4S] iron-sulphur clusters. Its activity is sensitive to oxygen and in particular to superoxide anions and H_2O_2. These reactive oxygen species can oxidise the iron-sulphur clusters which leads to step by step loss of the iron ions. To a certain extent, Fe^{++} can restore the activity of PFOR (Pineda et al. 2010).

In the current annotation of the HM-1:IMSS genome, there is only one ferredoxin gene (EHI_198670) encoding a 6.1-kDa protein; its sequence had been described before in a cloning paper. In addition, the genomes of the strains HM-3:IMSS and KU27 contain a second gene for a divergent 7.4-kDa ferredoxin. Several ferredoxin genes were deleted in the genome reannotation of 2010, among them a gene (XP_652694) also coding for the 7.4-kDa sequence. The ferredoxins from *E. histolytica* are annotated as being associated with two [4Fe-4S] clusters per molecule, as is the case in their bacterial homologues.

The reduced ferredoxin from the PFOR reaction is a strong reductant, but it has not been studied where exactly the electrons are going. One enzyme annotated as NAD(P)-dependent ferredoxin oxidoreductase (EHI_170000) could reduce $NAD(P)^+$. Under other conditions, the electrons could be used to generate molecular hydrogen. The amoebae appear to express a hydrogenase activity when they are in xenic culture with bacteria (Reeves et al. 1977), not in axenic culture. In the databases there are four genes coding for iron-only hydrogenase polypeptides, one of which was shown to have hydrogenase activity as a recombinant glutathione S-transferase fusion protein. A bacterial origin of the hydrogenase genes is highly probable. So far it is not known which of these gene products contributes most to hydrogen production, under which conditions this happens, and if it has any bearing on the pathogenicity of the amoebae. Finally, an important property of reduced ferredoxin is its ability to reduce and activate metronidazole to the nitroradical anion (Müller 1986), see below.

In *E. histolytica*, in the absence of the citric acid cycle and oxidative phosphorylation, the acetyl-CoA generated in the PFOR reaction cannot be used to generate large amounts of energy; however, the energy contained in its thioester bond can be

preserved, and one molecule of ATP can be generated. This is catalysed by an acetyl-CoA synthase (ADP forming, EC 6.2.1.13) (Reeves et al. 1977), which catalyses the reaction acetyl-CoA + ADP + Pi ↔ acetate + ATP + CoA. There are five annotated genes in the databases, one of which (EHI_178960) was studied biochemically (Jones and Ingram-Smith 2014). The alternative acetyl-CoA synthase that produces ATP from AMP and PPi (EC 6.2.1.1) has not been found in *E. histolytica*.

The generation of ATP is one positive use of acetyl-CoA. The problem of anaerobic glycolysis and fermentation is, however, that per molecule of glucose broken down, it generates two molecules of NADH and four molecules of reduced ferredoxin. The reduced ferredoxin can transfer its electrons to NAD^+, and the four NADH molecules can be used to reduce two molecules of acetyl-CoA via acetaldehyde to ethanol.

E. histolytica contains a complex system of alcohol/aldehyde dehydrogenases; even after deletion of some entries during the genome reannotation (Lorenzi et al. 2010), 17 entries are present. The NADPH-dependent alcohol dehydrogenase-1 (ADH1) (EHI_023110 and two related genes) was the first one to be characterised as a recombinant enzyme (Kumar et al. 1992). It is a Zn-containing enzyme which acts on ethanol as well as isopropanol and is highly expressed on the protein level. Interestingly, its homologue is downregulated in metronidazole-resistant *T. vaginalis*. The large bifunctional NADH-dependent ADH2 is probably the most important alcohol dehydrogenase in *E. histolytica* and is also important for the metabolic control (Pineda et al. 2015). It belongs to the ADHE family of bacterial alcohol dehydrogenases. Like in bacteria, the ADH2 appears to form helical rods in the cytoplasm which sediment with membrane fractions. The enzyme first reduces acetyl-CoA to an enzyme-bound hemiacetal which is then hydrolyzed to acetaldehyde and further reduced to ethanol which can be secreted. There are three ADH2 entries in the databases. In addition, there are three NADPH-dependent ADH3 genes, an aldehyde dehydrogenase gene and seven more putative alcohol dehydrogenases.

Under any conditions, the glycolytic pathway produces reduced metabolites, NADH from the GAPDH reaction, reduced ferredoxin and the molecules to which it transfers its electrons. Under anaerobic conditions, these must be used up to regenerate NAD^+ or oxidised ferredoxin. When this is done by reduction of acetyl-CoA, no extra ATP can be generated. In the presence of limited amounts of oxygen, the amoebae are able to spend the reducing metabolites on the reduction of oxygen, thus gaining an extra ATP; see section on redox metabolism. This means that *E. histolytica* has a limited aerobic metabolism in the absence of oxidative phosphorylation, a phenomenon known for a long time (Weinbach and Diamond 1974). Under these conditions, *E. histolytica* secretes acetate, rather than ethanol.

To compensate fluctuations in the supply of nutrients, *E. histolytica* stores glucose, its main energy source, in the form of glycogen. Glycogen is a polymer of α-1,4-linked glucose with α-1,6-linked branches. In the parasite, the glycogen structure appears to be very compact with branches every 5–6 glucose residues (Bakker-Grunwald et al. 1995). The cytoplasm of the amoebae contains numerous glycogen granules which are associated with glycogen phosphorylase. In the presence of

orthophosphate, this enzyme can cleave off the terminal glucose residue of glycogen to generate glucose 1-phosphate thus preserving the energy in the bonds between the glucose residues. The two full-length glycogen phosphorylases (EHI_138380 and EHI_096830) were found to lack the region responsible for the classical regulation by phosphorylation (Wu and Müller 2003). To remove the branches in the glycogen, *E. histolytica* possesses a large debranching enzyme (EHI_098360). To enter the glycolytic pathway, glucose 1-phosphate is isomerised to glucose 6-phosphate by phosphoglucomutase (EHI_110120) (Ortner et al. 1997b). Historically, this enzyme has been assayed in starch gels to distinguish *E. histolytica* from *E. dispar*. The databases also list two phosphoglucomutase/phosphomannomutase family members which have not been studied biochemically.

To synthesise glycogen, *E. histolytica* possesses a glycogen synthase (EHI_085980) and two putative branching enzymes (EHI_106090 and EHI_038160). The activated precursor UDP-glucose is generated from UTP and glucose 1-phosphate by UTP:glucose-1-phosphate uridylyltransferase (EHI_000440).

11.5.2 Drug Treatment and Redox Metabolism

This year, the treatment of *E. histolytica* with metronidazole, the most important nitroimidazole, celebrates its 50th birthday. During this time it has certainly saved millions of lives. The parent compound azomycin (2-nitroimidazole) (Fig. 11.4), already regarded as a candidate for an antibiotic, was isolated at the National Institute of Health, Tokyo, from a *Streptomyces* sp. from a soil sample (Maeda et al. 1953). Around the same time, scientists from the French company Rhône-Poulenc rediscovered the compound on Réunion Island. Several derivatives of the compound were synthesised with the aim to find a therapeutic agent against *T. vaginalis*, the most successful of which was R.P. 8823, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, now called metronidazole (Fig. 11.4) (Cosar and Julou 1959). Finally, it was discovered that this compound was also highly active against *E. histolytica*, and soon after it was used in humans (Powell et al. 1966). In addition, metronidazole is used to treat infections with *G. duodenalis* as well as anaerobic bacterial infections with *Bacteroides* spp, *Helicobacter pylori* or *Clostridium* spp. Two further nitroimidazoles, tinidazole and ornidazole (Fig. 11.4), are also active against *E. histolytica*; for tinidazole, there is some evidence that it may be slightly more effective in reducing clinical symptoms with slightly less adverse effects (Marie and Petri Jr 2013). The nitroimidazoles, which can be given orally, are readily absorbed into the bloodstream and are selected to treat an invasive amoebiasis, both dysentery and liver abscess. To eliminate the amoebae in the intestinal lumen, the therapy is usually supplemented with a luminal amoebicide such as paromomycin or diloxanide furoate.

The activity of metronidazole is closely associated with the redox metabolism of the target microorganisms. The drug needs to be reduced at its nitro group to become active. The reduction can proceed in several steps (Müller 1986), and the intermediates are chemically unstable, so their individual contribution to the toxicity of

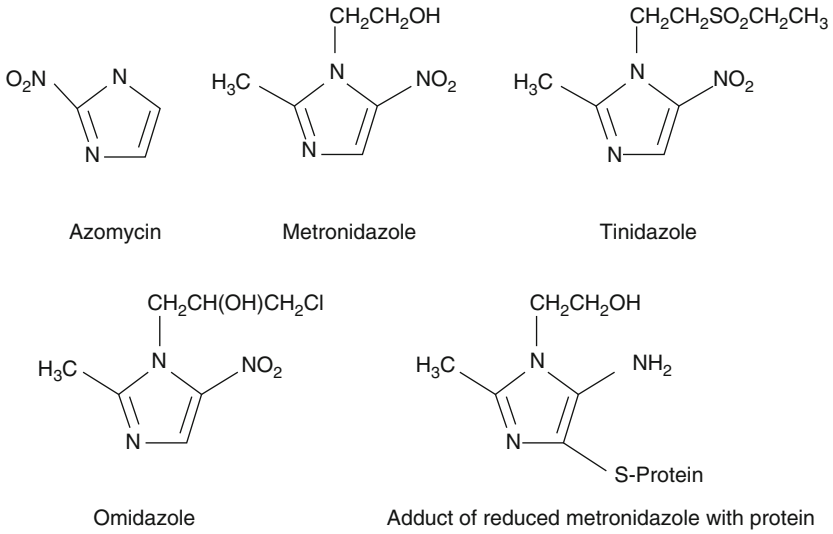

Fig. 11.4 The major nitroimidazoles used to treat *E. histolytica* infections, the parent compound azomycin and the way activated metronidazole is covalently bound to target proteins

metronidazole is difficult to study and still an open question. The first reduction step, supposedly by the transfer of an electron from reduced ferredoxin in the PFOR reaction to the nitro group of metronidazole, generates a nitro radical anion. One hypothesis sees this radical as mediating much of the toxicity of metronidazole such as by destroying DNA (Tocher and Edwards 1994); other studies doubt if the reduction products can break DNA strands (LaRusso et al. 1978). The nitro radical anion reacts quickly with molecular oxygen, however, producing a superoxide radical anion and unreduced metronidazole, a process called futile cycling (Mason and Holtzman 1975). In an aerobic organism, the nontoxic metronidazole is formed again, and the accompanying oxidative stress is not severe. The microaerophilic *E. histolytica* will be affected more by the superoxide radical anions; in addition, the reduction of metronidazole will proceed to the nitro and hydroxylamine derivatives (Müller 1986). These metabolites are very reactive with thiol groups in general and cysteine residues in proteins. In *E. histolytica* they modify covalently a specific small group of proteins, the flavin enzyme thioredoxin reductase (TrxR), thioredoxin (Trx), superoxide dismutase and purine nucleoside phosphorylase and a small putative glycan-associated protein (Leitsch et al. 2007). The cysteine residues are most likely bound to the 4-position of the reduced metronidazole (Fig. 11.4). Such protein modifications were also found in metronidazole-treated *T. vaginalis*, *G. duodenalis* and the fish parasite *Spironucleus vortens*.

In *E. histolytica*, TrxR was clearly inhibited in its thiol-reducing activity by modification with the reduced drug. This is a serious threat to the parasite, as it does not possess any of the components of the glutathione redox-protective system. Among the small redox-protective molecules, cysteine appears to play the major role assisted by other thiols such as thioproline which could serve storage functions

(Jeelani and Nozaki 2016). There has been a controversy in the past, whether *E. histolytica* could contain trypanothione, but this molecule is made up of two glutathione molecules linked by spermidine, and it would be hard to understand how it could be synthesised in the absence of glutathione.

The importance of the Trx system is underscored by a study, in which the protein targets of Trx were isolated. Trx contains a CXXC sequence; in its reduced form, one of the cysteine residues can attack an incorrect disulphide bond in another protein forming a mixed disulphide, and the second cysteine can attack this bond leading to oxidised Trx, later regenerated by TrxR, and the reduced target protein. With a mutated Trx with only one cysteine residue as bait (Schlosser et al. 2013), target proteins can be bound and isolated. In total, 234 putative Trx targets were resolved by two-dimensional electrophoresis, 16 of which could be identified by mass spectrometry.

E. histolytica TrxR (EHI_155440) can be a victim of metronidazole, but another twist of the matter is that TrxR can itself reduce and activate metronidazole (Leitsch et al. 2007). In this way it becomes understandable that typically those proteins become modified by the activated metronidazole, which interact with the Trx/TrxR system.

In the late phase of metronidazole toxicity, DNA degradation can be observed with several assays such as the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay, flow cytometry or simply agarose gel chromatography. The whole process of the killing of *E. histolytica* by metronidazole resembles apoptotic cell death in some aspects, but *E. histolytica* lacks caspases and caspase-dependent DNase, and no other DNases could be identified as responsible for the DNA degradation. So up to date, the molecular processes in metronidazole action against *E. histolytica* are far from being understood.

Although drug treatment of amoebiasis is not always successful, so far no clinical isolates have been found that are resistant to metronidazole. Emerging resistance cannot be excluded for any pathogen, and no second-line treatment of an alternative class of drugs would be available if the nitroimidazoles fail. In the laboratory, several attempts have been made to adapt *E. histolytica* to increasing concentrations of metronidazole, and the highest level attained was 40 μM (Wassmann et al. 1999), which is below the serum levels during oral therapy. In contrast, under anaerobic conditions, *T. vaginalis* can adapt to as much as 1000 μM of metronidazole (Kulda 1999). Whereas *T. vaginalis* is able to downregulate its PFOR and hydrogenosomes, PFOR expression appears to be unaltered in the partially resistant *E. histolytica* strains. Both organisms have to get rid of the reducing NADH from the first stage of glycolysis. Whereas *T. vaginalis* can use its lactate dehydrogenase to reduce pyruvate followed by the secretion of lactate, *E. histolytica* lacks this enzyme and has to go through the PFOR and ADH reactions to regenerate NAD^+. Instead, the partially resistant amoebae respond mainly to the oxidative stress accompanying metronidazole action (Wassmann et al. 1999); they upregulate superoxide dismutase and peroxiredoxin and downregulate TrxR (then called flavin reductase (Bruchhaus et al. 1998)). Although it appeared paradoxical at the time, it makes sense as TrxR activates metronidazole. The amoebae also downregulate the expression of one of the ferredoxin genes.

The major redox-protective enzymes in *E. histolytica* are the superoxide dismutase and the peroxiredoxins. There is only one gene coding for an iron-containing superoxide dismutase (EHI_159160); no manganese-containing enzyme was found. The peroxiredoxins were first called 29-kDa cysteine-rich surface antigen; then the protein (EHI_061980) was annotated as peroxide reductase (Bruchhaus and Tannich 1993). The peroxiredoxins, as they are widely called today, reduce H_2O_2, peroxynitrites and alkyl hydroperoxides with the electrons from reduced Trx. In the genome database, there are ten entries plus two annotated as peroxiredoxin family members. As described above, they are considered as vaccine component. *E. histolytica* does not possess a catalase.

In the human host, nitric oxide (NO) appears to be the major reactive nitrogen species reacting with cysteine residues to form S-nitrosothiols. TrxR is able to reduce S-nitrosothiols (Arias et al. 2012). In addition, TrxR can reduce molecular oxygen to H_2O_2 (Bruchhaus et al. 1998). The TrxR and Trx 41, one of the Trx isoforms, were localised to the inner side of the plasma membrane of *E. histolytica* close to the potential challenge by oxidising agents (Arias et al. 2008). A further anti-oxidative flavodiiron protein is able to reduce molecular oxygen ultimately to H_2O using NADH (Vicente et al. 2012). This protein is abundant in the cytoplasm of the amoebae and could play an important role in the aerobic metabolism. A recent high-throughput study investigated the activity of registered drugs against *E. histolytica* and found auranofin, an antirheumatic agent, to be a highly promising candidate (Debnath et al. 2012). This drug turned out to be an independent example of having TrxR as a target; not surprisingly, the drug enhanced the sensitivity of the amoebae against H_2O_2. Interestingly, the compound is also active against other important parasites such as *G. duodenalis*, *P. falciparum* and *Leishmania infantum*.

11.5.3 Lipid Metabolism: Fatty Acids and Phospholipids

For *E. histolytica* the major role of lipids is their presence in various membranes of the trophozoites which contain abundant phospholipids and cholesterol (Das et al. 2002; Sawyer et al. 1967). Proteins are attached to membranes by the addition of prenyl chains or by GPI anchors. Due to the lack of oxidative phosphorylation, the amoebae are unable to exploit the high energy content of lipids such as fatty acids.

In the classical pathway of type I fatty acid biosynthesis (Lynen 1980), the acetyl and malonyl groups from acetyl-CoA and malonyl-CoA are transferred to the acyl-carrier proteins (ACPs) in a large fatty acid synthase complex. Driven by the release of carbon dioxide, the two condense to form acetoacetyl ACP. Two steps of reduction and one step of dehydration lead to butyryl ACP. The fatty acid grows as the cycle is repeated a number of times each time adding two new carbon atoms. The type I fatty acid synthase complex was first characterised in the baker's yeast *S. cerevisiae* but is also present in animals. The component enzymes of type II fatty acid synthesis in bacteria and plants work in a similar way, but do not form large multienzyme complexes.

E. histolytica does not possess a conventional fatty acid synthase complex; fatty acids are largely acquired from the host. Nevertheless, there is some potential for fatty acid synthesis, or at least elongation (Das et al. 2002; Sawyer et al. 1967). There is an unusual large fusion protein of acetyl-CoA carboxylase and pyruvate carboxylase domains (EHI_082530), which has been characterised biochemically (Barbosa-Cabrera et al. 2012). It is able to remove a carboxyl group from oxaloacetate and transfer it to acetyl-CoA, thus activating it and forming malonyl-CoA and pyruvate. This fusion protein has not been identified in any organisms other than *Giardia* and *Entamoeba*. Fatty acid elongation could be carried out by a group of putative elongases which are similar to plant enzymes. Their number in the genome has been consolidated to five members. Neither these elongases nor the enzymes that could lead to the reduction of the beta keto groups left after the condensation of an acyl-CoA with malonyl-CoA have been studied at the biochemical level.

Most of the fatty acids acquired from the host or synthesised will go into phospholipids, which amount to 60–70 % of the total lipids in *E. histolytica* (Sawyer et al. 1967). So far little information is available at the biochemical level on how phospholipids could be synthesised or remodelled. The genome project revealed a number of genes associated with phospholipid biosynthesis, but gaps remain in the hypothetical pathways (Clark et al. 2007).

Finally, two interesting enzymes associated with *E. histolytica* lipid metabolism should be mentioned. One of them is a putative N-myristoyltransferase annotated as glycylpeptide N-tetradecanoyltransferase (EHI_159730), an enzyme transferring the C14 fatty acid myristate to proteins. Its homologue in *T. brucei* is a potential drug target (Frearson et al. 2010). The other enzyme, L-myoinositol 1-phosphate synthase (EHI_070720), has been studied at the biochemical level (Lohia et al. 1999). It catalyses the complex isomerisation of glucose 6-phosphate to L-myoinositol 1-phosphate. Inositol is found in phosphatidylinositol and its derivatives and in GPI anchors and is essential for these lipid anchors and for signal transduction.

11.5.4 Lipid Metabolism: Prenyl Metabolism and Cholesterol

The amoebae are not capable of de novo biosynthesis of cholesterol, but they exhibit some capabilities such as the extension of prenyl compounds and their attachment to proteins. The human host is able to synthesise cholesterol via five-carbon atom (C5) isoprene precursors. These are pieced together from acetyl-CoA first forming 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is reduced to mevalonic acid, a step inhibited by the statins, major drugs used to lower cholesterol levels in humans. Mevalonic acid is then phosphorylated and decarboxylated to the C5 compound isopentenyl diphosphate. This first stage of cholesterol biosynthesis is absent from *E. histolytica* as is an alternative pathway found in bacteria and plants (Clark et al. 2007). In the second stage, two C5 molecules, isopentenyl diphosphate and its isomer dimethylallyl diphosphate, are fused to form the C10 compound geranyl diphosphate; the addition of another C5 unit gives C15 farnesyl diphosphate. In

humans, the further steps in the final stage are the dimerisation of farnesyl diphosphate to C30 squalene followed by the formation of the four-ring sterol backbone and a number of further touch-up steps leading to cholesterol. The pathway from farnesyl diphosphate to cholesterol is absent from *E. histolytica*. As a consequence, the abundant cholesterol in the trophozoite membranes must be acquired from the host. Interestingly, hypercholesterinemia of the host appears to increase the severity of amoebic disease.

In *E. histolytica*, in the most recent annotation, there is one gene coding for the enzyme that isomerises isopentenyl diphosphate to dimethylallyl diphosphate (EHI_194410). In addition AmoebaDB lists genes encoding one putative bifunctional short-chain isoprenyl diphosphate synthase (EHI_142720) and two putative geranylgeranyl diphosphate synthases (EHI_028880 and EHI_105060). C20 geranylgeranyl diphosphate would be made from C15 farnesyl diphosphate by the addition of another C5 unit. So far, there are no reports on the biochemical characterisation of these enzymes; consequently their exact substrate specificities are not known.

Besides being important intermediates in the cholesterol biosynthesis pathway, C15 farnesyl diphosphate and C20 geranylgeranyl diphosphate have a major and ubiquitous function in the hydrophobic modification of proteins (McTaggart 2006). An important example is the modification of GTP-binding proteins allowing them to bind to membranes. The biochemical pathway has been proposed as a potential target for chemotherapy, as an example bisphosphonate inhibitors display activity against *E. histolytica* and *Plasmodium* spp. in vitro, as well as in vivo in rodent models (Ghosh et al. 2004).

The *E. histolytica* genome contains one gene each of the alpha and beta chains of protein farnesyltransferase (EHI_074760 and EHI_006190). The recombinant proteins were characterised on the biochemical level, and *E. histolytica* Ras4 was identified as the most likely target for modification (Kumagai et al. 2004). In addition, a protein geranylgeranyltransferase I beta-chain gene (EHI_077230) is present, however, no gene encoding the alpha chain. The beta chain was cloned and expressed together with the protein farnesyltransferase alpha chain (Makioka et al. 2006). In contrast to the protein farnesyltransferase, this hybrid molecule had a much broader specificity, demonstrating that a geranylgeranyl (C20) modification could play an important role.

The *E. histolytica* genome also possesses genes for two divergent CAAX prenyl proteases, enzymes for the trimming of prenylated proteins (EHI_187200 and EHI_075660). CAAX is the carboxy terminus of the substrate protein with C as the prenylated cysteine residue, A as an aliphatic amino acid and X as the terminal residue. The proteases cleave after the modified cysteine. After the processing step, a prenylcysteine carboxyl methyltransferase methylates the C residue; there are two divergent candidate genes (EHI_006100 and EHI_045150). So far, neither the protease nor the methyltransferase have been examined at the biochemical level.

Taken together, the cholesterol contained in *E. histolytica* membranes has to be acquired from the host, because major parts of the de novo cholesterol synthesis pathway are missing. Nevertheless, the amoebae are equipped with the enzymes to

elongate C5 prenyl precursors to C15 farnesyl- and C20 geranylgeranyl diphosphate and to generate membrane anchors of proteins such as Ras and Rho/Rac small GTPases. This is another aspect of the complex signalling mechanisms in *E. histolytica*.

11.5.5 Amino Acid and Nucleotide Metabolism

Whereas acanthamoebae are well equipped to synthesise amino acids themselves, *E. histolytica* can acquire them from its environment. Amino acids are consumed for protein synthesis; in addition, amino acids may be consumed as an alternative energy source when the level of glucose is low (Zuo and Coombs 1995). In simple media, asparagine, arginine, leucine and threonine are consumed more rapidly during lack of glucose. Aspartate is always consumed rapidly with much or little glucose in the medium.

There are some reactions which appear only at first sight to be involved in the biosynthesis of amino acids. Alanine can be generated directly from pyruvate by an alanine transaminase, for which two genes are annotated (EHI_096750 and EHI_159710). Interestingly, *E. histolytica* excretes alanine (Zuo and Coombs 1995), so this excess amino acid could be a way to dispose of some reducing power and a bound nitrogen at the expense of an acetyl-CoA generated by PFOR.

Cysteine is an extremely important amino acid for *E. histolytica*, and growth media are always supplemented with this amino acid. As glutathione is absent, cysteine is the main redox-protective small thiol (Fahey et al. 1984). Many *E. histolytica* proteins such as the lectins and peroxiredoxin contain a large number of cysteine residues. As a medium supplement, cysteine provides sulphur rather than only reducing power; it can be replaced by cystine, which the amoebae can reduce to cysteine (Jeelani et al. 2014). Under normal culture conditions, the level of cysteine is about twice as high as the level of cystine (Husain et al. 2010). The amoebae can also synthesise cysteine in two steps: in the first step, serine acetyltransferase (SAT) transfers an acetyl group from acetyl-CoA to the serine hydroxyl group (Nozaki et al. 1999), and in the second step cysteine synthase (CS) adds the thiol group from hydrogen sulphide (Nozaki et al. 1998). CS can also add the thiol group of methanethiol (CH_3SH) thus producing S-methylcysteine. SAT and CS are potential targets of chemotherapy (Jeelani and Nozaki 2016). Only some of the cysteine in *E. histolytica* exists as the free amino acid. By the reaction with aldehydes, it can be converted reversibly to adducts such as thiazolidine-4-carboxylic acid (T4C, thioproline), 2-methyl-thiazolidine-4-carboxylic acid (MT4C) and 2-ethyl-thiazolidine-4-carboxylic acid (ET4C), which can serve as a storage form of cysteine and as a defence against oxidative stress (Jeelani et al. 2014). Finally, cysteine is also the precursor of iron-sulphur clusters (Ali et al. 2004) which are generated exclusively by a NIF system comprising the NifS (EHI_136380) and NifU (EHI_049620) proteins found in the *E. histolytica* cytoplasm. Such a system is also found in nitrogen-fixing bacteria as well as ε-proteobacteria including *Campylobacter jejuni* and *Helicobacter pylori*.

A metabolomic investigation of the effect of cysteine depletion on the metabolism of *E. histolytica* (Husain et al. 2010) led to several surprising results: as expected, there was a rise in the level of O-acetylserine; this was mainly converted by CS to S-methylcysteine, not to cysteine. S-methylcysteine could potentially provide methyl or S-methyl groups, but its fate or function have not been defined. Also, it must be doubted that the SAT/CS pathway can provide lacking cysteine. A second unexpected result was that cysteine-depleted amoebae formed isopropylamine and the phospholipid phosphatidylisopropylamine. A general consequence of cysteine depletion was a suppression of energy metabolism, mostly the pyruvate to ethanol pathway. This could be caused by the lack of sulphur for the iron-sulphur proteins PFOR and ferredoxin.

In humans, cysteine can be generated from methionine via the transsulphuration pathway, but *E. histolytica* lacks this ability. Methionine is catabolised instead to 2-ketobutanoate, methanethiol and ammonia by methionine γ-lyase, a pyridoxal phosphate-dependent enzyme. *E. histolytica* possesses two methionine γ-lyase isoforms (EHI_144610 and EHI_142250) (Tokoro et al. 2003) which most likely have been horizontally transferred from the Archaea. The 2-ketobutanoate can potentially be used for the generation of one ATP molecule. Methionine γ-lyase is also able to degrade homoserine or cysteine. Due to its important role, the enzyme is also studied as a potential drug target. It will also be an interesting question, if the generated hydrogen sulphide or methanethiol could have any effect on the host.

The annotated enzymes from the genome project are potentially able to degrade a number of further amino acids (Clark et al. 2007), ultimately providing energy in the form of ATP as well as metabolites for proliferation of the amoebae. A large number of 174 transporters were identified among the putative *E. histolytica* gene products (Clark et al. 2007) which are able to acquire all molecules necessary from the environment. Detailed lists can be found at the website http://membranetransport.org (Ren et al. 2007).

Another consequence of the parasitic lifestyle of *E. histolytica* is the lack of purine as well as pyrimidine biosynthesis. Even a ribonucleotide reductase gene is missing in the genome. The salvage enzyme purine nucleoside phosphorylase (PNP) catalyses the reversible reaction purine bases + ribose-1-phosphate ↔ purine nucleosides + P_i. The genome contains five PNP genes. One of them was found to be covalently modified by the reduced drug during metronidazole treatment (Leitsch et al. 2007).

11.6 Open Questions

Many questions remain unanswered about the molecular biology of *E. histolytica*, a few of which are listed here. Definitively the methods have become available to make significant advances.

The structure of *E. histolytica* chromosomes, in particular of their ends, definitively needs more investigation. These questions are probably closely linked to the ploidy of the chromosomes and why the coupling of mitosis and cytokinesis is not as strict as in most other organisms. Genetic manipulation techniques have improved, but are far from the level in trypanosomatid research.

Even if it is very difficult to differentiate *E. histolytica* trophozoites into cysts, this should be tried, as it could open new venues to drug therapy or the establishment of a better animal model. Phagocytosis is central to the pathogenicity of *E. histolytica*, and much needs to be learnt about how the amoebae select what to eat, if and how the mechanisms to form and mature phagosomes depend on the food ingested, how nutrients are extracted and how amoebae cope with the waste products.

The chapter on immunology contains large gaps, and it suffers from the problem that small rodents do not develop amoebiasis as do humans. An important fundamental problem of the immune system is present in the defence against amoebiasis: too little response is not able to counter the parasites, but too much inflammatory response also harms the host. So any vaccine will have to be well balanced in this respect. The lack of extensive antigenic variation will be a strong help for the development of a vaccine. Finally, unusual immunomodulatory molecules from the amoebae could have other beneficial uses.

In the field of drug therapy, we are lucky to have metronidazole and other nitroimidazoles which continue to be active. Although we have learned more about their activation and interaction of their metabolites with cellular proteins, we still do not know which are the essential targets, what exactly causes the death of the amoebae and why *E. histolytica* has been so slow to develop resistance. As nitroimidazoles are not only used for treating infections with microaerophilic and anaerobic microorganisms but also are considered for anticancer therapy and tuberculosis treatment, these questions have a broad impact.

The generation of farnesyl- and geranylgeranyl precursors and their use as hydrophobic membrane anchors are important for *E. histolytica* and could be further developed into an alternative target for drug therapy; similarly the protein myristoyltransferase could be examined.

Why do the amoebic dysentery patients develop a hypocholesterinemia? It is impossible that the missing cholesterol is incorporated by the parasites, so is this a defence reaction of the host?

Finally the sequences of many putative transporters are known, but there are only few studies on the biochemical level. Definitively there are a number of ideas for drug development, but these should be worked at before *E. histolytica* learns to cope with the nitroimidazoles.

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Part III

Hot Topics

Phylogeny and Evolution

12

Christen M. Klinger, Anna Karnkowska, Emily K. Herman, Vladimir Hampl, and Joel B. Dacks

Abstract

The concept of a phylogeny of parasites is inextricably linked to that of the phylogeny of eukaryotes. Though it can be useful to infer functional principles from similar morphologies and trophic strategies, the evolutionary histories of parasites are most accurately viewed as independent shifts to this lifestyle from a free-living state. This chapter will describe the phylogeny of eukaryotes, the evolutionary positions of various prominent parasites within this framework, and the ways in which genomics has facilitated understanding of the free-living to parasitic transition, both in terms of phylogeny and function. Two major cellular systems of parasitological relevance, mitochondrion-related organelles and endocytic systems, will be explored, highlighting where considering the genomics and molecular cell biology of parasites in the context of their emergence from free-living relatives have helped us to better understand organelle evolution.

12.1 Introduction

Eukaryotes have conquered many environments in their ~2-billion-year history (Eme et al. 2014), but parasites have taken on one of the most challenging of all: other eukaryotes themselves. This poses many obstacles and a diversity of environmental conditions; indeed many parasites have developed complex life cycles and a number of cellular adaptations to them, including modification of genomes and organelles.

C.M. Klinger • E.K. Herman • J.B. Dacks (✉)
Department of Cell Biology, Canada Research Chair in Evolutionary Cell Biology, University of Alberta, 5-31 Medical Science Building, Edmonton, AB T6G 2H7, Canada
e-mail: cklinger@ualberta.ca; eherman@ualberta.ca; dacks@ualberta.ca

A. Karnkowska • V. Hampl
Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic
e-mail: anna.karnkowska@gmail.com; vlada@natur.cuni.cz

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The success of these organisms is fascinating, and at the same time deeply disturbing, as parasites wreak havoc on plant, animal, and human populations. Diseases such as malaria and Chagas' disease cause significant annual morbidity and mortality. Other parasites, such as *Theileria* and *Phytophthora*, affect livestock and crop populations and have adverse socioeconomic impacts. The study of parasitism has traditionally focused on understanding a single parasitic organism or lineage. There is a great deal, however, to be gained from taking a comparative evolutionary approach, which, we argue, engenders understanding of how parasitism arose and the underlying biology of pathogenesis in different parasites today. The mechanism by which parasites have evolved from free-living ancestral states underlies all aspects of parasitic infection and represents an important area of study, as rapidly acquired drug resistance challenges us to identify novel infection prevention and treatment strategies.

The key to this approach is the fact that parasitic organisms do not represent a single clade, nor do they represent basal lineages or any kind of "primitive" state. Parasites are found in all major clades of eukaryotic life, each sharing a common ancestor with free-living organisms. This suggests two conclusions regarding the evolution of parasitism: it has been arrived at multiple times through convergence, and it likely involves distinct mechanisms in each lineage.

The depth of comparison has been significantly enhanced by greater availability of genomic information from organisms across the eukaryotic tree. The genome of a given organism is a tremendously powerful resource in understanding its biology. It provides an extensive, though not always complete, part list allowing predictions of what cellular systems are functional. For cellular features that possess their own DNA, such as mitochondria and plastids, the organellar genome allows for a fairly complete study of their evolution. However, autogenously derived organelles (such as the endoplasmic reticulum (ER) and Golgi body) do not contain any genetic information of their own. Rather, protein markers characteristically associated with each organelle can be used to infer the organelle's presence in the absence of morphological data. By studying the retention of these factors in the nuclear genome, it is possible to hypothetically predict organelles and therefore cellular pathways present in an organism. Additionally, identifying these markers represents a starting point for molecular cell biological analyses, such as gene product localization and disruption. These can provide the impetus for launching downstream molecular parasitological studies, and therefore it is not surprising that parasites were among the first eukaryotes sequenced and that the list of sequenced parasite genomes continues to rapidly grow.

In this chapter we will explore ways in which the recent explosion of genomic information has informed our understanding of parasite evolution. We will start with an overview of eukaryotic phylogeny, emphasizing the position of parasitic taxa. We will then examine the evolution of two cellular systems often characteristic of parasitic organisms and key to their adaptation to the host environment and/or pathogenic mechanism: mitochondrion-related organelles (MROs) and organelles of the membrane-trafficking system. Though there are multiple evolutionary paths to parasitism, comparing the parasites to their closer, free-living relatives has revealed convergent evolution of these systems.

12.2 Phylogeny, Parasite Evolution, and the Last Eukaryotic Common Ancestor (LECA)

Gaining a phylogenetic framework of eukaryotes, parasitic or otherwise, hinges on two separate, though related, questions: Where is the root of the eukaryotic tree, and what are the relationships of eukaryotic taxa to one another? Early studies attempted to answer these questions simultaneously by including both eukaryotic and prokaryotic sequences in single-gene phylogenies using methods, like maximum parsimony and distance matrices with simple models of sequence evolution. The resulting phylogenies showed a ladder-like radiation of eukaryotes after the prokaryote-eukaryote split, with largely parasitic protist groups like diplomonads, microsporidians, and parabasalids all branching off before the radiation of "crown groups" such as animals, plants, brown algae, and ciliates (Sogin 1991). This artifactual grouping of parasitic taxa was supported by shared characteristics, namely, the lack of classical mitochondria but also an apparent lack of introns, Golgi bodies, peroxisomes, and a sexual life cycle. Combining this evidence resulted in the formalization of the Archezoa hypothesis, whereby it was proposed that these lineages represented "early" or "ancient" eukaryotes that diverged from the crown group taxa before the acquisition of canonical eukaryotic traits such as mitochondria (Cavalier-Smith 1987, 1989).

Over several decades, the Archezoa hypothesis was shown to be false. As discussed in much further detail below, the cell biological rationale of organelle absence was refuted by the discovery, first of genetic and later of molecular cell biological evidence evolutionarily linking MROs such as hydrogenosomes and mitosomes found in nearly every "mitochondrion-lacking" organism to classical mitochondria ((Burki 2014; Müller et al. 2012), inter alia). Likewise, these data have now been reported for sexual life cycles, Golgi bodies, and introns ((Koumandou et al. 2013), inter alia).

Additionally, the Archezoa hypothesis has been refuted due to the development of more robust techniques for phylogenetic inference and an improved understanding of eukaryotic systematics. Early phylogenies did not include enough characters to robustly place the taxa within a phylogeny and in addition were flooded with incorrect relationships caused by artifacts like long-branch attraction (LBA). LBA occurs when divergent and unrelated sequences in a dataset cluster regardless of whether divergence occurred due to a rapid increase over a short time or a steady increase over a long time (Philippe 2000). By including prokaryotic taxa and long-branching taxa of some parasitic groups, early phylogenies affected by LBA suggested that these parasitic taxa were closely related to prokaryotes (Stiller and Hall 1999).

Improvement in phylogenetic methods, combined with realization and mitigation of LBA, was quickly followed by the rise of phylogenomics as a method for improving resolution of eukaryotic relationships. Trees of one or a handful of genes may still suffer from a lack of support for internal nodes due to a paucity of informative positions. Increased genomic data for diverse eukaryotic lineages allows for inclusion of more genes and taxa, which serve to mitigate these issues. Concatenation of multiple genes into large multigene sequences for comparison ("phylogenomics") is the new method of choice for large-scale inference (Delsuc et al. 2005), but

is not without flaws. It allows for reduction of stochastic errors, but does not alleviate systematic errors such as model choice and inappropriate modeling of rate heterogeneity (Philippe et al. 2011; Rodríguez-Ezpeleta et al. 2007). Though powerful, phylogenomics techniques require careful execution to obtain meaningful results. Nonetheless, based on these large-scale concatenated phylogenies and incorporating ultrastructural information, eukaryotic diversity can be divided into five or six supergroups (Fig. 12.1), with parasites in each of the major clades of eukaryotic diversity ((Adl et al. 2012), inter alia).

The Amoebozoa and Opisthokonta comprise a larger clade, alternately named Unikonta, Amorphea, or most recently Opimoda. The Opisthokonta (Fig. 12.1, purple) includes animals and their unicellular relatives (Holozoa), along with fungi (Nucletmycea). Amoebozoa (Fig. 12.1, blue) contain a large number of amoeboid organisms along with slime molds. Parasites are found in numerous amorphean lineages; for example, microsporidians are basal fungi that parasitize animals, and *Entamoeba histolytica* is an important human parasite.

SAR and Archaeplastida, together with Cryptophyceae, Centrohelida, Telonemia, and Haptophyta, comprise the Diaphoretickes. The Archaeplastida (Fig. 12.1, teal) contain

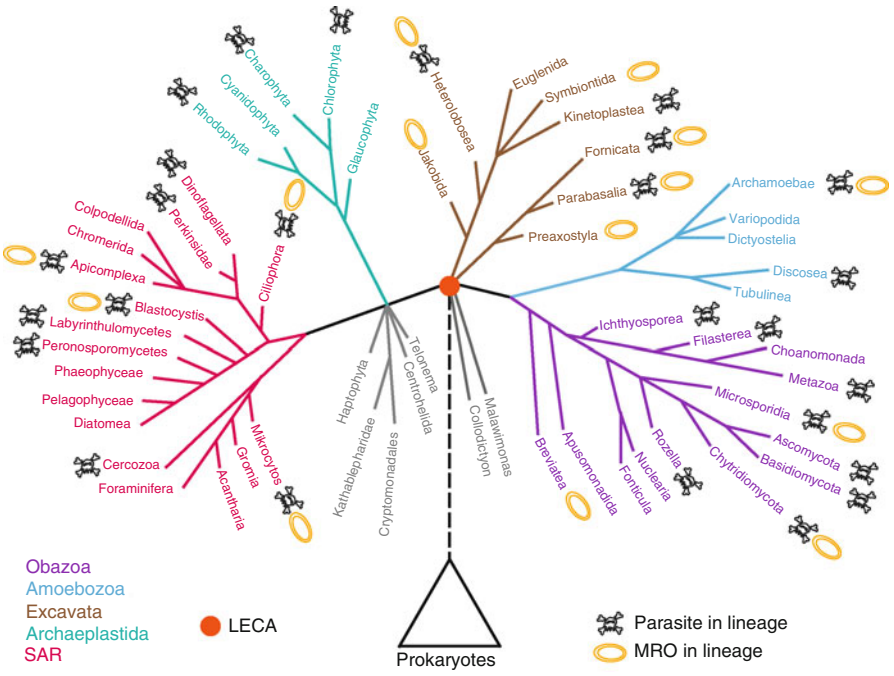


Fig. 12.1 Eukaryotic phylogeny. The current view of eukaryotic phylogeny, rooted as recently proposed on an excavate-like lineage. Large clades are color-coded and the distribution of mitochondrion-related organelles (MROs) and known parasitic organisms are denoted by *symbols* as per figure legend. Though these traits are often present in the same lineage, they do not demonstrate perfect correlation

land plants, mosses, as well as green, red, and glaucophyte algae, all united by the acquisition of a plastid through a single primary endosymbiotic event with a cyanobacterium.

SAR (Fig. 12.1, fuchsia) contains three main lineages, the stramenopiles, alveolates, and rhizarians. The rhizarians diverged first and are arguably the least understood major eukaryotic group. Stramenopiles can range from unicellular heterotrophs and photoautotrophs to multicellular kelps. Notable parasitic members include *Blastocystis*, an opportunistic pathogen of humans, along with *Phytophthora*, species of which cause potato blight and sudden oak death. Alveolates include the familiar ciliates *Tetrahymena* and *Paramecium* but also important fish pathogens like *Ichthyophthirius multifiliis*. Apicomplexa are almost exclusively parasitic, including the causative agents of malaria, *Plasmodium* spp., and cryptosporidiosis (diarrheal disease), *Cryptosporidium* spp.

The Excavata (Fig. 12.1, brown) is a diverse group of predominantly heterotrophic flagellates, many of which live in oxygen poor environments and/or are important parasites. Two main divisions exist: metamonads are generally endosymbionts/parasites and lack classical mitochondria, while discobids are generally free-living and have classical mitochondria. Within the metamonads reside some of the original Archezoa "amitochondriates," such as *Trichomonas vaginalis* and *Giardia intestinalis*. Within the discobids are important human parasites such as *Leishmania* and *Trypanosoma*. This group contains the only photosynthetic excavate lineage, euglenophytes, as well as the recently described deep-sea dwelling lineage of Symbiontida (e.g., *Calkinsia aureus*) and diplonemids, one of the most abundant groups in ocean plankton (Lukeš et al. 2015). The monophyly of Excavata was originally proposed based on shared morphological characteristics, essentially the presence of a ventral feeding groove "excavated" from the cell, along with the underlying cytoskeletal structures (Simpson 2003). Although the two main divisions of Excavata likely form a monophyletic clade, the relation of these to other lineages possessing the "excavate apparatus" is uncertain. This includes the originally proposed excavate *Malawimonas* and the newly characterized *Collodictyon* (Brugerolle et al. 2002; Zhao et al. 2012). Indeed, lineages such as apusomonads and breviates both possess two basal bodies, and their flagellar apparatuses are similar to the excavate-like state (Heiss et al. 2013a, b). These two lineages, however, are convincingly established as paraphyletically basal to the opisthokonts (Brown et al. 2013), forming the clade Obazoa (Fig. 12.1, purple).

Though our understanding of eukaryotic phylogeny is vastly improved from the days of the Archezoa hypothesis, the rooting remains uncertain. Various potential options are proposed, but none are overwhelmingly supported. The most recent evidence suggests a possible root either on an excavate lineage, between the Excavata and all others (He et al. 2014), or dividing Opimoda and Diphoda, consistent with the previous Bikont-Unikont hypothesis (Derelle et al. 2015). The lack of a concrete rooting hypothesis, however, does not change the notion that parasitism has multiple independent origins, nor does it hamper our ability to reconstruct the cellular state of the ancestral eukaryote. Consistent with current rooting hypotheses, a recent overview of ultrastructural data from microtubule organizing centers reconstructed a remarkably "excavate-like" state for the ancestral eukaryote (Yubuki and Leander 2013).

In the last decade, considerable effort has been put into sequencing and analyzing the genomes of diverse eukaryotes. These studies have resulted in a reconstruction of the last eukaryotic common ancestor (LECA), the last organism through which all extant eukaryotes draw their evolutionary path. This reconstruction has revealed a surprisingly sophisticated set of cellular components in the LECA, including nuclear architecture, the endomembrane system, mitochondria, cytoskeleton, and metabolism (reviewed in (Koumandou et al. 2013)).

Though the utility of such a reconstruction may not be immediately obvious to those outside the field of evolutionary cell biology, it is inherently applicable to the study of any cellular system of interest. The LECA can be thought to serve as a control group in the study of extant eukaryotes, allowing us to generate a null hypothesis about the evolution of a system: assuming no change to a system, it follows that the system should resemble that of the LECA, while changes represent potentially important innovations for that lineage. This evolutionary history provides essential context to the study of parasite/free-living pairs, including whether a given morphological or genomic character is an adaptive feature concurrent with parasitism or is preadaptive and hence present in both the parasite and its free-living relative(s).

12.3 Mitochondrion-Related Organelles (MRO) and Parasitism

One such system where a comparative evolutionary and genomic approach has been particularly insightful is the diversity of mitochondrion-related organelles. Dramatic differences in morphology of mitochondria in microbial eukaryotes under light and electron microscopy (shape, size, cristae morphology, number, etc.) have been known for decades. Textbook aerobic mitochondria are well characterized and act as the main sites of aerobic ATP generation, but also play pivotal roles in other aspects of cellular metabolism like apoptosis, amino acid metabolism, and synthesis of folate and iron-sulfur clusters, which are functional groups of some essential enzymes.

Some eukaryotes, including many prominent parasites, recognized as amitochondriates were classified as Archezoa (Cavalier-Smith 1987). However, the lack of mitochondria observed in Archezoa taxa was contradicted by the discovery of smaller double-membrane-bound enclosures lacking cristae, which were characterized as either hydrogenosomes or mitosomes. The notable exception is the lineage of oxymonads that appears to have lost the mitochondrion completely (Karnkowska et al. 2016). Accumulating genomic data have helped to decipher the evolutionary history of mitochondria, and it is now broadly accepted that mitosomes, hydrogenosomes, and other compartments, collectively known as MROs, originated from aerobic or facultatively aerobic mitochondria present in the LECA by adaptation to environments with low oxygen concentration (Embley and Martin 2006).

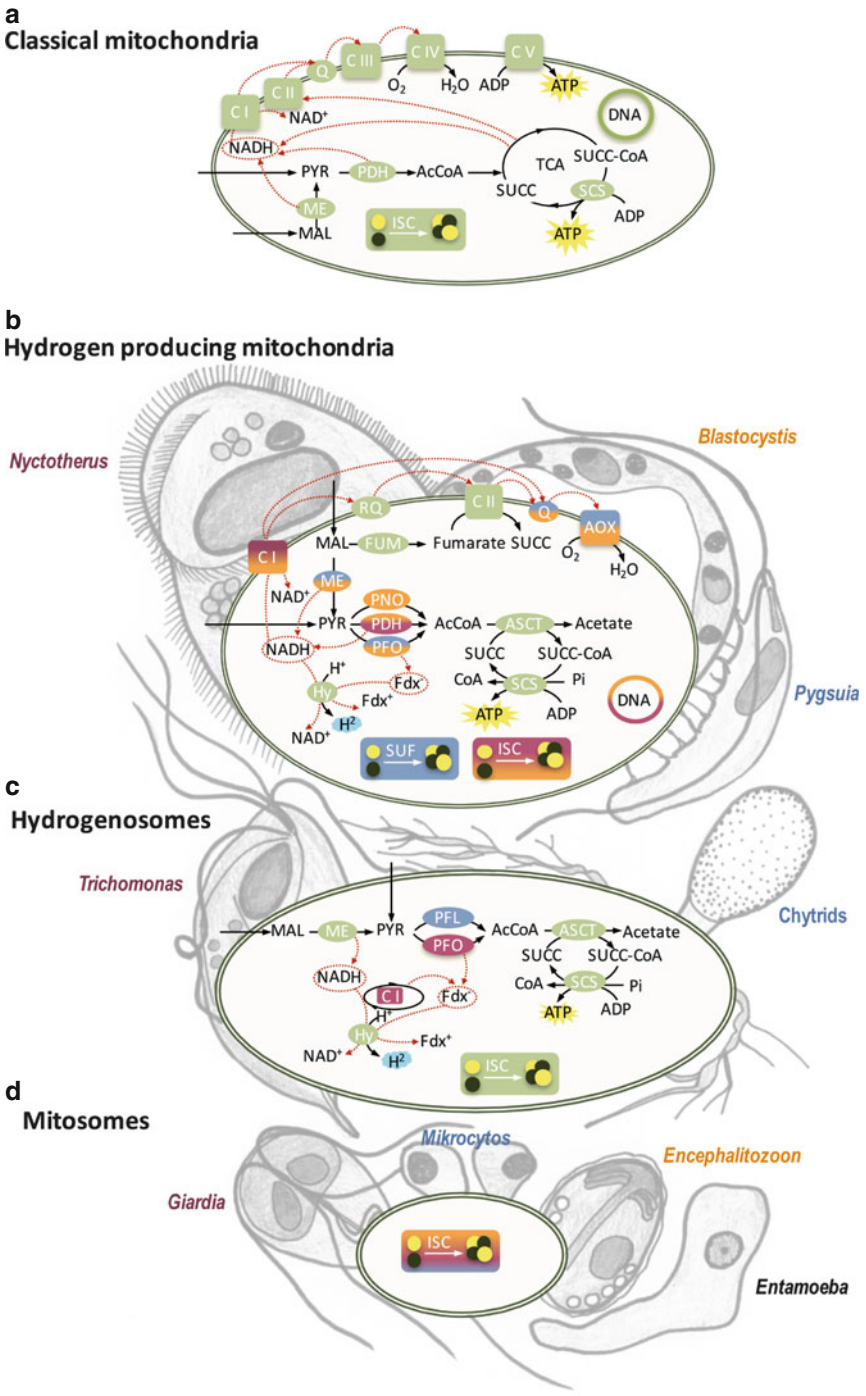
The patchy distribution of MROs on the tree of eukaryotes (Fig. 12.1) reflects the fact that several lineages have undergone such adaptation independently. Some clades contain only a few representatives with MROs (e.g., ciliophora, heterolobosea, Euglenozoa, apicomplexa), while other clades are composed entirely of

MRO-containing organisms (e.g., microsporidia, fornicata, parabasalia, archamoebae). Many organisms containing MROs are intestinal, mucosal, or intracellular parasites or commensals, but there is growing evidence of free-living lineages lacking classical mitochondria.

MROs represent various outcomes and transitional stages of a gradual evolutionary process, which greatly complicates our efforts to classify them. Müller et al. (2012) identify five classes of mitochondria and MROs: (1) classical mitochondria produce Adenosine Tri-Phosphate (ATP) by oxidative phosphorylation on an electron transport chain (ETC) using O_2 as the terminal electron acceptor (Fig. 12.2a); (2) anaerobic mitochondria produce ATP on an ETC in hypoxic environments and use other electron acceptors like fumarate or nitrate (not shown in Fig. 12.2); (3) hydrogen-producing mitochondria (HPMs) produce ATP by substrate-level phosphorylation (SLP) yet still possess a truncated ETC and produce H_2 (Fig. 12.2b); (4) hydrogenosomes produce ATP by SLP, lack an ETC, and produce H_2 (Fig. 12.2c); and (5) mitosomes do not produce ATP at all; organisms with mitosomes produce all ATP via cytosolic SLP (Fig. 12.2d). This classification reflects biochemical aspects of MRO diversity but does not reflect their evolutionary history; related taxa often possess different MROs and each class of MROs is distributed across unrelated clades. MROs in unrelated eukaryotes show striking convergent features, suggesting that there are a limited number of ways by which mitochondria may evolve to function under low oxygen concentration. These involve replacement of the pyruvate dehydrogenase complex (PDH) by anaerobic analogs, truncation or loss of the Electron Transport Chain (ETC), and involvement of [FeFe]-hydrogenase (H2ase) ((Hampl et al. 2011), inter alia).

The key process of mitochondrial biochemistry is the conversion of pyruvate to acetyl-CoA. In classical mitochondria this process is performed by the Pyruvate dehydrogenase PDH complex, but in MROs, three alternative enzymes are known to fulfill this role: pyruvate-ferredoxin oxidoreductase (PFO), pyruvate-NADP oxidoreductase (PNO), and pyruvate-formate lyase (PFL). The other key aspect is the reoxidation of reduced cofactors (NADH and ferredoxins). In classical and anaerobic mitochondria, this is done by the ETC, although the chain is truncated in the latter. In hydrogenosomes this function is performed by H2ase that reduces protons to H_2. HPMs represent a combination of the two ways. H2ases, PFOs, and PFLs of eukaryotes are apparently not directly related to homologues from the putative α-proteobacterial mitochondrial ancestor and have a patchy distribution across eukaryotes (Hackstein 2005; Hug et al. 2010; Meyer 2007; Stairs et al. 2011). The most probable explanation for these observations is one or a small number of gene transfer events from prokaryotes to eukaryotes followed by several eukaryote-to-eukaryote transfers.

In the course of evolution, many eukaryotic lineages adopted a parasitic lifestyle and entered new niches. Parasites, and gut endoparasites particularly, often have to survive in an environment that is low in oxygen, yet rich in organic nutrients. This is usually reflected by changes in their mitochondrial metabolism, as can be demonstrated by the anaerobic mitochondria of parasitic worms (Müller et al. 2012). These modifications are observed not only between species but also within lifecycles of a single species such as *Trypanosoma brucei*. The tsetse fly stage of *T. brucei* has a biochemically potent mitochondrion with effective ATP production. Though


a
Classical mitochondria
C I
C II
C III
C IV
C V
Q
NAD+
NADH
PYR
PDH
AcCoA
TCA
SUCC-CoA
SUCC
SCS
ADP
ATP
ME
MAL
ISC
DNA
b
Hydrogen producing mitochondria
Nyctotherus
Blastocystis
Pygsuia
RQ
MAL
FUM
Fumarate
SUCC
AOX
H2O
PNO
PFO
ASCT
Acetate
CoA
Pi
Hy
Fdx
Fdx+
H2
SUF
c
Hydrogenosomes
Trichomonas
Chytrids
PFL
d
Mitosomes
Mikrocytos
Encephalitozoon
Giardia
Entamoeba

unrelated to the presence/absence of oxygen, transformation to the bloodstream stage involves a downregulation of mitochondrial metabolism to such an extent that the mitochondrion no longer generates, and in fact consumes, ATP concurrent with a role in cytosolic NADH oxidation. The parasite's energetic demands are fully satisfied by glycolytic ATP production (Vanderheyden et al. 2000).

Further and permanent modifications can be seen in HPMs of the gut parasite/commensal stramenopile *Blastocystis* (Denoeud et al. 2011; Stechmann et al. 2008), the cockroach hindgut symbiont ciliate *Nyctotherus ovalis* (Boxma et al. 2005, 2007), and the free-living breviate *Pygsuia biforma* (Stairs et al. 2014) (Figure 12.2B). Unlike *Nyctotherus* that converts pyruvate to acetyl-CoA by the PDH complex, *Pygsuia* uses PFO and *Blastocystis* appears able to produce acetyl-CoA from pyruvate in three ways: by the PDH complex, PFO, and PNO. All three HPMs contain a partial Krebs cycle and similarly truncated respiratory chains with fumarate as the terminal acceptor. *Blastocystis* and *Pygsuia* also contain a gene encoding a noncanonical respiratory chain complex called alternative oxidase (AOX), known also from mitochondria of plants, trypanosomes, *Euglena*, and *Cryptosporidium parvum* (Putignani et al. 2004). AOX can receive electrons from complexes I and II and pass them to the terminal acceptor O_2, allowing adaptation to oxygen stress and maintenance of the NADH/NAD balance. These three examples represent modifications of classical mitochondria to an anaerobic lifestyle, but without concurrent loss of some features typical for mitochondria. Most notably some of these MROs still possess their own genomes. It is hypothesized that the genome has to be retained since it codes for particular pieces of the ETC that cannot be encoded entirely in the nucleus. Conditions under which an MRO may lose its genome are currently unclear (Allen 1993; Björkholm et al. 2015; Popot and de Vitry 1999), but hydrogenosomes and mitosomes, which we will discuss below, no longer possess any genetic material.

Hydrogenosomes (Fig. 12.2c) were discovered in parasitic trichomonads (Lindmark and Müller 1973), but are also found in other metamonads (e.g., *Paratrimastix*, formerly *Trimastix,* and *Spironucleus*), as well as in chytridiomycete fungi, ciliates, archamoebae, and heteroloboseans. In the *T. vaginalis* hydrogenosome, pyruvate is oxidized to Acetyl-CoA by a PFO; an H2ase reoxidizes the ferredoxin reduced in this reaction. Acetyl-CoA is then converted to acetate by an acetate-succinate CoA transferase (ASCT), and the resulting succinyl-CoA is used by succinyl-CoA synthetase (SCS) to generate ATP by SLP (Hrdy et al. 2004).

Fig. 12.2 Reductive evolution of mitochondria. In each panel a cartoon of the organelle and its accompanying biochemistry is shown, along with drawings of example organisms possessing the organelle. Mitochondrial evolution: (**a**) classical mitochondria, (**b**) hydrogen-producing mitochondria (HPM), (**c**) hydrogenosomes, and (**d**) mitosomes. Note that anaerobic mitochondria are not shown here as they are not yet described from microbial eukaryotic parasites. *AcCoA* acetyl-CoA; AOX, alternative oxidase, *ASCT* acetate/succinate CoA transferase, *C I* complex I, *C II* complex II, *C III* complex III, *C IV* complex IV, *C V* complex V (ATP synthase), *Fdx* ferredoxin, *FUM* fumarase, *Hy* [Fe]-hydrogenase, *ISC* iron-sulfur cluster pathway, *MAL* malate, *ME* malic enzyme, *PDH* pyruvate dehydrogenase complex, *PFO* pyruvate/ferredoxin oxidoreductase, *PNO* pyruvate/NADP oxidoreductase, *PYR* pyruvate, *Q* quinone, *RQ* rhodoquinone, *SCS* succinyl-CoA synthetase, *SUF* sulfur mobilization system, *SUCC* succinyl, *SucCoA* succinyl-CoA, *TCA* tricarboxylic acid cycle; *red dotted line* indicates electron transport

Other hydrogenosomes also produce H_2 and ATP via SLP, but the biochemistry varies. For example, hydrogenosomes of the symbiotic chytridiomycetes, *Neocallimastix* and *Piromyces*, lack PFO and instead contain PFL and their H2ase reoxidizes NADH produced by malic enzyme (Hackstein et al. 2008). The presence of hydrogenosomes not only in unrelated lineages of parasites but also in mutualists (e.g., *Neocallimastix*, the ciliate *Dasytricha*) and free-living organisms (e.g., the ciliate *Trimyema*, probably the excavate *Paratrimastix* and the holozoan *Loricifera*), indicates that, as with HPMs, hydrogenosomes have evolved multiple times by convergent evolution and do not represent a specific feature of parasites.

Mitosomes (Fig. 12.2d) represent the most highly and permanently reduced forms of MROs, typically not involved in energy generation (Katinka et al. 2001; Loftus et al. 2005; Morrison et al. 2007). Mitosomes not only lack genomes but also most known mitochondrial proteins. They probably lost their energy production capacity concurrently with adaptation to an endobiotic lifestyle and opportunity to scavenge energy-rich biomolecules from the host. This notion is supported by the fact that mitosomes, unlike other MROs, are not identified in free-living microbial eukaryotes. Some of them, like diplomonad *Giardia* and amoebozoan *Entamoeba*, are cavity or tissue parasites invading the intestinal tract, while others, like *Cryptosporidium*, Microsporidia, and *Mikrocytos mackini*, invade the cytoplasm of host cells. Due to their extreme simplification, examination of mitosomes may aid in answering the question of whether there are any universal functions of MROs and mitochondria.

Cryptosporidium spp. MROs produce ATP by SLP and, in the case of *Cryptosporidium muris*, also by oxidative phosphorylation using the Krebs cycle with an unusual respiratory chain and ATP synthase (reviewed in (Mogi and Kita 2010)). They lack a genome and H_2 production and so may be classified as mitosomes (Mogi and Kita 2010; Müller et al. 2012) or more generally as MROs (Keithly 2008), a view which we favor. The functions of mitosomes in other lineages are unclear. The mitosome of the microsporidian *Encephalitozoon cuniculi* probably contains two subunits of the E1 component of a PDH complex encoded by the nuclear genome, but this complex cannot be functional without the other two unidentified components (Katinka et al. 2001). They also likely contain glycerol 3-phosphate dehydrogenase, an enzyme that is involved in the transport of electrons from the cytosol to mitochondria, and manganese-containing superoxide dismutase, which forms part of the system that protects the cell from oxidative stress (Burri et al. 2006). A proteomic study of *E. histolytica* mitosomal fractions (Mi-ichi et al. 2009) revealed the presence of several enzymes involved in sulfate activation: ATP sulfurylase (AS), adenosine-5-phosphosulfate (APS) kinase (APSK), and inorganic pyrophosphatase (IPP), as well as a sodium/sulfate symporter involved in sulfate uptake. This suggests that sulfate activation is the major function of these mitosomes. Proteomic analysis of the mitosomal fraction of *Giardia* (Jedelský et al. 2011) revealed 139 proteins, but only 20 were confirmed to localize in the organelle. A study using biochemical tagging revealed presence of many other, mostly *Giardia*-specific, proteins in the mitosome (Martincová et al. 2015). Functions of these proteins are largely unknown and the only biochemical activity detected so far

is limited to the Fe-S cluster synthesis (Tovar et al. 2003). The recently discovered mitosome of the rhizarian *Mikrocytos mackini* is also most likely involved mainly in the Fe-S cluster assembly (Burki et al. 2013). Hence, formation of Fe-S clusters appears to be a common function of virtually all MROs with the exception of *E. histolytica* mitosomes.

Transcriptomic and genomic investigations of free-living relatives of some parasitic anaerobes or microaerophiles suggest that they also contain anaerobic derivates of mitochondria. Hence, free-living ancestors of these parasite/free-living pairs were already adapted to an anaerobic niche, allowing some extant lineages to colonize anaerobic environments such as intestinal tracts. Two major clades of microbial eukaryotes have been particularly useful in understanding the connection between the free-living and parasitic members.

Metamonads are a group of excavates known to inhabit anaerobic or microaerophilic niches like animal guts. Well-known metamonad parasites like *T. vaginalis* and the salmonid fish pathogen *Spironucleus salmonicida* (Jerlström-Hultqvist et al. 2013) contain hydrogenosomes, while *G. intestinalis* contains a mitosome. *Trichomonas* and *Spironucleus* hydrogenosomes have relatively similar biochemistry, despite the fact that *Spironucleus* is more closely related to *Giardia*. Comparison and phylogenetic analyses of *Trichomonas* and *Spironucleus* hydrogenosomal genes suggest derivation from a common ancestor, with *Giardia* mitosomes representing further degeneration (Jerlström-Hultqvist et al. 2013). A more complete understanding of the evolution of these parasite MROs has arisen due to recent sequencing projects focused on their free-living relative *Paratrimastix*. A transcriptome survey has revealed that it possesses a hydrogenosome-like MRO with a mitochondrial pathway involved in amino acid metabolism, as well as H2ase and a lack of all ETC complexes (Hampl et al. 2008; Zubáčová et al. 2013). Analysis of these four organisms clearly shows that hydrogenosomes appeared in the free-living ancestor of Metamonada.

The recently described hydrogenosome of the free-living archamoebae *Mastigamoeba balamuthi* (Nývltová et al. 2013, 2015) provides an opportunity for similar comparative studies with the mitosome-bearing *Entamoeba*. The *M. balamuthi* hydrogenosome contains complex II (but no other ETC complexes) as well as a H2ase and a PFO (Gill et al. 2007). Genomic data have also demonstrated the presence of genes involved in sulfate activation (AS, APSK, and IPP), a property shared with *E. histolytica.* The second unusual feature shared by those two taxa is the replacement of the mitochondrial type Fe-S cluster assembly machinery (ISC) by ε-proteobacterial enzymes NifS and NifU. Genes for these enzymes have been duplicated in *M. balamuthi*, with the product of one paralog localized in the cytosol and the other in the MRO. *E. histolytica* has only one copy, products of which are localized in the cytosol and putatively also in the mitosomes, but the evidence for mitosomal localization is ambiguous (Maralikova et al. 2010; Mi-ichi et al. 2009). The presence of common features between *E. histolytica* and *M. balamuthi* MROs suggests that the loss of aerobic mitochondrial metabolism and acquisition of anaerobic enzymes, sulfate activation pathways, and ε-proteobacterial Fe-S cluster assembly proteins occurred in a free-living ancestor of these amoebae.

Genomic and transcriptomic data, provided in increasing amounts by high-throughput sequencing techniques, can be used for in silico prediction of MROs based on their known properties (Burki et al. 2013), even for organisms without established cultures. Features related with adaptation for an anaerobic lifestyle, namely, modification or loss of ETC and presence of Fe-S cluster-containing proteins like PFO, are exploited in specific treatments by metronidazole. This nitroimidazole antibiotic is activated to its cytotoxic form by reduction, which takes place in the presence of low redox potential electron transporters (ferredoxins, PFO) and in the absence of the high redox potential acceptor O_2 and so has little effect on human cells (Upcroft and Upcroft 2001).

Comparative genomics of parasitic protists and their free-living relatives are profoundly impacting our understanding of reductive evolution of mitochondria. It endorses the idea that the evolution of parasitism was in some cases preceded by adaptation to anaerobic environments, including major mitochondrial modifications. A likely model is that free-living organisms first evolved the capacity to survive in anaerobic environments like animal guts, and some subsequently evolved further to become endobionts and, in some cases, parasites. However, extreme reduction exemplified by mitosomes is probably only possible in parasites or endobionts, as they are able to use their host to fulfill energetic requirements.

Reduction of classical mitochondria to MROs exemplifies a situation in which similar environmental pressures constrain evolution toward convergent features. The evolution of membrane trafficking in diverse parasites follows similar trends, including modification in gene complement and localization/function, but often results in very different systems depending on parasite, environmental, and host factors.

12.4 Membrane Trafficking in Parasites

Membrane trafficking is crucial to the ability of diverse parasites to grow and proliferate. It contributes to processes including motility, nutrient acquisition, host invasion, immune evasion, and secretion. Generally, membrane trafficking is the process by which protein and lipid components are shuttled between intracellular compartments and by which cells take up and release materials into their environment. These diverse roles follow a similar scheme, roughly divided into phases of cargo selection and coat recruitment, vesicle formation and scission, transport from donor to acceptor compartment, and tethering and fusion with the target membrane (Bonifacino and Glick 2004). Despite the importance of trafficking to parasite biology, knowledge of membrane trafficking function in many taxa lags behind that of model systems such as yeast and mammalian cells.

Studies in recent years have revealed that the LECA possessed a complex membrane trafficking system (MTS), including an ER, Golgi body, and at least one form of endocytic organelle, such as an endosome or lysosome ((Koumandou et al. 2013) inter alia). These studies have also revealed unexpected losses of ancestral MTS components in our model systems. Examples include three ancestral Rab proteins not found in the human genome (Elias et al. 2012), a novel ArfGAP family completely

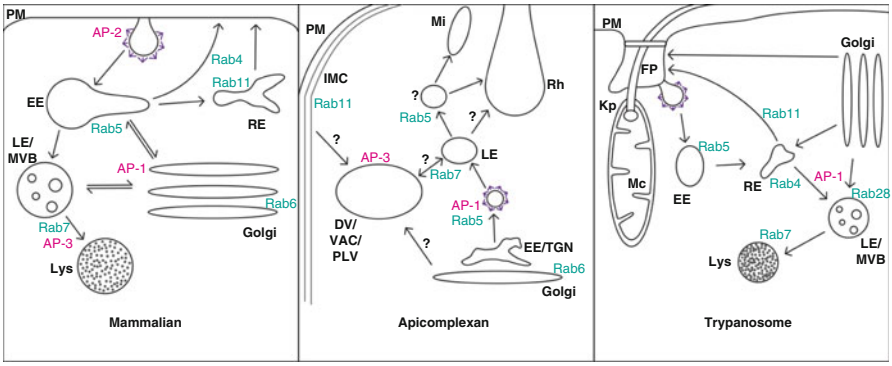


Fig. 12.3 Membrane trafficking in parasites. General trafficking pathways present in mammalian, apicomplexan, and trypanosomes are shown. *Bold* type labels organelles, while adaptor protein (*AP*) and Rab proteins are listed separately; *question marks* denote uncertainty in pathways and/or machinery. Clathrin coats are denoted by *purple triskelions*. *Abbreviations*: *PM* plasma membrane, *TGN* trans-Golgi network, *EE* early endosome, *RE* recycling endosome, *LE* late endosome, *MVB* multivesicular body, *Lys* lysosome, *IMC* inner membrane complex, *Mi* microneme, *Rh* rhoptry, *DV* digestive vacuole, *VAC* vacuolar compartment, *PLV* plant-like vacuole, *FP* flagellar pocket, *Kp* kinetoplast, *Mc* mitochondrion. Note that though apicomplexans like *T. gondii* possess stacked Golgi, morphology differs in other members of the phylum and a single cisterna is shown for simplicity. Also note that diagrams are not to scale

absent from animals and yeast (Schlacht et al. 2013), and a unique adaptor protein-related complex known as "TSET", one remaining component of which gave rise to the muniscin cargo adaptor in metazoa (Hirst et al. 2014). Thus, only by studying trafficking in diverse eukaryotes will we obtain a generalizable model of MTS function and be able to characterize divergent systems, including those of parasites.

One process of particular interest is post-Golgi trafficking, including endocytosis, exocytosis, and movement of cargo through endosomal organelles (Fig. 12.3). Uptake of extracellular material frequently occurs by clathrin-mediated endocytosis (CME), which involves numerous adaptor proteins that link cargo and clathrin at the plasma membrane (PM). Accessory proteins, together with clathrin, bend and stabilize the nascent vesicle, which eventually buds away from the PM and is separated by dynamin (Rao et al. 2012; Reider and Wendland 2011; Traub and Bonifacino 2013). Cargos are internalized and subsequently enter the endosomal system. All endocytic, as well as some exocytic, cargoes converge at the early endosome (EE) for sorting. Recycled cargo accumulates in tubular extensions for subsequent transport to the PM (either through recycling endosomes, or more rapid direct recycling), or back to the trans-Golgi network (TGN). Otherwise, it accumulates in larger volume domains for transit to the late endosomal system. Formation of intraluminal vesicles to form multivesicular bodies (MVBs) begins at the EE and continues throughout the endosomal maturation process. During this process, the organelles acidify and undergo changes in marker proteins. Late endosomes (LEs) form and subsequently fuse with lysosomes to form hybrid endolysosomes, in which cargo is finally degraded by acidic hydrolases (Huotari and Helenius 2011; Scott et al. 2014).

The study of parasites has led to a better understanding of the plasticity of this system. *T. vaginalis* adheres to host cells as a critical step in pathogenesis. This is mediated by adhesins such as lipoglycans that bind host galectin-1 and numerous other secreted components of the lipophosphoglycan surface matrix (Bastida-Corcuera et al. 2005; Okumura et al. 2008). The presence of prominent Golgi bodies (parabasal apparatus) is well documented (Honigberg et al. 1971), and multivesicular bodies (MVB)-derived exosomes were recently described (Twu et al. 2013). Consistent with secretion of many virulence factors, numerous trafficking families, including adaptor and coat proteins involved in vesicle formation, and tethering and fusion proteins involved in vesicle fusion are expanded several fold compared to humans (Carlton et al. 2007). *E. histolytica* lacks many identifiable endomembrane structures, such as stacked Golgi, but does possess numerous vacuolar structures of likely endolysosomal origin (Perdomo et al. 2015). Pathogenicity relies on efficient phagocytosis, and many proteins are recruited to the phagosome that function in endocytic trafficking in model systems ((Juárez-Hernández et al. 2013), inter alia). This amoeba encodes all common coat complexes, as expected, but similar to *T. vaginalis*, it possesses large expansions of GTPases known to regulate trafficking (Loftus et al. 2005). By contrast, the diplomonad *G. intestinalis* represents a case of extreme MTS divergence. Materials move from the ER to peripheral vacuoles, compartments that appear to perform all the functions of diverse endosomes/lysosomes, directly with no intermediate sorting compartment such as a Golgi (reviewed in (Faso and Hehl 2011)); enclosures termed encystation-specific vesicles present during part of the lifecycle have been suggested to represent a "Golgi-like" compartment. Unlike *T. vaginalis* and *E. histolytica*, *G. intestinalis* encodes a reduced set of trafficking factors compared to close relatives and the LECA.

The two parasitic lineages best studied, Apicomplexa and trypanosomes, have modified their MTS to facilitate very different modes of parasitism. Apicomplexa include obligate intracellular parasites of humans and domestic animals. They represent a system in which the endocytic functions of post-Golgi trafficking have been reduced in favor of increased secretive capabilities. Apicomplexans possess a cytoskeletal apparatus at the apical end of the cell known as the apical complex, along with secretory organelles involved in host cell invasion known as micronemes and rhoptries (Baum et al. 2008). These organelles are derived from endosomes/lysosomes (Klinger et al. 2013b; Ngô et al. 2004), yet the effect of these additional endosomal compartments on the parasite's MTS is incompletely understood. Despite extensively reduced metabolic functions, and a proposed reliance on uptake of host cell metabolites for growth and proliferation (Abrahamsen et al. 2004; Cassera et al. 2008; Plattner and Soldati-Favre 2008; Woodrow et al. 2000), classical endocytosis has yet to be demonstrated in these organisms (Pieperhoff et al. 2013). Indeed, it appears that apicomplexan parasites have co-opted machinery traditionally involved in endocytosis and recycling to function in building their invasion organelles (reviewed in (Tomavo 2014)).

The presence of a plant-like vacuolar lysosome (VAC) in *Toxoplasma gondii* suggested the potential for degradation of endocytosed materials (Miranda et al. 2010). Recent work has elucidated such a role for the VAC, yet the mechanism

through which uptake occurs remains elusive (Dou et al. 2014). There have been numerous reports of plasma membrane invaginations and endocytic activity (Botero-Kleiven et al. 2001; Coppens et al. 2000; Gross et al. 1993; Nichols et al. 1994), but no mechanistic data has yet emerged. Endocytic uptake has been visualized extensively in *P. falciparum*-infected red blood cells, where the process of internalization and digestion of hemoglobin in an acidified digestive vacuole (DV) represents a drug target (Sigala and Goldberg 2014).

In comparison, endocytosis is comparatively well studied in African trypanosomes and many mechanistic details have been elucidated. *T. brucei* relies extensively on endocytosis for its continued survival as an extracellular parasite, including extensive endocytosis and recycling of variable surface glycoproteins (VSGs) (Cross 1975; Field et al. 2009; Manna et al. 2014; Vickerman 1969). While Apicomplexa are polarized toward the apical end of the cell, trypanosomes are polarized posteriorly. All endocytic traffic occurs in the small region between the nucleus and the kinetoplast (the mitochondrial genome)/flagellar pocket, and all endo- and exocytic events occur in the pocket itself (reviewed in (Field and Carrington 2009)). All the hallmarks of a canonical endocytic system are present, including early, recycling, and late endosomes/MVBs, as well as a terminal lysosome for degradation. Endocytosis is essential to *T. brucei* survival, and perturbation of endocytic functions leads to pleiotropic effects on morphology and cytokinesis that are usually lethal (Allen et al. 2003; Manna et al. 2014).

Endocytic uptake between these two groups does share some features. Uptake of material involved in DV formation in *P. falciparum* and endocytosis in *T. brucei* both rely on actin, yet clathrin is dispensable for the former and not the latter (Elliott et al. 2008; García-Salcedo et al. 2004; Lazarus et al. 2008). Vesicle fission also differs from current models. Endocytosis in *T. brucei* occurs without dynamin or a dynamin-related protein (DRP) (Morgan et al. 2004), and though multiple DRPs have been found in Apicomplexa, these are involved in biogenesis of invasion organelles and fission of endosymbiotic organelles during cell division (Breinich et al. 2009; Charneau et al. 2007; Li et al. 2004; van Dooren et al. 2009). Trypanosomes and apicomplexans, like the vast majority of eukaryotes, lack Epsin, which is a specific innovation of the opisthokonts (Field et al. 2007). Instead, *T. brucei* uses a single "ENTH" domain-containing protein, EpsinR, to mediate cargo selection and clathrin recruitment (Gabernet-Castello et al. 2009). EpsinR proteins, although present in Apicomplexa, have not been functionally characterized.

Though this direct comparison clearly demonstrates a classical form versus function argument, comparison of genomic, morphological, and functional data between these groups and their close relatives can help to understand the evolutionary origins behind these fascinating MTS alterations. Two trafficking protein families, the adaptor protein (AP) complexes and Ras from brain (Rab) GTPases, have been particularly useful in this manner.

AP complexes are heterotetrameric complexes, composed of adaptin subunits, involved in cargo selection and coat recruitment during vesicle biogenesis (Robinson 2004). All five known AP complexes were present in the LECA, though AP-5 has been secondarily lost in numerous extant lineages (Hirst et al. 2011). In model

systems, AP-1 and AP-2 interact with clathrin, whereas AP-4 and AP-5 do not; interaction of AP-3 with clathrin is uncertain (Dell'Angelica 2009; Hirst et al. 2013; Robinson 2004). AP-1 is involved in trafficking between the Golgi and endosomes, AP-2 primarily functions in endocytosis at the plasma membrane, and AP-3 is involved in biogenesis and maintenance of lysosomes and related organelles. AP-4 and AP-5 functions are less well established, but appear to involve endosomal trafficking.

Salivarian trypanosomes encode AP-1, AP-3, and AP-4, though they lack AP-2 and AP-5 (Manna et al. 2013). The loss of AP-2 is associated with the gain of the Variant Surface Glycoprotein (VSG) coat, while the loss of AP-5 is more deeply derived within the kinetoplastids. *Leishmania*, another important disease-causing kinetoplastid, has independently lost the AP-4 complex. AP-1 was shown to be essential, yet it is dispensable for correct localization of the lysosomal marker protein p67 (Allen et al. 2007). AP evolution in Apicomplexa is somewhat more convoluted. AP-3 has been secondarily lost in the piroplasmids and *Cryptosporidium*, together with multiple losses of the complete AP-5 complex or AP-5 subunits in the above groups and *Plasmodium* (Nevin and Dacks 2009; Woo et al. 2015). The pattern of AP-3 loss suggests lineage-specific functions related to the *Plasmodium* DV and *T. gondii* VAC; this has been suggested by a recent study of chemotherapeutics in *T. gondii* (Fomovska et al. 2012). The only well-studied complex in Apicomplexa is AP-1. Consistent with model systems, where AP-1 is associated with TGN-endolysosome trafficking, early studies provided convincing localization for AP-1 at rhoptries and endosomal compartments in *T. gondii* (Ngô et al. 2003). More recent evidence points to a role for AP-1 in trafficking to micronemes and rhoptries, together with a sortilin-like receptor for cargo recognition (Kibria et al. 2015; Krai et al. 2014; Sloves et al. 2012; Tomavo et al. 2013).

Rabs are members of the Ras family of small GTPases involved in trafficking processes. They perform their roles by acting as organizational hubs for a multitude of upstream and downstream effectors, including tethers, SNAREs, SM proteins, PI kinases, and motor and cytoskeletal elements (Angers and Merz 2011; Brighouse et al. 2010; Stenmark 2009). Recent analyses demonstrate that the Rab complement of the LECA was extensive (23 Rabs), but multiple expansions and reductions have occurred post-LECA (Elias et al. 2012).

Apicomplexa and trypanosomes represent a reduction from the LECA Rab complement; trypanosomes have 16 Rabs (Ackers et al. 2005), whereas Apicomplexa possess between 15 (*T. gondii*) and eight (*C. parvum*) (Langsley et al. 2008). Three Rabs have been well characterized in both systems: Rab5, Rab7, and Rab11, which mediate EE, LE/lysosome, and recycling traffic in mammalian cells, respectively. *T. brucei* (Tb) Rab5 plays a role very similar to that in mammalian cells, being involved in facilitating EE traffic (Hall et al. 2004). *T. gondii* (Tg) Rab5A and 5C (two of three Rab5 paralogs encoded, an expansion from the primordial Rab5 in the LECA) play a different role and are essential for proper biogenesis of invasion organelles (Kremer et al. 2013). TbRab7 is involved in trafficking to lysosomes (Silverman et al. 2011), while Rab7 in *T. gondii* and *P. falciparum* localizes to putative LEs (Krai et al. 2014; Parussini et al. 2010). TbRab11, as well as TbRab4 in procyclic

parasites, is involved in recycling of material from intermediate endosomes to the PM (Hall et al. 2005; Pal et al. 2003). Apicomplexan homologues have been co-opted for a different function, as the Rab11 paralogs, Rab11A and 11B, are involved in formation and maintenance of the inner membrane complex (IMC) (Agop-Nersesian et al. 2009, 2010). Roles for additional *T. brucei* Rabs in membrane trafficking have been elucidated and appear to function similarly to their characterized homologues in model systems; TbRab28 is involved in retromer-dependent trafficking processes (Lumb et al. 2011) and TbRab21 is involved in intermediate endocytic trafficking (Ali et al. 2014). A detailed understanding of the function of other Rab proteins in Apicomplexa remains elusive. A recent screen in *T. gondii* failed to define precise roles for a large number of the remaining Rabs, but localized the majority in the early or late secretory system (Kremer et al. 2013).

Many of the unique features noted in Apicomplexa are shared with out-group taxa, such as ciliates and dinoflagellates, suggesting an origin in a common free-living ancestor. The alveolate-specific Rab11B mediates IMC formation in Apicomplexa and likely serves a similar role in maintenance of the homologous alveoli in other members of this group (Agop-Nersesian et al. 2010; Klinger et al. 2013b). Many alveolates have specialized secretory granules of uncertain origin referred to generally as extrusomes (reviewed in (Rosati and Modeo 2003)). Though it is unclear if these are homologous to secretory organelles of Apicomplexa, the use of sortilins as sorting receptors for secretory organelle proteins is a feature shared with the ciliate *T. thermophila* and likely with other alveolates as well (Briguglio et al. 2013). Additionally, *T. thermophila* uses CME for uptake of extracellular materials, but this process does not depend on actin and uses a DRP that does not group with classical dynamin in phylogenetic analyses (Elde et al. 2005). Due to a lack of knowledge for many related taxa, it is unclear if the free-living ancestor of Apicomplexa completed endocytic uptake with a DRP or not.

Closer still within the alveolates, Apicomplexa are sisters to the dinoflagellates, perkinsids, colpodellids, and chromerids, forming a single clade Myzozoa. Every group of myzozoans contains organisms that possess a structure morphologically similar, and potentially homologous, to the apical complex of Apicomplexa, including the conoid (peduncle in dinoflagellates), and organelles similar to micronemes and rhoptries ((Okamoto and Keeling 2014), inter alia). It is believed that the apical complex is a plesiomorphic feature of the Myzozoa and that it originally served for myzocytosis (phagocytosis of prey cytoplasm), as in extant lineages such as *Colpodella*. Apicomplexa later modified the function of this apparatus to facilitate host cell invasion (Cavalier-Smith and Chao 2004). Determining which changes in the apicomplexan MTS are concurrent with modification of this complex and which are specific adaptations to parasitism is important to understanding the evolution of the apicomplexan lifestyle. From our own results, it is clear that at least some changes, such as the loss of the exocyst complex (Klinger et al. 2013a), a tethering complex involved in regulated secretion, are common to all myzozoan taxa studied so far and may be associated with the acquisition of the apical complex. Other changes, such as widespread loss of AP-3, are restricted to Apicomplexa and hence may be concurrent with their parasitic lifestyle.

Analysis of the genome of *Naegleria gruberi,* a free-living heterolobosean related to kinetoplastids, revealed that the common ancestor of these taxa possessed a full complement of AP and Rab components, comparable to the LECA (Elias et al. 2012; Manna et al. 2013). Furthermore, the loss of AP-2 in salivarian trypanosomes is restricted to this subset of *Trypanosoma spp.*, suggesting this was a recent event. It is also concurrent with the acquisition of the Variant Surface Glycoprotein (VSG) family and has been suggested as a modification to enable extremely rapid, though less selective, endocytic processes (Manna et al. 2013). In addition to the aforementioned departures from classical CME, a recent proteomics-based study found novel clathrin-interacting proteins (CLASPS) specific to kinetoplastids of the genera *Trypanosoma* and *Leishmania* (Adung'a et al. 2013). Though further characterization is ongoing, it is clear that African trypanosomes have evolved to specialize in rapid, though not necessarily selective, endocytic events in order to survive the ravages of host immune responses. Only some of these traits are shared with their close relatives and may relate to mode of parasitic infection, as, for instance, *Leishmania* spp. are intracellular parasites.

One major challenge to understanding MTS evolution and function is the considerable plasticity in the system. Reductions and expansions of machinery do not seem to correlate well with functional complexity. *P. falciparum* encodes a set of membrane trafficking machinery that is reduced from, or at best comparable to, the LECA, and yet in infected red blood cells, the parasite must traffic components to at least ten distinct locations. *T. vaginalis* encodes large numbers of paralogs for most gene families, yet is not noticeably more complex than the human host it infects. Even within commonly used model systems, endocytic trafficking follows distinct pathways. Plants appear to lack an EE and use the TGN instead. *Saccharomyces* does not possess the same complexity of endosomal structures as mammalian cells and use distinct retromer-dependent trafficking pathways.

This extensive plasticity makes reconstructing an accurate ancestral MTS challenging and limits the use of any given organism as a model system. However, both hurdles may be overcome by continuing to grow our repertoire of genomic sequence and functional knowledge of cellular pathways in parasites and related free-living taxa. The study of parasite membrane trafficking has and will continue to lead to new avenues of discovery and understanding, not just of universal eukaryotic features but also of key differences between host and parasite biology. Through a thorough understanding of general eukaryotic cell biology, we gain a better appreciation for what is novel and hence therapeutically exploitable.

Conclusion

Comparison of parasites to multiple free-living out-groups improves the resolution of character evolution, but was infeasible prior to an increase in the number of available genomes. Throughout the mid-2000s chain-termination (Sanger) sequencing was gradually replaced by 454's pyrosequencing and Illumina's method of sequencing by synthesis, which were far less expensive and more practical for large sequencing projects. These technologies allowed for many genomes to be sequenced by individual labs or small consortia, increasing the number of taxonomic sampling points available. These technical advances have

not only increased the number of parasite/free-living pairs for direct evolutionary comparison but have also provided a wealth of sequence information for large-scale dissection of phylogenetic relationships, placing these pairs in context.

The recognition that parasites represent independent convergences on similar modes of life from a free-living ancestor has been an important factor in shaping the evolving landscape of parasitological research. It is crucial that parasitologists interpret parasite biology in the context of closely related, free-living organisms in order to understand how parasitism arose in any given lineage. Though some characteristics are shared, such as decreased aerobic function of mitochondria and modification of the membrane trafficking system, these represent cases of convergence through occupying similar environmental niches.

Recently published genomes of additional free-living relatives of parasitic groups, such as Chromera velia and Vitrella brassicaformis (Woo et al. 2015), as well as Bodo saltans (Jackson et al. 2016), in addition to others currently in progress including Mastigamoeba, Paratrimastix, and Carpediemonas, will allow for even more comparative studies of the transition to parasitism. Increased efforts to develop model parasitic systems show promise for molecular characterization, driven by the hypotheses produced from comparative studies. In this way, it will be possible to combine putative targets from bioinformatic studies with detailed biochemical studies to understand the modification of cellular systems that give parasites the ability to exploit their hosts. In the course of these studies, we expect to identify a number of ways in which parasites may gain traction in their new niches; these may well represent new opportunities to be exploited in combating parasitic threats to human health and well-being.

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Host-Parasite Interactions

13

Heinrich Körner, Shanshan Hu, and Christian Bogdan

Abstract

Ontogeny and differentiation of cells of the monocyte/macrophage lineage are currently subjects of intense research. The concept of the macrophage as "simple" phagocytic cell has undergone profound changes. It has now been established that this myeloid lineage of cells is phenotypically and functionally much more diverse and exerts a much wider influence on the immune response than previously thought. How have these new findings changed our perception of the role of monocytes and macrophages in parasitic diseases? There is now strong evidence that macrophages fulfill organ-specific differential functions, exert activating as well as inhibitory effects on the adaptive immune response, and exist in a whole range of different activation statuses which show a high degree of plasticity. In the present chapter, we will not only review the pertinent literature on these new developments but also bring it into relation to the anti-infectious immune response, focusing on four parasitic diseases (trypanosomiasis, toxoplasmosis, malaria, and leishmaniasis) as examples.

H. Körner (✉) • S. Hu
Menzies Institute for Medical Research, University of Tasmania,
17 Liverpool Street, Tasmania 7000, Australia
e-mail: heinrich.korner@utas.edu.au; shu2@postoffice.utas.edu.au

C. Bogdan
Mikrobiologisches Institut – Klinische Mikrobiologie, Immunologie und Hygiene,
Friederich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen,
Erlangen, Germany
e-mail: christian.bogdan@uk-erlangen.de

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13.1 Introduction

Metchnikoff's seminal observations on phagocytosis and the inflammatory recruitment of macrophages first reported in 1884 are the starting point of the concept of natural cellular immunity according to which phagocytic cells play a protective role during the inflammatory response to pathogens (Metchnikoff 1902). Since then we have learned that macrophages belong to the mononuclear phagocyte system (MPS) (Hume 2006; Van Furth et al. 1972), which contains different types of blood monocytes (classical, intermediate, and nonclassical) and a highly diverse set of tissue macrophages and dendritic cells. Furthermore, the function of MPS cells extends far beyond the uptake, killing, and digestion of microorganisms. In addition to their antimicrobial effector function, MPS cells are important sources of cytokines and other secretory products that activate lymphocytes and help to initiate immune responses (Nathan 1987, 2012). They can act as antigen-presenting cells and support the establishment of T-cell immunity (Martinez-Pomares and Gordon 2012; Roche and Furuta 2015; Savina and Amigorena 2007; Unanue 2002). As already recognized by Metchnikoff, macrophages are proficient in removing senescent as well as damaged cells which makes macrophages critical players in the process of tissue remodeling, repair, and healing (Brancato and Albina 2011). Finally, under steady-state conditions (e.g., in the intestine) (Mowat and Bain 2011) as well as during the resolution phase of inflammations, macrophages operate as immunosuppressors and thereby participate in the prevention or termination of immune responses, respectively (Buckley et al. 2014; Murray and Smale 2012; Nathan and Ding 2010). Besides these beneficial effects, macrophages also function as host cells and safe habitat for a wide spectrum of intracellular bacteria and protozoa (Price and Vance 2014). Tumor-associated macrophages at least partially account for the suppression of tumor-specific T-cell responses (Ostuni et al. 2015). Due to their strong inflammatory capacity, which increases vascular permeability and helps to attract other immune cells (e.g., neutrophils, Th17 cells), the generation of toxic reactive oxygen (ROS) and reactive nitrogen species (RNS), and the release of enzymes degrading extracellular matrix, macrophages are key players in tissue injury and chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, diabetes, or Alzheimer's disease (Abtin et al. 2014; Bogdan 2015; Murray and Wynn 2011).

Considering the phenotypic and functional diversity of MPS cells, the assessment of their contribution to the defense against protozoan parasites has become much more challenging. This is in part due to the complex developmental pathways of the cells of the MSP and to the ill-defined relationships of MPS subpopulations to each other and the cells of the adaptive immune response. Only recent molecular and genetic insights into the development of the entire myeloid lineage (Gautier et al. 2012; Ginhoux et al. 2010; Hashimoto et al. 2013; Martinez et al. 2013; Miller et al. 2012; Yona et al. 2013) have provided new tools for the analysis of the immunological role of monocytes, macrophages, and dendritic cells and set the basis for a uniform nomenclature of the subpopulations (Ginhoux and Jung 2014; Guilliams et al. 2014; Malissen et al. 2014; Murray et al. 2014; Ziegler-Heitbrock et al. 2010).

The published data clearly demonstrate that the phenotypic and functional spectrum of the cells of the MPS have previously been underestimated dramatically.

13.2 Biology of the Mononuclear Phagocyte System

Cells of the MPS have classically been defined as members of the hematopoietic lineage, which originate from committed precursor cells in the bone marrow, differentiate into peripheral cells circulating in the blood and the lymphatics with various immunological functions, and finally enter the tissue to turn into resident macrophages or dendritic cells (Hume et al. 2002; Van Furth and Cohn 1968). This dogma of a linear myeloid cell development has recently been disproved by fate mapping and transcriptional analyses that demonstrate independent waves of macrophage development during embryogenesis, tissue homeostasis, and inflammation (Ginhoux and Jung 2014). Additionally, the investigation of the interrelationship of subpopulations within the mouse and the human immune system, which had been controversial due to a limited number of markers, has recently been addressed in genomic studies, the results of which will be presented below (Gautier et al. 2012; Guilliams et al. 2014; Ingersoll et al. 2010; Martinez et al. 2013; Miller et al. 2012; Murray et al. 2014).

13.2.1 Surface Markers of Cells of the MPS

As the full breadth of conventional MPS surface molecules has been reviewed in detail (Taylor et al. 2005), we will restrict our discussion to the most important marker molecules (see Table 13.1). In mouse, two integrins involved in homing and recruitment of myeloid cells are most commonly used to define cells of the MPS. The heterodimeric adhesion molecules $\alpha_m\beta_2$ (CD11b-CD18) and $\alpha_x\beta_2$ (CD11c-CD18) are important for adhesion and extravasation and are used to separate cells of the MSP from other mononuclear cells (Imhof and Aurrand-Lions 2004). Both integrins are detectable predominantly on monocytes, macrophages, and dendritic cells. On alveolar macrophages, CD11c is more strongly expressed than CD11b when compared to bone marrow-derived macrophages (Prieto et al. 1994; Stent et al. 1995). Furthermore, CD11b and CD11c are also found on activated natural killer cells (Blasius et al. 2007; Chiossone et al. 2009) which makes it advisable not to rely exclusively on the expression of these two markers for the detection of myeloid cells. Therefore, a variety of additional markers are commonly used to define a MSP subpopulation. The F4/80 glycoprotein, a mouse homologue of the epidermal growth factor (EGF)-module-containing mucin-like hormone receptor (EMR) 1, is a class B seven-spanning transmembrane (TM7) receptor (Kwakkenbos et al. 2004) expressed on the surface of differentiated macrophages, which, however, is not essential for their development (Schaller et al. 2002). The granulocyte receptor-1 (Gr-1) antigen is constitutively expressed on early myeloid precursor cells of the mouse and is strongly upregulated in response to inflammatory signals. A widely

Table 13.1 Murine and human marker molecules of myeloid cells

System	Marker	Molecule family		References
Mouse	$\alpha_m\beta_2$ (CD11b-CD18)	Integrin	Cell adhesion and movement	Imhof and Aurrand-Lions (and 2004)
Mouse	$\alpha_x\beta_2$ (CD11c-CD18)	Integrin	Cell adhesion and movement	Imhof and Aurrand-Lions (and 2004)
Mouse	F4/80 antigen	EGF-module-containing mucin-like hormone receptor (EMR) 1)	Receptor	Kwakkenbos et al. (2004)
Mouse	Ly6C	phosphatidylinositol-anchored cell surface glycoproteins	Unknown function	Daley et al. (2008), Ribechini et al. (2010)
Mouse	Ly6G	phosphatidylinositol-anchored cell surface glycoproteins	Unknown function	Daley et al. (2008), Ribechini et al. (2010)
Mouse	CCR2	CC-Chemokine receptor	Cell movement	Kurihara et al. (1997)
Mouse	CX3CR1	CX3CR-chemokine receptor	Cell movement	Geissmann et al. (2003)
Mouse	iNOS	Metabolic enzyme	Marker of M1 macrophagen. Production of NO	Stuehr et al. (1989)
Mouse	Arginase	Metabolic enzyme	Marker of M2 macrophagen. Production of Urea and Ornithine	Green et al. (1990)
Human	CD14	Pattern recognition receptor	Co-receptor to TLR4 and MD2. Binds LPS	Passlick et al. (1989), Ziegler-Heitbrock (2014)
	CD16		FcγRIII, low-affinity Fc receptor	Passlick et al. (1989), Ziegler-Heitbrock (2014)
	TGM2	Metabolic enzyme	Transglutaminase	Martinez et al. (2006)

used monoclonal anti-Gr1-antibody (RB6-8C5) was found to stain neutrophils, eosinophils, myeloid-derived suppressor cells (MDSC), monocytes, certain macrophages, (plasmacytoid) dendritic cells, and $CD8^+$ T cells (Mordue and Sibley 2003). This is due to the fact that the Gr-1 epitope is present on two molecules of the Ly6-family of molecules, Ly6C and Ly6G. While Ly6G is expressed specifically on

Table 13.2 Macrophages, monocytes, and dendritic cells: New insights from genetic approaches (Guilliams et al. 2014)

Cell type	Origin	Tissue of residence	Function	Marker
Macrophages	Embryonic	Peripheral tissues	Surveillance, defense, immune regulation	CD45 F4/80 CD11b
Monocytes	Adult stem cells	Blood	Immune defense	CD45 Ly6C CD11b
Dendritic cells	Adult stem cells	Peripheral tissues	Immune defense and regulation (Antigen presentation)	CD45 CD11c

neutrophils (Daley et al. 2008; Wang et al. 2012) including granulocytic MDSC (Gabrilovich and Nagaraj 2009), Ly6C is much more widely distributed including neutrophils, dendritic cells, inflammatory monocytes, and peripheral $CD8^+$ T cells (Daley et al. 2008; Matsuzaki et al. 2003; Ribechini et al. 2010). Finally, CCR2 (Kurihara et al. 1997) and CX3CR1 (Geissmann et al. 2003), two members of the chemokine receptor family, are regulated during the differentiation of monocytes. Recently, CCR2 has been used in combination with CD64 (high affinity FcγRI) to define macrophage and dendritic cell populations (Malissen et al. 2014).

The two most important marker molecules that allow separation of human myeloid cells in subpopulations are CD14 (myeloid cell-specific leucine-rich glycoprotein), a glycosyl-phosphatidylinositol-anchored coreceptor of TLR4 (Simmons et al. 1989), and CD16, a low-affinity gamma Fc receptor (FcγRIII) (Qiu et al. 1990).

13.2.2 Ontogeny and Plasticity of Macrophage/Monocyte Subpopulations

The cells of the innate immune system are members of the hematopoietic system, and precursor cells of monocytes were thought to enter the circulation from the bone marrow, home to a tissue, and terminally differentiate into a tissue-resident macrophage (Van Furth and Cohn 1968). This view of a linear development has been proven to be too simplistic, and recent work has established that only a very small population of adult tissue macrophages originate from adult monocytic hematopoiesis with most arising from embryonic precursors that begin developing at different stages of embryonic development (Table 13.2). Fate mapping of macrophages has shown that primitive hematopoiesis (beginning at E7.5) in the yolk sac that produces a first wave of primitive macrophages is replaced by fetal liver hematopoiesis that generates a second wave of embryonic definitive monocytes (Davies et al. 2013; Ginhoux and Jung 2014; Wynn et al. 2013). These tissue macrophages are maintained by a slow rate of proliferation and persist in the tissue under

noninflammatory conditions throughout adult life (Malissen et al. 2014; Yona et al. 2013). Shortly before and after birth, committed myeloid precursors leave the bone marrow and differentiate to monocytes and common DC precursors (Geissmann et al. 2010). This leads to the situation that descendants of these waves reside in different tissues, sometimes side by side with embryonically derived macrophages (Hoeffel et al. 2012). In contrast, in the brain the entire population of microglial cells originates from an early recruitment of primitive macrophages (Ginhoux et al. 2010; Sheng et al. 2015), while heart or alveolar macrophages stem from fetal-liver-derived monocytes (Epelman et al. 2014; Guilliams et al. 2013; Naito et al. 1990). After birth, two forms of monocytes ("classical" Ly6C^{high} and "patrolling" Ly6C^{low}) leave the bone marrow (Geissmann et al. 2003) with the help of CCR2 (Serbina and Pamer 2006) and enter the blood stream. The presence of CCR2 is irreplaceable for CD11b^{+} Ly6c^{high} monocytes to be able to leave the bone marrow (Serbina and Pamer 2006). An absence of this chemokine receptor and, consequently, the inability of the monocytes to respond to chemokine signals, mostly CCL2, cause a profound lack of inflammatory monocytes in response to infection (Boring et al. 1998). Therefore, the CCR2$^{-/-}$ mouse strain has been a favorite model in studying the involvement of monocytes in the defense against parasitic disease as discussed later in this review (see Table 13.2).

Under inflammatory conditions, these cell types enter the tissue and develop into a multitude of subpopulations of inflammatory monocytes and DCs that contribute to the immune defense (Auffray et al. 2009). These various permutations of the basic monocytic cell type have to a large extent been defined within either an infection or a tumor model. For example, TNF and inducible nitric oxide (NO) synthase (iNOS or NOS2)-expressing DCs (TIP-DCs) are present during *L. monocytogenes* infection (Serbina et al. 2003), while monocyte-derived DCs have been identified after infection with *L. major* (Leon et al. 2007). However, a detailed analysis of the publications leaves the impression that some of the differences described are blurring and are based on poor consistency of experiments and definitions due to an individual interpretation of the data and a limited availability of marker molecules. This has recently led to the suggestion to unify and validate the nomenclature of this linage (Guilliams et al. 2014).

In their last step of differentiation under inflammatory conditions, monocytes enter the tissue and are now described as classically activated (M1) macrophages (Geissmann et al. 2010; Serbina and Pamer 2008) that express iNOS and TNF. The enzyme iNOS converts the amino acid L-arginine in a complex oxidoreductase reaction into citrulline and NO. NO not only exerts direct and indirect antiviral and antimicrobial effects but also has numerous immunoregulatory functions in the innate and adaptive arm of the immune system, affecting the activity of NK cells, DCs, macrophages, T cells, B lymphocytes, and plasma cells (Bogdan 2001, 2015). Under Th2 conditions in the presence of IL-4 and IL-13, e.g., during helminth infection, monocytes differentiate into M2 macrophages which are capable of in situ proliferation (Jenkins et al. 2011) and express a different spectrum of molecules such as arginase 1 (mouse) and transglutaminase 2 (mouse/human) (Martinez et al. 2013). Arginase 1 transforms L-arginine into urea and ornithine and can compete

with iNOS for the substrate. Ornithine is a precursor for the generation of polyamines as well as collagen, which are critical for cell proliferation and wound healing, but also involved in fibrotic processes. In addition, arginase 1 expression can entertain the growth of certain pathogens and negatively affect the proliferation of T lymphocytes (Bogdan 2015; Wynn et al. 2013). In the light of the multiple effects of arginase 1, the biological role of these M2 or alternatively activated macrophages is diverse ranging from tissue repair to the termination of immune responses and inflammations (Gause et al. 2013; Murray et al. 2014). It is interesting to note that during an antiparasitic Th2-type inflammation, tissue-resident macrophages strongly proliferate under control of IL-4 without further recruitment from the periphery (Jenkins et al. 2011).

A range of experimental observations in inflammatory models such as colitis point to fast tracks of differentiation between monocytes and DCs and imply plasticity between these stages (Rivollier et al. 2012). However, while the notion of true plasticity between monocytes and DCs has helped to identify PU.1 as master regulator that can intervene at several key points of myeloid differentiation (Geissmann et al. 2010), the role of plasticity in vivo is still unresolved and needs to be investigated in more detail.

The question which cells that have been generated in vitro in models of myeloid cell differentiation are a true representation of inflammatory DCs has important implications for the use of DCs in immunotherapy. In tissue culture, bone marrow cells are generally cultured in the presence of the cytokines GM-CSF and IL-4 or Flt3 (Naik 2008). A detailed study of both ensuing cell populations showed that the GM-CSF/IL-4 cultured cells expressed $CD11c^{+}MHC\text{-}II^{+}Mac\text{-}3^{+}$ and were capable to produce TNF and NO after challenge. This observation would mean that the in vitro correlate of TNF and iNOS-producing DC (TIP-DC) are bone marrow cells differentiated in the presence of GM-CSF/IL-4. This was confirmed by isolating $CD11b^{+}Ly6c^{+}$ bone marrow cells and culturing these cells in medium supplemented with either cytokine. While GM-CSF supported a development into DC-like cells, Flt-3 was not sufficient to support cell survival in culture (Xu et al. 2007).

Monocytic subpopulations have been analyzed mostly in mouse models. The availability of genetic modifications and genetic introduction of fluorochromes have allowed the phenotyping of subtypes and made it possible to allocate certain functions. However, human monocytic subpopulation has been identified, and more information has been gathered how the human MPS is structured. Based on the expression of CD14 and CD16, human peripheral monocytes can be divided into classical monocytes ($CD14^{++}$ $CD16^{-}$), intermediate monocytes ($CD14^{++}$ $CD16^{+}$), and nonclassical monocytes ($CD14^{+}CD16^{++}$) (Ziegler-Heitbrock 2014) (Table 13.1). Gene profiling of the classical and nonclassical subpopulation has established substantial differences and clearly demonstrated that these monocyte populations are distinct subsets and not different stages of monocyte differentiation (Ancuta et al. 2009; Frankenberger et al. 2012; Ingersoll et al. 2010; Wong et al. 2011, 2012). The nonclassical $CD14^{+}CD16^{++}$ population has been described as patrolling monocytes (Cros et al. 2010; Ingersoll et al. 2010; Wong et al. 2012) in analogy to the $CX3CR1^{+}$ monocytes in the mouse (Auffray et al. 2007).

13.3 Monocytic Cells in Diseases Caused by Protozoan Parasites

The different roles of myeloid cells such as DCs, monocytes, and macrophages were thought to be understood. Somewhat simplistically, DCs were viewed as professional antigen-presenting cells initiating a T-cell response, while monocytes and macrophages were regarded as primary effector cells that endozytose and subsequently kill intracellular bacteria and protozoa with the help of oxygen and nitrogen radicals. Additionally, and counterintuitively, macrophages were in certain cases also found to serve as reservoir of pathogens that had learned to survive the harsh conditions of the phagolysosome and were, thus, protected from the host's immune response inside their target cells. However, the recent redefinitions of monocytes and DCs as dynamic classes of effector cells and antigen-presenting cells and of macrophages as immunologically active regulatory cells have changed this picture and have started to influence the way we investigate myeloid populations and functions in parasitic disease.

13.3.1 Monocytes in Infection with Protozoan Parasites

The changing role of cells of the MPS has been documented in a variety of models of parasite infection. In infections by *Trypanosoma* spp., *Toxoplasma* spp., *Plasmodium* spp., and *Leishmania* spp., there is a considerable involvement of monocytes that will be discussed below.

Parasites of the genus *Trypanosoma* account for two of the most neglected human tropical diseases. In tropical sub-Saharan Africa, the African sleeping sickness is caused by subspecies of the *Trypanosoma (T.) brucei*, while in South and Middle America, *T. cruzi* causes Chagas' disease (Barrett et al. 2003). While the term "sleeping sickness" already points to CNS involvement in the African form of the disease, Chagas' disease in its chronic form frequently causes heart pathology. Both diseases are chronic and potentially fatal in humans. The major immunological difference between the two pathogens is the fact that *T. cruzi* invades host macrophages, while *T. brucei* exists exclusively extracellularly and, thus, is exposed to the full force of the humoral immune system (Barrett et al. 2003). Experimental models exist for both forms of the disease, and the role of the MPS has been investigated.

In models of the infection with *T. brucei*, inflammatory monocytes are almost exclusively of the M1 type due to the predominance of IFN-γ resulting in the production of large amounts of NO and TNF. These M1 cells are essential to control the pathogen in the liver but also in spleen and lymph nodes (Drennan et al. 2005; Magez et al. 1999, 2006). Among these monocytic cells, $CD11b^{+}Ly6c^{+}CD11c^{+}$ inflammatory DCs that can be considered to be analogous to TIP-DC (Serbina et al. 2003) have been identified as the main population producing these effector molecules during infection. The chronically activated TIP-DC-like cells lead to immunopathology, causing necrosis in liver tissue and resulting in exacerbated disease and a significantly reduced survival (Bosschaerts et al. 2010; Guilliams et al. 2009).

Interestingly, the immunopathogenic function of these cells is controlled by IL-10 (Guilliams et al. 2009) and is strongly reduced in CCR2$^{-/-}$ mice (Bosschaerts et al. 2010). In the CCR2$^{-/-}$ strain, M1 monocytes were significantly increased in the bone marrow, and TIP-DC-like cells were decreased in the liver correlating with a reduced tissue injury. This model is an interesting example of how a normally essential branch of the immune response can be turned by chronic inflammatory processes into an immunopathogenic executer leading to a reduced survival of the host.

The second *Trypanosoma* species, *T. cruzi*, results in a strong production of TNF in infected mice (Truyens et al. 1999). Recent work has shown that after infection, an inflammatory monocytic infiltrate accumulates in the hearts of affected mice. This infiltrate was in part granulocytic (Ly6G^{+}), but 70 % of cells were CD11b^{+} Ly6C^{+} and Ly6G^{-}. Because these cells could suppress T-cell responses, they were classified as myeloid suppressor cells, and as mechanism responsible for systemic immunosuppression, the authors identified arginase 1 expression (Cuervo et al. 2011).

Finally, the immune response to a *Trypanosoma* species that only infects livestock, *T. congolense*, depends on an early cellular inflammatory response by M1-like macrophages (Noel et al. 2002) and is characterized by large amounts of TNF (Magez et al. 2007).

Taken together, in trypanosomiasis the immune response needs to be finely balanced between parasite control and control of immunopathology. In these three models, both a beneficial role of M1 monocytes/macrophages and a detrimental role of M1-type TIP-DC-like cells have been shown, demonstrating a strong influence of the MSP on the clinical outcome of disease.

The protozoan parasite *Toxoplasma (T.) gondii* can infect all vertebrates and is highly prevalent in human populations. Transmission can be foodborne, zoonotic, or congenital. The pathogen can cause overt disease (in immunocompetent as well as in immunodeficient patients) but is mostly controlled by the immune system and remains in a state of dormant persistence. This pathogen has received considerable attention as model for parasitic infections eliciting Th1 responses and as pathogen with the potential to cause severe infections in immune compromised patients (Yarovinsky 2014).

In a mouse model of intraperitoneal infection, it was shown that GR-1^{+} myeloid cells entered the peritoneal cavity in large numbers in a MCP-1- and CCR2-dependent manner and where essential for the defense (Robben et al. 2005). These results were revisited in a more detailed study demonstrating that a large number of CD11b^{+} F4/80^{+} GR1^{+} Ly6G^{-} inflammatory monocytes infiltrated the gastrointestinal tract after intraoral infection with *T. gondii* (Dunay et al. 2008). The effector mechanisms of these cells were the expression of iNOS, IL-12, and TNF. This was to be expected because a critical role for NO had been demonstrated before (Scharton-Kersten et al. 1997). Furthermore, these studies proved a role of CCR2 for monocyte migration in infection. They convincingly showed that CCR2 was only needed for emigration of monocytes from the bone marrow but not for immigration of these cells into infected or inflamed tissues (Dunay et al. 2008). However,

in the absence of CCR2 or its chemokine ligand MCP1, the lack of tissue-infiltrating monocytes in the periphery resulted in an increased mortality (Robben et al. 2005). More recently, it was observed that the *T. gondii* antigen and TLR-ligand profilin governed Ly6C^{hi} CCR2^{+} monocyte recruitment in response to a lethal *L. monocytogenes* coinfection with *T. gondii* and helped the mice to resist this bacterial infection (Neal and Knoll 2014). In the model of cerebral toxoplasmosis, Ly6C^{hi} CCR2^{+} monocytes produced large amounts of proinflammatory cytokines and controlled parasite numbers (Biswas et al. 2015). An additional avenue of research in this model revealed that the regulatory functions of monocytes were elicited by the action of bone marrow-derived NK cells (Askenase et al. 2015). It will be a future challenge to weld together these seemingly unrelated observations into a coherent model.

The clinical disease resulting from an infection with parasites of the genus *Plasmodium (P.)* is called malaria. After the inoculation by the mosquito vector *Anopheles*, *Plasmodium* sporozoites first infect hepatocytes, before the parasite enters the erythrocytes. The erythrocytic infection cycle by merozoites accounts for the typical paroxysmal fever episodes.

In various mouse models, the parasite species *P. chabaudi*, *P. berghei*, *P. yoelii*, and *P. vinckei* are used to mimic some of the aspects of the human disease (blood stage vs. cerebral malaria) (Stevenson and Riley 2004). The involvement of monocytes has largely been neglected in these mouse models and has only been studied in detail in an infection by *P. chabaudi* (Sponaas et al. 2009). In this model, bone marrow-derived CD11b^{+} Ly6C^{hi} CCR2^{+} inflammatory monocytes were recruited to the spleen and expressed the inflammatory mediators iNOS and TNF. Only low levels of CD11c were detectable on the surface of these cells which were proficient in phagocytosing parasites, but were incapable of presenting antigen (Sponaas et al. 2009).

13.3.2 Monocytes in Cutaneous Leishmaniasis

Cutaneous leishmaniasis is endemic in the tropics and subtropics and has clinical manifestations ranging from small papular, plaque-like, or ulcerative skin lesions to necrotic tissue destruction of the mucosa (Reithinger et al. 2007). The pathogens that cause cutaneous leishmaniasis are intracellular parasites of the genus *Leishmania (L.)* spp which are transmitted in a promastigote (flagellated) form by blood-sucking female sand flies from reservoir animals or infected humans to new hosts. The mouse model, experimental cutaneous leishmaniasis, is caused by infection with *L. major* or a comparable species. Originally, the interest in this murine infection model was fueled by the dichotomy of symptoms and course of infection in different inbreed mouse strains. While the infection of C57BL/6 mice resulted in a strong production of IFN-γ and was resolved spontaneously, the infection of BALB/c mice was characterized by a persistent production of IL-4 and resulted in a progressive infection that ultimately was fatal (Heinzel et al. 1989). Early work in this model had identified macrophages and their production of iNOS-derived NO as a crucial

mechanism for the containment of the parasites and for the resolution of the disease (Diefenbach et al. 1998; Liew et al. 1991; Stenger et al. 1994). Furthermore, cytokines such as IL-12, IFN-γ, TNF, and type-1 interferons were implicated in the activation of NK cells and macrophages that allowed for a protective immune response (Diefenbach et al. 1998, 1999; Heinzel et al. 1993; Liew et al. 1990; Mattner et al. 1997, 2000, 2004; Wilhelm et al. 2001). However, the time line of activation, the exact cellular source of cytokines in situ, and the role of myeloid cell subsets (e.g., monocytes macrophages, dendritic cells, Langerhans cells) have been controversial despite huge efforts and the use of a variety of models (Mougneau et al. 2011).

An important observation has been that the reservoir of resident antigen-presenting cells ("sentinels") is starting to disappear approximately 48 hours after infection (Leon et al. 2007; Misslitz et al. 2004; Ritter et al. 2004b) and is replenished by $Ly6C^+$ monocytes that differentiate to $Ly6C^+CD11c^+CD11b^+$ monocytic antigen-presenting cells (moDCs) at the site of infection (Leon et al. 2007). These moDCs migrate into draining LNs in a CCR2-dependent fashion and drive locally an anti-leishmania $CD4^+$ Th1-type response. In these experiments, an absence of CCR2 caused mice to succumb rapidly to *L. major* infection, which indicated that moDCs are nonredundant in inflamed areas (Leon et al. 2007). In one study, it could be shown that these $Ly6C^+$ monocytes were recruited to the lesion as effector leukocytes in a CCR2-dependent fashion depending on the activation of platelets which were found to induce the local release of CCL2 (Goncalves et al. 2011). Other studies detected these $CCR2^+$ $Ly6C^+$ cells in the dermis and lymph node and showed that they differentiated to monocyte-derived DCs in both locations different from the "classical" tissue-resident skin DCs, such as Langerhans' or dermal DC (Leon and Ardavin 2008; Leon et al. 2007). Interestingly, DCs derived from monocytes that had been recruited to the dermis (dermal Mo-DCs) and subsequently migrated to the draining lymph nodes showed a mature phenotype ($CD11c^{int}$ $Ly\text{-}6C^{int}$ MHC $II^{hi}CD86^{hi}CD8\alpha^{lo}$ $DEC\text{-}205^+$) at the lymph node site and were responsible for the induction of protective Th1 responses against the parasite, but at the same time served as host cells for the parasites in addition to iNOS-negative macrophages and reticular fibroblasts (Bogdan et al. 2000; Leon et al. 2007; Stenger et al. 1996). In contrast, monocyte-derived DCs that had been recruited to the draining lymph nodes directly (LN moDCs) presented a subtly different, monocytic phenotype displaying $CD11c^{int}$ $Ly\text{-}6C^{hi}$ MHC II^{int} $CD86^-$ $CD8a^{lo}$ $DEC\text{-}205^-$ on their surface (Leon and Ardavin 2008; Leon et al. 2007).

These mature dermal moDCs or a phenotypically analogous cell type were dissected in more detail and found to be $iNOS^+$ and TNF^+, similar to the TIP-DCs previously described in *L. monocytogenes* infection (De Trez et al. 2009). The authors could show that IL-12p35, CD40, IFN-γ, and CCR2 expression were indispensable for recruitment/development of iNOS-expressing inflammatory moDC in the infected LN but claimed that TNF played a less important role in this process and IL-4/IL-13 were negative regulators of this TIP-DC-like cell population (De Trez et al. 2009). This could well be influenced by the *L. major* isolate used for

infection (Ritter et al. 2004a) because in other studies, TNF was shown to be a crucial component of the protective immune response, and in its absence, the infection proved to be rapidly fatal (Wilhelm et al. 2001). The actual mechanism(s) of the protective function of TNF in defense are still unclear, and the analysis of this cytokine in the *L. major* mouse model has yielded surprising results. It could be shown that despite the absence of TNF, the expression of iNOS protein was virtually unchanged (Wilhelm et al. 2001). Also, while the migration and differentiation of dendritic cells to the site of infection was impaired, the T-cell response was not (Ritter et al. 2008). Recently, a second Ly6C^{low} CD115^{+} monocyte population could be detected in the draining lymph nodes of *L. major*-infected TNF$^{-/-}$ mice (Fromm et al. 2012). These cells were not analogous to the CX3CR1^{high} Ly6C^{low} monocytes that have been described to patrol the vessels (Auffray et al. 2007), but were CD115^{+} and Ly6G^{-} and harbored large numbers of *L. major* parasites, thus probably facilitating the overwhelming infection in TNF-deficient mice. Whether a functional overlap exists between these two recently described cell populations needs to be tested.

Other *Leishmania* species showed a high variability in monocyte recruitment in mouse models. For example, after infection with *L. mexicana* C57BL/6 mice exhibited an impaired recruitment of monocytes and a defective Th1 response with fewer monocyte-derived DCs and a reduced production of NO (Petritus et al. 2012).

13.3.3 Monocytes in Visceral Leishmaniasis

Parasites of the *L. donovani* complex, *L. donovani* in East Africa and the Indian subcontinent and *L. infantum/chagasi* in Europe, North Africa, and Latin America (Chappuis et al. 2007; Ready 2014), are the causative agents of human visceral leishmaniasis. Like the related *Leishmania* species that cause mostly localized skin infections, these parasites are inoculated by sand flies and invade cells of the MPS, predominately macrophages and dendritic cells. Unlike the cutaneous *Leishmania* species, parasites of the *L. donovani* complex spread systemically via the lymphatics and blood stream after the death of their initial host cells and cause infection of new cells of the MPS in various tissues. The parasites reside in the spleen, liver, and bone marrow, but only a small percentage of infections result in clinical symptoms and overt disease (about 5 %). Without treatment, these patients will die within 2 years (Chappuis et al. 2007; Ready 2014).

In the mouse model of visceral leishmaniasis, the role of inflammatory monocytes as a distinct subset has not been investigated directly; however, there are observations that point to an involvement. It has been shown that chronic *L. donovani* infections remodel structure of the spleen and Ly6C/G^{+} cells accumulate in the red pulp (Yurdakul et al. 2011). A depletion of either Ly6C/G^{+} cells or Ly6C^{+} cells increase the parasitic burden (Yurdakul et al. 2011). Furthermore, *L. donovani* infection leads to a depletion of follicular dendritic cells in the

germinal centers (Smelt et al. 1997) and interferes with the intrasplenic migration of DCs and consequently reduces the immune response to the pathogens (Ato et al. 2002).

13.3.4 Monocytes in Human Infectious Diseases Caused by Protozoan Parasites

In human populations, parasitic infections are common, but information on the role of monocytes in the human response to parasitic infections is scarce because of the limited access to blood and tissue samples from patients. Furthermore, it has proven to be difficult to reconcile monocyte subsets observed in human infection with mouse subsets because the characterization relies on a different set of surface marker molecules such as CD14 and 16. The research in this area is ongoing and so far has yielded a constantly growing number of monocyte subsets and functions in the human immune system (Saha and Geissmann 2011).

Protection from toxoplasmosis depends on a strong production of IL-12 by monocytes and neutrophils which initiates the expression of IFN-γ by NK cells and T cells (Sher et al. 2003). In accordance with this observation, a tissue-specific gene deletion of suppressor of cytokine signaling molecule 3 (SOCS3) in mouse macrophages and neutrophils caused a reduced IL-12 response and $SOCS3^{-/-}$ mice succumbed to toxoplasmosis (Whitmarsh et al. 2011).

The best-investigated human parasitic disease with regard to the involvement of monocytes in the pathogenesis is human leishmaniasis (Pearson and Sousa 1996; Ready 2014; Reithinger et al. 2007). An expression analysis showed that monocyte chemotactic protein-1 (MCP-1) was strongly expressed in the skin of patients with self-healing lesions but was low in patients with chronic diffuse cutaneous leishmaniasis (Ritter and Moll 2000; Ritter et al. 1996). This was an early indication that the recruitment of monocytes to the site of infection was indeed important in the human immune response (Ritter and Körner 2002). In *L. mexicana*-infected patients, expression of iNOS at the skin infection site correlated inversely with the number of parasites (Qadoumi et al. 2002). Analysis of the pathology of cutaneous *L. braziliensis* patients shows a strong correlation of the size of the $CD16^{+}$ monocyte population and the severity of the disease (Soares et al. 2006). Furthermore, in patients with mucocutaneous leishmaniasis due to *L. braziliensis,* a strong immunoevasive behavior of this parasite was observed. These parasites are susceptible to the control by reactive oxygen species produced by monocytes (Novais et al. 2014) but at the same time induce a strong overexpression of CXCL10 and its receptor CXCR3 in human $CD14^{+}$ monocytes. This results in a strong recruitment of uninfected macrophages and the presence of a larger pool of potential host cells (Vargas-Inchaustegui et al. 2010). Furthermore, a strong TNF expression that originated from inflammatory monocytes were found at the site of the cutaneous lesions which accompanied

the ulceration of the skin. Before the skin exulcerated, an increased frequency of $CD16^+$ (both intermediate [$CD14^+CD16^+$] and nonclassical [$CD14^{dim}CD16^+$]) monocytes was detected in the peripheral blood of these patients. These cells were attracted to the skin via their chemokine receptor CCR2 and a lesion-specific expression of CCL2 enhancing the local expression (Passos et al. 2014).

In human visceral leishmaniasis, gene activation profiles obtained with macrophages taken from healthy donors infected with *L. donovani* showed a surprisingly quick cellular response to the infection challenge which refutes the notion of a silent entry of the parasite and a quiescent response (Ramirez et al. 2012).

Conclusion

Taken together, these findings summarized above (and in Table 13.3) show that monocytes are active in infections of murine and human hosts with protozoan parasites and represent an important part of both the local and the general protective immune response. There has been a variety of important changes in our knowledge of the ontogeny and the biology of monocytes and macrophages. This has significantly altered our appreciation of these cells, notably of their marker molecules and subpopulations as well as their function in infectious diseases. It will be important to incorporate the new insights into experimental approaches to parasitic infections. Most notably, we will have to design standardized experimental procedures in the future, which allow to compare results on monocyte and macrophage subpopulations obtained in different laboratories and model systems.

Table 13.3 Monocyte involvement in defense against parasitic diseases

Parasites	MSP involvement	Infection	References
T. brucei African trypanosomiasis "Sleeping sickness"	M1 monocyte; TIP-DC	Extracellular, liver, CNS	Magez et al. (2006), Bosschaerts et al. (2008)
T. cruzi South American trypanosomiasis "Chagas' disease"	$CD11b^+Ly6c^{hi}Ly6G^-$	Intracellular, different tissues, heart	Cuervo et al. (2011)
T. gondii Toxoplasmosis	$CD11b^+$ F4/80$^+$ $GR1^+$ $Ly6G^-$ $CCR2^+$	Intestinal	Dunay et al. (2008)
Plasmodium Malaria	$CD11b^+$ $Ly6c^{hi}$ $CCR2^+$	Erythrocytes, liver	Sponaas et al. (2009)
Leishmania major Cutaneous leishmaniasis	Dermal Mo-DC Lymph node Mo-DC	Skin	Leon et al. (2007)
Leishmania donovani Visceral leishmaniasis "Kala Azar"	Unclear	Liver, spleen	Engwerda et al. (2004)

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Parasite-Vector Interactions

14

Günter A. Schaub, Patric Vogel, and Carsten Balczun

Abstract

In the most important protozoan tropical diseases – malaria, leishmaniosis, sleeping sickness and Chagas disease – the parasites are transmitted to humans by specific vectors: the sporozoan *Plasmodium* spp. by female *Anopheles* mosquitoes, *Leishmania* spp. by female sandflies, *Trypanosoma brucei* ssp. by both gender of tsetse flies and *Trypanosoma cruzi* by all stages of triatomines. In the lumen of the vector´s gut, all parasites are confronted with the digestive enzymes of the insect and its intestinal immune reactions but also to the microbial fauna. If the parasites are transmitted via the saliva, they must also evade the immune reactions during their way to and in the salivary glands. On the molecular base, specific surface components of the parasites and respective receptors of the insect induce an attachment to or an invasion of the respective tissue, the development of infectious stages and/or immune reactions. In refractory vectors, the latter kill the parasites, but in susceptible vectors at least some parasites survive and can be transmitted. Since full genome sequences are available for many vector species and parasites, modern genomic and proteomic analyses will perhaps identify targets for control of parasite development in the vector.

G.A. Schaub (✉) • P. Vogel
Zoologie/Parasitologie, Ruhr-Universität-Bochum, Universitätsstrasse 150,
44801 Bochum, Germany
e-mail: Guenter.schaub@rub.de; patric.vogel@googlemail.com

C. Balczun
Central Institute of the Bundeswehr, Medical Service Koblenz, Koblenz, Germany
e-mail: carsten.balczun@rub.de

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14.1 Introductory Remarks

All parasite-vector systems show fascinating aspects of co-adaptations. In the following we will focus on the interactions in the most important protozoan tropical diseases – malaria, leishmaniosis, sleeping sickness, and Chagas disease. The parasites use specific groups of insects to be transmitted to humans, *Plasmodium* spp. female mosquitoes of the genus *Anopheles*, *Leishmania* spp. female sandflies, *Trypanosoma brucei* ssp. both gender of tsetse flies and only *Trypanosoma cruzi* non-dipteran insects but reduviid bugs of the subfamily Triatominae. Since dipterans are similar, their peculiarities will be mentioned in this introduction and those of triatomines below (see Sect. 14.5.2). All dipterans are holometabolous insects, thus possessing four developmental stages: egg, larva, pupa, and adult. These larvae develop in other substrates than adults, thereby avoiding food competition. The intestine of adults has some specialized regions. The distensible crop is part of the foregut and especially used to store ingested sugar solutions to kill microbiota. The foregut ends with the proventriculus. In tsetse flies, specialized cells of the proventriculus secrete the peritrophic matrix (PM); in mosquitoes and sandflies midgut epithelial cells produce it. In all Diptera, the PM envelopes the blood, which is digested in the midgut by serine proteases, especially trypsins and chymotrypsins, consistent with the neutral/alkaline pH in the midgut lumen (Kollien et al. 2004).

In all vectors, a genetic recombination occurs. Often parasites multiply in the lumen of the gut in which the parasites must not only be resistant to the intestinal immune reactions but also to digestive enzymes of the insect. In the gut of the vectors, the energy sources available for the parasites differ strongly from those in the human host. Whereas *Trypanosoma brucei* ssp. use large amounts of glucose in the human blood, sugars are rarely present in the gut of all these vectors (except sandflies), and the metabolism of the parasites depends on the digestion of amino acids. The duration of the intestinal phase can be short (*Plasmodium* spp.), transient (*T. brucei* ssp.), or permanent (*Leishmania* spp. and *T. cruzi*).

Whereas *Plasmodium* spp. are Sporozoa, the other three parasites, *Trypanosoma brucei* ssp., *Leishmania* spp., and *Trypanosoma cruzi*, belong to the order Kinetoplastida. These flagellates exhibit certain unusual biological features, like editing of mitochondrial transcripts and posttranscriptional regulation of gene expression (see Chap. 7) (Simpson et al. 2006). The infectious stages develop at different sites in the respective vector: In *Plasmodium* spp. infections, they develop between the gut cells and the basal lamina, in those with *Leishmania* spp. in the foregut, for *Trypanosoma brucei* ssp. in the salivary glands, and for *T. cruzi* in the rectum. Also the mode of infection shows peculiarities. Infectious stages of two of these parasites, *Plasmodium* spp. and *Trypanosoma brucei* ssp., are transmitted with the saliva during blood sucking of the insect. In the other two parasite-vector systems, they do not leave the gut and use opposite ways for the infection. In *Leishmania* spp., infectious stages are present in the foregut and are transmitted during blood ingestion. *T. cruzi* is defecated and initiates an infection if the feces gets into contact with little wounds or mucosal membranes. Since also the development in the vectors differs strongly, each of these systems will be presented separately, focusing on the molecular characteristics of the different stages, the development of the infectious stage, and the interactions of parasite and vector.

14.2 Interactions of *Plasmodium* and *Anopheles*

14.2.1 *Plasmodium* spp.

Globally, an estimated 3.3 billion people are at risk of being infected with malaria, and 1.2 billion are at high risk. In 2013, about 200 million cases of malaria occurred resulting in 584,000 deaths. An estimated 90 % of all fatal malaria infections burden African countries, and here, children aged less than 5 year account for 78 % of all deaths (WHO 2014). Parasites causing malaria are protozoans and parasitize not only mammals but also reptiles and birds. Five species of the genus *Plasmodium* infect only humans and some monkeys, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Until 2004 the latter, originating from monkeys, has been frequently misdiagnosed as *P. malariae* in microscopy in Southeast Asia (Ramasamy 2014). *P. falciparum* and *P. malariae* are prevalent in tropical and subtropical areas of Central and South America, Africa, and Southeast Asia. Infections with *P. vivax* occur in Central and South America, India, and Southeast Asia and those with *P. ovale* primarily in sub-Saharan Africa. *P. falciparum* is the predominant species in the world and causes the most severe form of malaria. Since investigations with the human pathogenic species require specific regulations, often other species of *Plasmodium* are considered as model systems, e.g., *P. gallinaceum* from chickens and *P. berghei* and *P. yoelii* from mice.

The life cycle of the parasite passes through three phases, schizogony, gamogony, and sporogony, including various stages of development in the *Anopheles* vector and the mammalian host. Schizonts develop in hepatocytes and red blood cells, gametocytes in red blood cells, and sporozoites in the vector. After transmission of sporozoites by the saliva of the vector, these parasites are transported in the blood and invade hepatocytes (see Chap. 9). They mature to schizonts, multiply, and develop merozoites that destroy the membrane of the host cell (exoerythrocytic schizogony). In *P. vivax* and perhaps *P. ovale* infections, dormant stages develop, termed hypnozoites that cause relapses even years later. Merozoites infect other hepatocytes or erythrocytes multiplying in the erythrocytic schizogony. Some of the parasites differentiate into sexual stages (gametocytes) within the erythrocytes and are ingested by *Anopheles* mosquitoes during a blood meal. Only these stages develop in the vector (see Sect. 14.2.3).

14.2.2 Anophelines

Female mosquitoes are the vectors of *Plasmodium* spp. Human pathogenic malaria is only transmitted by species of the genus *Anopheles*. More than 460 species of the genus *Anopheles* have been described so far. Approximately 70 of these species are potential vectors of human malaria, and 41 are dominant vector species capable of transmitting malaria at high level of major concern to public health (Sinka et al. 2012). Anophelines are found worldwide except Antarctica. *Anopheles* spp. that can transmit human malaria are not only found in areas where malaria is endemic but

also in areas where malaria has been eliminated. Thus, the latter areas are constantly at risk of reintroduction of the disease.

The larvae of these Diptera develop in water, feeding on algae and bacteria. The motile pupae remain in the water. After emergence, males and females suck nectar and honeydew, which are initially stored in a distensible region of the foregut, the crop. Only females ingest blood and digest it in the midgut. The period of time required for each developmental stage is temperature dependent. Adult females live up to 1 month (Schaub in press a).

14.2.3 Development of *Plasmodium* spp. in Mosquitoes

14.2.3.1 Gametogenesis and Development of Ookinetes

If mosquitoes ingest *Plasmodium*-infected blood, an average of 10^3 cell-cycle-arrested gametocytes within red blood cells are taken up (Sinden 1999). Within minutes these gametocytes mature and emerge from the erythrocytes. Inside the blood bolus in the midgut lumen, the gametocytes develop into macro- and microgametocytes (exflagellation) triggered by the drop of temperature, rise in pH, and the presence of a gametocyte-activating factor, xanthurenic acid. The signaling events of gametogenesis are controlled by a cGMP-dependent protein kinase (McRobert et al. 2008). The motile microgametes (male) fertilize macrogametes (female). Depending on the *Plasmodium* species, the round zygote develops within 16–20 h into a motile ookinete. Successful development of micro- and macrogametocytes as well as ookinetes is depending on the ability of the parasite to overcome the action of the digestive enzymes in the mosquito's midgut. Ookinetes in the border area of the blood bolus are suggested to be killed at first by these digestive enzymes secreted from the midgut epithelium, while ookinetes in the inner part of the blood bolus are more protected against the destructive action of the digestive enzymes and, hence, have more time to develop (Abraham and Jacobs-Lorena 2004).

The motile ookinete moves from the blood bolus to the midgut epithelium where it reaches the first barrier, the PM. It is composed of chitin, proteins, and proteoglycans and separates as a cylindrical sheet the midgut content from the single cell-layered midgut endothelium. Physical distension by the ingested blood and components of the blood meal itself induce the development of the PM. Depending on the mosquito species, it is secreted within hours (Billingsley 1990). This matrix is assumed to be a physical barrier for many parasites since it prevents the contact with the insects' tissues. Ookinetes move toward the midgut epithelium and invade the PM by introducing the anterior extremity into the fibrous layer (Pimenta et al. 2015). *P. falciparum* and *P. gallinaceum* ookinetes seem to secrete chitinases enabling the parasite to pass through the PM (Vinetz et al. 2000; Tsai et al. 2001). Infection of *Anopheles stephensi* by *P. berghei* is unaffected by the presence or absence of the PM, while a thick matrix in *Aedes aegypti* reduces midgut infections by *P. gallinaceum*. Therefore, the PM seems to be a partial, but not an absolute, barrier to midgut invasion by the ookinete (Billingsley and Rudin 1992).

Once the parasite has penetrated the PM, it has to transit the cells of the midgut epithelium to reach their basement membrane. Ookinete binding to the midgut and establishment of midgut invasion are most probably mediated by carbohydrate moieties of the midgut epithelium and parasite lectins (Zieler et al. 1999; Dinglasan et al. 2007). Salivary gland and midgut peptide 1, a dodecapeptide identified by phage-display, strongly inhibit *P. berghei* ookinete invasion via competitive binding to a putative midgut receptor, and in transgenic mosquitoes expressing salivary gland and midgut peptide 1 after blood feeding, transmission of the parasite is impaired (Ghosh et al. 2001; Ito et al. 2002). However, *P. berghei* ookinetes seem to use different invasion pathways of the midgut since the invasion of some parasite clones is strongly inhibited by salivary gland and midgut peptide 1 whereas the invasion of other clones is not (Vega-Rodríguez et al. 2014).

The parasites take an intercellular or an intracellular route across the epithelium. In the latter, the ookinete enters the epithelial cells without forming a parasitophorous vacuole, but aided by perforin-like molecules. *P. falciparum* perforin-like protein 4 gene is expressed in blood stages, female gametocytes, and ookinetes. Gene disruption results in no phenotypical changes during blood stages replication, gametocyte development, or gametogenesis. However, mosquitoes fed with perforin-like protein 4 deletion mutant gametocytes display an accumulation of ookinetes in the vector midgut and a severe reduction in oocyst numbers, indicating an inability of perforin-like protein 4 (−) ookinetes to transverse the midgut epithelium and the crucial participation in infection of the mosquito midgut (Wirth et al. 2015). The ookinete penetrates at clefts between the microvilli and invade many cells on their way to the basement membrane, causing every cell to begin apoptosis (Han et al. 2000; Pimenta et al. 2015). The epithelial responses in different mosquito-parasite combinations vary. While *A. stephensi* detaches apoptotic cells by an actin-mediated budding-off mechanism, in *A. aegypti* the midgut epithelium is repaired by a unique actin cone zipper mechanism including the formation of a cone-shaped actin aggregate that expels the cellular contents into the midgut lumen by sequentially closing as it brings together vital neighboring cells (Han et al. 2000; Gupta et al. 2005).

14.2.3.2 Development of Oocysts and Sporozoites

When the ookinete has crossed the epithelial layer of the midgut, it will remain between the intestinal epithelium and the basal lamina. Here, the ookinete will mature to the oocyst which undergoes considerable cytoplasmic subdivision (Simonetti 1996). The basal lamina consists mainly of laminin and collagen and covers the entire coelomic cavity, including the midgut. The development from a moving ookinete to a sessile oocyst is essentially induced by laminin (Adini and Warburg 1999). Its importance for ookinete transformation is emphasized by down-regulation of laminin γ1 of *Anopheles gambiae* leading to significant reduction of successfully developed oocysts (Arrighi et al. 2005). The paralogue surface proteins P25 and P28 from *P. berghei* trigger the laminin-mediated transformation (Vlachou et al. 2001). Two other surface proteins, Pbs21 and Pbs25, are also involved in the transformation process since single or double knockout of the respective genes

results in a substantial reduction of ookinetes successfully transforming into oocysts (Sidén-Kiamos et al. 2000). If two other binding partners of laminin at the surface of ookinetes, secreted ookinete adhesive protein and circumsporozoite and thrombospondin-related anonymous protein, are deleted from the parasite, the ability of ookinetes to invade midgut epithelium and transform into oocysts is reduced (Dessens et al. 1999, 2003).

Maturation to oocysts will be completed depending on the *Plasmodium* species within 10–24 days and up to thousands of sporozoites are released into the insect's hemocoel after rupture of the oocyst. The exact molecular events and interactions leading to sporozoite release are not exactly known. However, an important sporozoite protein named circumsporozoite protein, the putative cysteine protease egress cysteine protease 1, and further parasite proteins are involved in the egress process since deletion mutants and genetic manipulations of these factors are unable to release matured sporozoites from the oocyst (Aly et al. 2009).

Sporozoites within the hemocoel are distributed to many parts of the insects' body by the circulation of the hemolymph, even to legs and wing veins, but do not interact with the most tissues (Beier and Koros 1991). If sporozoites eventually pass by the basal membrane of the salivary glands and get into contact, parasite ligands recognize specific host receptors and sporozoites adhere to the basement membrane. The initial binding of sporozoites to the salivary gland tissue depends on several parasite ligands. The *Plasmodium* circumsporozoite protein plays a key role in the invasion of this organ. Circumsporozoite protein binds specifically to the salivary glands distal and lateral lobes in a similar fashion as the sporozoites within the salivary gland ducts. Antibodies to circumsporozoite protein and in competition assays the highly conserved N-terminus of circumsporozoite protein and full-length protein block the sporozoite invasion of salivary glands (Warburg et al. 1992; Sidjanski et al. 1997; Myung et al. 2004). An *Anopheles* salivary gland protein, named circumsporozoite protein-binding protein, interacts with the circumsporozoite protein. Downregulation by RNA interference (RNAi) in mosquito salivary glands blocks invasion by the parasite and feeding with blood containing circumsporozoite protein-binding protein antibodies reduces the parasite load by 25 % and 90 % at 14 and 18 days after feeding, respectively (Wang et al. 2013b). Another essential protein for attachment and invasion of salivary glands is the thrombospondin-related anonymous protein. In deletion mutants of *P. berghei*, sporozoites inside oocysts mature and the sporozoites are released into the hemocoel. However, these mutants are no longer motile and are unable to invade salivary glands (Sultan et al. 1997). The surface protein saglin of *Anopheles* salivary glands is a receptor for thrombospondin-related anonymous protein and interaction of these proteins is crucial for sporozoites salivary gland invasion since downregulation of saglin by RNAi resulted in up to 98 % inhibition of sporozoite invasion (Ghosh et al. 2009). The upregulated oocyst sporozoite 3-protein resembles thrombospondin-related anonymous protein and is also important for the invasion process (Mikolajczak et al. 2008). Initially identified in a microarray study to analyze differential expression of oocyst-derived and salivary gland sporozoites, a targeted gene deletion in *Plasmodium* prevents the parasites penetration of the salivary gland tissue. Since

function of different parasite ligands affects only invasion of salivary glands (upregulated in oocyst sporozoite 3-protein) and hepatocytes of mammalian hosts (thrombospondin-related anonymous protein), these genes might be good candidates for targets of vector-based transmission-blocking strategies, as well as strategies that prevent mammalian host infection.

After attachment to and crossing the basal lamina, the parasite invades the salivary cells by use of a vacuolar membrane of host origin (Pimenta et al. 1994). Inside the host cells, the sporozoites inside the vacuoles are attached to the membrane by their anterior end. The formation of this vacuole is only transient, and after disruption of the vacuole membrane, the parasite transits the cytoplasm, attaches to the apical plasma membrane of the cells, and invades the secretory cavity. Here, the parasites are again inside vacuoles from which they escape and form large bundles of parallel arranged sporozoites attached by multilamellar membrane junctions (Pimenta et al. 1994). Several sporozoites reach the secretory duct of the salivary gland and are now ready to be transmitted by the mosquitoes bite to a new vertebrate host. Upon release by the oocyst, sporozoites will reach the salivary glands within 1–2 days.

Malaria parasites go through radical reduction during their development in the mosquito vector (Smith et al. 2014). Each developmental step starting from maturation of micro- and macrogametocytes to oocyst formation is accompanied by population reduction, ending in a very low number of parasites. In a study on the early sporogonic cycle of *P. falciparum* in *A. gambiae*, ingestion of blood with an average gametocyte density of 433.5 per mm^3 results in a mean density of only 12.6 round zygotes, 5.5 ookinetes, 1.8 young oocysts, and 2 midsize oocysts. At 7 days post-infection, only 37.8 % of the mosquitoes are infected (Gouagna et al. 1998). Parasite numbers in the oocyst stage are lowest during entire *Plasmodium* life cycle. Therefore, the parasite development in the midgut of the insect vector is the key step for strategies to block malaria transmission.

14.2.4 Action of the Mosquito Immune Response Against *Plasmodium*

The insects' innate immune system consists of cellular and humoral components which contribute significantly to resistance against microbial infections (Hoffmann et al. 1999). Basically, the defense process of insects can be divided into two main stages: recognition and response. Both are joined by signaling pathways and regulated by modulating elements (Lehane et al. 2004).

Nitric oxide levels in the midgut of mosquitoes significantly increase upon ingestion of infected blood. Oxyhemoglobin and heme persist throughout blood digestion and should catalyze the synthesis of toxic nitric oxide metabolites which have a strong negative effect on parasite survival (Peterson et al. 2007). The production of nitric oxide synthase is activated before invasion of the intestinal epithelium and occurs while the parasite crosses the epithelial cells. The activity of this synthase triggers the production of different antimicrobial peptides which are responsible for the death of a large number of ookinetes in the midgut (Dimopoulos et al. 2001;

Herrera-Ortiz et al. 2011). In addition, the transition of ookinetes through midgut cells induces expression of nitric oxide synthase and thereby melanisation in a refractory strain of *A. gambiae* (Kumar et al. 2003, 2004). The enzyme is apoptotic cells are subject to detachment to the midgut lumen and, consequently, migrating parasites are hindered to cross the epithelium (see above).

As a consequence of nitric oxide synthase activity, increasing levels of nitric oxide are used for nitration of ookinete surface proteins, which cause the parasite to be tagged for thioester protein 1-mediated lysis or melanization when reaching the basement membrane (Blandin et al. 2004). Knockdown of thioester protein 1 abolishes lysis and melanization and increases the number of developing parasites (Blandin et al. 2004; Jaramillo-Gutierrez et al. 2009). Thioester protein 1 acts in a complex with the scaffold proteins *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1 and leucine-rich immune molecule 1, and loss of these components prevents ookinete recognition and drastically increases the number of parasites (Jaramillo-Gutierrez et al. 2009; Povelones et al. 2009). The non-catalytic serine protease is also required for accumulation of thioester protein 1 on microbial surfaces (Povelones et al. 2013).

When ookinetes cross the epithelium cells, the melanization cascade is activated, and the deposition of melanin and free radicals kills the parasite (Luckhart et al. 1998; Hoffmann et al. 1999). Melanization is a major process of insects' immune response. The recognition of non-self-compounds triggers the activation of factors present in the hemolymph that mediate melanin synthesis, especially phenoloxidase (Hillyer et al. 2003). Multiple clip-domain serine proteases regulate phenoloxidase activation as part of a complex cascade converting inactive pro-phenoloxidase into the active enzyme (Barillas-Mury 2007). Although the respective factors are found throughout the hemolymph and phenoloxidase is a very reactive enzyme, melanization is limited to the parasite. The activation of hemolymph pro-phenoloxidase is functional linked to parasite tagging by thioester protein 1 (Yassine et al. 2014).

Hemocytes are the main player of the insect cellular immunity. Mosquito hemocytes seem to be primary phagocytotic cells and directly involved in microbial clearance (Lavine and Strand 2002). However, they are also responsible for the production of several immune components, which mediate the immune response and pathogen clearance, such as thioester protein 1 (Blandin et al. 2004). Their special role in pathogen response is stressed by several studies analyzing the transcriptional profile of *A. gambiae* hemocytes which reveal specific responses to *Plasmodium* parasites and bacterial pathogens (Baton et al. 2009; Pinto et al. 2009). However, these studies do not distinguish between the different types of hemocytes. *Plasmodium* ookinete invasion of the mosquito midgut primes a robust and long-lived enhanced antibacterial and anti-*Plasmodium* response upon re-challenge which results in quantitative and qualitative differentiation of hemocytes that persist for the life span of the mosquito (Rodrigues et al. 2010). This recently explored phenomenon of immune priming illustrates that the biology and function of the different hemocytes have to be addressed.

The insects' innate immune responses are activated and regulated by intracellular immune signaling pathways, the Toll pathway, immunodeficiency (Imd) pathway, and the JAK/STAT pathway. Much of our knowledge on these pathways is transferred from the insights of *Drosophila* research and subsequent identification

of orthologous in mosquitoes. However, in comparison to *Drosophila*, mosquitoes immune gene families exhibit rapid expansions which might reflect a functional broadening of the mosquito defense systems (Waterhouse et al. 2007).

The Toll pathway in *Anopheles* mediates host response to *Plasmodium* challenge. Its activation by *P. berghei* restrains parasites survival in *A. gambiae* (Frolet et al. 2006). Concurrent silencing of *Relish 1* (Toll pathway) and *Relish 2* (Imd pathway) decreases the basal expression of the major antiparasitic genes encoding for thioester protein 1 and a leucine-rich repeat protein (see above) and abolishes resistance of *Anopheles*. Overactivation of the Toll pathway by silencing cactus, a negative regulator of this pathway, greatly reduces *P. berghei* infection in *A. gambiae* (Frolet et al. 2006; Garver et al. 2009). Hemocytes are suggested to be mediators of this antiparasitic response (Ramirez et al. 2014). Toll activation is more efficiently reducing *P. berghei* loads in *A. gambiae*, *A. stephensi*, and *A. albimanus* than *P. falciparum* loads (Garver et al. 2009).

The Imd pathway is effective in limiting *P. falciparum* infections. The transcription factor Relish 2 is negatively regulated by caspar and silencing of this regulator causes a near refractoriness to *P. falciparum* in *A. gambiae* (Garver et al. 2009). This phenotype is not achieved in a *P. berghei* infection. The Imd pathway seems to be most potent against the ookinete stage, but has also reasonable activity against early oocysts and lesser activity against late oocysts (Garver et al. 2012).

The JAK/STAT pathway is implicated in the immune response to *Plasmodium* parasites. The depletion of the transcription factor STAT results in increased *P. berghei* and *P. falciparum* development in *A. gambiae* and also numbers of *P. vivax* increase in *A. aquasalis* (Gupta et al. 2009; Bahia et al. 2011).

14.2.5 Evasion of the *Anopheles* Immune System by *Plasmodium*

The malaria parasite is able to counteract some of the immune response mechanisms of the *Anopheles* host. When the parasite rests between the basement membrane and the basal side of the midgut epithelium, where the ookinete differentiation to a sporozoite forming oocyst occurs, it is faced a multitude of immunogenic components. Nevertheless, *Plasmodium* might use laminin derived from the mosquito to coat its surface and hence prevent itself from being recognized as nonself by the vectors immune system (Arrighi et al. 2005). Moreover, laminin is not present only on the oocyst capsule but also in the membranes of the developing sporozoites inside the oocyst. Sporozoites might be covered during their formation with laminin to protect them from melanization in the hemocoel (Nacer et al. 2008). Most probably a parasite-derived transglutaminase cross-links parasite- and mosquito-derived proteins including laminin to the capsule (Smith and Jacobs-Lorena 2010).

The ookinete expresses PFS47 at its surface, an essential survival factor for *P. falciparum* to evade the immune response of *A. gambiae*. By a yet unknown mechanism, PFS47 suppresses the expression of the epithelial heme peroxidase and NADPH oxidase 5, which are key components of the nitration mechanism of ookinete surface proteins. Nitrification leads to thioester protein 1-mediated lysis or

melanization (see above). Disruption of *Pfs47* greatly reduces parasite survival in the mosquito (Molina-Cruz et al. 2013). However, this evasion mechanism seems to be specific for only some parasite-mosquito species (or strains) and presumably not functioning in all sporozoites.

14.2.6 Perspectives and Open Questions

Although much effort has been made in the last decades to elucidate the interactions of *Plasmodium* and its *Anopheles* hosts, much more needs to be explored to fully understand the interplay of parasite and host. Strategies to fight against *Plasmodium* transmission might be most promising if targeting parasite stages in the insect vector. Therefore, deciphering the recognition mechanisms, the key player of the immune signaling pathways and potential target sites for intervention is of great value.

Recently, the mosquito vector microbiota has experienced substantial attention. Larvae develop in water, and many bacteria survive metamorphosis and are present in the intestine of adult mosquitoes. The development of metagenomics tool made diversity screenings possible without the requirement for obtaining pure cultures for sequencing and hence profiling mosquitoes' microbiota. In addition, molecular studies on how bacteria interact with the hosts' immune system and respond to infection have been performed (Azambuja et al. 2005; Chouaia et al. 2010; Boissière et al. 2012). These efforts are reasonable since bacteria living in the midgut have been found to modulate the response of the mosquitoes toward *Plasmodium* infection (Boissière et al. 2012; Eappen et al. 2013). ROS not only modulate immunity against *Plasmodium* but also against bacteria (Molina-Cruz et al. 2008). Concurrent infections of adults with larvae-originating *Serratia marcescens* and *Plasmodium* increase the mortality rates. *Wolbachia*, an intracellular bacterium, significantly limits infections with *Plasmodium* (Smith et al. 2014). Therefore, it is of major concern to explore how microbial species could be used in paratransgenesis and malaria transmission-blocking strategies (Bongio and Lampe 2015).

The overwhelming progress in development of new and high-performance sequencing technologies has opened the door for broadly based studies on the interaction of vector and parasite. Sequencing and analyzing the genomes of multiple *Plasmodium* species as well as mosquito species are and will be a big step forward to understand the vector-parasite interactions and might enable possible manipulations in order to reduce disease burden. Starting in 2008 the *Anopheles* Genomes Cluster (AGC) has joined efforts to build the first anophelines comparative genomics consortium (Besansky 2015). Sixteen mosquito species were selected and their genomes were assembled (Neafsey et al. 2013) and made available through VectorBase (www.vectorbase.org). Together with the available genomes of several *Plasmodium* species (www.plasmodb.org), these data might be used to find new key player in the vector-parasite interaction by transcriptomic approaches as well as deciphering signaling network components. In addition, proteomic investigations are also valuable to provide insights into proteins involved in infection processes (Cázares-Raga et al. 2014; Cornelie et al. 2014).

14.3 Interactions of *Leishmania* and Sandflies

14.3.1 *Leishmania* spp.

Leishmania spp. are the causative agents of leishmaniasis, a neglected disease affecting more than 350 million people in 98 countries or territories on four continents. Two million new cases occur yearly (WHO 2010). The three clinical forms of leishmaniasis – visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis – differ in mortality and morbidity rates. The visceral manifestation is the most severe form and, if untreated, causes life-threatening systemic infections and about 50,000 deaths annually. The cutaneous and mucocutaneous forms cause transient or chronic skin sores and horrible facial disfigurement, respectively (Herwaldt 1999). Different *Leishmania* species induce these three forms in the Old World (e.g., *L. tropica*, *L. major*, *L. infantum*, *L. donovani*) as well as in the New World (e.g., *L. guyanensis*, *L. amazonensis*, *L. braziliensis*, *L. infantum*) (Bates et al. 2015). Only about ten *Leishmania* spp. out of 21 etiologic agents of leishmaniasis are of significant importance for public health. With some exceptions, human beings are incidental hosts, and infections base on other mammalian reservoir hosts (Ashford 1997, 2000). Human pathogenic *Leishmania* spp. are classified into two subgenera according to the preferred region of the gut of the vector, phlebotomine sandflies. Species of the subgenus *Viannia* colonize the hindgut, whereas species of the subgenus *Leishmania* develop in the mid and foregut of the vector (Bates and Rogers 2004; Lainson et al. 1977). In vitro cultivation of parasites of the subgenus *Leishmania* is easily done and hence most investigations (e.g., proteomic studies) have been done using this subgenus. However, during continuous in vitro cultivation, strains lose their infectivity properties (Araújo Soares et al. 2003).

Leishmania are Kinetoplastida (see Chap. 7) and go through a complex life cycle involving several developmental stages in the vertebrate host and the sandfly insect vector. These stages are adapted to the different conditions in the respective host. In mammals, the flagellate is phagocytized by or actively invades a macrophage and subsequently differentiates into an intracellular amastigote form. Amastigotes multiply, and after exhaustion of the host cell, they pass over into the blood and infect other cells of the reticulohistiocytic (reticuloendothelial) system or are transferred to the sandfly during blood feeding. In the insect, mainly promastigote forms develop.

14.3.2 Sandflies

Sandflies are tiny, only 2–3 mm long hairy insects. These Diptera are generally present in the warm zones of Asia, Africa, Australia, southern Europe, and the Americas (Killick-Kendrick 1999; Schaub in press b). Larvae live in humid soil and feed on organic detritus and its microorganisms. Males and females of many species feed on aphid honeydew. *Phlebotomus papatasi* also pierce plants to suck sap, and *Lutzomyia longipalpis* sucks nectar of flowers. Since the mouthparts of males are not suitable to pierce skin, only females suck blood, and only they act as vectors.

Blood feeding distends the midgut, and thereby, the cells secrete the PM. The structure of the PM is complex and varies during blood digestion. Several proteins are involved in formation and breakdown of the PM including the chitin-binding domain-bearing peritrophins (Sacks 2001).

In almost all species, females need a blood meal for egg development. Autogeny, i.e., the development of eggs without a previous blood meal, occurs only in a few species and only for the first oviposition. Thereafter, a blood meal is required for the next batch of eggs, laid about 3–8 days after a blood meal. The number of gonotrophic cycles and the lifespan of adult phlebotomine sandflies are species dependent and also affected by abiotic factors, e.g., temperature, a wild population of *P. ariasi* living about 29 days in France (Guzmán and Tesh 2000; WHO 2010).

14.3.3 Development of *Leishmania* spp. in Sandflies

Ninety-eight out of over 800 species of sandflies are proven or suspected vectors of human leishmaniases, including 42 *Phlebotomus* spp. in the Old World and 56 *Lutzomyia* spp. in the New World (Maroli et al. 2013). In the Old World, each species seems to transmit specifically only one *Leishmania* sp. Only *Phlebotomus sergenti* is a vector of two species, *L. tropica* and *L. aethiopica*. In contrast, some *Lutzomyia* species in the New World probably transmit more than one *Leishmania* species.

Although leishmaniases are severe diseases threatening the lives of so many people, knowledge on interactions of the parasites with their respective vectors and ecology of vectors is poor compared to malaria. However, studies elucidating the parasite-vector interactions are fundamental for the discovery of potential candidates for vector-based transmission-blocking vaccines. These vaccines aim at preventing the transmission of pathogens by targeting molecules expressed by the vector and promising candidates have recently been identified (Coutinho-Abreu and Ramalho-Ortigao 2010).

Leishmania colonize the alimentary tract as actively motile promastigotes. Similarly to *Trypanosoma cruzi* and *T. brucei* ssp., sexual recombination only occurs within the insect vector (Rogers et al. 2014). Replicative procyclic promastigotes found in the insects midgut in the blood meal phase along with non-dividing metacyclic promastigotes in the thoracic midgut and proboscis of the sandfly are the major forms in the insect host. However, several intermediate forms have been noticed (Bates and Rogers 2004; Gossage et al. 2003). Metacyclic promastigotes are infective when transferred into a mammalian host by sandfly bite.

When female sandflies take up blood, which contains macrophages infected with amastigotes, conditions for the development of the parasite change drastically, e.g., temperature decreases and pH increases. This induces the development from the small immotile and intracellular amastigote form to procyclic promastigotes. These are weekly motile with a short flagellum representing the first replicative forms in the insect vector (see Chap. 7). About 48–72 h post-infection, parasites begin to slow down their replication and differentiate into strongly motile long nectomonad

promastigotes. Since the PM disintegrates, these nectomonads can attach to the microvilli of the intestinal wall. Then they migrate anteriorly and transform into short proliferative nectomonad promastigotes (leptomonads). These finally develop into haptomonad promastigotes which attach to the cuticular lining of the stomodeal valve (Sacks and Kamhawi 2001). They secrete and are embedded in a filamentous proteophosphoglycan (Stierhof et al. 1999). Finally, metacyclic promastigotes develop, which are infective for mammals. Often the diameter of the foregut is reduced or blocked by the masses of parasites, inducing repeated feeding trials and thereby transmissions.

The proteolytic enzymes secreted by sandflies in order to digest the blood meal are the first obstacle for *Leishmania* development in the midgut of the vector. Sandflies use serine proteases for blood digestion, namely, trypsin- and chymotrypsin-like enzymes (Telleria et al. 2010). The activity of digestive enzymes strongly affects the development of *Leishmania* in sandflies. Specific knockdown of a blood feeding-induced trypsin supports the survival of *L. mexicana* (Sant'Anna et al. 2009). However, females of *L. longipalpis* fed on infected blood containing *L. infantum chagasi* show lower levels of trypsin activity, indicating a modulation of the expression by the parasite by a yet unknown mechanism (Telleria et al. 2010). There is evidence for both modulation of transcript abundance coding for digestive enzymes (Ramalho-Ortigão et al. 2007; Telleria et al. 2010) and directly affecting proteolytic activity in the midgut by serine protease inhibitors (Morrison et al. 2012). The latter is of special interest since the serine protease inhibitors have no target in the parasites genome and, hence, might be functional in affecting the mammal or insect host. Overall, parasites within an early-stage transition from amastigote to promastigote form seem to be highly susceptible to killing by digestive enzymes, while fully differentiated promastigotes are relatively resistant (Pimenta et al. 1997). Resistance might be ensured by phosphoglycans attached to the cell surface. *L. major* surface proteophosphoglycans seem to protect against digestion since a knockout mutant deficient in a specific surface phosphoglycan is more susceptible to killing than the wild type, and this effect can be restored by adding purified proteophosphoglycan (Secundino et al. 2010).

A *Leishmania* infection can increase as well as decrease the abundance of peritrophin-encoding transcripts of the PM (Ramalho-Ortigão et al. 2007; Jochim et al. 2008). The biological significance of this finding remains unclear. The PM traps parasites and act as a barrier to later development. The mechanism by which the parasite escapes from the PM is not fully understood, but the chitinolytic activity of the sandfly midgut at the end of blood meal digestion (and most likely based on the activity of vector chitinase) seems to be responsible for the escape. Indeed, reduction of *P. papatasi* chitinase by RNAi significantly reduces the number of *L. major* in the midgut following an infective blood meal (Coutinho-Abreu et al. 2010).

Subsequent the escape from the endoperitrophic space, the parasite must avoid being excreted with the blood remnants. Loss of infection within inappropriate vectors is predominantly associated with excretion of the digested blood meal (reviewed in Sacks 2001). Therefore, long nectomonads attach to the midgut epithelium by inserting their flagella between the epithelial microvilli. Attachment of *L. major* to

P. papatasi midgut epithelium is mediated by the parasites' abundant surface lipophosphoglycan binding to a galectin molecule at the sandfly's midgut epithelium (Bates 2008). However, this attachment mechanism is most studied in the *P. papatasi-L. major* interaction and is still to be determined in other vector-parasite systems where different mechanisms seem to be active (Jecna et al. 2013). In order to produce free-swimming metacyclic promastigotes, the parasite needs to detach from the midgut epithelium. In *P. papatasi*, *L. major* modifies the phosphoglycan side chains leading to the loss of affinity to the sandfly receptor (Pimenta et al. 1992).

Metacyclogenesis, the formation of small, rapid-swimming metacyclic promastigotes, is the final developmental step in the insect vector. These promastigotes are infective for mammals and transferred with the next bite. Metacyclogenesis is induced in vitro by low pH and nutrition depletion. The exact pH conditions in infected sandflies are unknown; however, a V-ATPase of *L. longipalpis* might contribute to gut acidification (Bates 2008). Two main mechanisms are presumably responsible for transmission of metacyclic promastigotes. Either these parasites occurring in the proboscis are deposited into the skin during feeding or a parasite population behind the stomodeal valve is regurgitated with a backflow of ingested blood (Dostálová and Volf 2012). Regurgitation is most likely an effect of the damage of the chitin layer of the stomodeal valve (proventriculus) and a gel-like plug built by *Leishmania*-secreted filamentous proteophosphoglycans containing large numbers of short nectomonads and metacyclic parasites. The time from an infected blood meal until a female sandfly may transmit *Leishmania* parasites is about 1–3 weeks. However, the precise period depends on the gonotrophic cycles of the different vector species and abiotic factors like ambient temperature (WHO 2010).

14.3.4 Interactions of the Innate Immunity of Sandflies and *Leishmania*

As for all blood-sucking insects, innate immunity is vitally important to control bacterial and parasitic infections in the midgut. However, as mentioned above, very few studies have focused on this topic in sandflies. Antimicrobial peptides are key components of the innate immunity. In *Phlebotomus duboscqi*, defensin is induced in midgut and hemolymph after challenge of the insect with both bacteria and *L. major* infection (Boulanger et al. 2004). The induction is specifically caused by the parasite, since the peptide is not found in either naïve insects or in insects receiving a non-infected blood meal. In addition, recombinant defensin possesses an antiparasitic activity on *Leishmania* promastigotes (insect form). Presumably lipophosphoglycans in the surface coat play a major role in upregulation of defensin expression because parasite mutants lacking this abundant promastigote surface glycoconjugate induce a significant lower expression level compared to infections with wild-type parasites. In contrast, feeding on blood containing *L. mexicana* does not induce a parasite-specific upregulation of defensin expression in *L. longipalpis* (Telleria et al. 2013). Hence, different phlebotomine sandflies and different *Leishmania* species may trigger diverse immune responses.

Feeding reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) or superoxide anion to infected sandflies is detrimental to *Leishmania* survival within the insects' gut (Diaz-Albiter et al. 2012). In addition, knockdown of the gene encoding for catalase, an antioxidant enzyme, decreases parasite survival in the midgut. Since ROS-activity is not increased in *Leishmania*-infected sandflies, *Leishmania* could evade the oxidative burst by an unknown mechanism or avoid eliciting a ROS-based response to assure survival within the midgut (Diaz-Albiter et al. 2012).

To date, only one component of immune signaling pathways is identified. *Caspar*, the negative regulator of immunodeficiency (Imd) signaling pathway, is significantly downregulated in *L. longipalpis* females after an *L. mexicana*-infected blood meal. Depletion of *caspar* expression by RNAi reduces sandfly gut populations of both *L. mexicana* and *L. infantum* indicating the participation of Imd pathway in immune homeostasis in phlebotomine sandflies (Telleria et al. 2012).

14.3.5 Interactions of Intestinal Microbiota and *Leishmania* in the Sandfly

According to standard identifications of isolated colony-forming bacteria and metagenomics analyses, wild sandfly populations contain many different intestinal bacteria, including *Serratia marcescens* (reviewed by Diaz-Albiter et al. 2012; Telleria et al. 2013). In vitro culture-derived promastigotes are lysed by *S. marcescens* (Moraes et al. 2008), but interactions in the sandfly can hardly be investigated, because the bacterium is insect pathogenic. However, it induces a differential response of the immune system than *Leishmania* and other bacteria. Since *Leishmania* can be affected by an immune response in the vector, these interactions should be considered. Therefore, the different bacteria have to be classified according to the immune response they induce.

14.3.6 Perspectives and Open Questions

There is obviously a great need for further research on the molecular interactions of *Leishmania* parasites and their respective phlebotomine vectors. Understanding these interactions might identify targets for control of parasite development and hence to block transmission in order to diminish the burden of leishmaniasis.

Deciphering molecular interactions between *Leishmania* and phlebotomine sandflies is boosted by genome projects of the different parasite species as well as for vector species. Since genome data for parasites of the *Leishmania* (*L. major*) subgenus is available for a decade, more recently the genome of a representative of the *Viannia* (*L. panamensis*) subgenus was published (Ivens et al. 2005; Llanes et al. 2015). In addition, genomes are available for the vector species *L. longipalpis* and *P. papatasi* (www.vectorbase.org). By complementary use of genomic, transcriptomic, and proteomic data, investigation of interacting factors between the

Leishmania parasites and their respective phlebotomine vector species might elucidate candidates for successful transmission-blocking strategies.

To date, the microbial ecology of phlebotomine sandflies is not well understood although the bacterial community most likely plays an important role in respect to the insects' biology and vector capacity (Peterkova-Koci et al. 2012). The establishment of an axenic rearing system for *L. longipalpis* might open new perspectives for studying the influence of microbiota on the midgut immunity and interaction of vector and parasite. In addition, it is very important to establish more colonies of other species of sandflies in the laboratory. So far, mainly *L. longipalpis*, *P. papatasi*, and *P. duboscqi* are considered. Similar efforts are required to include other species of *Leishmania*, especially the agents of visceral leishmaniasis. However, in these species the security requirements are much stronger. As a future objective, paratransgenic approaches might be a possible strategy for *Leishmania* control.

Recent advance in proteomic research is also promising for the identification of putative targets for control strategies. One of these approaches focuses on the saliva of the phlebotomines which plays an important role in the establishment of an infection and the pathogenicity. Although effects after inoculation of saliva by a needle and by phlebotomines differ in details of the immune response, cutaneous *Leishmania* infections are enhanced in both modes (Sacks and Kamhawi 2001). However, repeated exposure to bites of uninfected sandflies results in attenuated infections, explaining differences in the pathology of infections in indigenous inhabitants and immigrants or tourists (Sacks and Kamhawi 2001). Such a protection is also possible against visceral leishmaniasis, using gene knockout parasites and a salivary protein as an adjuvant (Fiuza et al. 2016).

Another approach considers exosomes, i.e., membrane surrounded vesicles with protein and nucleic acid contents, which are constitutively secreted by the flagellate within the lumen of the sandfly midgut containing numerous factors which are important for establishment of the disease. Technological enhancements in proteome research have enabled the investigation of the protein content of the exosomes (reviewed by de Jesus et al. 2014). *L. major* exosomes are part of the sandfly inocula, hence being delivered alongside with the saliva and the parasite during the sandfly bite. The results of a recent study on the function of egested exosomes suggest their ability to reach the host and influence the early events in the establishment of the infection (Atayde et al. 2015). Therefore, exosomes produced by *Leishmania* within the insect vectors might be a promising starting point for transmission blocking.

14.4 Interactions of *Trypanosoma brucei* ssp. and Tsetse Flies

14.4.1 *Trypanosoma brucei* ssp.

Two subspecies of *T. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, are the etiologic agents of Human African trypanosomiasis, commonly named sleeping sickness. Trypanosomes of a third subspecies, *T. b. brucei*, cause animal trypanosomiasis,

nagana, and are lysed by human sera. All three subspecies of these Kinetoplastida are exactly similar in morphology. The two human pathogenic subspecies are restricted to sub-Saharan Africa, *T. b. gambiense* in Western and Central Africa and *T. b. rhodesiense* in Eastern and Southern Africa, resulting in a chronic progressive or more acute course of the disease, respectively (Brun et al. 2010). Currently about 6000 new infections per year are estimated in 36 countries, in which 70 million people are at risk for acquiring the disease. About 98 % are infected by *T. b. gambiense* (WHO 2015a).

The trypanosomes have morphological distinct forms each representing a particular stage of their life cycle. In mammals, replicative blood trypomastigotes possess a slender, stumpy, or intermediate morphology and develop in the blood, lymph, or cerebrospinal fluid. In the vector, stumpy blood trypomastigotes transform into replicative procyclic trypomastigotes, replicative epimastigotes, and finally nonreplicative stumpy metacyclic trypomastigotes (Rodrigues et al. 2014).

T. brucei ssp. represents an ideal model organism for the study of fundamental processes of cell biology including cell morphology and division, cytological rearrangements, and protein trafficking. Such analyses are supported by the availability of the full genome sequence of these flagellates enabling researchers to design genetically modified strains by gene knockouts, gene replacements, and in situ epitope tagging through homologous recombination and RNA interference (Zhou et al. 2014) or on a global genome scale using new approaches such as RNA-seq de novo assembly (Hamidou Soumana et al. 2015). In consequence, the very basic molecular processes can be studied in vitro, but the in vivo analysis is still restricted by high costs and technical constraints. In addition, many isolates from humans do not develop in laboratory animals or after transfer into in vitro culture media. Another drawback of the research on vector-parasite interactions are laboratory-adapted strains that may display modified biochemical and cellular response if not exposed to their natural selecting microenvironments within the tsetse fly.

14.4.2 Tsetse Fly

T. brucei ssp. are transmitted by tsetse flies. The about 30 species and subspecies of these Diptera are 6–14 mm long (Schaub in press c). All are classified into the only genus of the insect family Glossinidae, *Glossina*, and are placed into three species groups, the fusca, palpalis, and morsitans group (Krafsur 2009). These groups are found in different habitats of the tsetse belt, sub-Saharan Africa between the latitudes of 14°N and 29°S. The palpalis group, including *G. palpalis palpalis* and *G. fuscipes*, are found in western and central Africa in riverside habitats (e.g., forests, riverbanks), but several species have adapted to high density urban centers in West Africa. The morsitans group, including *G. morsitans* and *G. swynnertoni*, is adapted to dry savanna habitats in East Africa, but requires shade of trees. The fusca group develops in lowland rain forests of West and Central Africa. Tsetse flies survival depends on an adequate temperature range between 16–38 °C and a relative humidity of 50–80 % (Franco et al. 2014). The life span is strongly affected by the season and lasts 1–9 months.

Glossinidae can be recognized by the forward-projected mouthparts. An additional peculiarity is the larval development which takes place in the uterus of the females. Only one egg/gonotrophic cycle develops, and the three larval stages are nourished by secretions from "milk glands." Full-grown third instar larvae are deposited and burrow into the earth and pupate. Due to this enormous investment of female, only about 10 larvae/female develop in intervals of about 10 days. This complicates the maintenance of colonies in the laboratory.

Both sexes are obligatory blood feeding (hematophagous), but they also ingest honeydew and nectar. Tsetse flies are pool feeders and inject saliva to stimulate vasodilation and to prevent blood coagulation (Ribeiro and Francischetti 2003; Champagne 2004). They store the blood in the anterior region of the midgut and a region of the foregut, the crop, and concentrate it. At the beginning of the midgut, the proventriculus, the PM is continuously synthesized and encloses the blood. Digestion takes place in the middle region of the midgut and absorption in the posterior region (Dyer et al. 2013). Within 4–5 days, most of the blood is digested (Gibson and Bailey 2003; Schaub in press c). Associated to the anterior midgut are special organs (mycetomes) that contain the primary obligate endosymbiont *Wigglesworthia glossinidia* which plays an essential role in tsetse reproduction and maturation of the immune system. Another often associated bacterium, *Sodalis*, is located extracellularly or intracellularly (Wang et al. 2013a).

14.4.3 Development of *Trypanosoma brucei* ssp. in Tsetse Flies

14.4.3.1 General Aspects

Only a few species are vectors of human sleeping sickness, but all are potential vectors of animal trypanosomiasis. According to the distribution of the tsetse flies, Glossinidae of the palpalis group are the most important vectors of *T. b. gambiense*. *T. b. rhodesiense* is mainly transmitted by species of the morsitans group. The prevalence of trypanosome-infected tsetse flies is generally low, with ≤1–15 % in different tsetse species and locations, and less than 1 % of tsetse flies are infected with *T. brucei* ssp. (Haines 2013; Franco et al. 2014). Therefore, a high proportion of the tsetse fly population seems to be refractory to these trypanosomes.

After ingestion with the blood, trypanosomes undergo specific development programs comprising differentiation, proliferation, and migration (Rotureau and Van Den Abbeele 2013). Differentially regulated gene expression is important in adaptation of trypanosomes from one life-cycle stage to the next, and 25–40 % of *T. brucei* genes are differentially expressed in bloodstream forms and procyclic forms in the tsetse midgut (Jensen et al. 2009; Nilsson et al. 2010). The genome of trypanosomes is organized into multi-gene polycistronic transcription units and differential regulation of a particular subset of mRNAs is achieved by posttranscriptional mechanisms (see Chap. 6). Positive and negative control elements regulate gene expression of transcripts derived from the same transcription unit by regulating mRNA stability and translational efficacy. The enormous complexity of cellular changes in the life cycle of trypanosomes is also presented on the proteome level. The proteome of *T.*

Table 14.1 Characteristics of different morphotypes of *T. brucei* ssp. and location in the tsetse fly

Morphotype	Location	Replication	Surface coat
Bloodstream trypomastigote (stumpy form)	Midgut	Nonreplicative	VSG
Procyclic trypomastigote (early)	Midgut, endoperitrophic space	Symmetric division	Procyclin (GPEET)
Procyclic trypomastigote (late)	Midgut, ectoperitrophic space	Symmetric division	Procyclin (EP 1 and EP 3)
Mesocyclic trypomastigote	Proventriculus	Asymmetric division	Procyclin (EP 1 and EP 3)
Epimastigote (free)	Proventriculus, foregut, hypopharynx	Nonreplicative	Unknown
Epimastigote (attached to microvilli)	Salivary glands	Symmetric and asymmetric division	BARP
Metacyclic trypomastigote	Salivary glands (lumen)	Finally nonreplicative	VSG

brucei is constituted by >3500 proteins of which >10 % are differentially synthesized during the life-cycle stages (Urbaniak et al. 2012; Butter et al. 2013). The proteomic analysis might fail to detect low-level proteins due to technical constraints, but the sheer quantity of proteins that are differentially synthesized highlights the complexity of adaptation trypanosomes have to accomplish in order to complete their life cycle.

The stepwise development of the *T. brucei* ssp. takes place in different compartments of the tsetse fly. In the midgut, blood trypomastigotes transform to procyclic trypomastigotes, which penetrate the PM, colonize the ectoperitrophic space, and migrate anteriorly to the proventriculus. There they transform to mesocyclic trypomastigotes and then epimastigotes, which penetrate the PM and migrate via the foregut, mouthparts, and salivary gland ducts to theses glands and differentiate into metacyclic trypomastigotes. Sometimes procyclic trypomastigotes seem to penetrate the gut wall and the epithelium of the salivary glands. These steps are correlated with different division forms and changes of the surface coat (Table 14.1).

14.4.3.2 Development in the Midgut

Preadaptions to Transformation

In the mammalian host, initially long slender trypomastigotes multiply. In response to the increasing parasite density, a "stumpy induction factor" is released resulting in the development of the nonproliferative stumpy form (see Chap. 6). This form is preadapted to differentiate within the tsetse fly. Besides the apparent morphological difference, this preadaptation is characterized by changes of the gene expression pattern represented by upregulation of genes encoding procyclins, proteins associated with differentiation, the metalloendoprotease MSP-B, as well as many enzymes required for oxidative phosphorylation (Rico et al. 2013). The multiple changes of

the gene expression levels are reflected by the increase in concentrations of more than 500 proteins in stumpy forms (Gunasekera et al. 2012). The stumpy form contains a mitochondrion with partially active mitochondrial enzymes that allow rapid switch to oxidative phosphorylation using the amino acid proline because the tsetse midgut lacks a significant glucose source used in the mammalian blood. Stumpy forms are arrested in the G0/G1 phases of the cell cycle which is a prerequisite to undergo the transformation. The molecular association between cell-cycle arrest and subsequent differentiation includes a protein tyrosine phosphatase (TbPTP1). Transcript downregulation of this phosphatase by RNAi induces spontaneous differentiation of procyclic forms; thus this enzyme acts as an inhibitor of differentiation before entering the tsetse midgut (reviewed by MacGregor and Matthews 2010).

Differentiation into Procyclic Trypomastigotes Within the Midgut

Once ingested by the tsetse fly, the parasites are exposed to a fastidious environment, especially the proteolytic activity of digestive enzymes and immune effector molecules. Only a small proportion of initially ingested trypanosomes (<10 %) are able to differentiate and proliferate. Since this also occurs during in vitro cultivation, it is parasite specific rather than insect host mediated (Gibson and Bailey 2003). Slender blood trypomastigotes are lysed, but stumpy forms differentiate into procyclic trypomastigotes in response to cold shock and citrate and cis-aconitate. These metabolites in tsetse's midgut are detected by receptors called "protein associated with differentiation," a family of surface carboxylate transporters (Dean et al. 2009). Two isoforms are expressed in the flagellar pocket of stumpy forms, but not in slender forms. Upon temperature drop from 37 °C to approximately 20–27 °C, these proteins are redirected and trafficked to the cell surface (Engstler and Boshardt 2004; Dean et al. 2009). The temperature shift mediates the accumulation of receptors on the trypanosome cell surface resulting in enhanced sensitivity of the stumpy forms to citrate and cis-aconitate, which would otherwise not be stimulated by citrate and cis-aconitate at physiological concentrations (Engstler and Boshardt 2004). Thus, this mechanism controls trypanosome development by assuring (1) that stumpy forms do not differentiate before they reach the insect vector and (2) that only stumpy forms can detect the differentiation signal (MacGregor and Matthews 2010; Rico et al. 2013). The tyrosine phosphatase TbPTP1 is the key element controlling the signal cascade of this temperature-regulated mechanism (Szoor et al. 2006). In the bloodstream it inhibits the differentiation of stumpy forms, but is inactivated after exposure to citrate and cis-aconitate. Another phosphatase, TbPIP39, is acting downstream of TbPTP1 in this signaling cascade (reviewed by Rico et al. 2013). Other signals also trigger this differentiation (Fenn and Matthews 2007). Mild acid conditions induce it just as citrate and cis-aconitate via the TbPTP1/TbPIP39 phosphatase signal cascade, whereas protease treatment effectively triggers differentiation without activating this cascade (Szoor et al. 2013). However, it is unknown whether or not these stimuli contribute to the differentiation process in vivo.

Within 4–8 h stumpy forms replace the variant surface glycoprotein (VSG) coat by procyclins, completely losing the infectivity to mammals. Mitochondrial activity

coincides with the upregulation of the cytochrome-oxidase complex transcripts (Kabani et al. 2009). The transition is completed within 14–24 h, and the procyclic forms multiply in the endoperitrophic space, i.e., in lumen of the midgut enclosed by the PM (reviewed by Matthews 2015). The procyclins are GPI anchored and belong to a family of glycoproteins characterized by multiple amino acids repeats, glu-pro (EP) or gly-pro-glu-glu-thr (GPEET). EP procyclin has three isoforms (EP1, EP2, and EP3) varying in lengths of the internal repeats and differing in the presence or absence of sugars linked to the N-terminus (Roditi and Lehane 2008). The N-termini of these proteins are processed by tsetse midgut proteases once exposed on the trypanosome surface, but the surface coat is resistant to inactivation by proteolytic cleavage (Acosta-Serrano et al. 2001). In an early phase, all isoforms of EP as well as GPEET are expressed at similar levels, followed by an upregulation of GPEET synthesis (Vassella et al. 2001), a process being under control of mitochondrial activities (Fenn and Matthews 2007). About 24–72 h after ingestion, the procyclic trypomastigotes continue to proliferate exponentially by asexual division. At about 48–72 h, first procyclic forms are attached to the PM (Gibson and Bailey 2003), and at 3 days post-infection, the surface coat of these procyclic forms is mainly composed of GPEET. During this period of time, an attrition of trypanosomes starts in many flies, coinciding with the digestion of the majority of blood.

Other potential factors in the gut acting on the trypanosomes are an alkaline pH gradient among the midgut and protease activity. The alkaline pH does not favor the growth but seems to be tolerated in vivo, as different strains that multiply in different regions of the midgut (anterior/posterior) have similar midgut infection rates during coinfection (Peacock et al. 2007). Trypsin is one of the most abundant proteases in the tsetse midgut, but inhibition does not affect midgut establishment, and the trypsin levels of susceptible and refractory flies are similar (Welburn and Maudlin 1999).

Another challenge for procyclic trypanosomes are complement factors of the mammalian blood. The complement cascade effectively lyses procyclic trypanosomes, but not bloodstream trypomastigotes. Besides a potential evasion by crossing the PM, other possible mechanisms could include inactivation by complement through host-derived serine protease inhibitors or protection through acquisition of sialic acids from blood glycoproteins by procyclic-specific surface trans-sialidases. The latter theory is favored because trans-sialidase negative mutants are unable to colonize the midgut (reviewed by Dyer et al. 2013).

14.4.3.3 Interactions with Compounds of the Midgut, Especially Tsetse's Immune Defense

At about 3–5 days post-infection, trypanosomes are eliminated in the majority of the tsetse flies (Gibson and Bailey 2003), and in approximately 90 % of flies which fed on infected blood, the trypanosomes fail to mature (Aksoy et al. 2003). Thus, the early midgut processes represent a first bottleneck for trypanosomes' establishment. It is unlikely that this phenomenon is caused solely by the inability to transform as the attrition also occurs when flies are fed with proliferative procyclic forms (reviewed by Oberle et al. 2010). This refractoriness is consistent with the low

prevalence observed in field populations (Haines 2013) and is most likely mediated through the integrity of the PM and compounds/immune effectors of the midgut.

Tsetse's PM acts as a biophysical barrier that protects the midgut epithelia from blood components, digestive enzymes, and microorganisms. In addition, it regulates the host immune response to foreign pathogens (Weiss et al. 2014). After eclosion, the PM is synthesized progressively and fully lines the midgut approximately 3–4 days later (Lehane and Msangi 1991). The analysis of PM´s proteome of teneral flies reveals >200 proteins, including proteins involved in stabilizing PM´s integrity, but also proteins relevant in immune responses, e.g. C-type lectins or CD36, a potential receptor for molecular patterns of pathogens (Rose et al. 2014). The importance of a robust wild-type PM is highlighted by RNAi-manipulated tsetse flies harboring a structurally impaired PM. These flies restrict bacterial growth by the production of antimicrobial peptides (AMPs), but do not increase attacin gene expression in response to trypanosomes. These flies are highly susceptible to midgut infections (Weiss et al. 2014). The effects of the PM and the immune system are elucidated in comparisons of refractory and susceptible fly populations also including teneral and starved flies. Less than 24 h post eclosion, teneral flies are twice as susceptible to trypanosome infection as flies aged 48 h, correlating with the increasing length and robustness of the PM (Walshe et al. 2011).

A strong indication for the suggested effect of the immune system is proteome analyses. Identifying >500 proteins in the midgut 3 days after ingestion of *T. b. brucei* with the blood meal, >10 proteins are differentially synthesized, either up- or downregulated, in infected flies. These proteins have putative functions including metabolic processes (e.g., antioxidants, detoxification) and agglutination but also immune modulation (Geiger et al. 2015). The innate immune system of insects contains different types of receptors, including peptidoglycan recognition proteins (PGRPs) that recognize molecular patterns of pathogens. Peptidoglycan recognition protein (PGRP-LB) is synthesized in the mycetome and the milk glands and maternally transferred to the progeny. PGRP-LB synthesis is induced in adult flies after the first ingestion of blood. Recombinant PGRP-LB has trypanocidal effect on bloodstream and procyclic forms (Wang and Aksoy 2012). Knockdown of expression by RNAi increases midgut infection prevalence (Wang et al. 2009). In turn, upregulation correlates with refractoriness to trypanosomes. Since teneral flies are unable to synthesize PGRP-LB, this might contribute to the higher susceptibility to trypanosome infections (Wang and Aksoy 2012; Haines 2013). Refractory fly populations also show higher expression levels of PGRP compared to teneral flies (Weiss et al. 2013). This correlation applies even within the same population, with significantly higher PGRP-LB levels in self-cured flies compared to those with established trypanosome infection. PGRP-LB and AMPs seem to act synergistically, with PGRP-LB levels being the main determinant, enforced by the secondary activity of AMPs (Wang et al. 2009).

The receptor-mediated pathogen detection activates the two main signaling cascades, Toll and immunodeficiency pathway (Imd) (Müller et al. 2008) resulting in production of effector molecules by the immunocompetent tissues midgut, proventriculus, and fat body (Lehane et al. 2004). Tsetse's immune system is able to

differentiate between different trypanosome life-cycle stages (Wang et al. 2009) and relies on more than one effector to prevent trypanosome establishment. Besides the protection provided by the PM, the immune factors that contribute to resistance to trypanosomes are ROS (see Sect. 14.3.4), tsetse EP protein, and AMPs. Lectins might also be potential effectors by inducing apoptosis (Welburn and Maudlin 1999).

The tsetse EP protein contains multiple dipeptide repeats of glutamic acid (E) and proline (P) and is localized in the midgut lumen and PM, hemolymph, and salivary glands, but not in the proventriculus (Chandra et al. 2004). It is a highly conserved protein, specifically synthesized in the genus *Glossina* (Haines et al. 2005, 2010). The expression is upregulated after trypanosome infections (Haines 2013) and refractory fly populations show higher expression levels than teneral and susceptible flies (Weiss et al. 2013). In turn, RNAi knockdown significantly increases trypanosome establishment in the midgut. Starvation also reduces the amount of tsetse EP and increases the susceptibility to midgut trypanosome infection (Haines et al. 2010; reviewed by Dyer et al. 2013). The exact mode how tsetse EP interferes with trypanosome development is unknown, but besides a potential agglutination of pathogens, an indirect role by strengthening the PM structure is suggested (Rose et al. 2014).

AMPs are effector molecules of the immune response (Müller et al. 2008), including attacin, cecropin, defensin, and diptericin. The bloodstream trypomastigotes do not induce a strong response in the first days, but afterward molecular detection of trypanosomes induces synthesis of specifically Imd-mediated AMPs (Hao et al. 2001, 2003). About 3 days post-infection, *G. m. morsitans* have attacin and defensin transcripts in the fat body and proventriculus, and 6 days post-infection, attacin, defensin, and cecropin are detectable in the hemolymph (Hao et al. 2001, 2003; Boulanger et al. 2002). This time point coincides with the attrition of trypanosomes from the endoperitrophic space and the appearance of procyclic forms in the ectoperitrophic space (Gibson and Bailey 2003), indicating that only procyclic trypanosomes are potent stimulators of the immune system. The molecular communication between the midgut epithelia and the immunocompetent tissues such as the fat body presumably involves nitric oxide (Hao et al. 2003). Afterward the level of AMP expression continues in flies with an established infection, but is downregulated in flies that successfully eliminated trypanosomes (Hao et al. 2001). Recombinant attacin kills procyclic forms in vitro, and in attacin knockouts susceptibility to trypanosome infection is increased (Hu and Aksoy 2006). In addition, feeding either bacteria or attacin in preceding blood meals significantly reduces the prevalence of midgut parasite infections, indicating the importance of an activated immune system for trypanosome clearance (Hao et al. 2001).

Species-specific differences of the regulation and kinetics of humoral defense molecules may be an important determinant of vector competence (Haddow et al. 2005). In contrast to the kinetics of AMP expression of *G. m. morsitans* upon trypanosome contact, *G. pallidipes*, that are less susceptible to *T. brucei* midgut infection, constitutively express attacin in the fat body and proventriculus and thus might have higher levels of early defense molecules (reviewed by Dyer et al. 2013).

However, AMPs cannot be the only determining factor as trypanosomes that survive initial host responses proliferate in the presence of host AMP (Hao et al. 2001).

Considering the orchestra of immune effectors that act together in order to clear parasitism, trypanosomes might have evolved strategies to bypass immune effectors. The switch of the surface coat (GPEET -> EP) of late procyclic forms may favor migration (Vassella et al. 2009) and/or parasite resistance to immune effectors secreted by the host gut epithelia (Weiss et al. 2014). In addition, procyclic forms secrete a variety of proteins, mainly proteases, through an exosome pathway. These species-specific proteins might play an important role in parasite-vector interactions, including immune evasion, but functional analysis remains to be done (Atyame Nten et al. 2010).

14.4.3.4 Migration to and Colonization of the Ectoperitrophic Space and Proventriculus

Development in the Ectoperitrophic Space and Proventriculus

Procyclic trypanosomes penetrate the PM 3–5 days post-infection and are detectable in the ectoperitrophic space at day 6 (Gibson and Bailey 2003). The penetration occurs at the anterior midgut presumably mechanically by disrupture of the PM, but the supporting use of proteases for degradation of the PM layer is possible (Dyer et al. 2013). In the ectoperitrophic space they continue to divide and migrate anteriorly toward the proventriculus, usually without invading the hemolymph. These procyclic trypomastigotes are significantly longer and thinner than in the midgut lumen (Sharma et al. 2008). In late procyclic forms, GPEET expression is downregulated (Urwyler et al. 2005), and the GPEET coat is replaced by EP1 and EP3 procyclins on the trypanosome surface (Vassella et al. 2001).

Six to 8 days after the blood meal, procyclic forms reach the proventriculus (Lehane et al. 2004). During migration they grow up to 40–60 μm length (reviewed by Sharma et al. 2009). They colonize the anterior midgut and proventriculus massively around days 9–14 (Gibson and Bailey 2003). These long forms become G2 arrested and transform into mesocyclic trypomastigotes (Sharma et al. 2009). At the proventriculus, trypomastigotes further transform into epimastigote by asymmetrical division producing one long and one short daughter epimastigote (Van Den Abbeele et al. 1999; Oberle et al. 2010). Both epimastigotes forms are nonproliferative in the proventriculus (Sharma et al. 2009). Trypanosomes penetrate the PM at the proventriculus and enter the gut lumen, and a mixture of morphotypes is found in the subsequent passage through the foregut (Van Den Abbeele et al. 1999).

Parasite-Vector Interactions During Migration to the Proventriculus

The signals and mechanisms that guide procyclic forms to cross the PM 3–5 days post-infection and to show directed migration to the anterior midgut are unknown. Among potential factors that might mediate migration are nitric oxide or L-cysteine (MacLeod et al. 2007). A behavior called social motility, the coordinated group migration of procyclic forms when cultured on semisolid surface, could explain the migration (Oberholzer et al. 2010). This social motility only occurs in early

procyclic forms (GPEET surface) and not in late procyclic forms (EP surface) (Imhof and Roditi 2015). An in vivo relevance is indicated by null mutants for *Rft1* (endoplasmatic reticular protein), in which social motility in vitro and midgut infection establishment are lower than in the wild type. This effect is observed 14 days but not 3 days post-infection (Imhof and Roditi 2015).

An active flagella-mediated motility to reach the foregut is indicated by deletion of the outer dynein arm component DNAI1. The mutants are viable and able to colonize the midgut, but are unable to reach the foregut or the salivary glands (Rotureau et al. 2013). In addition, the transmembrane protein PSSA-2 that is found in minor amounts on the surface coat of all insect stages, but not in bloodstream or metacyclic trypomastigotes, could be involved in either signal perception or migration. PSSA-2 null mutants establish midgut infections just as the wild type but show reduced salivary glands infection rates and a less efficient gland colonization (Fragoso et al. 2009). The differential regulation of cytoplasmatic proteins belonging to the ALBA family might be involved in the transformation of mesocyclic forms to epimastigotes through interaction with RNA-binding proteins. Knockdown of ALBA3/4 by RNAi in procyclic forms induces some of the changes that are usually observed during the differentiation process found in the proventriculus (Subota et al. 2011).

In the ectoperitrophic space, trypanosomes first multiply, but then population size remains constant indicating a density-dependent effect (Van den Abbeele et al. 1999; Oberle et al. 2010). Since trypanosomes in the mammalian blood sense parasite-intrinsic signals in response to the cell density (Rico et al. 2013), similar mechanisms might restrict the population size within the vector. There is a further reduction of trypanosomes at the proventriculus (Haines 2013). The induction of immune responses is likely as trypanosome proliferate in close proximity to the midgut epithelia. The epithelium secretes ROS that may eliminate trypanosomes and feeding antioxidants significantly increases trypanosome midgut survival and infection rates of salivary glands (MacLeod et al. 2007). In contrast, PM-compromised flies are highly susceptible to parasite infection, despite the ROS production. Therefore, ROS cannot be the sole determinant constituting trypanosome elimination after crossing the PM. The proventriculus is immunocompetent, but trypanosomes do not induce an AMP response at this stage (Hao et al. 2003). Therefore, a role of cellular immune responses by hemocytes in the proventriculus is discussed (Gibson and Bailey 2003; Hao et al. 2003).

14.4.3.5 Migration to and Colonization of Salivary Glands

Early Invasion/Colonization and Formation of Metacyclic Forms

The epimastigotes migrate via the esophagus and the mouthparts to the ducts of the salivary glands. About 14–21 days post-infection, epimastigotes are in the glands (Rotureau et al. 2012). In fact, only a few cells accomplish this way (Peacock et al. 2007; Oberle et al. 2010). In the glands, the long epimastigotes seem to die, and the short epimastigotes attach to the microvilli of the epithelium via the flagellum (Sharma et al. 2009). At the attachment zone, the flagellum enlarges and forms

junctional complexes (Vickerman 1985). In epimastigotes of *T. brucei*, EP procyclin mRNA is expressed but translation is suppressed. The new surface coat contains the brucei alanine-rich protein (BARP) that belongs to a family of glycosylphosphatidyl inositol-anchored proteins (reviewed by Fenn and Matthews 2007).

The attached epimastigotes undergo two different modes of development (Rotureau et al. 2012). In the first mode, they divide symmetrically in an early phase to colonize the glands. They multiply until the epithelium is overlayed with tightly packed epimastigotes (Oberle et al. 2010). The second mode, asymmetric divisions, produces two epimastigotes, but one daughter cell transforms into the infective metacyclic trypomastigote. In the premetacyclic epimastigote, levels of calflagin in the flagellum are increased. These two cell cycles continuously produce infectious parasites throughout the life span of the tsetse fly (Rotureau et al. 2012).

The premetacyclic epimastigotes undergo division arrest, build a new VSG coat that replaces the BARP coat, detach from the epithelia, and are the infective metacyclic forms that can complete the lifecycle once injected into a new mammalian host in the saliva. The first metacyclic trypomastigotes of *T. brucei* appear 18 days post-infection (Rotureau et al. 2012). The reacquisition of the VSG coat correlates with the infectivity for the mammalian host. In contrast to the VSG coats in the mammals, in the salivary glands only a low number of variable antigen types are synthesized. Sexual recombination between different trypanosome cells can occur within the insect vector, but it is not essential for metacyclogenesis. After infection of tsetse flies with two different isolates of *T. brucei* expressing different colored fluorescent proteins, synthesis of both fluorescent proteins occurs in some epimastigotes in the salivary glands, but not in the preceding development stages (Gibson et al. 2008). Hybrid metacyclic trypomastigotes develop. It is unknown how frequent sexual recombinations occur in vivo, but this contributes to the genetic diversity among trypanosomes as coinfections are not uncommon in the field. By coinfections in tsetse flies, serum resistance and thereby human pathogenicity are transferred from a *T. b. rhodesiense* strain to an animal pathogenic *T. b. brucei* strain (Gibson et al. 2015).

Parasite-Vector Interactions in the Salivary Glands

At present nothing is known about the underlying molecular mechanisms that mediate the differentiation to epimastigote forms, migration to the salivary glands, or the final differentiation step that results in metacyclic forms. It has been suggested that the BARP surface coat of epimastigotes could mediate tissue tropism, but its function remains unknown (Roditi and Lehane 2008). In addition, genes of several putative GPI-anchored surface proteins are upregulated in epimastigote forms in the salivary glands, implying a potential role in host-parasite interactions or preadaptation for transmission to the mammalian host, but functional analysis remains to be conducted (Savage et al. 2012). Some potential triggers for the switch between the two division cycles of epimastigotes have been suggested, e.g., cell-to-cell signaling or measurement of population density, but the underlying molecular mechanism is unknown (Rotureau et al. 2012).

In fact, the passage from the proventriculus to the salivary glands appears to be the most significant bottleneck as the trypanosome population is significantly reduced at this stage (Oberle et al. 2010; Rotureau and Van Den Abbeele 2013). Fitness and species- and sex-specific differences of tsetse flies affect the ability of trypanosomes to colonize the salivary glands. Starvation of adult flies for >3 days increases the rate of salivary gland infections. Since starvation deprives fat body reserves and reduces AMP production such as attacin, a potential immunosuppressive effect is possible in these stressed flies (reviewed by Haines 2013). In most *G. m. morsitans* flies with established midgut infection, trypanosomes reach the lumen of the foregut, but only a small proportion reach the salivary glands (Peacock et al. 2012a). *G. pallidipes* is much more effective to clear trypanosomes in the midgut, but is unable to restrict trypanosome once they cross the PM and move anteriorly. In contrast, *G. m. morsitans* continues to eliminate trypanosomes in subsequent tissues (proventriculus, passage to salivary glands) causing a low transmission index, presumably by immune effectors (Peacock et al. 2012b). *G. m. morsitans* sex has no impact on midgut infections, but males show significantly higher mature salivary gland infections (Peacock et al. 2012b).

The tsetse sialome is constituted by 250–300 proteins of which at least 20 are secreted into the gland lumen (Kariithi et al. 2011; Telleria et al. 2014). These components are enormously modulated by the parasite, including upregulation and downregulation of genes encoding essential salivary gland proteins. This modulation could include mechanisms to facilitate and maintain salivary glands colonization (Van Den Abbeele et al. 2010; Kariithi et al. 2011). Seventy-five percent of genes which are specifically associated with salivary gland function are upregulated by trypanosome infection. The encoded proteins are related to immune processes, stress tolerance, and proliferation, indicating tissue renewal in response to trypanosomes (Telleria et al. 2014).

Those proteins which are significantly downregulated in response to salivary gland infections include the anti-hemostatic saliva proteins. Reduction of anti-hemostatic potency seems to impact the feeding performance, increasing probing attempts and host contacts and thus facilitating the transmission to new mammalian hosts (Van Den Abbeele et al. 2010; Telleria et al. 2014). Once deposited into the bite site, metacyclic trypanosomes have to face innate immune reactions before an adaptive immune response raises parasite-specific antibodies. A modulation of saliva components in order to downregulate IL-6, IL-12, and tumor necrosis factor expression could facilitate immunosuppression. Thus, besides parasite-specific mechanisms to evade early trypanolytic factors, the modification of tsetse fly saliva composition could be beneficial for trypanosome survival in the mammalian host.

14.4.3.6 Interactions of Microbiota, Tsetse Flies, and Trypanosomes

Tsetse flies biology is strongly interwoven with the obligate endosymbiont *Wigglesworthia*. This endosymbiont is localized both intracellularly (bacteriome) and extracellularly (milk glands) and the dietary supplementation supports appropriate PM development, larval development, and maturation of the immune system. *Wigglesworthia* provides vitamin B metabolites that the fly cannot synthesize, e.g.,

riboflavin, thiamine, and pyridoxine (Wang et al. 2013a). These vitamin B metabolites are essential for proline homeostasis, and proline is the only source for ATP synthesis of both, tsetse fly and trypanosomes during their development in the insect vector (Aksoy et al. 2014). The nutrimental benefit is also linked to fecundity, as female tsetse flies free of *Wigglesworthia* are sterile. Such adults seem to exhibit structurally compromised PM that significantly increases trypanosome prevalence. The absence of *Wigglesworthia* during larval development causes severe deficiencies of the cellular immune system. *Wigglesworthia*-free tsetse flies exhibit significantly reduced hemocyte populations (30 % compared to the wild type) and AMP expression including attacin and cecropin (Weiss et al. 2011).

Wigglesworthia might modulate PGRP-LB expression to favor its own survival. PGRP-LB has putative amidase activity capable to degrade peptidoglycan fragments which are released as by-products during growth and division of the endosymbiont. These components would otherwise activate humoral immune responses through the Imd pathway as demonstrated by knockdown of PGRP-LB resulting in AMP production that significantly reduces the *Wigglesworthia* population (Wang et al. 2009). As mentioned before, recombinant PGRP-LB has trypanocidal activity (Wang and Aksoy 2012). Thus, by upregulation of PGRP-LB for immune evasion, it may affect tsetse fly refractoriness positively by stimulating tsetse's early immune response (Aksoy et al. 2014).

Another endosymbiont of tsetse flies is the Gram-negative bacterium *Sodalis glossinidius* (Dale and Maudlin 1999). In contrast to *Wigglesworthia*, the function of *Sodalis* and the interaction with tsetse are poorly understood (Wang et al. 2013a). Like *Wigglesworthia*, it is transmitted by maternal milk gland secretions to the progeny during larval development. It is not found in all natural tsetse fly populations and is also transmitted from male to female tsetse flies during copulation (De Vooght et al. 2015). A number of *Sodalis* proteins are found associated with tsetse PM, indicating that *Sodalis* either grows in association with the PM or secretes proteins which are translocated to the PM (Rose et al. 2014). A third bacterium, *Wolbachia*, resides mainly in the reproductive tissues, causing reproductive deficiencies, including cytoplasmic incompatibility (Alam et al. 2011). Aposymbiotic tsetse flies lacking both symbionts and *Wolbachia* show a more severe immunodeficiency compared to the wild type, indicating that either *Sodalis* or *Wolbachia* or both may have a minor role in the maturation of the immune system (Weiss et al. 2012). This effect can be restored with blood supplemented with *Wigglesworthia* cell extracts, indicating that either symbiont factors or their recognition seems to be essential for immune system maturation during larval development (Weiss et al. 2012). In addition to its potential minor role for immune maturation, *Sodalis* seems to modulate the vector competence. Several mechanisms have been suggested, including an inhibition of the trypanocidal activity of midgut lectins (Welburn et al. 1993), and a modulation of the PM structure by cleaving chitin fibrils which would facilitate PM penetration by trypanosome. The latter theory is supported by a putative chitinase of *Sodalis* which is found in association with the teneral PM (Rose et al. 2014). Selective clearance of *Sodalis* using antibiotics increases the resistance to trypanosomes (Dale and Welburn 2001). In addition it shows a high degree of

genetic diversity and the presence of some genotypes of *Sodalis* correlates with the ability of tsetse flies to establish transmissible infections (Geiger et al. 2007).

Besides their interaction with the host, the microorganisms of the tsetse's microbiome can also interact with each other to gain benefits, but studies on their interactions have only recently begun (Synder and Rio 2013). In addition to these two endosymbionts, tsetse flies also harbor other gut-associated bacteria, mainly Gram-negative, covering a broad spectrum that was found among different geographical regions. However, it is unknown whether or not these midgut microorganisms affect the development of trypanosomes in the tsetse (Geiger et al. 2013).

14.4.4 Perspectives and Open Questions

The accessibility of tsetse's full genome sequence together with new tools enabling to manipulate strains genetically has provided enormous insights into the trypanosome development cycle in tsetse. This includes increasing knowledge of cellular processes which are required to allow rapid morphological changes as well as their regulation. Trypanosomes encode a battery of potential RNA-regulating factors. Their functional analysis as well as the signaling cascades that underpin these mechanisms will improve the understanding of molecular interactions between trypanosomes and tsetse. According to proteome analyses, several host proteins are differentially regulated after infection with these trypanosomes. The further functional characterization could reveal potential targets determining the vector competence of tsetse flies and thus improving control strategies of African trypanosomiasis (Geiger et al. 2015).

A major breakthrough in the investigations of development of trypanosomes offers the overexpression of the RNA-binding protein TbRBP6 in vitro. Such manipulations of a single protein will help to elucidate the underlying mechanism of formation of metacyclic trypomastigotes (reviewed by Matthews 2015). However, nothing is known about the mechanism to measure and maintain the density of procyclic forms at the proventriculus and to achieve a specific level of infection there. Also signals that guide the different development stages to finally reach the PM and salivary glands are unknown. In addition, the signals triggering the differentiation into epimastigotes and subsequently metacyclic trypomastigotes remain to be investigated. The same applies for those factors that induce epimastigote attachment and control the switch between the different modes of proliferation that assure both permanent colonization of salivary glands and the presence of infectious forms. Another open question is whether these differentiation signals are parasite derived or provided by the host and how these signals are detected by trypanosomes.

Interactions with the microbiota are another open field. As indicated by the interactions of *Trypanosoma cruzi* and triatomines, not only symbionts but also other bacteria are engaged. The transfer of *Sodalis* from male to female flies during mating and subsequently to the offspring indicates the possibility to use genetically modified symbionts that interfere with pathogen development in the insect host and thus offering another promising tool for disease control (Sassera et al. 2013; De Vooght et al. 2015).

14.5 Interactions of *Trypanosoma cruzi* and Triatominae

14.5.1 *Trypanosoma cruzi*

14.5.1.1 General Aspects

Trypanosoma cruzi is the etiologic agent of Chagas disease, a neglected disease, mainly relevant in Latin America. However, infected immigrants are increasingly introducing it to the United States and Europe. It is mainly connected to rural poverty. Insecticide spraying campaigns against the vectors colonizing the houses have strongly reduced the prevalence of the disease within the last 25 years from about 30 million infected people in 1990 to recent estimations of about 6–7 million (WHO 2008, 2015b).

All strains of *T. cruzi* only infect mammals. The populations of these flagellates possess predominantly a clonal genetic structure. Most recently the statement that only restricted genetic recombinations occur is again discussed controversially (Messenger and Miles 2015). The recent classification based on multilocus sequence typing classifies six groups, and these discrete typing units are named TcI–VI (Zingales et al. 2012). The geographic distribution of these units in Latin America differs, but in Bolivia all discrete typing units occur and in Brazil nearly all. Strains of all discrete typing units differ in biological characteristics, e.g., virulence and pathogenicity for laboratory mammals, multiplication rate during in vitro cultivation, or metacyclogenesis rate in the vector (reviewed by Schaub et al. 2011). Some discrete typing units are associated with infections in humans and vectors adapted to colonize houses, sylvatic vectors, and humans and sylvatic vectors and wild mammals. Under natural conditions the populations of *T. cruzi* in mammalian hosts and vectors are often a mixture of clones. After experimental infections with strains belonging to different typing units, the number of flagellates can be higher than in single infections and the percentages of the clones change in the course of time (reviewed by Schaub et al. 2011). In mixed in vitro cultures, the proteomic analysis reveals an interaction of two stocks of parasites a few minutes after performing the mixture. In mixed cultures, about 60 % of the proteins are lower concentrated than in cultures of pure stocks and about 30 % higher concentrated (Machin et al. 2014).

In the mammalian host and the vector, different stages develop. In mammals, intracellularly multiplying amastigotes develop to nonreplicative blood trypomastigotes possessing a slender, stumpy, or intermediate morphology. In the gut of the vector, they transform to spheromastigotes and epimastigotes and multiply and develop to nonreplicative metacyclic trypomastigotes. Their kinetoplast remains in a more subterminal position than in blood trypomastigotes (Fig. 14.1). Epimastigotes and metacyclic trypomastigotes are well adapted to the use of histidine as an energy source (Atwood et al. 2005).

14.5.1.2 Use of In Vivo-Derived Versus in Vitro-Derived Parasites

Blood Trypomastigotes

Investigations of blood trypomastigotes are impeded by the restrictions to use laboratory animals and the number of parasites developing in them. From mice as

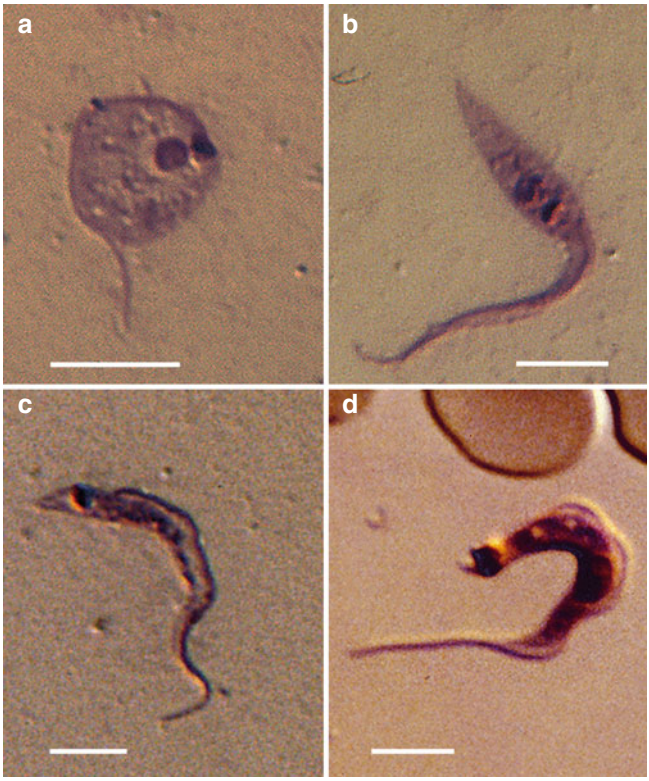

Fig. 14.1 Different developmental stages of *Trypanosoma cruzi*: spheromastigote (**a**), epimastigote (**b**), metacyclic trypomastigote (**c**), blood trypomastigote (**d**); Giemsa stained (scale bar: 5 μm) (Schaub et al. 2011)

mainly used laboratory animal, about 2 ml blood can be obtained, from rats about 10 ml. In rats, many strains of *T. cruzi* develop only low and transient parasitemias. This can be increased by a treatment with immunosuppressive drugs, but the variation within a group of rats is high. The best possibility offer is athymic nude rats in which a low-virulent strain of *T. cruzi* develops to an average of 1.5×10^8 blood trypomastigotes/ml blood, i.e., about 1.5×10^9 blood trypomastigotes are obtained from one rat (Schaub et al. 2001). Using a very careful separation from blood cells by differential centrifugation, only thrombocytes "contaminate" the flagellates. To eliminate contaminations, trypomastigotes are separated from epimastigotes by chromatography using different substrates, e.g., DEAE-Sephacel or octacosane-coated beads (Kleffmann et al. 1998; Hölscher et al. 2003). However, after ion-exchange chromatography, only an elution into a buffer supplemented with proteins avoids changes on the surface of the trypomastigotes, and centrifugation forces ≤400 g avoid a shedding of surface proteins (Hölscher et al. 2003).

In vitro cocultivation with mammalian cells offers the possibility to obtain cell culture-derived trypomastigotes which possess only slightly different characteristics compared to blood trypomastigotes. As in the mammalian host, the parasites enter the host cell, transform to the amastigote stage, multiply, transform to trypomastigotes, destroy the host cell membrane, and are present in the medium about 4–5 days after infection. These cell culture-derived trypomastigotes are not present as pure culture. The medium also contains transition stages of *T. cruzi* and remnants of the host cells.

Epimastigotes

The insect stage of *T. cruzi* can hardly be obtained from the vector. To avoid contaminations by remains of digestion, the population attached to the rectal cuticle could be isolated after the urine has flushed out the rectal content. Thereby, low numbers of epimastigotes can be obtained. However, these samples also contain blood trypomastigotes and intermediate stages (Schaub and Lösch 1988).

Epimastigotes can much easier be investigated using large numbers from cell free in vitro cultures. Fortunately, this parasite grows rapidly in many culture media, especially after supplementation with fetal calf sera. However, special conditions are required to exclude the development of metacyclic trypomastigotes, which develop in aging cultures (see section "Blood trypomastigotes"). Pure epimastigotes can be obtained using intervals of 3–4 days to passage the parasites to fresh medium. In addition, a shaking of culture flasks reduces attachment rates and thereby metacyclogenesis rates (Kleffmann et al. 1998; Goldenberg and Ávila 2011). However, the different multiplication rates of clones/strains require a careful control. To determine the schedule for the respective strain, aliquots should be used in complement-induced lysis assays, in which only trypomastigotes survive.

In harvesting of epimastigotes from in vitro cultures, one pitfall seems to exist, the effects of protein-free-washing salines. Washing and an incubation for 30 min in protein-free medium induce stress by serum deprivation. The release of apoptotic vesicles by the flagellate into the medium might have increased the number of proteins present in a proteomic analysis of cell surface proteins (reviewed by Queiroz et al. 2013). An incubation of epimastigotes and metacyclic trypomastigotes for 6 h in protein-free medium induces the formation of plasma membrane-derived vesicles (Bayer-Santos et al. 2013). The effects of centrifugation forces are not considered and often the respective data are not included. Therefore, many investigations do not analyze the surface coat, but the exoproteome, a term used for proteins found in the extracellular proximity of cells (Armengaud et al. 2012). Before a detailed investigation of epimastigotes, we recommend a centrifugation of $\leq$400 g, which excludes effects on the surface coat of blood trypomastigotes (see section "Blood trypomastigotes").

Metacyclic Trypomastigotes

It is very difficult to obtain high numbers of metacyclic trypomastigotes from the vector. After feeding of the bugs, the urine contains mainly metacyclic

trypomastigotes, but only relatively low numbers (Schaub and Lösch 1988). In addition, pure samples can only be obtained using isolation procedures, which might affect the surface coat (see below).

In vitro cultivation offers better possibilities. Using cell free in vitro cultures, many factors increase metacyclogenesis rates, e.g., reduction of nutrients in old cultures, changes of pH, osmolality, components of hemolymph and gut, metabolic stress, and cAMP. The development of a standardized procedure for induction of metacyclogenesis has enabled detailed investigations of this process (Contreras et al. 1985). The highest yields of trypomastigotes are obtained after incubation of late exponential growth phase epimastigotes for 2 h in a stress medium containing no proteins, amino acids, or sugars, followed by an incubation in the same medium supplemented with proline or glutamate, aspartate, and glucose. Avoiding a shaking of the culture flasks supports the attachment and thereby the transformation. Within 6 days, 95 % of the flagellates are metacyclic trypomastigotes. However, only the development from epimastigotes occurs, in which the kinetoplast migrates to the posterior end of the cell. This is also the main mode in the vector, but there metacyclic trypomastigotes also originate from other stages: ring-like forms, giant cells, spheromastigotes, and epimastigotes. In unequal divisions, the daughter cells are a trypomastigote and an epimastigote (Kollien and Schaub 2000).

The standardized procedure for induction of metacyclogenesis is strongly adapted to one clone of *T. cruzi*, Dm28c. Other clones/strains often develop only lower percentages of trypomastigotes. Thirty years after the publication of the procedure, an enormous amount of data has been collected especially by the group of Goldenberg (reviewed by Goldenberg and Ávila 2011). Between 6 and 12 h after transfer into the supplemented medium resistance to complement-mediated lysis increases from 0.5 to 60 % of the flagellates, indicating the establishment of the surface coat of trypomastigotes. During this period of time, in light microscopy no morphological changes are evident. Comparing the expression of genes in flagellates collected at different time points after induction of metacyclogenesis, stage-specific genes are expressed before morphological changes are visible (reviewed by Ávila et al. 2003). However, differences in the expression level do not correlate to differences in the concentrations of the respective protein, e.g., in the major cysteine proteinase cruzipain (Tomás et al. 1997). Since not all epimastigotes transform, trypomastigotes have to be isolated. This can be done by a complement-induced lysis of epimastigotes and a spinning down of the trypomastigotes.

14.5.2 Triatomines

Of the >140 species of Triatominae the majority lives in Latin America and mainly in sylvatic areas. Only some species are strongly adapted to houses, especially *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongylus megistus*, and *T. dimidiata* (Fig. 14.2) (reviewed by Schaub et al. 2011; Stadler et al. 2011). These biggest

Fig. 14.2 Adults of *Triatoma infestans* (**a**), *Panstrongylus megistus* (**b**), *Rhodnius prolixus* (**c**), and *Triatoma dimidiata* (**d**) (scale bar: 1 cm) (Schaub et al. 2011)

blood-sucking insects are night active and hemimetabolous, i.e., all postembryonic stages suck blood from all warm-blooded animals. Some pre-adult instars can starve for up to about 1 year. Each preadult instar requires one full engorgement of about 6–12 times their own body weight or several smaller volumes of blood for the development to the next of the five pre-adult instars. They store the blood in the

distensible midgut region, the stomach, and concentrate it. During the first 4 h after feeding, >50 % of the total weight of ingested blood is excreted. In the posterior region of the midgut, the small intestine, the pH is slightly acidic and the blood is mainly digested by cysteine and/or aspartic proteases (Balczun et al. 2012). In contrast to Diptera, the midgut cells are not covered by a PM, but all midgut cells secrete perimicrovillar membranes into the gut lumen. This synthesis is strongly reduced during starvation of bugs and increases after feeding (summarized by Gutiérrez-Cabrera et al. 2014).

Feeding affects the milieu in the rectum dramatically. Remains of digestion are replaced by clear colorless urine (mainly a sodium chloride solution). For several days, yellow white urate spheres from the Malpighian tubules are present in the rectum. Then dark-brown remains of digestion appear. Within 1 h after feeding, the pH increases from pH 6 to pH 8.5, returning to the old level within an additional day. Osmolality triples after appearance of urate spheres, and concentrations of anions and cations change strongly (Kollien et al. 2001). Concentrations of free amino acids in the urine are about 60 % lower than in first dark drop of feces and those of protein/peptide-bound amino acids 90 % lower (AH Kollien 2016, unpublished). In the feces, free taurine predominates, followed by proline and histidine. In the urine, proline is the dominant free amino acid.

14.5.3 Development of *Trypanosoma cruzi* in Triatomines

14.5.3.1 General Aspects

The development of the parasite in the vector depends on the strain/clone of the parasite and the species/strain of the vector. Mainly in two species, *Rhodnius prolixus* and *Triatoma infestans*, parasite-vector interactions have been investigated. Generally, after ingestion of infectious blood, the blood trypomastigotes transform in the midgut to spheromastigotes and epimastigotes, especially the latter multiplying intensively (Schaub et al. 2011). After passage to the rectum within <1 week, they also multiply there. In the most detailed investigated parasite-vector system from Chile, an initial uptake of 8000–10,000 blood trypomastigotes/second instar of *T. infestans* results in a threefold increase within 1 week and in about 240,000 parasites 3 weeks later (Schaub 1989a). After three feedings in monthly intervals and three molts, the final preadult stage contains up to two and three million parasites in the midgut and the rectum, respectively. In the rectum, about two-thirds of the population are attached to the rectal cuticle. During long periods of starvation, sometimes the whole population of flagellates is passed to the rectum, but in other bugs that die of starvation, all parts of the intestine remain to be colonized at a low level.

Only the development of four stages in the vector can be correlated to specific conditions. During starvation periods of the bugs up to 3 months, the percentages of drop-like intermediate stages between sphero- and epi- or trypomastigotes and those of spheromastigotes in the population of the rectum are increased tenfold (Kollien and Schaub 2000). Feeding such long-term starved bugs results in the rapid growth of epimastigotes to giant cells, a multiple division stage containing many

sets of organelles. Three days after feeding, this stage represents 30–50 % of the total population but then disappears. After feeding of short-term starved bugs, no giant cells develop, but metacyclogenesis is induced in epimastigotes, not in other precursor stages. Whereas the compounds that induce the development of round stages and giant cells are unknown, this way of metacyclogenesis is induced by hemolymph proteins of about 17 kDa that pass into the urine at the beginning of urine secretion (Kollien and Schaub 2000; T Kleffmann 2016, unpublished).

14.5.3.2 The Surface Coat of Epimastigotes and Metacyclic Trypomastigotes

Since the posttranscriptional regulation of gene expression occurs at the level of primary transcript processing by trans-splicing and polyadenylation, and the stability of mRNA and/or the translation product, only proteomics offer the methodology to identify relevant proteins (see Chap. 6). Proteome analyses have created an enormous bulk of information on proteins present in *T. cruzi*. Including the major life-cycle stages, many proteins have been identified, the concentrations of some of them differing between the stages (Atwood et al. 2005; de Godoy et al. 2012; Brunoro et al. 2015). Identifying 5900 proteins of blood trypomastigotes, 2200 are not present in metacyclic trypomastigotes (Brunoro et al. 2015). Of these proteins, in the interaction with the vector, mainly surface coat components are relevant.

The components of the surface coat are typically anchored to the plasma membrane by glycosylphosphatidylinositol. The sequences of the glycoproteins are extensively polymorph (Buscaglia et al. 2006). They are synthesized in all developmental stages and encoded by thousands of members of multigene families. About 80 % of these proteins are mucin-associated proteins, mucins, and trans-sialidase/gp85 proteins (Nakayasu et al. 2009). About 850 mucin-encoding genes comprise about 1 % of the parasite genome, but up to 25 % of these genes are pseudogenes (El-Sayed et al. 2005; Buscaglia et al. 2006; Goldenberg and Ávila 2011). About 60 % of the molecular mass of mucins are carbohydrates. According to their composition, mucins of *T. cruzi* can be classified into TcSMUG L and TcSMUG S, both possessing >80 % identity. Mucins are present in the coat in a mosaic-like pattern, differing between the stages of *T. cruzi*, and also the lipid moiety of mucin anchors differs (Mattos et al. 2014). The number of mucin molecules per surface area is similar in cell-derived trypomastigotes and epimastigotes, although the latter possess about 4-fold more glycosylinositol phospholipids (Pereira-Chioccola et al. 2000).

Mucins of epimastigotes possess a lower molecular mass than those on cell culture-derived trypomastigotes, 35–50 kDa versus 60–200 kDa, and are classified as invariant mucins (Buscaglia et al. 2006). These epimastigote-specific mucins differ between major groups of *T. cruzi*, epimastigotes of one discrete typing unit mainly containing galactofuranose. TcSMUG S are the major proteins of the surface coat of epimastigotes. These mucins are composed of 56–85 amino acids and highly O-glycosylated. The second type of mucins, TcSMUG L, possesses a remarkably conserved structure (reviewed by Gonzalez et al. 2013). Presumably due to the

absence of terminal β-Gal residues, no sialic acid residues are connected to these mucins. In addition, the activity of trans-sialidases is much lower than in metacyclic trypomastigotes and the structure differs from the enzyme of the trypomastigotes (Giorgi and de Lederkremer 2011).

The surface coat of metacyclic trypomastigotes has tasks which are identical to those of epimastigotes: protection against the digestive enzymes of the vector and its immunity compounds. However, they must also be prepared for the mammalian host, and there tasks of the surface coat are identical with those of blood trypomastigotes. None of the components of the surface coat of metacyclic trypomastigotes has been correlated to its tasks in the vector. The molecular masses of mucins of the surface coat of metacyclic trypomastigotes are similar to those of epimastigotes (Buscaglia et al. 2006). However, their compositions differ, because only those isolated from trypomastigotes bind to host cells. In contrast to trypomastigotes, epimastigotes do not or much less expose molecules that are relevant for infection of mammalian cells (Table 14.2). The majority of surface coat proteins of metacyclic trypomastigotes are linked to tasks in the mammalian host, e.g., phosphatidylserine, gp82, and trans-sialidases (DaMatta et al. 2007; Giorgi and de Lederkremer 2011; Cortez et al. 2014). Phosphatidylserine is exposed in the different trypomastigotes (DaMatta et al. 2007). Gp82 and gp90 are present in the new surface coat replaced during metacyclogenesis in *Rhodnius* (Cordero et al. 2008). Gp82 is essential in infections of mammals by the oral route (Cortez et al. 2014). Trans-sialidase genes are linked in the genome to a proportion of mucin genes. These enzymes are abundant in the surface coat of metacyclic trypomastigotes and transfer sialic acid, which cannot be synthesized by *T. cruzi*, from host cells to the mucins. In the mammalian host the rapid sialylation of mucins protects the parasite against complement-mediated lysis (Giorgi and de Lederkremer 2011). Also many investigations of the surface coat of metacyclic trypomastigotes do not consider the difficulties of protein-free media, high centrifugation forces, and effects of isolation columns, although some of them mention a shedding of proteins (e.g., Baida et al. 2006). Similarly to epimastigotes, ectosomes and exosomes also bud from metacyclic trypomastigotes, but are also released while in contact with or internalized into mammalian host cells (Bayer-Santos et al. 2013).

14.5.3.3 Development in Midgut

After an ingestion of infectious blood, in the stomach many blood trypomastigotes die. Also after infections using epimastigotes of the Dm28 clone in decomplemented blood, most of the parasites are lysed during the first day independently of the gut bacteria (Dias Fde et al. 2015). This cannot be attributed to digestive enzymes, because only glycosidases and no digestive proteases are present in this region of the gut. In contrast to *Trypanosoma brucei* death seems not to be restricted to long blood trypomastigotes. In the stomach, *T. cruzi* interacts with a hemolytic factor which lyses erythrocytes but also *T. cruzi*. Lysis in the stomach differs between strains and is connected with the action of agglutinins. In *R. prolixus*, the epimastigotes of the Dm28 clone are agglutinated and lysis resistant, whereas those of the Y

Table 14.2 Presence or activity of surface coat molecules in the different stages of *Trypanosoma cruzi*

Component	Blood trypomastigote	Cell-derived trypomastigote	Epimastigote	Metacyclic trypomastigote	Reference
Trans-sialidase			(+)	++	Giorgi and de Lederkremer (2011)
Calpain			+	–	Ennes-Vidal et al. (2011)
Cruzipain		–	++	–	Uehara et al. (2012)
Phosphatidylserine	+	+	–	+	DaMatta et al. (2007)
gp63			+	+	d'Avila-Levy et al. (2014)
gp82			–	+	Cortez et al. (2014)
gp85	++++	+++	+	++	Mattos et al. (2014)
gp90			–	+	Cordero et al. (2008)
Ecto-phosphatases			+	++	Freitas-Mesquita and Meyer-Fernandes (2014)
Ecto-nucleotidases			+	++	Freitas-Mesquita and Meyer-Fernandes (2014)

strain behave just in the opposite way (reviewed by Kollien and Schaub 2000). However, the Y strain is strongly attenuated and the relevance of this phenomenon has to be investigated in fresh isolates of *T. cruzi* and the respective vector.

During transformation to epimastigotes, the surface coat is replaced. Recent proteomic analyses of surface components are complicated by the reactions of the flagellates to incubations in protein-free salines and perhaps to centrifugation forces (see section "Blood trypomastigotes"). Comparing the protein groups obtained after "shaving" via trypsin and after chromatography of biotinylated surface proteins, 1365 and 1488 proteins, respectively, only about half of them are present in both samples (Queiroz et al. 2013). About 700 proteins in the control sample indicate presumably the importance of incubations in serum-free saline and/or of centrifugation, inducing a microvesicular release of proteins from exosomes. This can also be the reason for the development of large vesicles budding from the plasma membrane resembling ectosomes (Bayer-Santos et al. 2013). These and smaller vesicles within the flagellar pocket, resembling endosomes, contain many proteins involved in different tasks, such as metabolism, signaling, and parasite survival and virulence. These vesicles are linked to life-cycle regulation and are much stronger induced in media free of proteins or containing low concentrations (Garcia-Silva et al. 2013).

After passage to the digestive part of the midgut, the surface coat must protect the cell against the digestive enzymes of the vector and its immunity compounds and enable an attachment to the intestinal wall. The resistance to proteases of the triatomines seems to be caused by the mucins. According to liquid chromatography-tandem mass spectrometry, they contain only few proteolytic sites (Nakayasu et al. 2009). Zinc metalloproteases, gp63, are suggested to digest nutrients in the gut and/or to protect the flagellate against molecules of insect immunity. Genes encoding gp63 are very abundant in the genome of *T. cruzi*, but more of these genes are expressed stronger in intracellular amastigotes than in epimastigotes. Another relevant compound, heme, is produced by the digestion of hemoglobin (reviewed by Paes et al. 2011; Nogueira et al. 2015). Since heme is ingested by the flagellate and metabolized, epimastigotes proliferate, but metacyclogenesis rate is reduced.

In the midgut, epimastigotes attach to the perimicrovillar membranes, a phenomenon that seems to be explainable at the molecular level (reviewed by Alves et al. 2007; Nogueira et al. 2007). In electron microscopy many parasites are orientated parallel to the matrix and the flagellum shows no enlargements. Epimastigotes attach via glycoinositol phospholipids at their surface to the perimicrovillar membranes of the intestinal cells (Alves et al. 2007; Nogueira et al. 2007). Avoiding the development of these membranes by decapitation of the bug or feeding an antiserum strongly interferes with a colonization of this region (Gonzalez et al. 1999, 2006). The membranes contain a variety of glycoconjugates, e.g., mannose, galactosamine, *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, and sialic acid. Epimastigotes possess mannose-specific carbohydrate-binding proteins and exhibit a strong affinity for higher mannose oligomers that are present in the midgut (Bonay et al. 2001). These binding

proteins are mucins. After feeding blood supplemented with TcSMUG S, parasite levels are similar to those of controls without supplement. However, TcSMUG L inhibits or strongly reduces the development. These peptides bind to specific binding sites of the gut (Gonzalez et al. 2013).

Also other surface coat components are suggested to be involved in the attachment, multiplication, and metacyclogenesis in the vector (reviewed by d'Avila-Levy et al. 2014). Cruzipain, a cysteine peptidase, is exposed abundantly on the surface of epimastigotes and also interacts in vitro with the adhesion in the isolated midgut as indicated by the level of adhesion after incubation of epimastigotes with anti-cruzipain antibodies or a panel of cysteine peptidase inhibitors (Uehara et al. 2012). Inhibition of the activity of gp63 and 80 kDa calcium-regulated cysteine peptidases (calpains) also interferes with the attachment (Ennes-Vidal et al. 2011; d'Avila-Levy et al. 2014). However, in these exposures of the luminal surface of the midgut to epimastigotes, the flagellates were washed in salines without proteins and centrifuged strongly. This might have induced changes in the surface coat and reaction of components which are not accessible in untreated epimastigotes.

14.5.3.4 Development in the Rectum

In the rectum, the behavior of epimastigotes differs strongly from that in the midgut. In strong infections, carpets of flagellates cover the cuticle, and the flagellates are only in contact with the cuticle with the tip of the flagellum. Epimastigotes attach with a specific region of the flagellum by hydrophobic interactions to the wax layer of the cuticle. This flagellar region enlarges and increases the attachment by hemidesmosome-like material beneath the plasma membrane (Schmidt et al. 1998; Kleffmann et al. 1998; Kollien et al. 1998). Searching for the binding receptors, chitin is not accessible and there are no indications for a heparin receptor on the flagellum and a galactose-binding lectin on the rectal cuticle. Although the attachment to the cuticle in the rectum is long known and relevant for all trypanosomatids (e.g., Wenyon 1926), the components of the flagellum that enable this have not been identified.

The development of the non-dividing metacyclic trypomastigotes is mainly restricted to the rectum. These parasites do not attach to the cuticle, presumably due to the replacement of the surface coat of epimastigotes during metacyclogenesis (Kollien and Schaub 2000). In the final preadult stage of *T. infestans* from Chile mentioned above, 50 % of the rectal population are metacyclic trypomastigotes, i.e., about one million. About 50 % of the total population in the rectum is flushed out during or after blood ingestion of the bug when the urine passes the rectum excreting also the remains of digestion. The excreted urine contains mainly trypomastigotes (Kollien and Schaub 2000). In addition, proteins from the hemolymph, which are present in the urine, induce metacyclogenesis from epimastigotes within 20 min after feeding. Only the attached epimastigotes remain in a thick layer, and trypomastigotes between them near the cuticle are not flushed out. One week later, the rectum contains a similar population density and a similar percentage of trypomastigotes. Molting is another vector-borne phenomenon that strongly affects the rectal

population. Besides the cuticle at the surface of the bug also that lining foregut and hindgut is shed. Thereby, the attached population of *T. cruzi* is also drawn out of the anus. However, after the separation of the old cuticle from the new one and before ecdysis, the new one is already colonized.

In investigations of in vitro metacyclogenesis, many changes are obvious (reviewed by Goldenberg and Ávila 2011). The volume of reservosomes, which store proteins and lipids, decreases during metacyclogenesis. Specific glycoproteins are included in the surface coat of in vitro-derived metacyclic trypomastigotes (Table 14.2). The abundance of other proteins decreases, e.g., that of cruzipain. An overexpression of this proteinase enhances metacyclogenesis (Tomás et al. 1997). Since the level of gene expression does not correlate to the synthesis of the respective protein, only one aspect of stage-specific gene expression will be mentioned. Untranslated mRNAs are stabilized by specific proteins and stored as P bodies. In the cytoplasm of epimastigotes, mRNAs of several metacyclic trypomastigote-specific proteins wait for the transformation of the parasite (reviewed by Goldenberg and Ávila 2011). The strong changes of the protein profiles are reflected in a phosphoproteome analysis (Marchini et al. 2011).

14.5.4 Interactions of the Intestinal Immune System, the Microbiota, and *Trypanosoma cruzi*

Also in triatomines the innate immune system is not restricted to the hemolymph (Müller et al. 2008). However, in the intestine only humoral components are present and no immunity cells. In contrast to the dipteran vectors, also all preadult instars ingest sterile blood. Therefore, the intestinal microbiota should be restricted. However, triatomines obtain the obligatory mutualistic symbionts via coprophagy, and also other bacteria are deposited in the feces. Therefore, also triatomines harbor a variety of bacteria and fungi in the intestinal tract, some of them competing with the host for resources in the gut (Eichler et al. 1996; Vallejo et al. 2009; da Mota et al. 2012). The cardia, a short region of the anterior midgut before the stomach, seems to act as refuge for symbionts. Although blood is sterile, after blood ingestion the expression of genes encoding lysozymes, defensins, and prolixicin and the antibacterial activity are increased in the cardia and stomach (reviewed by Garcia et al. 2010; Vieira et al. 2014; Soares et al. 2015), presumably to exclude a growth of bacteria originating from the skin of the host. The response differs according to bacteria fed in the blood (Vieira et al. 2014). Curiously, after blood ingestion, the population of the mutualistic symbionts grows in the stomach. In the small intestine, the expression of these genes and the activity is much lower, but there symbionts are lysed by so far unknown factors (Eichler and Schaub 2002). In addition to these AMPs, a Kazal-type inhibitor is secreted into the stomach and modulates the microbiota in *R. prolixus* (Soares et al. 2015). These inhibitors are usually anticoagulants. In different triatomines, the genes encode long precursor proteins containing 6, 7, or 8 Kazal-like domains (Meiser et al. 2010). The coagulation is inhibited by

double-domain proteins, but some and also single domains possess other characteristics, acting against bacteria (Soares et al. 2015). In addition to AMPs and Kazal-type inhibitors, other protease inhibitors are produced in response to bacteria (reviewed by Soares et al. 2015). In the anterior midgut (stomach plus cardia), the expression of genes of a most recently identified AMP is increased by an infection with *T. cruzi* and affects the microbiota (Buarque et al. 2016). Knocking down this AMP strongly increases the number of bacteria and decreases those of *T. cruzi*.

Not only bacteria but also *T. cruzi* induces the expression of genes of AMPs, e.g., those of defensins in *Triatoma brasiliensis* (Waniek et al. 2011). In *R. prolixus*, this is evident for one of two lysozyme genes, but the viability of the flagellate seems to be unaffected. In this triatomine, a knockdown of a gene of the Imd pathway, *rpRelish*, decreases the expression of *defensin A* in the midgut, but not of lysozyme B and A, the expression of the latter even increases. Whereas the population level of the symbiont *Rhodococcus rhodnii* increases, the level of *T. cruzi* remains unaffected, also after silencing the transcription factor *rpDorsal* of the Toll pathway (Mesquita et al. 2015). A clear response of the vector is evident for the radical nitric oxide. The expression rate of genes encoding nitric oxide synthase is upregulated in the stomach during the first 2 days after infection (reviewed by Garcia et al. 2010). In the small intestine, much higher concentrations of nitrite, a metabolite of nitric oxide, occur in infected *R. prolixus*. In the rectum levels of nitrite are also massively increased, but already before the flagellates has passed to this region. In a similar approach, the level of nitrite in the stomach is reduced at 9 days post-infection (Castro et al. 2012). All these immune reactions do not affect *T. cruzi* in a long-term infection. Only agglutinins interfere with the development in a *T. cruzi* strain-dependent manner (see Sect. 14.5.3.3).

The induction of the immune response by *T. cruzi* does not affect the flagellate, but supports its growth. At least the upregulation of the Kazal-type inhibitor by *T. cruzi* reduces the population of bacteria (Soares et al. 2015). After reduction of the concentration of transcripts of the inhibitor by RNAi prior to infection with the blood trypomastigotes, less *T. cruzi* of the CL strain but more bacteria develop in the stomach. However, the bacteria should be identified, because not all bacteria are pathogenic for *T. cruzi* (Azambuja et al. 2005). Using axenic nymphs of *T. infestans* and *R. prolixus*, which obtained the respective symbiont in the first instar, during 10 days after infection of fifth instar nymphs with *T. cruzi*, the number of the colony-forming units of the respective symbiont is similar in the different regions of the gut of uninfected and infected nymphs (Eichler and Schaub 2002). In triatomines captured in the field, an analysis of gut metagenomic DNA indicates no effects of the infection on the microbiota (Gumiel et al. 2015). Also these triatomines from the field contained *Serratia marcescens*. Using in vitro and vector assays, this bacterium lyses epimastigotes of *T. cruzi* Y strain but not those of the Dm28c clone (reviewed by Castro et al. 2012).

Differences between strains of *T. cruzi* are also evident in the interaction with bacteria in the vector. After infection with epimastigotes of the Y strain and the clone Dm28c, in the latter infections nitrite and nitrate concentrations are lower, the

activities of phenoloxidase and antibacterial compounds are higher, and less bacteria but more flagellates develop. The Y strain does not establish in *R. prolixus* and the numbers of bacteria are only slightly decreased (Castro et al. 2012). Therefore, some strains/clones modulate the immune reactions to decrease the population of microbiota.

These data indicate a rapid induction of the immune response of the vector to the infection. However, the parasite or at least some strains possess not only immunosuppression mechanisms in the infections of humans but also in the vector. Investigating the interactions of *T. cruzi* and intestinal bacteria and symbionts in long-lasting infections of *T. infestans*, different bacteria only develop in infected bugs (S Eichler 2016, unpublished).

14.5.5 Perspectives and Open Questions

Identifications of surface coat glycoproteins and investigations of the interactions with the vector should consider the fragility of the parasites. Cells should not be incubated in protein-free saline. Pure proteins should be used that can be separated after isolation of the surface coat. Using fine needles and syringes, a mechanical shedding can be obtained and compared to the data obtained after trypsin "shaving" and the effects of different centrifugation forces, e.g., >400 to 2000 g (Hölscher et al. 2003). Thereby, the occurrence and protein composition of vesicles (Marcilla et al. 2014) can be compared with those obtained by more stressful procedures. The proteomic map of *T. cruzi* should not only be evaluated for the development of new drugs (Menna-Barreto and Perales 2014; Menna-Barreto et al. 2014) but also for compounds relevant for the interaction with the vector. Perhaps this offers a chance to identify the lipid-binding molecules or hydrophobic domains at the flagellum of epimastigotes. Investigations of interactions of *T. cruzi* and the vector should use strains belonging to different discrete typing units and possessing different biological capacities. The use of systems in which parasite and vector originate from the same location includes aspects of the long-lasting coevolution. Using more systems, also the pathogenicity of *T. cruzi* for the vector can be compared (Schaub 1989b).

Investigations of the vectors should consider the variability between the different species and include sylvatic species. So far, only the transcriptome of *R. prolixus* is available (Ribeiro et al. 2014). The spectrum of antibacterial compounds remains to be completed. Especially the factors determining the development and lysis of symbionts are an important open question. Bacteria and symbionts have to be identified separately not only by identifying colony-forming units but also using metagenomics like next-generation sequencing of the 16S rRNA gene (Vieira et al. 2015). This is also relevant for approaches using genetically modified symbionts to kill *T. cruzi* in the vector (Pennington 2015). Investigations of interactions with *T. cruzi* should include standardized infections with different bacteria plus the respective symbiont and an axenic maintenance of the bugs.

14.6 General Perspectives and Open Questions

In these four parasite-vector systems, mainly strains of parasites are used which can easily be cultivated in the laboratory. However, in the populations of parasites in the field, many strains possess a low virulence and are difficult to be cultivated. In addition, many strains lose the virulence during permanent in vitro cultivation or permanent mammal-mammal infections. Fresh strains from the field offer more information which is relevant in the natural cycle. During coevolution, parasites had to adapt to vectors and humans, and both act as filters killing those parasites which are less adapted and vulnerable. Harvesting of parasites from in vitro cultures is another problem. The medium must contain proteins, because within their life-cycle parasites are never confronted with a protein-free environment. We have to consider that parasites are living cells and not inorganic material. Therefore, high centrifugation forces are critical. Using low centrifugation forces will result in a loss of about 20 % of parasites, but the parasites will not be damaged. If in vitro cultures are used, only the number of cultures has to be increased.

Also the rearing of vectors contains pitfalls. After transfer of a population into the laboratory, usually a low percentage of insects will breed. Some species of vectors totally refuse to breed in the laboratory. Thus, the genetic variability is reduced, and the interactions described above cannot be generalized. In addition, the official pressure to use less mammals is crucial. If colonies of blood-sucking insects are permanently fed in vitro through artificial membranes, specific strains of vectors are selected, showing a different behavior. Therefore, data obtained with laboratory colonies have to be confirmed using uninfected and infected vectors directly after capture in the field. Such investigations automatically contain coevolved parasite-vector strains. The enormous variability within vector populations impedes approaches to attack the vector or to kill the parasites in the vector. Sequencing and analyzing the genomes of the parasites as well as the respective vector will be a big step forward to understand the interactions. However, all the data obtained by bioinformatics analyses have to be verified whether or not genes are expressed.

Recent approaches considering the microbiota result in interesting information of other tesserae in the parasite-vector interactions. Often the Gram-negative bacterium *Serratia marcescens* colonizes vectors. However, it is not only pathogenic for many parasites and vectors but also for humans and cannot be used for a biological control. Metagenomics like next-generation sequencing of the 16S rRNA gene will provide us with an enormous bulk of data, which are species of bacteria the respective vector hosts. However, the relevance for the parasite in the intestinal tract can only be estimated if we get the information whether they also colonize the lumen or affect the conditions in the lumen. Also the size of the respective bacteria population is relevant. It will be very labor intensive but necessary to investigate the interactions of parasites and each species of the microbiota separately and as a mixture in axenically maintained uninfected and infected vectors.

The modern genomic and proteomic analyses will help us to understand the interactions and perhaps to identify targets for control of parasite development in the vector. If the aim to control diseases via the vector will not be achieved, this information offers at least an insight into amazing and fascinating aspects of biology.

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From Molecule to Drug

15

Pascal Mäser and Reto Brun

Abstract

Drug development for protozoan parasites has gained a renewed impetus at the turn of the century with the foundation of product development partnerships such as the Drugs for Neglected Diseases initiative (DNDi) or Medicines for Malaria Venture (MMV). These organizations have established the required target-product profiles for new drugs and have set up research and development consortia involving partners from the academic, the pharmaceutical and the governmental sector. This has successfully replenished the drug development pipelines for malaria, human African trypanosomiasis and other parasitic diseases. In spite of recent breakthroughs in drug target identification and validation, most of the new drug candidates have been found through cell-based screens. Several new chemical entities against *Plasmodium falciparum* or *Trypanosoma brucei* are being tested in the clinical phase of development. However, a large need for new leads persists for *Leishmania* spp. and *Trypanosoma cruzi*. Here we give an overview on antiparasitic drug targets and illustrate the research and development pipeline with recent success stories for malaria and human African trypanosomiasis.

15.1 Historical Background

Drug discovery for protozoan parasites has been one of the cornerstones of pharmaceutical industry. Historically, the discoveries of protozoa as etiological agents of diseases such as malaria or sleeping sickness coincided with the inventions of

P. Mäser • R. Brun (✉)
Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Socinstrasse 57, CH-4051 Basel, Switzerland
e-mail: pascal.maeser@unibas.ch; reto.brun@unibas.ch

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the first synthetic dye stuffs. It was Paul Ehrlich who linked the two emerging fields of research at the end of the nineteenth century. Having observed that certain organic dyes exhibit greater affinity for parasites than for host cells, Ehrlich concluded that selective antiparasitic chemotherapy was possible if such dyes are cytotoxic. He put the idea into practice in 1891 by successfully treating two autochthonous malaria patients in Berlin with methylene blue (Guttmann and Ehrlich 1891), a synthetic dye developed in 1876 by Heinrich Caro of BASF. Ehrlich also experimented with African trypanosomes, studying drug action and drug resistance (Ehrlich 1907). At that time, African trypanosomes were not only good models for drug efficacy testing – observable in the microscope and infective to mice – but also of economic importance. In what was one of the first lead-to-drug development programmes, the cotton dye Congo red, synthesized in 1883 by Paul Böttiger of Bayer, was optimized to trypan red (1903), trypan blue (1904) and finally to suramin (1916), also known as Bayer 205. After the independence of the African colonies in the 1960s and 1970s, however, the interest of pharmaceutical industries in the parasitic protozoa began to wane. The resulting regression in drug R&D and shortage of new drug candidates were severe for sleeping sickness and malaria alike. The existing chemotherapeutics for *Plasmodium falciparum* were being jeopardized by the global spread of drug resistance, while those for *Trypanosoma brucei* spp. had been inadequate all along – in particular for the cerebral stage of the disease. The situation aggravated in the 1990s with rising prevalences of malaria and sleeping sickness. A possible solution to the crisis emerged only at the turn of the century, more than 100 years after Ehrlich's pioneering work. This was the foundation of public–private partnerships (PPP), which subsequently matured to product-development-partnerships (PDPs). PDPs such as the Drugs for Neglected Diseases initiative (DNDi, launched in 2003) and Medicines for Malaria Venture (MMV, launched in 1999) have by now proven to be successful instruments for antiparasitic drug development. Finally, new chemotypes are being developed towards the next generation of antimalarials and trypanocides. Here we summarize drug R&D pipelines and highlight for *Plasmodium falciparum* and the trypanosomatids.

15.2 Target Product Profiles

The PDPs not only raised the funds for preclinical and clinical development of drug candidates, they also contributed a product-oriented mind-set that greatly supported academic research (which is usually very innovative but less target oriented). In particular, the definition of target product profiles (TPPs) allowed to prioritize the hits from early discovery programmes and provided guidance for further development of the most promising molecules. Several TPPs may apply to the same disease, depending on the prevalence, the envisaged group of patients and the available clinical facilities. In any case, a TPP must be established bottom-up, starting from the needs of the patient and the medical personnel at the point of care. Sample TPPs

Table 15.1 Target product profiles – examples for malaria and HAT therapy based on the recommendations by MMV and DNDi, respectively

Treatment property	Human African trypanosomiasis	Malaria
Composition	Monotherapy	Combination of two co-formulated compounds
Mode of action	Cidal, multitarget	Very fast acting
Drug resistance	Effective in melarsoprol refractory patients	Effective against all drug-resistant reference isolates. Very low susceptibility to acquired resistance in vitro. Resistance markers identified
Stage-specificity	Effective against stages 1 and 2	Effective against all asexual blood stages and liver stages. Also effective against gametocytes and *P. vivax* hypnozoites
Bioavailability	>50 %	>50 %, no food effects
Treatment schedule	<7 days p.o. once daily	Single dose p.o.
Clinical efficacy	>95 % at 18-month follow-up	100 % at day 7, >95 % at day 28 Fever decreased at 24 h
Adverse effects	Safe during pregnancy and for lactating women	Minimal drug-related adverse effects. Safe during pregnancy and for lactating women. No enhanced risk in G6PD-deficient patients
Mortality	<1 % drug-related mortality	No drug-related mortality
Stability	3 years at 30 °C and 65 % relative humidity	5 years at 30 °C and 65 % relative humidity
Cost	<30 €	0.80 € for adults 0.20 € for infants

for HAT and malaria are shown in Table 15.1. Given the very high incidence of malaria of 200 million cases in 2014 (WHO 2014), imperative points for a new antimalarial are outstanding tolerability, oral bioavailability and low cost of goods. In addition, full efficacy against the known drug-resistant *P. falciparum* mutants is a must. In the light of the malaria eradication agenda (Alonso et al. 2011), transmission-blocking activity (i.e. gametocytocidality) and efficacy against the *P. vivax* hypnozoites are desirable. HAT, which has a much lower incidence but a higher mortality of the infected patients (Brun et al. 2010), has somewhat less stringent requirements concerning safety and price. Penetration through the blood–brain barrier into the cerebrospinal fluid and the brain, however, is a prerequisite for a new drug against HAT to ensure efficacy also for the late stage of the disease. A direct implication of the TPPs with their various – and frequently conflicting – requirements is that the molecule that becomes a drug will be a compromise. It will not be the most active molecule of a series but the one with an optimized profile regarding efficacy, safety, pharmacokinetics, cost, stability, formulability, etc. Such a molecule is usually not discovered in a screen but developed in a long process from an initial hit (see Sect. 4.2).

15.3 Mechanisms of Drug Action

In a cell-based in vitro screen, a hit may be defined as a molecule that exhibits high activity against a given parasite and low toxicity to mammalian cell lines. This can be assessed with the in vitro selectivity index, i.e. the 50 % inhibitory concentration (IC_{50}) to mammalian cell lines divided by the IC_{50} towards the parasite (Kaminsky et al. 1996). Drugs like artemisinin for *P. falciparum* or pentamidine for *Trypanosoma brucei* have in vitro selectivity indices of several thousand. What is the molecular basis of such striking parasite-specific activity? In the following we try to dissect the molecular mechanisms of antiparasitic drug action and selectivity. Concentrating on the nature of the difference between the drug target in the parasite and its closest orthologue in the human host, we distinguish five different scenarios that create opportunities for antiparasitic drug selectivity (Table 15.2).

15.3.1 Parasite-Specific Presence of Drug Targets

The classical definition of an antiparasitic drug target is an enzyme or receptor that is essential to the pathogen but absent in the host. Typical examples are the folate biosynthetic enzymes, dihydropteroate synthase and dihydrofolate synthase in *Plasmodium* spp., rendering the malaria parasites susceptible to sulfonamides. Drug targets in the apicoplast belong to the same category. The apicoplast is the remnant of a secondary endosymbiont of cyanobacterial origin (Ralph et al. 2004) that has no counterpart in mammalian cells. Elucidation of the apicoplast metabolic pathways has allowed the rational repurposing of antibiotics for malaria. Among the druggable targets in the apicoplast are the enzymes of the deoxyxylulose-phosphate (i.e. non-mevalonate) pathway for isoprenoid synthesis such as DOX-P

Table 15.2 Molecular mechanisms of antiprotozoal drug selectivity

Parasite specific	Parasite	Key pathway or enzyme	Drug or inhibitor
Presence of target	*P. falciparum*	Folate synthesis	Sulfadoxine
	P. falciparum	Non-mevalonate pathway	Fosmidomycin
	P. falciparum	Fatty acid synthase type II	Triclosan
	T. brucei	Trypanothione metabolism	Melarsoprol
	Leishmania	Ergosterol synthesis	Amphotericin B
Stability of target	*P. falciparum*	Dihydrofolate reductase	WR99210
	T. brucei	Ornithine decarboxylase	Eflornithine
Essentiality of target	*P. falciparum*	Pyrimidine synthesis	DSM265
Uptake of drug	*T. brucei*	Adenosine transporter 1	MelB, pentamidine
	T. brucei	Aquaglyceroporin 2	MelB, pentamidine
Activation of drug	*P. falciparum*	Interaction with haem	Artemisinin
	P. falciparum	Interaction with haem	OZ277
	T. cruzi	Nitroreductase type I	Nifurtimox
	T. brucei	Nitroreductase type I	Fexinidazole

reductoisomerase, the target of fosmidomycin. Developed as an antibiotic in the 1980s, fosmidomycin has been repositioned for malaria in 1999 (Jomaa et al. 1999). Fosmidomycin–clindamycin combination therapy is currently in clinical development (Lanaspa et al. 2012). The apicoplast also hosts a type II pathway for fatty acid synthesis, whereas mammals have type I fatty acid synthases. Triclosan was shown to be active against *P. falciparum* in vitro (Surolia and Surolia 2001). However, initial reports of triclosan efficacy in a malaria mouse model (Surolia and Surolia 2001) were not reproducible later on (Baschong et al. 2011).

The trypanosomatids, too, possess various biochemical peculiarities that are absent in their hosts and that lend themselves to chemotherapeutic attack (Opperdoes 1985). Trypanothione (bis-glutathionyl spermidine (Fairlamb et al. 1985)) and its associated enzymes trypanothione synthase, trypanothione reductase and trypanothione S-transferase comprise an essential system for defence against oxidative stress. Lacking catalase, African trypanosomes rely on trypanothione to detoxify H_2O_2. Melarsoprol (MelB) forms a complex with trypanothione, MelT, which then inhibits trypanothione reductase (Cunningham et al. 1994). Trypanothione biosynthesis is of particular interest because it connects polyamine synthesis with S-adenosylmethionine metabolism, a link that is absent in the metabolic networks of mammalian cells. Trypanosomatid sterol biosynthesis is special as well. Unlike most protozoan parasites, *T. brucei* and *T. cruzi* are able to synthesize sterols (Fügi et al. 2014). However, they make ergosterol while mammals make cholesterol. The presence of ergosterol in their membranes is thought to render *Leishmania* susceptible to amphotericin B; however, this has been questioned (Chattopadhyay and Jafurulla 2011). Triazole inhibitors of sterol C14 demethylase (CYP51) are the most advanced drug candidates in the pipeline against *T. cruzi* (Urbina 2009). Posaconazole, marketed as an antifungal drug, has been in clinical Phase II trials for chronic Chagas disease, but it revealed disappointingly high relapse rates (Molina et al. 2014); see Sect. 5.7.

15.3.2 Parasite-Specific Lack of Target Turnover

A different type of therapeutic window may result from divergent regulation of the potential targets in host and pathogen. Dihydrofolate reductase (DHFR), required to make thymidine from uridine, is an essential enzyme in all cells. *Plasmodium falciparum* DHFR is the target of antifolates such as pyrimethamine or WR99210, an experimental compound with an in vitro selectivity index of 60,000 (Zhang and Rathod 2002). This high degree of selectivity is caused, at least in part, by divergent regulation of gene expression by means of a negative feedback loop: DHFR binds its own mRNA, preventing it from being translated. In the case of the human enzyme, this block is mediated by the active site and will be suspended upon inhibition by WR99210. This is not the case for *P. falciparum* DHFR, which remains bound to its own mRNA even in the presence of WR99210 (Zhang and Rathod 2002). Thus human DHFR is replenished after inhibition, but *P. falciparum* DHFR is not.

A similar phenomenon might explain the selectivity of eflornithine (difluoromethylornithine, DFMO) towards *T. b. gambiense*. Eflornithine covalently binds to ornithine decarboxylase (ODC), inhibiting the first and dedicating step of polyamine synthesis. ODC is an essential enzyme in trypanosomes as well as in their mammalian hosts. Moreover, the ODC activities in trypanosomal and murine cell extracts were equally sensitive to eflornithine (Iten et al. 1997). However, mammalian ODC has an extremely high turnover with a half-life of 15–30 min, whereas the half-life of *T. b. gambiense* ODC was determined to be 18–19 h (Iten et al. 1997). Thus, due to higher turnover, human ODC gets replenished faster than parasite ODC after binding of eflornithine (Phillips et al. 1987).

15.3.3 Parasite-Specific Essentiality of Drug Targets

If an antiparasitic drug target is an essential enzyme but its human orthologue is not, that will increase the parasite-specific selectivity of an inhibitor. This rather theoretical scenario is scarcely documented by actual drugs. Pyrimidine synthesis in *P. falciparum* may be one example. In contrast to human cells, the malaria parasite has no pyrimidine salvage pathway and exclusively relies on de novo synthesis of pyrimidines to make nucleic acids. Thus dihydroorotate dehydrogenase (DHODH) is an essential enzyme for *P. falciparum* but not necessarily for mammals, which can take up and interconvert pyrimidines from their diet. One DHODH inhibitor, DSM265, is in clinical development for malaria. DSM265 clearly is selective for *P. falciparum* DHOD over human DHOD (Coteron et al. 2011). Possibly, human pyrimidine salvage contributes to the excellent safety profile of DSM265 (www.mmv.org) as well.

15.3.4 Parasite-Specific Drug Uptake

Transport processes are important determinants of drug susceptibility. Hydrophilic drugs cannot diffuse across biological membranes and are unable to reach cytosolic targets in the absence of transporter proteins. Cross resistance between melamine-based arsenicals and diamidines (e.g. melarsoprol–pentamidine cross-resistance, MPXR) is a well-known phenomenon in African trypanosomes (Baker et al. 2013) that has been attributed to the loss of transport functions (Carter et al. 1995). Two different transporters have been identified from *T. brucei* that transport melarsoprol and pentamidine in addition to their physiological substrates: the adenosine transporter TbAT1 (Mäser et al. 1999) and the aquaglyceroporin TbAQP2 (Baker et al. 2012). Homozygous genetic disruption of either gene causes MPXR in *T. brucei* (Baker et al. 2012; Matovu et al. 2003). Heterologous expression of *TbAT1* and *TbAQP2* in yeast and *L. mexicana*, respectively, caused drug hypersensitivity (Mäser et al. 1999; Munday et al. 2014). No human nucleoside transporter or aquaglyceroporin is known to transport melarsoprol and pentamidine, indicating that human cells might be protected from the toxic effect of these drugs by lack of import.

15.3.5 Parasite-Specific Activation of Drugs

A number of antiprotozoal drugs are not activated until they are inside the parasite. Artemisinins become reactive and alkylate haem and target proteins upon chemical reduction of the peroxide bridge (Fügi et al. 2010), which requires a strong reducing agent. In *P. falciparum* this is thought to be ferrous haem, the side product of haemoglobin degradation in the parasite's food vacuole. Human cells do not reduce artemisinins. The same mode of action can be assumed for synthetic ozonides such as OZ277 (see Sect. 5.1). This is supported by the finding that artemisinin and OZ277 are over 1000-fold more active against *P. falciparum* than against *Babesia divergens*, which also proliferates in erythrocytes but does not consume haemoglobin (Kaiser et al. 2007).

The nitroimidazoles also belong to the drugs that need to be chemically reduced to become reactive. Nifurtimox is being used against trypanosomes, and fexinidazole is in clinical development against *T. b. gambiense* (see Sect. 5.4). Fexinidazole, like most nitroimidazoles, has a positive Ames test. It was nevertheless approved for studies in man because it was not mutagenic to mammalian cells which, unlike bacteria, cannot reduce fexinidazole (Torreele et al. 2011; Tweats et al. 2012). *T. brucei* and *T. cruzi* possess type I nitroreductases that are necessary for nifurtimox susceptibility (Wilkinson et al. 2008). The presence of these bacterial-type enzymes in trypanosomes is likely to be a prerequisite for fexinidazole selectivity, too.

15.3.6 Implications for Hit Discovery

In summary, the question of how a drug can selectively kill a protozoan parasite but not its host has at least five mechanistically different answers, all of which can be documented by drugs or experimental compounds. The different scenarios are of importance to hit discovery since they are not equally amenable to 'postgenomic' approaches. Cases of scenario 3.1 are straightforward to detect by comparative genomics between host and parasite. Other proven antiparasitic targets, in particular those of scenario 3.2, can hardly be predicted by bioinformatics and are usually found through cell-based screening. We conclude that it is impossible to replace phenotypic screening with target-based, 'rational' screens. Wet- and dry-lab approaches towards new parasiticides should be carried out in parallel.

15.4 Drug Discovery

15.4.1 Target Assay Versus Whole-Cell Assay

When drug discovery for protozoan parasites was scaled up some 30 years ago, emphasis was put on whole-cell assays. During the first 10 years, the cultivation conditions were improved, and defined culture media with a mammalian serum component became the gold standard. Intracellular parasites were grown in the

corresponding host cell, and all parameters were adjusted to mimic the natural environment of the protozoan parasite. Inspired by the approach of pharmaceutical industry to start drug discovery with a target and to optimize on the target, target assays became popular and were soon after considered state of the art. Knowing the target and having an assay at hand which is high-throughput screening (HTS) compatible have obvious advantages and allow the use of structure–activity relationship (SAR) analysis for lead optimization. However, the translation of hits from target assays to whole-cell assay was often poor, and valuable time was wasted. Would it not make more sense to screen a given library of compounds once against the whole parasite instead of doing hundreds of enzyme assays with the same library again and again? In recent years there was a shift in paradigm and a trend to get back to do the first screen on the whole parasite. In a second step, it can be attempted to identify the targets of the resulting hits, and, once identified, further optimization can be done on the target. In summary, a whole-cell assay offers many advantages over a target assay: It covers all possible targets and allows to measure multitarget activity, and it filters out compounds that do not enter the target cell.

15.4.2 In Vitro Assays

In vitro assays for protozoan parasites have to be multiwell plate-based, 96-well format for medium throughput and IC_{50} (inhibitory concentration 50 %) determination or 384-well format for HTS. A single concentration (or two concentrations at 5x difference) assay serves as a prescreen to identify primary hits which then can be followed up in a serial dilution assay with IC_{50} determination. Controls are important for quality control: (1) a positive control with parasites without any compound (=100 % growth); (2) a negative control, culture medium without parasites (=background); and (3) standard drugs for reproducibility and comparison. The endpoint for reading has to be selected carefully; the signal to background ratio should be as high as possible or the background as stable as possible.

There are numerous critical parameters of an assay that have to be considered: (1) parasite strain and population (Laboratory strain or recent field isolate? Drug sensitive or drug resistant? Is it the relevant life cycle stage of the parasite?), (2) cultivation conditions (pH, serum component, temperature and gas composition), (3) assay duration (should be long enough to result in a stable IC_{50} value) and (4) read-out (photometric, fluorometric, β-counting, luminescence). Standard drugs serve as quality control, and their IC_{50} values should be in a defined range; otherwise, the assay has to be discarded. Additional information can be obtained from in vitro assays regarding, e.g. protein binding by determining the IC_{50} in the presence of increasing serum concentrations. An increase of the IC_{50} values with increasing serum concentrations is an indication of strong protein binding. We can also get information on fast- or slow-acting compounds by comparing the IC_{50} values of different assay durations. A slow-acting compound shows a significant decrease in IC_{50} values from 24 to 48 to 72 h assay duration, while a fast-acting one does not show a further decrease after 24 h.

15.4.3 Rodent Models

The principle of an in vivo rodent model is to reduce the parasite load of an infected animal or even to cure the animal. Ideally the human-pathogenic parasite is used for infection; however, some of the human parasites do not infect rodents (e.g. the human-pathogenic *Plasmodium* species) and have therefore to be replaced by surrogate models, e.g. the rodent malaria parasites *P. berghei* and *P. chabaudi*. Related, non-human-pathogenic strains of parasites can be used (e.g. *Trypanosoma brucei brucei* instead of *T. b. rhodesiense*) to reduce the risk of infection for laboratory personnel.

For African trypanosomes there is an excellent array of animal models available covering the disease stages and the two different forms of human African trypanosomiasis. There are acute models of *T. b. rhodesiense* and *T. b. gambiense* (using immunosuppressed mice) infection with treatment on days 3–6 postinfection and monitoring of parasitaemia for 2–3 months. A model mimicking the second stage of infection uses a chronic *T. b. brucei* isolate and a delayed treatment on days 25–30 postinfection with a 6-month monitoring period. Various field isolates of *T. b. rhodesiense* and *T. b. gambiense* are available to reach a better coverage of the strains circulating in endemic areas.

For malaria (as an anthroponosis not infective for rodents), we have to use rodent malaria species such as *Plasmodium berghei, P. chabaudi, P. vinckei* or *P. yoelii*. P. berghei infects mice and leads to a fatal infection within a few days, while the other species show more chronic but detectable infections. Drug treatment is similar to the one used for mouse models for African trypanosomes with a 4-day treatment but also a single dose treatment is possible. Efficacy is assessed by counting the parasite number 24 h after treatment and calculating the percentage reduction versus the untreated control animals. Cure is very difficult to reach in these models. There is a mouse model for *P. falciparum* which is very laborious and expensive (Angulo-Barturen et al. 2008). It uses SCID mice which are injected with washed human red blood cells (RBCs) over several days before being infected with *P. falciparum*. Injection of RBCs has to be maintained during treatment and monitoring; otherwise, the proportion of red cells will shift back to mouse RBCs and thus distort the outcome of the efficacy experiment.

15.4.4 Hit to Lead to Preclinical Candidate

A hit is a compound which shows specific in vitro activity in a whole-cell assay. The activity has to be below a threshold which is defined for each assay and each parasite. The activity should be specific for the target parasite and is determined by the selectivity index (SI = IC_{50} value for mammalian cells/IC_{50} for the parasite). In vitro activity on the parasite and selectivity are prerequisites for proceeding into an animal model of infection experiment. Cure or a significant reduction of parasitaemia in the absence of the signs of acute toxicity promotes compounds from hit to lead. A lead compound is still far from a preclinical candidate and just at the beginning

of a long and costly process. In vitro and in vivo activity/efficacy have to be evaluated more in depth, also toxicity, metabolic stability, protein binding and pharmacokinetic parameters. The lead molecule has to be improved for activity but also for safety and pharmacological parameters, a process called lead optimization. This is an iterative process between medicinal chemistry and biological assays and can be guided by structure–activity relationship analyses. Not only efficacy can be optimized but also reduction of toxicity, bioavailability, metabolic stability and other physico-chemical parameters. This optimization process is also guided by the target product profiles (see Sect. 15.2).

15.5 Examples of Drug Development Projects

15.5.1 Synthetic Peroxides

Artemisinin and its derivatives are first-line drugs for malaria in combination with a partner drug with a longer half-life (artemisinin combination therapy, ACT). A natural product from *Artemisia annua*, artemisinin, is a sesquiterpene lactone with an endoperoxide bridge as the active centre. Artemisinins cannot be produced in sufficient quantities from the plant, and synthesis of the molecule is too complex and expensive. To develop simpler and fully synthetic peroxides was the goal of a consortium with the University of Nebraska Medical Center, the Swiss Tropical and Public Health Institute and the Centre for Drug Candidate Optimisation (Melbourne). Over 700 adamantane-based peroxides were synthesized and studied in vitro and in rodent models. The first clinical candidate, OZ277 (Arterolane), (Dong et al. 2010) was registered by Ranbaxy Laboratories (India) in 2012 as a combination therapy with piperaquine. The next-generation peroxide OZ439 has improved characteristics, e.g. a longer half-life than OZ277, and resulted in the cure of the *P. berghei* mouse model with a single oral dose of 20 mg/kg (Charman et al. 2011). It is currently in Phase II clinical trials in combination with either piperaquine or ferroquine. OZ439 is chemically sufficiently different from the artemisinins to raise optimism that it will not cross react with emerging artemisinin-resistant parasites.

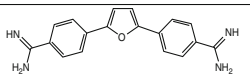
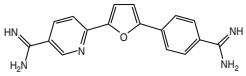
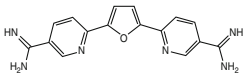
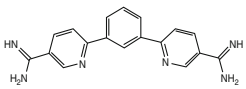
15.5.2 Spiroindolones

The NGBS Consortium consisting of the Novartis Institute for Tropical Diseases (Singapore), the Genomics Institute of the Novartis Foundation (San Diego), the Biomedical Primate Research Centre (the Netherlands) and the Swiss Tropical and Public Health Institute conducted a screen of a natural product library yielding the spiroindolones as a new antiplasmodial class. A lead optimization programme resulted in KAE609, which proved to have excellent oral bioavailability and was curative in the *P. berghei* mouse model at a single dose of 100 mg/kg (Rottmann et al. 2010). Laboratory studies showed that KAE609 rapidly inhibited protein

synthesis in *P. falciparum* parasites suggesting a new mode of action compared to the antimalarials in use. The molecule is in the pipeline of the Medicines for Malaria Venture (MMV) foundation and currently in Phase II clinical trials. It is hoped that KAE609 will become a next-generation treatment possibly with a single treatment dose.

15.5.3 Aromatic Diamidines

The Consortium for Parasitic Drug Development under the leadership of the University of North Carolina (USA) has screened over 2000 new diamidines against *T. brucei* in vitro and many hundreds in mouse models of infection. The diphenyl-furan DB75 (Fig. 15.1) was an initial lead, and its methoxime prodrug DB289 became an oral clinical candidate for first stage human African trypanosomiasis. It passed clinical trials up to Phase III and cured hundreds of patients. In an additional Phase I trial with an extended treatment time, renal toxicity was observed which led to the stop of further development. A backup programme revealed two pyridyl-derivatives of DB75 which were able to pass the blood–brain barrier of mice and to result in the cure of animals with a CNS infection. DB829 (Fig. 15.1) (Wenzler et al. 2013) could cure a second stage monkey model in Kenya with a 5-day treatment schedule at the low dose of 2.5 mg/kg administered intramuscularly. A related molecule, 28DAP010 (Fig. 15.1), was similarly efficacious in CNS-positive mice (Wenzler et al. 2014), but by that time, the programme was stopped because of two molecules with superior characteristics (see Sects. 5.4 and 5.5).

Designation	Structure	IC_{50} in ng/mL	Protein binding in mouse plasma at 1μM	Curative dose i.p. Acute model	Curative dose i.p. CNS model
DB 75		1.2	93.2%	4x 20 mg/kg (3/4)*	No cure possible
DB 820		3.0	79.0%	4x 10 mg/kg 4x 5 mg/kg (3/4)	No cure possible
DB 829		9.0	60.2%	1x 10 mg/kg 4x 5 mg/kg	10x 20 mg/kg
28DAP010		7.5	73.7%	1x 10 mg/kg 4x 5 mg/kg	10x 20 mg/kg

* This treatment schedule only cured 3 out of 4 mice

Fig. 15.1 Selected trypanocidal diamidines, their structures, in vitro and in vivo efficacy and protein binding

15.5.4 Nitroimidazoles

Nitroimidazoles are an old class of antimicrobials. Metronidazole and tinidazole are examples of existing drugs for intestinal protozoa, and megazole is a potent molecule in animal models of HAT (Bouteille et al. 1995). It was never developed for this indication because of its mutagenic potential. The Drugs for Neglected Diseases initiative (DNDi) revisited the nitroimidazoles and provided its partner, the Swiss Tropical and Public Health Institute, with over 800 such molecules. The in vitro screening revealed 35 active nitroimidazoles which were further tested in mouse models of acute and CNS infection (Fig. 15.2). In this way fexinidazole was rediscovered, originally under development as a broad-spectrum antimicrobial agent by Hoechst AG without making it to human trials. Despite a positive Ames test, it is not mutagenic for mammalian cells. Fexinidazole has a good oral absorption and is rapidly metabolized to a sulfoxide and further to a sulfone (Torreele et al. 2011). While fexinidazole disappears within hours from the plasma of the host, the metabolites reach high plasma levels, especially the sulfone. All three molecules are able to pass the BBB. Mice with a CNS infection of *T. b. rhodesiense* and *T. b. gambiense* could be cured with a 5-day treatment at 100 mg/kg given orally twice daily. Safety was assessed in healthy volunteers, and daily doses up to 3600 mg were well

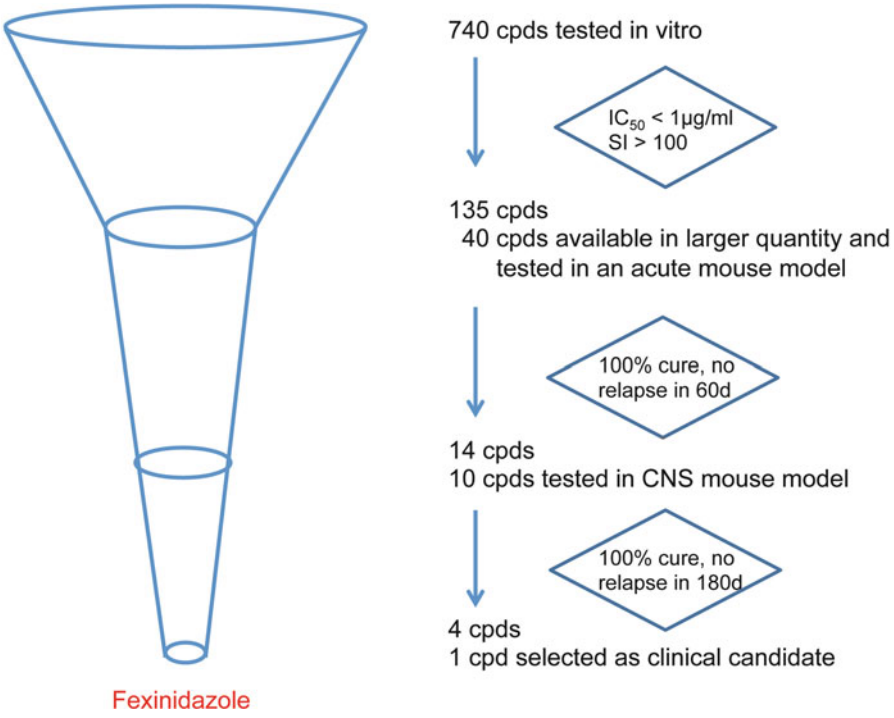


Fig. 15.2 The identification of fexinidazole from a set of 740 nitroimidazoles as an example for a straightforward cell-based drug discovery programme for HAT

tolerated (Tarral et al. 2014). Over 200 patients have already been treated for 10 days successfully; however, cure can only be declared after a follow-up of 12–18 months.

15.5.5 Benzoxaboroles

The benzoxaboroles are a new class of antimicrobial compounds synthesized by Anacor Pharmaceuticals (Palo Alto, CA, USA). These small molecules contain a boron atom and show remarkable activity against a variety of protozoan parasites. Their pronounced activity against African trypanosomes encouraged a lead optimization programme in collaboration with DNDi to develop an oral drug against second stage human African trypanosomiasis. The effort to improve CNS penetration and pharmacokinetic properties was successful and resulted in SCYX-7158 as a clinical candidate. It has a good oral bioavailability, is fast acting and cures mice with a CNS infection with an oral 7-day treatment at 12.5 mg/kg (Jacobs et al. 2011). Brain levels in mice are almost as high as plasma levels and far above therapeutic concentrations. Toxicity and ADME studies did not reveal any liabilities. SCYX-7158 passed Phase I clinical trials successfully and will soon be administered to sleeping sickness patients in studies executed by DNDi and partners.

15.5.6 Drug Development for Visceral Leishmaniasis

DNDi has today the most advanced pipeline for new drugs for visceral leishmaniasis (VL). Fexinidazole, a clinical candidate for HAT currently in Phase III studies in the Democratic Republic of Congo, showed also promising activity against *L. donovani* in vitro and in a mouse model of infection. A Phase II proof-of-concept study is ongoing with fexinidazole in VL patients in Sudan and will be expanded, if successful, to other countries in South Asia, East Africa and South America. Another nitroimidazole, VL-2098, was selected based on positive efficacy, safety and pharmacokinetic studies for further preclinical investigations. If the results are satisfactory, then a new clinical candidate might be available for further development.

The oxaboroles, having yielded a clinical candidate for HAT, are also being exploited for use against VL. One lead oxaborole has shown excellent efficacy in a hamster model of *L. infantum* infection and is followed up as preclinical candidate.

The oligodeoxynucleotide CpG D35 is an immune modulator that reduced the skin lesions in treated macaques challenged with *L. major,* thus demonstrating immunoprotective activity (Puig et al. 2006). The idea is to combine it with a therapeutic drug to treat post-kala-azar dermal leishmaniasis and cutaneous leishmaniasis.

The antitubercular drug PA-824 (a nitroimidazopyran) occurs as two enantiomers. While the (S)-enantiomer is the active form for tuberculosis, the (R)-enantiomer

showed excellent in vivo efficacy in a murine model of VL after a 5-day oral administration of 100 mg/kg twice daily. In vitro drug combination experiments showed an additive effect with fexinidazole which could be seen as an indication for a combination therapy of (R)-PA-824 and fexinidazole.

15.5.7 Drug Development for Chagas Disease

Also for Chagas disease, DNDi holds the most interesting pipeline with several projects in development. Fexinidazole and the oxaboroles, drug development candidates for HAT and VL, also show promise for Chagas disease. The good safety profile of fexinidazole combined with its efficacy in acute and chronic rodent models of Chagas disease encouraged the launch of a proof-of-concept study in Chagas patients using different oral dosing regimens. Efficacy and safety are the primary measure and will determine whether further studies are being undertaken. Among the oxaboroles three molecules produced promising results in a mouse model of infection. Further profiling in laboratory animals will be necessary before clinical studies can be envisaged.

Clinical trials with two azoles, posaconazole and E1224 (a prodrug of ravuconazole) resulted in disappointing results with treatment failures reaching 70–90 %, while the standard drug benznidazole had a much lower failure rate (<30 %) (Chatelain 2015). While posaconazole dropped out as clinical candidate, E1224 is being followed up as a combination partner with benznidazole in a Phase I trial for pharmacokinetic interaction and safety.

Conclusions

The gap in translational research for new drugs for tropical parasitic diseases which opened up in the 1990s was successfully bridged by product-development-partnerships (PDPs). This new model brings together partners from academic, private and governmental institutions and funding from large foundations and governments. Enormous progress could also be made with the identification of new drug targets through comparative genomics; however, most targets still have to be validated. This approach has so far not translated into new drugs despite its great potential. An excellent drug pipeline is available for malaria where a constant flow of new drugs is needed to overcome drug resistance which inevitably develops against new molecules after a few years in use. Also for human African trypanosomiasis, the future is bright with two oral drug candidates in the pipeline. If one of the two candidates makes it to the market, then this will add the required tool for elimination which is envisaged by WHO and the Bill & Melinda Gates Foundation (Brun et al. 2011; Holmes 2014). Greatest need for new drugs among the protozoan diseases is for Chagas disease. After the failure of new azoles (posaconazole and ravuconazole), the pipeline for Chagas is very weak and leaves a gap of new treatment options for the chronic stage of disease.

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Vaccine Development

16

Julie Healer and Alan F. Cowman

Abstract

Parasitic protozoa exact an enormous toll on human life, with immediate medical and economic consequences. The heaviest burden of these diseases is borne by the poor, and it is recognised that they contribute to global poverty. Although most infections are treatable with drugs, these are often expensive and toxic and require multiple doses and induction of resistance problematic. Vaccines are recognised as highly cost-effective public health tools and are the only means by which global eradication of any disease has been achieved. Despite the urgent need, no vaccine is available against any human parasite. Reasons for this include the relative complexity and different species of the causative organisms compared with most viruses and bacteria; their cryptic habitats, often intracellular and inaccessible to immune mediators; a lack of understanding of the mechanisms conferring immune protection; and the lengthy and costly process of developing any therapeutic, particularly when the target population includes pregnant women, babies and young children.

This chapter outlines some of the promising avenues for vaccine development against the most widespread and serious pathogenic protozoa, *Trichomonas*, *Leishmania* and *Plasmodium*. Whatever the species, there are three main vaccine types aimed at inducing protective immunity. These are whole cell, DNA and subunit vaccines, and we describe some of the candidates currently being assessed as well as the challenges facing researchers in this field.

J. Healer
Walter and Eliza Hall Institute of Medical Research,
1G Royal Parade, Parkville, Victoria 3070, Australia
e-mail: healer@wehi.EDU.AU

A.F. Cowman (✉)
Head, Division of Infection and Immunity, The Walter and Eliza Hall institute of Medical Research, 1G Royal Parade, Parkville, VIC 3051, Australia

Department of Medical Biology, Faculty of Medicine, Dentistry and Health Science,
The University of Melbourne, Parkville, VIC 3052, Australia
e-mail: cowman@wehi.edu.au

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16.1 Introductory Remarks

Vaccines function by the induction of immunological memory against specific antigens to mount a protective effector response to infection by a pathogenic organism that prevents its establishment or replication in the host and causing a disease state. Following the discovery that microorganisms were the causative agents of disease, Louis Pasteur introduced the principles of vaccinology, proposing the isolation, inactivation and injection of pathogenic organisms for disease prevention. These basic rules have been followed with much success, leading to the control of some of the world's most devastating infectious diseases.

Over the past 20 years, with the publication of the genomes of many pathogenic organisms, as well as that of humans, the emergence of recombinant DNA technology and a greater understanding of the complexity of the human immune system together with the accessibility of computer technology have all led to a more systematic vaccine development approach using bioinformatics and without the requirement for pathogen growth and isolation. This reverse vaccinology approach (Rappuoli et al. 2002) has been successful in the production of several antibacterial vaccines that were previously refractory to development using traditional methods.

Compared with most viruses and bacteria however, even the simplest protozoan parasites have complex life cycles, mostly involving sexual reproduction with associated exchanges of genetic material that result in antigenic diversity. Also, there is often differential gene expression during successive life cycle stages, with different antigens presented to the host during the course of infection. Furthermore, to add to the antigenic diversity, many parasite species that infect humans are comprised of immunologically distinct strains, and some, such as trypanosomes, *Giardia* and *Plasmodium*, use antigenic variation as a means of evasion of detrimental immune responses. Antigenic complexity is one reason why there has not yet been a single antiparasitic vaccine marketed for use in humans. Other challenges include limited understanding of the exact mechanisms behind protective immunity, which can be compounded by the host-specificity of the pathogen, often ruling out animal models of the development of immunity.

Depending on which niche a parasite species exploits within the host, it will have evolved complex mechanisms of immune evasion to enable its persistence. Whether it is an extracellular parasite circulating in the bloodstream bathed in a ready supply of nutrients, such as trypanosomes that constantly and randomly vary surface antigens that are directly exposed to immune effector mechanisms, or resident on a mucosal surface, such as *Trichomonas vaginalis*, or intracellular parasites that exploit self-/non-self immune surveillance such as *Leishmania* and *Plasmodium* species, each has adapted to its particular niche environment in different ways, which require individually designed vaccines that induce specific immune responses to counter each infection.

It is outside the scope of this overview to describe all of the current vaccine candidates and strategies in development against even a single parasite species such as *Plasmodium falciparum*, never mind all of the protozoan parasite species of humans, but we will describe some of the recent advances in vaccines to tackle important diseases causing the highest mortality and morbidity and the most common infections of humans.

16.2 Recent Advances

16.2.1 A Vaccine Against Sexually Transmitted Infection?

Trichomonas vaginalis ranks as the most common curable and non-viral sexually transmissible infection (STI) worldwide and is among the most common protozoan infections of humans in industrialised countries (Soper 2004). Due to a lack of awareness and screening programmes, this pathogen is most often unreported and undetected in millions of people globally. Despite equal numbers of men and women infected, women bear the brunt of the disease, with around ten times higher disease prevalence than men. Even so, women often remain asymptomatic and transmit the infection to sexual partners, sometimes even with the use of condoms (*Trichomonas* fact sheet CDC 2007). The symptoms of trichomoniasis can vary from mild to severe, with effects on foetal development, infertility, increased mortality and a predisposition to HIV infection, AIDS and cervical cancer. Recent research suggests a link between male *Trichomonas* infection and aggressive prostate cancer (Stark et al. 2009).

Despite improvements over the past decade, diagnosis of *T. vaginalis* is difficult and unaffordable for most low-income countries with the highest burden of disease. A rapid point of care test is available, but it is restricted to women, and sensitivity is low. The current treatment is antibiotics, but resistance is spreading and reinfection is common. A vaccine would be a cheap, easily administered prophylactic to reduce or prevent incidence and would be of particular use in low-income settings. Towards this goal, a murine model has been established using vaginal infection and significant protection induced with a live, whole-cell vaccine administered with Freund's adjuvant. Using Alhydrogel, an FDA-approved adjuvant, this live cell vaccine reduced infection and induced parasite clearance (Smith and Garber 2014), an encouraging start on the road to vaccine development. Since the publication of the *T. vaginalis* genome in 2007 (Carlton et al. 2007) and subsequent proteomic studies reviewed by Hirt et al. (2011), several antigens have been suggested as potential subunit vaccine candidates, such as lactoferrin-binding protein (see below) (Sehgal et al. 2012) and a 63KDa cysteine protease whose activity is required for host cell recognition and adhesion. Immunisation of mice with this antigen elicited specific IgG in serum and IgA in the vaginal tract and was associated with enhanced elimination of *Trichomonas* following vaginal challenge (Hernandez et al. 2005). A study in the bovine model *T. foetus* identified a heavily glycosylated surface antigen (Corbeil et al. 2001). In a study comparing the outcome of pregnancy in infected women, those with significantly higher vaginal IgG level to the *T. vaginalis* antigen, *T. vaginalis* lipophosphoglycan (TvLPG) were more likely to have a normal pregnancy than those with lower IgG levels (Bastida-Corcuera et al. 2013). Each of these candidates warrant further investigation – lactoferrin-binding protein is an essential component in iron-scavenging which in turn is very important in *T. vaginalis* survival. *Trichomonas*, like other amitochondriate protozoa (e.g. *Giardia*, *Entamoeba*), requires a higher extracellular iron concentration (50–200 uM) compared to other eukaryotic cells (0.4–4 uM) (Weinberg 1974). To protect against invading pathogens, the mammalian host

has evolved scavenging mechanisms to limit the availability of iron, which for *Trichomonas* is at mucosal surfaces and becomes more readily available during menstruation. Lactoferrin is an extracellular glycoprotein and part of the innate immune system; however, pathogens can obtain iron from host lactoferrin (Weinberg 1974). *Trichomonas* binds iron directly from host lactoferrin in a surface protein receptor-mediated fashion. Approximately 90,000 of these molecules are present on each parasite surface (Lehker and Alderete 1992), making these proteins attractive vaccine candidates by virtue of their surface localization and their essential role in parasite viability. Interestingly, as another immune evasion strategy, *Trichomonas* has also co-opted surface-bound lactoferrin as a means of preventing complement-mediated lysis (Alderete et al. 1995). It has been shown that *T. vaginalis* can degrade immunoglobulin, and so targeting an Ig-degrading protease may disable a crucial immune escape mechanism of the parasite; however, any successful vaccine must mediate parasite clearance and not simply reduce symptoms, since this may exacerbate transmission of the parasite.

16.2.2 Challenges in Development of a *T. vaginalis* Vaccine

Translation from animal model to human clinical trial is an expensive process and the fact is that most vaccines fail to progress past Phase 1/2a efficacy trials. This is a particular challenge for *T. vaginalis* because of its extracellular (mucosal surface) site of infection. A more detailed understanding of the immunology of the genital tract would be of much benefit to STI vaccine design, and perhaps much can be learned from the successful anti-STI vaccines, Cervarix and Gardasil, which protect against cervical cancer caused by HPV, about induction of appropriate protective immune responses (Einstein et al. 2014; Schiller and Lowy 2006).

16.2.3 Leishmaniasis

Leishmaniasis is caused by 20 species of *Leishmania* parasites, each causing one of three main disease manifestations with different geographic distributions. Around thirty species of sandflies (*Phlebotomus* spp. in the old world and *Lutzomyia* spp. in the new world) transmit the extracellular promastigotes that are phagocytosed by human macrophages wherein they transform into amastigotes that replicate and are released upon host cell rupture (Pearson and Sousa 1996; Sacks and Kamhawi 2001). Sandflies can also transmit the parasites to zoonotic reservoir hosts such as dogs and rodents (Alvar et al. 2004; Reithinger et al. 2007). There are approximately 1.5–2 million new cases of leishmaniasis per year (Alvar et al. 2012), and visceral disease (or Kala Azar) that infects the splenic, liver and bone marrow macrophages, if left untreated, can be fatal (Kumar and Engwerda 2014), making leishmaniasis the second most lethal parasite infection after malaria. The current control measures rely on drugs with severe toxic side effects and increasing development of drug resistance (Croft et al. 2006) (see Chap. 7 for an extensive introduction to *Leishmania*).

There is a strong rationale for vaccine development against *Leishmania* since people cured of infection develop life-long immunity. Historically, the process of leishmanization was used in countries of Southern Europe and the Middle East, by which uninfected people were inoculated with live parasites from skin lesions, but this has been mostly abandoned due to safety concerns (McCall et al. 2013). Killed or live-attenuated parasites were used in the first generation of manufactured *Leishmania* vaccines; however, these failed to induce protective immunity. Most vaccines against human pathogens are designed to induce high antibody levels that prevent infection or block activity of toxins; however, antibodies alone are not effective against *Leishmania*. Second-generation vaccine design has turned towards genetically modified *Leishmania*, DNA vaccines (see below for more details) and recombinant subunit vaccines as protein in adjuvant delivery systems (Kumar and Engwerda 2014). Although a number of *Leishmania* recombinant antigens have been tested in mice, only a few have progressed to clinical trials in non-human primates or dogs or to preclinical studies in humans (Kumar et al. 2010; Singh et al. 2012). Other examples of recombinant antigens yet to be validated include sterol 24-c-methyltransferase (Goto et al. 2007), amastigote-specific protein A2 (Ghosh et al. 2001), K26 hydrophilic acidic surface protein B (HASPB) (Stager et al. 2000), Leishmania-activated c-kinase (Benhnini et al. 2009), promastigote surface antigen 2 (Handman et al. 1995), nucleoside hydrolase (Al-Wabel et al. 2007) and surface glycoprotein gp63 (Connell et al. 1993). The first defined recombinant vaccine to progress to human phase I/II clinical trials is Leish-111 F, a multisubunit vaccine containing the *L. major* stress-inducible protein 1, a homologue of eukaryotic thiol-specific antioxidant along with *L. brasiliensis* elongation and initiation factor. In preclinical studies, Leish-111 F provided protection against CL and VL in mice, but failed to protect against canine VL with *L. infantum*; nevertheless it has been tested in both healthy volunteers and leishmaniasis patients in several endemic countries (Chakravarty et al. 2011; Llanos-Cuentas et al. 2010; Nascimento et al. 2010; Velez et al. 2009).

Challenges in *Leishmania* vaccine development include:

- Intracellular habitat that is inaccessible to antibodies, thus relying on T-cell immunity
- Efficient presentation to T cells by diverse HLA molecules on the surface of antigen-presenting cells
- Antigenic diversity of multiple species and strains

The HLA-restricted mode of antigen recognition requires previous experience of the immunogen and its introduction into processing pathways in antigen-presenting cells (APCs). This is particularly important in virulent infections where rapid and high-frequency responses are required of effector T cells to bring the infection under control. Nonetheless, a large pool of potential vaccine candidates have emerged from preclinical studies in animal models (Sundar and Singh 2014), although none have yet proven successful in clinical trial.

A novel approach that has successfully induced protective T-cell responses in mice, combined sequences of five candidate antigens derived from homology-screening

against human strains, and were immunogenic in individuals cured of leishmaniasis or who are asymptomatic for the disease (Chakravarty et al. 2011; Das et al. 2014). These antigens were chosen from a shortlist of candidates selected on the basis of their reported induction of effector T cells in either humans or animal models and include a 92 amino acid (AA) peptide from the kinetoplastid membrane protein (KMP) 11 (Basu et al. 2007; Bhaumik et al. 2009), 199 AA peptide from the thiol-specific antioxidant antigen (TSA) (Chakravarty et al. 2011), 354 AA from cysteine protease A (CPA) (Rafati et al. 2006) and a 160AA region of CPB as well as 449 AA sequence from the elongation factor 1-α (P74) (Chenik et al. 2006). The sequences encoding these antigen peptides were combined in MIDGE (minimalistic immunogenically defined gene expression) DNA construct vaccine known as LEISHDNAVAX engineered to specifically induce TH1 cells at high frequency (Das et al. 2014). This strategy exploits the utility of DNA vaccines to address the issues of HLA polymorphism and parasite strain diversity through rapid sequencing and selection of immunogenic conserved peptide epitopes and has the potential to adapt to changing host-parasite landscapes through the opportunity to easily introduce or switch out sequences as necessary to cope with emerging strains or tolerance among immunised individuals. A recent publication reports favourable safety and tolerability in a preclinical study in rodents (Riede et al. 2015), paving the way for application to regulatory bodies for a clinical trial in humans.

For a more comprehensive review of current vaccines in development against *Leishmania*, see *further reading*, Jain & Jain Vaccines for visceral Leishmaniasis: a review.

16.2.4 Malaria

As the parasitic disease that poses the greatest global public health problem, it is arguably correct that malaria has received by far the most research attention and funding of all anti-protozoan vaccines. We are on the cusp of seeing the first licensed vaccine for use against any human parasitic organism, with the RTS,S (or Mosquirix) vaccine against malaria (RTS 2015), developed by GSK in partnership with the Malaria Vaccine Initiative and the Bill and Melinda Gates Foundation likely to be approved within the next year.

Formulated with the hepatitis B surface antigen (S), RTS,S is a recombinant vaccine produced as a virus-like particle in yeast, which contains a repeat (R) region and T-cell epitope (T) from the circumsporozoite (CSP) protein present on the surface of the parasite form injected by mosquitoes (see Chap. 9 for details of the different forms in the life cycle of *Plasmodium*). RTS,S is the first malaria vaccine to undergo phase III evaluation in Africa (RTS 2014). The randomised, controlled, double-blind Phase III efficacy trial of RTS,S enrolled 15,460 children in two age groups in seven countries in sub-Saharan Africa: group (1) 5–17 months at first immunisation without co-administration and group (2) 6–12 weeks at first immunisation in co-administration with DTwP/HepB/Hib and OPV. The follow-up period was >30 months following the third dose of RTS,S/AS01. The three intramuscular doses were given in 1-month intervals followed by an 18-month booster dose in one

of the three trial arms. The control groups received rabies vaccine (group 1, 5–17-month-olds) and meningococcal C conjugate vaccine (group 2, 6–12-week-olds). The trial occurred in the context of insecticide-treated bednet (ITN) use by 80 % of trial participants.

Vaccine efficacy at the 18-month postvaccination follow-up was 46 % against clinical malaria in the older age group, but significantly lower in the infant group (27 %), and this was associated with significantly lower antibody titres (ELISA units) in the younger cohort. In this trial, an average of 829 and 449 malaria cases per thousand children and infants respectively were averted by RTS,S. However, a concerning outcome of the trial was that meningitis, a serious adverse event, occurred more frequently in the vaccine group. The immune mechanism by which RTS,S protects vaccines is by induction of high titres of antibody that protect against sporozoite infection of hepatocytes (Foquet et al. 2014), thereby reducing the load of infectious merozoites that cause the pathogenic blood stages of malaria (Campo et al. 2015). Lessons from the RTS,S progression from antigen discovery of CSP (Nussenzweig and Nussenzweig 1989) through to clinical trials is that its development has been lengthy and iterative (Cohen et al. 2010), with protection dependent on strong adjuvants.

This vaccine as it stands would undoubtedly provide significant cost-effective reductions in the malaria burden across Africa, but falls somewhat short of the landmark 80 % efficacy for 4 years by 2025 called for in the malaria vaccine technology roadmap (see *further reading: WHO immunization*). The 2013 update of this document calls for prioritisation of vaccine development against *P. falciparum* and also *P. vivax*, which has been somewhat neglected by vaccinologists to date. Of the 30 malaria vaccines in clinical development, only two target *P. vivax* antigens (Table 16.1). A number of explanations are posited for this dearth of research into vaccines against *P. vivax*; the assumption that this species causes a benign form of malaria has been challenged (Mendis et al. 2001; Rogerson and Carter 2008; Price et al. 2009); slow progress on in vitro culture systems (Noulin et al. 2013) has certainly impeded discovery of new candidates, as has the cost of product development (Reyes-Sandoval et al. 2010); therefore, the new strategic goals of the WHO and the malaria vaccine community in general can only lead to a greater understanding of the biology of this parasite and therefore to its control.

16.3 Perspectives and Open Questions

A common roadblock to vaccine development against all protozoan organisms appears to be the lack of a clear understanding of immune correlates of protection, since those derived from testing in small animal and even non-human primate models often cannot be extrapolated to humans. Due to limited success of subunit or DNA vaccines, a return to the attenuated whole organism approach appears to offer a wider range of benefits, including the numbers of antigens exposed to the immune system and induction of a wider range of potentially protective responses, and it is hoped that these will lead to a more efficacious vaccine. Of particular concern for such vaccines however is the potential for induction of pathogenic responses,

Table 16.1 Global malaria vaccines in clinical development

Phase 1a	Phase 2a	Phase 1b	Phase 2b	Phase 3
ChAd63/MVA ME-TRAP+ MatrixM[54] (University of Oxford)	RTS,S-AS01 delayed fractional dose (GSK)	Ad35.CS01 (NIAID)	ChAd63/MVA ME-TRAP[54-60] (University of Oxford)	RTS,S AS01[61] (GSK)
Polyepitope DNA EP 1300[62] (NIH/NIAID)	Ad35.CS/RTS,S-AS01 (GSK/Crucell/WRAIR)	EBA175.F2[63, 64] (NIAID)	GMZ2[65, 66] (EVI)	
PfCelTOSFMP012[67] (USAMRMC)	Ad35.CS/Ad26.CS[68, 69] (Crucell/SeattleBioMed)	SE36[70] (Osaka University)	MSP3 [181-276][71-75] (EVI)	
CSVAC[76] University of Oxford	NMRC-M3V-D/Ad-PfCA Prime/Boost[77] (NMRC)	PfSPZ[78-80] (Sanaria)		
Ad63.AMA1/MVA.AMA1 +AI/CPG7909[81-83] (University of Oxford)	ChAd63/MVA(CS/TRAP/AMA1) University of Oxford			
SR11.1 (Institute Pasteur)	M3V.D/Ad.PfCA (University of Oxford)			
ChAd63/MVA PvDBP (University of Oxford)	ChAd63 /MVA.AMA1[81-83] (University of Oxford)			
PfAMA1-DiCo[84] (Inserm)	ChAd63/MVA MSP1[85](University of Oxford)			
ChAd63PfRH5±MVA RH5[86-88] (University of Oxford)	FMP2.1-AS01B (AMA1) (USMRMC)			
Pfs25-EPA (NIAID)	*VMP001-AS01B[89](CSP) (WRAIR)*			
Pfs25-VLP (Fraunhofer CMB)				
PfGAP (P52/P36/SAP1)[90] (Seattle BioMed)				
PfSpz-Cvac				

	pre-erythrocytic(P. vivax)		Transmission-blocking
	Blood stage (P. vivax)		Live attenuated sporozoite
	Multistage		

http://www.who.int/vaccine_research/links/Rainbow/en/index.html
www.malariavaccine.org

reversion to an infectious state and the logistics of manufacture and delivery of live vaccines. To address the first issue, there is a necessity for clear biomarkers that can easily determine the immune versus pathogenic or non-immune state.

The most extensively tested live-attenuated malaria vaccine is the Sanaria PfSPZ vaccine, composed of aseptic, purified, cryopreserved, attenuated (non-replicating), metabolically active *Plasmodium falciparum* (Pf) sporozoites (SPZ) (Hoffman et al. 2010). This vaccine provided complete protection against Pf infection challenge with the same parasite strain in 100 % (6/6) volunteers, who received five doses of 1.35×10^5 PfSPZ administered intravenously (Seder et al. 2013). Based on these data, investigators have developed a clinical development plan that maps out a 4–5-year timeline to licensure and a large-scale demonstration project to eliminate malaria from an island population in Africa. Six different clinical trials of PfSPZ vaccine at seven clinical sites in the United States, Mali, Tanzania, Equatorial Guinea and Germany were initiated in 2014. These trials are designed to assess the reproducibility of the data generated in the original small-scale study and to assess and optimise durability of protection, protection against heterologous strains of *P. falciparum*, reduction in numbers of doses and immune assays that predict protection, implementation of immunisation and alternative route of administration. The development of large scale, aseptic, cryopreserved sporozoites for injection has already provided the malaria research community with an immensely valuable tool for assessment of the kinetics of malaria infection and parasite growth rate in individuals of different prior immune status, and the hope is that such studies will continue to shed light on the acquisition of immunity to malaria (Hodgson et al. 2015).

A criticism of the radiation-attenuation approach has been the quality control issue that the relevant gene expression profile is suitably achieved in each sporozoite batch. These concerns have led to the development of a genetic approach to attenuation (GAP), where genes that have been linked to parasite developmental stages in the liver stages in rodent models of malaria are deleted, therefore providing an effective quality control for attenuation of every parasite (Labaied et al. 2007). However, the risk of relying on a single rodent model of infection to inform the necessary developmental gene deletions in *P. falciparum* was highlighted when the first phase 1 clinical trial of P52$^-$/P36$^-$ genetically attenuated sporozoites was terminated, due to a breakthrough malaria infection in one of the trial volunteers receiving the second higher dose of the vaccine via mosquito bite (Spring et al. 2013). A subsequent study of the *P. berghei* P52$^-$/P36$^-$ GAP parasite line demonstrated that some of these parasites develop in the absence of a parasitophorous vacuole membrane (see Chap. 9 for an in-depth description of intra-hepatocytic development of *Plasmodium*), which may explain the breakthrough from the human liver to bloodstream in the first GAP vaccine trial (Ploemen et al. 2012). A second-generation 3KO GAP, with an additional liver-stage gene deletion (Sap1$^-$/P52$^-$/P36$^-$), has been developed without any exogenous drug-resistance marker (Mikolajczak et al. 2014). SAP 1 protein functions in an independent biological role from P52/P36 in regulation of RNA stability and as such improves on the robustness of the attenuation. Phase 1 trials of this next-gen GAP vaccine have commenced,

and with the deletion of *SAP1*, no adverse parasitaemic events occurred (S. Kappe, personal communication, 2015). A second GAP parasite line has been created with deletion of *B9*, a newly identified member of the 6-CYS protein family to which P52 and P36 are members, together with *SLARP*, the gene also known as *SAP 1*. Preliminary studies in hepatocytes and humanised mice indicate that these GAP parasites invade, but full development of liver schizonts does not occur; clinical trials are also planned to confirm the safety and immunogenicity profile of this vaccine (van Schaijk et al. 2014).

There are, however, logistical issues that pose significant challenges to the large-scale deployment of live-attenuated malaria sporozoite vaccines. These include the necessity for a liquid nitrogen cold chain to maintain the viability of the vaccine product, the large numbers of sporozoites that currently must be obtained from infected mosquitoes, incurring significant labour costs, and the intravenous delivery route that has been proven necessary for induction of optimum immunity (Seder et al. 2013). An automated platform for sporozoite removal from infected mosquitoes is in development at Sanaria, which may increase the availability of the large numbers required for large-scale vaccination campaigns. It is hoped that with the support of the vaccine community, these technical issues can be overcome; however, the true value of whole-cell vaccines may be in focusing research on promising candidate antigens that can be cheaply manufactured to GMP standard in large scale, providing a fully protective malaria vaccine that can be offered to everyone at risk.

16.3.1 How the Eradication Agenda Affects Malaria Vaccine Research

In 2007, Bill and Melinda Gates spearheaded the call for a renewal of effort to eradicate malaria (Roberts and Enserink 2007). Although a huge impact has already been made in reducing the global malaria problem using existing tools such as insecticide-treated bednets and mass drug administration (Owens 2015), it is widely held that eradication will require an effective vaccine (Plowe et al. 2009) since the current tools are susceptible to constraints such as insecticide and drug resistance, as well as sustainability concerns over cost and implementation in resource-poor settings. Current thought is that multiple interventions, acting at different points in the parasites' life cycles in humans and mosquitoes, will be necessary to realise eradication (Plowe et al. 2009; Hall and Fauci 2009). The goal of malaria vaccine development until this point had focused primarily on reducing the burden of morbidity and disease, as is partly reflected in the current cohort of candidates in clinical development (Table 16.1), with emphasis on only two blood-stage antigens (MSP1 and AMA1) in various formulations and eight iterations of a CSP-based vaccine. The rationale for development of a blood-stage vaccine was initially based on observations that naturally acquired immunity to *P. falciparum* was associated with antibody responses to blood-stage antigens; transfer of

hyperimmune gamma-globulin is able to reduce the parasitaemia and disease in severely ill children infected with malaria parasites (Cohen et al. 1961; Sabchaeron et al. 1991). The current strategic view for malaria vaccine development is that the eradication agenda will be unmet by a blood-stage-only vaccine, since sterile immunity is unlikely to result from such a vaccine and its impact on transmission to mosquitoes is unknown. A partially protective pre-erythrocytic vaccine (like RTS,S) alone is also unlikely to meet the necessary requirements for eradication, since there is no immune protection afforded against breakthrough blood-stage infection. A necessary component is believed to be one that interrupts malaria transmission, and Pfs25-based vaccines that target the zygote/ookinete stage in the mosquito are furthest along in clinical development, although another two transmission-blocking candidate antigens are also being considered (Pfs230 and Pfs48/48 (Sinden et al. 2012)); with increased funding driving the strategic objective, we can expect an expansion in the number of candidates for vaccines that interrupt transmission, which can also include pre-erythrocytic and blood-stage targets if significant reductions in gametocyte densities result from these.

Decades ago as students of parasitology, we were told that an ideal malaria vaccine would target conserved antigens in multiple life cycle stages, thereby reducing infectious load, morbidity and transmission, and in fact there are three such vaccines in feasibility testing or clinical development (pink boxes in Tables 16.1 and 16.2). A greater understanding of the genetic complexity of *Plasmodium* complicates the development of a vaccine that is broadly protective against genetic variants, but this must be a high priority. However, for eradication, necessary additions will include components of *P. vivax*, *P. knowlesi*, *P. ovale* and *P. malariae.*

Table 16.2 Lead development/optimisation preclinical projects

Vaccine	Formulation
PvDBPII[91, 92] *(ICGEB)*	Protein in adjuvant
PfEBA/Rh[93, 94] (WEHI/Gennova)	Protein in adjuvant
VMP002 CSP (WRAIR)	Protein in adjuvant
Novel B cell targets (Seattle BioMed/JHU/NIAID/NMRC)	
Novel T cell targets (NIAID)	
Multistage Malaria vaccine (R21, ME-TRAP[95], RH5[86,87], Pfs230[96], Pfs25[97]) (Oxford/EVI/ReiThera/UPMC/Novartis/GSK	ChAd63, MVA + protein in adjuvant

Sources: www.malariavaccine.org, WHO rainbow table Jan 2015 additions

16.3.2 Antigen Discovery

Publication of the genomes of *P. falciparum* (Gardner et al. 2002), *vivax* (Carlton et al. 2008) and *knowlesi* (Pain et al. 2008) has presented the research community with an unparalleled opportunity for antigen discovery and prioritisation. It is worth remembering, however, that despite decades of research, few of the existing clinical candidates (Table 16.1) have been ruled out (Doolan et al. 2014). To ensure that rational design of vaccines is adopted over the more traditional empirical methods that we have relied upon to fill the current clinical development pipeline, the vaccine development pathway may be improved upon. Specific developments need to include a deeper understanding of correlates of immune protection, standardisation of assays to expedite the down-selection of candidates, revision of clinical trial protocols to fast-track go/no-go decisions on antigen progression and perhaps, most importantly, a firm commitment from funders for sustainable advancement of research priority areas.

Further Reading

Cohen (2010) From the Circumsporozoite Protein to the RTS,S/AS candidate vaccine (Cohen et al. 2010)
Doolan (2014) IJP Genome-based vaccine design: the promise for malaria and other infectious diseases (Doolan et al. 2014)
Ellis (2010) Blood stage vaccines for *Plasmodium falciparum* (Ellis et al. 2010)
Jain & Jain (2015) Vaccines for visceral Leishmaniasis: a review (2015).
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http://www.who.int/immunization/topics/malaria/vaccine_roadmap/en/
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