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Editors

Amebiasis

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ISBN 978-4-431-55199-7 ISBN 978-4-431-55200-0 (eBook)
DOI 10.1007/978-4-431-55200-0
Springer Tokyo Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014956867

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Preface

Amebiasis is one of the major neglected tropical diseases. Nearly 50 million people worldwide are infected with the pathogen *Entamoeba histolytica*, causing large-scale morbidity and mortality particularly in developing countries. Unfortunately, there is not much awareness about the disease and the pathogen among clinicians, biomedical scientists, and students. In recent years there have been major advances in our understanding of the parasite and the disease as a result of rapid progress in genomic and other biomedical technologies, such as visualization of molecular processes. In order to increase awareness and understanding, an EMBO Lecture Course was organized March 2–3, 2012, in New Delhi, India, as a satellite meeting of an International Conference "Amebiasis: Exploring the Biology and Pathogenesis of *Entamoeba*", March 4–7, 2012, in Khajuraho, India. The major objective of the lecture course was to provide a basic understanding of the pathogen *E. histolytica* and the disease amebiasis. The lecture course was attended by students, researchers, and clinicians from India and a number of other countries around the world. During this lecture course, the lack of a recent publication documenting the current state of knowledge in this field was felt by both faculty and participants. The concept of this book arose after several discussions with participants of the Khajuraho Conference, and the enthusiasm with which this idea was received helped us to make the decision to publish this book.

The book has been planned to document and present new developments in this field in order to help clinicians for better diagnosis and management of patients, researchers for initiating research projects in areas that are poorly understood, and students for updating their knowledge. The most recent book in this area was published more than a decade ago, before sequencing of the *E. histolytica* genome had been completed and published. The genome sequence helped us to understand not only the biology of the organism, but also to identify diagnostic markers along with new drug and vaccine candidates. Subjects covered by the book range from genomics and molecular and cell biology to drug resistance and new drug development. Most of the chapters provide recent information based on the latest publications. Some of the chapters describe critical methods that have helped to develop sophisticated reagents and tools for both fundamental and applied studies.

The contributing authors include almost all the active researchers and clinicians from all over the world. This book will be useful primary material and a valuable source of information for anyone interested in understanding amebiasis, its diagnosis, and its treatment. We believe that this will also be useful to those who are interested in learning about the biology of early branching eukaryotes and protist pathogens. We sincerely hope that young scientists will take up new projects in understanding amebiasis and also will use *E. histolytica* as a model system to study some of the fundamental biological processes. We believe that many of these processes in *E. histolytica* follow novel mechanisms.

Large numbers of colleagues and others have helped to make publication of this book possible. First and foremost are the contributors. Almost everyone we approached readily agreed to contribute and enthusiastically responded to all our demands for submitting on time different versions of their chapters. We are also grateful to all our reviewers. Without their timely and critical comments it would not have been possible for us to get this book published on time. We greatly appreciate the valuable time they spent in getting this book into shape. We are also indebted to Springer for agreeing to publish this book and the large number of their staff for their contributions in different phases, from editing to printing. Particularly, we thank Mr. Kaoru Hashimoto and Ms. Haruka Imai at Springer for their continuous encouragement and assistance. Finally, many members of our laboratories have helped us in editing this book. We would like to acknowledge the contributions of Madhu Baldodia, Somlata, and Reiko Nakagawa in bringing out this book and acceding to our demands beyond the call of duty.

Tokyo, Japan — Tomoyoshi Nozaki
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Contents

Chapter 1
Introduction

Tomoyoshi Nozaki and Alok Bhattacharya

Amebiasis, the result of infection with the protist parasite *Entamoeba histolytica*, is manifested as either commensal or invasive forms of the disease in humans. The majority of infected people do not display any pathology, and the parasite exists as a commensal, continuing to multiply and spread. Only a small fraction of the infected individuals show overt symptoms of amebiasis with invasion in the intestinal tissues or in some extraintestinal sites, such as liver. Therefore, one of the major problems in this field is to understand the mechanisms that make *E. histolytica* invasive in some individuals. *E. histolytica* displays a simple life cycle with two forms, infective cyst and vegetative trophozoites. The cysts are secreted by individuals that harbor the parasite along with fecal material and enter a human through contaminated food and water. Cysts convert into trophozoites in the intestine, and the trophozoites either produce cysts or multiply to produce more trophozoites, or both. Trophozoites can either invade epithelial tissue through the intestinal barrier or enter the circulation by penetrating through the basement membrane, lodging and multiplying in one of the organs, for example, the liver.

A large number of cellular and molecular processes are involved in the survival and pathogenesis of *E. histolytica*. In this book, a number of chapters describe many of these processes and give insight into the mechanisms. The genomes of *E. histolytica* and many other *Entamoeba* species have been recently explored. Moreover, many isolates of *E. histolytica* have been sequenced using "next-generation sequencing" (NGS) platforms. C. Graham Clark and Rune Stensvold describe genetic and genomic variations among different *Entamoeba* species and suggest that continuous future discovery of new divergent *Entamoeba* species will improve our understanding of this important group of anaerobic single-celled eukaryotes (Chap. 2). In the chapter by

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_1

Gareth Weedall (Chap. 3), the results and analysis from these genome projects are summarized and the relationship between genome variations and virulence potential elaborated. They point out the importance of studying genetic polymorphisms at the whole genome level. Over the years a number of methods have been developed that are useful in *E. histolytica* genotyping. After genome sequencing was completed and published, newer methods were developed for *Entamoeba* genotyping taking advantage of sequence information. Carol Gilchrist and Ibne Ali describe two different approaches for genotyping, the multilocus sequence typing system (Chap. 4) and the STR-based genome typing (Chap. 5). These methods are useful in typing large number of isolates and identification of pathogenic genotype.

The availability of NGS technology has not only helped to understand genome organization and genome-wide variations but also provides a powerful technique to qualitatively and quantitatively analyze total mRNA and small RNA transcriptome (RNA-seq). RNA-seq is particularly useful to identify expressed genes, introns, and transcription start sites. If the studies are done under different conditions, changes in gene expression can be measured and correlated with the biology of the organism. These concepts are elaborated in the chapter by Chung-Chau Hon and Nancy Guillen et al., in which the methodology and results from RNA-seq of *E. histolytica* are described (Chap. 7). Their analysis clearly shows the occurrence of a large number of splicings through a stochastic process. Small RNAs, such as microRNAs and siRNAs, have revolutionized our concept of gene regulation and helped to shift the paradigm from protein centric to both RNA- and protein-based regulatory networks. Typical microRNAs are found in higher eukaryotes and may not be present in *E. histolytica* as some of the genes involved in its biogenesis are thought to be missing. However, *E. histolytica* expresses a class of 27 nucleotide novel RNAs. Laura Morf and Upinder Singh summarize current knowledge about generation and functions of these small RNAs and describe the regulatory role of these small RNAs in *E. histolytica* gene expression (Chap. 9). They also show that the machinery for biogenesis of these RNAs can be useful in gene silencing.

Many systems such as *Entamoeba* are not amenable for genetic studies because of their complex life cycle, biology, and the culture conditions used for their cultivation. Therefore, methods are needed for introduction of genes from outside so that alternate techniques, such as RNAi and the dominant-negative approach, can be used for genetic analysis. David Mirelman and Rivka Bracha summarize all the studies that have helped to develop methods for introduction of genes and their use in downregulation of specific gene expression in *E. histolytica* (Chap. 6). These methods have helped in our understanding of some of the molecular processes in this organism. For example, it has been possible to investigate in detail the molecular mechanism of rRNA transcription. The chapter by Abhishek K. Gupta and Sudha Bhattacharya explores this area and gives a glimpse into "how gene expression is regulated in this organism" (Chap. 8). In particular, they point out generation of novel circular RNAs as part of processing intermediates of rRNA precursors. Circular noncoding RNAs have recently been observed in metazoans, and their importance in gene regulation is being highlighted. Vijay Pal Yadav and Sudha Bhattacharya summarize our current understanding of retrotransposable elements

of *E. histolytica* (Chap. 10). These elements constitute a substantial part (about 6 %) of the *Entamoeba* genome and are likely to be important for their biology. This chapter also includes mechanism of transcription and expansion of these elements in the genome. Epigenetic mechanisms are attracting increasing attention as a major regulatory key to different biological processes, such as gene expression and development. In *E. histolytica*, a methyl transferase that modifies tRNA has been identified and studied in relationship to environmental changes (Chap. 11). Preferential modification of a retrotransposable element has also been observed, and a protein that recognizes methylated cytosine has also been identified by Michael Kirschenbaum and Serge Ankri. DNA replication constitutes one of the fundamental processes involved in cell proliferation. A number of enzymes participate in the process so that replication is initiated and terminated at the right time, maintaining fidelity and control. One way to study replication is to understand the participating enzymes. Guillermo Pastor-Palacios and Luis Brieba et al. describe some of the DNA polymerases of *E. histolytica* in their chapter (Chap. 22) and attempt to correlate the structural and biochemical properties with replication machinery.

Cell biology of *E. histolytica* has been extensively studied because many of the cellular processes are intimately related to pathogenesis, such as phagocytosis and movement. *E. histolytica* displays an extensive signaling system made up of a large number of kinases, calcium-binding proteins, and GTP-binding proteins. A combination of these molecules appears to control and co regulate different cellular processes, such as stress response, phagocytosis, and motility. Somlata and Alok Bhattacharya summarize our current understanding about the mechanism of phagocytosis in *E. histolytica*. The molecular process of initiation and formation of phagocytic cups appears to be distinctly different in this organism compared to other eukaryotic systems; however, the subsequent steps involving actin dynamics, pseudopod movement, and scission are likely to be similar (Chap. 12). Direct participation of calcium-binding proteins in actin dynamics has been seen so far only in this organism. Further, Saima Aslam, Shahid Mansuri, and Alok Bhattacharya describe the cellular signaling pathways, highlighting those that are mediated through Ca^{2+} (Chap. 13). They also point out the involvement of other second messengers, such as cyclic AMP in amoebic signaling systems, although not much work has been done to understand the involvement of second messengers other than Ca^{2+}. Protein kinases constitute the major part of the signaling proteome of *E. histolytica*. Different classes of protein kinases make up the kinome, and in this respect the *E. histolytica* kinome is similar to other eukaryotic systems. Transmembrane protein kinases (TMK) represent one of the largest families of the kinome and have been studied more extensively compared to other kinases. TMKs are single-pass membrane proteins containing both extracellular and intracellular domains that can be classified into nine families and are thought to be involved in phagocytosis, proliferation, and virulence. Nathaniel Christy and William A. Petri describe our current understanding on the structure and function of this large group of protein kinases (Chap. 14). Regulated trafficking and secretion of pathogenic factors, such as cysteine proteases, has been extensively studied and is summarized by Kumiko Nakada-Tsukui and Tomoyoshi Nozaki (Chap. 17). During survival in different

niches of the host body, such as the intestinal lumen, liver, lung, and brain, as well as the external environment, *Entamoeba* must acclimate itself to a variety of stresses. Thus, stress-sensing mechanisms are important to study, particularly in relationship to pathogenesis and cell death. Daniela Faust and Nancy Guillen describe a newly characterized global stress-sensing mechanism in *Entamoeba* and elaborate on their importance in amoebic biology (Chap. 15). Regulation of cell cycle and cell division is promiscuous in *Entamoeba*. The unusual mechanism of cell-cycle control generates polynucleated cells. Our current view of the mechanism is described by Jaspreet Singh Grewal and Anuradha Lohia (Chap. 16). *Entamoeba* does not reveal discernible organelles in a form typical for other eukaryotes. The mitochondria have become highly divergent from that of other aerobic eukaryotes and are referred to as mitosomes. Although mitosomes do not have any DNA, they still have unique functional features and have been retained by the cell. Takashi Makiuchi, Fumika Mi-ichi, and Tomoyoshi Nozaki (Chap. 18) describe the mitosomes with respect to the components and their participation in different functions, particularly in protein transport mechanisms.

As a consequence of reductive evolution and secondary loss of aerobic respiration in mitosomes, *Entamoeba* relies solely on glycolysis and fermentation for ATP generation. Thus, understanding glucose metabolism and its regulation is essential for understanding the biology and pathophysiology of *Entamoeba*. This topic is summarized by Erika Pineda and Emma Saavedra et al. (Chap. 20). To comprehensively understand metabolism, combined omics approaches, such as transcriptomics and metabolomics, are becoming essential. Ghulam Jeelani, Dan Sato, and Tomoyoshi Nozaki summarize new key findings on metabolism using these integrated approaches (Chap. 19). Moreover, similar omics-based strategy can also help to identify new pathways involved in differentiation of trophozoites into cysts and the mechanism that initiates and regulates this process. Amino acid metabolism, particularly sulfur-containing amino acid metabolism in *Entamoeba*, is remarkably different from that in the hosts, thus providing a rationale approach for drug discovery. This aspect is clearly evident from the chapter by Isha Raj and Samudrala Gourinath et al. that highlights structural biology-based analyses of the two major enzymes of the cysteine/*S*-methylcysteine biosynthetic pathway and their usefulness in rational drug design (Chap. 21).

It is not clear if the immune system plays any role in initiation and development of invasive amebiasis. This question has been addressed in a number of chapters in which the authors have described roles of different host factors including different arms of the immune system in amebiasis. A large number of parasite and host factors contributes to amoebic pathogenesis, and some of the key questions, such as why only a fraction of infected people have invasive disease and why some people have liver infection, have not yet been satisfactorily answered. Overall pathogenesis in relationship to amebiasis is reviewed by Mineko Shibayama and Victor Tsutsumi (Chap. 23). Innate and acquired immunity against amebiasis and the pathology of amebiasis have been extensively studied. Leanne Mortimer and Kris Chadee describe innate host immunity and defense in the intestine (Chap. 24). Because mucins play a critical role during the entry of parasites from the intestine, the

destruction of the mucin layer can act as an accessory in establishing invasive disease, as highlighted by V. Kissoon-Singh, E. Trusevych, and K. Chadee (Chap. 27). Cysteine proteinases have been implicated in the pathology of a number of infectious diseases, and ever since their discovery in *E. histolytica* cysteine proteases (CP) have been thought to have an important role in invasion, cytolysis, and tissue destruction in amebiasis. *E. histolytica* has a large repertoire of CPs, and their role are described in two chapters by Iris Bruchhaus and Jenny Matthiesen (Chap. 25) and Elena Helk, Hannah Bernin, and Hanna Lotter (Chap. 26).

Infection by *Entamoeba* results in a range of outcomes from drastic symptoms, such as dysentery and liver abscess, to no symptom. Outcome of infections depends in part on the host genotype, which regulates the nature of the host–parasite relationship. So far, only parasite factors have been investigated; efforts have been made recently to also understand host factors. Shannon N. Moonah, Nona M. Jiang, and William A. Petri, Jr. summarize many studies on the role of human genetic polymorphism on susceptibility to amebiasis. They describe various polymorphisms of leptin receptor and their association with disease outcome (Chap. 28). Contributions of HLA polymorphism are highlighted by Cecilia Ximenez et al. (Chap. 29).

Metronidazole has been a drug of choice against amebiasis for decades. Unfortunately, there is no major second-line drug at present, and there is concern that widespread emergence of drug resistance will cause a major public health problem worldwide. Therefore it is important to study metronidazole action, resistance, and new drug development. Michael Duchene summarizes our current view on the mode of action of metronidazole and its relationship to redox regulation affected by the drug in *Entamoeba* (Chap. 30). Development of potential new drugs is covered in a number of chapters, and it appears that it will be possible to develop alternate drugs very soon. Rosa M. Andrade and Sharon Reed describe a new drug target, thioredoxin reductase, identified using a high-throughput technology (Chap. 31). Anjan Debnath summarizes the discovery of a new anti-amebic drug among FDA-approved molecules (Chap. 32), and a description of a range of heterocyclic anti-amebic compounds is given by Amir Azam (Chap. 33).

Part I
Genetics and Genomics

Chapter 2
The Continuously Expanding Universe of *Entamoeba*

C. Graham Clark and C. Rune Stensvold

Abstract In 1919, Clifford Dobell concluded that all the descriptions of *Entamoeba* in humans could be ascribed to three species: *Entamoeba histolytica*, *Entamoeba coli*, and *Entamoeba gingivalis*. At this time, morphology and host were the primary bases for naming species. We now know that both are unreliable, because host ranges can be broad and identical morphology can hide substantial genetic differences. Since Dobell, the number of accepted *Entamoeba* species in humans has continuously increased, with the most recent being identified in 2012. The application of molecular tools, especially DNA sequencing, has greatly increased our understanding of variation within the genus *Entamoeba*, but initial reliance on cultures gave us only a limited insight. For the past few years DNA extracted directly from feces from a wide range of hosts has been used to explore previously hidden *Entamoeba* diversity. Recent data include discovery of a uninucleate-cyst clade in nonhuman primates that is related to *Entamoeba bovis* and demonstration of substantial diversity within *E. coli*. Host ranges for some species are also expanding, with *E. coli* being found in rodents and an *E. muris*-like organism in primates. These results suggest that our picture of *Entamoeba* diversity is still incomplete and that further sampling is certain to uncover novel lineages.

2.1 Background

Amebae of the genus *Entamoeba* are easily recognized by their distinctive nucleus, which is usually described as being a "ring and dot" in appearance. The ring describes "peripheral chromatin" that lines the inside of the nuclear membrane, and the dot is generally referred to as the karyosome. The function of the karyosome

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_2

remains unknown, but in contrast to most eukaryotes this central body is not the nucleolus, as the functional equivalent of this organelle in *Entamoeba* is actually the peripheral chromatin [1]. With most stains this characteristic nuclear appearance is immediately recognizable, and an unidentified organism can be assigned to the genus *Entamoeba* on the basis of nuclear appearance alone.

It is the taxonomic ranks below that of genus that cause the majority of problems in the nomenclature and taxonomy of *Entamoeba*. The simplicity of the cell structure and lack of a fixed shape undermine the use of morphological features in species descriptions that is traditional in eukaryotic taxonomy. As a result, *Entamoeba* taxonomy has been subject to two types of "error" in the naming of species: (1) reliance on a character that does not reflect underlying genetic divergence, leading to overestimation of diversity and the naming of invalid species: examples include relying on the host as a species-specific character when in fact some *Entamoeba* species have quite a broad host range; and (2) morphological simplicity means that genetic divergence is not always reflected in morphological differences, which leads to underestimation of diversity and assigning the same species name to quite different organisms. Examples of both these errors follow.

Much of the interest in the taxonomy of *Entamoeba* arises from the need to positively identify *E. histolytica*, and the story of this species name is therefore a focus of the chapter. However, the taxonomy and nomenclature of the genus are currently in a state of flux, and this is also explored.

2.1.1 History of Entamoeba *Taxonomy from 1875 to 1919: Lösch to Dobell*

Although intestinal amebae had undoubtedly been seen by earlier workers, the first depiction of what is definitely *E. histolytica* is in the 1875 publication of Fedor Lösch [2], who was working in St. Petersburg, Russia. He describes the case of a peasant farmer, J. Markow, from the Archangel Territory of northern Russia, who was suffering from dysentery. Lösch's diagrams clearly show cells with the ring-and-dot nucleus characteristic of *Entamoeba*, and his description of the disease in Markow (and Lösch's animal model experiments) is fully compatible with invasive intestinal amebiasis. Lösch named the organism "Ameba coli" but whether he intended this as a descriptive name or a taxonomic name is unclear. The genus *Entamoeba* was named in 1895 by Casagrandi and Barbagallo [3], who were studying *E. coli*, and for reasons that are unclear they (and others) concluded that Lösch's ameba was the same organism. It fell to Fritz Schaudinn [4] to officially name *E. histolytica* in 1903 and distinguish it from the nonpathogenic *E. coli*, although he should actually have named the pathogen "*E. coli*" if he had followed the rules of nomenclature. Nevertheless, with a few exceptions, *E. histolytica* has been accepted as the name for the pathogenic species of *Entamoeba* in humans since that time.

By the time Dobell came to write his 1919 monograph "The amebae living in man" [5], the number of names for amebae that he considered to be wholly or partly synonyms of *E. histolytica* had risen to more than 30 and for *E. coli* to over 20. Some of these names were the result of disagreement over priority for the genus and species name, but many were the result of assigning species names to minor morphological variants of the amebae (sometimes imagined) or to amebae that were morphologically indistinguishable but from different hosts. Although we now know that Dobell made a number of wrong decisions, in his monograph he recognized only three species of *Entamoeba* in humans, namely *E. gingivalis* in the mouth plus *E. histolytica* and *E. coli* in the intestine. His strongly worded but well-reasoned case for this was highly influential in the field for many decades. By doing this "cull" of names he greatly simplified the nomenclature and placed the onus on future workers to justify why their "new" ameba should be considered a separate species by setting a standard against which others' descriptions should be compared.

2.1.2 *Taxonomy of* Entamoeba *in the Pre-molecular Era:* Entamoeba dispar, Entamoeba moshkovskii, Entamoeba hartmanni, *etc.*

The first "challenge" to Dobell's nomenclature was not long in arriving. In 1925 the great French parasitologist Emile Brumpt described *Entamoeba dispar* [6]. He concluded that this was a new species based on two observations. First, he observed an infection that persisted for many months without the infected individual developing symptoms. Second, he infected kittens, a very sensitive animal model for invasive amebiasis that was widely used at the time, and again no disease developed. He concluded that, despite the fact he could not distinguish them based on morphology or host, *E. dispar* and *E. histolytica* were distinct species [6]. No one believed him, or at least no one was willing to support this view publically. The Royal Society for Tropical Medicine and Hygiene invited Brumpt to give an address in 1926, and the record of the discussion that followed his presentation gives a fascinating insight into the general thinking at the time [7]. The issue was not so much whether *E. dispar* existed as a distinct species, but rather that it would be problematic because there was no way for them to be distinguished and therefore it would not inform medical decisions. For the next 50 years the species name virtually disappeared from the literature, only being used by Brumpt himself [8] and one of his students, Simic [9–13].

During the Second World War, a paper was published in Russian describing a new species of *Entamoeba* that was indistinguishable from *E. histolytica* by morphology but that had distinct physiological characteristics, namely, it was able to grow over a wide temperature range [14]. *Entamoeba moshkovskii* was isolated from sewage. Its isolation not from a host but from the environment raised questions

about whether it was a free-living organism or a parasite, an issue that still has not been fully resolved. Following the end of the war, environmental surveys across the world repeatedly isolated *E. moshkovskii* not only from sewage but also from freshwater sediments (see Clark and Diamond [15] for references). Clearly this organism is widespread if not ubiquitous. In the mid-1950s, an *Entamoeba* was isolated from a patient in Laredo, Texas, that was by chance found to grow at room temperature as well as at body temperature [16]. A few additional isolations of such amebae were made over the following years, but these "*E. histolytica*-like" ameba infections appeared to be rare (see Clark and Diamond [17] for references). The physiological similarity with *E. moshkovskii* did not escape notice, but proving they were the same organism was not possible in the absence of other criteria (see Sect. 2.2).

One of the synonyms of *E. histolytica* that Dobell had discarded in 1919 [5] was *Entamoeba hartmanni*. This species was described as closely resembling *E. histolytica* but significantly smaller: the cyst of *E. histolytica* is generally around 12 μm in diameter, while that of *E. hartmanni* is less than 10 μm, often around 7 μm, and the trophozoites are proportionately smaller also. Dobell believed that *E. hartmanni* represented one end of a size continuum and so was not distinct from *E. histolytica*. Nevertheless, "small race" *E. histolytica* was often referred to in the literature, indicating that other workers recognized it as an identifiable entity. In the 1950s, Burrows [18, 19] showed that the cyst size distribution in mixed infections showed two discrete peaks that coincided with the known mean sizes of the small and large "race" organisms. Therefore, although there was some overlap at the size extremes, "small race" *E. histolytica* was a stable and distinct entity. Burrows also noted some minor but consistent morphological differences in the nucleus to support his view and revived the name *E. hartmanni* for the small organism. This change was rapidly accepted in the field, suggesting that most workers already accepted that it was a distinct organism.

Thus, by the 1970s, *E. hartmanni* had joined *E. histolytica*, *E. coli*, and *E. gingivalis* as a member of the human *Entamoeba* flora, but the other proposed members had either been rejected (*E. dispar*) or were in limbo for lack of evidence (*E. moshkovskii*). The next steps required the development and application of new methodologies to the investigation of *Entamoeba*.

2.1.3 *The Early Use of Molecular but Non-sequence Data in Taxonomic Analysis: Isoenzymes, DNA Hybridization, and Antibody*

The first indication that Brumpt may have been correct about *E. dispar* being a distinct organism came in 1972. Martínez-Palomo and colleagues in Mexico used lectins to investigate the cell-surface sugars of *E. histolytica* growing in vitro. They observed that the effect of concanavilin A on agglutination of *E. histolytica*

divided strains into two groups and that these groups were correlated with whether the ameba had been isolated from an individual with disease or one without symptoms [20]: this was interpreted as revealing a virulence marker. A few years later, Sargeaunt and Williams started using the method of isoenzyme analysis, originally developed for and applied to bacteria, to investigate variation in *Entamoeba*. They initially showed that the method could distinguish between different species of *Entamoeba* [21] and uncovered intraspecific pattern variation. They later found that *E. histolytica* isolates fell into two groups (later called "pathogenic" and "nonpathogenic") that correlated with the disease status of the patient from whom the ameba was isolated [22]. They were later the first to revive Brumpt's proposal that two distinct species were involved. Soon thereafter, the first monoclonal antibodies against *E. histolytica* were developed; some of these also showed differential reactivity with strains depending on their origin [23]. Eventually, DNA analysis came on the scene, with Tannich et al. [24] showing that restriction fragment length polymorphisms (RFLP) revealed through Southern blotting also correlated with the isoenzyme patterns and strain origins. At about the same time, investigations using repetitive DNA of *E. histolytica* showed that some repeats only hybridized to one of the two isoenzyme groups [25].

2.2 The Impact of Ribosomal DNA on *Entamoeba* Taxonomy: Riboprinting, Sequences from Cultures, and Sequences from Stool DNA

Although evidence was accumulating in support of Brumpt's hypothesis, alternative explanations were still possible. For example, all the protein data could also be explained by differential expression of genes or different posttranslational modifications of proteins. The DNA data were trickier to explain, but differential gene amplification could not be ruled out, for example, if all strains had the same gene complement but one gene or genome "variant" was amplified in strains that caused disease. To investigate these alternative explanations, the ribosomal RNA (rRNA) genes were used. The small subunit (SSU) rRNA gene had emerged as a useful target for investigating phylogeny and molecular taxonomy because of its relatively slow evolutionary rate and high conservation within species. The SSU rRNA genes of "pathogenic" and "nonpathogenic" *E. histolytica* were first investigated in 1991 by Clark and Diamond [26] when it was shown that sequence differences detected using restriction enzymes (riboprinting) could again divide *E. histolytica* into two groups that correlated with the other markers reported previously. The basis of some of these restriction enzyme digestion differences at the sequence level was also investigated, and differential gene amplification using primer pairs that only amplified one of the two SSU rRNA gene variants found no evidence for the presence of the "other" variant: in other words, differential gene amplification could not be the explanation. Combined with accumulating sequences for other genes, by 1993 the

evidence was thought to be sufficient to warrant the redescription of *E. histolytica* to separate it from *E. dispar* and to recognize the existence of the latter species [27]. Brumpt was thus vindicated almost 70 years after his original proposal, and the existence of *E. dispar* appears to have been universally accepted. Nevertheless, the difficulty identified in 1926 [7] still exists: namely, differentiating the two species to inform medical decisions is still problematic. Microscopy is still the mainstay of parasite diagnosis in most parts of the world but does not allow the differentiation of *E. histolytica* and *E. dispar*.

Riboprinting also gave the first insight into the relationships among a wide range of species in the genus *Entamoeba*. This approach also showed the existence of cryptic diversity within several species, most notably *E. coli* and *E. moshkovskii* [15], and finally forged a link between *E. moshkovskii* and the rare "*E. histolytica*-like" infections represented by the Laredo isolate [17]. Crude estimates of similarity can be generated from the proportions of shared restriction sites, and these in turn can be used to generate phylogenetic trees. The vast majority of relationships identified using riboprinting estimates have been confirmed subsequently through sequencing of the SSU rRNA genes and the use of increasingly sophisticated phylogenetic analyses [28].

For many years the investigation of *Entamoeba* at the DNA level was limited to those species for which organisms growing in culture were available. This limitation also restricted the number of isolates of each that could be investigated as establishing an organism in culture is labor-intensive, and expensive if the culture is maintained for any length of time. Limitation of investigations to cultured organisms was in part a reflection of the difficulty of extracting usable DNA from fecal specimens. Feces is notorious for the presence of inhibitors of DNA analysis enzymes, and it was not until specific commercial kits were developed for fecal DNA extraction, eliminating this as a problem, that a full exploration of the genetic diversity of *Entamoeba* became possible.

That reliance on culture was possibly limiting our view of *Entamoeba* diversity was suspected early on, as an inability to grow certain species commonly found in livestock was noted. Whether the uninucleate cysts of *E. bovis* seen in cattle were genetically distinct from those of *E. polecki* seen in pigs, for example, could not be addressed because the former would not grow in culture. The advent of fecal DNA kits changed this, and it soon became clear that our view of *Entamoeba* diversity based on cultured amebae was woefully incomplete. The first SSU rRNA gene obtained from fecal DNA was from Vietnamese pigs and proved to be a new organism. Although expected to be *E. polecki* because of its uninucleate cysts, it proved to be unrelated and was given the name *E. suis* [29]. This finding was just the beginning, however: as new hosts are sampled, the known diversity of *Entamoeba* is still expanding rapidly. Even when more samples from previously investigated hosts are analyzed, it is not uncommon to detect new and genetically distinct sequence variants and even new branches of the *Entamoeba* tree. We have still only scratched the surface.

2.3 The Current Situation: Phylogeny, Diversity, Nomenclature, Host Specificity, and Impact of the Lack of Morphology

2.3.1 Recent Surge in Reports of Novel Ribosomal Lineages

The recent application of sequencing to polymerase chain reaction (PCR) products amplified from DNA extracted directly from feces has resulted in the discovery of a large number of novel *Entamoeba* lineages; to date, 29 distinct lineages have been identified (Table 2.1; Fig. 2.1) [30]. Some of these organisms were named a long time ago, while others appear to be distinct genetic variants (subtypes, ST) of species that have already been named (e.g., *E. coli* ST1 and ST2); however, further sampling may challenge this view—it is possible that the discreteness of lineages may disappear as further sequences become available. In addition, there are seven well-supported ribosomal lineages in the most recent phylogenetic trees that do not show a strong affinity with established species; these have been named by allocating a number to each ribosomal lineage (RL) (Fig. 2.1; Table 2.1) rather than a traditional Latin binomial. This terminology was introduced by Stensvold et al. [30] to enable a working nomenclature for *Entamoeba* organisms in the absence of morphological data and to respect the fact that names applicable to these lineages may already exist, but no data are available to link the two.

The recent and continuing surge in the discovery of new RLs and STs has made it clear that substantial additional sampling is needed to analyze the host specificity and genetic diversity of each potentially new lineage, and hence establishment of species names for novel lineages is still premature. An exception to this has been the recent finding of *Entamoeba bangladeshi* [36]. A variety of primers of varying specificity were applied to amplify DNA of *Entamoeba* directly from feces [30, 36], among them a genus-specific primer pair, ENTAGEN-F and ENTAGEN-R. This primer pair was used to analyze fecal DNAs from Bangladeshi children with and without diarrhea who were microscopy positive for four-nucleated cysts but PCR negative for *E. histolytica*, *E. dispar*, and *E. moshkovskii* [36]. Sequencing of PCR products produced evidence of a novel species, named *E. bangladeshi*. The morphology of cysts and trophozoite stages of *E. bangladeshi* appear similar to that of *E. histolytica* [36]. Phylogenetic analysis of the relationship between *E. bangladeshi* and other *Entamoeba* parasites reveals that, although distinct, *E. bangladeshi* clearly groups with the clade of *Entamoeba* infecting humans that includes *E. histolytica* and *E. dispar* (Fig. 2.1). *E. bangladeshi* is more distantly related than *E. dispar*, but closer than *E. moshkovskii,* to *E. histolytica* [36]: our more recent analyses suggest that it is specifically related to (but distinct from) *E. ecuadoriensis*, previously isolated only once, from sewage (unpublished observations). Hence, humans are now known to be hosts of four different but related species of *Entamoeba* (*E. histolytica, E. dispar, E. moshkovskii*, and *E. bangladeshi*), at least one of which is pathogenic, that cannot be differentiated using cyst morphology. Phylogenetically, this "complex" moreover contains an additional two species that have not been found in

Table 2.1 Host specificity of *Entamoeba*

Species/lineage	Subtype	Potentially invasive	Cyst nuclei[a]	Environment	Humans	Nonhuman primates	Ungulates	Rodents	Birds	Reptiles	Amphibia
E. histolytica	–	X	4	–	X	–	–	–	–	–	–
E. dispar	–	–	4	–	X	X	–	–	–	–	–
E. bangladeshi	–	–	4	–	X	–	–	–	–	–	–
E. moshkovskii	–	–	4	X	X	–	X[a]	–	–	X	–
E. nuttalli	–	X	4	–	–	X	–	–	–	–	–
E. ecuadoriensis	–	–	4	X	–	–	–	–	–	–	–
E. bovis	–	–	1	–	–	–	X	–	–	–	–
Entamoeba RL1	–	–	1	–	–	–	X	–	–	–	–
Entamoeba RL2	–	–	NA[b]	–	–	–	X	–	–	–	–
Entamoeba RL3	–	–	1	–	–	X	–	–	–	–	–
Entamobea RL4	–	–	NA	–	–	–	X	–	–	–	–
Entamoeba RL5	–	–	4	–	–	–	–	–	–	X	–
Entamoeba RL6	–	–	4	–	–	–	–	–	–	X	–
E. terrapinae	–	–	4	–	–	–	–	–	–	X	–
E. insolita	–	–	4	–	–	–	–	–	–	X	–
E. hartmanni	–	–	4	–	X	X	–	–	–	–	–
E. equi	–	–	NA	–	–	–	X	–	–	–	–
E. ranarum	–	–	1	–	–	–	–	–	–	–	X
E. invadens	–	X	1	–	–	–	–	–	–	X	–
E. suis	–	–	1	–	–	X	X	–	–	–	–
E. gingivalis	–	–	None	–	X	–	–	–	–	–	–

E. polecki	ST1	–	1	–	X	–	X	–	X	–	–
	ST2	–	1	–	X	X	–	–	–	–	–
	ST3	–	1	–	X	–	X	–	X	–	–
	ST4	–	1	–	X	–	–	–	–	–	–
Entamoeba RL7	–	–	8[c]	–	X[a]	X	X[d]	–	–	–	–
E. muris	–	–	8	–	–	–	–	X	–	–	–
E. coli	ST1	–	8	–	X	X	–	–	–	–	–
	ST2	–	8	–	X	X	–	X	–	–	–

For each species, subtype (ST) and ribosomal lineage (RL) the hosts recorded to data are indicated (X)
Based on references [29–35] and unpublished observations
[a]Unpublished observations
[b]Possibly four nuclei per cyst [30]
[c]Probably eight nuclei per cyst (Vidal-Lapiedra, unpublished observations)
[d]Lebbad et al., unpublished observations (1,306-bp sequence with 99 % identity to RL7)
NA - not available

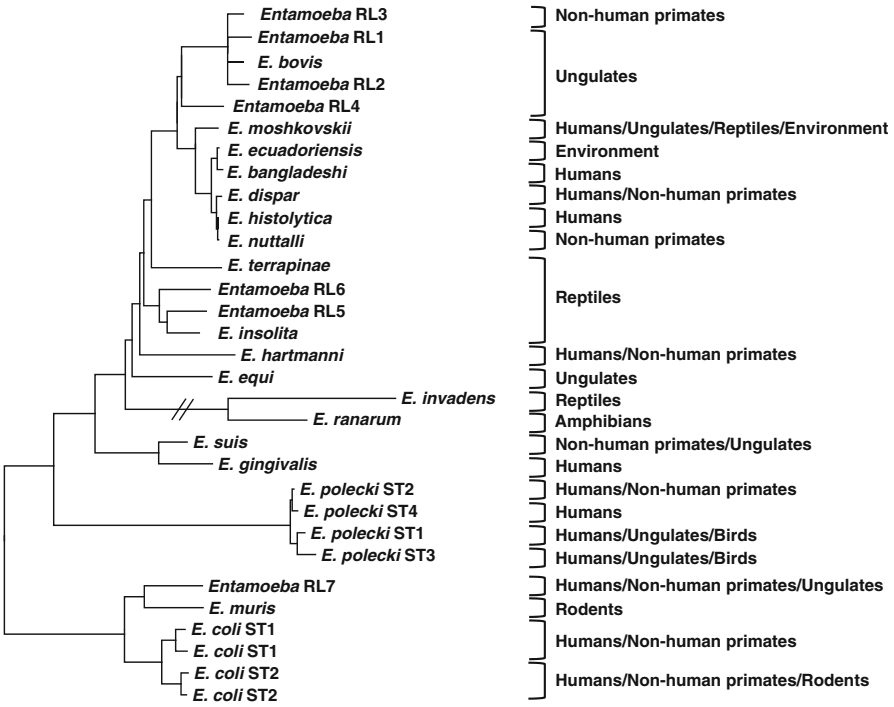

Fig. 2.1 Phylogenetic relationships among SSU rRNA gene sequences of *Entamoeba* species. The tree shown has been redrawn from Fig. 2.1 in Stensvold et al. [30], with the addition of *E. bangladeshi*. The cross-hatched branch has been shortened for convenience

humans so far, namely, *E. ecuadoriensis* (sewage) and *E. nuttalli* (nonhuman primates) [29, 31].

Designing PCR primers specific for a genus that keeps expanding in genetic diversity is challenging. Amplification of novel ribosomal lineages from fecal DNAs has been approached using the primers ENTAM1 and ENTAM2 [37], and more recently ENTAGEN-F and ENTAGEN-R [30]. The ENTAGEN primers amplify a PCR product of approximately 400 bp, and from the sequence of this product lineage-specific additional primers can be designed. An alternative approach is to apply low-specificity primers targeting all eukaryotic SSU rRNA genes, such as the RD5 + RD3 primer pair, with subsequent product cloning and clone sequencing; although this is even more sensitive, at least in theory, it can pose practical problems, such as nonspecific primer binding and the need for the screening of many clones that do not contain *Entamoeba* sequences to find those that do.

When screening for *Entamoeba*-specific DNA by PCR using genomic DNA extracted directly from feces, it appears, perhaps not surprisingly, that the choice of primers influences the results obtained in cases of mixed *Entamoeba* infection, as was recently observed during the screening of two samples from cattle [30].

Initially, unambiguous *E. bovis* SSU rRNA gene sequences were obtained using broad-specificity primers. Later, the application of primers of higher specificity led to the amplification and sequencing of a distinct lineage, RL2, from these two samples. Importantly, morphological data had been obtained before the molecular work, and although most of the cysts in the samples were uninucleate (consistent with *E. bovis*), a few tetranucleate cysts were seen as well; it is speculated that the tetranucleated cysts might represent the RL2 lineage. Hence, using primers of stricter specificity may be of great utility in cases where mixed infections are suspected.

Meanwhile, when designing diagnostic primers for a species the primers obviously need to take into account any intraspecies diversity that exists. There is evidence that published primers used for the detection of *E. hartmanni* target regions that exhibit sequence variation within this species, and that only some genetic variants of this species are being detected using that particular primer pair [30, 32]. A similar problem may exist in *E. moshkovskii*, where diagnostic primers have been designed based on the sequence of a single variant when many are known to exist.

2.3.2 Phylogeny and Host Specificity

Major host groups of *Entamoeba* identified to date include human and nonhuman primates, ungulates, rodents, reptiles, birds, and amphibians (Table 2.1; Fig. 2.1). An important observation is the fact that many lineages of great genetic diversity have been isolated from members of all host groups apart from birds and amphibians.

Entamoeba phylogeny indicates that the genus has not exclusively coevolved with its host but instead the parasite has repeatedly jumped host species barriers: how often is difficult to estimate. This variation is exemplified by the fact that *Entamoeba* lineages from, for example, humans and ungulates are scattered across the entire phylogenetic tree. Additionally, within the group of "ungulate-specific" lineages (in the topmost part of the tree in Fig. 2.1) is embedded RL3, which so far has only been found in various species of langur, arboreal monkeys from Asia. Moreover, although all four subtypes of *E. polecki* have been found in humans, only *E. polecki* ST2 (previously "*E. chattoni*") has been found in nonhuman primates; whether this is the result of insufficient sampling remains to be seen. Also of interest is the detection of a lineage related to *E. muris* (RL7) in a langur, *E. suis* in a gorilla, and *E. coli* in a chinchilla [30]. These observations taken together indicate that the host specificity of several lineages of *Entamoeba* may be only moderate, and some examples suggest a need to revisit and revise species' taxonomic descriptions.

Humans are natural hosts to at least eight well-established, genetically extremely diverse species of *Entamoeba*, and possibly more than 13 separate lineages (Table 2.1). Despite limited sampling, the relatively recent introduction of the use of PCR-based detection of *Entamoeba* directly from stool DNA has led to a total of 11 lineages being found in ungulates so far (Table 2.1). Most potential hosts remain to be investigated, and so it seems likely that the genetic universe of *Entamoeba* will continue expanding for the foreseeable future.

2.3.3 *Intraspecies Diversity of* Entamoeba

Studies of genetic diversity within *Entamoeba* lineages are pivotal for identification of differences in transmission patterns, including host specificity, evolutionary and taxonomic inferences, and for efforts to predict virulence and design relevant nucleic acid-based diagnostic tools. Intraspecies, and intra-RL, genetic diversity has been studied using SSU rRNA genes [15, 29, 30, 37–39], but multilocus markers are really needed for efforts to obtain high-resolution data (genotyping). The latter are starting to emerge and are addressed in another chapter of this book.

So far, genetic variation in species of *Entamoeba* other than *E. histolytica*, *E. dispar*, and *E. moshkovskii* has been little investigated, and this has consisted mainly of SSU rRNA gene analyses of other species of *Entamoeba* infecting humans.

E. polecki comprises four subtypes, that is, four clearly independent clusters within this well-defined species. All four subtypes have been found in humans, and humans are the only known host of ST4, which also appears to be the most common human *E. polecki* subtype. The host specificity of *E. polecki* subtypes is shown in Table 2.1. Interestingly, a mixed *E. polecki* infection was found for the first time in a sample from a rhea; the bird harbored both ST1 and ST3 [30]. *E. polecki* ST2 was formerly known as *E. chattoni* and *E. polecki* ST3 as *E. struthionis* [29, 30, 37, 40].

Similarly, *E. coli* has been divided into two STs based on phylogenetic analysis. ST1 has been detected only in samples from humans and nonhuman primates, whereas ST2 has also been found in one rodent so far (Table 2.1; [30]). Other distinct lineages are also characterized by octonucleate cysts, namely *E. muris* [41] and *Entamoeba* RL7 (A. Vidal-Lapiedra, unpublished observations). Here again it is not possible to ascribe species names to the RL, because there are a number of octonucleate cyst-producing taxa described for which no sequence data are available, including *E. caviae*, *E. cuniculi*, *E. gallinarum*, and *E. wenyoni* [41, 42], and the host spectrum of these lineages is still to be resolved; indeed, the possibility exists that these may be synonyms of *E. muris* or *E. coli*.

Quadrinucleate cyst-producing species of *Entamoeba* such as *E. hartmanni*, *E. dispar*, and *E. histolytica* appear to exhibit much less genetic variability [30, 43], except for *E. moshkovskii* ([15]; unpublished observations). Parija and Khairnar [43] studied the diversity in SSU rRNA genes of *E. histolytica*, *E. dispar*, and *E. moshkovskii* detected by nested multiplex PCR from various types of patient samples in Puducherry, India. Screening for polymorphisms employed riboprinting and single-strand conformation polymorphism (SSCP), and nucleotide sequencing was used for confirmation and identification of polymorphisms. A substantial number of isolates were screened, but RFLP analyses detected variation in only one *E. histolytica* isolate, suggesting a low degree of diversity in the SSU rRNA gene region studied. By SSCP, polymorphism was found in 7–12 % of the *E. histolytica* and *E. moshkovskii* PCR products, whereas no variation was found in the 174-bp SSU rRNA gene region of 171 *E. dispar* samples studied.

Substantial variation in *E. moshkovskii* was identified by riboprinting studies carried out by Clark and Diamond [15], who identified at least six ribodemes among 25 isolates; in situations where nucleic acid-based methods are used for primary detection (cysts of *E. moshkovskii* have only rarely been found in humans), problems with primer specificity may have led to detection of only certain variants of the species as mentioned earlier (Sect. 2.3.1; [44]).

Although the genetic diversity across the *E. dispar* SSU rRNA gene is probably <1 %, sequences from *E. coli* (including both subtypes) on the other hand exhibit 18 % polymorphic positions [45]; these estimates were based on data available in GenBank in 2010. It is worth noting that *E. coli* appears to exist in different "strains" distinguished by cyst size [46]; no attempt has yet been made to link this morphological difference to the SSU rRNA gene-based subtypes.

Few attempts have yet been made to compare variation in SSU rRNA genes to variation in protein-coding genes. The internal transcribed spacer (ITS) region, widely used to detect diversity in some organisms, has been studied only to a very limited extent in *E. histolytica* [47, 48], but data suggest that variation is substantially lower than in genes encoding repeat-containing proteins.

2.3.4 Impact of the Lack of Morphology

Detection, differentiation, and naming of species of *Entamoeba* based on morphological data are affected by at least three major problems.

1. For those ribosomal lineages only recently been discovered, very little information on morphology, if any, is available. Although this is often simply because of a lack of microscopic study, there have been cases in which *Entamoeba* sequences have been obtained from samples that were microscopy negative [30]. Traditionally, cysts have been isolated from fecal samples and analyzed by a number of techniques to produce the morphological description (see following). Although at least one species of *Entamoeba*, *E. gingivalis*, does not produce a cyst stage, the life cycles of many novel lineages are incompletely known. For instance, there are at least two different *Entamoeba* lineages infecting horses (unpublished data), and for neither of these has a cyst stage been identified despite careful microscopy having been performed.
2. Many infections involve multiple *Entamoeba* species, making the link between morphology and ribosomal sequences tenuous even when microscopy has been performed, as mentioned earlier for RL2 (Sect. 2.3.1), for example.
3. Apart from size and the number and appearance of nuclei and chromatoid bars, there are few morphological hallmarks that differentiate cysts, and the lack of features leads to two potential problems as mentioned in the earlier "Background" section. If we use minor morphological differences between cysts to separate species, these may turn out not to be reliable. Conversely, we may group together

distantly related organisms because we cannot tell them apart. Thus, we can either underestimate or overestimate diversity quite easily unless we supplement our morphological findings with molecular data. One such example is represented by the finding of uninucleate cysts in fecal samples from nonhuman primates. Such cysts have traditionally and collectively been referred to as "*Entamoeba chattoni*" (now *E. polecki* ST2), but recent sequencing of PCR products obtained from certain primate fecal samples containing only uninucleate cysts revealed the presence of a novel ribosomal lineage, *Entamoeba* RL3 [30].

Phylogenetic relationships observed between ribosomal lineages of *Entamoeba* analyzed by riboprinting [15] and, later, sequencing of cultured isolates [28, 29], until recently appeared to directly reflect the number of nuclei present in mature cysts. However, after the discovery of additional ribosomal lineages, primarily from mammals [30, 45], we now know that although lineages within most clades share the same number of nuclei in mature cysts, cyst nuclear number alone is not an indicator of phylogenetic relatedness among clades.

Nevertheless, detailed morphological analysis of trophozoites and cysts is still important. Erythrophagocytosis has been observed in a number of species, including *E. histolytica*, *E. invadens*, and *E. moshkovskii* [49–51], and is best visualised by permanent staining of fixed fecal smears. DAPI staining is one way of visualizing DNA and therefore enables counting of nuclei, and Calcofluor can be used for identification of a cyst wall in cases where it is necessary to differentiate trophozoites from cysts [38]. Immunofluorescence using a monoclonal antibody known to react with antigens in cysts of *E. histolytica* and *E. dispar*, but not *E. hartmanni*, *E. coli*, and *E. polecki*, remains to be tested on a wider range of lineages, and the significance of the specificity of the antibody is incompletely understood [38]. Conventional microscopy using iodine staining is helpful in the general morphological examination of cysts. The example taken from screening cattle samples for *Entamoeba* (see Sect. 2.3.1) emphasizes that thorough purification, staining, and morphological description is essential for cases in which mixed infections are present, to provide data critical to the interpretation of molecular data.

2.4 The Future

The identification of new *Entamoeba* lineages is likely to continue by "traditional" molecular methods as described here. However, a new source of data is likely to emerge in the near future, namely, the identification of *Entamoeba* by chance in environmental and fecal samples as part of metagenomic analyses and massive parallel sequencing of PCR products amplified by broad-specificity primers targeting the eukaryotic SSU rRNA gene. At present this is rare, because the approach used in most studies specifically targets bacterial ribosomal genes, but it seems likely that as costs decrease and applications broaden more and more eukaryote-oriented studies will be performed. We look forward with great interest to the new insights into *Entamoeba* diversity that such studies will provide!

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Chapter 3
The Genomics of *Entamoebae*: Insights and Challenges

Gareth D. Weedall

Abstract An organism's genome provides vital information about its biology and evolution. The genome of *Entamoeba histolytica* was sequenced and assembled in 2005 and provided many insights into the biology of the pathogen. A number of additional *E. histolytica* genomes and those of pathogenic and nonpathogenic relatives have followed, a process aided in no small part by the advent of second-generation sequencing technology. However, assembling *Entamoeba* genomes has proven particularly challenging. A number of factors contribute to this, including the large number of repetitive elements in *Entamoeba* genomes, their complex karyotypes, and their highly biased nucleotide compositions. In spite of the challenges, these genomes have been highly informative about the biology and evolution of *Entamoebae* and comparative analyses of pathogenic and nonpathogenic species (in particular, pathogenic *E. histolytica* and nonpathogenic *E. dispar*) have shed light on key virulence genes and on important gene families. Further advances in genome sequencing technology may improve our ability to sequence these challenging genomes, which will advance our knowledge of the evolution of the genus and of the pathogenicity of *E. histolytica*.

3.1 Introduction

Whole genome sequencing of human pathogens has an important role in research to combat diseases. Annotated genome sequences provide a rich store of data to be mined, for instance, to discover candidate targets for drugs or vaccines. They allow a range of postgenomic analyses to be undertaken, such as the analysis of gene expression using microarrays or whole transcriptome sequencing. More generally, the genome can provide a framework with which to coordinate knowledge of the organism's biology and to allow fast integration and dissemination of research. A good example of this is the online database EuPathDB, which acts as a resource

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_3

for integrating numerous "-omic" datasets of eukaryotic protist pathogens and provides tools to explore and mine these datasets [1].

The genomes of a growing number of important human parasites have been sequenced, including species of *Plasmodium* [2, 3], *Trypanosoma* [4, 5], *Leishmania* [6], *Giardia* [7, 8], *Cryptosporidia* [9, 10] and *Trichomonas* [11]. Information gained from these projects has greatly increased our understanding of the biology of these organisms, knowledge which may be used to combat the diseases they cause.

Entamoeba histolytica is the causative agent of amoebic dysentery in humans, although other species of *Entamoeba* infect humans and some may also cause disease [12–14]. The draft genome sequence of *Entamoeba histolytica* was published in 2005 [15] and the sequencing of several additional *Entamoeba* species genomes has been carried out since then. These genomes have allowed genomic and comparative genomic analyses that are reviewed here and have clarified some of the technical challenges faced when trying to sequence these complex genomes.

3.2 Phylogeny of the *Entamoebae*

Entamoebae are part of the *Amoebazoa*, which separated from the opisthokont lineage (animals and fungi) after the divergence of plants [16]. Figure 3.1a illustrates the phylogenetic position of *Entamoeba. Amoebazoa* are themselves divided into two lineages: the free-living *Mycetozoa* (which include the slime-mold *Dictyostelium discoideum*) and the *Archamoebae*, which lack mitochondria and can be divided into the *Mastigamoeba* and *Entamoeba* lineages [16]. The *Amoebazoa* are a poorly sampled group within the tree of life and may be highly diverse: for instance, divergence between the *Archamoebae* and the *Mycetozoa* may be as great as that between animals and fungi [18].

The *Entamoeba* genus is diverse and contains a large number of species found to parasitize a broad range of hosts from amphibians to mammals [19–21]. It is worth noting here that, as with many unicellular organisms, defining species can be problematic. Species are often morphologically indistinguishable [22] and must be defined by genetic divergence, yet distinguishing between intraspecific diversity and interspecific divergence is not straightforward. In response to this, Stensvold et al. [17] introduced the "ribosomal lineage" to describe an individual based on its 18S small subunit ribosomal RNA (18S SSU rRNA) sequence, without formally defining a species. The diversity seen within some *Entamoeba* species, such as within *Entamoeba coli* [17] or *Entamoeba moshkovskii* [23], may indicate that in fact two or more species have been classified under the same name.

Figure 3.1b illustrates the phylogenetic relationships among a small sample of *Entamoeba* species. The most closely related species to *E. histolytica* currently identified is *Entamoeba nuttalli*, a pathogen that infects captive and wild macaques, the main host species identified so far [24–26]. The most closely related species to *E. histolytica* that is known to infect humans is *Entamoeba dispar*, although it shows a broader host range than *E. histolytica* and can infect nonhuman primates [25].

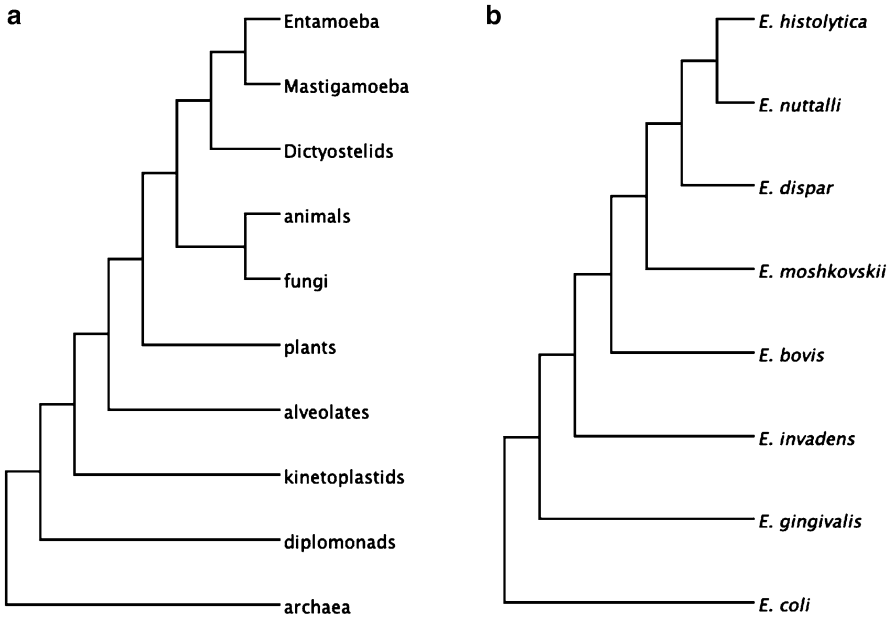

Fig. 3.1 Phylogenetic position and diversity of *Entamoeba*. **a** Phylogenetic topology of the evolutionary position of *Entamoeba*, showing the divergence of the *Amoebazoa* (containing *Entamoeba*, *Mastigamoeba*, and *Mycetozoa*) from the opisthokonts (animals and fungi) after the divergence of the plants. Also shown are the relative positions of other protists: the alveolates (including *Plasmodium*), kinetoplastids (including *Trypanosoma* and *Leishmania*), and diplomonads (including *Giardia*). The tree is rooted by Archaea. Branches are not to scale. The tree is based on data from a phylogenetic analysis of 100 genes [16]. **b** Phylogenetic relationships within *Entamoeba*. The tree shows the relationships among a set of *Entamoeba* species (details in main text). Branches are not to scale. The tree is based on data from a phylogenetic analysis of 18S small subunit ribosomal RNA [17]

Also unlike *E. histolytica*, *E. dispar* is not virulent in humans, although this has been challenged [12]. A putative novel species, *Entamoeba bangladeshi*, was recently isolated from humans in Bangladesh [27]. The evolutionary distance between *E. histolytica* and *E. bangladeshi* is greater than that between *E. histolytica* and *E. dispar* but less than that between *E. histolytica* and *Entamoeba moshkovskii*. *E. moshkovskii* has been found to infect humans in several countries [13, 14, 28, 29]. However, in contrast to *E. histolytica* it has been isolated from a wide range of sources including saltwater and freshwater environments, lake sediments, and sewage, in addition to human stools [23]. Perhaps related to this, *E. moshkovskii* can tolerate temperatures as low as 10 °C, a feature shared with *E. bangladeshi* and suggesting that both could be primarily free living. Also, as already noted, *E. moshkovskii* may in fact be a species complex, which could explain some of this diversity. The range of hosts infected by *Entamoeba* species is broad, and the genus includes parasites of ruminants (*Entamoeba bovis*) and reptiles (*Entamoeba invadens*). However, *Entamoeba* phylogeny does not strictly follow host phylogeny and humans

are infected by species only distantly related to *E. histolytica*, such as *Entamoeba coli* and *Entamoeba gingivalis*, the latter being unusual in parasitizing the oral cavity rather than the gut and in contrast to other species apparently forming no cysts. Many *Entamoeba* species are barely studied or, such as *E. bangladeshi* and *E. nuttalli*, only very recently described. Given the likelihood that only a small proportion of *Entamoeba* diversity can even be cultured, the true diversity of the genus is probably vast. The still small, but increasing, number of genomes sequenced (described in the next section) may help us to understand the evolution of the genus and particularly that of its more virulent members.

3.3 Genome Sequencing and Annotation of *Entamoeba histolytica*

The most important *Entamoeba* species from the perspective of human health, *E. histolytica*, had its draft genome sequence published and annotated in 2005 [15, 30]. The sequenced strain, HM-1:IMSS, is the most widely studied laboratory culture-adapted strain. It was isolated from a rectal ulcer of an adult human male with amoebic dysentery in Mexico and was originally called ABRM (isolation information in Diamond et al. [31]). After the first assembly and annotation, the genome was reassembled with additional sequence data and was reannotated in 2010 [32]. The assembly of whole genome shotgun Sanger sequenced reads (unguided by physical mapping) consists of 20,800,560 base pairs of DNA assembled in 1,496 scaffolds. The genome is extremely AT rich (approximately 75 % AT), which makes sequencing and assembly difficult. For instance, the AT richness prevented the generation of large-insert BAC (bacterial artificial chromosome) libraries, which can aid the assembly process by allowing the sequencing of long stretches of contiguous DNA that retain more spatial information than whole genome shotgun sequencing alone.

The genome was found to be gene rich (approximately half the assembled sequence was predicted to be coding sequence) and highly repetitive: the estimated number of annotated genes was 8,333, many of which are members of large multigene families [30, 32]. Features of the biology of *E. histolytica* may be reflected in its genome organization and architecture. For instance, mechanisms of gene regulation based on gene structure, such as alternative splicing of introns or upstream open reading frames in 3′-untranslated regions of genes, are unlikely to be major modes of gene regulation as introns are rare [30] and the untranslated regions very short [33, 34]. Whole transcriptome sequencing of *E. histolytica* confirms the lack of alternative splicing [35].

As already noted, computational prediction yielded 8,333 genes [15, 30, 32]. However, the annotation of the *E. histolytica* genome is a dynamic process requiring identification, correction, and confirmation of models of gene structure and association of functional information with genomic loci (i.e., curation). High-throughput genomic technologies can speed up this process: expressed sequence tag (EST) and

whole transcriptome sequencing have been used to identify previously unannotated genes, correct misannotated introns, and identify 5′- and 3′-untranslated regions [34, 35]. The nonprotein coding portion of the transcriptome is of great biological importance, but it often remains unidentified because computational gene prediction usually focuses on protein-coding genes. Genome mining has identified approximately 100 previously unannotated small nucleolar RNAs (snoRNAs) in the *E. histolytica* genome [36]. Perhaps the most important part of genome annotation, the functional annotation of genes, remains largely incomplete because functional genetic analysis is by its nature low throughput. It is a particular problem in organisms such as *E. histolytica*, which are highly divergent from well-described "model" organisms and for which genetic tools are not readily available. Figure 3.2 illustrates the state of functional gene annotation at the time of writing (January 2012). These data were extracted from the amoebaDB website, part of the EuPathDB suite, which hosts the most up-to-date annotations and many genomic datasets ([38], www.amoebadb.org). AmoebaDB is a resource for the integration of genome-scale data and annotation curation. It has proved to have a useful role as a forum for community-driven curation, by which researchers can link annotation to genomic loci, allowing manual correction of gene models, functional annotation, and linking

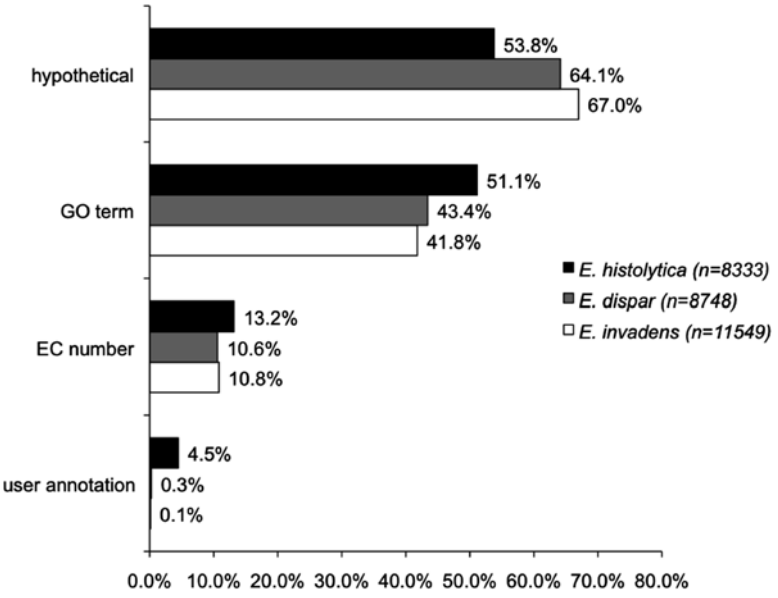

Fig. 3.2 Functional gene annotation of *Entamoeba histolytica*, *E. dispar*, and *E. invadens*. The plot shows the proportions of genes whose products are annotated as "hypothetical" (note that this does not include genes annotated with relatively uninformative descriptions such as "X-domain-containing protein"). Also plotted are the proportions of genes associated with a gene ontology (GO) term of any class (cellular component, molecular function, or biological process); genes associated with an enzyme commission (EC) number; and genes with user-added annotations. The plot is based on data from amoebaDB and a previous article [37]

of gene models to relevant literature. Three hundred and seventy three *E. histolytica* gene models have user comments added at the time of writing, 59 of which had only "hypothetical protein" annotations. All genome sequencing and annotation efforts ultimately require some human curation if they are to be useful research tools.

The *E. histolytica* genome was sequenced before the arrival of second-generation sequencing technology. This technology has had a transformative effect on many areas of biology by allowing rapid sequencing of entire genomes or transcriptomes. The technologies work by parallelizing the sequencing process so that millions of short sequences can be determined simultaneously. Rapid improvements in performance and reductions in cost mean that whole genome sequencing may become as commonplace as polymerase chain reaction (PCR) in the near future. A number of *E. histolytica* genomes in addition to the HM-1:IMSS have been sequenced using second-generation sequencing technology, allowing the study of intraspecific genomic diversity [39, 40]. These studies have identified single-nucleotide polymorphism throughout the genome (discussed in Sect. 3.7) as well as polymorphism in gene copy number. The occurrence of this duplication of genes [39] and genome regions [32] hints at the plasticity and complexity of the *E. histolytica* genome, which also presents a challenge for genome sequencing, as is discussed in the next section (Sect. 3.4).

3.4 Why Is *Entamoeba* Genome Assembly So Challenging?

All *Entamoeba* genomes sequenced to date should be considered draft assemblies. They generally consist of a large number of scaffolds (>1,000) that are not assigned to chromosomes. Although *E. histolytica* and its relatives are not uniquely difficult genomes to sequence and assemble, they do present considerable challenges, which are discussed next.

Several genetic techniques can be used to help the assembly of genomes. Sequencing of large-insert BAC (bacterial artificial chromosome) libraries can help genome assembly because of their large insert sizes (hundreds of kilobases). Unfortunately, the high AT content of the *E. histolytica* genome prevented the sequencing of large-insert BAC libraries in the original sequencing project [15]. HAPPY mapping can be used to define linkage between markers based on the frequency of co-occurrence of the markers in samples generated by fragmentation and limiting dilution of genomic DNA [41]. HAPPY mapping has been applied to *E. histolytica*, but the results were difficult to interpret, largely because of the extreme repetitiveness of the *E. histolytica* genome (Dr. Elisabet Caler, personal communication). Optical mapping is a technique to produce ordered restriction maps of single DNA molecules [42]. Optical mapping has been applied to *E. histolytica*, with some success (Dr. Elisabet Caler, personal communication). A small number of putative linkage groups (23) could be determined. However, it remains difficult to map sequence scaffolds, particularly the many short scaffolds in the *E. histolytica* genome assembly, to such restriction maps.

It is instructive to compare the genome assembly of *E. histolytica* with that of the mycetozoon *Dictyostelium discoideum*, which was also published in 2005 [43]. *D. discoideum* is the closest relative of the *Entamoebae* with a fully sequenced genome, although the evolutionary divergence between *Entamoeba* and *Dictyostelia* is huge. The genome of *D. discoideum* is similarly AT rich (77.6 % AT), which precluded the generation of large-insert BAC libraries, and is rich in repetitive DNA. The approach taken was whole chromosome shotgun sequencing (WCS), in which chromosomes were separated by pulsed-field gel electrophoresis (PFGE), isolated, and shotgun Sanger sequenced [43], undertaken in combination with HAPPY mapping to guide the sequence assembly. This combination of physical mapping and sequencing allowed assembly of the six chromosomes of the *D. discoideum* genome, totaling approximately 34 million bases.

Unfortunately, the molecular karyotype of *E. histolytica* appears to be particularly complex. In contrast to many organisms whose chromosomes can be well separated by pulsed-field gel electrophoresis (e.g., *Saccharomyces cerevisiae*; Fig. 3.3), this method does not clearly separate the chromosomes of *E. histolytica*, as illustrated in Fig. 3.3 [44]. Probe hybridization defined 14 linkage groups,

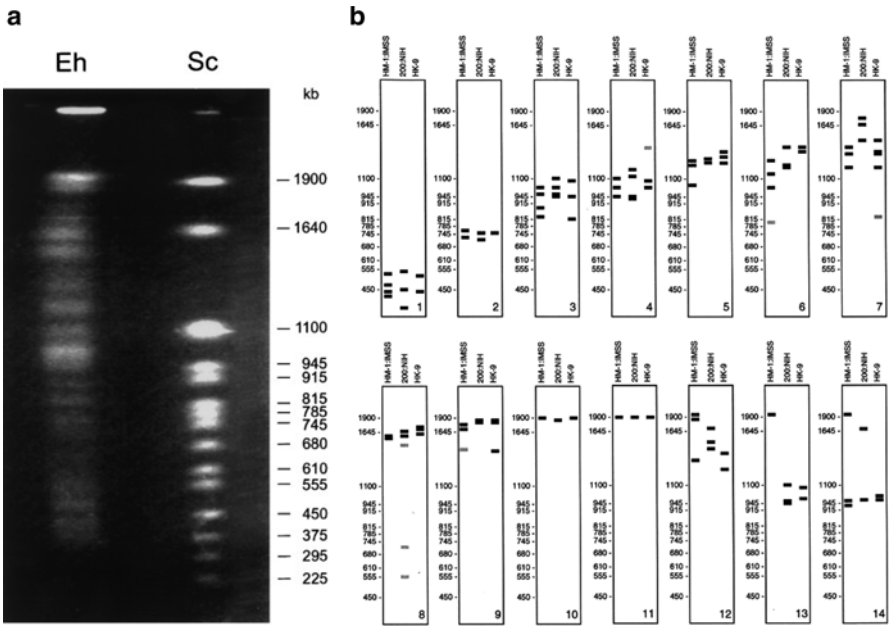

Fig. 3.3 The complex karyotype of *Entamoeba histolytica*. **a** Pulsed-field gel electrophoresis (PFGE) of *Saccharomyces cerevisiae* and *Entamoeba histolytica* shows clear separation of the *S. cerevisiae* chromosomes but a much more complex pattern in *E. histolytica*. **b** Illustration of 14 linkage groups defined using a large set of molecular probes hybridized to PFGE gels. Three *E. histolytica* strains (HM-1:IMSS, 200:NIH, HK-9) were probed and show different-sized chromosomes. Based on the number of bands seen for each putative linkage group in each strain, the authors estimated a ploidy of at least four. (Figures reproduced with permission from Willhoeft and Tannich [44])

although the sizes of these putative chromosomes varied between *E. histoytica* strains, and multiple bands (often four) were frequently present within a single strain, which led the authors to suggest a ploidy of four or more [44]. The natural ploidy of *E. histolytica* is unknown. It appears to be variable under different growth conditions, with up to tenfold more genome copies per cell under axenic growth conditions than is common when cells are grown in the presence of bacteria [45]. Variation in the size of putatively allelic chromosomes within and among strains has been noted in other single-celled eukaryotes, and this may be caused by variations in repetitive DNA or duplications of genomic regions, both of which are seen in the *E. histolytica* genome [20, 21, 32, 46–48].

To add to the complexity of the *E. histolytica* karyotype, it has been proposed that the genome contains a mixture of linear and circular molecules [49–52]. A circular molecule contains the *E. histolytica* ribosomal RNA genes, and multiple copies of this molecule exist in the nucleus [53]. In *D. discoideum*, a chromosomal region containing rRNA genes appears to act as a "master copy" to generate many linear extrachromosomal molecules encoding these genes [43, 54]. No obvious candidate for such a "master copy" of rRNA genes has been found in the *E. histolytica* genome, but the incompleteness of the genome assembly means that the existence of one cannot be ruled out.

Transfer RNA genes show an unusual organization in *Entamoeba*. The genes occur in mixed sets, each gene separated by DNA that may contain short tandem repeats; the entire set forms a repeat unit that is tandemly duplicated in many copies [20, 21]. The arrangement of tRNA arrays varies among species [55]. It has been suggested that these arrays of tRNA genes could cap the chromosomes and act as telomeres, as no specific *Entamoeba* telomeric DNA has been identified. This configuration would again be analogous to the situation in *D. discoideum*, in which ribosomal DNA repeats appear to act as telomeric sequences. However, although the sequences of *E. histolytica* tRNA repeat units have been determined, they have not been linked to nonrepetitive chromosomal sequences, so their genomic location in relationship to other genes remains unknown.

The occurrence of multiple rRNA episomes and long tRNA array repeats both cause problems for genome assembly, as together they can represent a large proportion of the sequence library at the expense of the nonrepetitive genomic fraction. However, the *E. histolytica* genome contains even more repetitive DNA in its very large number of transposable and repetitive elements [32]. These repetitive elements may lead to chromosomal variability by providing unstable regions of chromosomes where breakage and rejoining may occur [32]. They also provide a considerable obstacle for genome assembly as, unless they are spanned by a sequence read or read pair, they can prevent unambiguous joining of adjacent nonrepetitive genome regions. Some of these elements are several kilobases long, making them much longer than the average read length of most sequencing technologies. Many of the assembled *E. histolytica* genomic scaffolds end in repetitive DNA, indicating that the presence of these repetitive elements does indeed lead to fragmented assemblies. Figure 3.4 illustrates some of the possible effects that genomic complexity in *E. histolytica* might have on genome assembly.

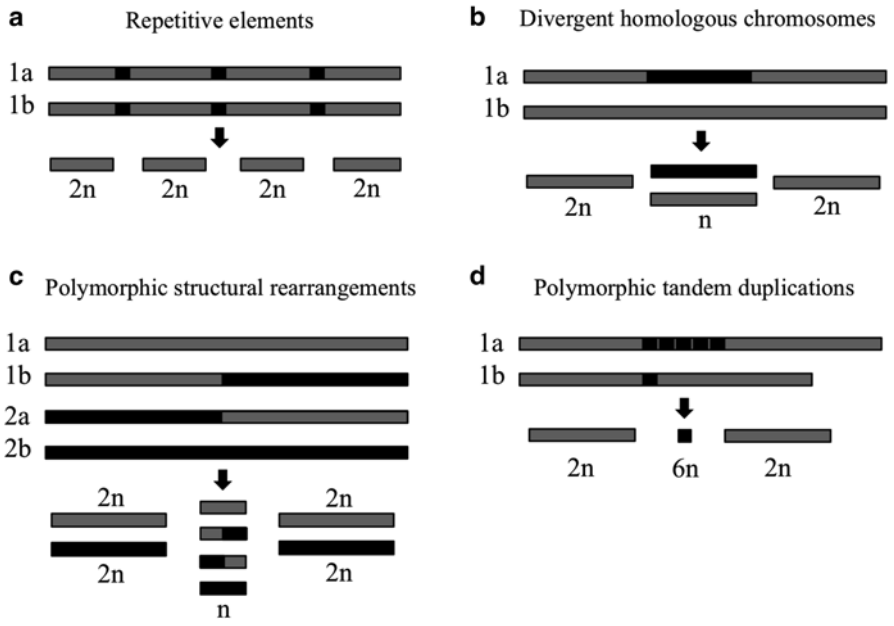

Fig. 3.4 Diagrams illustrating the effects of genotypic complexity on genome assembly. In each case, homologous chromosomal regions (*1a* and *1b*) are shown, assuming diploidy for ease of presentation. The *arrows* represent sequencing and assembly of the chromosomal regions and the assembled scaffolds are displayed, with labels indicating the estimated depth of coverage, in units "*n*", across the scaffold. **a** Repetitive elements that are not spanned by reads or read pairs cause a fragmented assembly. **b** Highly divergent regions of homologus chromosomes will cause breaks in the assembly where sequences diverge. **c** Structural rearrangement among chromosomes and tandem duplications (**d**) may break assemblies and produce scaffolds with differing depths of coverage

Many aspects of the genomic biology of *Entamoeba* remain mysterious. What makes these genomes so interesting also makes it technically challenging to reconstruct their sequences. However, it may soon be possible to overcome some of these challenges using techniques such as optical mapping and second- (and soon third-) generation sequencing technologies that produce long-insert paired reads or long single reads.

3.5 The Genomes of Other *Entamoeba* Species

Comparative genomic analyses can be a useful way to investigate the common and unique features of the biology of different species. In addition to the genome of *E. histolytica* HM-1:IMSS (and 15 other *E. histolytica* strains or derived cell lines), genomes from four other *Entamoeba* species have been sequenced and assembled in draft form. For some of these species, the genome sequence has been made publicly available via sequence repositories or the amoebaDB web resource, but descriptions

and analyses of the assembly and annotation have not yet been published. They are briefly described here, in order of increasing divergence from *E. histolytica*.

Entamoeba nuttalli is a pathogenic parasite isolated from captive and wild macaques [24–26]. *E. nuttalli* strain P19, isolated from a rhesus macaque (*Macaca mulatta*) in Nepal, was sequenced using Illumina sequencing technology (Dr. Elisabet Caler, personal communication). The annotated genome assembly is publicly available via GenBank (accession number PRJNA72167) and amoebaDB. The genome assembly represents approximately 14 million base pairs of DNA in 5,233 scaffolds and contains 6,187 annotated protein-coding genes. Compared to *E. histolytica*, the *E. nuttalli* genome assembly is smaller and more fragmentary, which might reflect real genomic differences or differences in the sequencing technology.

Entamoeba dispar is the described species next most closely related to *E. histolytica*. The two are morphologically identical, although their genomes may be evolutionarily quite distant [56]. Consequent to its morphological similarity to *E. histolytica*, *E. dispar* was only designated a distinct species in 1993 [22]. The genome of the axenized strain SAW760 was sequenced (to lower coverage depth than *E. histolytica*) using whole genome shotgun Sanger sequencing. The assembly (available via GenBank under accession number PRJNA12914 and also hosted by amoebaDB) consists of approximately 23 million base pairs of DNA in 3,312 scaffolds and shows similar AT content (approximately 76.5 % AT), proportion of coding sequence, and number of annotated genes (8,749) to *E. histolytica*. The more fragmentary genome assembly of *E. dispar* relative to *E. histolytica* (3,312 scaffolds compared to 1,496) is likely to be the result of the lower depth of sequence coverage. Despite this, it is still possible to observe large-scale genomic rearrangements between *E. histolytica* and *E. dispar* (discussed in Sect. 3.6).

The genome of the axenized Laredo strain of *E. moshkovskii* (originally derived from a human infection) has been sequenced using 454 sequencing technology. The genome assembly is currently available via amoebaDB and covers approximately 23 million base pairs in 1,147 scaffolds.

The reptile parasite *Entamoeba invadens* is more distantly related to *E. histolytica*. It is primarily studied as a model for the process of cyst formation because, in contrast to *E. histolytica*, it can be induced to encyst in axenic laboratory culture. The axenized IP-1 strain has been sequenced by whole genome shotgun Sanger sequencing, and the assembly is available via GenBank (under accession PRJNA12926) and amoebaDB. The genome is larger than that of *E. histolytica* or *E. dispar* (approximately 41 million base pairs of DNA in 1,149 scaffolds) and is slightly less AT rich (approximately 70 % AT); 11,549 annotated genes span approximately 38 % of the genome.

3.6 Interspecific Variation Among *Entamoeba* Genomes

Given the amount of sequence data used in the *E. histolytica* genome assembly, it is likely that the majority of genes of HM-1:IMSS genes are represented in the assembly. The gene content of the organism reflects its biology and has been reviewed in

detail [30]. For instance, expanded multigene families encode large numbers of protein kinases and phosphatases, highlighting the importance of cellular signaling in response to environmental signals [30, 57]. A family of approximately 100 transmembrane kinases, which consist of an extracellular domain, a single transmembrane domain, and an intracellular kinase domain, has been described [58, 59]. Members of this family of kinases may have roles in phagocytosis and amoebic virulence [60–62]. Another *Entamoeba* kinase containing a C2 domain (*Eh*C2PK) has been shown to be important during the initiation of phagocytosis [63]. Rab GTPases, controlling vesicular trafficking in the cell, are also encoded in large numbers (>100) in the genomes of *E. histolytica* and *E. invadens* [64, 65]. Despite the evident importance of these genes, the precise functions of only a few have been discovered [66–70]. The functions of other large gene families, including a family of AIG1-like GTPases [32] and BspA-like proteins (proteins homologous to a bacterial fibronectin-binding protein that contain a leucine-rich repeat domain) [32, 71], remain unknown. Differential expression among the AIG1-like proteins suggests the family may have a role in virulence or in adaptation to the intestinal environment [72–74].

There are few comparative genomic studies of the gene content of *Entamoeba*. Most relevant studies have investigated small numbers of loci in two or more species. Analyses to detect the presence of absence of genes implicated in virulence can help explain the ability of *E. histolytica* to cause disease. Secreted cysteine proteases play a role in amoebic virulence, and a key cysteine protease virulence factor, CP-A5, is a pseudogene in *E. dispar* [75]. Another key virulence factor, the Gal/GalNAc lectin complex, consists of heavy, intermediate, and light subunits encoded by multigene families. Families encoding heavy and light subunit genes differ in size between *E. histolytica*, *E. dispar*, and *E. invadens*, and intermediate subunit genes are found only in *E. histolytica* and *E. dispar* but not in *E. invadens* [30]. The lysine- and glutamic acid-rich protein (KERP1) is a virulence factor reported to be unique to *E. histolytica* [76]. However, although no KERP1 orthologue is annotated in *E. dispar*, a partial sequence match to the 3′-half of the gene (scaffold accession DS550082, position 1-251, in frame) suggests that one may exist but is currently not annotated because of the incompleteness of the sequence assembly.

A comparative analysis on a very broad scale compared the predicted proteomes of *E. histolytica* and *D. discoideum* and identified 3,833 *E. histolytica* proteins with putative orthologues in *D. discoideum* [18]. Of these, all but 62 also had putative orthologues in other eukaryotic proteomes (representing animals, fungi, and plants), indicating very few genes are specific to all members of the *Amoebazoa*. Because less than half of the *E. histolytica* proteome is shared with *D. discoideum* and their biology is quite different, it is highly likely that that many *Entamoeba*-specific genes exist. One way in which the metabolism of *Entamoeba* differs markedly from *Mycetozoa* is that *Entamoeba* are microaerophilic. As a consequence, they have no mitochondria and rely on fermentation for energy generation [15]. Genes encoding fermentation enzymes in *E. histolytica* are among a set of genes (at least 68) that were apparently acquired by horizontal gene transfer from bacteria [15, 30, 77–79]. Some genes appear to have been acquired before to the divergence of *Entamoeba*

and other major amoebazoan lineages, some afterward, although most are shared by divergent *Entamoeba* species *E. histolytica* and *E. invadens*, indicating relatively ancient gene acquisition [80, 81].

Several comparative analyses of the abundant transposons and repetitive DNA of *Entamoeba* have been undertaken [82–86]. Approximately 20 % of the *E. histolytica* genome assembly is estimated to consist repetitive elements, which tend to be found in clusters in the genome [84]. Many of the genome sequence scaffolds are flanked by these elements, which might partly explain its fragmentary nature. Transposable elements might also act as sites of chromosomal instability, leading to structural changes in the chromosomes that can be seen as breaks in the synteny of genes between *E. histolytica* and *E. dispar* [84]. Among *Entamoeba* species, striking differences in transposon content are seen. *E. histolytica* and *E. dispar* contain vary large numbers of class I LINE and SINE retrotransposons. In contrast, *E. invadens* and *E. moshkovskii* are rich in class II DNA transposons such as DDE and mariner elements [82, 84]. An *Entamoeba*-specific repetitive element (ERE1) is common in *E. histolytica*, *E. dispar*, and *E. invadens*, and a second (ERE2) is found only in *E. histolytica* [84]. Analyses of the *E. histolytica* and *E. dispar* genomes indicate recent active transposition [83, 85, 86]. The positions of SINE retrotransposons commonly differ between *E. histolytica* and *E. dispar* genomes, and even those occurring in orthologous positions appear to have arisen from independent insertions in the two species lineages [86]. However, relatively little is known about intraspecific variation among transposons.

3.7 Intraspecific Variation Among *Entamoeba* Genomes

Among *E. histolytica*, a number of studies have described intraspecific and intrapopulation diversity. Information-rich sets of genetic markers are essential for population genetic analysis. This information richness is determined by both depth and breadth of marker information: that is, many markers of limited individual diversity (single-nucleotide polymorphism, SNPs) or fewer markers of high diversity (short tandem repeats/microsatellites).

Unusually, the *E. histolytica* genome appears not to contain microsatellites. Although simple sequence repeats are present, these are often within protein-coding genes, so may be unsuitable as population genetic markers, being under selection. The tRNA-STR loci have been used as genetic markers in *E. histolytica* and *E. dispar* [87–94], and (along with studies using internal repeats of genes encoding the serine-rich *Entamoeba histolytica/dispar* protein and chitinase) they show very high levels of diversity in parasite populations [88, 90–93, 95–101]. The extensive polymorphism among tRNA-STR loci makes them very good markers for showing local "epidemic" transmission among people in close contact, such as family members in the same household [88], people residing in the same institution [96, 101], and recent sexual partners [102]. However, the diversity and complexity of these markers do make it difficult to determine the relatedness of individuals, so they are less

effective for determining population structures more broadly, particularly in endemic settings. For instance, in the study of population diversity in South Africa using tRNA-STR, the diversity of parasites among households was great but cannot be effectively quantified [88]. An added complication when using tRNA-STR markers is that each is actually the consensus of multiple copies of the marker in the same individual. How this affects the evolution of these sequences (for instance, variants spreading among copies by gene conversion and concerted evolution) is not known. Therefore, a note of caution is necessary regarding their use as population genetic markers.

Quantitative measures of diversity among individual parasites in a population may be gained by using larger sets of (binary) SNP markers. However, a major obstacle to the use of SNPs to genotype parasites for population studies is that stool samples typically contain very little parasite DNA: this limits the number of SNPs that can be determined per individual, thus limiting the resolution of typing. If a suitable isolation method could be devised, whole genome amplification and whole genome sequencing of parasites from field isolates could be applied to allow population genomic studies to be carried out.

A number of studies indicate that allelic SNP diversity is low among *E. histolytica* [39, 40, 103–105] although haplotype diversity appears to be high [40]. Such seemingly paradoxical results could be explained by sexual recombination and reassortment of alleles. This idea is supported by patterns of SNP polymorphism among whole genome-sequenced laboratory-adapted *E. histolytica* strains, which display decreasing linkage disequilibrium with physical distance between nucleotide positions, indicative of meiotic recombination [39]. The high haplotype diversity seen among SNP markers on different sequence scaffolds (possibly from different chromosomes) typed in a large sample of parasites from an endemic population showed no linkage disequilibrium, suggesting sexual reassortment of chromosomes [40]. Both studies, along with the observation that the genome encodes genes necessary for meiosis, point toward *E. histolytica* sexuality, although the mechanism is unknown [106]. Analyses of population structure in *E. histolytica* would benefit from improved genome assembly, particularly the determination of chromosomal sequences. This advance, with improved methods to type large numbers of markers, will allow the application of linkage disequilibrium-based genome-wide association methods and greatly increase our understanding of the population biology of the parasite.

3.8 Conclusions and Future Prospects

The draft genome assemblies of the *Entamoeba* species sequenced so far remain fragmentary and have, in general, stubbornly refused to cooperate in attempts at improvement and "finishing." However, the application of techniques such as optical mapping to define linkage groups, or long read sequencing to span the many clusters of repetitive elements, offer hope of improving the quality of the genome

assemblies. Second-generation sequencing technology is rapidly increasing the rate at which whole genomes can be sequenced, generating a wealth of comparative data that will allow a more thorough understanding of the biology and evolution of *Entamoeba*. As these genome sequencing technologies continue to advance, both the cost and the quantity of genomic DNA required to sequence an entire genome are becoming less. It may soon be possible, although challenging, to sequence *Entamoeba* genomes isolated directly from human stools, allowing detailed analyses of population genomics, genome-wide association studies of virulence, and other traits and analyses of the microepidemiology of amebiasis. Many questions remain about the complex genomic biology and evolution of *Entamoeba* species. However, what makes it most challenging also makes it most interesting.

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Chapter 4
Multilocus Sequence Typing System (MLST): Genetic Diversity and Genetic Components to Virulence

Carol A. Gilchrist

Abstract Data from whole genome sequencing of *Entamoeba histolytica* strains have been used to identify polymorphic loci. Sixteen of these loci were used to genotype 63 *E. histolytica* strains obtained from an area in Bangladesh where amebiasis is endemic. These markers seemed to segregate autonomously and exhibit extensive haplotype diversity. Despite the genetic heterogeneity, a strong association was observed between two single-nucleotide polymorphisms (SNPs) in the cylicin-2 gene and disease phenotype. This observation suggests that there are genetic differences between virulent and avirulent parasites and that, because of the naturally occurring high background reassortment, multilocus sequence typing in *E. histolytica* will permit us to link genetic markers with disease outcome in clinical samples.

4.1 Introduction

Entamoeba histolytica infection can result in asymptomatic colonization, diarrhea, dysentery, or amebic liver abscess. Human susceptibility to the disease of amebiasis is influenced by host genetics (HLA Dr/Dq haplotype and leptin receptor Q223R mutation) and host malnutrition (as described in Chap. 28) [1–4]. Parasite genetic factors are also implicated in the development of disease; however, these are not as well defined [1, 3, 5, 6]. Although progress has been made in characterization of virulence factors through direct manipulation of the *E. histolytica* genome and the development of animal models for amebiasis, the gold standard for virulence remains the outcome of infection in humans [7–12].

Technical limitations exist in obtaining the whole genome sequence of pathogens using the currently available technology for massively parallel sequencing, as these infectious organisms are often present at very low numbers in complex samples. Although it is usually isolated as a culture with a fecal bacterial flora,

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_4

E. histolytica can be axenized (mono-cultured) to eliminate contaminating bacteria and yield pure *E. histolytica* DNA for sequencing; however, this process is challenging, labor intensive, and potentially selective [13, 14]. Short tandem repeats specific to *E. histolytica* have therefore been used in genotyping schema to type population variation [5, 15–17]; these have successfully linked parasite genetics to the phenotype of the disease occurring in the host, as described elsewhere in this volume (Chap. 5) [5, 17]. The drawbacks to this genotyping method, however, are that the evolutionary relationship between strains cannot be inferred (because of the potential for both expansion and contraction in the number of repeats); moreover, the virulence traits associated with a particular repeat motif are not known and cannot be mapped within the *E. histolytica* genome [5, 15–17].

4.2 Multilocus Sequence Typing (MLST): A Balance Between Variability and Stability

A misleading impression of pathogen clonality can occur if insufficiently polymorphic markers are used in genotyping [18]. In *E. histolytica*, however, the levels of diversity observed in short tandem repeat markers, both those associated with the *E. histolytica* tRNA genes and those within the serine-rich *E. histolytica* protein (SREHP), suggest that polymorphisms occur frequently even in limited geographic areas [5, 15, 19–22]. The level of intraspecies genetic variation is therefore sufficient to permit discrimination between stains using even a few genetic loci.

A genotyping system also requires that the chosen genetic markers be sufficiently stable to allow strains to be typed accurately and placed in the correct molecular phylogenetic context. Compared with other DNA markers, single-nucleotide polymorphisms (SNPs) are usually stable. Earlier studies had evaluated the stability of *E. histolytica* SNPs by surveying *E. histolytica* SNP frequency in 9 kb of coding and noncoding DNA amplified from 14 Bangladeshi strains [23, 24]. The data suggested that the *E. histolytica* A/T-rich intergenic regions were highly polymorphic, whereas nonsynonymous SNPs (which resulted in a change in the encoded amino acid) tended to be more conserved [23]. Nonsynonymous SNPs therefore were selected as markers for MLST [14].

The sequencing in parallel of *E. histolytica* amplicons that span the location of several common SNPs (multilocus sequence typing) was therefore evaluated for its utility in *E. histolytica* molecular epidemiological studies by Gilchrist et al. [14].

4.2.1 E. histolytica *Multilocus Sequence Typing Schema*

Multilocus sequence typing (MLST) systems have been used widely to discriminate between pathogen species and strains. Although these systems have most commonly been applied to bacterial species, MLST has also been used to characterize micro-eukaryotes [25–28]. In bacteria, a common approach has been to select and

sequence several housekeeping genes. An example of this was the application of a nontyphoidal *Salmonella* MLST schema to investigate the source of disease outbreaks in Gambia. Seven housekeeping genes were sequenced to type the *Salmonella* infections occurring in children and their companion animals. The *Salmonella* strains were distinct in each host species, suggesting human-to-human transmission of *Salmonella* was primarily occurring in this population [29]. In the microeukaryotes, however, a more diverse range of multilocus genotyping strategies have been applied, partly because of the limited number of whole genome-sequenced strains available to guide marker selection and the need to capture sufficient phylogenetic signal to observe measurable variation between strains [25, 30, 31].

The *E. histolytica* genome resequencing effort led by the Hall and Caler laboratories has resulted in the sequences of 14 *E. histolytica* genomes and is described in another section of this volume (Chap. 3) [14, 32–34]. Each sequenced strain when compared to the reference HM1:IMSS strain contained approximately 1,500 homozygous and 1,000 heterozygous SNPs within its coding DNA: half of all the SNPs identified were unique and present in only one strain.

Six collection-specific SNPs occurring within 9 kb of DNA were associated with symptomatic disease development by Bhattacharya et al., and their work showed that SNPs could potentially be used as markers of virulent amebas [23]. However, these specific SNPs were particular to the Bangladesh strains sequenced during this study and not present in the genome sequences derived from the wider international collection. A new set of *E. histolytica* marker SNPs was selected to establish a MLST typing strategy for detecting virulence association [14].

To make certain that none of the selected marker SNPs were sequencing artifacts, SNPs that occurred only once in the sequence data were removed from consideration, which accounted for approximately half the SNPs identified in any given strain. Also removed from the pool of candidates were SNPs occurring in the 58 *E. histolytica* protein families composed of five or more members to reduce the possibility that misalignment would result in a "false-positive" SNP assignment in later work [33].

As some of the strains were sequenced using the 454/Roche next-generation sequencing platform at relatively low coverage (i.e., the number of times a particular DNA was sequenced), 12 SNPs identified in the strain MS96-3382, DS4-868, and the reference sequence HM-1:IMSS were verified by conventional Sanger sequencing. The Sanger sequences of the MS96-3382 and DS4-868 strains matched the sequence produced by the next-generation sequencing (NGS). The Sanger data from HM-1:IMSS also matched the reference genome; however, one SNP in the alcohol dehydrogenase gene was found by NGS to be heterozygous in the HM-1:IMSS reference strain, which was not previously known. Confident, therefore, in the predicted SNPs, the next requirement was the identification of evolutionarily stable SNPs postulated to be the best candidates for inclusion in an internationally applicable MLST schema.

To increase the likelihood that the selected SNPs could be linked to virulence traits, those that occurred either where orthologous sequences were not present in the closely related species *E. dispar* or where the SNPs were within genes thought to be potentially virulence associated or virulence regulated were selected [7, 35–39].

If a common SNP is connected to a disease phenotype, sufficient statistical power for an association to be identified will exist even in relatively low sample numbers. The second selection criterion, therefore, was that the MLST SNPs should occur at a frequency of 0.5–0.3 in the sequenced genomes, a "common" SNP (in this chapter, any SNP present in more than or fewer than 30 % of isolates is defined as "common" or "less common," respectively).

4.3 Evaluation of the *Entamoeba histolytica* MLST System

E. histolytica isolates collected during an ongoing longitudinal study of enteric pathogens infecting children living in an urban slum of Dhaka, Bangladesh, were made available for multilocus sequence typing [2–4, 40, 41]. The study samples included both DNA isolated from stools that were *E. histolytica* enzyme-linked immunosorbent assay (ELISA) positive and DNA from *E. histolytica* strains established in xenic culture from these stools. Both, therefore, represented strains in circulation at the Dhaka study locale. In this study cohort, 20 % of *E. histolytica* infections are associated with diarrhea [4]. To include invasive extraintestinal samples, the aspirates from amebic liver abscesses were collected for this study at the Rajshahi Medical College, Rajshahi, Bangladesh [14, 17, 42].

4.4 Massive Parallel Sequencing

The goal of the work was to design a high-throughput assay applicable for processing a large number of samples. The advent of inexpensive second-generation, ultrahigh-throughput, DNA sequencing technologies (DNA fragment-based "sequence by synthesis") has transformed parallel sequencing of amplicons into a routine exercise in standard benchtop molecular biology. Illumina DNA sequence "reads" are generally 35–150 bp per contiguous read, with the option of obtaining either a single read or pairs of reads from the ends of 200- to 300-bp DNA fragments.

A nested strategy to amplify the DNA isolated from the previously discussed sources (stool samples, short-term xenic cultures of parasites from stool, and amebic liver abscess aspirates) at the selected SNP loci was used. To make use of direct Illumina next-generation amplicon sequencing, the size of the final amplicons was restricted to 300 bp or less, which were then sequenced from each end (paired end reads) using the Illumina GAIIx sequence platform [43]. Illumina error rates are generally between 0.5 % and 1 % overall, and standard protocols in SNP discovery require 15 to 40× read coverage to compensate for this [44]; in this case, however, a lower 8× coverage standard for the identification of previously discovered SNPs in Illumina sequence data was used [45].

The sequences required for correct annealing to the oligonucleotides coating the Illumina flow cell and to the Illumina sequencing primers were added to the

amplifying polymerase chain reaction (PCR) oligonucleotides to produce amplified barcoded Illumina "libraries" [43]. The 7-bp index barcode was used in the PCR amplification step to "tag" the fragments with a sequence unique to the original sample (a barcode). This barcode was then obtained as part of the sequence generated during the next-generation sequencing run and used to bin the sample reads according to the original sample, before alignment to a reference genome and SNP detection (demultiplexing). The introduction of sample-specific barcodes during the amplification process allows concurrent sequencing of a larger number of samples. The number of samples that can be combined depends on the complexity of the Illumina library, the number of indexed samples, and the depth of desired sequence coverage. In the paper by Gilchrist et al. [14], 21 initial targets were selected and 84 barcoded samples were combined and run on a single Illumina GAII sequencer lane. As described in the next section, subsequent quality control metrics reduced the number of targets to 16 and samples to 64.

4.5 Illumina Library Complexity

It was discovered upon demultiplexing that an equivalent number of sequence reads was obtained from each of the 84 *E. histolytica* samples whether the libraries were generated from stool samples, polyxenic cultures, or liver aspirates. However, in the sequences generated from stool samples and polyxenic cultures a greater number of the reads did not derive from the *E. histolytica* amplicons. It is posited that this is caused by off-target amplification of DNA from gut flora (organisms not present in the amebic liver aspirates but which are in co-culture with the amebic trophozoites and are, obviously, present in stool samples), or a reduction in specificity in the culture DNA, as most of those samples did not need to undergo nested PCR amplification before library preparation.

A lack of primer specificity may have been particularly acute at five of the loci, as these frequently failed to amplify, and these loci were conservatively removed from the study. However, it is possible that absence of the amplicon was caused by deletion of the target DNA or divergence at the primer binding sites in some isolates. The final data set consisted of 63 of the original 84 samples (63 % of stool samples from asymptomatically colonized individuals, 80 % of diarrheal stools, 73 % of xenic cultures, and 84 % of amebic liver aspirates): all 16 remaining target sequences seemed to be efficiently amplified, and hence ≥8× sequence coverage of the SNP loci was obtained.

4.6 MLST Analysis

Amplicon sequences were identified as "homozygous Reference" (if the same as the reference HM-1:IMSS sequence at this position), "heterozygous" (containing both the HM-1:IMSS nucleotide and the variant nucleotide at this position), or

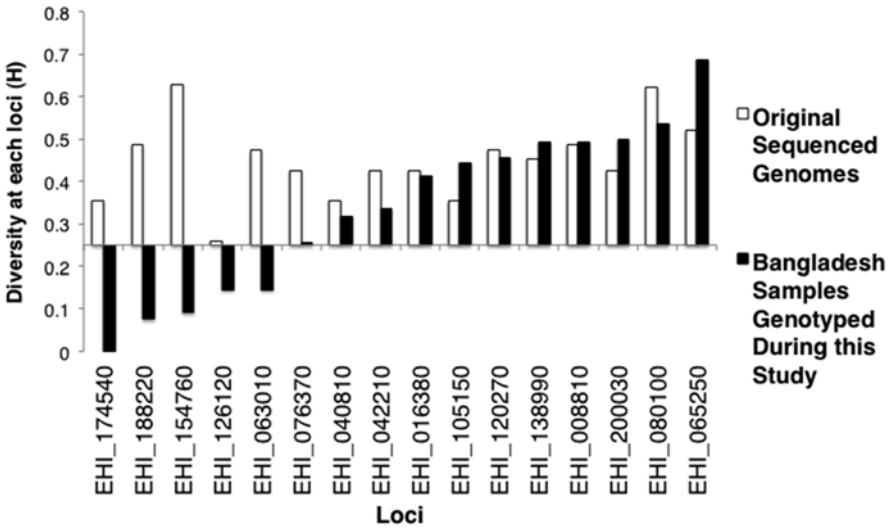

Fig. 4.1 Similarity of *Entamoeba histolytica* diversity in Bangladeshi and whole genome-sequenced strains. Shown on the *y*-axis (*H*) is the calculated heterozygosity, representing the sum of the squared allele frequencies subtracted from 1. On the *x*-axis are the loci containing the single-nucleotide polymorphisms (SNPs) genotyped by the multilocus sequence typing system (MSLT). *Filled bars* represent value in Bangladesh samples genotyped during this study; *open bars* are value in the sequenced genomes. (Figure reproduced with permission from Gilchrist et al.[14])

"homozygous Non-Reference" (having only the variant base at this location). The frequency of 11 of the final 16 alleles in the Bangladesh multilocus typed samples was similar to their occurrence in the original genome-sequenced strains, whereas 5 were invariant or nearly so in Bangladesh (Fig. 4.1).

In phylogenetic trees, only 4 of the 63 samples could be linked with greater than 50 % confidence (Fig. 4.2). *E. histolytica*, therefore, does not exist as a small number of lineages where linkage disequilibrium occurs between markers [47]. The extensive haplotype diversity even in a single geographic location suggests that genetic reassortment is extensive.

Monitoring of *E. histolytica* trophozoites growing in culture has not provided any experimental evidence for the occurrence of recombination in *E. histolytica* [15]. The MLST data suggest, however, that either a very low rate of recombination does actually occur, resulting over millennia of evolution in the diversity observed, or recombination is repressed in culture. *E. histolytica* trophozoites do not normally encyst in culture, and recombination may be an event occurring in the encystation or excystation phase of the parasite life cycle [48]. The extensive endo-reduplicative cycling observed in *E. histolytica* could also favor recombination or activation of the bioinformatically identified meiotic *E. histolytica* genes in the response to the correct stimuli [32, 49–53]. The potential for a rapid rate of evolution should be considered when comparing differences in *E. histolytica* genotypes from distinct geographic regions.

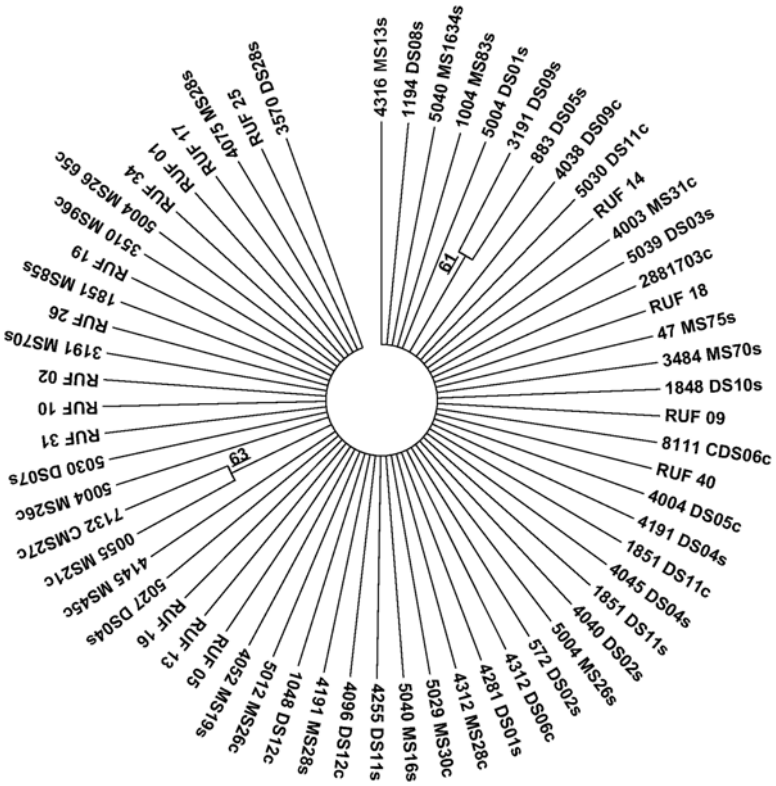

Fig. 4.2 Lack of consistent patterns of descent among SNP markers from Bangladeshi *E. histolytica* isolates suggests they segregate independently. Consensus phylogeny inferred from 100 bootstrap replicates of polymorphic SNP markers, constructed using MEGA 5 and the maximum-likelihood method based on the Tamura–Nei model [14, 46]. Branches produced in fewer than 50 % of the bootstrap phylogenies were collapsed. Sequences from stool have the suffix *s*, culture *c*, monthly survey stools begin with *MS* or *CMS*, diarrheal stools *DS* or *CDS*, amebic liver abscess samples *RUF*. (Figure reproduced with permission from Gilchrist et al. [14])

4.7 Identification of SNP Markers That Are Informative Regarding the Virulence Potential of These Parasites

Two closely linked SNPs (SNPs $2725^{C/T}$ and $2730^{A/G}$) within the cylicin-2 gene (AmoebaDB ID EHI_080100; XM_001914351) were associated with invasive amebiasis. In the Dhaka region, both the Reference and Non-Reference SNPs were present at approximately equal levels in asymptomatically colonized hosts, but the Non-Reference SNP was present in only 16 % of the samples isolated from diarrheal patients (Fig. 4.3). The Non-Reference SNP was present in 75 % of amebic liver aspirates isolated 160 miles away in Rajshahi [14]. It is important to recognize,

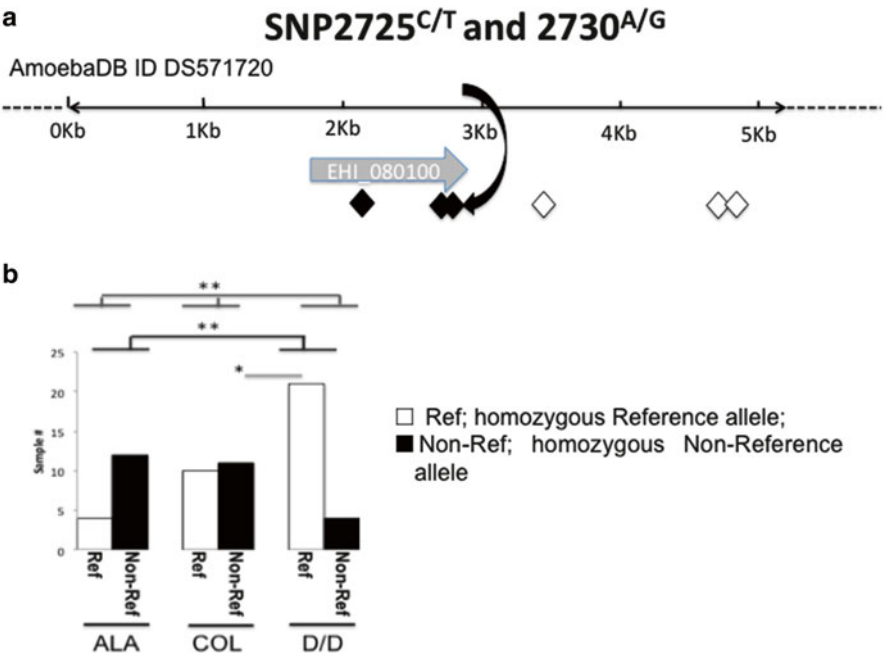

Fig. 4.3 Association of SNP2725$^{C/T}$ and 2730$^{A/G}$ in diarrheal/dysentery or amebic liver abscess. **a** Genomic map of the contiguous DNA (AmoebaDB ID DS571720), containing the Cylicin-2 gene (AmoebaDB ID EHI_080100; XM_001914351 open reading frame indicated by *arrow* in the cartoon). Nonsynonymous (*filled diamonds*) and intergenic (*open diamonds*) SNPs. (Adapted from the AmoebaDB website http://amoebadb.org/amoeba/ [54]). **b** SNP2725$^{C/T}$ genotype: *open squares* Ref: homozygous Reference allele; *filled squares* Non-Ref: homozygous Non-Reference allele, *ALA* amebic liver abscess, *COL* asymptomatic colonization, *D/D* dysentery/diarrhea. Three-way chi-squared contingency analysis of all samples: $p=0.002$, $q=0.032$ (**). Fisher's pairwise comparison between ALA and D/D: $p=0.0003$, $q=0.0144$ (**); D/D and COL: $p=0.0182$ (*). (Figure 4.3b adapted and reproduced with permission from Gilchrist et al. [14])

therefore, that some of the genetic differences between the *E. histolytica* infections that resulted in child diarrhea and those resulting in liver abscesses could be the result of this geographic separation.

4.8 Future Directions

In *Cryptosporidium* sp., a number of multilocus schema have been applied to enable higher phylogenetic resolution of isolates in this sexually recombining organism [55–57]. Although it has been generally agreed that the multilocus analysis is superior to the common single polymorphic *gp60* locus analysis, the proliferation of multilocus fragment size-based typing (MLFT) schemes has made it difficult to compare studies. However, the meta-analysis of Robinson et al. has suggested that

some of the loci in the disparate schema are particularly informative, and they postulate that the combination of markers linked to different virulence determinants could result in an improved, extended, multilocus typing system with a superior capacity to assess the virulence potential of an isolate [57].

A key consideration for the evaluation of disparate schema developed in response to the availability of the genomic sequence is a data repository that will allow the comparison of multilocus markers and the identification of informative targets. Fortunately, the development of pathogen-specific websites combined with the increasing availability of cloud-based computing tools will facilitate the comparison and sharing of genotype project databases and the development of collaborative web-enabled studies [54, 58, 59].

It will be important to evaluate whether the genotype–phenotype associations detected vary over time as a result of the development of genotype-specific immunity and whether they are applicable in all human populations. We hypothesize, as discussed at the beginning of this chapter, that the outcome of an *E. histolytica* infection (e.g., colonization vs. diarrhea) is the result of a combination of parasite, host, and environmental factors; however, concerns over the privacy and security of patient information in an era of big data biology need to be addressed before we can fully grapple with these issues [60, 61].

Acknowledgments The work discussed here was supported by grant 5R01AI043596 from NIAID to W.A.P. This project has also been funded in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract numbers N01-AI30071 and/or HHSN272200900007.

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Chapter 5
The tRNA Gene-Linked STRs and Other Genetic Typing Methods

Ibne Karim M. Ali

Abstract Only one in ten *Entamoeba histolytica* infections is invasive but the bacterium is responsible for an annual death toll of up to 100,000 people. A key question in amebiasis is, therefore, what is responsible for the variable outcome of infection. To address whether it is linked to the genotype of the infecting strain, several protein-coding and noncoding genes have been investigated. However, with the use of only tRNA gene-linked short tandem repeat (STR) loci, a significant difference among the genotypes of three different clinical populations—asymptomatic, diarrheal/dysenteric, and liver abscess—was detected. This multilocus genotyping method is simple and reliable as it amplifies DNA extracted from culture, stool samples, and liver abscess pus samples. As a result, these markers are useful in studying the patterns of transmission of this important disease and the epidemiological links between individual infections. Because tRNA-linked STRs are noncoding DNAs, one caveat of this system is that they may not be directly linked to parasite virulence and may work only as surrogate markers to predict infection outcomes. A novel genotyping method to identify nonsynonymous SNPs that may directly correlate with the parasite virulence is currently underway.

5.1 Introduction

Entamoeba histolytica is an intestinal protozoan parasite and the causative agent of amebiasis. According to the one of the most cited references [1], one-tenth of the world population is infected with *E. histolytica*, resulting in up to 100,000 deaths worldwide each year [2, 3]. However, this estimate predates the formal separation of pathogenic *E. histolytica* from nonpathogenic *Entamoeba dispar* [4] and is now being reassessed. Current data indicate that *E. dispar* is perhaps ten times more

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_5

common than *E. histolytica* worldwide [5], but local prevalence may vary significantly. However, it is already clear that not all *E. histolytica* infections lead to disease in the host but that at most one in ten *E. histolytica* infections progresses to development of clinical symptoms [6]. Nevertheless, *E. histolytica* remains a significant source of morbidity and mortality in developing countries [7]. A key question in amebiasis is what determines the outcome of an *E. histolytica* infection. Both parasite genotype [8] and host genetics [9], plus host nutritional status [10–13] (described in Chap. 28), appear to contribute to this process. In this chapter, genotyping methods for *E. histolytica*, especially tRNA gene-linked short tandem repeats (STRs) that identified a positive association between parasite genotype and infection outcome, are discussed.

5.2 Genotyping Methods for *Entamoeba histolytica*

Infection outcomes are variable and unpredictable with *E. histolytica*, making this an exciting organism for the study of genetic diversity. A few polymerase chain reaction (PCR)-based DNA typing methods have been reported for *E. histolytica*, making use of repetitive elements contained within both protein-coding genes and noncoding DNAs.

In *E. histolytica*, the rRNA genes are present on a highly repetitive extrachromosomal circular DNA, about 25 kb in size and with 200 copies per genome. and the strain-specific gene (SSG) [14] or *Tr* [15] has been described in the upstream region of the rDNA circle [16–18]. *E. dispar* strains do not possess this gene, and in some *E. histolytica* strains this gene is completely absent. In SSG-positive strains, a noncoding transcript of unknown function has been detected. The SSG gene contains tandem repeats, and PCR amplification shows variations in the number of repeat units among *E. histolytica* strains [19]. Absence of this gene in some *E. histolytica* strains has limited its application as a polymorphic marker.

Polymorphism has been investigated in the repeat-containing protein-coding chitinase gene in *E. histolytica*. The chitinase gene, expressed only in the cyst form of the amoeba, shows only very limited diversity [20–24]. Similarly, the Gal/GalNAc lectin, a major amoebic virulence protein, showed only very limited diversity among strains of *E. histolytica* [25]. Perhaps the most widely studied protein-coding gene is the serine-rich *E. histolytica* protein (SREHP) gene, which encodes an immunodominant surface antigen containing tandem repeats of related dodeca- and octapeptides [26, 27]. SREHP is thought to play an important role in parasite adherence to apoptotic cells [1]. Repeat number, sequence, and restriction site-derived variations have been observed in the SREHP gene among strains of *E. histolytica* [19, 22–24, 28–30]. A study in Bangladesh with *E. histolytica* DNA from clinical strains showed extensive SREHP polymorphism, and the polymorphic patterns observed in amebic liver abscess strains were different from those of intestinal strains [28].

Polymorphism has been investigated using random amplified polymorphic DNA (RAPD)-PCR in a few north Indian isolates [31]. Although this method showed a

good degree of polymorphism, each isolate invariably produced multiple products in PCR, which is not ideal for large-scale surveys of genetic epidemiology as the analysis of results will be difficult. Also, RAPD may amplify DNA from host and associated bacteria in clinical samples. Because of this potential problem, its use remains limited.

Shah et al. [32] used a comparative genomic hybridization approach to investigate the genotypic differences among four reference strains of *E. histolytica* and two of *E. dispar*. One of the drawbacks of this approach is that it requires a large amount of genomic DNA (5–10 μg), which has so far restricted its use to laboratory strains only. However, between *E. histolytica* and *E. dispar* strains they identified a number of genes restricted to *E. histolytica*. Some of these genes have been implicated to the virulence of *E. histolytica* HM-1:IMSS. However, intraspecies variation in these genes in context with virulence differences in *E. histolytica* strains has not been studied.

5.2.1 The tRNA Gene-Linked STRs in E. histolytica

One of the striking findings in the *E. histolytica* genome sequence is the abundance and unique organization of the tRNA genes in the genome [33, 34]. More than 10 % of all the sequence reads contained tRNA genes that are 71 to 87 bp long and rich in Gs and Cs. Almost all of them are organized in tandem arrays with intergenic regions being rich in A+T (about 80 %) and containing short tandem repeats (STRs). A total of 25 distinct arrays were identified, containing one to five tRNA genes in each. The unit length of arrays varies between 490 and 1,775 bp. In 4 arrays, G+C-rich regions other than tRNA genes were identified. Three of these have been identified as the previously unknown 5S RNA genes in *E. histolytica*; the fourth is a suspected small nuclear RNA-encoding gene [35]. Outside the coding regions, apart from being A+T rich, there are no significant sequence similarities between the arrays. However, there exists a compositional bias; that is, one strand contains on average about 70 % pyrimidine.

5.2.1.1 Initial Studies of Polymorphism in Repeat Loci Containing tRNA Genes

A number of noncoding loci in *E. histolytica* and *E. dispar* have been identified by different groups of researchers, and each locus contains tandem repeat units. All these polymorphic loci were later found to contain genes for tRNA. Three of these repeat loci were originally identified separately by other groups but their potential as polymorphic markers was initially investigated by Zaki and Clark [36]: locus 3-4, a 978-bp element described by Michel et al. [37] (GenBank accession number M77091); locus 9-4, a 931-bp DNA element isolated by Rosales-Encina and Eichinger (GenBank accession number AF265348); locus 16-17, a 964-bp element reported by Huang et al. [38]. There was close similarity between locus 3-4 and

locus 9-4. In addition, Zaki and Clark [36] also isolated and tested locus 1-2 (GenBank accession numbers AF276055-9) and locus 5-6 (GenBank accession numbers AF276060-5).

Haghighi et al. [23, 24] compared the polymorphic potential of loci 1-2 (now known as locus D-A) and 5-6 (now known as locus I-W) with those of the protein-coding chitinase and SREHP genes using samples from institutionalized and homosexual people from Japan and clinical and reference strains from some other countries. In their studies, SREHP showed the most polymorphic patterns while chitinase was the least polymorphic. However, they observed that none of these loci alone could detect all the genotypes of the *E. histolytica* strains they tested, and concluded that more than one locus should be used for strain typing.

Zaki et al. [39] have shown that amplification of tRNA loci using DNA prepared from cultures reflects the patterns seen with DNA extracted from the original stool sample, indicating that although culture is not necessary it is not misleading either. However, there is a danger that one genotype may outgrow the others in culture when the infected person was originally infected with more than genotype of *E. histolytica*. In another study, Zaki et al. [40] used some of the STR loci to investigate the genotypes of *E. histolytica* and *E. dispar* in South Africa. Among other things this study showed that the genotypes remained stable over time in the same infection. No changes in the patterns obtained at any of the STR loci using DNAs extracted from *E. histolytica* HM-1:IMSS or *E. dispar* SAW760 at various times over the past several years were noticed. The markers therefore appear to be sufficiently stable to use for epidemiological studies.

A considerable degree of STR length polymorphism was observed not only among axenic strains of *E. histolytica* but also among strains isolated from a restricted geographic location [23, 24, 40]. Comparison of nucleotide sequences among *E. histolytica* strains at many of the loci studied in these studies revealed that the differences in PCR product sizes are mainly the result of variable number of STRs, in agreement with earlier observations at two loci [36]. However, no association between the parasite genotypes and the outcome of infections has been reported for *E. histolytica* in any of these studies.

5.3 Development of the tRNA Gene-Linked Six-Loci Genotyping System

Ali et al. [41] developed an extended set of six polymorphic markers making use of all the 46 STRs adjacent to flanking regions of tRNA genes in *E. histolytica* [33, 34]. The STRs selected for this optimal panel of markers were chosen on the basis of their reliability, sensitivity, and degree of polymorphism detected in PCR (Fig. 5.1). They have also designed *E. histolytica* and *E. dispar* species-specific primer pairs for all the selected loci, which eliminate the potential problems caused by mixed infections. The primers have been tested using DNA samples from diverse geographic locations, and they have successfully amplified DNA isolated directly

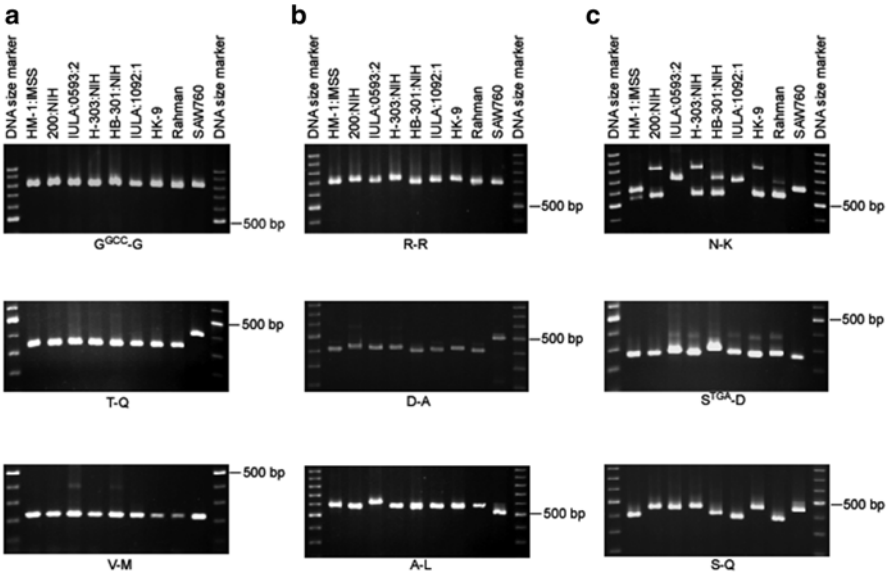

Fig. 5.1 Examples of polymorphism observed in tRNA-linked short tandem repeats (STRs) of *Entamoeba histolytica*. **a** Three STRs show little or no polymerase chain reaction (PCR) product-size polymorphism. **b** Three of the selected STRs show low to moderate polymorphism. **c** The remaining three selected STRs show moderate to high polymorphism. All samples are *E. histolytica* except for *Entamoeba dispar* SAW760

from liver abscess pus and stool samples, eliminating the need for culture of the amoebae. The DNA can be prepared for analysis using either a commercial kit or a modification of an existing purification method.

5.3.1 A Nonrandom Distribution of Parasite Genotype with Respect to Infection Outcome Was Detected

Ali et al. [8] used the selected markers to investigate the possibility of a correlation between parasite genotype and clinical outcome of infection using samples from Bangladesh. A total of 111 *E. histolytica*-positive DNA samples were included (excluding the follow-up samples) for this study: 38 in the asymptomatic group, 30 in the diarrhea/dysentery group, and 43 in the liver abscess group. Both the asymptomatic and diarrhea/dysentery samples were from children from Mirpur, Dhaka; the liver abscess (LA) samples were from adult patients from different clinics and hospital across Dhaka. Using the PCR patterns at six loci, a total of 26, 18, and 41 genotypes were identified in the asymptomatic, diarrhea/dysentery, and liver abscess sample groups, respectively. Overall, a total of 85 distinct genotypes (1 through 85) were detected across all samples. In the asymptomatic group, the

most common genotypes were 51 (infecting 4 children) and 13 and 19 (infecting 3 children each). In the diarrhea/dysentery group, the most common genotypes were 66 (infecting 7 patients) and 45 and 50 (infecting 3 patients each). Only 2 genotypes, genotypes 23 and 39, were found in more than 1 ALA patient, and these were found in 2 patients each. The households of the children infected with the most common genotype, genotype 66, had a widespread distribution in Mirpur. Because the total number of genotypes detected was very high, 85 among 111 samples, the number of samples represented by a single genotype was low. Nevertheless, the most prevalent genotype, 66, was significantly more common in samples from the diarrhea/dysentery group ($P = 0.0005$), although it was found in only 7 of the 30 samples from the diarrhea/dysentery group (23.3 %).

Genotype distribution among samples from three different clinical groups showed a strong statistically significant difference. For examples, some PCR patterns at D-A and N-K2 showed differential distributions among the two sample groups (asymptomatic versus diarrhea/dysentery) from Mirpur. Similarly, between the asymptomatic and the liver abscess sample groups, PCR patterns showing the greatest difference were A-L, N-K2, and S-Q, whereas between the diarrhea/dysentery and liver abscess groups, the greatest differences were in loci A-L, S-Q, and D-A.

5.3.1.1 Limitations of the Foregoing Study

Although evidence of a nonrandom distribution of parasite genotypes among the sample groups was seen, there were, however, some limitations in that study. One, all the asymptomatic and diarrhea/dysentery samples were from preschool children from Mirpur, so it is not known whether they represent genotypes from all age groups and the strain diversity across Bangladesh. All ALA samples were from adults in other parts of Dhaka, and no adult or geographically matched asymptomatic or diarrhea/dysentery control groups were available. So, comparisons between the Mirpur and ALA samples must be interpreted with caution. Two, the genotypes were assigned on the basis of the PCR product sizes in gels, and as a result some assignments could be inaccurate. Three, the genotype assignment is based on PCR product size differences, but same-size products do not necessarily mean the identical sequence.

5.3.1.2 Other Limitations of tRNA Gene-Linked STR Markers

Two cell lines with identical patterns at six STRs derived from the most widely used *E. histolytica* HM-1:IMSS isolate showed two different virulence phenotypes: one was highly virulent in experimental animals while the other was not [42]. In a recent study in China, Feng et al. [43] identified two patients with different outcome of infections who were infected with the same genotype of *E. histolytica*. These data suggest that the six STRs have their limitations in predicting parasite virulence in some cases.

Although the tRNA-linked STR loci showed a statistically significant difference among the genotypes representing the three sample populations, it is highly unlikely that they are directly responsible for parasite virulence; other approaches are needed to identify the genes responsible for this result. It is also important to test whether these statistically significant observations made in Bangladesh can be replicated in other parts of the world. A recent study investigated all the D-A and N-K2 sequence types reported in the literature in context to finding an association with infection outcomes [44]. They identified a total of 103 D-A sequences (from 9 countries) and 106 N-K2 sequences (from 13 countries) that produced a total of 13 and 29 sequence or haplotypes, respectively. A haplotype network using a common panel of 58 samples for both loci for which clinical information was available, however, showed no association with a particular geographic distribution or infection outcome. The *E. histolytica* HM-1:IMSS-derived D-A and N-K2 were most widely distributed sequence types worldwide.

5.3.2 Application of the tRNA Gene-Linked STRs

5.3.2.1 Detection of a New Infection or Genotype in Follow-Up Samples

Ali et al. [8] investigated the genotype patterns in multiple (follow-up) samples from each of 23 children, which included a maximum of seven follow-up samples from 1 child over a period of 11 months, and the same genotype was detected in all except one transiently infected different genotype. Six and five follow-up samples were available from 1 child each, and both these children carried the same original genotype throughout the follow-up period. Thirteen and 7 children gave two and three follow-up samples, respectively. The genotypes changed in 7 children, whereas in the remaining 13 children the same genotypes were detected at all loci. On average, half the six loci changed when a follow-up genotype was different, indicating that a reinfection is easily detectable. In 4 children, four different genotypes remained the same for more than 10 months, indicating that the ability to produce a long-term infection is not restricted to a particular genotype.

5.3.2.2 Detection of Genetic Selection or Organ Tropism in the Same ALA Patients

The genotypes of *E. histolytica* in stool- and liver abscess-derived 18 paired samples (16 from Bangladesh, 1 from the USA, and 1 from Italy) from the same ALA patients revealed that the intestinal and liver abscess amoebae are genetically distinct [45]. This finding suggests either that *E. histolytica* subpopulations in the same infection show varying organ tropism, or that a DNA reorganization event takes place before or during metastasis from intestine to liver. Although a second infection is a reasonable possibility in an endemic country such as Bangladesh, and

could indeed be true in some of these cases, it seems extremely unlikely to be a realistic explanation for the cases from Italy and the USA where *E. histolytica* infection is rare.

5.3.2.3 Identification of a Unique Avirulent *E. histolytica* Strain

Escueta-de Cadiz et al. [46] used the six-loci tRNA gene-linked genotyping system to genotype 37 Japanese *E. histolytica* samples from three clinical groups (5 asymptomatic, 12 diarrhea/dysenteric, and 20 ALA cases). Based on six STR data, 23 distinct genotypes were identified in those samples. Importantly, one asymptomatic isolate appeared to be inherently avirulent as it failed to induce liver abscess formation in experimental animals. This strain possesses four distinct STRs compared to any other strains of the study.

5.3.2.4 Tracking the Disease Transmission Within Family Members (in South Africa)

Zaki et al. [40] earlier investigated the diversity, stability, and transmission patterns of *E. histolytica* genotypes within families in a geographically restricted region in South Africa using the tRNA gene-linked loci. They concluded that the PCR patterns were stable over time in the same infection, and that infected family members carried, almost always, the same genotype of *E. histolytica*. This result further strengthens the usefulness of this typing method for identifying genotypes that may show a period of asymptomatic infections before causing disease in the infected individuals.

5.3.3 E. histolytica *Diversity Based on Sequence Types in tRNA Gene-Linked Loci*

Because same-size PCR product does not necessarily mean same sequence, it is advantageous to sequence PCR products to detect all the variation in a given locus. Although several groups [23, 24, 47] have sequenced PCR products in different tRNA gene-linked loci, only one has identified statistically significant sequence types that correlated with infection outcome [48]. In this study, among 112 clinical samples (21 asymptomatic, 20 diarrhea/dysentery, 71 liver abscesses) they identified 11 sequence types in locus R–R (Fig. 5.2). Sequence type 5RR was significantly associated with asymptomatic samples compared to either diarrhea/dysenteric samples ($p = 0.0148$) or combined symptomatic (diarrhea/dysentery and liver abscess) samples ($p = 0.0241$) (Fig. 5.3). Similarly, sequence type 10RR was significantly associated with the symptomatic samples in comparison with asymptomatic samples ($p = 0.0123$). However, the coverage of asymptomatic samples by sequence type 5RR (10/21) and symptomatic samples by 10RR (29/91) were low (Fig. 5.3).

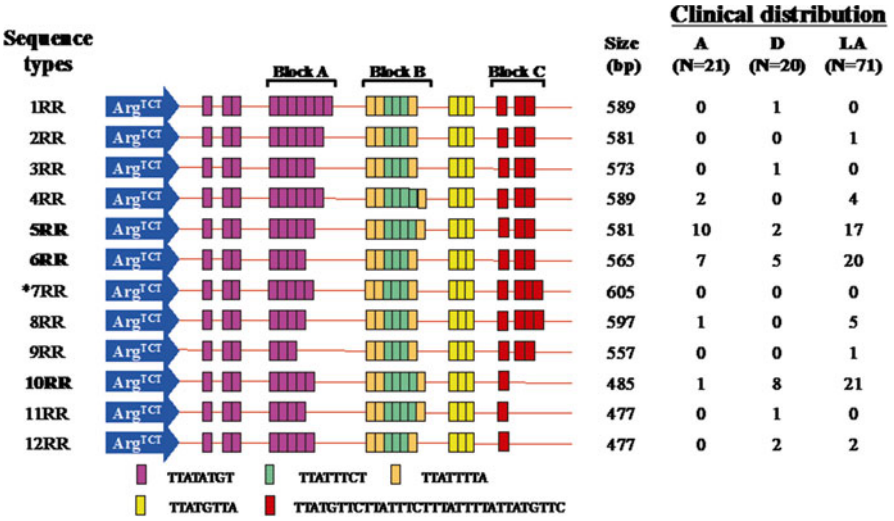

Fig. 5.2 Schematic representation of locus R–R sequence types and their distribution in clinical samples. The tRNA gene sequence is shown by an *arrow* and the short tandem repeat sequences are shown with *colored bars*. Three blocks (Block *A*, Block *B*, Block *C*) of short tandem repeats showed variability in number of repeats. Among all 112 samples, 11 different locus R–R sequence types were detected. *Sequence type 7RR was not detected in this study population although it was previously identified in a Japanese *E. histolytica* strain from an individual with unknown clinical manifestation [47]. The three most common sequence types (5RR, 6RR, 10RR) are shown in *bold font*. In the clinical distribution: *A* asymptomatic, *D* diarrhea/dysentery, *LA* liver abscess; *N* represents the total number of samples in a particular clinical population. (Figure adapted from Ali et al. [48])

5.3.4 Future Work: Genome-Wide SNPs in E. histolytica

Most genotyping systems in *E. histolytica* have focused on repetitive sequences. One of the major drawbacks of using repetitive sequences is that they may overestimate genetic diversity because the repetitive regions are prone to incorporate polymorphisms from DNA slippage [49]. However, only a handful of studies have investigated polymorphisms in nonrepetitive regions of the *E. histolytica* genome. Bhattacharya et al. [50] sequenced more than 9-kb coding and noncoding regions in each of 14 *E. histolytica* strains (4 laboratory strains and 10 clinical isolates) in 13 different loci. They detected a total of 14 SNPs: 5 within coding regions and 9 in noncoding regions. Strikingly, all the SNPs in coding regions were synonymous SNP, in sharp contrast to what has been seen in *Plasmodium falciparum*, where most SNPs were nonsynonymous [51]. Six SNPs were exclusively detected in the asymptomatic strains, including the only significant SNP in the coding region of a lectin gene ($p = 0.015$). This study by Bhattacharya et al. [50] showed promise for investigation of genome-wide SNP detection in clinical strains. In fact, Weedall et al. [52] sequenced genomes of 10 *E. histolytica* clinical strains (originating from Bangladesh, Italy, Korea, Mexico, UK, and Venezuela) using the next-generation

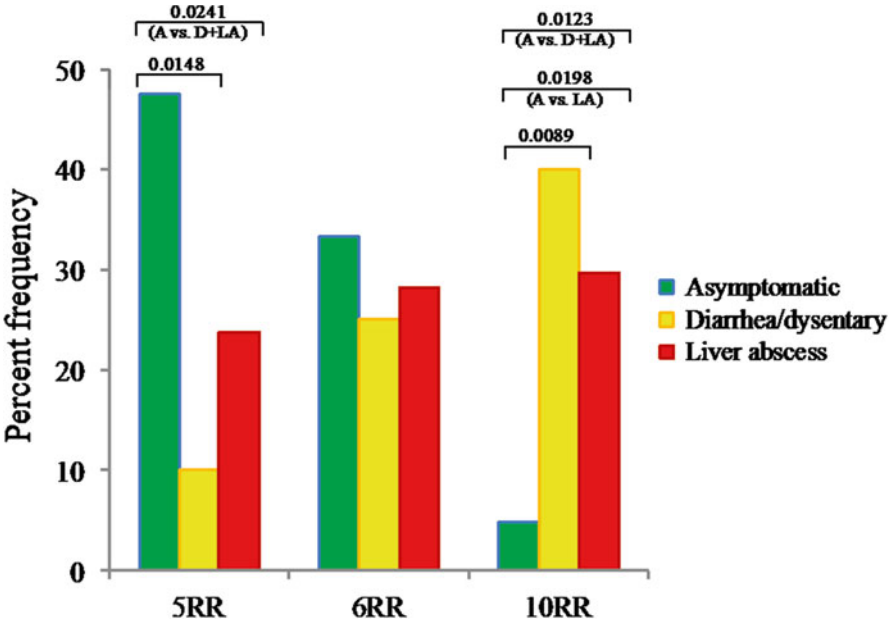

Fig. 5.3 Percent frequency of the three major locus R–R sequence types among the clinical samples. The sequence type 5RR is significantly associated with the asymptomatic sample group (*p* values between asymptomatic and diarrhea/dysentery, and between asymptomatic and diarrhea/dysentery plus liver abscess sample groups are 0.0148 and 0.0241, respectively). In contrast, the sequence type 10RR is significantly associated with the symptomatic sample group (i.e., diarrhea/dysentery and/or liver abscess sample groups) (*p* values between asymptomatic and diarrhea/dysentery, asymptomatic and liver abscess, and asymptomatic and diarrhea/dysentery plus liver abscess sample groups are 0.0089, 0.0198, and 0.0123, respectively). The sequence type 6RR is distributed almost evenly across all clinical sample groups. *A* asymptomatic, *D* diarrhea/dysentery, *LA* liver abscess. (Figure adapted from Ali et al. [48])

SOLiD platform. Three of the strains were from asymptomatic individuals and the remaining 7 were from diarrhea/dysenteric patients. Compared to the reference genome of HM-1:IMSS, ~2,000 to ~10,000 SNPs were detected in the clinical strains, accounting for 0.6439–0.8573 SNPs per kilobase, which was rather low compared to SNPs detected in *Plasmodium* genomes [53]. Fifty-three genes contained 5 or more nonsynonymous SNPs, including more than one member of virulence-associated lectin genes. Recently, Gilchrist et al. [54] utilized the genomic data from Weedall et al. [52] as well as from the John Craig Venter Institute (Rockville, MD, USA) led by Dr. Elisabet Caler, and developed a multilocus sequence typing (MLST) system (discussed in Chap. 4). They used the next-generation sequencing platforms to identify nonsynonymous SNPs in 16 selected loci in 63 samples from Bangladesh representing three clinical groups: asymptomatic, diarrhea/dysentery, and liver abscess. Strikingly, 2 SNPs identified in their study in cylicin-2 gene (EHI_080100) showed a significant association with disease outcome [asymptomatic/diarrhea ($p = 0.0162$) or dysentery/amebic liver abscess

($p = 0.0003$)]. Perhaps the SNP detection using the next-generation sequencing platforms would be the method of choice to identify meaningful SNPs that show a direct association with the virulence phenotype in this important human parasite.

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Chapter 6
Genetic Manipulation Techniques

David Mirelman and Rivka Bracha

Abstract In the past two decades we have seen a very important advance in the research of the biology of *Entamoeba histolytica*. This dramatic progress has been mostly the result of (1) the introduction of a transfection system to express exogenous genes and to up- and downregulate the expression of selected genes, (2) the completion of the parasite's genome sequencing, including the genomes of various *Entamoeba* spp. and some of its strains, and (3) the development of microarrays both for genomic and for transcriptional profiling. The introduction of these molecular tools has significantly expanded our ability to investigate many important questions such as the role and molecular mechanisms of different virulence factors, transport, and metabolic systems, as well in the cell cycle and differentiation of the parasite. In addition, it enabled the better understanding of the molecular differences among the various *Entamoeba* species and strains. Nevertheless, there are still many open questions that need to be answered.

6.1 Introduction

The application of modern genetic techniques to *Entamoeba histolytica* research has encountered considerable difficulties because the organism is multiploid and has no known sexual cycle and no genetic system. Weedall et al. [1] have recently suggested that the differences in gene family content and gene copy number among the sequenced genomes indicates that sexual reproduction probably takes place from time to time or did in the past.

Genetic research on the amoeba parasite can be done in two main ways, by comparing the genomes and transcriptomes of various *Entamoeba* isolates that were found to differ in their phenotype and then following the leads of such analysis. Using this approach, Gilchrist et al. [2] have shown that *E. histolytica* isolates obtained from patients in the same geographic region, who were either asymptomatic or symptomatic, could be differentiated by nonsynonymous single-nucleotide polymorphisms (SNPs) of some genes predicted to be involved in virulence.

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_6

Another group [3] has compared the transcriptome of the well-known virulent strain HM-1:IMSS with that of the virulence-attenuated *E. histolytica* strain, Rahman, in an attempt to identify potential virulent factors. Differences were indeed found that could be attributed to the differences in virulence, such as in the relative expressions of the cysteine proteinase family [4–7]. In a similar way the *Entamoeba histolytica* serine-, threonine-, and isoleucine-rich protein (EhSTIRP) was found to be exclusively expressed in virulent but not in nonvirulent *Entamoeba* strains [8, 9]. Others have used this approach to identify genes involved in the process of encystation and excystation in *Entamoeba invadens* [10, 11].

The second approach has been more reductionist and tries to explore the role of different genes in cultured parasites by changing their content using molecular biology techniques that were previously developed for other eukaryotic organisms. This approach has not been very easy because *Entamoeba* trophozoites are almost not amenable to genetic manipulations due to their unique genome organization, and classical techniques such as homologous recombination (HR) have not worked; it has been impossible to generate gene knockouts or to integrate exogenous genes into its genome. Evidence for the existence of an HR machinery in the parasite has been reported [12–14], together with the characterization of the *EhRAD51* gene as a recombinase [12]. This discovery is very encouraging as it suggests that perhaps in the not too distant future the technologies for gene targeting as well as gene knockouts and knock-ins will become applicable.

This chapter summarizes our present knowledge and the techniques used for the manipulation of gene expression in *Entamoeba* trophozoites, pinpoints the advantages and weaknesses of each, and considers some of their contributions to the understanding of the parasite biology.

6.2 Transfections and Episomal Expression of Exogenous Genes

The ability to manipulate the expression of genes of interest is a very important tool for understanding the particular function and role of a gene in the parasite life cycle, metabolism, or pathogenesis. Unfortunately, as already mentioned, all attempts to date to integrate exogenous genes into the amoeba genome have failed and the only success in up- or downregulating genes has been by episomal expression. The initial breakthrough for this approach was the introduction, about 19 years ago, of methods for transient episomal transfection in *Entamoeba histolytica* [15, 16]. The first plasmid vectors that were constructed for transient expression had reporter genes cloned under promoters that were widely used for expression in eukaryotic cells, such as viral promoters. Such plasmid constructs were prepared to test for the efficiency of the transfection methods that were mainly conducted by electroporation. These initial plasmid constructs failed, however, to express the reporter gene, and only plasmids in which the reporter gene was placed under the 3′- and 5′-regulating elements of *E. histolytica* genes, such as those of the Gal/GalNAc lectin heavy

subunit (*Ehhgl*) or actin (*Ehact*), displayed any transient expression. Transient expression in *E. histolytica* leads to very low levels of protein because of the low efficiency of the transfection. To achieve higher levels of expression, a selection system based on antibiotic sensitivity was developed [17, 18]. In those days *E. histolytica* was known to be resistant to most of the common antibiotics, and only two drugs were found to be useful: the neomycin derivative geneticin (G418) and hygromycin. Plasmids containing the corresponding genes that confer resistance to such antibiotics, under the promoters of *E. histolytica* genes, were constructed and enabled the selection of stable transfectants that expressed high levels of the reporter gene [17, 18]. In 2001 Ramakrishnan et al. [19] reported that *E. histolytica* was sensitive to low concentrations of blasticidin, and they described plasmids coding for blasticidin *S*-deaminase with which they also expressed the green fluorescence protein (GFP). These basic expression systems have become widely used by numerous investigators in the field, and improvements in the constructs are constantly reported such as the Gateway technology (Invitrogene) to generate more efficient expression vectors [20]. The analysis of promoter regions of different genes of *E. histolytica* have shown that the majority have short 5′-UTR regions and in many of them there are upstream enhancer sequences such as segments termed URE4 that have to be included to avoid a significant drop in the expression [21, 22]. Most of the transfections are nowadays performed either by electroporation or by using membrane active reagents such as lipofectin [23], and several modifications have been added to plasmid constructs to improve their expression levels. Even with the best transfection protocols, the mortality of trophozoites is very high; the percentage of successfully transfected trophozoites is quite low in comparison to other eukaryotic cells, and recovery after transfection takes at least 48 h until growth and division resume.

The establishment of efficient episomal expression systems has enabled the study of the role and function of numerous genes. The effects of overexpression of many families of *E. histolytica* genes on the different activities and phenotypes of the parasite have been investigated by many groups and it is impossible to review all of them in the scope of this chapter. After much effort, recently there has also been some success in the transfection and expression of exogenous genes in the reptile parasite *Entamoeba invadens* [24, 25]. Investigators should take into consideration that the upregulation of expression of certain genes could also be toxic for the trophozoites. Examples for such an effect were seen when genes such as the amoebapores [26], or of certain members of the cysteine proteinase family, were overexpressed and found to be very toxic to the trophozoites [27]. Modification of expression of other genes such as myosin affected the cellular functions of the parasite such as their phagocytic capacity [28–30], whereas simultaneous overexpression of antioxidant enzymes, such as the iron-containing superoxide dismutase Fe-SOD and peroxiredoxin, showed significant decrease in the susceptibility of parasites to the antiamoebic drug metronidazole [31]. In addition, the upregulation of two other genes recently found to be involved in oxidative and nitrosative stress (*EhSIAF* and *EhPTPA*) enhanced parasite survival and increased their adherence to mammalian cells [32]. In recent years there has been significant progress in the

ability to label exogenously expressed genes with either a fluorescent or luminescent tag [33–36] or an antibody epitope [37], which significantly contributed to identify the cellular localization of the expressed proteins. Another approach to assign function to genes has used forward genetics [38] to identify negative regulating genes of phagocytosis. *E. histolytica* trophozoites were transfected with cDNA from an overexpressing library and then exposed to human red blood cells loaded with the toxin tubercidin. The surviving trophozoites were identified as phagocytosis mutants. Further characterization of the episomal overexpressed genes revealed three negative regulating genes of phagocytosis [38]. The genes that were identified encoded profilin, a cytoskeleton-regulating protein, a hypothetical protein-H644, and EhLimA, which was previously discovered as a protein in the plasma membrane that is associated with the trophozoite cytoskeleton and was also found in membrane lipid rafts [39].

Vectors for inducible gene expression based on the tetracycline (Tet) repressor system have also been developed for *E. histolytica*. The first Tet-induced plasmid constructs, prepared by Hamann et al. [40] and Ramakrishnan et al. [41], were used for the short-term induction of overexpression of genes coding for virulence factors to minimize the toxicity associated with the accumulation of the upregulated proteins overexpressed in the parasite. The advantage of the inducible expression vector is that it can provide an appropriate control in its noninduced state and its biological activities can be compared to those of the induced trophozoites. The effects of additions of tetracycline to the parasite cultures during a long period of induction should also be taken into consideration.

6.3 Downregulation of Gene Expression

Downregulation of the expression of certain genes is another technique very useful to study the role of a particular gene in the biological activities of the parasitic cell. Methods for the expression of antisense transcripts, which can disrupt the endogenous gene transcript, have been used in many organisms to downregulate the expression of genes of interest. The successful use of the antisense method took advantage of the finding that the parasite possesses two copies of the gene encoding the ribosomal protein L21 (*EhRPL21*). One of these copies, *EhRP-L21-g34*, which is highly transcribed but not translated in *E. histolytica*, contains a longer than usual upstream 5′-UTR of 34 nt [42, 43]. Transfection of plasmid constructs containing the CAT reporter gene under this particularly long 5′-UTR element into *E. histolytica HM-1*:*IMSS* trophozoites produced high levels of CAT transcripts without expressing the CAT protein. This finding gave us the idea to exploit this unusual 5′-UTR element for the expression and accumulation of high levels of antisense mRNA of a gene of interest to increase the disruption of the endogenous sense transcript. This antisense principle proved to be effective for downregulating gene expression of numerous genes of *E. histolytica*. Three genes coding for different virulence factors, cysteine proteinase 5 (*EhCP-5*) [44, 45], the light subunit (35 kDa) of the Gal/GalNac lectin, *Ehlgl*) [46], and amoebapore A (*Ehap-a*) [47], were each cloned in the antisense

orientation under the 5′-promoter sequences of the *EhRP-L21-g34* gene, and after transfection and selection each of the three transfected trophozoites showed a significant reduction in the level of both the sense transcript and the level of the respective protein of the endogenous gene. The levels of the reduction varied from 60 % for the amoebapore protein to 90 % for the cysteine proteinase inhibition. The reduction in the expression of each of these three important virulence factors caused phenotypic changes in the transfectants and enabled investigations that have shed light on their role in the parasite pathogenicity [44, 45, 47]. In a model system that uses human intestinal xenografts in SCID mice, the cysteine proteinase-deficient amoeba failed to induce intestinal epithelial cell production of the inflammatory cytokines interleukin IL-1B and IL-8, and caused significantly less gut inflammation and damage to the intestinal permeability barrier [48]. These amoebae also failed to produce liver abscesses in hamsters [44] and had reduced ability to degrade human intestinal mucus [49]. Antisense inhibition of the light subunit (35 kDa) of the Gal/GalNac lectin resulted in reduction of the trophozoite cytopathic and cytotoxic activities and in their ability to induce the formation of liver lesions in hamsters [46]. The antisense downregulation of the amoebapore A gene proved to be more problematic because it required the participation of the 5′- and 3′-UTR regions of the *Ehap-a* gene to be included in the antisense plasmid construct [50]. However, once the transfectant was produced and the endogenous level of amoebapore protein became reduced, the amoebae were found to be significantly less pathogenic. Cytopathic and cytolytic activities as well as lysis of red blood cells were markedly inhibited [47]. Moreover, trophozoite extracts of those transfectants displayed lower pore-forming activity and were less potent in inhibiting bacterial growth compared with controls. In addition, their ability to induce the formation of liver abscesses in hamsters was totally impaired. These experiments provided the first direct evidence for the crucial role that amoebapores play in parasite virulence [47]. The antisense principle was also applied to the downregulation of a gene coding for a lysine-rich surface antigen in *Entamoeba dispar* [51, 52].

Using the same antisense principle, the expression of the gene coding for aldehyde dehydrogenase (*EhADH2*) was also significantly reduced [53]. The NAD-dependent ADH activity of the transfected trophozoites was 60 % to 70 % lower, accompanied by a marked decrease in amoebic growth and viability. In addition, the downregulation of *EhMLBP*, a protein that specifically binds to methylated long interspersed elements (LINE), resulted in trophozoites with impaired growth and cytopathic activity [54].

The Tet-inducible system mentioned previously [40] has also been successfully adapted to express, upon induction, antisense transcripts using other amoebic promoters. This system was first adapted to inhibit the expression of *EhCaBP1*, one of the calcium-binding protein genes of *E. histolytica* [55]. A 50 % reduction in the level of the protein was obtained under tetracycline induction, which resulted in inhibition of cellular proliferation. The investigators also found that *EhCaBP1* was involved in dynamic changes of the actin cytoskeleton, and that downregulation of *EhCaBP1* resulted in severely impaired endocytosis and phagocytosis. The Tet antisense system has also been used to downregulate the expression of the *E. histolytica* GlcNAc-phosphatidylinositol deacetylase, which participates in the biosynthesis of

glycosyl-phosphatidyl-inositol (GPI) anchors [56]. Induction of the expression of the antisense transcript resulted in reduced cell proliferation, endocytosis, and adhesion to target cells. Further evidence for the importance of GPI anchoring for amoebic pathogenesis was obtained by the downregulation of the expression of mannosyl-transferase (*EhPIG-M1*), which is another gene required for synthesis of GPI. Lower amounts of GPI and proteo-phospho-glycan (PPG) [57] were found on the cell surfaces, and the transfected trophozoites had higher sensitivity to complement and displayed a dramatic reduction in their ability to induce liver abscesses in hamsters.

The antisense technique used to downregulate the expression of *E. histolytica* genes has significantly contributed to clarifying the roles and functions that many genes have in the various activities of the parasite, especially in its pathogenicity. The method has, however, a number of limitations. First, in most cases, only a partial inhibition of expression (~60 %) was achieved [46, 47]. Second, to achieve optimal and stable episomal expression of antisense transcripts, there is a need to grow the transfected parasites at high concentrations of the selective antibiotic [geneticin (G418), hygromycin with or without tetracycline]. Prolonged growth of the parasites under such conditions causes some toxicity and affects their growth, and proper controls with similar, sham-selective plasmids have to be designed and included in the studies. In addition, it has been observed that the levels of downregulation of gene expression, seen in transfected trophozoites that express antisense transcripts, sometimes decrease after several weeks of cultivation. The reason for the resumption of higher expression of the downregulated gene is not well understood and may indicate that trophozoites slowly adapt and compensate for the reduced level of expressed protein.

The downregulation of gene expression was also achieved in several cases by using synthetic oligomers of antisense peptide nucleic acids (PNA) [58] The expression of *EhErd2*, a chromosomal gene, which codes for a homologue of a Golgi membrane receptor, was reduced 45 % (in protein levels), which caused a reduction in parasite proliferation [59]. *EhSec61*, a conserved essential integral component of the endoplasmic reticulum [60], was also treated with the corresponding PNA; it achieved an 80 % decrease in the expression of Sec61 protein and caused a 50 % decrease in proliferation. Unfortunately, this approach has not become popular, probably because of the costs of the PNA synthetic compounds that must be constantly added to the trophozoite growth media at a concentration of 20 μM.

6.4 Modulation of Gene Expression by a Dominant-Negative Effect

The overexpression of a mutated or truncated form of a gene, which then competes with the endogenous protein for its activity and cellular location, is termed a dominant-negative downregulation. This approach has also been successfully used in *E. histolytica* to study the role of certain important proteins, especially those that have a crucial function and whose downregulation by other methods had been unsuccessful. The overexpression of mutated or truncated genes has also been

performed in some cases with vectors containing Tet-inducible elements [40] as well as with labeled tags such as GFP [28, 33–36] and HA epitope tags [37].

A number of examples given next demonstrate the usefulness of the dominant-negative approach for understanding the function of certain proteins. The role of myosin II in the amoebic capping process [28, 61] was investigated by overexpressing the light meromyosin domain (LMM), which is essential for myosin II filament formation. The presence of high levels of this domain in the transfected trophozoites resulted in a dominant-negative effect similar to a myosin II null phenotype seen in other eukaryotic cells, which, as mentioned earlier, cannot be knocked out in *Entamoeba*. The transfectants that overproduced LMM grew at a slower rate, polynucleation was observed in numerous cells, they had slower motility and shorter pseudopods, and the formation of caps and uroids was dramatically reduced. In addition, LMM-overexpressing parasites were unable to form liver abscesses and were highly sensitive to the immune response [29].

Another protein that was subjected to a dominant-negative investigation was the heavy subunit of the Gal/GalNAc lectin, which plays a very important role in adherence of the parasite to other cells and matrixes. The heavy lectin subunit consists of exo-, trans-, and intramembrane domains, and its cytoplasmic tail is suspected to be involved in signal transduction. Tranfectants in which a segment (224 aa) of the cytoplasmic tail was overexpressed were found to have a decreased extracellular Gal-lectin-dependent adhesion activity [33]. Furthermore, the transfectants that overexpressed the lectin cytoplasmic peptide were impaired in their motility and could not induce the formation of liver abscesses [33]. Interestingly, when a mutation was introduced into the sequence of the overexpressed cytoplasmic peptide, it resulted in the loss of the dominant-negative phenotype [33]. Other modifications in the heavy subunit of the Gal-specific lectin also caused phenotypic changes [62, 63]. The dominant-negative approach was also used to study the role of the lipid-anchored 35-kDa light subunit (*Ehlgl1*) of the Gal/GalNAc lectin complex [64] in amoebic adhesion and in the cap formation phenomenon of the heavy subunit. Overproduction of a 31-kDa *N*-terminal truncated protein (−55 aa) caused the replacement of most of the endogenous 35-kDa light subunits in the disulfide-linked Gal-lectin heterodimeric complex. The formation of heterodimers between the mutated light subunit and the heavy lectin subunit exerted a dominant-negative effect that included a significant decrease in the ability of the amoebae to adhere and kill mammalian cells as well as in their capacity to form rosettes and phagocytose erythrocytes. Trophozoites expressing the truncated light subunit were also impaired in their ability to cap the heavy subunit of the Gal/GalNAc lectin to the uroid region following the binding of antilectin antibodies [64].

The amoebic protein alcohol dehydrogenase 2 (*EhADH2*) was shown to be necessary for amoebic growth and survival [53]. It had been reported that *EhADH2* is a fusion protein, with a separate N-terminal acetaldehyde dehydrogenase activity (*EhALDH*) and a C-terminal alcohol dehydrogenase (*EhADH*) domain. To learn more about the role of this bifunctional protein, trophozoites were transfected with a plasmid containing a copy of the *EhADH* gene in which a single histidine in the putative active site of the ADH domain was mutated. The overexpression of the mutated protein completely eliminated both the ADH and the ALDH activities [53].

Overexpression of a mutated *EhRacG-V12* protein that is homologous to the human Rac1 small GTPases resulted in a dominant-negative phenotype which had an altered polarization of cells and alterations in the actin cytoskeleton [65]. Several other virulence functions were found to be defective, including the ability to adhere to host cells, destroy host cells, and release cysteine proteases. Using the dominant-negative approach, Labruyère and colleagues [66] showed that overproduction of the C-terminal domain of *EhPAK1*, which contains a constitutive kinase activity, caused a significant reduction in amoeboid migration, as measured by dynamic image analysis. Interestingly, these transfected amoebae displayed increased erythrophagocytosis rates. In addition, the independent functions of the two representative Rab7 small GTPase isotypes, *EhRab7A* and *EhRab7B*, which are crucial in the regulation of trafficking to late endosomes, lysosomes, and phagosomes, were investigated [67]. Overexpression of both *EhRab7A* and *EhRab7B* resulted in increased late endosome/lysosome acidification. In contrast, expression of the permanently activated *EhRab7B-GTP* mutant gave rise to a dominant-negative phenotype that included a decrease in late endosome/lysosome acidification and defective secretion of lysosomal proteins. On the other hand, overexpression of *EhRab7A-GTP* enhanced acidification but had no effect on cysteine protease activity, either intracellular or secreted. Together, these data indicate that both Rab7 isotypes are involved in lysosome and phagosome biogenesis but have distinct, although coordinated, roles [67]. Recently, it was elegantly shown that *E. histolytica* trophozoites overexpressing wild-type G-protein subunit *EhGα1* in an inducible manner exhibit an enhanced ability to kill host cells that may be wholly or partially the result of enhanced host cell attachment. EhGα1-overexpressing trophozoites also display enhanced transmigration across a Matrigel barrier, an effect that may result from altered baseline migration. Inducible expression of a dominant-negative EhGα1 variant resulted in the converse phenotypes [68]. Overexpression of the methyl-binding protein EhMLBP protects trophozoites against heat shock and reduces protein aggregation. This protective function is lost in trophozoites that overexpress a mutated form of EhMLBP which is devoid of its heat-shock domain [69].

The limitations of downregulation by antisense transcripts also hold true for the overexpression of dominant-negative modified or truncated proteins. Investigators need to consider the effects of the high concentrations of antibiotics required for maintaining the high level of overexpression of the mutated or truncated gene products in the transfectants as well as the length of time that the modified phenotype should be maintained.

6.5 Gene Silencing in *Entamoeba histolytica*

As already mentioned, one of the most elegant ways to knock out genes is by homologous recombination, Unfortunately, this has not been possible to achieve in *Entamoeba* because integration of exogenous genes to the genome has failed so far. Two main pathways for gene silencing in eukaryotic cells have been characterized:

(1) posttranscriptional gene silencing (PTGS), in which the mRNA is inactivated by degradation or translational repression, and (2) transcriptional gene silencing (TGS), which occurs by DNA methylation, chromatin modifications, or RNA interference.

6.5.1 RNAi-Based Methods for Silencing of Gene Expression

A number of possibilities for gene silencing by the RNAi principle have been described: (1) the introduction of dsRNA to a target gene [8, 70, 71], (2) the short hairpin-based method [72], (3) the external administration of synthetic siRNAi molecules to the media of growing trophozoites [70, 73], or (4) by allowing trophozoites to ingest bacteria that harbor a plasmid which produces the desired dsRNA [74]. There is a report, however, that loss of the dsRNA-based gene silencing can occur and the parasites resume, by a still-unknown mechanism, expression of the protein [75].

Although there are variations in the components of the RNAi machinery among different organisms, the principle is conserved and includes the following steps: double-stranded RNA (dsRNA) molecules are cleaved into short RNAs (siRNAs) that interact with endogenous homologous mRNAs and these guide the RNase-mediated cleavage complex to the target RNA. Bioinformatic studies of the *E. histolytica* genome revealed that most of the genes participating in this process exist in *E histolytica* [76]. For more details on the RNAi methods and uses, see recent reviews [77, 78] as well as in Chap. 9: "AntiSense Small RNA in the Regulation of Gene Expression" by Upinder Singh.

6.5.2 Epigenetic Gene Silencing *in* E. histolytica

Transcriptional gene silencing was incidentally discovered in *E. histolytica* during an attempt to overexpress the amoebapore gene (*Ehap-a*) by transfecting trophozoites of the virulent strain HM-1:IMSS with a plasmid (psAP-1, Fig. 6.1) that contained a genomic copy of the *Ehap-a* gene, with its original genomic upstream and downstream flanking elements [79]. Surprisingly, instead of overexpressing the amoebapore (AP-A) protein, the transfectants were found to be completely devoid of this protein, indicating that both the episomal and the endogenous gene became silenced. Overexpression of the amoebapore A protein was observed, however, when transfection was done with a plasmid (pA-7) [26] in which the ORF of the *Ehap-a* gene was flanked by the 5′- and 3′-regulating elements of another *E. histolytica* gene (*EhRP-L21gLE-3*) [42, 43]. The DNA sequences that were important for triggering the silencing of the *Ehap-a* gene were found to reside in the 5′-upstream flanking segment (473 bp) of the *Ehap-a* gene. Plasmid psAP-2 (Fig. 6.1), containing only such sequences (without the ORF of *Ehap-a*), was able to induce silencing even faster. Sequence analysis of the 473 bp upstream flanking element revealed

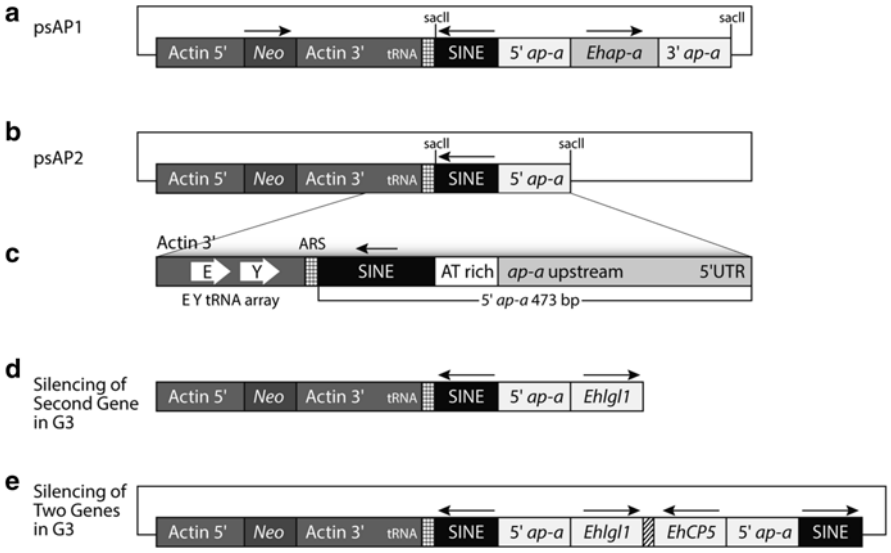

Fig. 6.1 Schematic representation of the plasmids used for gene silencing: The basic vector, *pEhActNeo*, possesses the autonomous replicating sequences (ARS) from *Entamoeba histolytica* [51, 79, 80], the neomycin phosphotransferase gene (*Neo*) flanked by the 5′- and 3′-*Ehactin* gene regulatory sequences, and tRNA arrays for glutamic acid (*E*) and tyrosine (*Y*) [81]. **a** Plasmid psAP-1 contains the *Ehap-a* gene flanked by its 5′- and 3′-regulatory sequences, which were inserted into the *pEhActNeo* basic vector. **b** Plasmid psAP-2 contains only the 5′-regulatory sequences of the *Ehap-a* gene (473 bp). **c** Details of the important sequences required for gene silencing, the 473 bp of the upstream region of *Ehap-a* gene, which includes the truncated SINE1 retroposon element, and the two tRNA arrays for glutamic acid (E) and tyrosine (Y). **d** Plasmid construct for silencing of a second gene of choice in G3 trophozoites: the second gene (*Ehlgl1*) is fused to the 473-bp 5′-regulatory sequences of *Ehap-a*. **e** Plasmid for the simultaneous silencing of two additional genes in G3 trophozoites : the construct contains two genes of choice (*EhCP-5* and *Ehlgl1*), each cloned under the 5′-upstream sequences of *Ehap-a* and inserted into the basic vector [82]

that it contained, in addition to the *Ehap-a* promoter motifs, a 5′-truncated segment (140 bp) of a SINE1 retroposon element that is transcribed in the opposite orientation and is preceded by a unique, T-rich stretch of 48 bp [83]. Plasmids constructs of psAP-2 in which a deletion was made of at least 60 bp from the 5′-end of the 473-bp element or plasmids that included the entire sequence of the SINE1 element (550 bp), together with its 3′-polyadenylation signal sequences, were incapable of inducing the silencing of the amoebapore gene [83]. Run-on experiments revealed that no transcription initiation occurred for the *Ehap-a* gene, so this type of silencing is considered being a transcriptional gene silencing (TGS) event [79]. Silencing of the *Ehap-a* gene remained in effect even after curing of the plasmid by continuing the culturing of the transfected trophozoites for several weeks without the selective antibiotic. A plasmid-less clone (termed G3) was isolated from the silenced culture. Interestingly, the plasmid-less G3 trophozoites could not express the *Ehap-a* gene even when retransfected with plasmid pA7 (see earlier), which caused the overexpression of the amoebapore gene in trophozoites of strain HM-1:IMSS.

The expression of the *Ehap-a* gene was also successfully silenced with a slightly different plasmid construct in the same *E. histolytica* strain HM-1:IMSS (isolate 2411) by Irmer et al. [81] (see also below in Sect. 6.5.4).

6.5.3 Silencing of Additional Genes in the Plasmid-Less G3 Trophozoites

As we had found that in G3 trophozoites the silencing of the *Ehap-a* gene was accompanied by the silencing of additional genes related to the pore-forming protein family (*Ehap-b* and SAPLIP1) [79], it was interesting to try to see if other non-related genes cloned under the control of the 5′-upstream sequence of the *Ehap-a* gene, which was used to generate the silenced G3 amoebae, could be silenced as well. Constructs (Fig. 6.1) in which the light subunit of the Gal/GalNAc lectin (*Ehlgl1*) was placed under the 473 bp of the 5′ upstream of the *Ehap-a* gene and its 3′-regulatory sequence and transfected into the parent strain HM-1:IMSS resulted, as expected, in the overexpression of *Ehlgl1* gene, because the promoter of the *Ehap-a* gene is functional. However, transfection of the same construct into G3 trophozoites resulted in complete silencing of both the episomal as well as the genomic copy of the *Ehlgl1* gene [82]. Further analysis revealed that cloning a truncated 5′-ORF (>120 bases) of the *Ehlgl1* gene into the plasmid was sufficient to cause the complete silencing of this gene. Plasmid constructs harboring two genes, for example, the *EhCP-5* and *Ehlgl1* (Fig. 6.1), each cloned under the 5′ upstream of the *Ehap-a* gene transfected into G3, resulted in cultures silenced in three genes [84].

In all the silenced cultures it was possible to remove the plasmid following the discontinuation of the selection antibiotic while keeping the silenced state of those genes. Although the mechanism of the trigger and maintenance of the silenced state were not completely understood at that time, this process created a stable amoeba culture in which there was almost complete abrogation of the chosen gene product with no need to use antibiotics to maintain the silenced state. This method opened the door for many investigators to try and silence genes of choice to learn more about the role and function they have in different pathways of the parasite physiology. It is important to remember, however, that in those silenced cultures there are two silenced genes: the *Ehap-a* gene, which is the background, and the gene of choice. Analysis of the function of the second gene has to be compared to that of the G3 strain.

The G3 trophozoites in which the silencing of additional genes was performed were freely distributed to the research community and are available at the ATCC strain collection. A summary of the genes, which were silenced by different investigators using the G3 trophozoites, is briefly described next. Among them are some that have already been mentioned, such as the light subunit of the Gal/GalNAc lectin (*Ehlgl1*) [82], which revealed its importance in the capping of the heavy subunit of the Gal/GalNAc lectin to the uroid region. Analysis of the other members of the *Ehlgl* family revealed two groups: silencing of *Ehlgl1* caused the silencing of genes *Ehlgl2* and *-3*, and silencing of the *Ehlgl5* gene copy also silenced *Ehlgl4* [85].

In the cysteine proteinase gene family, silencing of *EhCP5* lowered the proteinase activity by 30 % [82]. This isolate also served to investigate the role of this particular proteinase in an ex vivo human intestinal model [86, 87]. Silencing of other members of the cysteine proteinase family elucidated the physiological role that many of them play in the physiology and virulence of the parasite [88]. Silencing was also performed on two of its endogenous inhibitors (*EhICP1* and *EhICP2*), but this did not have any effect on morphology or viability of the trophozoites [89]. Silencing of the rhomboid protease (*EhROM1*) caused decrease in adhesion to host cells and decreased phagocytic ability [90]. The silencing of another protease, EhMSP-1, an M8 family surface metalloprotease, resulted in an increase in adherence and a decrease in tissue culture destruction and mobility [91]. Two genes belonging to the cysteine protease-binding protein family were also silenced. Silencing of CPBF8 resulted in the reduction of lysozyme and β-hexosaminidase activity in phagosomes [92], and silencing of CPBF1 caused accumulation of unprocessed EhCP-A5 in the nonacidic compartment of the amoeba and its missecretion, indicating the role of the gene in the trafficking and processing of EhCP-A5 [93]. Silencing of the methionine γ-lyase (*EhMGL*) gene resulted in resistance to trifluoromethionine, revealing a novel mechanism of drug resistance in *E. histolytica* [94]. The role of sulfate activation in mitosomes was demonstrated by silencing two of the important genes involved in sulfate activation, MCF and Cpn60. Silencing of these genes impaired sulfolipid synthesis and proliferation [95]. During silencing of the serine-, threonine-, and isoleucine-rich protein gene, *EhSTRIP*, the presence of small RNA molecules with an antisense orientation to this gene were identified [96]. Furthermore, it was shown that such small antisense RNA molecules, with antisense orientation to the silenced gene, appeared in each of the silenced cultures. Those sRNA molecules continued to be produced even when drug selection was discontinued, although in somewhat lower amounts [96]. Silencing of the EhLimA gene did not show any phenotypic changes [39]. As mentioned earlier, the silencing method in the G3 trophozoites also allowed for the simultaneous downregulation of three genes of choice. Such a strain, RB9, was silenced in addition to the *Ehap-a* gene in the *Ehlgl1* and *Eh-CP5* genes [97] (Fig. 6.1). The plasmid-less RB9 trophozoites are virulence attenuated and could perhaps serve as a candidate for live vaccination. We are aware that we may have not mentioned all the genes that were successfully silenced in strain *E. histolytica* G3 and whose roles and functions were investigated by the different investigators. We apologize to those whose work was not cited here.

6.5.4 *Molecular Mechanism of Gene Silencing in* Entamoeba histolytica

Understanding the molecular mechanism of the silencing process has encountered many difficulties. An interesting question is whether all genes could be silenced using this method. Naturally we have much less information regarding this question

as failures have not often been mentioned. Three of the genes that in our laboratory failed to become silenced were *EhSOD*, *EhSUMO*, and the heavy subunit of the Gal/GalNAc lectin *Ehhgl1* (R. Bracha, unpublished results). It is possible that such genes are essential for parasite viability.

A second question relates to the fact that not all the HM-1:IMSS isolates, among the many that are maintained in different labs, could be silenced in the *Ehap-a* gene using the plasmid psAP-2. The HM-1:IMSS strain from which the G3 silenced strain was generated is a virulent isolate, termed 2411, which was obtained from Dr. G.D. Burchard from the Bernhard Nocht Institute in Hamburg. Analysis by PCR using strain specific primers has confirmed that all the isolates tested, including isolate 2411, belonged to the HM-1:IMSS strain [98, 99]. Recently, interesting findings have indicated that retrotranspositions could generate diversity among the SINE retroposon elements [13, 100] and it could be that some of such changes have contributed to the ability or inability of certain HM-1:IMSS isolates or clones to become silenced. A third issue is whether all plasmids commonly used for transfection in *E. histolytica* could be used to induce gene silencing. Irmer et al. [81] have reported that to silence the amoebapore gene the plasmid sequence has to contain two tRNA arrays for glutamic acid and tyrosine. These two arrays are present in plasmids psAP1 and psAP2 [79], which were originally constructed for the silencing of the amoebapore gene (Fig. 6.1), but their removal prevents silencing. They also stated that the truncated SINE is not essential for silencing. We have found, however, that gene silencing was more stable and complete when the psAP-2 plasmid contained the upstream truncated SINE1 sequences: if it was shortened too much, silencing did not occur [83].

As already mentioned, the silencing of *Ehap-a* is of the TGS type. In many cases such a process involved DNA methylation, heterochromatinization, or both. DNA methylation was analyzed by bisulfite sequencing in the promoter region of the *Ehap-a* gene, revealing that there were no changes in the methylation status of the G3, the silenced strain, in comparison with HM-1:IMSS [79]. Moreover, no DNA rearrangement or changes in the restriction pattern of the genomic DNA were found. Treatment of G3 with 5′-azacytidine, a potent DNA methylation inhibitor, did not resume *Ehap-a* transcription. The status of the chromatin in G3 versus HM-1:IMSS was analyzed by several methods. Chromatin immunoprecipitation (ChIP) analysis utilized antibodies raised against a synthetic peptide (15-mer) of the *E. histolytica* histone H3 N-terminal sequence containing a mixture of di/tri-methylated lysine 4 (H3mK4). The immunoprecipitation experiments showed demethylation of K4 in the *Ehap-a* genomic region of G3 [83, 101], indicating transcriptional inactivation in that loci. In addition, the levels of core histone H3 were found to be consistently higher in the silenced genes. Because of the significant differences in histone H3 amino acid sequences between *E. histolytica* and those of higher eukaryotes [for example, in *E. histolytica*, instead of lysine at position 9 (K9) there is arginine (R9)] [102], no other histone H3 modifications were investigated at the silenced gene loci. Nucleosome compaction, determined by controlled chromatin digestion with micrococcal nuclease, revealed a significant resistance to digestion in the promoter region of the silenced *Ehap-a* loci as compared to the parental strain that expresses the

gene [101]. Cultures of the G3 trophozoite in the presence of trichostatin A, an inhibitor of histone deacetylase, did not revert the silencing, but nicotinamide, an inhibitor of Sir2 histone deacetylase, caused a small resumption of *Ehap-a* transcription (R. Bracha, unpublished results). The genomic region affected by the silencing of *Ehap-a* is quite limited in size, and the genes downstream to *Ehap-a*, EHI-159470 and the upstream SINE1, are both transcribed in G3 trophozoites [83].

Small RNA molecules have been shown to participate in many silencing processes [77]. As already mentioned, bioinformatic analysis of the *E. histolytica* genome identified many components of the RNAi machinery but not all. Dicer, the enzyme responsible for chopping the RNA into small segments, was not found in *E. histolytica* [103]. Even so, many small RNA fragments of 27 nt that have 5′-polyphosphate termini and map antisense to genes were identified and recently reported [104]. It was only natural to look for small RNA molecules in the G3 silenced trophozoites corresponding to the silenced *Ehap-a* gene. Preliminary experiments failed to detect them, but recently it was reported that antisense small RNAs (sRNAs) specific to the silenced *Ehap-a* gene were identified in G3 trophozoites [96]. Moreover, when additional genes were silenced in G3 trophozoites, antisense small RNAs to those newly silenced genes were also detected. These small RNAs are located in the nucleus, associated with the argonaut protein EhAGO2-2, and as already mentioned they are 27 nt long with 5′-polyphosphate termini [96]. In addition to those sRNA molecules that were identified and characterized in the silenced strains, a nuclear accumulation of ~140 nt ssRNA with sequence homology to the 5′ of the SINE1 was observed in the G3 strain but not in HM-1:IMSS, the parent strain [83]. At present we do not know what role these noncoding, truncated SINE1 single-stranded RNA transcripts may play, especially in the first silencing event of the amoebapore gene. One possibility is that because of their accumulation they may bind to the homologous genomic regions of SINE1 that are located upstream to the *Ehap-a* gene, triggering the binding of a silencing complex which then spreads downstream and is responsible for the chromatin modification, as has been earlier proposed [105–107]. Such a molecular mechanism could perhaps also explain how a second gene may be silenced when it is cloned in a plasmid under the 473-bp 5′-*Ehap-a* gene promoter region. Transfection of such a hybrid plasmid into the G3 trophozoites may trigger, by sequence homology, the transfer of the silencing machinery from the genomic *Ehap-a* upstream region where it continues to silence the *Ehap-a* gene, to the episomal construct that contains the same *Ehap-a* upstream sequences and which include the truncated SINE element [79, 83]. Such an in-trans transfer of the silencing heterochromatin complex may then spread the chromatin modification downstream to silence the ligated second gene on the plasmid because there is no boundary or insulator region between them [84, 85, 107]. An open question is how the silencing complex is then transferred back from the episomal location of the gene to the genomic locus of the second endogenous gene. This action perhaps may be guided by the small RNAs that were recently discovered also when silencing a second gene of interest [96].

Taken together, the molecular mechanism of the unusual silencing process in *Entamoeba histolytica* is still not completely understood. The heterochromatin

formation over the silenced genes loci as well as the finding of antisense sRNAs to those genes suggests that they are probably involved in the maintenance and stability of the silenced state. The role of the tRNA arrays for glutamic acid and tyrosin in the region of the transfecting plasmid, which were shown to be essential for the initiation of the silencing process, is also not yet clear.

6.6 Concluding Remarks

The ability to manipulate gene expression is undoubtedly one of the most effective ways to learn about the roles of different genes in the physiology and metabolism of the amoeba. One has to keep in mind, however, that most genetic manipulations are accompanied by changes in the transcriptome and these are often not directly related to the gene examined. It is not yet clear why or how this happens. There are many examples where numerous and unexpected changes were observed, for example, following transfection with a vector containing only the G418-resistant gene (U. Singh, personal communication). Changes were also observed after overexpression of the URE3 transcription factor [22], and significant differences were seen in the expression levels on many genes between the G3, the silenced strain, and its parent strain HM-1:IMSS (Gilchrist and Bracha, unpublished results). Significant changes were also observed in different clones of the same strain, for example, between a virulent and a nonvirulent strain of *E. histolytica* HM-1:IMSS [7, 9]. This fact illustrates the need for careful analysis and appropriate and relevant controls for each genetic manipulation that caused changes in parasite gene expression.

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Part II
Regulation of Gene Expression

Chapter 7
Surveying *Entamoeba histolytica* Transcriptome Using Massively Parallel cDNA Sequencing

Chung-Chau Hon, Christian Weber, Mikael Koutero, Marc Deloger, Jean-Yves Coppee, and Nancy Guillen

Abstract Massively parallel cDNA sequencing, known as RNA-Seq, is a recently developed approach to interrogate transcriptomes. Its rapid and ongoing development promises to revolutionize transcriptomics over the next decade. In this chapter, we first review the transcriptome research of *Entamoeba histolytica* during the past 25 years in retrospect: from cDNA library screening in the 1980s to the latest RNA-Seq analyses. Then, we share our experiences on applying RNA-Seq to the *Entamoeba* transcriptome, aiming to provide useful caveats and tips. Next, we summarize the results of our latest RNA-Seq analyses of *E. histolytica*, including identification of unannotated transcripts, revision of existing gene models, reconstruction of alternative isoforms, and quantification of stochastic noise in splicing and polyadenylation. Finally, we outline a number of advanced applications of RNA-Seq that could potentially be applied to *E. histolytica*, hoping to shed light on the future directions of *Entamoeba* transcriptomics.

7.1 From a Few Transcripts to an Atlas of Transcriptome: 25 Years in Retrospect

7.1.1 Full-Length cDNA Sequencing: Started Out Small

A transcriptome refers to the dynamic collection of all RNA transcripts expressed from the genome of a cell [1]. In 1980s, surveys on transcriptomes were conducted mainly through low-throughput Sanger sequencing of cDNA libraries [2]. At the time, constructing a comprehensive transcriptome map for any eukaryote was

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_7

extremely tedious, if not impossible [3]. In 1987, the first *Entamoeba* transcripts (actin mRNAs) were sequenced [4, 5]. These landmark studies revealed, for the first time, some unusual properties of *Entamoeba* transcripts, for example, extremely short untranslated regions when compared with other well-studied eukaryotes. In the following years, a cascade of transcript hunting from these full-length cDNA libraries began and a number of important transcripts were subsequently identified, including the surface lectin [6], elongation factor-1 alpha [7], ferredoxin [8], amoebapore [9], etc.

7.1.2 Large-Scale EST Sequencing: Kick It Up a Notch

In the following decade, when Sanger sequencing throughput was improved by automation, a number of more efficient sequencing strategies were developed to survey the notably random sequencing of expressed sequenced tags (EST) libraries [10]. In the late 1990s, through sequencing of *Entamoeba histolytica* EST libraries [11–14], a large number of transcripts were identified, which led to the discovery of a number of protein-coding transcripts that are seemingly specific to *Entamoeba* [11]. Interestingly, other than coding transcripts, two families of highly abundant polyadenylated noncoding transcripts were also identified [12], named ehapt1 and ehapt2. Although ehapt2 family was later identified as transcripts of retrotransposons [15, 16], the identity of ehapt1 remains unknown [17]. These findings hinted the existence of a large number of yet to be described noncoding transcripts in the *Entamoeba* transcriptome, and the hunt for transcripts continued.

7.1.3 Genome Sequence Unleashed: Budding Blossoms of Spring

Until 2005, when the sequencing project of the *E. histolytica* genome was completed and in silico annotation predicted at least 8,000 coding transcripts [18, 19], the long hunt for coding transcripts from cDNA libraries was brought to a close. Combining the whole genome sequence with the accumulated EST data, fundamental characteristics of *Entamoeba* transcripts, for example, sequence motifs for splicing and polyadenylation of pre-mRNA, were unveiled. Davis and coworkers mined the EST data sets to provide evidence for 164 predicted introns and 35 novel introns, supporting the amoebic consensus-splicing site (5′-GUUUGU-UAG 3′) summarized previously [20]. Zamorano and coworkers further analyzed the available EST and genomic sequences and identified the sequence motifs involved in pre-mRNA polyadenylation, including an AU-rich domain corresponding to the consensus UA(A/U)UU polyadenylation signal [21]. Other than coding transcripts, transcripts expressed from transposable elements were also characterized. First, distribution of genomic copies of long interspersed elements (LINEs) and short interspersed

elements (SINEs) was defined [22, 23]. Later, Huntley and coworkers further analyzed the expression of EhSINE1, a category of SINEs previously named ehapt2 and found to be one of the most abundant transcripts in *E. histolytica* [16], based on ~20,000 EST sequences [24]. Their analyses suggested at least 142 of the 393 EhSINE1 elements were transcribed, providing evidence to support the previous speculation that transposable elements might be extensively transcribed and contribute a significant proportion of the *Entamoeba* transcriptome [17].

7.1.4 Microarrays Took Off: Ripening Fruits of Summer

The release of the *E. histolytica* genomic sequence and resources in 2005 initiated a series of genome-wide microarray gene expression profiling of the parasite under a wide range of conditions [25]. The first microarray was based on genomic DNA clones sequenced in the genome project, covering 1,971 annotated genes [26]. This initial study detected 47 % of the examined genes to be transcribed under standard culture conditions and concluded that host–parasite interactions in the Caco-2 cell model did not appear to induce major transcriptional changes in the parasites [26]. Soon after, a custom oligonucleotide microarray, designed to cover the entire collection of predicted coding transcripts (~10,000), detected the expression of more than 80 % of these transcripts [27], which is significantly higher than the previous estimation of 47 % [26]. Later, Hackney and coworkers utilized expression profiles to identify sequence motifs in promoters of genes with very high or very low gene expression levels, representing the first genome-wide characterization of promoter motifs based on gene expression profiles [28]. Instead of relying on coding transcripts predicted from genomic sequences, Weber and coworkers designed an 70-mer oligonucleotide microarray based on 1,300 bona fide transcripts identified from a cDNA library of virulent strain of HM-1:IMSS and a liver-specific cDNA subtraction library [29]. The cDNA sequences derived from this study assisted the coding gene model prediction in the original [19] and revised [18] genome annotation. Upon the gene model reannotations in 2010 [18], the microarrays were then redesigned based on these improved gene models [30–32]. Since the introduction of microarrays in 2005, numerous studies on differential gene expression of the parasite of different strain backgrounds or under different culturing conditions were published, leading to the discovery of transcripts potentially related to the life cycle and the virulence of the parasite [25]. For example, by comparing the transcriptome of *E. histolytica* in cyst and trophozoite stages, developmentally regulated genes, which might participate in life-stage conversion, were identified (reviewed by Singh and Ehrenkaufer [33]). In addition, comparisons of expression profile of trophozoites in vitro and in vivo, for example, amebic liver abscess in hamster [34] and colitis in mouse [27], have facilitated the identification of transcripts expressed during colonization and invasion. The applications of microarrays to study *E. histolytica* pathogenesis were extensively reviewed [25]. Currently, microarrays still remain the mainstream platform for transcriptional profiling in *Entamoeba*.

7.1.5 RNA-Seq Takes Over: The Future Is Here

In the late 2000s, rapid advancement of massively parallel DNA sequencing technologies led to the development of the whole transcriptome shotgun sequencing approach, known as RNA-Seq [35], which revolutionized the way transcriptomes being studied and would likely make microarrays obsolete. At the time of writing, two publications have applied RNA-Seq on the *E. histolytica* transcriptome. Bosch and coworkers used RNA-Seq as a microarray replacement to identify differentially expressed transcripts in a G-protein mutant versus the wild-type strain [36]. Our group used RNA-Seq to revise the gene models in AmoebaDB and to analyze the alternative usage of splicing and polyadenylation sites [37]. These two articles marked the beginning of a new era on the transcriptomics of *Entamoeba*, and more applications of RNA-Seq on the *Entamoeba* transcriptome are expected to emerge soon.

7.2 RNA-Seq: Mapping the Transcriptome at Its Highest Resolution

RNA-Seq is a revolutionary approach to interrogate the transcriptome on a global scale at single-nucleotide resolution [38]. The magic of RNA-Seq lies in its capability to sequence millions of cDNA fragments in a reasonably short period of time [39]. Although numerous protocols have been developed for various platforms (e.g., Illumina) and specific purposes, such as transcription start site mapping [40], their workflows are generally similar. First, pools of RNA were fragmented and adaptors were incorporated through either RNA ligation or priming during reverse transcription. Then, the RNA fragments were reversely transcribed into cDNA fragments flanked by adaptors containing specific sequences for sequencing primers. Then, these cDNA fragments were randomly sequenced on a nanoscale in a massively parallel fashion, generating a considerably large number (usually measured in millions or billions) of relatively short reads (usually tens to a few hundreds of nucleotides) [35]. These short reads were either mapped to the reference genome to identify transcripts [41], or assembled de novo to reconstruct the transcripts if a reference genome was not available [42].

Expression level of transcripts can be simply quantified by counting the number of mapped reads normalized by transcript length and library size, that is, reads per kilobase per million mapped reads (RPKM) [43], which is analogous to the normalized hybridization intensity in microarrays despite its digital nature. Later, more statistically robust quantification methods were developed on top of the oversimplified RPKM methods [44, 45]. Owing to the digital nature of transcript quantification in RNA-Seq, the dynamic range of transcript detection in RNA-Seq is theoretically unlimited (at least 8,000 fold), that is, able to simultaneously quantify the expression levels of rare and abundant transcript accurately [46]. Comparing to the relatively limited dynamic range of microarrays (usually a few hundredfold,

because of background noise and saturation of hybridization intensity), RNA-Seq is theoretically a superior tool for transcriptome-wide quantification, because transcript abundances usually span across several orders of magnitude. What truly made RNA-Seq revolutionary is not its superior dynamic range, but rather its ability to define the boundaries (e.g., transcription start sites and polyadenylation site) and complexity (e.g., alternative splicing and polyadenylation isoforms) of all transcripts in a transcriptome, given an adequate sequencing depth. This information is extremely useful for revising gene models, discovering new transcripts, and defining alternative isoforms. In the following sections, we first share our experiences on applying RNA-Seq to the *E. histolytica* transcriptome and then summarize our findings on the transcriptome survey.

7.3 Think Before You Start Sequencing! Caveats and Tips of RNA-Seq for *Entamoeba*

In this section, we summarize our experiences of applying RNA-Seq to the *E. histolytica* transcriptome. In particular, we have sequenced both the poly(A)+ and total transcriptome of *E. histolytica* strain HM1:IMSS and Rahman using pair-end non-strand-specific protocol [37] and single-end strand-specific protocol (unpublished) on the Illumina platform. We aimed to provide useful suggestions on a number of technical aspects for RNA-Seq in *Entamoeba*.

7.3.1 RNA Fractionation and Depletion of Ribosomal RNA

To ensure adequate sequencing coverage and depth on the target transcript populations (e.g., mRNAs), total RNA is often fractionated to enrich a particular population of transcript for sequencing, for example, poly(A)+ transcripts. In fact, our analyses on total transcriptome RNA-Seq data suggest that more than 95 % of RNAs in the *E. histolytica* transcriptome are ribosomal RNAs (rRNAs). Therefore, separating the desired fraction of transcripts from rRNAs is crucial. The standard method is affinity purification of poly(A)+ transcripts using the oligo(dT) matrix. A previous report of RNA-Seq on *Plasmodium falciparum* claimed this methodology did not work effectively because of the A+T-rich genome (~80 % A+T) [47]. Although the genome of *E. histolytica* has a comparable but slightly lower A+T content (~75 % A+T), purification of poly(A)+ transcripts using Sera-Mag Oligo(dT) Magnetic Particles (Thermo Scientific) seems to work efficiently, with only about 1 % rRNA reads in our published pair-end libraries [37]. However, we found that RNA fragment-size selection seems to be crucial to rRNA contamination: inclusion of smaller RNA fragments (<150 nt) seems to substantially increase the level rRNA contamination. There are two other common options for rRNA depletion, which are designed to enrich both poly(A)+ and non-poly(A)+ transcripts against rRNA, namely, subtractive hybridization and exonuclease digestion [48].

First, subtractive hybridization, which is based on sequence-specific binding of rRNAs, can be achieved with commercially available kits, such as RiboMinus Eukaryote Kit for RNA-Seq (Ambion). The probes are designed to hybridize with highly conserved regions of rRNAs of eukaryotic species including human, mouse, rat, *Drosophila*, and yeast. However, given the divergence of *Entamoeba* rRNAs from rRNAs of these species, the efficiency of these probes are in doubt. Therefore, we do not recommend applying these commercially available rRNA subtractive hybridization kits to *Entamoeba* RNA-Seq. Instead, custom probes designed based on *Entamoeba* rRNA could be considered [47]. The second option is exonuclease digestion, which depends on the activity of a 5′-phosphate-dependent exonuclease that digests RNA containing a free 5′-monophosphate end, and thereby selectively removing rRNAs but retaining other non 5′-monophosphate transcripts including coding mRNAs.

7.3.2 Strand Specificity and Overestimation of Coding mRNA Abundance

We performed both strand-nonspecific [37] and strand-specific (RNA ligation-based; unpublished data) RNA-Seq on the *E. histolytica* transcriptome. Our strand-specific RNA-Seq data suggested that natural antisense transcripts, that is, transcripts that are transcribed from the opposite strand of coding genes, are pervasive. In fact, we found at least 1,000 coding genes having more than 10 % of its mapped reads in the antisense direction (unpublished data). In fact, the widespread presence of natural antisense transcripts has been well documented in other models organisms, such as mammalians [49] and fungi [50].Therefore, in non-strand-specific RNA-Seq datasets, abundance of these coding mRNAs might be overestimated because reads originating from their overlapping natural antisense transcripts are indistinguishable from those that originate from the coding mRNA. The overestimated abundance might thus affect accuracy of differential expression analyses. Therefore, we strongly recommend strand-specific protocols [51] if differential expression of coding mRNAs is the primary objective of the RNA-Seq analyses.

7.3.3 Depth of Sequencing and Multiplexing

How many reads are enough for RNA-Seq profiling of *E. histolytica* transcriptome? A previous study suggested approximately 50 million reads is necessary to accurately quantify mRNA expression levels for approximately 95 % of genes in a mammalian cell line [52]. Another study suggested that approximately 5 million is enough to quantify most of the transcriptional activity in diverse bacterial species [53]. To investigate the effect of sequencing depth on the coverage of the *E. histolytica* transcriptome, we summarized the percentage of genes being covered under

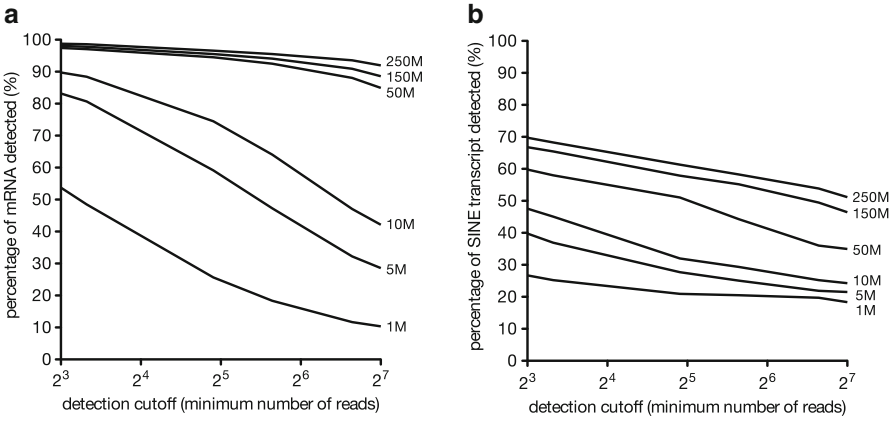

Fig. 7.1 Percentage of mRNA (**a**) and SINE (**b**) transcripts being detected at various sequencing depths. Each series in the plots represents a re-sampled library at a particular depth. The re-sampled depths are labeled on the *right end* of the lines, in unit of million reads (*M*). The plots thus showed the percentage of transcripts (i.e., *y*-axis) that can be detected upon a particular detection cutoff (i.e., *x*-axis) at a particular sequencing depth (i.e., series)

various sequencing depth. This dataset is composed of strand-specific RNA-Seq reads pooled from HM1:IMSS and Rahman strain. If we choose the detection cutoff at 2^3 reads, approximately 10–50 million reads are able to detect about 90 % to 98 % of the bona fide coding transcripts, and further increase of sequencing depth does not substantially increase the coverage (Fig. 7.1a). Alternatively speaking, approximately 10 to 50 million reads should be enough for differential expression analyses of coding mRNA in *E. histolytica*. Deeper sequencing would be wasteful if differential expression of coding mRNA is the primary objective. Considering the current throughput of several sequencing platforms (e.g., Illumina HiSeq 2500) already exceeded 150 million reads per lane and most of them support multiplex with barcoding, simultaneous sequencing of multiple samples in one lane is thus possible. However, if less abundant transcripts (e.g., rare alternative splicing isoforms or low-abundance noncoding RNA) are matters of concern, deeper sequencing depth should be considered. For example, about 60 % of SINE transcripts can be detected at the depth of 50 million reads, and further increase of sequencing depth to 250 million reads substantially increased the detection rate to approximately 70 % (Fig. 7.1b), suggesting the coverage of SINE transcripts is not saturated at 50 million reads and deeper sequencing is recommended if SINE transcripts are the subject of study. Another example is rare alternative splicing isoforms. We sequenced the poly(A)+ transcriptome of *E. histolytica* at the depth of approximately 500 million pair-end reads and discovered the existence of a larger number of low-abundance splicing isoforms [37]. If quantitative analyses of the dynamics of rare alternative splicing isoforms in *E. histolytica* is the subject of study, our rough estimation for a comprehensive coverage would be at least 2,000 million reads.

7.3.4 *Quality of Predicted Gene Models for Downstream Analyses*

The quality of the reference genome and gene models would certainly affect the downstream analyses of RNA-Seq data. The current genome assembly of *E. histolytica* (AmoebaDB version 1.7) consists of 1,496 scaffolds with 8,201 coding gene models, which is often considered as "unfinished:" that is, it might contain numerous misassembled regions and thus wrongly annotated gene models, and a portion of the genome might be missing from the assembly [54]. Nonetheless, the percentages of mappable reads in our RNA-Seq datasets are around 95 % [37], suggesting most of the genomic regions encoding for the poly(A)+ transcripts exist in the current genome assembly. However, about 900 coding gene models in AmoebaDB version 1.7 were found adjacent to ambiguous regions of the scaffold (i.e., within 100 nt to scaffold ends and regions with "Ns") or lack of a complete open reading frame (possibly pseudogenes) [37]. These predicted gene models are likely to be incomplete or erroneous, and thus conclusions derived from these gene models could be misleading, for example, in surveying small RNAs derived from intronic and exonic regions [55]. In this example, the exonic and intronic small RNA counts could be misleading if exonic regions were wrongly annotated as intronic regions. Therefore, to provide high-quality gene models for downstream analyses, we defined a set of bona fide gene models ($n = 7{,}312$) based our RNA-Seq data [37]. With the anticipated rapid growth of RNA-Seq data deposition, the number and quality of these bona fide gene models is expected to improve over time. We therefore recommend colleagues to restrict their downstream analyses to these bona fide gene models for well-drawn conclusions.

In the following sections, we describe how we used the RNA-Seq to answer the following questions about the *E. histolytica* transcriptome:

1. How much of the genome is transcribed?
2. How good (or bad) are the predicted gene models?
3. How much of the alternative splicing and polyadenylation events are functionally relevant?

7.4 How Much of the Genome Is Transcribed? Hunting for Hidden Transcripts

7.4.1 *Overall Composition of the* E. histolytica *Transcriptome*

To investigate the overall composition of the *E. histolytica* transcriptome, we sequenced the total unfractionated RNA of both HM1:IMSS and Rahman strains (unpublished data, ~170 million single-end reads of 38 nt). As expected, more than 94 % of reads was mapped to structural RNAs (i.e., rRNA and tRNA) (Table 7.1).

Table 7.1 Composition of *Entamoeba histolytica* transcriptome

RNA type	Number of reads	Percentage (%)
rRNA	160,618,479	94.0225
unknown	5,459,912	3.1961
mRNA	3,821,545	2.237
rDNA-Tr	460,002	0.2693
SINE	242,036	0.1417
tRNA	212,097	0.1242
LINE	9,421	0.0055
ERE	6,318	0.0037

"Number of reads" and "percentage (%)" indicate the number and percentage of reads mapped to the genomic region annotated with the corresponding "RNA type:" including *rRNA* ribosomal RNA, *unknown* unannotated genomic region, *mRNA* annotated coding RNA, *rDNA-Tr* transcripts derived from ribosomal DNA episome, *SINE* transcripts from short interspersed elements, *tRNA* transfer RNA, *LINE* transcripts from long interspersed elements, *ERE* transcripts from *Entamoeba* repetitive element

Although about 2.23 % of the reads were mapped to coding mRNA, about 0.14 % and 0.26 % of reads were mapped to repetitive elements SINE retrotransposons and transcripts from ribosomal DNA (rDNA-Tr), respectively, supporting the previous observations that EhSINE1s [24] and rDNA-Tr [17] are extensively transcribed. Interestingly, about 3.2 % of the reads were mapped to unannotated genomic regions, suggesting the existence of a large population of unknown transcripts in the *E. histolytica* transcriptome. It should be noted that the sequencing depth of this dataset on the nonstructural RNA is relatively shallow because more than 94 % of reads mapped to structural RNAs. Further sequencing of the rRNA-depleted RNA fractions might provide more insight to the existence and overall abundance of these unknown transcripts.

7.4.2 Genomic Regions Being Transcribed as Poly(A)+ Transcripts

To quantify the portions of genome being transcribed as poly(A)+ RNA, we sequenced the poly(A)+ RNA of both HM1:IMSS and Rahman strains [37]. This dataset covered about 95 % of the coding regions at the depth of at least ten reads (i.e., saturated on mRNA coverage). Based on this dataset, we concluded about 86 % of the "nonrepetitive and nonstructural RNA" genome (i.e., genomic regions excluding ribosomal RNA, transfer RNA, and repetitive elements) was transcribed (covered by at least two reads). We noted that approximately 24 % of these transcribed genomic regions are unannotated, reflecting the incompleteness of the current gene model annotation and the existence of a large number (by genomic area) of unknown transcripts [37].

7.4.3 *Hunting for Unannotated Transcripts*

To characterize these unannotated transcribed regions, we defined transfrags, that is, continuously transcribed fragments (>100 nt) that are at least 100 nt away from any annotated transcripts (including structural RNAs, coding RNAs, and repetitive elements) and ambiguous genomic regions [37], and examined their properties. In this way, we identified 1,995 novel transfrags. About 9 % of these novel transcripts ($n = 181$) were found to contain a complete open reading frame (ORF), which were then annotated using InterProScan and BLAST and incorporated into the bona fide gene models [37]. A number of small peptides with well-known functions were identified, such as G-protein subunit gamma, amoebapore, ferredoxin, and thioredoxin. Most of these novel ORFs (i.e., 159 of 181) are shorter than 300 nt (average, ~268 nt), reflecting the fact that ORFs of less than 100 codons were basically ignored in previous gene predictions [19]. The mean GC content of these novel coding transcripts are comparable to that of the existing coding transcripts and readily distinguishable from that of the other noncoding features, supporting the validity of these novel coding transcripts (Fig. 7.2a). The mean GC content of the novel

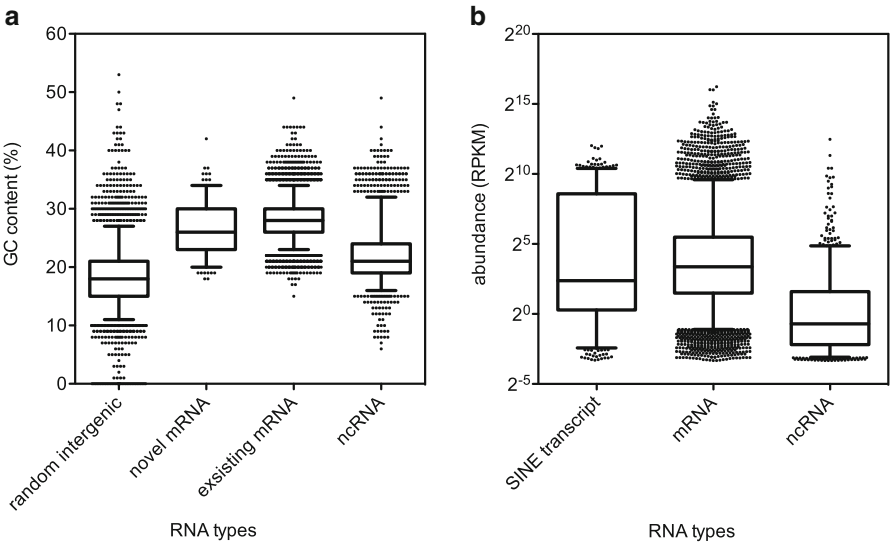

Fig. 7.2 GC content and abundance of various RNA types. **a** GC content. Random intergenic refers to randomly chosen unannotated genomic regions (500 bp each, $n = 5{,}000$). Novel mRNA refers to newly identified coding mRNA that is not annotated in AmoebaDB v1.7; existing mRNA refers to coding mRNA is annotated in AmoebaDB v1.7; ncRNA refers to the noncoding RNA identified from the RNA-Seq. **b** Abundance, measured in reads per kilobases per million mapped reads (RPKM). SINE transcripts refers to the transcripts derived from annotated SINE genomic regions; mRNA refers to both novel and existing mRNA in **a**. ncRNA refers to ncRNA in **a**. It should be noted that transcripts with RPKM value less than 0.1 were excluded in **b**. The "*boxes* and *whiskers*" represent the 5th, 25th, 50th, 75th, and 95th percentiles. The *dots* represent data points beyond the 5th and 95th percentiles

noncoding transcripts (ncRNA, $n = 1{,}814$) is slightly lower than that the coding transcripts but readily distinguishable from that of the random unannotated genomic regions. The elevated GC content of these ncRNAs suggests these transcripts are unlikely to be solely transcriptional noises. Although the mean abundance of these ncRNAs is much lower than that of the coding mRNA and SINE transcripts (Fig. 7.2b), 124 ncRNAs are considered to be abundant (>10 RPKM; the median of coding mRNA is 9.7 RPKM). These 124 ncRNAs include the previously reported unknown abundant noncoding transcript, ehapt1 [12]. Our RNA analyses offer a genome-wide catalogue of ncRNAs, which is the first step toward the understanding of its functions.

7.5 How Good (or Bad) Are the Predicted Gene Models? Defining Bona Fide Gene Models

7.5.1 Genome Quality and Gene Model Accuracy

Another application of RNA-Seq is for revision of gene models. Initial analyses o*f E. histolytica* genome in 2005 (assembly of ~23 Mb with 888 scaffolds) predicted 9,938 coding genes [19]. Nonetheless, the genome is AT rich and highly repetitive, and thus this initial assembly might contain misassembled regions and partially sequenced or unidentified genes [54]. Therefore, the genome was reassembled 5 years after its initial analyses, with more than 100 artifactual tandem duplications eliminated, reducing the assembly size to approximately 20 Mb with 1,496 scaffolds and predicted gene number to 8,201 with 40 % of the original gene models revised [18]. Even so, most of the gene models were solely based on in silico prediction and lack of supporting experimental data. How good are these predicted gene models? Roy and Penny investigated the correlation between intron length bias and accuracy of gene models, using the initial *E. histolytica* assembly as an example [56]. They suggested that skewed ratio of intron length categories of $3n$, $3n+1$, and $3n+2$ could be indicators of intron prediction errors, as intron lengths are not expected to respect coding frame. They found a significant excess of $3n+2$ introns in the initial *E. histolytica* assembly (Fig. 7.3a), and such a skewed ratio might actually represent errors in assembly that led to frameshift and affected the accuracy of intron prediction. Their results suggested that at least some 20 % of the initial *E. histolytica* introns predictions in 2005 are not in fact introns but instead coding sequences [56].

7.5.2 How Many of the Splicing Junctions Can be Confirmed?

Our RNA-Seq data confirmed 2,089 of 2,557 junctions annotated in AmoebaDB version 1.7. Conversely speaking, 468 of these were left unconfirmed. These unconfirmed junctions might result from either poor data coverage or intron prediction errors. Based on the aforementioned intron length bias in wrongly predicted introns [56],

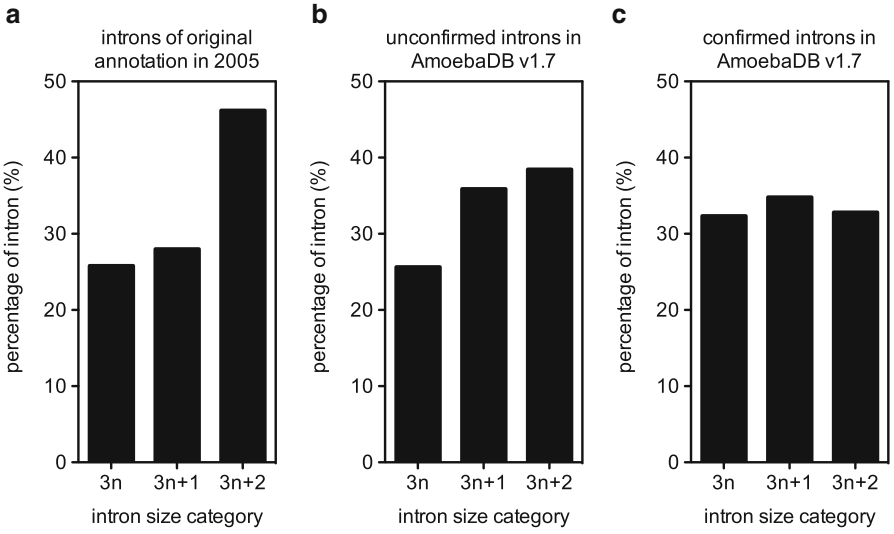

Fig. 7.3 Intron size category bias in various subsets of intron: all introns annotated in the original genome annotation in 2005 (**a**); introns in AmoebaDB v1.7 that cannot be confirmed by our RNA-Seq (**b**); introns in AmoebaDB v1.7 that are confirmed by our RNA-Seq (**c**). "3*n*", "3*n*+1", and "3*n*+2" refer to introns with length of multiple of 3, multiple of 3 plus 1, and multiple of 3 plus 2, respectively

we thus compared the intron length category ratio of the confirmed and unconfirmed AmoebaDB junctions. In unconfirmed junctions, both 3*n*+1 (~36 %) and 3*n*+2 (~38 %) are substantially more frequent than 3*n* introns (~25 %) (Fig. 7.3b), whereas in the confirmed junctions, the ratio of 3*n*+1 (~34 %), 3*n*+2 (~35 %), and 3*n* introns (~31 %) is mostly unbiased (Fig. 7.3c). For instance, about 50 % of these unconfirmed junctions are located either in proximity to the ambiguous genomic regions ($n=154$), or within repetitive regions ($n=77$), which are prone to assembly errors. These data suggest a substantial proportion of these unconfirmed junctions might be errors of intron prediction and do not exist. In fact, 30 % of unconfirmed junctions ($n=163$) were corrected and led to modifications of gene models.

7.5.3 Defining a Set of Bona Fide Gene Models

Based on the coverage and junction data from our RNA-Seq analyses, we validated 6,411 and revised 720 existing gene models [37]. The gene model changes include incorporation of new junctions, modification of existing junctions, fusion of or split into multiple gene models, and extension or shortening of transcript ends. Along with the 181 coding genes, we defined a set of bona fide gene models ($n=7,312$), which can be considered as high-quality gene models that can be used for downstream analyses with minimal uncertainty. Alternatively speaking, 1,220 of the

existing gene models (~15 %) could not be validated, although 892 of these "invalid" models were not validated either because of the lack of a complete ORF (e.g., pseudogene) or located at the proximity of ambiguous genomic regions (e.g., scaffold breaks). In fact, most of these unvalidated gene models are repetitive, with less than 25 % of them being unique. It thus agrees with the fact that repetitive sequences tend to create breaks in the scaffolds and pseudogenes are often repetitive. We believe most of these invalid gene models are either pseudogenes or incompletely sequenced in the genome assembly, and therefore deeper RNA-Seq data are unlikely to substantially increase the number of bona fide gene models. Finally, we identified the poly(A) tails of 4,509 coding transcripts and therefore annotated the 3′-untranslated regions of their gene models, adding valuable information to the bona fide gene models.

7.6 How Many of the Alternative mRNA Isoforms Are Functionally Relevant? Quantifying the Stochastic Noise of RNA Processing

7.6.1 Alternatively Splicing and Polyadenylation Events Are Pervasive

Alternative splicing and polyadenylation events, which generate multiple isoforms from a single mRNA precursor, are pervasive in eukaryotic transcriptomes. Using our RNA-Seq datasets, we reconstructed and quantified the alternative splicing and polyadenylation events in *E. histolytica* transcriptome [37]. First, we identified a large number of alternative junctions on coding mRNAs ($n = 3{,}413$, ~1.5 times more than the annotated junctions, $n = 2{,}089$), although these alternative junctions are rarely spliced when compared with the constitutive junctions. Second, we observed ubiquitous alternative polyadenylation cleavage events surrounding the constitutive polyadenylation cleavage site of coding mRNAs, although these alternative cleavage events are much less frequent than the constitutive cleavage events. These observations suggested these rare alternative splicing and polyadenylation events are pervasive in coding mRNA of *E. histolytica*, similar to the observations in higher eukaryotes [57].

7.6.2 Quantifying the Stochastic Noise to Identify the Nonstochastic Events

These alternative isoforms could be either consequences of physiological regulation or the stochastic noise of RNA processing [58–60]. How many of the observed alternative splicing and polyadenylation events are consequences of stochastic noise

of RNA processing? Understanding the properties of the stochastic erroneous splicing and polyadenylation events certainly helps us to identify the physiologically regulated events that are more likely to be functionally relevant [60]. Given the depth and resolution of RNA-Seq technology, we were able to analyze these alternative events quantitatively. We first showed that the occurrence of alternative splicing events is correlated with splicing site sequence, occurrence of constitutive splicing events, and mRNA abundance, implying the majority of these alternative splicing events are likely to be stochastic error of splicing machineries. We then estimated the corresponding error rates to medians of 0.1 % to 0.4 %. Next, we showed that the extent of microheterogeneity in polyadenylation cleavage sites is correlated with the occurrence of constitutive cleavage events, suggesting most of the microheterogeneity in polyadenylation is likely to be stochastic. We then estimated the inherent limit of stochastic microheterogeneity in polyadenylation cleavage to be within 25 nt. Overall, we only identified a small fraction of alternative splicing and polyadenylation events that are unlikely to be solely stochastic, implying the functional relevance of alternative splicing and polyadenylation in *E. histolytica* might be limited to only a small number of genes.

7.7 Conclusions and Perspectives

RNA-Seq is gradually replacing microarrays as a major platform for transcriptomics, but it is definitely much more than just a microarray replacement. We have discussed the application of RNA-Seq in measuring gene expression, identifying unannotated transcripts, revising existing gene models, and reconstructing alternative isoforms, which are considered as the "standard" applications of RNA-Seq. In fact, in combination with other technologies and tweaks in standard library preparations, applications of RNA-Seq could be much more powerful. Here we outline a number of advanced applications of RNA-Seq that could potentially be applied to *E. histolytica* transcriptome.

7.7.1 Mapping the Start and End of Transcripts

A genome-wide atlas of the exact start and end sites of transcripts is the stepping stone for understanding the regulatory mechanism of transcription in an organism. Theoretically, given enough sequencing depth, data generated from standard RNA-Seq libraries could be used to define the start and end of nonoverlapping transcripts simply by looking for the boundaries of the corresponding transcribed fragments. However, in reality, because data coverage of transcripts is not always deep enough (and thus the coverage is "broken") and some transcripts are overlapping on the same strand, boundaries of transcribed fragments often do not correspond to the start and end of the transcripts. Therefore, various protocols were developed specifically for mapping transcript ends [61]. To map transcription start site, the general

strategy is to enrich the 5′-termini of the transcript by either affinity purification of the 5′-cap structure [62, 63] or selective degradation of the uncapped RNA fragments [64, 65]. To map the ends of poly(A)+ transcripts (i.e., polyadenylation sites), the general strategy is to enrich the 3′-termini of the transcripts by using poly(T) oilgo for cDNA synthesis [66, 67], followed by in silico detection of polyadenylation cleavage site based on sequence composition [68]. Genome-wide mapping of transcript start sites revealed the complexity of transcriptional activities around transcript start sites and led to the discovery of several new categories of promoter-associated transcripts [61].

7.7.2 Simultaneous Profiling of Long and Small RNAs

An unusual class of endogenous PIWI-associated 5′-polyphosphated small RNA was first identified in *Entamoeba* with Sanger sequencing [69] and further characterized using 454-pyrosequencing [55]. Details of small RNA populations in *Entamoeba* are discussed in another chapter of this book. The genomic origins of these small RNAs are currently unknown, but simultaneously analyses of RNA-Seq data from long transcripts and small RNA would certainly shed light on this aspect. For example, Lasa and coworkers sequenced both the long and short RNA fractions (<50 nucleotides) from *Staphylococcus aureus* and found that short RNAs are generated through the digestion of overlapping sense/antisense transcripts by RNase III endoribonuclease [70].

7.7.3 CLIP-Seq: Characterization of RNA-Binding Protein

CLIP-Seq, that is, crosslinking immunoprecipitation high-throughput sequencing, is a RNA-Seq approach to identify RNA sequences that interact with a particular RNA-binding protein. This approach relies on UV-crosslinking between RNA and the protein bound to it, followed by immunoprecipitation for the protein, and finally high-throughput sequencing of the RNA molecules bound to the protein. A typical application is to characterize argonaute proteins bound to mRNA and microRNA molecules [71]. Mapping of splicing factor-binding sites on pre-mRNA is another common application of CLIP-Seq [72, 73].

With the rapidly expanding availability of high-throughput sequencing platforms, we expect, soon after, these advanced RNA-Seq approaches will be applied to the *E. histolytica* transcriptome. In addition, other exciting and innovative RNA-Seq approaches, for example, single-cell transcriptome sequencing [74] and dual RNA-Seq of host and pathogen [75], are continuously being developed and refined. Such rapid and ongoing development of RNA-Seq technologies is likely to soon bring the decade-long microarray era to an end. This seemingly promising technology is expected to shed more light on the poorly characterized transcriptome of *Entamoeba*.

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Chapter 8
Ribosomal RNA Genes and Their Regulation in *Entamoeba histolytica*

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Abstract The transcription of ribosomal RNA genes is tightly regulated in response to different environmental and growth stress conditions. This chapter summarizes our current understanding of such regulation in *Entamoeba histolytica*. The rRNA genes of *E. histolytica* are located exclusively on extrachromosomal plasmids, which may have one transcription unit (rDNA I) or two units (rDNA I and rDNA II) per circle. These plasmids are localized to the nuclear periphery where the nucleolus has been mapped using antibodies against RNA polymerase I and a known nucleolar marker, fibrillarin. Transcription of rDNA I is driven by two promoters, P1 and P2, which are 1.5 kb apart. Pre-rRNAs are transcribed from both promoters under normal growth conditions, although P1 is a weaker promoter. Upon growth stress (serum starvation and cycloheximide treatment), pre-rRNAs accumulate from promoter P2 but not P1, showing that the two promoters respond differentially to stress. Surprisingly, we found that transcripts of 0.7–0.9 kb also accumulated along with pre-rRNA under stress. These transcripts map to the 5′-external transcribed spacer (ETS) of pre-rRNA from promoter P2. These novel transcripts are heterogeneously sized circular molecules and accumulate as noncoding RNA. They can spontaneously self-circularize in vitro in the absence of cellular proteins. Because the 5′-ETS has binding sites for pre-rRNA processing factors, we speculate that these circular transcripts inhibit processing of pre-rRNA by sequestering the processing factors. Thus, ribosome biogenesis during growth stress in *E. histolytica* seems to be controlled posttranscriptionally by downregulating the processing of pre-rRNA.

Abbreviations

ETS	External transcribed spacer
IGS	Intergenic spacer
ITS	Internal transcribed spacer
NoRC	Nucleolar remodeling complex

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_8

ori	Origin
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RNA Pol	RNA polymerase
tsp	Transcription start point

8.1 Introduction

Transcription of ribosomal RNA genes, and thus ribosome production, which determines the potential for cellular proliferation, is highly regulated in response to general metabolism as well as specific environmental conditions [1, 2]. Although Bacteria and Archaea have one RNA polymerase (Pol) that transcribes all genes, eukaryotes have three RNA Pols, each responsible for transcription of different genes; RNA Pol I for the synthesis of rRNA, RNA Pol II for messenger RNA, and RNA Pol III for small RNAs including transfer RNAs. Ribosome production in eukaryotes involves the function of all three RNA Pols. RNA Pol I, located in the nucleolus, transcribes rRNA genes into nascent pre-rRNA, which is processed into the mature 18S, 5.8S and 28S/25S rRNA species. RNA Pol II transcribes mRNAs for ribosomal proteins and RNA Pol III transcribes precursor to 5S rRNA. Transcription of rRNA genes by RNA Pol I is regulated by diverse mechanisms. In most organisms these genes are present in very high copy number [3], of which a fraction (about 50 % in mammals) is transcriptionally active [1]. The rest are in a heterochromatic configuration refractory to transcription (reviewed in [4]). Ribosomal RNA synthesis can therefore be modulated either by varying the transcription rate per gene or by varying the number of active genes. Studies in yeast and mammals show that the overall initiation rate rather than the number of active genes is likely to determine the rate of rDNA transcription during exponential growth [5]. In mammals the transcriptionally silent state of rDNA is established by the nucleolar remodeling complex (NoRC), whose function requires association with specific noncoding RNAs [6–8]: these are intergenic transcripts of 150–300 nucleotides and are complementary in sequence to the rDNA promoter. They can silence rDNA genes in *trans*. They are of low abundance, do not accumulate in vivo unless stabilized by interaction with NoRC, and are thought to form a triple-helical DNA:RNA structure at the rDNA promoter that recruits DNA methyltransferases [4]. In yeast, derepression of Pol I under stress conditions in a mutant strain has been shown to simultaneously derepress Pol II transcription of ribosomal protein genes and 5S rRNA synthesis by Pol III [9], suggesting the central role of Pol I in regulation of all ribosomal components.

8.2 The Transcriptional Apparatus of *Entamoeba histolytica*: An Overview

The main components of any transcriptional apparatus are the RNA polymerases and their regulatory factors. All RNA polymerases, whether from Bacteria, Archaea, or eukaryotes, are multisubunit enzymes. It is thought that each subunit of the single RNA Pol of Bacteria/Archaea may have diverged to give rise to the three eukaryotic RNA Pols. The current nomenclature system used for describing genes encoding subunits of eukaryotic RNA Pols employs RPA, RPB, and RPC for RNA Pols I, II, and III, respectively [10]. In *Saccharomyces cerevisiae*, RNA Pol I contains 14 subunits, Pol II has 12 subunits, and Pol III has 17 subunits, of which 4, 5, and 8 subunits are unique to the respective RNA polymerase with no counterpart in the other Pols. Five subunits (RPB 5, 6, 8, 10, and 12) are common to all three RNA polymerases [11]. The remaining subunits show variable levels of divergence between the three Pols. The exact same pattern is seen for RNA Pol II and III of *Mus musculus*. However the RNA Pol I of *M. musculus* lacks two of the unique subunits found in *S. cerevisiae* Pol I.

Biochemical analysis of the RNA Pols of *E. histolytica* has not been attempted except for a preliminary report [12]. A comprehensive search for subunits of all three RNA Pols was performed by DNA sequence analysis of the *E. histolytica* genome using PSI-BLAST with genomes of *S. cerevisiae* and *M. musculus* as queries [13]. Most of the subunits, including all five common subunits, could be identified because of the high degree of conservation. The presence of all core subunits suggests a common structural assembly of RNA polymerases in *E. histolytica*. All Pol I subunits of *S. cerevisiae* were found in *E. histolytica*, except two unique subunits (RPA 34.5 and RPA 14, which are also absent in mouse). In RNA Pol II all subunits were identified in *E. histolytica* except one unique subunit (RPB3). However, in Pol III, five unique subunits (RPC37, 34, 31, 17, and 11) could not be identified in *E. histolytica*. This lack may result from sequence divergence, so that the subunits could not be identified, or occur because *E. histolytica* Pol III lacks these subunits.

Multiple sequence alignment of amino acid sequence of major RNA Pol subunits of *E. histolytica* with other organisms (*S. cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *M. musculus*) showed high level of conservation [13]. For example, RPA1 (the largest subunit of RNA Pol I) is homologous to the largest subunits of RNA Pol II and III, that is, RPB1 and RPC1, respectively. Its amino acid sequence shows eight conserved domains (A–H) [14, 15]. These eight domains were found to be well conserved in *Eh*RPA1 as well as in *Eh*RPB1 and *Eh*RPC1. Similarly, Zn-binding motifs found in several RNA Pol subunits were conserved in *Eh*RPA1, 2, and 12, and in *Eh*RPB10 and 12. A region of homology (about 50 aa) called the α-motif, which is present in the α-subunit of *Escherichia coli* RNA Pol [16], is also conserved in eukaryotic RNA Pols. *Eh*RPAC40 and *Eh*RPAC19 showed good sequence conservation in the α-motif when compared with other organisms. Sequence comparison of *Eh*RPA1, *Eh*RPB1, and *Eh*RPC1 identified a region

(aa 1,321–1,560) which was least conserved amongst them. This method was used successfully to raise *Eh*RPA1-specific antibodies for nucleolar localization [17].

It is well known that the three nuclear RNA polymerases vary in their sensitivities to the cyclic octapepetide-α-amanitin, which is exploited to study the transcription of genes by the three different polymerases. RNA Pol I is resistant, whereas RNA Pol II is 50 % inhibited by 5–20 μg/ml and RNA Pol III is 50 % inhibited by 250 μg/ml α-amanitin [18]. The α-amanitin sensitivity of Pol II occurs because it contacts residues in two helices of the Pol II largest subunit RPB1, the amanitin motif and the bridge helix [19]. However, the RNA Pol II of *E. histolytica* is resistant to α-amanitin [20]. When the α-amanitin motif of *Eh*RPB1 was compared with that of mouse, it was found that of seven key residues that bind to α-amanitin only one residue was retained, although the other six residues were not conserved [13, 21]. The bridge helix motif of *Eh*RPB1 also has two substitutions that are conserved in most of the RPB1 orthologues. Thus, changes in key residues of α-amanitin motif and the bridge helix domain in *Eh*RPB1 may contribute to the observed α-amanitin resistance in *E. histolytica*.

8.3 The rRNA Genes in *Entamoeba* Are Extrachromosomal

The rRNA genes in eukaryotes are highly repetitive, and hundreds to thousands of copies are generally present as tandem arrays on one or more chromosomal loci [3]. However, in some organisms these genes are predominantly found on extrachromosomal locations. The slime molds (e.g., *Dictyostelium* and *Physarum*) and ciliates (e.g., *Tetrahymena* and *Paramecium*) carry their rRNA genes on large palindromes [3, 22], which are linear molecules with telomeric ends. In certain protozoa, such as *Naegleria gruberi* [23], *Euglena gracilis* [24], and *Entamoeba* [25–27], the rDNA exists as circular molecules. Ribosomal DNA circles are also found in oocytes from a variety of organisms, including *Xenopus laevis*. Here, the circular rDNA is an amplified product of chromosomally integrated genes and, presumably, is needed to fulfill the enhanced requirement for ribosomes in the developing oocyte. By contrast, the rRNA genes in *Entamoeba* are present exclusively extrachromosomally without any chromosomal copy reported so far. The cellular logic of maintaining essential genes such as the rRNA genes extrachromosomally is not clear. It is possible that extrachromosomal rRNA genes are evolutionary intermediates in the amplification process that generated hundreds of copies; these were integrated in the chromosomes in most organisms, but remained extrachromosomal in a few cases.

The best characterized circular rDNA molecule in *E. histolytica* is called EhR1 from strain HM-1:IMSS. This plasmid of 24.5 kb is present in about 200 copies per haploid genome equivalent [26–29]. Sequence data show that each molecule of EhR1 (Fig. 8.1) contains two rRNA transcription units (rDNA I and rDNA II) arranged as inverted repeats. A variant of EhR1 called EhR2 is also

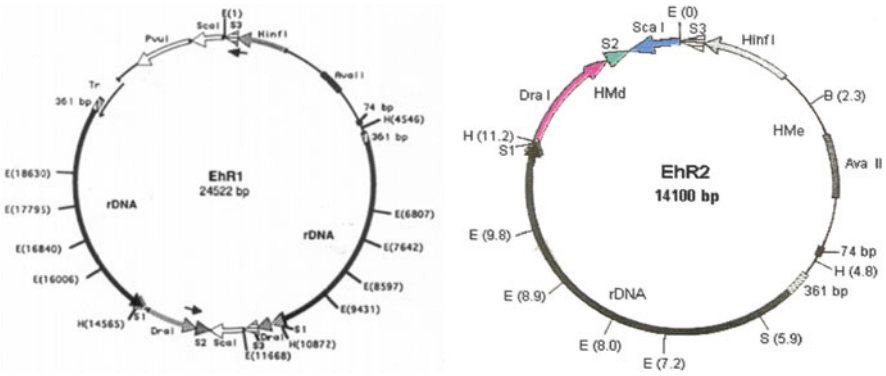

Fig. 8.1 Ribosomal RNA genes in *Entamoeba histolytica* are located extrachromosomally on circular DNA molecules. rDNA circles from *E. histolytica* strain HM-1:IMSS (EhR1 and EhR2) are presented. The *Eco*R1 (*E*) and *Hin*dIII (*H*) sites are indicated. *Arrow* marked rDNA gives the orientation of rRNA genes. Location of various families of short tandem repeats in clockwise direction (*Hin*fI, *Ava*II, 74 bp, *Dra*I, *Sca*I, and *Pvu*I) shown in *Eh*R1. The 361-bp stretch upstream of the 18S gene in both rDNA units is identical in sequence. *Small arrows* near 12 o'clock and 6 o'clock positions indicated location of 346-bp direct repeat sequence in EhR1 that may be involved in homologous recombination to generate EhR2 [28, 29]

found in strain HM-1:IMSS. This 14-kb molecule, having one transcription unit (rDNA I), is formed by intramolecular recombination of direct repeats in EhR1, resulting in two half molecules of which only one (containing rDNA I) is retained by the cell (Fig. 8.1) [30]. rDNA circles with one or two rDNA units have been found in all examined strains of *E. histolytica* (200:NIH, HK-9, and Rahman), and in *Entamoeba moshkovskii* and *Entamoeba invadens* [29]. The rDNA circle of the sibling species *E. dispar* is almost identical in sequence to *E. histolytica* [31, 32].

Studies to map the replication origin (ori) in the rDNA plasmid showed that replication could initiate in EhR1 from multiple, dispersed sites rather than a fixed origin. The activation or silencing of oris in this episome appears to be context dependent [33, 34]. The primary ori in exponentially growing cells was mapped close to the promoter of rRNA genes in the upstream intergenic spacer (IGS). However, when cells were allowed to resume growth after a period of stress, the early oris that became activated were located throughout the molecule. Later the ori in the upstream IGS was preferentially used, with concomitant silencing of the early oris; this is strikingly similar to the dispersed oris in the rDNA of the *Xenopus laevis* oocyte system [35]. Early embryos, in which rDNA is not transcribed, can initiate replication throughout the rDNA. At later stages, when transcription resumes, replication initiates primarily in the intergenic spacers and initiation in the transcribed regions is repressed [36].

It will be interesting to understand the factors that govern replication initiation in this plasmid and the possible role of rDNA transcription in this process.

8.4 The Nucleolus in *Entamoeba* Is at the Nuclear Periphery

The nucleolus is a well-defined compartment of the nucleus and is the site of rDNA transcription, pre-rRNA processing and modification, and pre-ribosome assembly [37]. As viewed by electron microscopy the nucleolus in model organisms is composed of three regions: the fibrillar center, the dense fibrillar component, and the granular component [38]. Although it is best known as the site for nascent ribosome synthesis, the nucleolus may, in fact, have many other roles, including biosynthesis of the signal recognition particle, regulation of cell-cycle progression, and as a locus of mRNA and microRNA traffic [39]. In this context it is fascinating to note that nucleolar organization in *Entamoeba* appears to be completely different from that seen in model organisms. Localization studies showed that the rDNA circles mapped to the inner membrane of the nucleus [40]. Using fluorescence microscopy with antibodies against the *E. histolytica* homologue of fibrillarin (a known nucleolar marker) and *E. histolytica* RNA Pol I subunit, it was shown that both antibodies colocalized to the nuclear periphery with very little labeling in the nucleoplasm and cytoplasm (Fig. 8.2) [17]. This organization was disrupted during serum starvation in *E. histolytica* and encystation in *E. invadens*, showing the dynamic response of nucleolus to stress as reported in other systems [41].

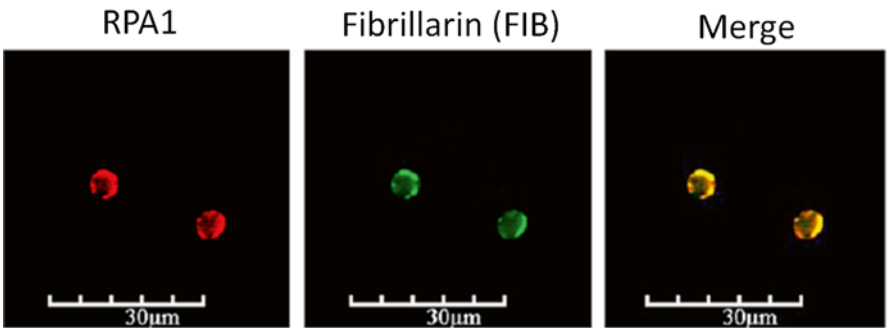

Fig. 8.2 Nucleolus in *E. histolytica* is located at the nuclear periphery. Trophozoites were double labeled with anti-EhFIB and anti-EhRPA1. RPA 1 was labeled with *Alexa-red* conjugated secondary antibody (mouse) and FIB with *Alexa-green* secondary antibody (rabbit). Confocal laser-scanning microscopy shows colocalization as *yellow signal* in the merged panel [17]

8.5 Organization of the rRNA Gene Promoter in *E. histolytica*

Studies with a number of model systems have revealed that each rDNA transcription unit consists of a coding region that is transcribed into a long pre-rRNA that is further processed into mature rRNAs [42]. During this processing the external and internal transcribed spacers (ETS and ITS, respectively) play crucial roles as sites of specific endonucleolytic cleavages. After pre rRNA processing, the ETS and ITS RNA fragments are lost. Consecutive coding regions in the rDNA repeats are separated by a nontranscribed region called the intergenic spacer (IGS) that contains gene regulatory sequences. Transcription of rRNA genes is regulated by three important regions in the IGS: promoters, enhancers, and terminators. The core promoter generally resides between +10 and −40 with respect to the RNA Pol I initiation site. The promoter also contains an upstream element (about −150), which has a modulatory role; the spacing between these sequences and their relative orientation is crucial [43, 44]. Promoter sequences between species are markedly diverged, which may partly explain the species-specific transcription of rRNA genes. Analysis of structural parameters of ribosomal gene promoters from human to lower plants revealed that specific structural features, in addition to base sequence, are fundamental for promoter function and transcriptional control by directing specific DNA–protein interactions [1]. In addition to the core promoter, another DNA element, the spacer promoter, is located in the IGS. The transcripts from this promoter appear to be relatively unstable and seem to enhance transcription from the adjacent core promoter.

The enhancer sequences are generally tandem repetitive elements (60- or 80-bp repeats in the frog and 130-bp repeats in the rat) that can stimulate transcription from the promoter, independent of orientation or distance. The terminator element consists of approximately 18-bp sequences in rodent, human, and frog. In addition to its location at the 3′-end of the pre-rRNA-coding region, the terminator is also found just upstream of the gene promoter. In facilitating release of polymerase, the terminator indirectly stimulates reinitiation of transcription by increasing the availability of initiation-competent RNA Pol I [44].

Studies with the rDNA transcription unit in *E. histolytica* show that it follows the general organization of regulatory sequences of rRNA genes found in most eukaryotes. For example, (1) the transcription start point (tsp) maps 1–2 kb upstream of the mature 18S rRNA (2.627 kb upstream of the mature 18S rRNA in rDNA I, and 1.224 kb upstream of the mature 18S rRNA in rDNA II) [45, 46]; (2) the IGS of both rDNAs contains tandem repetitive elements, which may have regulatory roles (Fig. 8.3); and (3) the promoter is located within 100 nucleotides upstream of the tsp. Inspection of the sequence immediately upstream of the tsps in rDNA I and II showed that a 51-nucleotide sequence was highly conserved (86 % identity; Fig. 8.3), which is significant because the rest of the sequence upstream and downstream of the tsps in the two rDNAs is very divergent [28]. This 51-nucleotide sequence could correspond with the core promoter by analogy with rRNA genes in other systems. For promoter mapping, different deletion fragments were cloned upstream of the luciferase reporter sequence, and the plasmids transiently

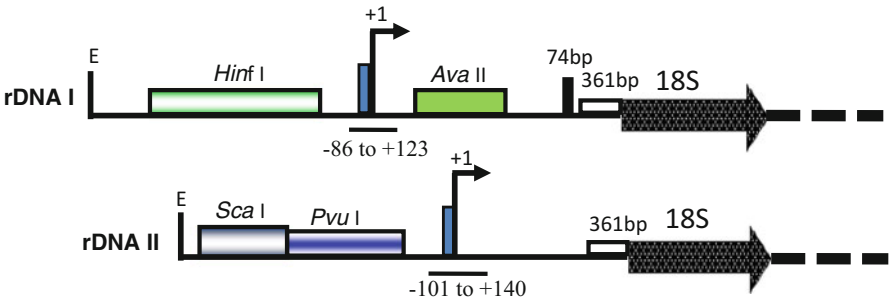

Fig. 8.3 Comparison of upstream sequences between rDNA I and II of EhR1. The upstream regions of both rDNAs are different from each other except the 361-bp sequence (shown as *blank box*) immediately upstream to 18S, and a 51-nt stretch (shown in *blue*) immediately upstream to +1 (TSP). Region spanning nucleotides −86 to +123 (in rDNA I) and nucleotides −101 to +140 (in rDNA II) shown below the TSP is required to support transcription

transfected into *E. histolytica* [46]. Luciferase transcript levels measured by reverse transcriptase-polymerase chain reaction (RT-PCR) showed that a region spanning nucleotides −86 to +123 (in rDNA I) and nucleotides −101 to +140 (in rDNA II) was required to support transcription. These data showed that, apart from the conserved 51-nucleotide putative core promoter, some upstream sequence was also required for transcription. Although this sequence is not conserved in the two units, it is highly A + T rich in both rDNAs. A minimum of 80 bp downstream of tsp was also essential for promoter activity.

The efficiency of rDNA I and II promoters measured by nuclear run on was found to be comparable [46]. Both promoters could bind and compete for similar nuclear proteins. By competition gel shift assay it was found that a minimum region of rDNA I promoter essential for protein binding was located between positions −73 and −32 with respect to the tsp. Site-directed mutagenesis showed that protein binding was actually enhanced when the sequence between positions −73 to −69 was mutated, although mutating the sequence between positions −68 and −53 reduced the protein binding very strongly. The latter region is almost completely composed of As and Ts: it is possible that this region may adopt a favorable conformation required for DNA–protein interaction further downstream. A- and T-rich tracts are associated with bent DNA found in rRNA promoter-upstream sequences in a variety of organisms, including *Physarum polycephalum* [47], *Arabidopsis thaliana* [48], *Escherichia coli* [49], and humans [50].

As already mentioned, most RNA Pol I promoters contain two distinct sequence elements: a core promoter and an upstream element. However, this bipartite arrangement is not seen in *Acanthamoeba castellani* [51] and *A. thaliana* [52]. In these organisms, the upstream element is absent, which appears to be true in *E. histolytica* as well. A highly conserved sequence element is found in plants between nucleotide positions −6 to +6 immediately surrounding the tsp [52]. This sequence, which is A + T rich from positions −6 to −1 and G (or A + G) rich from positions +2 to +6, is also quite conserved in other organisms, including *E. histolytica* [46]. It is proposed that this sequence may be equivalent to the RNA Pol II initiator (INR) (which can

assemble the Pol II transcription complex and determine transcription start site in the absence of other promoter domains) [52]. The requirement for tsp downstream sequences in rRNA promoter activity in *E. histolytica* could be related to the U3 proteins that are part of the single-subunit (SSU) processome: these associate with this region in *S. cerevisiae* and stimulate transcription, possibly by influencing chromatin structure [53].

8.6 The rRNA Genes in *E. histolytica* Are Transcribed from Two Promoters

During the mapping of *E. histolytica* rDNA promoter described here, the transcripts were measured by RT-PCR, which would detect even low-level transcription. Further analysis of the rDNA I core promoter by Northern blotting showed that it may, in fact, be a weak promoter because transcripts were not detectable. Instead, these studies showed the presence of a second stronger promoter downstream to the previously mapped promoter [54]. This difference was discovered when the expression of the luciferase reporter gene (1.6 kb) cloned downstream of the full-length 5′-ETS was checked by Northern blot analysis in a stably transfected cell line. The size of transcript expected from the originally mapped core promoter (now called P1) was 4.27 kb. However, the observed size was 2.7 kb. It was concluded that a second promoter (P2) existed downstream of P1. As evident from Northern blot analysis of total RNA, nascent pre-rRNA molecules corresponding to promoter P1 and P2 were readily detected in normal cells, indicating that both these promoters are engaged in transcription of rRNA genes. It is possible that the low-level transcription seen with P1 in the luciferase construct may be caused by lack of upstream IGS sequences in this construct. These sequences, which include the *Hin*fI repeats [28], may act as enhancers and may be required for efficient transcription from P1. The tsp of P2, mapped by primer extension, was located 1.1 kb upstream of the 5′-end of 18S rRNA [54] (Fig. 8.4). The relative role of these two promoters in rDNA transcription is not known. However, it was observed that the two promoters responded differently to growth stress.

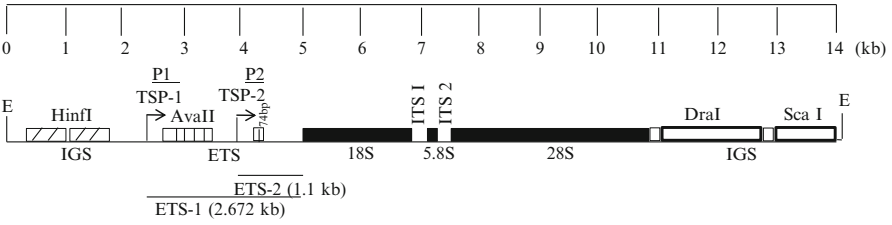

Fig. 8.4 Schematic linear view of rDNA transcription unit and flanking sequences of EhR2: coding regions of 18S, 5.8S, and 28S rRNA. *IGS* intergenic spacer, *ETS* external transcribed spacer, *ITS* internal transcribed spacer, *E Eco*RI site (as reference point). *Bent arrows* represent transcription start points (TSP). TSP-1, TSP-2, ETS-1, and ETS-2 correspond to the two promoters, P1 and P2. Different classes of repeats in IGS and ETS are marked (as in Fig. 8.1)

8.7 Unprocessed Pre-rRNA Accumulates During Growth Stress

As mentioned earlier, transcription of rRNA genes is highly regulated in response to both general metabolism and growth stress. Conditions that are harmful to cellular metabolism, such as nutrient starvation, environmental challenges, and specific inhibition of protein synthesis, cause downregulation of rDNA transcription; and subsequent upregulation is observed upon reversal of adverse conditions [55, 56]. To study the mechanisms by which *E. histolytica* regulates rRNA synthesis, the cells were starved of serum and the levels of pre-rRNA were measured by Northern hybridization.

In contrast to expectations from the model systems, it was found that in *E. histolytica* the 8.5-kb pre-rRNA accumulated 2.5 fold during serum starvation, and 1.5 fold when protein synthesis was inhibited by cycloheximide treatment [54]; this was particularly so for transcripts originating from P2, as cell lines stably transfected with the luc reporter construct driven by P2 promoter also showed accumulation of luc transcript during serum starvation. Pre-rRNA accumulation was not the result of increased transcription of rRNA genes, as the incorporation of ^{3}H-methyl methionine into RNA did not increase during starvation (Bhattacharya et al., unpublished data). It appears that the accumulation of pre-rRNA may occur because of inhibition of processing rather than upregulation of transcription. In addition to the full-length pre rRNA, there was also accumulation of some partially processed intermediates. Strikingly, a heterogeneous population of RNA molecules (0.7–0.9 kb) corresponding to the 5′-ETS also accumulated during stress. These RNAs and their possible link with pre rRNA accumulation are described next.

8.8 The 5′-ETS RNA Accumulates as Circular Molecules Under Stress

The 5′-ETS is the first part of pre rRNA to be transcribed and is generally the longest spacer. It varies in length from species to species: in the mouse, it is approximately 4 kb [57], whereas in yeast it is 696 nt [58]. It contains conserved regions of sequence complementarity with the box C+D snoRNA U3 and serves a crucial role in initiating the correct processing and assembly of preribosomal particles [59]. It is itself cleaved endonucleotically at defined sites (A′, A_0, A_1). Cleavage at site A_1 leads to the formation of the 40S preribosomal particle, following which the 5′-ETS is rapidly degraded, presumably by the exosome, which has 3′–5′ exonuclease activity [60, 61].

It is therefore interesting that in *E. histolytica* a part of the 1.1 kb 5′-ETS2 (the 5′-ETS of transcripts from promoter P2) was found to accumulate during stress. A broad band of 0.7–0.9 kb hybridizing with the 5′-ETS2 probe showed threefold greater intensity in RNA from serum-starved cells compared with normal cells.

No such intense band was obtained with ETS1 probe (specific for transcripts from promoter P1) or from ITS and 5.8 S probes [54]. Further analysis of this RNA class, referred to as "etsRNA" showed that it was not polyadenylated. It was transcribed in the same direction as rRNA, and it was nuclear localized. When the endpoints of this RNA class were mapped, it was found that it consisted of two major families of circular RNAs of size 766 nt and 912 nt. The 5′-junction of the circularization event in the 766-nt family was at position +102G (with respect to the tsp of transcript from promoter P2); the 3′-junction was at +867A. In the 912-nt family the 5′-junction was again at +102G whereas the 3′-junction was at +1013A. In each family there were shorter circles that had suffered short internal deletions (97 and 63 nt in the 766 and 912 families, respectively). The circular nature of these RNAs was first detected by RT-PCR with out-facing primers; this was further confirmed by resistance to digestion with exonuclease T (which requires a free 3′-terminus), and to nicking conditions (90 °C, with $NaHCO_3$) in which linear RNA species rapidly disappeared, but the etsRNA was resistant. Circularization of etsRNAs did not seem to require protein factors, as linear transcripts obtained by in vitro transcription could spontaneously circularize.

By primer extension it was shown that ETS2 is processed at +94 (site A′) and +1030 (site A_0). It is interesting that the A′–A_0 ETS fragment that may be released during pre-rRNA processing contains the junction sequences of all the circles found in vivo and in vitro. However the A′–A_0 linear RNA failed to circularize in vitro. RNA circles were obtained when the 3′-end of this linear RNA was shifted to +1013, the 3′-junction seen in the in vivo circle (A′, −1013 fragment, Fig. 8.5). It appears that a number of subfragments of the A′–A_0 RNA segment may possess an inherent propensity to circularize. However, the quantity of circles of different sizes formed under growth stress may depend on the concentration of the linear precursors (which would be targeted by the degradation machinery) or the generation of suitable ends

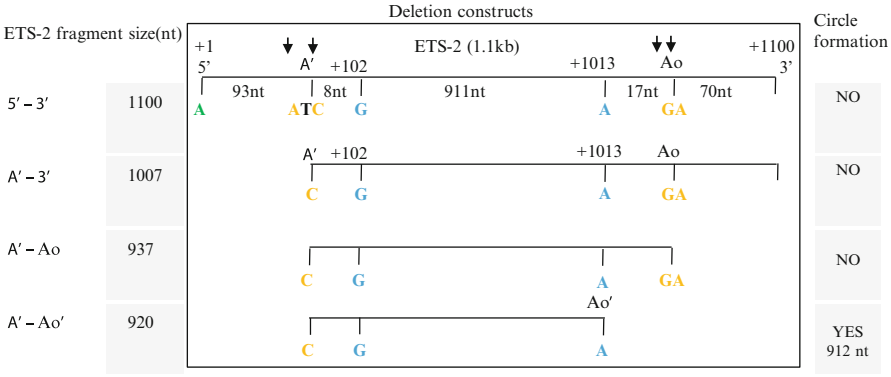

Fig. 8.5 In vitro self-circularizing ability of 5′ ETS-2 subfragments. *Top line* is schematic representation of ETS-2 with the processing sites A′ and A_0 highlighted in *yellow*. +102G and +1013A are the junctions of the 912-nt circle in vivo. A_0′ represents the 3′-end (+1013A) of 912 nt etsRNA. Molecules ending at this position could circularize in vitro

(for example, A_0' at position +1013) in the linear precursors conducive to circularization. Once formed, the circles would be much more stable than the linear precursors and may serve regulatory functions, for example, to inhibit pre-rRNA processing.

8.9 Circular Noncoding RNAs May Regulate Pre-rRNA Levels

As already mentioned, noncoding RNAs with regulatory functions have been reported from the rDNA locus. Transcripts arising from the rDNA IGS in mouse are processed into 150–300 nt RNAs which interact with TIP5, the large subunit of the chromatin remodeling complex, NoRC. This association is essential for the epigenetic silencing of the rDNA locus [6]. In another study, an IGS transcript originating 1 kb upstream of the rRNA tsp was reported in lung cancer cells. The levels of this transcript correlated negatively with those of the 45 S pre rRNA, indicating a regulatory role for this RNA [62].

It is possible that the etsRNAs reported in *E. histolytica* also have regulatory roles. Their self-circularization ability would lead to their stabilization and consequent accumulation, which seems to happen during stress, possibly when the linear 5′-ETS processing intermediate exposes the correct 3′-end that is necessary for circularization. Because the 5′-ETS region of pre-rRNA is the primary site for assembly of the pre-rRNA processing machinery, it is possible that accumulated ETS RNA in the form of circles may trap or store the processing factors in an inactive state under stress conditions when synthesis of new ribosomes is stopped. Accumulation of unprocessed pre-rRNA may permit the starving trophozoite to rapidly assemble new ribosomes when normal nutrient conditions are restored. It is interesting that the accumulated circular etsRNAs rapidly disappear when serum is restored to serum-starved cells [54], further suggesting a regulatory role for these RNAs during stress.

Accumulation of pre-rRNA has also been reported in mitotic cells [63]. This pre-rRNA is partially processed and is found in nucleolar-derived foci in association with processing factors. It is thought that this RNA may reenter the new nuclei and assist in the rapid processing of pre-rRNAs for generation of mature rRNAs.

Another class of circular RNAs have been reported from exons. Most of these are associated with exon skipping whereby the skipped exons are retained as circular molecules as in the CytP450 gene [64], the long noncoding RNA, ANRIL [65], and the Sry transcript in mouse testis [66]. It appears that these circular exons may not be mere by-products of splicing because they are transported to the cytoplasm and accumulate there. In a general sense it may be stated that RNAs with the propensity to circularize occur in many different contexts in the cell. In their linear state these RNAs have a transient existence, but as circular molecules they are stabilized and may be recruited for a variety of functions, such as intron mobility in the case of full-length circular introns [67], or regulatory functions yet to be assigned in other cases.

8.10 Summary and Conclusions

Genome sequence analysis shows that *E. histolytica* possesses all three RNA polymerases found in eukaryotes. Its RNA Pol I (which transcribes rRNA genes in eukaryotes) is well conserved as judged by amino acid sequence comparison with yeast and mouse. Nucleolar organization in *E. histolytica* is unique, being located at the nuclear periphery. The rRNA genes are present in about 200 copies per haploid genome, on extrachromosomal circles, which are capable of independent replication from multiple oris. The oris are activated or silenced in a context-dependent manner in response to growth stress. Two kinds of circles exist: those with one rDNA transcription unit (rDNA I) per circle and those with two units (rDNA I and II) arranged as inverted repeats.

The rDNA transcription unit of *E. histolytica* follows the general organization of regulatory sequences of rRNA genes found in most eukaryotes. The tsp mapped 1–2 kb upstream of the mature 18S rRNA (2.627 kb upstream of the mature 18S rRNA in rDNA I, and 1.224 kb upstream of it in rDNA II). The IGS of both rDNAs contained tandem repetitive elements, which may have regulatory roles. The promoter was located within 100 nucleotides upstream of the tsp. A 51-nucleotide sequence immediately upstream of the tsps in rDNA I and II was highly conserved in the two rDNAs (which are otherwise very divergent in their IGS sequences) and could correspond with the core promoter. A minimum of 80 bp downstream of tsp was also essential for promoter activity. Further analysis of the rDNA I core promoter (P1) showed that it may, in fact, be a weak promoter, and a second stronger promoter (P2) was found downstream to P1. The tsp of P2 was located 1.1 kb upstream of the 5′-end of 18S rRNA, and it was observed that the two promoters responded differently to growth stress.

In contrast to expectations from model systems, it was found that in *E. histolytica* the 8.5-kb pre-rRNA accumulated 2.5 fold during serum starvation, and this was particularly so for transcripts originating from P2. In addition to the full-length pre-rRNA, there was accumulation of some partially processed intermediates. Strikingly, a heterogeneous population of RNA molecules (0.7–0.9 kb) corresponding to the 5′-ETS2 also accumulated during stress. This RNA class, referred to as "etsRNA," was not polyadenylated. It was transcribed in the same direction as rRNA, and it was nuclear localized. When the endpoints of this RNA class were mapped, it was found that it consisted of two major families of circular RNAs of size 766 and 912 nt. Circularization did not seem to require protein factors, as linear transcripts of etsRNAs obtained by in vitro transcription could spontaneously circularize. Circle formation required specific sequences at the ends of the linear precursors.

Although the function of circular etsRNAs remains to be explored, it is possible that they may have regulatory roles. Because the 5′-ETS region of pre-rRNA is the primary site for assembly of the pre-rRNA processing machinery, it is possible that accumulated ETS RNA in the form of circles may serve as a scaffold to trap or store the processing factors in an inactive state under stress conditions when synthesis of new ribosomes is stopped. Accumulation of unprocessed pre-rRNA may permit the starving trophozoite to rapidly assemble new ribosomes when normal nutrient

conditions are restored. Noncoding RNAs with regulatory functions have been reported from the rDNA locus in mouse where transcripts arising from the IGS are processed into 150- to 300-nt RNAs that interact with the chromatin remodeling complex and cause epigenetic silencing of the rDNA locus. The circular etsRNAs of *E. histolytica* may add to the repertoire of regulatory RNAs involved in regulation of rRNA levels in the cell.

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Chapter 9
Small RNAs and Regulation of Gene Expression in *Entamoeba histolytica*

Laura Morf and Upinder Singh

Abstract *Entamoeba histolytica* harbors a variety of small RNA populations as well as homologues of proteins of the RNA interference (RNAi) pathway, suggesting the presence of a functional RNAi pathway in this unicellular eukaryote. Additionally, several attempts in adapting RNAi-based gene-silencing techniques in *E. histolytica* were successful, proving that a functional RNAi pathway is indeed present in this protozoan parasite. The most abundant small RNA population has a 5′-polyphosphate structure indicating a Dicer-independent biogenesis pathway. Bioinformatic and functional analyses of small RNAs highlights a role in gene silencing, which contributes to strain-specific gene expression patterns. However, whether the small RNAs have regulatory roles contributing to gene expression changes during tissue invasion or developmental changes is not known. Furthermore, the exact mechanism by which the small RNAs mediate gene expression regulation is also not known. In this chapter we give an overview on the known elements of the *Entamoeba* RNAi pathway, the characteristics of the small RNA populations, and summarize the insights we have gained from studying gene expression regulation by small RNAs.

9.1 RNA Interference and Small RNAs

RNA interference (RNAi) was first observed in plants, where RNAi accounted for the silencing of pigmentation genes in petunias [1]. In the late 1990s RNAi was also identified in an animal model, the nematode *Caenorhabditis elegans*, and since then RNAi has been discovered in a wide variety of organisms. At the center of the RNAi pathways are the small RNAs that associate with the highly conserved Argonaut

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_9

proteins and regulate gene expression sequence specifically at either a transcriptional or posttranscriptional level. The small RNAs range typically between 18 and 30 nt, and an expanding universe of different small RNAs has been recently described [2]. As many of the characteristics of the small RNAs differ among organisms, the pathways and cofactors with which these small RNAs function also diverge highly among organisms [2].

One of the first protozoans described to encode an RNAi pathway is *Trypanosoma brucei* [3]. Interestingly, the related species *Trypansosma cruzi* and *Leishmania major* appear not to encode an RNAi pathway [4]. In apicomplexan parasites, genome sequencing revealed an Argonaut homologue in *Toxoplasma gondii*, and cloning efforts confirmed the presence of siRNAs and microRNAs [5]. However, in the related apicomplexan parasites *Plasmodium* and *Cryptosporidium* neither the RNAi-related protein homologues nor siRNA or microRNAs could be identified [6, 7]. Thus far, most efforts in RNAi/miRNAs in protozoan parasites have focused on parasite-encoded small RNAs. Interestingly, however, some recent data indicate that some parasites, such as *Cryptosporidium* and *Toxoplasma*, are able to maneuver the host cell miRNA pathways in a way that favors parasite intracellular development [8, 9].

The protozoan parasite *Entamoeba histolytica* harbors small RNAs of different lengths as well as encoding homologues of genes of the RNAi pathway. In the last few years, research has clearly demonstrated that small RNA-mediated gene expression regulation plays a role in the biology of *E. histolytica* and that a robust RNAi pathway is present in this organism. Also, RNAi can serve as a highly valuable genetic tool to achieve gene knockdown in *E. histolytica*. In this chapter we summarize what is known on small RNAs and the RNAi pathway in *Entamoeba*.

9.2 Primary and Secondary Small RNAs

Exogenous as well as endogenous RNAs can serve as source for the generation of small RNAs in an RNAi pathway. In *C. elegans* exogenously added double-stranded RNA (dsRNA) is processed into small RNA duplexes, 20–30 bp in length, by the Dicer protein (an RNAseIII enzyme). These primary siRNAs harbor 5′- monophosphate termini (indicating their generation by an RNAseIII enzyme) and are loaded into a RNA-induced silencing complex (RISC) to target genes for sequence specific silencing at either the transcriptional or posttranscriptional level (Fig. 9.1). Subsequently the Argonaut protein, a key component of the RISC complex, cleaves the mRNA target, thereby mediating gene silencing. In certain organisms, RNA-dependent RNA polymerase (RdRP) can further amplify the silencing signal by generation of "secondary" small RNAs. A secondary RNAi pathway, which mediates a process of "amplified silencing," has been described in plants and nematodes [2, 10, 11]. In nematodes such as *C. elegans*, RdRP synthesizes short secondary small RNAs that are structurally distinct from the Dicer-derived small RNA counterparts in that they contain 5′-triphosphate termini. An interesting issue is how the amplified silencing mediated by secondary small RNAs is controlled and does not become rampant in the genome. A recent study in *C. elegans* addressed this question and defined that the

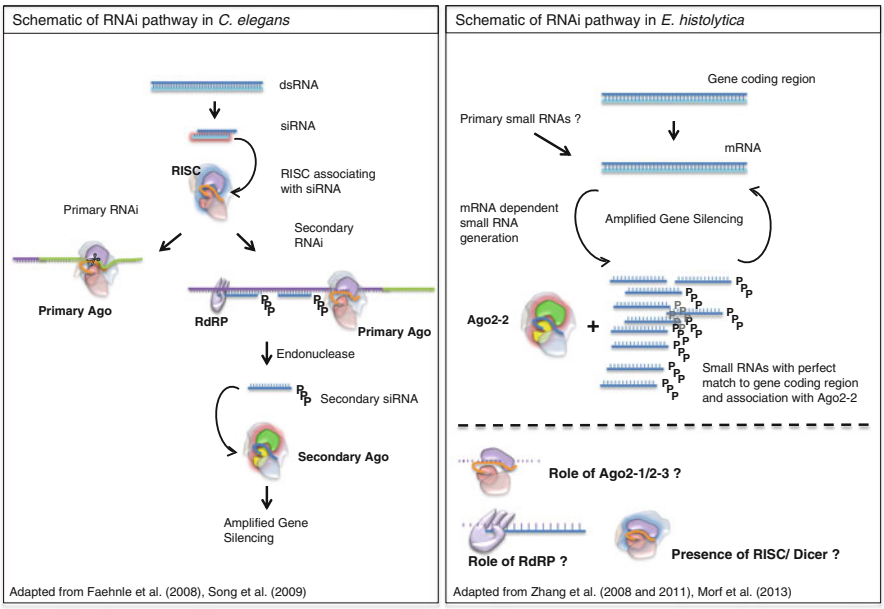

Fig. 9.1 Schematic of the RNAi pathway in *Caenorhabditis elegans* and *Entamoeba histolytica*. Briefly, in *C. elegans* dsRNA is cleaved by DICER into siRNAs that associate with RISC to lead to either gene silencing by transcriptional or posttranscriptional gene silencing or to small RNA amplification by a RdRP-dependent pathway. In *E. histolytica* small RNAs associate with Argonaut to mediate gene silencing as well as amplification of small RNAs from mature and nascent RNA

primary siRNAs can act as triggers to initiate amplified silencing but cannot function as templates upon which further amplified silencing can occur. Thus, they are involved in attenuating RdRP-dependent amplification to avoid indefinitely amplified silencing [12]. This study clearly revealed that an elaborate pathway is involved in controlled gene silencing by secondary small RNAs. Importantly, the pathway of amplified silencing with small RNAs with 5′-triphosphate termini has only been described in nematodes (*C. elegans* and *Ascaris*) and in *E. histolytica*.

Interestingly, thus far all small RNA populations characterized in *E. histolytica* harbor 5′-triphosphate termini [13]. Thus, a pathway resembling the secondary small RNA pathway in nematodes is present in *E. histolytica* and is the major contributor to small RNA-mediated gene expression regulation in this deep branching eukaryotic parasite.

9.3 RNAi Pathway-Related Protein Homologues in *E. histolytica*

The RNAi machinery typically consists of several core proteins including the highly conserved Argonaut proteins, the RNaseIII enzyme Dicer, and RdRP that contribute to the generation of primary and secondary small RNAs and gene

silencing (Fig. 9.1). The homologues of RNAi pathway-related genes in *E. histolytica* are summarized next.

Argonauts The core element of all RNAi pathways consists of the highly conserved family of Argonaut proteins, which associate with small RNAs to mediate sequence-specific gene silencing. An Argonaut protein is typically characterized by four domains: N-terminal, PAZ, Mid, and Piwi, with the PAZ, Mid, and Piwi domains playing the greatest functional roles [14]. Often Argonaut proteins prefer small RNAs with a specific first nucleotide (e.g., G) and the Mid domain determines this specificity [15]. In some cases Argonaut proteins cleave the target mRNA through its Piwi domain that is composed of an RNAseH-like fold which contains the conserved DDH catalytic residue. However, not all Argonauts posses this RNA cleavage activity [15].

Three Argonaut gene homologues are encoded by the *E. histolytica* genome Ago2-1 (EHI_186850), Ago2-2 (EHI_125650), Ago2-3 (EHI_177170), which contain both PAZ and PIWI domains [16]. Of these three genes only Ago2-2 is highly expressed; the other two have very low expression in *Entamoeba* trophozoites. In *E. histolytica* trophozoites Ago2-2 is localized to the nucleus [17] and associates with an abundant 27-nt small RNA population. These Ago2-2-associating small RNAs were analyzed by pyrosequencing and are further discussed in Sect. 9.5. However, no RNA cleavage activity has yet been demonstrated for any of the amebic Argonaut proteins.

Dicer In many organisms, such as *C. elegans* and *Drosophila melanogaster*, a Dicer enzyme is involved in cleaving the dsRNA targets into primary small RNAs (characterized by 5′-monophosphate and 3′-OH termini). Dicer is a member of the RNaseIII family protein, which is composed of three classes: the bacterial RNAseIII enzymes, Drosha enzyme, and Dicer enzymes [18]. Although human and Drosophila Dicer, for example, are highly complex enzymes encoding a number of functional domains, the Dicer enzymes in *T. brucei* contain only two RNAseIII domains [19, 20], and the *Sacharomyces castelli* Dicer contains only a single RNAseIII domain and two dsRNA-binding domains (dsRBD), rendering it the most minimal Dicer thus far identified [21].

Thus far no canonical Dicer enzyme could be detected in *E. histolytica* and only one gene with a single conserved RNaseIII domain could be identified. The fact that dsRBD domains appear not to be essential for Dicer activity suggests that this EhRNasIII could function as a Dicer enzyme [19, 20]. Extensive efforts to analyze the function of this single RNAseIII protein have been done in *E. histolytica* using cleavage assays as well as knock-down techniques, but no Dicer activity could be identified, raising the question of whether the *E. histolytica* RNAi pathway acts without a Dicer enzyme (Pompey and Singh, unpublished data). RNAi pathways acting without Dicer are not uncommon and are described in other organisms such as the Piwi-interacting small RNAs (piRNAs), which are shown to be specifically expressed in germline cells, as well as secondary small RNAs in *C. elegans* and in the nematode *Ascaris* [22, 23]. However, further studies are needed to define the role of the single conserved RNAseIII enzyme in *E. histolytica* and whether the RNAi pathway of *E. histolytica* functions in a Dicer-independent manner.

RNA-Dependent RNA Polymerase In RNAi pathways, RdRPs contribute to amplified gene silencing by synthesizing dsRNA that generates secondary small RNAs in either a Dicer-dependent or Dicer-independent manner. Found in many eukaryotes, RdRP has a role in posttranscriptional and transcriptional gene silencing as well as in antiviral defense. However, thus far it has only been implicated in amplified silencing in plants and nematodes [24]. *E. histolytica* encodes one RdRP homologue (EHI_139420) and one gene encoding a partial RdRP domain (EHI_179800). Both genes show low expression levels among all strains analyzed. Studies characterizing these RdRPs and their potential role in RNAi in *E. histolytica* are ongoing.

Drosha and Exportin Proteins The Drosha enzyme is responsible for processing of microRNA populations inside the nucleus and Exportin proteins are responsible for the transport of RNA from the nucleus to the cytoplasm [2].

No homologues of Drosha have been identified in the *E. histolytica* genome. Using a bioinformatics approach, microRNAs have been predicted in *E. histolytica* [25]; however, functional proof is lacking to date. Searches of the *E. histolytica* genome database reveal two likely Exportin proteins (EHI_029040 and EHI_107080).

Taken together, *E. histolytica* encodes three Argonaut homologues, of which Ago2-2 associates with small RNAs, two homologues of RdRP, no identifiable Dicer, and two potential Exportin proteins, which might act to transfer small RNAs from the nucleus to the cytoplasm.

9.4 Endogenous Small RNA Populations in *Entamoeba*

E. histolytica harbors endogenous small RNA of different lengths that can vary among life-cycle stages. Visualization by Northern blot analysis revealed three distinct small RNA populations (27, 22, and 16 nt) in trophozoites of the virulent *E. histolytica* strain HM-1:IMSS, the nonvirulent strain Rahman, the noninvasive human parasite *Entamoeba dispar*, and the reptilian parasite *Entamoeba invadens* [13]. Thus, it appears that *E. histolytica* harbors distinct populations of small RNAs and the overall pattern of small RNAs is conserved among *E. histolytica* strains and *Entamoeba* species; however, detailed characterization and functional proof of the 22- and 16-nt small RNA populations are still missing.

E. histolytica **HM-I:IMSS** The three most abundant small RNA populations readily detected in the trophozoite stage of the virulent *E. histolytica* laboratory strain HM-1:IMSS are at 27, 22, and 16 nt. The 27-nt small RNA population is the most abundant one, associated with a conserved Argonaut protein (EhAgo2-2); it is mentioned in detail in a separate section below. The 16- and 22-nt small RNAs are not yet well described because of their low abundance. A fourth population of 32-nt small RNAs was found to be highly abundant in the cyst stage of *E. histolytica* and increases in oxidative stress, indicating a possible biological role in response to stress.

E. histolytica **Rahman** The nonvirulent *E. histolytica* laboratory strain Rahman harbors a similar pattern of small RNAs. Interestingly, a size-fractionated small RNA library identified strain-specific patterns of antisense small RNAs mapping to certain genes, including the virulence factor STIRP, which is highly expressed in the virulent strain HM-1:IMSS but that is not expressed in the *E. histolytica* Rahman strain [26]. This finding indicates a crucial role of small RNAs in strain-specific gene expression patterns and thus a biological role in regulating strain-specific virulence phenotypes.

Entamoeba invadens *E. invadens*, a reptile parasite, also has a population of small RNAs associated with Argonaut proteins. These small RNAs and their biological roles in regulating parasite development are currently under investigation (Zhang, Ehrenkaufer, and Singh, unpublished data).

9.5 Characteristics of Argonaut-Associated Small RNAs in *E. histolytica* Trophozoites

Immunoprecipitation studies revealed that a distinct population of small RNAs associates with the highly expressed *E. histolytica* Argonaut protein Ago2-2 [13]. To characterize this population a pyrosequencing run of the small RNA that associate with Ago2-2 was performed [26]. Data analysis clearly showed that the small RNAs peak at 27 nt and match the most abundant small RNA population detected in *E. histolytica* trophozoites. These small RNAs have a 5′-G predominance and thus resemble secondary small RNAs in the nematodes *C. elegans* and *Ascaris* [22, 26]. In other systems, including Drosophila, Ago-associated small RNAs map largely to transposons with relatively few mapping to coding regions (<7 %), but in *E. histolytica* the majority of these small RNAs map to gene open reading frames in either the sense or antisense direction. In detail, small RNAs reads map to about 4 % of annotated protein-coding genes in the *E. histolytica* genome, of these about 45 % mapped antisense to the annotated protein-coding region, approximately 20 % mapped sense to annotated protein coding, and 25 % mapped to intergenic regions. Only about 7 % of the reads mapped to SINE/LINE retrotransposons and other potential repetitive regions in the genome [26]. Also, the small RNAs reads did not distribute equally over the genome but rather cluster in a small number of genomic locations, indicating that small RNAs might be derived from specific genomic regions, such as centromeric or telomeric regions as seen in other systems [27, 28].

Interestingly, the small RNAs map to both introns as well as to exon–exon junctions, indicating that they can be generated from either nascent or mature mRNA. Some loci with both sense and antisense small RNAs were noted. Strand-specific RT-PCR at such small RNA rich loci demonstrated bi-directional transcripts suggesting that these transcripts may serve as template for small RNAs to both strands. Biochemical characterization of the sense as well as antisense small RNAs revealed that both sets of small RNAs have 5′-polyphosphate termini, indicating that they are not derived by an RNAseIII enzyme, such as a Dicer [26].

Taken together, the 27-nt small RNAs in *E. histolytica* trophozoites resemble typical secondary small RNAs as described in nematodes: they have 5′-polyphosphate termini, associate with an Argonaut protein, and have a 5′-G bias [13, 26]. Further, as described in detail next, *E. histolytica* 27-nt small RNAs have a function in gene silencing [29], as described for secondary small RNAs in nematodes [12].

9.6 Small RNAs Map to Amebic Genes in Varying Patterns

Pyrosequencing data revealed that small RNAs in *E. histolytica* trophozoites map either sense (S) or antisense (AS) to gene-coding regions (Fig. 9.2). Interestingly, gene expression levels correlate with the pattern by which small RNAs map to genes.

Genes with Antisense Small RNAs Based on pyrosequencing data, a total of 226 open reading frames have small RNAs mapping to them in the antisense direction (only genes with ≥50 AS small RNAs were considered). The antisense small RNAs mapped toward the 5′-end of the gene in 77 % of these genes. Additionally, genes with antisense small RNAs were biased toward low gene expression and was the first indication that antisense small RNAs have a direct role in gene silencing [26].

Genes with Sense and Antisense Small RNAs A total of 45 genes were identified that had both sense and antisense small RNAs mapping to the open reading frame (only genes with ≥50 AS and S small RNAs were considered). Also, for these genes the antisense small RNAs are biased toward the 5′-end of the gene whereas the sense small RNAs show a more heterogeneous distribution with no clear bias. Comparison with microarray data showed that most of the genes in this group had low gene expression [26].

Genes with Sense Small RNAs A total of 87 genes were identified with only sense small RNAs (only genes with ≥50 S small RNAs were considered). In contrast to the 5′-bias of antisense small RNAs, the small RNAs that mapped sense to genes

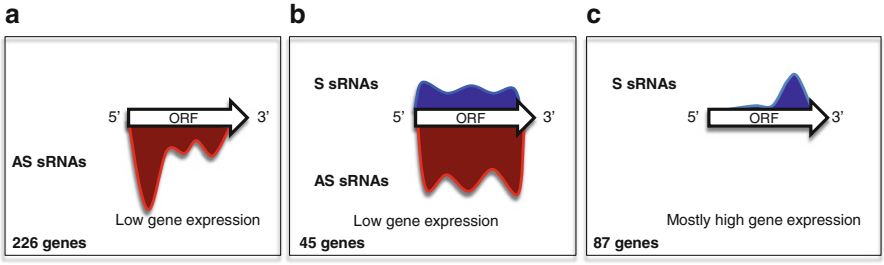

Fig. 9.2 Schematic of distribution of small RNAs to *E. histolytica* protein-coding genes (*ORF* open reading frame). **a** Small RNA distribution on genes with only antisense small RNAs. **b** Small RNA distribution on genes with both antisense (*red*) and sense small (*blue*) RNAs. **c** Small RNA distribution on genes with only sense small RNAs. Genes with antisense or sense/antisense small RNAs have low gene expression

showed a clear bias toward the 3′-end of the gene-coding regions. Also, in contrast to the low expression of genes with antisense small RNAs, most genes to which only sense small RNAs map show high gene expression and belong overall to four gene families (40S ribosomal protein S16; Gal/GalNAc lectin; three protein kinase families; and hypothetical proteins) [26].

Interestingly, there was a strain-specific pattern of small RNA abundance in *E. histolytica* strains with varying virulence profiles. A number of genes were identified that are not expressed in one *E. histolytica* strain but are highly expressed in another strain. The level of gene expression correlated inversely with the abundance of antisense small RNAs mapping to the gene (genes with high expression had no small RNA expression; genes with low expression had high abundance of antisense small RNAs mapping to them). A number of these genes are known to have virulence-related phenotypes; thus, it appears that gene expression regulation by antisense small RNA abundance is important for virulence potential in *E. histolytica*. Additionally, a number of stress-regulated genes had high numbers of small RNAs mapping to them, indicating that small RNAs may be involved in stress regulation of genes.

9.7 Gene Knockdown Technique Based on the Endogenous RNAi Pathway

To study the biology of *Entamoeba*, several attempts have been taken to utilize RNAi-based gene knockdown techniques to achieve gene silencing [30–33].

Gene silencing triggered by long dsRNA is well established in many organisms including *C. elegans* [34], *Drosophila* [35], and *Trypanosoma* [3]. This approach was adapted for *E. histolytica* in using a 340- to 790-bp-long region of a gene that was placed head to head on both sides of an unrelated DNA linker for stable expression of a ds-hairpin RNA. This approach was applied to knock down a number of genes in *E. histolytica* [30], such as Diaphanous [30], and proteins of the kinesin 5 family [36, 37]. Although knockdown efficiency greatly varied from gene to gene, the partial success of the technique indicated the presence of a functional RNAi pathway in *E. histolytica*.

Gene knockdown by short hairpin RNA is also well established in several model systems [38–40]. This method was successfully adapted in *E. histolytica* by expression of hairpin stem resulting in efficient silencing [31]. In most other systems, 22-nt small RNAs showed the highest silencing efficiency; however, in *E. histolytica* the 25- to 29-nt hairpin was most efficient. Considering that *E. histolytica* 27-nt small RNAs are associated with gene silencing, this does not appear surprising. As in *C. elegans*, soaking or bacterially expressed small RNA feeding resulted in gene knockdown in *E. histolytica* [33, 41].

Although all the studies mentioned here adapted the knockdown techniques used in other organisms for use in *E. histolytica*, the focus was largely on the technique

itself. Little insight was gained on how the RNAi pathway uses the exogenously added RNA or on the mechanisms of gene silencing in *E. histolytica*.

Entamoeba histolytica **G3** A unique small RNA-mediated gene silencing mechanism has been described in *E. histolytica*, resulting in persistent knockdown of the amebapore gene (*AP-A*); this cell line is named G3 [42]. This silencing mechanism was coincidentally discovered and is dependent on the presence of a truncated segment of SINE1, an upstream element of the *ap-a* gene [43]. A detailed analysis revealed that the gene silencing in G3 is not dependent on DNA methylation but a significant change in the level of histone methylation (H3K4) at the silenced gene loci. A recent study demonstrated that AS small RNAs to the silenced genes are generated in the G3 cell line and that these small RNAs locate in the nucleus and associate with Ago2-2 [17]. These findings indicate that the G3 silencing method is dependent of the generation of small RNAs and that small RNA-mediated gene silencing might act via histone modifications. Further efforts are needed in this area to definitively and functionally associate the small RNA pathway with histone modifications and chromatin changes.

9.8 Functional Analysis of Small RNAs That Associate with Argonaut Proteins

AS Small RNAs Are Functional in Gene Silencing Observational data from the pyrosequencing analysis showed that genes with AS RNAs are biased toward low gene expression in *E. histolytica*. However, promoter analysis of these "silent" genes revealed that their promoters are constitutively active [29]. When a portion of a gene that has abundant AS small RNAs (heretofore referred to as a "trigger") was fused to a luciferase reporter and stably transfected into *E. histolytica* trophozoites, luciferase expression was abolished, indicating that AS small RNAs are indeed functional and silence the gene fused to it [29]. Fusion of the trigger to the luciferase gene resulted in small RNAs being generated to luciferase; the small RNAs have 5′-polyphosphate termini and are 27 nt in size. This direct proof of the functionality of AS small RNAs suggests that genes are silenced via AS small RNAs after transcription resulting from active promoters.

In addition to silencing a luciferase gene, the trigger-gene fusion was also capable of silencing a chromosomally encoded amebic gene fused to the trigger, which implies that the newly generated AS small RNAs were functional in efficiently silencing the endogenous copy of the gene at the chromosomal location. This silencing remained even after plasmid removal, indicating that the endogenous copy of the gene is used thereafter as template to amplify the small RNAs. Importantly, robust silencing persisted over months. Additional analysis revealed that the silencing is sequence specific and that a related gene with 56 % sequence identity was not silenced and had unchanged expression levels [29].

Transcription Is Needed for the Generation of Small RNAs A trigger construct with no promoter was designed to determine if transcription is needed for the generation of small RNAs or if the presence of the trigger DNA alone might be enough to induce the generation of small RNAs. In the cell line stably harboring the promoter-less construct there was no downregulation of the endogenous gene fused to the trigger, nor the presence of AS small RNAs to this gene, indicating that transcription is indeed needed for trigger-gene-mediated generation of small RNAs and resulting gene silencing [29].

Furthermore, Northern blot analysis revealed a population of AS small RNAs to the intronic region of a gene fused to the trigger, indicating that these AS small RNAs are derived from nascent mRNA [29]. These data are in contradiction to data from *C. elegans* in which the secondary AS small RNAs were shown to be derived from mature mRNA. Furthermore, pyrosequencing data revealed small RNAs to intronic regions of a number of genes as well as exon–exon junctions, supporting the hypothesis that transcription is needed for the generation of small RNAs and the biosynthesis pathway may rely on relaxed specificity on either nascent or mature mRNA [26].

RNAi-Related Genes Could Not Be Targeted for Silencing Extensive attempts to target the RNAi-related genes (EhAgo2-1/ 2-2/ 2-3, EhRNAseIII, EhRdRP1) for gene silencing using the trigger-mediated approach failed to silence the target gene (Pompey and Singh, unpublished data). Interestingly, however, despite lack of silencing, the trigger-gene fusion did induce generation of small RNAs to these genes. Furthermore, the small RNAs generated to these genes are functional, despite the inability to silence the chromosomal loci. These results may indicate that these genes are indeed involved in the RNAi pathway and can therefore not be silenced by an RNAi-related mechanism, or that these genes are essential and thus cannot be silenced, or that these specific genomic loci are resistant to small RNA-mediated silencing. Further studies are currently ongoing to explain these findings and to investigate if changes in genomic loci might be involved in this resistance to RNAi-based silencing phenomenon.

9.9 Final Remarks

E. histolytica harbors a robust RNAi pathway consisting of multiple populations of small RNAs as well as homologues of Argonaut, RdRP, and Exportin as well as RNAseIII enzyme. Ago2-2 immunoprecipitated RNA pyrosequencing revealed that small RNAs associate with Argonaut, peak at 27-nt sequence, and locate to specific genomic loci as well as open reading frames. Further, functional analyses showed that small RNAs are indeed key in gene silencing in *E. histolytica* and responsible for an elaborate small RNA amplification mechanism of gene silencing in this protozoan. Despite this great progress in studying the role of small RNAs in gene expression regulation in *E. histolytica*, many important questions still remain to be addressed (Box 9.1). Thus, we are looking forward to future research in the field of RNAi in *E. histolytica*.

Box 9.1: Important Unanswered Question on the *Entamoeba histolytica* RNAi Pathway

- Does the *E. histolytica* pathway act without a Dicer enzyme?
- Are the small RNAs generated by RdRP?
- Which factors control the amplified gene silencing?
- Can the small RNAs induce changes on the DNA level (e.g., DNA methylation or chromatin modification)?
- Is the RNAi pathway also capable of defending the cell from foreign RNA/DNA (e.g., viruses)?
- What controls the process of amplified silencing? Why is the process not rampant? What protects the genome?
- What are the roles of the other two Argonaute proteins?
- What aspects of amebic biology does this pathway regulate? Virulence and tissue invasion? Development?
- What is the evolutionary advantage of maintaining the amplified silencing pathway?
- Is the endogenous RNAi pathway a way for the parasite to interact with or affect host cells?

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Chapter 10
The Biology of Retrotransposition in *Entamoeba histolytica*

Vijay Pal Yadav and Sudha Bhattacharya

Abstract Retrotransposons move into nonhomologous insertion sites within the genome via an RNA intermediate that is reverse transcribed into DNA. Those that lack long terminal repeats include long and short interspersed nuclear elements (LINEs and SINEs). LINEs are autonomous; partner SINEs retrotranspose using the LINE machinery. *Entamoeba histolytica* contains three classes of LINEs (EhLINE1, 2, 3) and SINEs (EhSINE1, 2, 3), which constitute approximately 11 % of the genome. EhLINE1 (4.8 kb) and EhSINE1 (550 bp) are the most abundant. They insert at AT-rich sites on all chromosomes, are not telomeric, and are close to protein-coding genes.

EhLINEs typically encode two open reading frames (ORFs). The N-terminal one-third of EhLINE1 contains ORF1, which has nucleic acid-binding properties. The ORF2 contains the reverse transcriptase (RT) domain and the endonuclease (EN) domain, which resembles type IIS restriction endonucleases. The purified EN domain protein could nick pBluescript DNA, and lacked strict sequence specificity. It displayed low K_m, suggesting high affinity for DNA, and a low turnover number that could limit retrotransposition. Although EhLINE1 ORF1p is expressed in cultured *E. histolytica* cells, ORF2p is not detected. A cell line was obtained that expressed ORF2p by tetracycline induction and also contained an EhSINE1 copy marked with a GC-rich tag. In the presence of tetracycline, mobilization of the marked EhSINE1 was observed. Interestingly, mobilized EhSINE1 copies engaged in active sequence exchange during retrotransposition, probably brought about by multiple template jumping of RT, leading to rapid spread of sequence tag to the EhSINE1 population and generation of diversity.

10.1 Transposable Elements: An Overview

Transposable elements (TEs), or mobile genetic elements, are discrete DNA sequences with the unusual ability to jump into nonhomologous insertion sites within the genome [1]. TEs have emerged as diverse, abundant, and ubiquitous

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© Springer Japan 2015

T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_10

components of eukaryotic genomes, constituting up to 80 % of nuclear DNA in plants, up to 20 % in fungi, and up to 50 % in metazoans [2, 3]. By virtue of their ability to move to new chromosomal locations, they are important in shaping the evolutionary course of the genome and contribute to phenotypic diversity of a population. It is well established that these elements play an important role in nuclear architecture, genome stability, gene amplification, and altered gene regulation [4–7]. TEs conventionally belong to two broad classes, I and II. Class I transposons, also called retrotransposons, transpose via an RNA intermediate that is reverse transcribed into DNA. On the basis of presence or absence of long terminal repeats (LTRs), all retrotransposons can be divided into two major groups, LTR and non-LTR. The LTR elements share similarity with retroviruses. The second group, that is, non-LTR retrotransposons, include the well-studied long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). Class II TEs comprise DNA-based transposons that mobilize by moving or copying their DNA sequence. All TEs that encode the functions required for their own transposition are called autonomous elements. Their transposition functions can sometimes be utilized by other TEs (nonautonomous elements) that lack the necessary coding capacity but can transpose equally efficiently. Thus, among the non-LTR retrotransposons, LINEs are autonomous elements whereas partner SINEs retrotranspose using the LINE machinery.

10.1.1 TEs in Protozoan Parasites

TEs have been reported in many protozoan parasites including trypanosomes, *Giardia*, *Crithidia*, *Trichomonas*, and *Entamoeba*. The genomes of these parasites, as in all eukaryotic genomes, have been colonized by diverse repetitive elements (reviewed in [8–10]). Of all classes of TEs, the non-LTR retrotransposons appear to be the most abundant in these organisms (except *Trichomonas vaginalis*, in which DNA transposons are highly abundant). Both autonomous and nonautonomous elements are present, and they occupy specific genomic locations in each parasite. Full-length TEs could not be found in species of *Leishmania* and *Plasmodium*. Small degenerate retroposons (0.55 kb) containing the "79-bp signature," named LmSIDERs (for *s*hort *i*nterspersed *de*generate *r*etroposons), have been identified in the genomes of *Leishmania major* [11], *L. infantum*, and *L. braziliensis* [12]. Although no transposons have been reported from the current *Plasmodium* species, evidence of significant retrotransposon activity in the lineage leading to *Theileria* and *Plasmodium* has been proposed [13].

The *T. vaginalis* genome contains a large number of DNA transposons (amounting to ~7 Mb in the 160-Mb genome) and a much smaller number of retrotransposons (~23 kb) [10]. Interestingly the first report of mariner transposon outside of animals has come from this organism. Besides mariner, *T. vaginalis* also contains transposons belonging to the maverick [14] and mutator [15] families.

The genomes of *Trypanosoma brucei* and *T. cruzi* contain several types of retrotransposons but no DNA transposons [16]. The non-LTR retrotransposons in these organisms deserve special mention as they are, so far, the best characterized elements in protozoan parasites. In *T. brucei* the autonomous LINE, which may be an active element, is called *ingi*; its nonautonomous SINE is RIME. In *T. cruzi* the active LINE element is L1Tc and its nonautonomous SINE is NARTc. L1Tc is widely distributed in the *T. cruzi* genome. This 5-kb element has been biochemically characterized and shown to encode apurinic/apyrimidinic endonuclease [17], reverse transcriptase [18], RNase H [19], and nucleic acid chaperone activities [20], which are all required for retrotransposition. The first 77 nt of the element are highly conserved and shared with NARTc, as also with Tb*ingi* and RIME elements. This sequence not only contains an internal promoter for the transcription of these elements but also has a hepatitis delta virus-like ribozyme [21]. The ribozyme activity presumably enables the correct 5′-end of the mature transcript to be generated in case the element is transcribed as a polycistronic unit (because genes in trypanosomatids are generally organized in large polycistronic clusters from which monocistronic mRNAs are later released by *trans*-splicing). Thus, these elements have acquired the necessary activities to maintain their functional properties while coevolving with the host genome.

10.1.2 TEs in Entamoeba

The recent availability of genome sequence data has made it possible to identify TEs in Entamoebae. The *Entamoeba* species that have been sequenced include *E. histolytica*, *E. dispar*, *E. invadens*, and *E. moshkovskii. E. histolytica* is the etiological agent responsible for amoebic dysentery and liver abscess; *E. dispar* is morphologically identical to *E. histolytica* but is nonpathogenic. *E. moshkovskii* is free living. *E. invadens* is a reptilian parasite and used as a model system for encystation studies because, in contrast to *E. histolytica*, it can form cysts in axenic cultures.

Genome sequence analysis showed that *E. dispar* and *E. histolytica* are practically devoid of known eukaryotic DNA transposons but have an abundance of retrotransposons [22–27]. Conversely, the genomes of *E. invadens* and *E. moshkovskii* contain few retrotransposons (0.1 % of the genome), but have an abundance of DNA transposons belonging to four superfamilies (hAT, Mutator, Tc1/mariner, and piggyback); these are estimated to constitute about 9 % of the genomes [28, 29].

Among the class I TEs, LTR retrotransposons can be identified by the presence of sequences homologous to the Integrase encoded by the element. No sequences homologous to this domain were identified in any of the *Entamoeba* species examined. In addition, all *Entamoeba* reverse transcriptase (RT) sequences show strongest similarity to those encoded by non-LTR retrotransposons and only weak similarity to those of LTR retrotransposons. These results suggest the absence of LTR retrotransposons in these genomes [28].

Even before the *Entamoeba* genome sequence data were available, it was already clear from limited sequence data of a repetitive DNA (which had very close match with RT) that the *E. histolytica* genome harbored non-LTR retrotransposons [22]. Whole genome sequence analysis has now revealed that these elements occupy 11 % and 7 %, respectively of the *E. histolytica* and *E. dispar* genomes, whereas the DNA transposons (all belonging to the Mutator subfamily) account for less than 0.1 % of the genome [29].

The only elements for which biochemical data regarding their transposition functions are available are the non-LTR retrotransposons of *E. histolytica* and *E. dispar*. The rest of this chapter therefore focuses on these elements and reviews our current understanding of their mode of retrotransposition.

10.2 The LINEs and SINEs of *Entamoeba histolytica*

Studies with repetitive DNAs of *E. histolytica* revealed a 4.8-kb element, part of which had a very close match with the RT of non-LTR retrotransposons [22]. Another repetitive and highly transcribed 0.55-kb element was discovered that lacked an open reading frame (ORF) [27, 30]. This element shared a 70-nt sequence at the 3′-end with the 4.8-kb element, and the two were proposed to be a LINE/SINE pair [8, 31]. Subsequent genome sequence analysis revealed that *E. histolytica* contains three classes of LINEs (EhLINE1, 2, 3) and three classes of SINEs (EhSINE1, 2, 3) [23, 24] (Fig. 10.1). Similar to non-LTR retrotransposons of other organisms, EhLINEs also code for two ORFs. Of these, the ORF2 contains the very well conserved RT and endonuclease (EN) domains. Analysis of the *E. dispar* genome showed that it too contains three families of LINEs and SINEs [29].

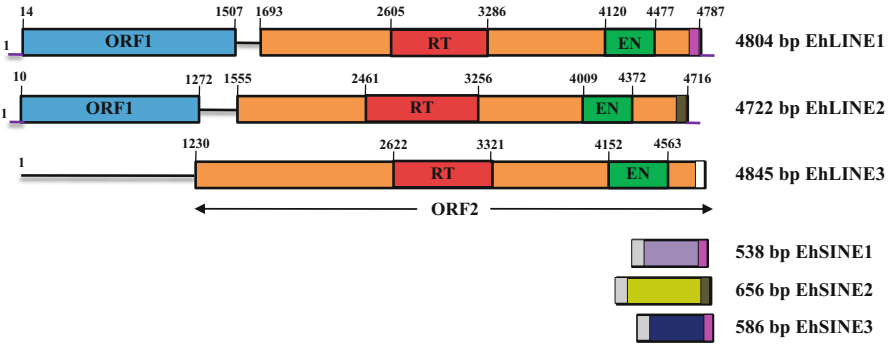

Fig. 10.1 Sequence organization of full-length EhLINEs and EhSINEs. Consensus sequence of each EhLINE family derived by comparative analysis of all database entries was used to mark the ORFs and other features, including the RT and EN domains in ORF2. The sizes of each consensus element are indicated. *Numbers* on top of each LINE family denote nucleotide positions. Regions identical between EhLINEs and EhSINEs at their 3′-ends, and between EhSINEs at their 5′-ends, are shown by similar shading

10.2.1 Sequence Comparison of the Three LINE/SINE Families

The size and copy number of each LINE/SINE as deduced from the genome sequence are shown in Table 10.1. Most copies of each element are truncated at the 5′- or 3′-end or at both ends. Of the full-length copies, none was found to contain a complete ORF because of the many point mutations [23]. EhLINE1 constitutes the largest family of TEs in *E. histolytica* with a total of 742 elements, including 88 complete copies and 46 putative complete elements, truncated by their location at the end of assemblies. Similarly, EdLINE1 is the most frequent LINE in *E. dispar*, having 573 copies with 63 complete and 64 putative complete elements [29].

Phylogenetic analysis based on manual reconstruction of the RT consensus sequence indicated that all LINEs found in *Entamoeba* species were derived from a single ancestral sequence. At a later point in evolution, in the ancestral line that led to *E. histolytica* and *E. dispar*, LINE TEs split into two separate lineages, giving rise to the Eh/EdLINE1 and Eh/EdLINE2 subfamilies. Subsequently, in this same ancestor, a third LINE subfamily diverged from LINE1, giving rise to EhLINE3 in *E. histolytica* and EdLINE3 in *E. dispar* [29].

Although EhLINE2 and 3 are present in fewer copies than EhLINE1, their overall sequence organization is very similar, and the RT and EN functional domains are well conserved. However, the consensus sequence reconstructed for EhLINE3 does not have ORF1, possibly because too many mutations have accumulated in this part of the element [23].

Table 10.1 The LINE/SINE families of *Entamoeba histolytica* and *Entamoeba dispar*

LINE/SINE	Size (bp)	Copy no.[a]		Percentage of genome[a] (%)
		Complete	Incomplete	
EhLINE1	4,804[b]	88	654	5.2
EhLINE2	4,722[b]	73	442	3.7
EhLINE3	4,845[b]	10	87	0.8
EhSINE1	538[b]	264	181	0.9
EhSINE2	656[b]	94	162	0.5
EhSINE3	586[b]	9	40	0.1
EdLINE1	4,825[c]	63	510	3.7
EdLINE2	4,735[a]	28	449	2.2
EdLINE3	4,406[a]	2	42	0.2
EdSINE1	601[d]	282	143	0.9
EdSINE2	604[a]	53	136	0.3
EdSINE3	579[a]	2	16	<0.1

[a]Lorenzi et al. [29]
[b]Bakre et al. [23]
[c]Bhattacharya et al., unpublished data
[d]Shire and Ackers [25]

By virtue of the sequence identity at the 3′-end between EhLINEs and EhSINEs (87 % identity in a 73-nt stretch between EhLINE1 and EhSINE1, and 76 % identity in a 84-nt stretch between EhLINE2 and EhSINE2), EhSINE1 and 2 may be considered as partner SINEs of EhLINE1 and 2, respectively.

Phylogenetic analysis indicates that the nonautonomous SINE elements also already existed in the common ancestor before the speciation process that gave rise to *E. histolytica* and *E. dispar* [29]. The most abundant EhSINE, EhSINE1, is closely related to EdSINE3. Because only two full-length copies of EdSINE3 are found in *E. dispar*, this suggests that this element is probably no longer functional in *E. dispar*. The most abundant EdSINE, EdSINE1, is closely related to EhSINE3, which is present in very few copies in *E. histolytica*. The 5′-end of EdSINE1/EhSINE3 is more similar to EhSINE2 whereas its 3′-end resembles EhSINE1. These findings suggest that in the common ancestor of *E. histolytica* and *E. dispar*, EdSINE1/EhSINE3 originated as a chimeric element, its 5′-end derived from the precursor sequence of EhSINE2/EdSINE2 and its 3′-end originated from an ancestral EhSINE1-like element [29].

10.2.2 Genomic Location of LINEs/SINEs in E. histolytica *and* E. dispar

Previous Southern hybridization studies of pulsed-field gel electrophoresis (PFGE)-separated chromosomes of *E. histolytica* with EhLINE1 and EhSINE1 probes showed that these elements reside on all chromosomal bands, do not seem to be telomeric, and might be dispersed in the *E. histolytica* genome [32]. Sequence analysis of the immediate upstream and downstream flanking sequences for each element supported this view, as no conserved sequences could be found at the sites of insertion of any of the elements. However, all the elements seemed to insert in AT-rich sequences, with a clear preponderance of T-residues in a 50-nt stretch upstream of the site of insertion of each element [23]. A computational analysis of preinsertion loci was done to detect unique features based on DNA structure, thermodynamic considerations, and protein interaction measures. Target sites could readily be distinguished from other genomic sequences based on these criteria [33]. Regions of favorable DNA structure appeared to be preferentially recognized for retrotransposition. Importantly, no instance was encountered of an element inserted within a gene. Analysis of a 2-kb region surrounding each element showed that in 80 % cases a gene was found near an element, within the first 0.5 kb, whereas another element was found within the first 0.1 kb. Such close proximity of elements to one another could be the result of clustering of sequences that serve as favourable target sites for insertion. Elements were found in both orientations with respect to each other. No clear bias of any pairs of elements occurring near each other could be discerned [23].

An analysis of TE dispersion along the scaffolds revealed that 90 % of the genomic sequence in *E. histolytica* has a repeat density below the mean value for the

genome [29]. A similar pattern was observed for *E. dispar* and *E. invadens* as well, which demonstrates that, although dispersed in the genome, TEs are not uniformly distributed along these genomes but are enriched in relatively small genomic regions, which also harbor many protein-coding genes.

Among the genes present near the TEs in *E. histolytica*, most (about 63 %) were found to be hypothetical. Of the genes that gave a match in the database, the most common were protein kinases, GTPases, heat-shock proteins, AIG family (which is associated with resistance to bacteria [34]), and BspA [23]. Housekeeping genes were rarely found. A cluster of 31 members of the Hsp70 protein family was associated 30 % of the time with TEs within 1 kb of the gene [35]. It has been previously reported that multiple natural TE insertions in *Drosophila* reduce the level of expression of hsp70 genes by inserting near gene promoter regions [36]. In this sense, TEs could be playing an adaptive role in microevolution by manipulating the expression of genes.

Earlier investigations with a limited number of genomic loci showed that the sites occupied by EhSINE1 in the *E. histolytica* genome were empty at homologous regions in *E. dispar* [31], and conversely the sites occupied by EdSINE1 in the *E. dispar* genome were empty in *E. histolytica* [25]. Subsequently, a genome-wide search was done by extracting all genomic occurrences of full-length copies of EhSINE1 in the *E. histolytica* genome and matching them with the homologous regions in *E. dispar*, and vice versa, wherever it was possible to establish synteny [37]. The data confirmed the earlier observations because only about 20 % of syntenic sites were occupied by SINE1 in both species. This difference occurred in spite of the fact that the physical properties of DNA sequences adjoining the insertion sites were similar in these sibling species, and the basic retrotransposition machinery appears to be conserved in them as well. The difference in occupancy may have arisen from secondary loss of SINEs from selected loci. Alternatively, SINE expansion might have occurred after the divergence of the two species. The absence of SINE1 in 80 % of syntenic loci could affect the phenotype of the two species, including their pathogenic properties, which needs to be explored.

10.3 Sequence Organization of the Major *E. histolytica* Retrotransposon: EhLINE1

The consensus sequence of EhLINE1, with complete ORFs, was reconstructed manually by selecting the most common nucleotide at each position. Analysis of the consensus sequence showed that EhLINE1 had a length of 4,804 bp (Fig. 10.1). The RT domain showed the closest match with RTs encoded by the R4 clade of non-LTR retrotransposons, most notably the R4 element of *Ascaris lumbricoides* and the Dong element of *Bombyx mori*. The EN domain had sequence features resembling Type IIS restriction endonucleases, and was very similar to the domains in R2, R4, and CRE clades of non-LTR elements [8, 24]. The N-terminal one third of the element encoded ORF1, which has matches with proteins containing coiled-coil domains.

Two types of EhLINE1 were found: one with a single ORF and one with two ORFs [23]. A 5-nt sequence (AAGCA) was duplicated at position 1442 in the element containing two ORFs, resulting in a stop codon at position 1507. The putative start of the second ORF was assigned at the AUG at position 1693. Interestingly, EhLINE2 also had copies with a single ORF or with two ORFs. In both types of element the stop codon disrupted the single ORF at a distance of about one-third of the length from the N-terminus. Closely related non-LTR retrotransposons with either one or two ORFs have been reported. SLACS in *Trypanosoma brucei* and CZAR in *Trypanosoma cruzi* have two ORFs whereas CRE1 and CRE2 of *Crithidia fasciculata* have a single ORF [8]. However, the occurrence of one-ORF and two-ORF elements of the same type in a single organism as seen in *E. histolytica* has not been reported so far.

10.3.1 Properties of ORF1p and RT Domain of ORF2p

Among the non-LTR retrotransposons, ORF1 (or the amino-terminal part of elements with a single ORF) shows considerable sequence diversity in different organisms, but there appears to be functional conservation. ORF1p has nucleic acid-binding property [38], which in some elements (e.g., I and Jockey) is associated with three cysteine-histidine motifs (CCHC type) similar to that found in the gag protein of many LTR retrotransposons. Other elements (e.g., R2) contain conserved C_2H_2 zinc-finger and/or c-myb DNA-binding motifs [39]. The ORF1p of human L1 element lacks such CCHC motifs. Its C-terminus is highly basic and contains conserved amino acid residues thought to function in RNA binding. It contains a centrally located leucine zipper domain, which is required for multimer formation [40]. Some noncanonical RRM (RNA recognition motif) domains have been identified in ORF1p [41]. Missense mutations in either the leucine zipper or conserved C-terminal amino acids of ORF1p abolish L1 retrotransposition in cultured mammalian cells [42], showing the functional importance of this polypeptide. The colocalization of L1 ORF1p with L1 RNA in ribonucleoprotein particles (RNPs) indicates that the possible role of this protein is to associate with the RNA template after translation and import the template back into the nucleus for reverse transcription.

The EhLINE1 ORF1 encodes a 498-amino-acid-long polypeptide of 60.5 kDa. Its sequence shows the presence of a "coiled-coil domain" at C-terminus, and the most basic region is located at the N-terminus (pI = 10.67). Our preliminary analysis of recombinant EhLINE1 ORF1p shows that it binds ssRNA, ssDNA, and dsDNA in a cooperative, nonsequence-specific manner as previously reported for mouse L1 [43]. The nucleic acid-binding activity is located at the N-terminus; the C-terminus appears to promote formation of multimeric complexes.

ORF2 (or the carboxy-terminal part of elements with a single ORF) encodes a multifunctional protein consisting of reverse transcriptase and endonuclease activities. Both in vitro and in vivo RT activity has been detected for many non-LTR elements.

Phylogenetic analysis demonstrated that RTs encoded by non-LTR retrotransposons represent a lineage that is distinct from the RTs encoded by LTR retrotransposons and retroviruses [44]. The non-LTR retrotransposon-encoded RT has both RNA- and DNA-dependent polymerase activities [18, 45]. The existence of DNA polymerase activity associated with this RT would allow synthesis of both the complementary DNA and second-strand DNA necessary to complete the elemental integration starting from RNA. A highly conserved YXDD motif is found in the RT domain, and mutations in Asp residues eliminate RT activity in both LTR and non-LTR retrotransposons [39, 42, 46].

Studies with the RT encoded by *Bombyx mori* R2 element show that it is capable of adding nontemplate nucleotides (usually T residues). It has high processivity, which could enable it to synthesize both DNA strands during a retrotransposition reaction [47], and has low fidelity [48]. Although R2 RT lacks RNaseH activity, it can efficiently displace the RNA or DNA template strand. Another unusual property of RT (also seen with RT of *Trypanosoma cruzi* L1) is synthesis of a continuous cDNA strand on discontinuous template RNA because of jumping of the RT from the 5′-end of one RNA template to the 3′-end of another RNA template [18, 49]. The RT encoded by human L1 also shares these properties. Studies with this RT have become easier after solving the problem of expression and purification of the active enzyme [50]. RNP particles containing L1 RT have been isolated from cultured mammalian cells [51].

Our preliminary work showed that the ORF2 protein encoded by EhLINE1 (recombinant full-length ORF2p) has RT activity with various substrates, including poly (rA)-olgo(dT), and a 120-nt RNA template from the 3′-end of EhSINE1. EhLINE1-ORF2p seems to have considerably high processivity in vitro as full-length cDNA was the major product with very few lower-sized products. A mutation in the two Asp residues in the conserved YXDD motif (YMDD to YMYY) abolished RT activity.

10.3.2 Properties of the EhLINE1 Endonuclease

The general model that has served to explain LINE retrotransposition is target-primed reverse transcription, proposed by Eickbush and coworkers from their work on the silk moth element R2Bm [52]. According to this model, retrotransposition is initiated when the LINE-encoded polypeptides (ORF1p and ORF2p) associate with the element RNA to form a RNP particle. The endonuclease activity residing in the RNP nicks the bottom strand of the target site, generating a 3′-OH group that primes reverse transcription of the element RNA. The non-LTR retrotransposons described so far can be classified into two broad categories based on the nature of the endonucleases encoded by the elements. One class encodes the apurinic endonuclease (APE) and the other encodes a restriction enzyme-like endonuclease (EN). All the elements of the latter class (including EhLINEs) belong to the R2 group, which is considered to be of ancient origin [44].

It is generally believed that target site selection is determined by the element-encoded endonuclease [53] and that consequently, elements with the restriction enzyme-like endonuclease would be site specific. All well-characterized members of the R2 group do show some level of site specificity and insert into defined sites (28S rRNA gene, spliced leader gene, TAA repeats, telomere). However, EhLINEs are widely dispersed in the genome of their host, with no apparent site specificity.

To better understand the mode of transmission of EhLINE1 in the *E. histolytica* genome, the EN domain was cloned and expressed in *Escherichia coli* and its properties were studied with respect to target site specificity of nicking in vitro [54]. The purified protein could nick a completely unrelated substrate, supercoiled pBluescript DNA, to yield open circular and linear DNAs, showing that the enzyme was, at best, loosely sequence specific. The conserved $PDX_{12-14}D$ motif was required for activity. To determine whether the enzyme exhibited any nicking hotspots in the *E. histolytica* genome, an empty target site was searched and one such site, known to be occupied by EhSINE1, was identified. When a 176-bp fragment containing this empty site was used as a substrate for EN, it was prominently nicked on the bottom strand at the precise point of insertion of EhSINE1, showing that the enzyme indeed preferentially nicked selected genomic sequences. These data also confirmed that EhSINE1 could use the EhLINE1-encoded endonuclease for its insertion. The sequence preference of the EN was determined in vitro with a variety of mutated substrates. It was possible to assign a consensus sequence, 5′-GCATT-3′, which was efficiently nicked between A-T and T-T [33]. The upstream G residue enhanced EN activity, possibly serving to limit retrotransposition in the A*/*T-rich *E. histolytica* genome.

Studies on the kinetics of EhLINE1 EN-catalyzed reaction, determined under steady-state and single-turnover conditions, revealed a significant burst phase followed by a slower steady-state phase, indicating that release of product could be the slower step in this reaction [55]. The enzyme behaved as a monomer. Although Mg^{2+} was required for activity, 60 % activity was seen with Mn^{2+} and none with other divalent metal ions. Substitution of $PDX_{12-14}D$ (a metal-binding motif) with $PAX_{12-14}D$ caused local conformational change in the protein tertiary structure, which could contribute to reduced enzyme activity observed in the mutated protein. The protein underwent conformational change upon the addition of DNA, which is consistent with the known behavior of restriction endonucleases. In fact, the endonuclease encoded by EhLINE1 is similar to restriction endonucleases in many respects. As mentioned, it contains the conserved $PDX_{12-14}D$ motif similar to that found in the active site of type IIS restriction endonucleases [56]. Similar to restriction endonucleases, it displays a low K_m, suggesting high affinity for DNA. It has a low turnover number that could be an evolutionary advantage to limit retrotransposition. The binding of the enzyme to DNA is accompanied by major conformational change. These similarities with bacterial restriction endonucleases suggest that the endonuclease encoded by EhLINE1 and other related non-LTR retrotransposons was possibly acquired from bacteria, through horizontal gene transfer. The loss of strict sequence specificity for nicking may have been subsequently selected to facilitate the spread of the retrotransposon to many intergenic regions of the *E. histolytica* genome. Analysis of more such endonucleases

would reveal how these enzymes may have been engineered to suit the needs of retrotransposition. An understanding of the role of this endonuclease and other factors involved in target site selection would help in designing vectors for targeted gene insertion and genetic manipulation in *E. histolytica*.

10.4 Induction of De Novo Retrotransposition in Cultured Cells

Although LINEs are typically present in high copy number in a large variety of genomes, it is widely seen that most LINE copies are, in fact, inactive because of the accumulation of multiple mutations. In the human genome most L1 copies (>99.8 %) are inactive. About 80 to 100 L1s remain retrotransposition competent (i.e., active) [57], and a number of human diseases are caused by recent insertions of L1s [58, 59]. To study de novo retrotransposition, episomal systems were developed [42] in cultured mammalian cells in which a full-length L1 element was expressed. Retrotransposition was scored using a mneoI reporter cassette that consists of an antisense copy of neo gene disrupted by an intron and is transcribed in the same direction as L1. The arrangement ensures that G418-resistant cells will arise only when a L1 transcript is spliced, reverse transcribed, and reintegrated into chromosomal DNA. Retrotransposition events were recovered, and the flanking sequences demonstrated typical L1 structural hallmarks. This system could also retrotranspose SINEs, such as Alu and SVA [60, 61].

10.4.1 Use of the Cell Culture System to Understand Biology of Retrotransposition

The cell culture assay has established conclusively the requirement of L1-encoded proteins ORF1p, and ORF2p for retrotransposition [51, 62–64]. Using this system to study the translation of mammalian L1 it was found that ORF2p is translated by an unconventional mechanism whereby translation of an upstream ORF (ORF1) is needed for translation of ORF2 [65]. This system also revealed the role of cytosine methylation in suppressing retrotransposition [66] and the regulation of L1 by human APOBEC3 cytidine deaminases [67, 68]. SiRNA and piRNA based silencing of transposons is well established in mammals, zebrafish, *Drosophila*, and *Tetrahymena* [69]. It was found that co-transfection of the L1 construct with L1-specific siRNA reduced retrotransposition [70]. Thus, the cell culture assay system has been extensively used to understand the regulation of retrotransposition, especially in the mammalian system.

Such an assay would be similarly beneficial to study the retrotransposition of *Entamoeba* LINEs/SINEs and to address the role of DNA methylation and RNA silencing in the modulation of retrotransposition. It has been shown in *E. histolytica*

that the methylated base 5-methylcytosine is localized predominantly in repetitive DNA elements, including LINEs [71], and the protein EhMLBP, which binds to methylated LINEs, has been identified [72]. In addition, sequence analysis of 27-nt small RNAs of *E. histolytica* showed that a small population of these RNAs (0.5 %) mapped to retrotransposons [73]. To analyze the functional role of DNA methylation and RNA interference in the control of retrotransposition in *E. histolytica*, a system for de novo induction of retrotransposition is required. The construction of such a system for *E. histolytica* is described next.

10.4.2 Construction of a Retrotransposition-Competent Cell Line of E. histolytica

Although EhLINE1 ORF1p is abundantly expressed in cultured *E. histolytica* cells, full-length transcripts of EhLINE1 and ORF2p polypeptide are not detected; hence, these cells are not expected to be retrotransposition competent. A cell line was obtained by transfecting *E. histolytica* cells with a plasmid construct containing the complete EhLINE1 ORF2 (reconstructed to remove the stop codons in resident EhLINE1 copies) [74]. To avoid possible adverse effects of active retrotransposition, the ORF2 was cloned in a tetracycline (tet)-inducible expression vector. It expressed the 2.9-kb ORF2 transcript and ORF2p (111 kDa) in a tet-inducible manner (Fig. 10.2). In this cell line a second plasmid was introduced that contained a constitutively expressed, marked EhSINE1 copy (with a 25-bp GC-rich tag) and a 176-bp fragment containing the target site sequence where EhSINE1 is known to insert in the *E. histolytica* genome [54]. Retrotransposition events occurring at this target site in the plasmid can be scored without using selection pressure by a PCR-based approach (Fig. 10.2). In the presence of tet, this cell line is expected to be retrotransposition competent. Tet was added to cultures in early log phase and cells were harvested after 48 h (late log). Retrotransposition of the marked SINE copy to the insertion target site was scored by PCR amplification of total genomic DNA, followed by Southern hybridization with the marked SINE probe (Fig. 10.2). Specific amplicons expected from the mobilization of the marked SINE to the insertion site were obtained in the presence of tet, although no amplicons were obtained when tet was added to a cell line containing the marked SINE and insertion hotspot but lacking ORF2. A hallmark of retrotransposition is the generation of target site duplications (TSD). The flanking sequences of 13 randomly selected clones of the amplicons were determined, and a 22-bp TSD was found in all cases (Fig. 10.2), which matched exactly in size and sequence with the TSD found at this insertion hotspot at its genomic location [54]. No insertion event was found at any region in the 176-bp fragment other than the hotspot. Therefore, based on three criteria, namely, strict requirement of ORF2p expression for mobilization, specific insertion into the retrotransposition hotspot, and the 22-bp TSDs accompanying the insertion, it was concluded that the scored events were the result of retrotransposition and not processes such as DNA recombination [74]. This was a first demonstration of its type in an early branching eukaryote.

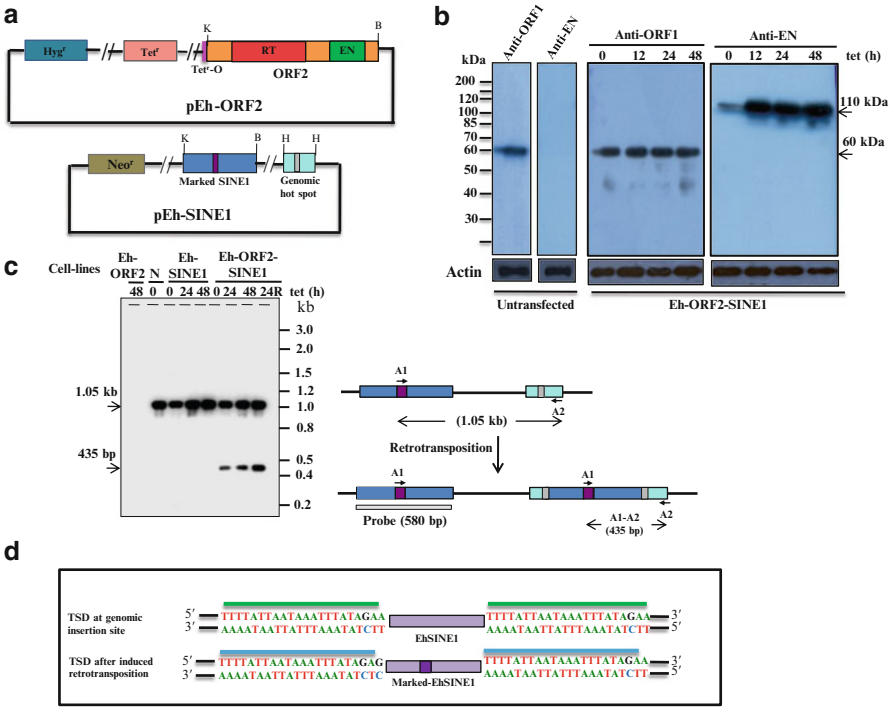

Fig. 10.2 **a** The plasmid in the doubly transfected retrotransposition-competent cell line [74]. pEh-ORF2 provides the RT and EN activities required for retrotransposition. pEh-SINE1 contains the marked EhSINE1 with a 25-bp GC-rich tag (*purple*), and a 176-bp DNA fragment containing the genomic hot spot of EhSINE1 insertion (*grey box*). Restriction enzymes: K, *Kpn*I; B, *Bam*HI; H, *Hin*dIII. **b** ORF1p (60 kDa) is expressed continuously in untransfected cells; while ORF2p (110 kDa) is expressed only upon tet induction of cells transformed with pEh-ORF2. Western blots were performed with indicated antibodies. **c** Mobilization of the marked-EhSINE1 copy to the genomic hot spot was detected by 435-bp amplicon obtained with primer pair A1/B1 in the doubly-transfected cells. The amplicon was visualized by Southern hybridization with marked EhSINE1 probe. **d** Retrotransposition is accompanied by 22-bp target site duplication (TSD)

10.4.3 Evidence of Recombination Between SINEs During Retrotransposition in E. histolytica

To obtain some insight into the process of retrotransposition, the question was asked whether the marked SINE copy suffered any changes consequent to retrotransposition [74]; this was accomplished by checking the sequences of the newly retrotransposed copies. Retrotransposition events at the target site were retrieved by PCR amplification with primers flanking the target site. All amplicons obtained had the size expected from events where a full-length SINE copy had retrotransposed at the insertion hotspot, which shows that in this system the

predominant retrotransposition events are contributed by full-length SINEs. This pattern is expected, as truncated SINE transcripts are not seen in *E. histolytica* [30, 31], and also shows that 5′-truncations are not common during EhSINE retrotransposition, although truncated copies of SINEs are abundant in the *E. histolytica* genome [29].

For sequence analysis the amplicons were cloned and 23 randomly selected clones were sequenced. The data showed that the sequences belonged to three different categories (Fig. 10.3). Set I consisted of ten sequences matching completely with the marked SINE. Seven of these were 100 % identical to the marked SINE, and three had one mismatch each. Set II contained eight sequences lacking the tag and showing 98–99 % identity with various genomic SINE copies but not with the marked SINE copy. It is estimated that 142 SINE copies are transcribed in *E. histolytica* [26], some of which were mobilized upon tet induction. Set III consisted of five sequences containing the 25-bp tag at the expected location but, surprisingly, showed only 94–95 % overall sequence identity (22–27 mismatches) with the marked SINE. They also showed at best 94–98 % matches with the genomic SINE copies. However, when the sequence on either side of the tag was searched separately (5′-half and 3′-half of each SINE separately), 98–100 % matches were obtained, and each side matched with different genomic SINE sequences [74]. In these five instances of set III, the tag had associated itself with genomic SINE sequences to result in recombinants derived from at least three different SINE sequences, one of them being the marked SINE and two belonging to different genomic SINEs (Fig. 10.3).

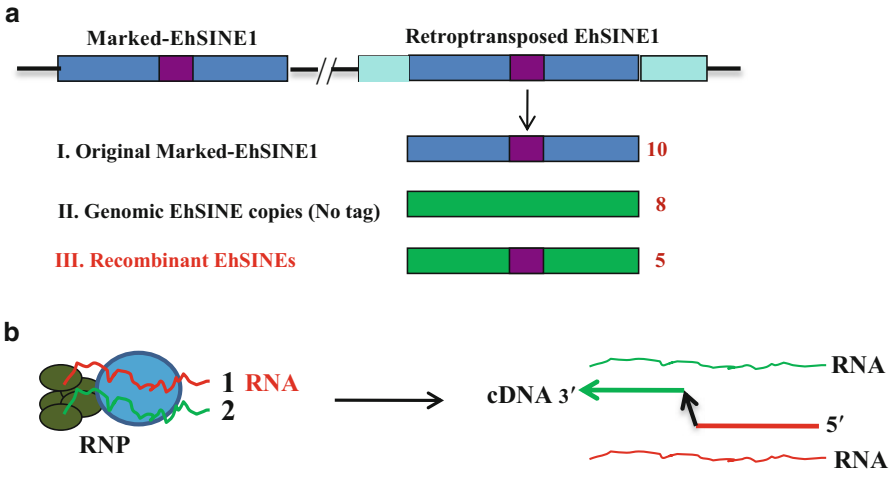

Fig. 10.3 **a** Sequencing of the newly retrotransposed EhSINE copies at the genomic hot spot revealed three categories. **b** Hypothetical model for generation of recombinant SINEs. During cDNA synthesis the RT may jump from one SINE template to the next if the two are in close proximity in the RNP particles

Control experiments confirmed that the acquisition of tag by the genomic SINE copies was not through a DNA recombination event before retrotransposition and that the recombinants did not exist before the induction of retrotransposition. This study therefore shows that recombinant SINEs are formed consequent to retrotransposition. The process is rapid, as these events were scored within 48 h of retrotransposition induction and occurred at high frequency (>20 % of total events scored). Some of the events in set II might also be recombinants, as the number of mismatches reduced when the 5′- and 3′-halves of each sequence were searched separately with the database.

Chimeric molecules arising from reverse transcripts have been observed in yeast Ty elements and were attributed to gene conversion [75], whereas in retroviruses high-frequency recombination occurs during reverse transcription of the two co-packaged RNAs in the virion as a result of template switching [76]. In non-LTR elements, tripartite chimeric LINEs have earlier been reported in a fungal genome [77]. In mammalian genomes U6/L1 pseudogene chimeras have been experimentally demonstrated [78]. However, recombination between multiple copies of the same SINE family during retrotransposition as observed in *E. histolytica* is a novel observation.

These results are the first direct demonstration that SINE copies engage in active sequence exchange during retrotransposition, leading to the rapid spread of the sequence tag to the SINE population, and generation of diversity. The demonstrated properties of RT to displace the RNA template during cDNA synthesis, and to perform multiple template jumping [49, 79], could lead to these recombinants. It is hypothesized that the RNP particle (formed when the EhLINE1-encoded polypeptides associate with EhSINE1 RNA) contains more than one RNA molecule per RNP. Reverse transcription of these closely associated RNAs could generate recombinant cDNAs by template jumping according to the model shown (Fig. 10.3). Human Alu subfamilies show the existence of mosaic elements [80], which could arise from such a mechanism. Messenger RNA transcripts in the cell are also templates of the same retrotransposition machinery during retropseudogene formation. If they too engage in similar recombination during reverse transcription, the sequence diversity thus generated may provide selective advantage to the host.

10.5 Summary and Conclusions

TEs are common inhabitants of all organisms, including the *Entamoeba* spp. The sequenced *Entamoeba* genomes show that LTR retrotransposons are absent in all of them. The genomes of *E. invadens* and *E. moshkovskii* contain few retrotransposons, but have an abundance of DNA transposons. Conversely, *E. dispar* and *E. histolytica* are practically devoid of DNA transposons but have an abundance of non-LTR retrotransposons. These elements occupy 11 % and 7 %, respectively, of the *E. histolytica* and *E. dispar* genomes; they belong to three related families of LINEs and SINEs and insert mainly in intergenic regions. The genomic hotspots

for retrotransposition appear to be determined by a combination of favourable DNA structure and preferred endonuclease nicking sequence in the vicinity of this structure. Although the basic retrotransposition machinery is conserved in *E. histolytica* and *E. dispar*, analysis of SINE loci showed that 80 % of occupied loci in one species were unoccupied in the other. As TEs are known to affect the expression of genes in their vicinity, this difference could affect the phenotype of the two species, including their pathogenic properties. LINE/SINE occupancy can also vary between the strains of the same species, and this has been utilized to develop sensitive methods for strain differentiation in *E. histolytica* [81, 82].

Sequence analysis of EhLINEs showed that they belong to the R4 clade of non-LTR retrotransposons. The most abundant of these, the 4.8-kb EhLINE1, encodes a putative nucleic acid-binding protein, ORF1, and reverse transcriptase and a restriction enzyme-like endonuclease, contained in ORF2. The EhLINE1-encoded endonuclease does not nick in a strictly sequence specific manner, but displays sequence preferences. It shares kinetic properties with restriction endonucleases, suggesting that it may have been acquired by horizontal gene transfer. The EhLINE1 machinery can be used by partner EhSINE1 for its own retrotransposition.

Although EhLINEs are present in hundreds of copies, most of these have accumulated multiple mutations and are no longer active. EhLINE1 ORF1p is abundantly present in cultured *E. histolytica* cells, but ORF2p is undetectable. A retrotransposition-competent cell line was obtained by expressing exogenously provided ORF2p in *E. histolytica*. This cell line could retrotranspose a marked EhSINE1 copy to a specific insertion site provided on a plasmid. Using this system it was demonstrated that SINE copies engage in active sequence exchange during retrotransposition, probably brought about by the property of reverse transcriptase to perform multiple template jumping. This phenomenon could explain the unusual sequence diversity observed in SINE elements. The retrotransposition-competent cell line will be very useful for future experiments to understand the mechanism of retrotransposition in *E. histolytica* and its regulation by a variety of mechanisms including DNA methylation and RNA interference.

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Chapter 11
Entamoeba histolytica: Bridging the Gap Between Environmental Stress and Epigenetic Regulation

Kirschenbaum Michael and Ankri Serge

Abstract Increasing evidence indicates that parasites display unique and diverse mechanisms of epigenetic regulation. In this chapter we present the current state of knowledge about the *Entamoeba histolytica* DNA/tRNA methyltransferase (Dnmt2) machinery and the related EhMLBP, a protein involved in the recognition of methylated DNA targets. The regulation of these epigenetic components by environmental challenges relevant to the biology of the parasite (including heat shock, glucose starvation, oxidative and nitrosative stresses) is also discussed.

11.1 *Entamoeba histolytica*: Life Cycle and Environmental Challenges

Entamoeba histolytica, the protozoan parasite responsible for amebiasis, is a dimorphic organism whose life cycle consists of two stages: the infective cyst and the invasive trophozoite. During its development the parasite moves through a series of different localized microenvironments and biological niches to which it must adapt. Initial infection begins with ingestion of nascent cysts as obtained from contaminated water supplies or food. Upon passage through the upper gastrointestinal tract, the parasites excyst in the terminal ileum, whereupon they migrate to and colonize the large intestine. Here, the parasite's life cycle takes a series of divergent paths depending on the ultimate pathophysiology of the disease. Ninety percent of *E. histolytica* infections are asymptomatic and the parasite remains a commensal organism feeding on the various flora and microbiota of the colon [1]. The trophozoites multiply and divide through binary fission, encyst, and pass through the stools, perpetuating the life cycle. However, in the other 10 % of cases in which symptomatic infection occurs, the trophozoites invade the mucosal lining of the colon, burrowing and coalescing into flask-shaped ulcers, with resultant colitis or dysentery of the host. Disease progression may end here, resolving with the

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_11

infection; or it may continue, with final emergence occurring in distal organs, generally the liver. Other more rare manifestations include pulmonary, cardiologic, and brain involvement [2].

Among the various environmental challenges encountered by *E. histolytica* are drastic changes in pH, pO_2, glucose concentration, biofilm substrate, the surrounding biome, nutrient availability, and the numerous assaults of the host immune system; including oxidative stress, heat shock, complement activation, and phagocytosis ([1, 3–5]). The initial environment, the colonic lumen, is host to a diverse array of resident microflora comprising more than 50 genera and 400 species [3]. Oxygen content is low, pH fluctuates markedly, glucose levels are low (but variant in accordance with the nutritional status of the host), and the metabolic environment consists mostly of acetogenic sugars and short-chain fatty acids [3]. The mucus overlaying the colonic epithelium is a complex gel of glycolipids, glycoproteins, and sugar residues including *N*-acetylglucosamine, *N*-acetylgalactosamine, D-GALACTOSE, fucose, and sialic acids [3]. As mentioned previously, *E. histolytica* begins as a commensal, feeding off this rich diversity of microorganisms in the large intestine. Indeed, it is a voracious predator, and the trophozoites are capable of consuming up to 1,000 bacteria per hour, individually [6]. Regarding the host immune system, it, too, is initially tolerogenic, utilizing both T regulatory cell activation and secretory immunoglobulin A to suppress inflammatory responses and prevent parasitic contact with the colonic mucus, respectively [1]. When the parasite does invade the colonic mucosa, however, it is henceforth subjugated to radically different environments (depending on the bodily compartment/reservoir), most notably characterized as being oxygenated, composed of an extracellular matrix (collagen, elastin, laminin, and fibrinogen) [7], and hostile, resulting from the activated inflammatory immune response.

As such, the amoeba must be capable of adapting to the demands of its surrounding environment. Numerous questions abound, and we find ourselves questioning the precise mechanisms controlling these transitions; which enable the parasite to so perfectly adapt to such a broad range of different situations. Of particular concern is determination of the triggers that change the ultimate pathophysiology of the organism, as it abandons its role as a commensal and becomes an agonist pathogen.

11.2 Epigenetics as a Tool for Adaptation

Epigenetic regulation of protein expression has long been recognized to be a key component in the cellular development, adaptability, and physiology of all living things, ranging from the simple prokaryotes and Archaebacteria to plants, animals, and human beings. Epigenetics specifically refers to chemical or structural modifications to DNA that preserve the genetic code but ultimately result in altered RNA transcription and protein expression. This trait may, in fact, be heritable, resulting in altered phenotype/differentiation of all descendant cells, despite the fact that they all share the same genotype and overall genetic code. Numerous epigenetic signals have already been elucidated, most prominently featuring DNA methylation and covalent modifications of histone proteins (e.g., acetylation, phosphorylation).

These modifications result in overall changes in chromatin structure and accessibility to transcription factors [8] and other nuclear proteins, such as methyl binding domain proteins [9].

In recent years, epigenetic regulation of gene expression has emerged as a crucial aspect of parasite biology. Indeed, this genomic plasticity has been demonstrated as a key factor in the virulence, differentiation, and lifecycles of protozoa as varied as *Toxoplasma gondii*, *Plasmodium falciparum*, and *Trypanosoma brucei* [10–13]. Alternative transcriptomes have also been obtained for the virulent HM1:IMSS *E. histolytica* strain versus the avirulent Rahman strain, with differential protein expression profiles for key virulence genes including the cysteine proteases, Gal/GalNAc lectins, and the protective peroxiredoxin [14]. Although many of the fundamental principles of epigenetic gene regulation are similar to those in mammalian cells and model systems, protozoan parasites also display unique and diverse mechanisms of epigenetic gene regulation [15–17]. This chapter presents our current state of knowledge about Dnmt2-mediated methylation in the parasite *E. histolytica* and its regulation by the environment.

11.3 Evidence for 5-Methylcytosine in the Genome of *E. histolytica*

DNA methylation is associated with gene silencing and transposon control [18, 19]. In mammals, 3 % to 8 % of cytosine residues are methylated, generally in a CpG context [20]. Typically, DNA methylation leads to recruitment of methylated CpG binding domain (MBD) proteins, which themselves interact with histone deacetylase to alter chromatin structure; condensing it, and silence gene expression [21]. The first clue about the presence of m5C in *E. histolytica* came 13 years ago when transfected *E. coli* activated their *mrr* methylation-restricting systems in response to exogenous *E. histolytica* transfectant plasmids (unpublished results). Direct evidence of the presence of methylated cytosine in the parasite's genome was then achieved via immunoblotting with m5C-specific antibody [22]. Recently, high pressure liquid chromatography (HPLC) coupled to mass spectrometry revealed low amounts of m5C in *E. histolytica* DNA (about 0.05 %) but definitely more than the detection level of the method (unpublished data). This presence of m5C in the genome of the parasite raises questions regarding its formation, the cellular/signaling events regulating this phenomenon, and its role or roles in the parasite life cycle and virulence.

11.4 *E. histolytica* Dnmt2 (Ehmeth) is a DNA Methyltransferase

The formation of m5C is catalyzed by 5-cytosine methyltransferase (m5C-MTase) with *S*-adenosylmethionine as a cofactor. The mammalian DNA machinery consists of three active DNA MTases: Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 has a high

preference for hemi-methylated DNA [23, 24] as a substrate, functioning as a "maintenance" DNA MTase, which preserves epigenetic differentiation throughout descendent cell lines during mitotic events. Dnmt3a and Dnmt3b, however, are de novo DNA MTases acting on nonmethylated DNA (for review, see Jeltsch [20]) and initiate active epigenetic regulation. A fourth enzyme, Dnmt2, is the most conserved of all DNA MTases; belonging to a large family of proteins conserved in all species from *Schizosaccharomyces pombe* to humans [25]. It is also the most enigmatic, however. The enzyme has very weak methylation activity on DNA [26–28]. More recently, methylation of $tRNA^{Asp}$ could be attributed to Dnmt2 [29]. Although this could indicate a biological function of the enzyme, the phenotype of knockout (KO) mutants is usually very mild or not detectable [29, 30]. Remarkably, the tRNA methylation activity follows a DNA methylation motif (utilizing cysteine79 present in motif IV of the catalytic site) and not the one employed by the structurally similar tRNA methyltransferases (which use an alternative cysteine to stabilize the Michael addition of a methyl group) [31]. Indeed, what distinguishes Dnmt2 from the other DNA MTases is its comparatively shorter N-terminal regulatory domain, which may play a role in its highly discriminate DNA-binding activity. The catalytic C-terminal domain is shared by all DNA methyltransferases; and structural analysis of human Dnmt2 showed a high similarity to the M.*Hha*I methyltransferase from *Haemophilus haemolyticus* [32].

E. histolytica belongs to the so-called Dnmt2 only organisms and does not contain any of the canonical DNA methyltransferases (Dnmt1 and Dnmt3). Substantial evidence supports *E. histolytica* Dnmt2 (called Ehmeth), as a genuine DNA MT. First, a number of DNA sequences have been identified via methylated DNA immunoprecipitation (MedIP) using the 5mC antibodies. These sequences include ribosomal DNA (*rDNA*), heat-shock genes (HSP70 and HSP 100), and retrotransposons [22, 33, 34]. Further analysis of these sequences utilizing bisulfite sequencing indicated that, in contrast to mammals, where cytosine is methylated predominantly within the CpG dinucleotides, the DNA methylation pattern in *E. histolytica* is not restricted to a CpG context, but can also occur at non-CpG sites [22]. Interestingly, non-CpG-methylation in mammals is primarily found in viral or stably integrated plasmid sequences [35], as well as in the endogenous long interspersed nuclear element, LINE-1 [36]. In higher plants, DNA methylation is commonly found not only in the symmetrical motifs, CpG and CpNpG, but also in some asymmetrical contexts, such as CpN, and is needed for normal development [37]. Therefore, it has been proposed that non-CpG methylation may reflect the substrate specificity of Dnmt2.

The role of the Dnmt2 protein family is still under investigation. Conventionally, DNA methylation in higher eukaryotes is linked with the silencing of gene expression. A correlation between DNA methylation and gene expression has been reported for the HSP100 gene of *E. histolytica* [34]. This apparently is not its most important function in *E. histolytica* because treatment with 5-azacytidine (a potent inhibitor of DNA methyltransferase) has a limited effect on gene expression in the parasite [38]. Remarkably, however, the ability of 5-azacytidine (23 μM)-treated *E. histolytica* trophozoites to form liver abscesses in infected hamsters is significantly reduced [22], suggesting that Ehmeth activity [39] regulates *E. histolytica* virulence.

One of the other functions of DNA methylation in higher eukaryotes is to provide protection from selfish DNAs that include retroelements [40]. The non-long-terminal repeat (non-LTR) retrotransposons encode a reverse transcriptase (RT) and other proteins that are needed for transposition. Non-LTR retrotransposons consist of short interspersed nuclear elements (SINEs) or long interspersed nuclear elements (LINEs), which are also transposed by reverse transcription of mRNA directly into the site of integration [41]. The sequencing of the *E. histolytica* genome revealed multiple LINE families and SINE elements that are also abundantly transcribed [42, 43]. Nevertheless, most of the LINEs have lost their transposition ability, probably because of mutations in some of their essential genes, such as reverse transcriptase [44]. It has been proposed that these mutations are the result of the accelerated deamination that occurs to the methylated cytosines that are present in the LINEs [45]. Newly emergent biotechniques may enable us to explore this phenomenon. Recent work has established a retrotransposition-competent cell line in *E. histolytica*, that is, reconstructed ORF2 (reverse transcriptase and accompanying endonuclease) serving as an activated LINE element, coupled with a secondary vector consisting of marked SINE linked to a targeted hotspot of integration [46]. Double transfectants displayed retrotransposition capability, mobilizing the marked SINEs and inserting them into the neighboring hotspot. It will be interesting to examine the relationship between Ehmeth expression and the frequency of retrotransposition, the stability of these activated SINEs in both their respective mosaics and tendency to accumulate polymorphisms (as correlated to Ehmeth expression), and, finally, the methylation status of the newly mobilized SINEs. This control of retrotransposons via Dnmt2-mediated DNA methylation has been demonstrated in other Dnmt2 only organisms including *Dictyostelium* and *Drosophila* spp. [28, 47], and the control of repetitive DNA elements by the trematode *Schistosoma mansoni* Dnmt2 has also been recently proposed [48].

11.5 Ehmeth is a tRNA MT

The observation that Dnmt2 methylates tRNA was first reported by Goll et al. [29]. In this seminal paper, the authors showed that Dnmt2 has a strong methylation activity at C38 of $tRNA^{Asp}$ in mice, *Drosophila melanogaster*, and *Arabidopsis thaliana* [29]. In addition to $tRNA^{Asp}$, $tRNA^{Val}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ are also methylated by Dnmt2 [49, 50]. Interestingly, Dnmt2 modifies these tRNAs at cytosine 38 following the reaction mechanism established for 5-cytosine DNA methyltransferases [29, 49]. This observation extends to *E. histolytica* as well. Recombinant Ehmeth prepared from *E. coli* was able to methylate synthetic $tRNA^{Asp}$. Concurrently, global $tRNA^{Asp}$ methylation in *E. histolytica* was measured via incorporation of radioactive methyl group into the tRNA of the parasite, utilizing hDnmt2. In this assay, the amount of SAM incorporated is proportional to the amount of unmethylated tRNA [39]. Recently, bisulfite sequencing of tRNA has been developed as well. This method offers direct detection of cytosine methylation in tRNA, accurately

localizing the methylated cytosines within the sequence. In applying this method to *E. histolytica*, we showed that Ehmeth, as do other Dnmt2 proteins [29], methylates $tRNA^{asp}$ at C38 (manuscript in preparation).

Therefore, in contrast to human Dnmt2, which apparently has a strong substrate preference for tRNA, Ehmeth can use both DNA and tRNA as substrates. This dual specificity for DNA and tRNA has also been proposed for the Dnmt2 homologue in *Drosophila melanogaster* [31]. The exact biological role of Dnmt2-mediated tRNA methylation is not yet known in *E. histolytica*, however. The position next to the anticodon loop suggests a role in the basic transcriptional process, but influence on tRNA folding and stability is also possible. A recent work points to the role of *Drosophila* Dnmt2 in the regulation of tRNA degradation. In this work, stress-induced cleavage of tRNAs was Dnmt2 dependent, and Dnmt2-mediated methylation protected tRNAs against ribonuclease cleavage [49]. Additionally, Dnmt2 has been implicated as an agent in the *Drosophila* innate immune response; intercepting exogenous viral RNA and labeling it for disposal utilizing the Dicer/Argonaute RNAi machinery [51]. In *Saccharomyces cerevisiae*, Trm9-mediated tRNA methylation is linked to the translation enhancement of genes related to stress response, DNA damage, and other cellular functions. These results together with previously published data support a role of tRNA methylation in the control of tRNA stability and consequently protein synthesis [52]. Recently, it has been shown that disrupting both the Dnmt2 and the NSun2 tRNA methyltransferases in mice led to the complete loss of tRNA methylation, reduced protein synthesis, and lethality [30].

11.6 Regulation of Ehmeth Activity and the Role of the Environment

It is well documented that long-term culture of pathogens, particularly parasites, leads to virulence attenuation [53, 54]. This observation also applies to *E. histolytica* for which regular passage through hamster liver is necessary to keep functional the ability of the parasite to form a liver abscess [55]. Similarly, we observed that continuous culture of *E. histolytica* in *TYI*-S-33 media progressively lowers the expression of Ehmeth to a barely detectable level (Fig. 11.1). This observation raises new and interesting questions about the regulation of Ehmeth expression and the impact of environment on this regulation. An analogous observation about the effect of growth conditions on the expression of Dnmt2 in *Saccharomyces pombe* was reported stating that nutrition (peptone) regulates *Schizosaccharomyces pombe* Dnmt2-dependent tRNA methylation [50].

Glucose starvation (GS), one of the most studied metabolic stresses, has been investigated in the malaria parasite *Plasmodium falciparum*. Interestingly, the PfEMP (*var*) genes, key components in malaria pathogenesis, are among the genes upregulated by GS [56]. Accordingly, it has been proposed that ambient glucose concentration is a good indicator of the environmental changes to which the parasite is exposed during its life cycle. This regulatory role of glucose is particularly

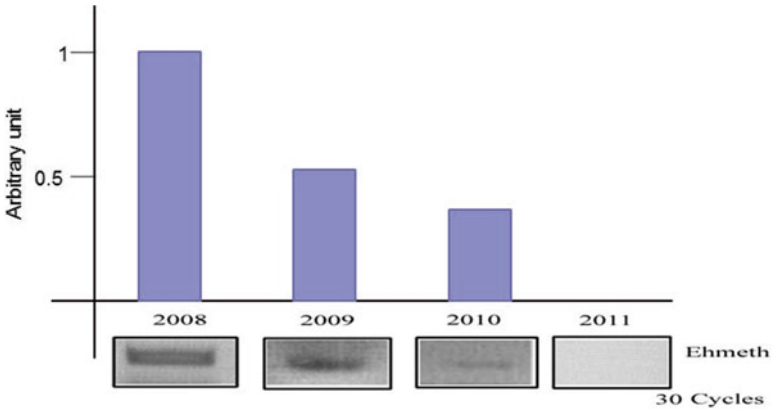

Fig. 11.1 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Ehmeth expression in an HMI:MSS strain that has been kept in continuous culture for more than 4 years (2008–2011). The HMI strain (a gift of Prof. Mirelman, Weizmann Institute) used in this study was originally isolated from a hamster that developed a liver abscess following injection of trophozoites in its liver. The strain was kept under continuous culture in TYI media without further passage in hamster liver. Semiquantitative *RT-PCR* was used for measuring Ehmeth expression

relevant to *E. histolytica* because, as already mentioned, it lives in the colon, a niche where the amount of available glucose for fermentation is usually small (about 0.2 g/kg tissue) because of the high absorptive capacity of the glucose transporters in the small intestine [57–59]. On rare occasions, it has been reported that *E. histolytica* trophozoites leave the colon and migrate to the liver. In this organ, the concentration of glucose was estimated to be twice that of perfusing blood (about 2.0 g/kg tissue) [60–62]. We recently reported that *E. histolytica* is capable of responding to changes in its surrounding glucose concentration: short-term glucose starvation (12 h) led to the accumulation of enolase, a glycolytic enzyme, and the inhibition of the Ehmeth activity in its nucleus [39]. Extending the condition of glucose starvation beyond 12 h led to the progressive death of most of the parasite population. Surprisingly, some individual clones survived and adapted to this absence of glucose in the media. Adaptations included a number of metabolic changes. Specifically, the increased expression of various catabolic enzymes involved in amino acid regulation; in particular, methionine gamma lyase, aspartate ammonia lyase, and dihydropyrimidine dehydrogenase (DPD), an important effector of the pyrimidine catabolism pathway. Indeed, DPD is crucial for parasite growth when the availability of glucose is limited [5]. Undergoing experiments also point toward increased $tRNA^{asp}$ methylation levels in these glucose-starved parasites (unpublished data). This result raises many intriguing questions about the role of tRNA methylation in the adaptive mechanism to glucose starvation.

Until recently, no interacting partner had been identified for Dnmt2. We have identified that enolase interacts with the catalytic site of Ehmeth, subsequently inhibiting both its DNA and tRNA methyltransferase activity [39]. Additionally, short-term glucose starvation (12 h) triggers the accumulation of enolase from the

cytoplasm to the nucleus, resulting in activated Ehmeth inhibiton [39]. We recently analyzed the crystal structures of both *E. histolytica* enolase and of Ehmeth [63, 64], but the molecular details on the Ehmeth–enolase hybrid remained elusive. Hence, the three-dimensional structure of the Ehmeth–enolase complex still needs to be elucidated.

11.7 Ehmeth Protects *E. histolytica* from Oxidative and Nitrosative Stresses

Although the overall biological function of Dnmt2/Ehmeth is not yet completely understood, recent work has enabled us to view their expression, pleiomorphically, in a broader context; particularly in terms of survival, longevity, and adaptability to oxidative stresses. Dnmt2 expression has been implicated as a necessary component to maintaining the normal lifespan in *D. melanogaster*; and, indeed, overexpression induces longevity in fruit flies [65]. It has been proposed that the underlying mechanism behind this observation is an increased resistance to oxidative damage; which has a well-established association with both degenerative diseases and aging [66]. Dnmt2 overexpression induces small heat-shock protein (Hsp) expression in *Drosophila melanogaster* [65], which facilitates the stabilization/sequestration of damaged or misfolded proteins [67]. Similarly, our group has demonstrated Hsp 70 upregulation in Ehmeth overexpressing *E. histolytica* transfectant [68]. Moreover, these Ehmeth-overexpressing trophozoites exhibit significantly greater resistance/survivability to H_2O_2 exposure. H_2O_2 is one of the principal convergent intermediate metabolites in oxidative stress. Activated resistance to oxidative damage is not surprising when considered in the context of *E. histolytica* virulence. Passage from the anoxic luminal colon into the tissues or bloodstream of the human host necessitates a dramatic change in environmental pO_2. Moreover, the parasite must now withstand the assaults of the human immune system, including oxidative bursts of superoxide anion and nitric oxide. Hsp induction, coupled with the upregulation of other protective antioxidant proteins (e.g., peroxiredoxin, iron containing superoxide dismutase), is thus seen in virulent [14] and even in laboratory-made drug-resistant strains of *E. histolytica* [69]. Puzzlingly, however, Ehmeth expression does not seem to directly induce Hsp 70 expression via methylation of its promoter, implying that there are other agents or mediators involved in the process [68].

Nitric oxide (NO) is the major cytotoxic molecule released by activated macrophages for defense against *E. histolytica* [70]. It is synthesized from L-arginine utilizing the calmodulin dependent iNOS dimer and has been implicated as a major effector for immunomediated antimicrobial defense. *E. histolytica* actually responds to NO and initiates fragmentation/mobilization of its proto-endoplasmic reticulum-like mitosomes, in addition to upregulating numerous genes involved in oxidative control and glycolysis [71]. *S*-Nitrosylation is an emerging redox-based posttranslational modification. *S*-Nitrosylation of crucial virulence factors and metabolic enzymes has been reported [72, 73]. There is increasing evidence to

support NO as a regulator of key epigenetic events. NO can have direct or indirect effects on the nucleosome assembly and chromatin structure by inhibiting or activating transcription factors, histone deacetylases, histones, and nuclear receptors. In addition, NO can disrupt the binding of transcription factors with their interacting proteins and can inhibit their nuclear localization (for a recent review, see Illi et al. [74]). The regulation of DNA methylation pattern by "stress" in some specific loci in plants, basal chordates, and mammals, including humans, has been well documented. However, the mechanisms that control this regulation are not well understood [75]. Thus, nothing is known about the effect of NO on Dnmt activity in general and on Dnmt2 in particular. Indeed, we do not know if the same protective effect of Ehmeth against oxidative stress applies to nitrosative stress [68]. Our ongoing research to address these issues indicates that Ehmeth protects the parasite from nitrosative stress, although the mechanism behind this protective effect is still under study.

11.8 Recognition of Methylated Cytosine by EhMLBP

Conventional methyl-CpG-binding proteins contain the conserved DNA-binding motif methyl-cytosine binding domain (MBD), which preferentially binds to methylated CpG dinucleotides. These proteins serve as transcriptional repressors, mediating gene silencing via DNA cytosine methylation (for a recent review, see Clouaire and Stancheva [76]). Information about methylated DNA-binding proteins in protozoa, however, was nonexistent. Indeed, bioinformatics analysis of the *E. histolytica* genome revealed an absence of MBD homologues, raising the very important question of how *E. histolytica* senses the aforementioned methylated regions in its DNA. Research initiated 3 years ago has established that a protein named *E. histolytica* methylated LINE binding protein (EhMLBP) [77] is involved in DNA methylation recognition. Specifically, it has a tendency to interact with those portions of the genome known already to be methylated (e.g., RT LINE DNA, rDNA) but competitive DNA probe binding assays have shown it to be a strong sensor of DNA methylation in a variety of genes including dihydrouridine synthetases, RAP GTPase-activating protein, serine/threonine protein kinase, and leucine-rich repeat containing protein. The common ground is that EhMLBP binds with a much higher affinity to methylated DNA over its nonmethylated counterpart. Further characterization of EhMLBP revealed that its C-terminal DNA-binding region has strong homology with histone H1 of *Xanthomonas oryzae* and *Trypanosoma brucei gambiense*; however, it shares no homology with the *E. histolytica* histone H1, or any of the other "classical MBDs" in mammals, plants, or insects. Thus, an in-depth analysis of EhMLBP localization, cognate protein partners, and DNA targets was carried out. The results revealed EhMLBP to be a perinuclear protein with strong preference for "kinked" DNA containing adenine stretches as present in LINE and SINE retrotransposons at their 3′-ends, and a consensus motif shared by the aforementioned genes [77, 78].

Regarding downregulation of EhMLBP, antisense technology, peptide targeting, and the lexotropic agent distamycin A (shown to be a potent inhibitor of EhMLBP) [79] all resulted in trophozoites with impaired growth and virulence; this finding identified EhMLBP as an essential constituent of the parasite *E. histolytica* and a possible target for anti-amebic chemotherapy. Interestingly, functional analysis revealed that EhMLBP also contains heat-shock domains, heat-shock transcriptional elements, and an N-terminal fibrinogen α-chain. What it lacks, however, is the conserved α-crystallin domain shared by Hsps in all three domains of life: Archea, Bacteria, and Eukarya. This lack indicates convergent evolution and a possible link between environmental heat stress and epigenetic control of transcription. Indeed, heat shock has been shown to induce EhMLBP expression both in vitro and in vivo, and the heat-shock element promoter (shared with the other Hsps) is induced by the same transcription factor [80]. Moreover, heat shock also induces pan-nuclear mobilization of EhMLBP along with its appearance in cytoplasmic vesicles that appear as putative stress granules. Not surprisingly, EhMLBP overexpression has been shown to protect heat-shocked trophozoites and even reduces overall protein aggregation in both control and heat-shocked trophozoites [80].

The fundamental question confronting us is whether EhMLBP is a sensor of DNA methylation initiating an adaptive response to methylated portions of the genome or whether it may, in fact, induce DNA methylation via recruitment of proteins such as Ehmeth. A study in EhMLBP overexpression revealed increased transcription of RT LINE DNA [80]. It would be interesting to investigate further the methylation status of this DNA, as well as concurrent overexpression/underexpression of Ehmeth. Conversely, what happens to Ehmeth/EhMLBP expression under overexpression of the cognate DNA targets? Finally, further research may reveal details about a putative protein scaffold, interactions with *S*-MARs, and/or cytoplasmic interactions with proteins and their expression/degradation.

11.9 Concluding Remarks

During the past few years, we have improved our knowledge on the biochemistry of Ehmeth, its mode of action, its targets, and the effects of their respective interactions. The data we have obtained thus far imply Ehmeth activity is induced under conditions threatening the genomic integrity of the parasite (i.e., external challenges such as stress, nutrients, and foreign genetic material). That Ehmeth expression seems less vital or pervasive under laboratory conditions suggests that this artificial atmosphere (in vitro) favors the emergence of strains with more lethargic phenotypes. Alternative, demanding environments may reveal more about Ehmeth expression, activity, and virulence. The identification of these conditions constitutes an important challenge for the coming years. The adage “Tell me who your friends are and I’ll tell you who you are” has been shown to be true when we identified enolase as the first Dnmt2-interacting protein implicated in both epigenetic regulation and metabolism in *E. histolytica*. More of these interacting proteins must be identified

in the future if we want to understand the full mechanism of Ehmeth expression as it relates to ultimate proteome expression.

Although most of our research is fundamental and tends to focus on the characterization of the epigenetic components in the parasite, we cannot ignore that several epigenetic drugs are being tested in clinical trials or even already being used (e.g., anticancer or antiepileptic drugs). It may thus be possible to test epigenetic targets as putative drugs for the treatment of amebiasis. Indeed, we may extend this philosophy toward treatment of other parasitic infections as well. From a clinical perspective, this possibility is very attractive because of the lack of homology between parasitic proteins such as EhMLBP (which has no mammalian counterpart) and human epigenetics. This possibility is particularly relevant because of emergent reports of amebiasis refractive to pharmaceutical treatment [81, 82] and various laboratory strains with existing metronidazole and even multidrug resistance [83, 84]. Furthermore, a host of adverse effects is associated with some of the conventional treatments. Possible side effects for metronidazole, for example, include nausea, diarrhea, thrombophlebitis, and even CNS toxicity [85]. Our previous work on EhMLBP has shown that it is possible to find an inhibitory peptide that blocks specifically the activity of this protein, which highlights the idea that epigenetics may be exploited for the development of alternative pharmaceutical agents that will serve as novel drugs, targeting a parasite's unique metabolism or reproductive niche that is not manifested in human physiology.

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Part III
Cell Biology and Signaling

Chapter 12
Phagocytosis in *Entamoeba histolytica*

Somlata and Alok Bhattacharya

Abstract Phagocytosis is one of the essential processes that is necessary for the survival and pathogenicity of *Entamoeba histolytica. E. histolytica* is known to phagocytose red blood cells (RBC), bacteria and other unicellular organisms, immune cells, and apoptotic cells during either proliferation in the gut or invasion in intestinal and extraintestinal tissues. Molecular pathways are not clear about molecular details in *E. histolytica* and are thought to be different from those known in other systems. In this chapter we describe our current understanding of molecular mechanisms of phagocytosis in *E. histolytica* and highlight new avenues for research for future drug development.

12.1 Introduction

Phagocytosis is a conserved and an essential process in majority of eukaryotic cells, ranging from protists to mammals. It plays a critical role in many processes, varying from uptake of nutrients to defense against the host immune system in parasitic protists. In particular, phagocytosis has been thought to be a virulence marker for *Entamoeba histolytica* [1]. Trophozoites mainly source their nutrients by phagocytosis of bacterial and other cells in the intestine, followed by digestion and reutilization of the nutrients [2]. Erythrophagocytosis leads to bloody dysentery in many patients with intestinal invasive disease. Phagocytosis of dead epithelial, immune, and nonimmune cells is also thought to be one of the important features of amoebic pathogenesis [3, 4]. Because a mutant cell line defective in phagocytosis showed both growth defects and lower virulence, it is thought that phagocytosis is one of the important cellular processes in amebic pathogenesis. There are three steps in tissue destruction: adherence to host cells, cytolysis, and phagocytosis of dead cells. Adherence to target cells is a prerequisite as blocking this process results in inhibition of target cell killing by amoebae [5]. The process involves initial recognition of target particles followed by activation of a cytolytic pathway. Dead cells are targets of amoebic phagocytosis, which again requires initial recognition.

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_12

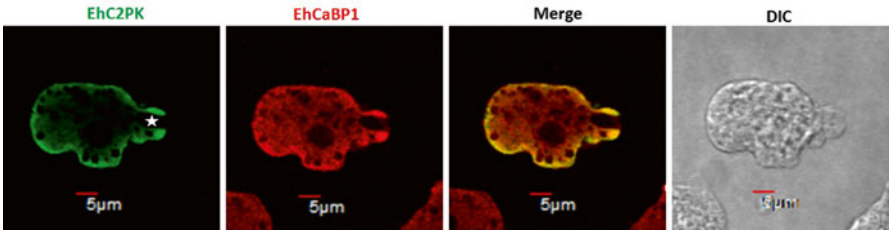

Fig. 12.1 *Entamoeba histolytica* trophozoite actively phagocytosing human erythrocyte. Immunofluorescence micrograph shows the prominent phagocytic cup and localization of two proteins EhC2PK (labeled with Alexa488) and EhCaBP1 (labeled with Alexa546) during the process. *Bar* 5 μm

Subsequent steps are initiation of signaling event, cytoskeleton reorganization, and cessation with pinching of vesicles. We do not have detailed information about most of these steps. For example, defined receptor(s) or ligand(s) that initiate phagocytosis have not been identified; although there is some evidence to suggest that Gal/GalNAc lectin is likely to be one of the phagocytosis receptors, engagement of the lectin does not always lead to phagocytosis [6]. In this chapter we describe our current understanding on the different stages of phagocytosis in *E. histolytica* that can be observed using various microscopic techniques (Fig. 12.1). Moreover, molecules that participate in the phagocytic pathways of *E. histolytica* are likely to be useful target of new drug development because inhibition of the pathway by down-regulation of some of the components, such as EhCaBP1, causes inhibition of cell proliferation [7]. Therefore, it is important to understand molecular mechanisms involved in phagocytosis in *E. histolytica*.

12.2 Mechanism of Phagocytosis in Other Organisms

12.2.1 Definition and Variation of Phagocytosis

By definition, phagocytosis is ingestion of large particles (>0.5 μm) by cells, [8] and differs from other endocytic processes, such as macropinocytosis, as it involves recognition and binding of particle by receptors on the cell surface followed by internalization. Phagocytosis is required to clear the bacteria and fungi from the site of infection by neutrophils, macrophages, and dendritic cells. This mechanism also helps in initiation of adaptive immune response by release of proinflammatory cytokines and by presenting antigens derived from these pathogens to lymphoid cells [9]. Apart from professional phagocytes, fibroblasts, epithelial cells, and endothelial cells also perform phagocytosis of apoptotic bodies. Although the major function of phagocytosis is to mediate removal of infectious agents, many pathogens such as *Salmonella typhimurium*, *Mycobacterium tuberculosis*, and *Legionella pneumophila* are ingested by a variety of heterogeneous mechanisms and are

capable of surviving in vacuoles of macrophages [10]. The phagocytosis for these organisms is not a process for clearing but a chance for gaining access to an environment favorable for their replication and pathogenesis. The virulent strain of *S. typhimurium* induces membrane ruffling upon binding to the macrophage surface, which results in internalization of bacterium in a compartment resembling macropinosomes. *L. pneumophila* recruits C3 complement component on its surface and adheres to macrophages through C3 receptors. The binding of parasites induces a structure called coiling phagosomes and the parasite is internalized [10]. Similarly, *M. tuberculosis* also uses deposition of C3b and C3bi on the bacterial surface, which are recognized by specific receptors on the host cell surface. Moreover, another pathway also exists that depends on C4b-like protein on the bacterial surface that can bind C2b and form C3 convertase, catalyzing the deposition of C3b on the bacterial cell surface [11]. All these organisms have evolved mechanisms to bypass degradation in the vacuoles and are, therefore, able to grow and survive successfully in the host cells.

12.2.2 The Molecular Mechanism of Phagocytosis and Cytoskeletal Rearrangements

The most studied phagocytic system is the Fc receptor (FcR)-mediated phagocytosis in macrophages [8]. The process is initiated when FcR on the surface of a phagocyte bind to immunoglobulin-coated bacterial and other cells. At sites of phagocytosis the plasma membrane is pulled around the target particles, leading to the formation of phagocytic cups [8]; this is followed by constriction at the distal margins of the cup that results in closure of the phagosome [12]. Transient assembly of actin filaments along with myosin plays a critical role in different steps of this process [12]. Initiation of phagocytosis is achieved by ligand-induced Fcγ receptor clustering that leads to activation of the membrane-associated Src family of kinases, such as Hck, Fgr, and Lyn that phosphorylates ITAM motif (immune-receptor tyrosine-based activation motif) of FcR, which in turn recruits the syk family of tyrosine kinases [13]. Syk kinase phosphorylates p85, which is a regulatory subunit of PI3K (3-phosphatidylinositiol kinase), resulting in activation of catalytic subunit p110 that leads to generation of PtdIns(3,4,5)P3 close to the clustered FcR region of the membrane [14]. A nonphagocytic cell can be induced to initiate phagocytosis by expression of FcR and syk, suggesting that some of the signaling molecules, such as syk, play an important role in phagocytic signal transduction from FcR [15]. Phospholipases are also important in phagocytosis and macropinocytosis, and FcR-mediated phagocytosis requires PLCγI (phospholipase Cγ, PLCγ), which hydrolyzes PtdIns(4,5)P2 to DAG (diacylglycerol) and Ins(3,4,5)P3, resulting in Ca^{2+} release from the endoplasmic reticulum. All these results suggest that Ca^{2+} is necessary for at least some forms of phagocytosis [8]. DAG remains in the plasma membrane and recruits PKCα or PKCε to the site of phagocytosis [8]. In the presence of PI3K inhibitors, incomplete phagosomes appear as actin-rich phagocytic cups,

suggesting that PI3K may be involved in organizing the later stages of phagocytosis. Syk kinase is believed to regulate actin remodeling at the site by two pathways, the first step being the activation of p85 leading to the generation of PtdIns(3,4,5)P3 by class I PI3K [16]. The increased level of 3 helps to recruit RhoGEFs Tiam1 (T-lymphoma invasion and metastasis), Vav1, and ARF leading to the recruitment of Rho proteins that are needed for actin dynamics during engulfment [17–19]. In the second pathway, Vav and Rac proteins are phosphorylated, resulting in activation of actin remodeling machinery through WASP (Wiskott–Aldrich syndrome protein) and Arp2/3 [20]. Moreover, cdc42-dependent actin remodeling and PI3K activation are two parallel signaling pathways that are linked during phagocytosis. When a macrophage cell line is transfected with a GFP-tagged PH domain that binds PI3K products, PtdIns(3,4,5)P3 accumulates rapidly in phagocytic cups and disappears when the cups are sealed [16]. Blocking PI3K activity by wortmannin (wtn) and LY294002 led to significant reduction in ingestion of large particles although uptake of smaller particles remained unaffected [21]. However, PtdIns(3,4,5)P3 was not essential for actin assembly in FcR-mediated phagocytosis [21, 22], suggesting that PI3K may be involved in focal delivery of the internal membrane to the site of phagocytosis to compensate for the loss of membrane during phagosome formation as a result of pseudopod extension [21, 23]. Several myosin isoforms such as myosin Ic, II, Ixb, and V are differentially distributed in the phagosome and may be involved in force generation [24, 25]. Cox and colleagues have shown the involvement of unconventional myosin X in phagocytosis [26]. This myosin isoform has three PH domains: a conventional motor domain, MyTH4 (myosin tail homology 4), and a FERM (4.1, ezrin, radixin, moesin) [27], which accumulate at the site of phagocytosis in a PI3K-dependent manner similar to that of F-actin. Moreover, expression of only the tail region containing the intact PH domain leads to a significant decrease in phagocytosis whereas a similar experiment using the mutated PH domain did not. Interestingly, the tail region did not inhibit F-actin recruitment. All these observations suggest that myosin-X was necessary for membrane extension [28]. Although myosin-X is a vertebrate-specific myosin, myosin VII of *Dictyostelium discoideum* is thought to have similar properties as it shares some conserved features in the tail region [29]. In *Dictyostelium*, actin dynamics and myosin contractile elements are necessary for formation and closure of the phagocytic cups. Myosin VII null mutants showed defects in phagocytosis while other actin-mediated processes are unaffected. Cells containing the mutant protein also exhibited a defect in adherence to particles as well as substratum [30].

During phagocytosis, the organized movement of actin cytoskeleton and membrane is coordinated by Ras superfamily of GTPases [31]. These molecules interact with membrane and protein in a regulated manner by noncovalent interactions. GTP-bound forms of these proteins activate various effector enzymes that regulate actin polymerization, myosin contractility, and membrane trafficking. These protein are regulated by GEFs (guanine nucleotide exchange factor) favoring GTP binding (activation) and GAPs (GTPase-activating protein) that stimulate GTP hydrolysis (inactivation) [32]. GTPases activate RAC1 and CDC42, initiating actin polymerization through activation of WAVE (WASP-family verprolin-homologous

protein family) that leads to crosslinked actin filaments involving the Arp2/3 complex [33]. It is also thought that there are multiple pathways for actin polymerization, such as Rac-mediated actin polymerization through activation of PtdIns4P 5-kinase (PI5K) and PAK1 [34], and by phosphorylation of LIM kinase by PAK1-inhibiting actin-filament-binding protein cofilin, resulting in increase of actin-filament turnover [35]. Some of the other controlling pathways in phagocytosis are regulation of myosin contractibility through phosphorylation of myosin light chain (MLC) by rho kinase and activation of PI5K and PLD2 by the GTP-bound form of ARF6 (ADP-ribosylation factor 6). PI5K and PLD2 are recruited by RAC1-GTP at the site of phagocytosis [36, 37].

12.3 Phagocytosis in *Entamoeba histolytica*

12.3.1 Surface Molecules Involved in Target Recognition

Many molecules have been implicated in adherence of particles (bacteria, RBC, dead cells, etc.) to trophozoites, but so far no defined receptor responsible for initiating phagocytosis has been identified. One of the likely cell-surface receptors is Gal/GalNAc lectin. However, it is not clear if these molecules are responsible for recognition of diverse ligands because binding of some ligands to lectins does not always lead to phagocytosis [38]. KERP1 was identified as one of the cell-surface proteins involved in binding human enterocytes [39]. Because it appears to be specific for *E. histolytica*, it may be involved in its pathogenesis as its expression is found to be elevated in trophozoites isolated from liver abscess [40]. EhCAPDH, a complex of two proteins, EhCP112 (cysteine proteinase) and EhADH112 (adhesion), is an adhesin and was identified as one of the proteins that bind RBC [41, 42]. The complex is involved in many functions related to pathogenesis, such as adherence, destruction of host cells, and phagocytosis as specific antibodies can block all these processes. Glycosylphosphoinositol (GPI) containing cell-surface molecules is thought to play an important role in pathogenesis including endocytosis/phagocytosis. Blocking the GPI biosynthetic pathway by antisense RNA directed against participating enzymes resulted in a reduction in adherence and endocytosis [43]. Phagocytosis of apoptotic cells is not inhibited after blocking adherence mediated by Gal/GalNAc lectin, suggesting that there may be specific surface receptors on *E. histolytica* that recognize ligand(s) on apoptotic cells. One of the possible receptors is serine-rich *E. histolytica* protein (SREHP) that can recognize apoptotic host cells. The defect in apoptotic cell ingestion upon blocking SREHP by a specific antibody was shown to be independent of Gal/GalNAc-mediated adherence [44]. Laughlin et al. have demonstrated the existence of lipid raft-like membrane domains that are involved in adhesion and phagocytosis in *E. histolytica* [45]. Further, segregation of Hgl and Lgl subunits of Gal/GalNAc lectin in a PtdIns(4, 5)P2 and Ca^{2+}-dependent manner has been observed during phagocytosis of human erythrocytes [46]. All molecules mentioned here are suspected to be

Table 12.1 Putative receptors known to participate in phagocytosis in *Entamoeba histolytica*

Receptor	Structural feature	Functions	Target	Ligand/coreceptor
Gal/GalNAc lectin [47]	Heterotrimer of GPI-anchored transmembrane peptide	Adherence	Erythrocytes, Bacteria, and apoptotic cells	Galactosamine-containing carbohydrates
SREHP [43]	Transmembrane protein with no cytoplasmic catalytic domain	Receptor for phagocytosis	Apoptotic host cells	
TMK96 [48]	N-terminal extracellular domain and cytoplasmic kinase domain	Receptor for phagocytosis	Erythrocyte	
TMK39 [49]	N-terminal extracellular domain and putative cytoplasmic kinase domain	Receptor for phagocytosis	Specific for apoptotic cells	
C1q/collectins [44]	Cytosolic protein with carbohydrate recognition domain	Adaptors protein	Apoptotic cells	Mannose and phospholipids
EhROM1 [50]	Transmembrane protease	Regulates receptor localization	Apoptotic host cell	

involved in the recognition and initiation of phagocytosis although there is no direct evidence to suggest that indeed these molecules recognize cells that are destined for phagocytosis. The molecules that are putatively receptors for various ligands and known to participate in phagocytosis are summarized in Table 12.1.

12.3.2 Initiation of Signaling Event and Cytoskeleton Reorganization

It is apparent from the previous section that cell-surface receptors that are involved in the recognition of phagocytic ligands and initiation of signaling have not been clearly identified. Therefore, it is difficult to postulate the nature of the signals that are involved in the process. Moreover, the nature of the signaling system is likely to be dependent on the phagocytic ligand, such as RBC and apoptotic cells. SREHP has been suggested to participate in the phagocytosis of apoptotic cells. Because it lacks any conserved catalytic or docking site in the cytoplasmic portion of the molecule, it is difficult to speculate on the nature of signal transduced after attachment of the ligand [44]. Phagosome-associated transmembrane kinase (PATMK) is involved in phagocytosis of apoptotic cells (see Sect.… for details). PATMK has a predicted cytoplasmic tyrosine kinase domain that may be responsible for signal transduction. However, activity of this kinase domain has not been shown,

and it is not clear if it is a functional enzyme [48]. Apoptotic cells display exposed phosphatidylserine (PS) on the cell surface. PS is an important ligand for recognition of apoptotic as well as aging cells by trophozoites, but the receptor specific for this ligand is still unknown. A signaling cascade initiated by Gal/GalNAc lectins is not known because the cytoplasmic part of the molecule does not have any known signaling-associated domains nor any molecule that binds to the cytoplasmic tail after receptor occupation has been identified. The presence of a GPI anchor in the small subunit of the Gal/GalNAc lectin and association with a lipid raft do suggest that this subunit may have an important role in signal transduction, although the nature of the signaling cannot be predicted at present.

12.3.3 Role of Ca^{2+} in Phagocytosis

Erythrophagocytosis has been demonstrated to be a receptor-mediated process in this organism [51]. The mechanism of phagocytosis of RBC by *E. histolytica* has been recently investigated to understand the phagocytic process in this organism. Ca^{2+} signaling was found to be important for erythrophagocytosis in *E. histolytica* as chelation of intracellular Ca^{2+} blocked erythrophagocytosis [52]. Becuase the *E. histolytica* genome encodes 27 calcium-binding proteins, it was thought that one or some of these proteins may be involved in transducing the Ca^{2+} signal during phagocytosis. EhCaBP1 was identified as one of the key molecules during erythrophagocytosis and was found to be recruited early to the phagocytic cups. It binds directly both G- and F-actin and modulates actin bundling [7].

Recent work by Somlata et al. suggests that recruitment of a C2 domain containing protein kinase (EhC2PK) at the ligand attachment site is one of the early signaling events that is necessary for phagocytosis to take place. It was shown that the process also requires enrichment of calcium-binding protein EhCaBP1, and subsequently actin, before the phagocytic cups are stabilized [53]. It appears that Ca^{2+} plays a critical role as EhC2PK binds the PS-containing inner leaflet of the plasma membrane in the presence of Ca^{2+} (for details about Ca^{2+} signaling, see chapter on kinases and signaling). The kinase activity of EhC2PK is shown to be essential for the progression of phagocytic cups to the phagosome as overexpression of a kinase-dead mutant resulted in a reduction of erythrophagocytosis [54]. Because the substrate for EhC2PK has not been identified, the nature of the downstream signaling pathway propagated through EhC2PK is not clear. As trophozoites overexpressing the kinase-dead mutant show slower initiation of actin accumulation and polymerization at the site of phagocytic cups, we can speculate that protein(s) involved in initiation of actin polymerization may likely to be direct substrate(s) of EhC2PK. Apart from EhCaBP1, another calcium-binding protein, EhCaBP3, is also involved in erythrophagocytosis in *E. histolytica* [55]. This calcium-binding protein colocalizes with myosin I and is believed to be involved in closure of phagosomes.

12.3.4 *Actin Cytoskeleton Remodeling in* E. histolytica

Although phagocytic receptors and signaling pathways are still elusive and may be unique in *E. histolytica*, several molecules involved in actin cytoskeleton remodeling are conserved in this organism. A list of different *E. histolytica* actin remodeling proteins involved in phagocytosis is summarized in Table 12.2; details about these proteins are given in Chapter…. Although the number of these proteins found to be involved in phagocytosis is large, it is likely that their involvement is nonspecific, mostly through modulation of actin dynamics and not specifically phagocytosis. This distinction is evident as in the dominant-negative situation or on expression blocking of these proteins, both phagocytosis and pseudopod formation are inhibited.

In many systems, phosphoinositides (PIs) are major signaling intermediates during receptor-mediated endocytosis. PtdIns(4,5)P2 has been extensively studied for its role in modulation of actin cytoskeletal dynamics. As mentioned previously, PtdIns(4,5)P2 binds several actin-binding proteins (ABPs), such as N-WASP, profilin, and vinculin and regulates actin dynamics. Byekova et al. have shown that during phagocytosis the PtdIns(3,4,5)P3-specific probes accumulate in extending pseudopods and phagocytic cups but the accumulation is absent from pinocytic compartments. Moreover, overexpression of PH domain led to an inhibition of erythrophagocytosis and increased motility [79]. PI-binding FYVE domains are known for binding PtdIns(3)P, and there are 11 RhoGEF proteins containing FYVE domain in *E. histolytica* and among them, EhFP4 was found at the site of phagocytosis [65]. *E. histolytica* genome encodes class I and class III PI3Ks. Participation of these kinases in phagocytosis has not been demonstrated directly. It appears that lipid mediators and effectors are involved in cytoskeleton dynamics and phagocytosis in *E. histolytica* similar to that in other systems. However, detailed mechanisms are far from clear, and much work needs to be carried out before a clear picture emerges.

12.3.5 *Phagosome Maturation*

The newly formed phagosomes undergo various steps to form mature endosomes and facilitate the digestion of ingested material. The *E. histolytica* genome encodes 91 Rab proteins, which are small GTPases and widely known to be involved in vesicular trafficking, as and are described in detail in Chap. XX "Molecular basis for the trafficking of cysteine proteases and soluble lysosomal proteins in *Entamoeba histolytica*." Phylogenetic analysis and multiple alignment show that 22 amoebic Rabs are highly similar to Rabs in other organisms and the remaining 69 are moderately similar [80]. Biological functions of such a large family of Rabs in *E. histolytica* have not been investigated. One of the Rab family members, Rab11B, has been shown to be involved in secretion of important virulence marker cysteine proteases. Overexpression of this gene led to increased cysteine protease

Table 12.2 The molecules known to play role in cytoskeletal dynamics in *E. histolytica*

S. No.	Protein	Function	Evidence	Localization	Over/under expression	References
1	RacA (196 a.a. protein)	Involved in membrane ruffling, receptor capping	Experiments done with transformants, genome analysis	Cytoplasm of unstimulated cell and localizes to uroid after induction of capping	Overexpression of p21racA-V12 leads to decreased rate of cytokinesis and formation of actin cups	Ghosh and Samuelson [56]
2	RacG (GTP-binding protein belonging to Rho family, 85 % similar to human Rac1)	Involved in uroid formation, capping activity and distribution of actin filaments	Immunolocalization of RacG in *E. histolytica* cells induced for capping by ConA treatment	Cytoplasm of unstimulated cell and localizes to uroid after induction of capping	Overexpression of an activated mutant leads to uncontrolled uroid formation, disturbing the polarization	Guillen et al. [57]
3	EhGEF1 (69-kDa protein contains Dbl homology domain, PH domain)	EhRacG is a preferential target, may provide link between F-actin dynamics and RacG signaling	Biochemical, cellular characterization of EhGEF1	EhGEF1 is localized at plasma membrane and pseudopod region, localizes to uroids when induced by ConA	Threefold overexpression of GEF1-HSV-tagged mutant leads to decrease in cell motility and ability to damage cells	Aguilar-Rojasa et al. [58]
4	EhGEF2 (belongs to Dbl family of proteins, has Arm repeats, has conserved catalytic domain)	Can activate in vitro EhRacA, EhRacB, EhRacC, EhRacD, EhRacG, EhRacH, and EhCdc42	In vitro guanine nucleotide exchange assay with recombinant proteins, immunofluorescence	N-terminal region and DH domain direct the membrane localization of EhGEF2	Overexpression of catalytically inactive version of protein led to 30 % decrease in phagocytosis and chemoattractive response	González De la Rosa et al. [59]
5	EhGEF3 (110-kDa protein containing Dbl homology domain in tandem with plecktrin homology domain)	Capable of stimulating nucleotide exchange on EhRacA and EhRho1, partial activity toward cdc42	In vitro nucleotide exchange, confocal localization	Colocalizes with EhRacA in uroid region of cells	Overexpression of dominant-negative EhGEF3 impairs ConA-dependent RacA activation, overexpression of WT protein has a negative effect on F-actin content, cell migration, and monolayer destruction	Arias-Romero et al. [60]

(continued)

Table 12.2 (continued)

S. No.	Protein	Function	Evidence	Localization	Over/under expression	References
6	ARP2/3 complex (involved in actin nucleation process)	Associates with newly formed phagosome	Identified by LC-MS in phagosome membrane isolated from *E. histolytica* cells phagocytosing human serum-coated beads			Hon et al. [38]
7	EhCaBP1 (EF-hand containing protein)	Transiently associates with newly forming phagocytic cups, binds actin in vitro	Immunolocalization, time-lapse imaging, co-sedimentation assay	Unstimulated amoeba shows cytoplasmic localization, stimulation with RBC leads to enrichment in phagocytic cups and pseudopods	Knockin expression leads to decrease in rate of phagocytosis	Sahoo et al. [7] and Bailey et al. [51]
8	Actin (conserved, binds phalloidin but not DNase I)	Involved in all processes related with cytoskeleton	First evidence of existence of actin was shown by immunostaining with antihuman actin antibody			Kettis et al. [61] and Meza et al. [62]
9	Myosin IB (unique unconventional myosin)	Associated with early phagosome, may be involved in actin crosslinking	Immunolocalization of molecules on uptake of beads coated with human serum	Concentrate in pseudopods as well as under plasma membrane, in unpolarized cells in membrane of vesicle and in cytosol in well-defined dots, also seen in phagocytic cups	Overexpression of protein leads to decrease in RBC uptake	Voigt et al. [63]
10	Myosin II	Abundantly recruited around nascent phagosome, involved in capping and uroid formation	Immunolocalization of cells engulfing beads, dominant-negative approach	Localized underneath receptor-capped structures, localization is diffused beneath the membrane after treating cells with kinase inhibitors	Kinase inhibitors and other inhibitors of protein phosphorylation prevented formation of capping and uroid	Arhets et al. [64]

11	PI3Ks (six orthologues of class I PI3K are present with RBD, PI3Ka, and C-terminal PI3K domains	Involved in endocytosis, phagocytosis, and chemotaxis	Treating trophozoites with PI3K inhibitors and study immunolocalization of actin and other proteins	PI3K inhibition leads to decrease in phagocytosis but actin-rich cups are found that do not form phagosomes	No direct experimental data available	Hon et al. [38]
12	EhFP4 (contains FYVE domain, RhoGEF/DH domain)	EhFP4 with PtdIns(3)P, PtdIns(4)P coordinately regulate phagocytosis and phagosome maturation	Immunolocalization, live cell imaging, and immunoprecipitation	During initiation of phagocytosis GFP-Hrs-FYVE translocated to phagocytic cup, proximal region of phagosome, and tunnel-like structure connecting plasma membrane to phagosome	Overexpression of FYVE domain from EhFP4 inhibited phagocytosis while enhancement was observed when mammalian Hrs-FYVE domain was expressed	Nakada-Tsukui et al. [65]
13	EhFLN (EhABP-120, belong to filamin family of proteins)	Crosslinking and actin binding proteins that organize filamentous actin in networks and stress fibers, forms link between cytoskeleton and plasma membrane	In vitro associates with PA and PI(3)P, immunolocalization of protein and various peptide	Localization is cytosolic as well as protein is enriched in membrane	Overexpression of short regions of EhFLN displays increased motility and chemotactic response	Díaz-Valencia et al. [66]
14	EhRho1 (has unusual isoleucine instead of phenylalanine at 45)	Not significantly ADP-ribosylated by Rho-specific C3 exoenzyme	In vitro biochemical characterization			Godbold et al. [67] and Bosch et al. [68]
15	EhPAK (lacks CRIB motif, binds Rac1)	Implicated in cell polarization, motility, and phagocytosis	Interaction with Rac1 and WASP by immunoprecipitation, immunolocalization of molecule in amoeba	Shown to be enriched in uroid region of cell	Cell loses polarity if C-terminal (187–457 a.a.) overexpressed	Labruyère et al. [69]

(continued)

Table 12.2 (continued)

S. No.	Protein	Function	Evidence	Localization	Over/under expression	References
16	PAK 2 (has PH domain, highly conserved CRIB domain, kinase domain, and interacts with EhRacA)	Involved in cell morphology, cell cycle, and gene transcription	In vitro Rac A activation assay, immunoprecipitation assay, collagen invasion assay	PAK2 colocalizes with ConA-induced caps, before induction with ConA localization is cytosolic, localization of N-terminal and WT protein is similar	Overexpression of C-terminal-kinase domain leads to defects in collagen infiltration, deregulation of cytokinesis	Arias Romero et al. [70]
17	PAK3 (contains N-terminal PH-like domain, a PBD/CRIB domain, C-terminal kinase domain)	Involved in receptor capping, may be involved in cell motility	Full-length protein is active demonstrating kinase activity independent of any small GTPase	Distributed in cytoplasm of unstimulated cells, localizes to caps after induction with ConA		Dutta et al. [71]
18	α-Actinin (63-kDa Protein, has N-terminus actin binding, and C-terminus Ca^{2+} domain and central helical rod domain)	Crosslink filamentous actin in Ca^{2+}-dependent manner	In vitro characterization of protein			Virel and Backman [72]
19	α-Actinin2 (70-kDa protein with all conserved motifs with a longer rod domain to encompass two spectrin repeats)	Binds Ca^{2+} and crosslinks actin filaments in vitro	In vitro biochemical characterization, co-sedimentation assay, EM of actin crosslinked fibers			Virel et al. [73] and Addario et al. [74]

20	EhRab5 and EhRab7A	Exclusively involved in phagocytosis	Immunolocalization, time-lapse imaging with vesicle-permeable dyes	Localized to prephagosomal vacuoles after priming with RBC	Overexpression of GTO from mutant-impaired formation of PPV and phagocytosis	Saito-Nakano et al. [75]
21	Formins (contains conserved Formin homology domain 2, GTPase-binding domain, and loosely defined FH1 and FH3 domain)	Binds actin directly and is sufficient to nucleate actin monomers	Immunolocalization, mass spectrometric analysis	Ehformin-1 and Ehformin-2 are associated with F-actin in pseudopodia, Ehformin-3 along with -1 and- 2 is found in phagosomes, Ehformin-1 and 2 colocalize with microtubular assembly in nucleus	Overexpression of Ehformin-1 and -2 delays the process of cell division	Marion et al. [76], Majumder and Lohia [77] and Bosch et al. [78]

and cytolytic activities. Overexpression of Rab11B is also associated with enhanced exocytosis, which indicates its involvement in recycling of endosomes [81]. Moreover, Rab7 has nine members, of which Rab7A and Rab7B display distinct localization in trophozoites. Rab7B is found in acidic compartments, and overexpression leads to increased acidification of late endosomes. In contrast, overexpression of Rab7B-GTP mutant resulted in defects in phagocytosis, disassembly of prephagosomal vacuoles, and mis-secretion of lysosomal proteins [82]. Interestingly, Rab7A is seen in nonacidic compartments and partially colocalizes with lysosomal proteins that make up post-Golgi compartments. Overexpression of the Rab7A-GTP mutant did not affect the secretion of cysteine proteases significantly. There are many members of this large family of proteins that have not been assigned any specific biological role, and further research is required to explore more about the function of these proteins in vivo.

12.4 Conclusion

Entamoeba histolytica depends on phagocytosis for its survival in the host. Although phagocytosis is a conserved process, the mechanisms in *E. histolytica* appear to be different as many of the participating molecules do not have any homologue in other systems. On the other hand, some of the known homologues of molecules involved in phagocytosis in other systems exhibit modified structural organization and also show different regulatory mechanisms. The endocytosis is mediated by a complex signaling pathway that includes various families of receptors, transmembrane kinases, a large number of calcium-binding proteins, and mediators of cytoskeletal dynamics. The large family of receptor molecules on *E. histolytica* help the organism to identify the variety of cells it can ingest and may be by different pathways. Precise roles of different participants of the process are not very clear, requiring further work before it is possible to derive pathways. More knowledge about the mechanism of phagocytosis in *E. histolytica* will not only help in understanding the evolution of the phagocytic process but also in developing novel inhibitors with potential for new drugs. *E. histolytica* is also a good model system for studying phagocytosis as it is highly phagocytic and has a large cell size that is helpful in tracking the process using imaging techniques.

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Chapter 13
Signaling Pathways in *Entamoeba histolytica*

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Abstract A variety of functions in eukaryotes, such as cell migration, contraction, secretion, proliferation, differentiation, and exocytosis, are initiated and sustained by signaling processes. Most of the signalling pathways have been described in detail for many eukaryotic systems, particularly for mammalian systems. Signaling pathways quite often consist of cell-surface receptors, intracellular components that function as adaptors and transducers including those that generate second messengers leading to either alteration in gene expression or cytoskeleton dynamics. We have rudimentary knowledge about the organization of signaling systems in *Entamoeba histolytica* and the mechanisms by which initiation is coupled with functional readout, in spite of identification of a number of molecules known to participate in these pathways in other organisms. In this chapter we provide a summary of our current understanding on calcium and G-protein signaling pathways of *E. histolytica* and their role in different biological processes.

13.1 Introduction

A major feature of amebiasis is the absence of an overt invasive form of the disease in a large number of infected individuals. It is generally estimated that more than 90 % of the infected individuals are asymptomatic and it is not clear why some individuals develop invasive amebiasis. Moreover, only a small fraction of patients display extraintestinal infection, particularly liver abscess. It is believed that both parasitic and host factors have important roles in deciding the fate of infection, varying from no symptoms to extraintestinal invasion. The intricate relationship between host and parasite is governed by their various inherent signaling systems. The gut microbiome is also known to influence the behavior of *E. histolytica*, although there are a few studies that indicate the importance of this relationship in amoebic

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_13

pathogenesis [1]. In this chapter we plan to explore and present our current understanding of different signaling systems present in *E. histolytica* as an attempt to look more closely at the *E. histolytica*–human relationship.

All living organisms have developed extensive mechanisms to respond to environmental signals. These signals are perceived through an array of receptors present mainly on the cell surface. The diversity of these receptors helps organisms to interact with different extracellular signals and respond appropriately. Signals perceived on the surface are transduced intracellularly through a number of intricate pathways that results in the alteration of gene expression and consequently cellular decision-making ability. A variety of signaling pathways may be present in *E. histolytica*, but the detailed mechanisms of these signaling pathways are still unknown. Molecular characteristics of many surface receptors are still not known, and studies are needed to understand the mode by which these signaling pathways are initiated or activated. The best characterized putative surface receptors are transmembrane kinases (TMKs), covered in another chapter. Among secondary messenger-mediated signaling systems, Calcium (Ca^{2+}) signaling has been studied in detail. It is likely that some of the other secondary messengers, such as cyclic AMP, may be important in amoebic biology and there may be a crosstalk between the two pathways. Therefore, in this chapter we have mainly covered the Ca^{2+} signaling system and provided information about other signaling systems such as those mediated by G proteins and cAMP. Because regulation of cytoskeleton dynamics is one of the key features of *E. histolytica*, we have also covered signaling mechanisms that control the cytoskeleton.

Ca^{2+} activation events have not been fully studied in *E. histolytica*, although it is clear that it is important in many processes including pathogenesis and cytopathic activity [2–4]. Ca^{2+} is a prominent regulator that can exert multiple effects on the structure and dynamics of the actin cytoskeleton (please see the chapter on Phagocytosis for details). The actin cytoskeleton makes essential contributions to diverse processes, including endocytosis, phagocytosis [5], cytolysis [6], and virulence [7, 8]. Precise spatial and temporal balance of the polymerized and unpolymerized pools of actin is a key to production of coordinated cell movement. Ca^{2+} is involved in initiating the amebic cytolytic activity [9], which can be blocked by Ca^{2+} channel blockers or by treatment with EGTA [2]. On the other hand, stimulation of amebic PKC activity with phorbol esters enhanced lysis of target epithelial cells [10]. Further, Ca^{2+} and phorbol esters have been found to influence the activities and release of many digestive enzymes, such as proteinases and lipases, some of the molecules thought to be involved in pathogenesis [9, 10]. Changes in the Ca^{2+} profile were also related to the cell cycle and the developmental stages of the parasite, that is, the cyst or the trophozoite stage [11]. Depletion of intracellular Ca^{2+} by BAPTA-AM led to a decrease in erythrocyte uptake, suggesting that Ca^{2+} is involved in phagocytosis [12]. Amebae also possess a Ca^{2+}-dependent phospholipase activity that appears to contribute to cytotoxicity [13]. These studies point out the importance of Ca^{2+} signaling in amoebic biology.

13.2 Ca^{2+} Signaling in Eukaryotes

Cells sense many different external signals through specific receptors, mostly present on the cell surface. and these signals are converted into secondary messengers for signal transduction. Ca^{2+}-mediated signaling pathways are present in almost all eukaryotes and are used to control processes as diverse as fertilization, proliferation, development, learning and memory, contraction, and secretion. However, any deviations, such as sustained high Ca^{2+} concentration can result in cell death through both necrosis and apoptosis [14]. Thus, cells have evolved mechanisms to release and distribute Ca^{2+} in different cellular organelles in a controlled manner and thereby regulate the spatial and temporal boundary of Ca^{2+} concentration. Hundreds of cellular proteins have been adapted to bind Ca^{2+} over a million-fold range of affinities (nM to mM), in some cases simply to buffer or lower Ca^{2+} levels, and in others to trigger cellular processes. The spatial distribution of Ca^{2+} signaling in turn depends upon the cellular localization and distribution of these calcium-binding proteins (CaBP) which exhibit large range of affinities [15]. A transient rise in the resting cytosolic Ca^{2+} levels is enough to trigger rapid cellular responses, such as contraction, secretion, or changes in the function of key metabolic enzymes. More sustained Ca^{2+} signals mediate crucial longer ter m responses including cell growth, cell division, and cell death (apoptosis).

13.2.1 Calcium Homeostasis

13.2.1.1 Regulation of the Intracellular Ca^{2+} Concentration

Eukaryotic cells maintain their intracellular Ca^{2+} concentration at a level 10,000- to 20,000 fold lower than that of the extracellular environment [14]. This steep Ca^{2+} gradient is maintained by several mechanisms that operate through the combined action of the plasma membrane and intracellular organelles. In eukaryotic cells, cytoplasmic Ca^{2+} levels are maintained either through the entry of extracellular Ca^{2+} into the cytoplasm through plasma membrane transporters or channels mostly regulated by cell-surface receptors (receptor-operated Ca^{2+} channels), or an electrical potential across the plasma membrane (voltage-gated Ca^{2+} channels), or released from intracellular Ca^{2+} stores such as the endoplasmic reticulum (ER), mitochondria, glycosomes, and acidocalcisomes via store-operated Ca^{2+} channels, in addition to nonselective channels [16]. These channels are essential components for refilling of intracellular Ca^{2+} stores following intracellular Ca^{2+} release and play an important role in a multitude of signalling pathways. Depleted intracellular Ca^{2+} stores are refilled by pumping Ca^{2+} back into the stores after initiation of the signaling pathway. Intracellular Ca^{2+} release occurs through four main families of intracellular Ca^{2+} channels in mammals, including inositol 1,4,5-trisphosphate receptors (IP3Rs), ryanodine receptors (RyRs), two-pore channels (TPCs), and some transient receptor

potential (Trp) channels. In response to the intracellular messengers, IP3, mammalian IP3Rs, and RyRs are responsible for release of Ca^{2+} from the ER [17, 18]. Ca^{2+} release from acidic organelles such as lysosomes occurs via nicotinic acid adenine dinucleotide phosphate (NAADP)-activated channels (TPCs) [19, 20]. Moreover, release of Ca^{2+} from intracellular stores in mammalian systems has been shown to occur through intracellular Trp channels, such as TrpM, TrpML and TrpP2 (polycystin-2) [21, 22]. Many protist parasites have evolved novel Ca^{2+} homeostasis pathways for their survival as they encounter differential ionic conditions during their life cycles. Moreover, mechanisms of Ca^{2+} influx and requirement differ during different stages of the parasite life cycle. It has been shown that Ca^{2+} is essential for parasite function, invasion, virulence, and survival. Some human parasitic protists, such as *Toxoplasma gondii*, tightly control their intracellular Ca^{2+} concentration and exhibit Ca^{2+} signals in response to physiological stimuli. Mammalian intracellular parasites employ novel Ca^{2+} entry pathways for their survival inside mammalian host cells where Ca^{2+} concentration is maintained at low levels. In the extracellular trematode *Schistosoma mansoni*, Ca^{2+} influx channels are important for muscle contraction and viability [23]. Intracellular Trp channels in various parasites have been identified, and three *S. mansoni* Ca^{2+} channels have been cloned and characterized [24, 25]. Many parasites store their intracellular Ca^{2+} in ER, mitochondria, glycosomes, and acidocalcisomes [26–29]. However, it is not clear if homologues of mammalian intracellular Ca^{2+} channels is present in parasites and if mediation of Ca^{2+} release via these channels takes place.

In eukaryotic cells, active export of Ca^{2+} into the extracellular space is accomplished by the action of plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchanger. In different protist parasites there are several reports of PMCA-type Ca^{2+}-ATPases, but there is no molecular evidence for the presence of a Na^{+}/Ca^{2+} exchanger in any parasitic protist. Ca^{2+}-binding proteins, such as calmodulin (CaM) in mammals, activate PMCA Ca^{2+}-ATPases, and biochemical evidence for CaM stimulation has been reported for the enzymes from *Trypanosoma cruzi* [30], *Leishmania braziliensis* [31], and *Leishmania donovani* [32]. At the molecular level the PMCA-type Ca^{2+} ATPases described to date lack a typical CaM-binding domain, suggesting either the presence of a different domain that is able to bind CaM or non-CaM-dependent regulation in protist parasites.

Most of the Ca^{2+} is sequestered by cytosolic buffer proteins, such as calbindin-D9k, calretinin, and parvalbumin, thus resulting in a small fraction of free Ca^{2+} [33]. Amplitude and duration of Ca^{2+} signals are maintained by these cytosolic buffers besides playing an important role in limiting the spatial spreading of local Ca^{2+} signals. The level of intracellular Ca^{2+} inside the cytoplasm at any given point of time is maintained by a dynamic balance between reactions that introduce Ca^{2+} and reactions which remove Ca^{2+}. Removal of Ca^{2+} usually occurs by combined action of buffers, pumps, and exchangers. If a cell becomes activated by an external signal, this often results in an increase up to 100 fold in the intracellular free Ca^{2+} concentration because of uptake of extracellular Ca^{2+} or the release of Ca^{2+} from intracellular stores. These changes in free Ca^{2+} concentration can cause significant oscillations of Ca^{2+} in the cytosol, providing the possibility of signal transduction for a number of different cellular activities.

It is as yet not clear if the pathway just described for maintaining Ca^{2+} homeostasis is present in a similar form in *E. histolytica*. However, many of the components are present, although generation of Ca^{2+} oscillations on activation has not been shown so far. Some of the components, such as IP_3 and IP_4 that mobilize Ca^{2+} to the cytoplasm from internal stores and P-type Ca^{2+}-ATPases, are present in *E. histolytica*. Intracellular Ca^{2+} is stored in internal vesicles (presumably ER) and these vesicles display IP_3 and IP_4 receptors [34–36]. P-type Ca^{2+}-ATPase of *E. histolytica* is a homologue of the human plasma membrane Ca^{2+} transporter *and Saccharomyces cerevisiae* vacuolar Ca^{2+} transporting ATPase [37, 38] and is likely to be involved in entry of Ca^{2+} from external sources. Genome analysis of *E. histolytica* has revealed six putative P-type Ca^{2+}-ATPases. However, none of the Ca^{2+}-ATPases or store-operated channels in *E. histolytica* has been investigated so far.

13.2.2 Intracellular Calcium-Binding Proteins

In eukaryotic cells transduction of signaling events in response to changes in intracellular Ca^{2+} levels are generally mediated by proteins possessing Ca^{2+}-binding motifs, such as C2 domains, EF-hands, and endonexin folds. C2 domains are 120-residue motifs that confer phospholipid-binding activity on the majority of proteins that possess them [39, 40] and participate in a variety of Ca^{2+}-regulated processes including signal transduction, membrane trafficking, and lipid second-messenger generation. An important example of a C2 domain-containing protein is protein kinase PKC. The most common Ca^{2+}-binding motif is the EF-hand motif. The basic structural/functional unit of an EF-hand protein is a pair of EF-hand motifs with helix-loop-helix (HLH) motif [41]. An EF-hand consists of an N-terminal helix (the E-helix, 10–12 residues) immediately followed by a centrally located, Ca^{2+}-coordinating loop (12 residues) and a C-terminal helix (the F-helix, 10–12 residues). Generally, the alpha helices (E and F-helix) are found to be perpendicular to each other.

The *E. histolytica* genome encodes 27 novel CaBPs with no homologue in other organisms. Different CaBPs may participate in different functions that are spatially and temporally separated. The majority of these have multiple EF hand domains [42]. A list of CaBPs containing EF-hand and C2 domains along with their putative functions is given in Table 13.1. Only a few of *E. histolytica* CaBPs (EhCaBPs) have been structurally and functionally characterized. The most studied molecules are EhCaBP1, EhCaBP2, EhCaBP3, and URE3BP. Here we summarize our current understanding of these molecules. All these molecules may help to couple Ca^{2+} signal transduction with downstream pathways, such as activation of kinases. A likely activation of PKC can lead to adhesion plate formation and adhesion to fibronectin [53], a process essential for interaction of the parasite with extracellular matrix components (ECM) and its subsequent degradation. Furthermore, extracellular Ca^{2+}, amoebic intracellular Ca^{2+} flux, bepridil-sensitive Ca^{2+} channels, and a putative CaM-dependent signal transduction pathway have been implicated in growth and encystation of *Entamoeba* [54] and also in excystation of *E. invadens* [55].

Table 13.1 List of EF-hand and C2-domain containing proteins and their functions

Proteins	Function	References
EhCaBP1 (EF-hand containing protein)	Involved in actin-bundling Overexpression increases the rate of phagocytosis Knockdown by anti-sense RNA decreases the rate of phagocytosis and cell proliferation.	Jain et al. [12] and Sahoo et al. [43, 44]
EhCaBP2 (EF-hand containing protein)	Possibly involved in Ca^{2+} signaling	Chakrabarty et al. [45] and Mustafi et al. [46]
EhCaBP3 (EF-hand containing protein)	Involved in actin polymerization and bundling Associates with phagosome scission machinery	Aslam et al. [47]
Granin 1 and 2 (EF-hand proteins)	Hypothetical proteins Possibly involved in the control of endocytotic pathways and Ca^{2+}-dependent granular discharge	Nickel et al. [48]
URE3-BP (EF-hand containing protein)	Involved in transcription regulation	Gilchrist et al.[49]
CaM (EF-hand containing protein)	Might be involved in the release of collagenases through degranulation of electron-dense granules	Muñoz et al. [50]
EhC2PK (C2 domain containing protein)	Involved in initiation of phagocytosis	Somlata et al. [51]
EhC2A (C2 domain containing protein)	Role in translocation of URE-3BP to the plasma membrane in a Ca^{2+}-regulated manner	Moreno et al. [52]

13.2.2.1 EhCaBP1

Two closely related EF-hand-containing proteins, EhCaBP1 and EhCaBP2, were characterized from *E. histolytica* [44, 45]. EhCaBP1, a 14.7-kDa protein, shares 29 % sequence identity with the well-studied eukaryotic EF-hand-containing protein, calmodulin (CaM) [56]. However, this protein is functionally distinct from CaM as it does not activate c-AMP phosphodiesterase [57]. Downregulation of EhCaBP1 blocked proliferation of the parasite [43]. Detailed analysis suggested that EhCaBP1 is involved in different forms of endocytosis, such as pinocytosis and erythrophagocytosis [44]. EhCaBP1 associates transiently with phagocytic cups but is absent from phagosomes and may participate in the initiation step of endocytosis by directly interacting with both F- and G-actin [12, 44]. NMR studies showed that EhCaBP1 contains two globular domains connected by a flexible linker region spanning eight amino acid residues [58]. Each domain consists of a pair of canonical Ca^{2+}-binding EF-hand motifs. This arrangement allows certain movement of the domains with respect to the central linker region. The unusual organized structure of EhCaBP1 was ascertained by X-ray crystallography [59]. The two glycines (G63, G67) present in the central linker region make it more flexible than CaM, although the region connecting EF-hands I and II was found to be less flexible with extended conformation. The N-terminal domains of three molecules of EhCaBP1 interact in a head-to-tail manner to form a trimer. Each trimeric interface is characterized

by hydrophobic pockets, and interpocket distance is almost equal to the distance between the hydrophobic pockets in the extended structure of CaM. Hence, it is highly plausible that both the domains carry distinct functional properties, thus conferring several/additional functional features to the protein. Both N- and C-terminal domains of CaBP1 have been investigated [60]. The N-terminal domain appears to be involved in the localization in phagocytic cups and activation of kinase although the C-terminal domain did not show any transitional property. However, the importance of the C-terminal domain became apparent when it was overexpressed in amoebic cells, as the cells display a dominant-negative phenotype. It appears that both domains of EhCaBP1 are required for its function [60].

13.2.2.2 EhCaBP2

EhCaBP2, an isoform of CaBP1, shows 79 % sequence similarity with calcium-binding protein EhCaBP1. Confocal microscopy revealed that EhCaBP2 is mainly localized in the plasma membrane, in contrast to EhCaBP1. Moreover, it does not bind actin or colocalize with actin in actin-rich structures [46]. These results suggest that these two proteins may play different functional roles in *E. histolytica*. The multidimensional nuclear magnetic resonance spectroscopic technique was used to study the structure of this molecule. The results showed that the protein consists of two globular domains connected by a short flexible linker region of four residues. Further, comparison of the three-dimensional (3D) structure and dynamics of EhCaBP2 with that of EhCaBP1 revealed that these two proteins vary significantly in the structure and organization of N-terminal domains and interdomain linker [46].

13.2.2.3 EhCaBP3

CaM-like calcium-binding protein from *E. histolytica* (abbreviated as EhCaM or EhCaBP3) is a 17.23-kDa monomeric protein. Although it displays highest sequence identity with CaM, it is functionally different. Detailed analysis of this protein showed its involvement in erythrophagocytosis. It is recruited to the phagocytic cups during initiation but unlike EhCaBP1 it stays until the phagosomes are closed and is even present in early phagosomes [47]. EhCaBP3 is a cytoskeleton-remodeling protein that enhances actin polymerization in the presence of Ca^{2+} and binds to G-actin in a Ca^{2+}-dependent manner. It also interacts with myosin 1B in the presence of Ca^{2+} and is thought to be involved in phagosome closure. Downregulation of EhCaBP3 led to the absence of myosin 1B at the site of cup closure, affecting the phagosome scission machinery and consequently decreasing the rate of phagocytosis [47]. Nuclear magnetic spectroscopic (NMR) studies have revealed that EhCaM is partially folded with a well-folded N-terminal domain and an unstructured C-terminal counterpart displaying an unusual structure for a CaBP [61].

13.2.2.4 URE3BP

The URE3 (upstream regulatory element 3) was originally identified as a negative regulatory sequence by linker-scanner mutagenesis of the promoter of the *E. histolytica* Gal/GalNAc lectin gene *hgl5* [62]. The transcription factor binding to the URE3 motif, URE3-BP, was identified in a yeast one-hybrid screen using the URE3 sequence as bait [63]. URE3-BP contains two Ca^{2+}-binding motifs (EF hands), and it dissociates from DNA in the presence of Ca^{2+} [49]. Mutation in one of the URE3-BP EF-hand motifs resulted in a dominant-positive mutant protein that remained bound to the URE3 motif in the presence of an intracellular [Ca^{2+}] signal. Amoebae expressing the dominant-positive mutant form of URE3-BP were more virulent in two different animal models of amebiasis [64].

13.2.2.5 EhC2PK

EhC2PK, a C2 domain-containing protein kinase, is distinctly different from other C2 domain containing protein kinases including PKC. It shares only 30 % sequence similarity with known C2 domains of PKCα and PKCγ but residues involved in Ca^{2+}-binding are conserved. EhC2PK differs from typical PKCs with respect to the absence of a C1 domain, necessary for interaction with diacylglycerol (DAG). Sequence similarity searches using the kinase domain showed maximum identity (45 %) with Ca^{2+}/CaM-dependent protein kinases of humans, but not with other C2 domain-containing kinases. The functional analysis of EhC2PK showed its involvement in the initial stage of phagocytosis. EhC2PK accumulates in the phagocytic cups in a Ca^{2+}-dependent step and recruits EhCaBP1, which in turn provides an anchoring point for many proteins including actin filaments, resulting in initiation of phagocytosis [51]. Ca^{2+} plays an important role for the membrane localization of EhC2PK and for phagocytic cups to progress toward phagosomes [51].

13.2.2.6 EhC2A Protein

One of the first C2 domain-containing proteins that have been characterized in *E. histolytica* is EhC2A. EhC2A contains an N-terminal Ca^{2+}-binding C2 domain and a proline-rich C-terminal region. EhC2A was originally identified as URE3-BP binding protein. In amoebic trophozoites, EhC2A and URE3-BP translocated to the plasma membrane upon exposure to increased intracellular Ca^{2+} concentrations. Ca^{2+}-induced mobilization of URE3-BP represents a novel mechanism in which a C2 domain protein is involved in the anchoring of a transcription factor to the plasma membrane [52].

Apart from these molecules, a few others have been partially characterized. CaM was shown to be present in *E. histolytica* by partial purification [65], although the gene has not been identified yet. It was demonstrated that Ca^{2+}/CaM plays a role in the secretion of collagenase containing electron-dense granules as several CaM antagonists such as TFP, R24571, W-7, and DL-propranolol inhibited EDG secretion [50].

Two related EF-hand proteins, Granin1 and Granin2, are found in intracellular granules in *E. histolytica* [48], and the authors postulated that these may be involved in control of endocytotic pathways and Ca^{2+}-dependent granular discharge. However, there is no experimental evidence to support any of the suspected functions.

13.2.3 Ca^{2+}-Independent Function of EhCaBPs

Some of the functions of CaBPs do not require their ability to bind Ca^{2+}. In yeast it has been shown that the growth defect of a CaM null mutant can be complemented by a mutant CaM that cannot bind Ca^{2+} [66]. CaM can bind microtubules in a Ca^{2+}-independent manner [67], suggesting that Ca^{2+} is not necessary for all functions of CaBPs. The most common binding partners for the apo-S100 proteins are enzymes. For example, S100B and S100A1 bind glycogen phosphorylase [68], whereas S100A10 and S100A11 interact with transglutaminase in the absence of Ca^{2+} [69]. Using mutants that are defective in binding Ca^{2+}, it was shown that EhCaBP1 and EhCaBP3 bind F-actin in a Ca^{2+}-independent manner [12, 47]. Moreover, EhCaBP3 is able to bundle F-actin, and EhCaBP1 is recruited at the phagocytic cups through binding EhC2PK in the absence of Ca^{2+} [47, 51].

13.3 G-Protein-Mediated Signaling

GTP-binding proteins (G proteins) are important transducers of cellular signaling (Oldham and Hamm [70]. Heterotrimeric G proteins are composed of three distinct subunits (Gα, Gβ, and Gγ) and are associated with G-protein-coupled receptors (GPCRs). Gα binds guanine nucleotide, and Gβ and Gγ form an obligate heterodimer [70]. Gα has a binding site for Gβγ when Gα is in its inactive GDP-bound state. The activated receptor acts as a guanine nucleotide exchange factor (GEF) for Gα, releasing GDP and allowing subsequent GTP binding. The binding of GTP causes a conformational change in three flexible "switch" regions within Gα, resulting in Gβγ dissociation. Gα-GTP and freed Gβγ independently activate downstream effectors, such as adenylyl cyclases, phospholipase C isoforms, and Rho-family guanine nucleotide exchange factors (RhoGEFs) to modulate levels of intracellular second messengers [70, 71]. "Regulator of G-protein signaling" (RGS) proteins generally serve as inhibitors of Gα-mediated signaling [72]. However, one class of RGS proteins, RGS-RhoGEFs, serve as positive "effectors" for activated Gα signal transduction [71, 73]. The *E. histolytica* genome encodes 307 putative protein kinases representing all seven eukaryotic kinases including receptor tyrosine kinases [74, 75]. Functional heterotrimeric G-protein subunits in *E. histolytica* have been characterized, and a signaling pathway constituted by this receptor when perturbed is seen to regulate multiple cellular processes required for pathogenesis. The *E. histolytica* Gα subunit EhGα1 interacts with EhGβγ dimer and exhibits conventional nucleotide cycling properties. EhGα1 binds and hydrolyzes GTP, and its switch

regions undergo a conserved conformational change. When in an activated state, EhGα1 interacts with an RGS domain containing RhoGEF (EhRGS-RhoGEF). EhRGS-RhoGEF is likely to represent a functional signaling link between heterotrimeric G proteins and Rho family GTPases in *E. histolytica*. However, crystal structure suggests that EhGα1 shows distinct structural features and cannot be classified within mammalian Gα subfamilies [76]. *E. histolytica* trophozoites overexpressing wild-type EhGα1 exhibit an enhanced ability to kill host cells and also display enhanced migration. When Ser-37 of EhGα1 was mutated to cysteine, this mutant EhGα (S37C) displayed dominant-negative behavior as it was unable to bind GTPγS and could not activate downstream signaling. Thus, perturbation of heterotrimeric G-protein signaling via the dominant-negative mutant affects the expression of transcriptional regulation of virulence factors and alters trafficking of cysteine proteases.

13.4 Cyclic AMP Signaling

3′–5′-Cyclic adenosine monophosphate (cAMP) plays an important role in the growth and differentiation of many organisms. cAMP acts as a differentiation promoting factor in *Dictyostelium discoideum*. Aggregate and spore formation of the amoeba depends upon chemotactic mobility, which in turn is controlled by cAMP levels [77]. As with other eukaryotes, the growth of *E. invadens* is inhibited by the increase in cAMP levels. In *Entamoeba*, cAMP affects both transcription of actin mRNA and actin cytoskeleton dynamics [78]. Furthermore, activation of PKA by cAMP results in nuclear translocation of its catalytic subunit and phosphorylation of proteins involved in the reorganization of the actin cytoskeleton [79]. *E. invadens* increases the production of cAMP in response to the presence of epinephrine. Further, epinephrine caused an increased binding of nonhydrolyzable GTP analogues to membrane fragments. Increase in cAMP levels in whole cells and membrane fragments also occurs in the presence of modulators of heterotrimeric G-protein signaling, such as forskolin (FK), pertussis toxin (PTX), and cholera toxin (CTX). Specific blockers of adenylyl cyclase (AC) inhibit the increases in cAMP [80]. Amoebic cells therefore appear to contain G-protein-regulated adenylyl cyclase that functions downstream of an adrenergic receptor. So far the receptor has not been either functionally or structurally characterized.

13.5 Cytoskeleton Dynamics and Signaling

Actin is one of the most conserved and ubiquitous eukaryotic proteins that is a major component of the cytoskeleton. Actin-like proteins have also been reported from bacterial systems [81]. The actin cytoskeleton is required in multiple processes, such as endocytosis and motility, and is an indispensable component of

many subcellular structures, such as lamellopodia, filopodia, stress fibers, and focal adhesions [82, 83]. In highly motile organisms actin constitutes a major fraction of cellular proteins and in them actin is quite often encoded by several genes. *E. histolytica* displays a high motility and endocytic activity, which are thought to be related to its pathogenesis [84]. Therefore, cytoskeleton dynamics play an important role in amoebic biology [44, 84], also evident from the fact that, there are six actin genes encoded in the *E. histolytica* genome. Because actin cytoskeleton dynamics is regulated and controlled through signaling pathways, here we have summarized our current understanding of the signaling pathways of *E. histolytica* that are involved in regulating actin dynamics.

13.5.1 Regulation of Actin Dynamics

Formation of actin filaments from actin monomers requires a number of different steps including nucleation, polymerization, and branching. Effective dynamics also mean the ability to depolymerize when required, which is evident from the fact that blocking depolymerization can also inhibit many processes where the actin cytoskeleton is involved [85].

13.5.1.1 Nucleation

Nucleation of actin filaments is a crucial step as it helps in formation of actin filaments upon activation of the appropriate signaling pathway. Nucleation requires specific proteins comprising three classes: the actin-related protein complex (Arp2/3), formins, and Spire [86, 87]. The Arp2/3 complex consists of seven subunits: actin-related protein 2 (Arp2, 44 kDa), actin-related protein 3 (Arp3, 47 kDa), ARPC1 (40 kDa), ARPC2 (35 kDa), ARPC3 (21 kDa), ARPC4 (20 kDa), and ARPC5 (16 kDa). It appears that actin-related proteins of the Arp2/3 complex mimic an actin dimer to which actin monomers bind and help to form actin filaments (daughter filaments) on the side of the existing mother filaments, leading to formation of Y-shaped branches [88]. Although this complex is highly conserved in eukaryotes, many of these proteins have not yet been identified in the genomes of a few unicellular eukaryotes, such as algae, microsporidia, and apicomplexa [89].

Orthologues of the Arp2/3 complex and formins have been found in *E. histolytica*, but no evidence is available for spire family proteins. The functional role of these proteins in actin nucleation of *E. histolytica* is not as yet clear although there are reports that suggest that these may function in *E. histolytica* in the same way as in other organisms. For example, Arp2 of *E. histolytica* can functionally complement that of *D. discoideum* [90]. Because some of the proteins of this complex have been found in the *E. histolytica* phagosome proteome, there is a strong possibility that the Arp2/3 complex participates in the phagocytic process in *E. histolytica* [91].

The Arp2/3 complex requires nucleation-promoting factors for its activation, including Wiskott–Aldrich syndrome protein (WASP) and suppressor of cAMP receptor (SCAR) [92]. Usually WASP/SCAR family proteins have a C-terminal WASP homology 2 domain (WH2), a cofilin homology domain (C), and an acidic domain (A), making a VCA domain. WASP homology 2 domain and cofilin homology domains of VCA bind actin whereas the acidic domain along with the cofilin homology domain associate with the Arp2/3 complex, thereby bringing the Arp2/3 complex and actin together [93]. In *E. histolytica*, orthologues of WASP and SCAR family proteins are not found. Recently, a new family of WASP/SCAR proteins has been identified from the human subtelomeric region known as WASH (Wiskott–Aldrich scar homology). WASH has a conserved VCA domain at its C-terminus and colocalizes with actin in humans. WASH is required for viability of *Drosophila* [94]. A homologue of WASH has been found in the proteome of *E. histolytica*, suggesting that the function of the WASP/SCAR family may be carried out by WASH that is helping actin nucleation in an Arp2/3-dependent pathway [94].

In addition to Arp2/3 complex, formin also plays important role in actin cytoskeleton dynamics. Formin is an actin nucleation protein that binds to growing ends of actin leading to the formation of actin filaments, in response to extracellular signals and independent of the Arp2/3 complex [95]. Formin has multiple domains (FH1, FH2, FH3, GBD, and DID) that are functionally different from each other. The FH2 domain is involved in rapid nucleation by binding to the positive end and promoting addition of actin monomers to the growing end. The FH1 domain consists of proline repeats that interact with profilin and also with adapter proteins, such as SH3 and WW-domain containing proteins. FH3, GBD, and DID domains are involved in cellular localization of these proteins and Rho GTPase signaling [96–98]. *E. histolytica* encodes three classes of formin homology proteins (Table 13.2). Microscopic studies have shown that Ehformin 1 and 2 are associated with F-actin and participate in signal transduction mediated by actin. For example, during endocytosis these are found to colocalize with F-actin [96]. On overexpression of these proteins, a number of parameters related to mitosis, cytokinesis, and genome segregation in *E. histolytica* are altered, suggesting their role in regulating these processes.

In general, there is not yet a clear picture about the mechanism of initiation of actin filament formation. However, there are three possibilities by which fast-growing barbed ends are generated for initiating actin polymerization in response to extracellular signals: uncapping existing filaments, severing existing filaments ,and de novo actin nucleation. Capping proteins and gelsolin are present in the barbed ends as masks; these are dissociated by polyphosphoinositides [100, 101] in

Table 13.2 Domain architecture of *Entamoeba histolytica* formin-1 to formin-8

Class of formins	Domains	Remarks
Ehformin 1 to 3	FH1, FH2, FH3, GBD	Closest homologues of a group of *D. discoidium* formins DRFs [98]
Ehformin 4	FH1, FH2, FH3	Similar to formin ForC and ForG of *D. discoidium* [99]
Ehformin 5 to 8	FH1	Contain uncharacterized region [97]

response to a signal, thereby unmasking the barbed ends. Actin depolymerizing factor (ADF)/cofilin protein family can sever actin filaments without capping but this activity causes filament disassembly rather than polymerization [102]. In platelets, a change in Ca^{2+} levels stimulates gelsolin either to sever or cap actin filaments and when this is coupled with uncapping, leads to new barbed ends [103]. To date no cellular factor is known that can make new barbed ends.

13.5.2 Cytoskeleton Signaling

13.5.2.1 Rho Family GTPases

Rho family GTPases are involved in coupling signal transduction with rearrangement of the actin cytoskeleton. In particular, these proteins transmit signals from cell-surface receptors to cytoskeleton effector proteins, thereby participating in a number of processes, such as formation of phagocytic cups, lamellipodia, stress fibers, filopodia, and cell migration [99, 104]. Rho GTPases are regulated by GEFs (guanine nucleotide exchange factor) and GAP (GTPase activating protein). GEF exchanges the bound GDP of the Rho to GTP, thereby activating Rho-GTPases. GAP activates GTPases leading to hydrolysis of bound GTP to GDP, switching Rho GTPase from its active to an inactive form. Thus, GEF and GAP act as molecular switches controlling the activity of Rho proteins.

Different classes of Rho-GTPases and their predicted homologues of *E. histolytica* along with functional assignment are listed in Table 13.3 [105]. EhRho1 is a novel Rho GTPase that has diverged from its mammalian counterparts. An aromatic amino acid at the 45th position regulates transition of the mammalian molecules between active and inactive states. However, *E. histolytica* protein has an isoleucine in this position and this position does not appear to have any overt functional significance [106]. EhRho1 is not a substrate of C3 exoenzyme of *Clostridium botulinum*, unusual as the C3 exoenzyme specifically inactivates Rho proteins by ADP-ribosylating aspargine residues present in the effector domain. EhRho1 also lacks the signature Rho insert helix, which is conserved in mammalian counterparts and participates in enhancing the rate of nucleotide exchange in the absence of GEF. In spite of sequence differences, EhRho1 is involved in serum-stimulated actin reorganization and microtubule formation during mitosis along with Ehformin1 and other effectors [106].

Generally, diaphanous-related formins (DRF) of mammals are activated by Rho GTPases. DRF displays an auto inhibitory conformation resulting from internal interaction between the dimerization domain (DID) at the N-terminus and the diaphanous autoregulatory domain (DAD) at the C-terminus. Rho GTPase binds a domain near DID at GBD (G protein-binding domain), and this binding causes the DRFs to be in active state initiating polymerization activity [95, 107]. The crystal structure of the EhRho1-γSGTPase-Ehformin1 complex suggested that the mechanism of Ehformin1 autoinhibition and activation in *E. histolytica* is different from that of the mammalian counterpart [108].

Table 13.3 Rho subfamily [105]

Subfamily	Species	Function
RND	**Human (Hs)**	Stress fiber formation, focal adhesions
	HsRND1(Rho6)	
	HsRND2(RhoN)	
	HsRND3(RhoE)	
Rho	**Human (Hs)**	Stress fiber formation, focal adhesions
	HsRhoA	
	HsRhoB	
	HsRhoC	
	***Saccharomyces cerevisiae* (Sc)**	
	ScRho1	
	ScRho2	
	ScRho3	
	E. histolytica	
	EhRho1	
RhoDF	**Human (Hs)**	Filopodium formation
	HsRhoD	
	HsRhoF(Rif)	
Rac	**Human (Hs)**	Membrane ruffle formation, lamellopodium-formation at leading edge, phagocytosis
	HsRac1	
	HsRac2	
	HsRac3	
	HsRhoG	
	E. histolytica	
	EhRacA	
	EhRacB	
	EhRacC	
	EhRacD	
CDC42	**Human (Hs)**	Cellular polarity and actin cytoskeleton, filopodium formation
	HsCDC42	
	HsRhoJ(TCL)	
	HsRhoQ(TC10)	
	***S. cerevisiae* (Sc)**	
	ScCDC42	
RhoUV	**Human (Hs)**	Filopodium formation
	HsRhoU(Wrch)	
	HsRhoV(Chp)	
RhoH	**Human (Hs)**	
	HsRhoH(TTF)	
RhoBTB	**Human (Hs)**	
	HsRhoBTB1	
	HsRhoBTB2(DBC-2)	

RhoA activates Rho-associated kinase (ROCK), resulting in a cascade involving Lim kinase (LIMK) and cofilin, which in turn regulates actin cytoskeleton dynamics [109, 110]. When *E. histolytica* trophozoites were incubated with fibronectin and lysophosphatidic acid, ROCK-2-like protein was found to be involved in stress fiber formation along with EhRho1 [111]. Rac is a subfamily of the Rho family GTPases that has also been described in *E. histolytica*. Although functional participation of this molecule in cytoskeleton dynamics is not known, it is clear that it does participate in surface processes and pathogenesis as overexpression of a constitutively active EhRacA or EhRacG resulted in inhibition of phagocytosis and surface-receptor capping [111, 112].

Dbl family proteins function as GEF, initially identified as a transforming factor from a diffuse B-cell lymphoma: these carry a Dbl homolgy domain (DH) and a Pleckstrin homology domain (PH). The DH domain is involved in GTP/GDP exchange [113], whereas PH domain anchors the protein to the membrane through phosphoinositides [114]. Three GEF belonging to the Db1family (EhGEF1, EhGEF2, and EhGEF3) have been identified in *E. histolytica*. EhGEF1 encodes a 69-kDa protein that preferentially activates and colocalizes with EhRacG at the plasma membrane on treatment with concanavalin A, suggesting that EhGEF1 plays a role in cytoskeleton dynamics, capping, and uroid formation. Therefore, it appears that EhGEF1 may act as a bridge between EhRacG signaling and cytoskeleton dynamics [115]. In vitro studies have shown that EhGEF2 can activate EhRacA, EhRacB, EhRacC, EhRacD, EhRacG, EhRacH, and EhCdc42, although EhRacG is the main target. EhGEF2 displays novel features not present in other Db1 family proteins, such as the presence of five armadillo (Arm) repeats, the absence of a PH domain, and the presence of a DH domain that binds phosphatidic acid (PA) [116]. EhGEF3 encodes a 110-kDa protein containing DH and PH domains. The DH domain of EhGEF3 is closely related to the DH domain of the Vav3 protein. It activates EhRacA and EhRho1 in vitro and colocalizes with EhRacA at the rear end of migrating cells [117].

These observations suggest that intricate signaling networks are involved in regulating actin dynamics and cell proliferation although the overall features are similar with respect to other eukaryotes. There are some distinctive differences, probably caused by the need for a high level of actin dynamics.

13.5.2.2 p21 Activated Kinases (PAK)

P21-activated kinases are serine/threonine kinases that were initially identified as a RhoGTPase-binding partners in rat brain cytosol [118]. Rac and Cdc42, but not RhoA, interact with 62-, 65-, and 68-kDa proteins in a gel overlay assay. These three proteins were later identified as isoforms of PAK (PAK1, PAK2, PAK3) [119]. Six isoforms of PAK are classified into two groups (group I and group II) on the basis of their regulatory mechanism and domain architecture [120, 121]. Group I PAKs (PAK1–3) are similar to ste20, a kinase from budding yeast, and are activated upon binding to Rho GTPases, Cdc42, and Rac1. Group II PAKs (PAK4–6) are not

similar to ste20 and do not require GTPase for activity, but presence of GTPases influences their localization [122].

In general, PAK has an N-terminal regulatory domain and a C-terminal kinase domain. The regulatory domain of group I PAKs contains a Cdc42 and Rac-interactive binding domain (CRIB) and an autoinhibitory switch domain (AID), which is absent in group II PAKs. The regulatory domain of PAKs consists of a canonical PXXP SH3-binding motif and a nonclassical (PXXP) SH3-binding site that interacts with adapter proteins, leading to recruitment of PAK at the membrane in response to tyrosine kinase receptor activation [122, 123]. Different isoforms of PAK proteins are expressed differently in various tissues. PAK1, PAK3, and PAK5 are predominantly expressed in brain, whereas PAK4 is highly expressed in prostate, colon, and testis, PAK6 in testis and prostate, and PAK2 ubiquitously [124–126]. Gene knockout mouse models of Pak2 or Pak4 showed embryonic lethality in contrast to deficiency in Pak1, Pak3, and Pak5, which are not lethal [120, 125, 127]. PAK phosphorylation at threonine and serine residues are an essential requirement for its activation; it has been shown that 3-phosphoinositide-dependent kinase-1 (PDK1) activates PAK1 by phosphorylating Thr423 residue [128]. PAK1 and PAK4 play a role in cytoskeleton dynamics. Activation of PAK1 leads to formation of lamellipodia, filopodia, and membrane ruffles and phosphorylates components of cytoskeleton machinery, such as P41-ARC (ARP 2/3 complex 41-kDa subunit) [129]. PAK4 is a member of a multiprotein complex that consists of slingshot phosphatase (SSH-1L), LIMK, actin, and the scaffold protein 14-3-3z that regulates cofilin [130]. PAK-regulated cytoskeleton dynamics by phosphorylating key components of cytoskeleton is thought to be one of the key control features in actin cytoskeleton dynamics. For example, PAK phosphorylates myosin I isoforms and regulatory light chain (RLC) of nonmuscle myosin in *Acanthamoeba*. In *Dictyostelium*, DdPAKB phosphorylates the heavy chain of myosin Ib, resulting in the activation of myosin.

Fourteen putative PAKs and two PAK-like proteins are encoded by the *E. histolytica* genome. EhPAK1 has a kinase domain but no CRIB-binding domain. However, a fragment of 150 amino acid residues at the N-terminus binds to activated Rac1, but not to Cdc42. EhPAK1 is involved in cell migration, cell polarity, and erythrophagocytosis. The requirement of the kinase domain of PAK in these functions has been recognized. The overexpression of EhPAK1 causes a significant reduction in cell mobility, and an increase in the rate of erythrophagocytosis has been observed [131].

EhPAK2 contains an N-terminal PH domain and a highly conserved CRIB domain. The EhPAK2 CRIB domain is similar to DdPAKC from *D. discoideum* and Cla4p from *Saccharomyces cerevisiae*. It binds EhRacA GTPase both in vitro and in vivo. EhPAK2 is required by trophozoites for invasion and also plays a role in capping. This role was recognized when trophozoites were transfected with a vector containing pExEhNeo/EhPAK2-myc, pExEhNeo/Nterminal-myc, and pExEhNeo/kinase-myc (that is, myc-tagged full-length EhPAK2, myc-tagged N-terminal domain, and myc-tagged kinase domain of EhPAK2, respectively, under G418 selection). Overexpression of kinase-myc displayed a 90 % decrease in its ability to

invade collagen matrix and a defect in capping. Instead of a single cap, multiple concanavalin A cap sites were observed on the same cell. Impairment in cytokinesis was also observed as giant and multinucleated cells were visible. The cells expressing either myc-tagged full-length EhPAK2 and myc-N-terminal domain of EhPAK2 did not show any alterations in these properties, suggesting the importance of the kinase domain in the function of EhPAK2 [132].

EhPAK3 contains a GTPase-interacting (CRIB), an N-terminal PH, and a C-terminal kinase domains. In unstimulated trophozoites, EhPAK3 is uniformly distributed in cytoplasm; however, it accumulates at caps upon concanavalin A stimulation, suggesting its involvement in defence mechanism against host immune response. EhPAK3 shows maximum similarity with *D. discoideum* PAKC (DdPAKC) and also forms a group with DdPAKA, Dd Myosin I heavy-chain kinase (DdMIHCK), and EhPAK2 [133].

13.6 Conclusion

It is quite clear that *Entamoeba histolytica* displays an extensive signaling network managed by a vast array of components, mainly consisting of calcium-binding proteins and protein kinases. Although it has retained many features of eukaryotic signaling systems exemplified by the presence of conserved molecules, it also uses some unique mechanisms that are found only in this organism.

In *E. histolytica*, Ca^{2+} has been shown to play an important role in pathogenesis and cytopathic activity. Ca^{2+} also controls actin dynamics and regulates processes such as endocytosis, phagocytosis, adherence, cytolysis, and virulence. The components, such as IP_3, IP_4 and P-type Ca^{2+} ATPases, are present in *E. histolytica*. None of the Ca^{2+} ATPases or store-operated channels has been investigated although its genome analysis has revealed six putative P-type Ca^{2+}-ATPases. Of 27 putative EhCaBPs, only 4 have been functionally characterized in *E. histolytica*. EhCaBP1, EhCaBP3, and EhC2PK participate in phagocytosis; URE-3B is a negative transcription regulator controlling expression of pathogenesis-related genes. The existence of signaling through G-protein-coupled receptors and second-messenger cAMP has been documented. However, detailed mechanisms involved in different signal transduction pathways have not yet been elucidated. The *E. histolytica* genome encodes a large repertoire of protein kinases of different types. TMKs make a sizable part of the kinome and are involved in multiple functions, such as cell proliferation, endocytosis, and virulence. As *E. histolytica* shows a very high rate of actin-dependent processes (pseudopod formation, phagocytosis), signaling pathways that regulate actin cytoskeleton dynamics have an essential role. Therefore, it is not surprising that many of the conserved actin dynamics regulatory molecules are present in these cells. Unfortunately, molecular mechanisms of only a handful of signaling molecules have been studied so far, and there is a need to expand this line of research for the clear understanding of amebiasis.

Acknowledgments The authors thank Department of Biotechnology and BNP Paribas for financial support for carrying out some of the authors' work described here. A.B. also thanks the Department of Science & Technology for the JC Bose Fellowship.

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Chapter 14
Transmembrane Kinases and Their Role in *Entamoeba histolytica* Pathogenesis

Nathaniel C.V. Christy and William A. Petri Jr.

Abstract The transmembrane kinases (TMKs) of *Entamoeba histolytica* are a recently discovered family of cell-surface proteins that bear sequence similarity to the intermediate subunit of the Gal/GalNAc adherence lectin. The TMKs have been implicated in growth response to serum components as well as the phagocytosis of host cells and bacteria, processes that are critical to amebic pathogenesis during infection of the human host. Members of the TMK family appear to function variously as active kinases or pseudo-kinases depending on the structure of their intracellular domains and likely interact with intracellular signaling networks during signal transduction.

14.1 Introduction

"It would, perhaps, be one step towards the establishment of a rational mode of treatment in this very destructive form of dysentery, if the nature of the connection subsisting between the affection of the bowels and that of the liver were clearly ascertained, and the manner in which the one supervenes to the other were closely observed." (Annesley, p. 385) This quote, from an 1841 description of hepatic dysentery in India by the physician James Annesley, highlights the perplexing dual nature of infection of both the intestine and the liver in human hosts by *Entamoeba histolytica* [1]. Known today as amebiasis, this disease has been observed since ancient times [2], but Annesley's report was an early observation of the link between liver abscesses and the pathology seen during intestinal dysentery caused by *E. histolytica*. The molecular and cellular mechanisms that permit *E. histolytica* to infect such distinct physiological niches of the host remain in part a mystery to this day. Because of the nature of *E. histolytica* as a single-celled organism, the receptors and cell signaling components on the surface of the parasite represent an important interface with the host during survival and replication in differing compartments. Regardless of its location, the parasite must be able to adhere to host cells and

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_14

compartments such as the colonic epithelium, induce widespread contact-dependent host cell death, and ingest these cells and other host components to survive, as well as to respond to a myriad of other signals that inform the parasite as to its external environment [3, 4]. It can easily be speculated that the parasite modulates its survival during different stages of host exposure at least partially through its diverse repertoire of cell-surface sensors and their ability to multitask in these various environments. Although a number of amoebic surface molecules that are important in pathogenicity have been discovered and characterized, the discovery of a novel family of cell-surface proteins known as the transmembrane kinases (TMK) promises to shed further light on how *E. histolytica* interacts with the host during its varied stages of infection. This section focuses on the characterization to date of members of the TMK family as signaling molecules during amebic pathogenesis as well as speculate on the signaling mechanisms that TMK family members may use during signal transduction.

14.2 Discovery of the TMK Family

14.2.1 Initial Characterization

The discovery of the TMK family stems from a key event that has itself contributed to our understanding of the pathogenic biology of *E. histolytica*: the identification of the amebic cell-surface Gal/GalNAc adherence lectin. It was known that pathogenicity was linked with the ability of trophozoites to adhere to mucins in the colonic epithelium and that this was likely caused by a parasite galactosamine-binding lectin [5]. However, the identity of this molecule remained undiscovered until the purification of the galactose/*N*-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin [6, 7]. The 260-kDa Gal/GalNAc lectin complex was also found to be an immunodominant surface antigen expressed during parasitic infection of both the intestine and the liver [8]. This adhesin was further characterized as having two unique subunits: a "heavy" lectin (Hgl) of 170 kDa and a "light" subunit (Lgl) of 35 kDa [9]. The Hgl lectin was observed to have a cysteine-enriched extracellular region with a carbohydrate recognition domain (CRD) [10]. A third "intermediate" subunit (Igl) of 150 kDa was also discovered by biochemical purification [11]. When purified together, both the Hgl and Igl subunits were able to bind galactose and galactosamine-containing residues via the cysteine-rich regions of their extracellular domains [12, 13]. In contrast to the biochemical approaches used to discover the Gal/GalNAc lectin, the recent rapid expansion of genetic information leading to the completion of sequencing of the *E. histolytica* genome [14] also led to the identification of a large family of more than 80 putative cell-surface proteins during sequence analysis of the Igl lectin subunit [11]. These open reading frames (ORF) were unique in that they shared significant sequence similarity to the Igl lectin with a number of CXXC-based motifs in their extracellular domains.

Group[a]	Transmembrane kinase no.	Size[b]	No. of amino acids, TM to kinase[c]	Signature motif in kinase	No. of amino acids, kinase to end[d]	No. of motifs[e]			
						CXC	CXXC	CXXXC	CXXCXXGYY
A	4, 17, 23, 25, 52, 53, 55, 61, 65, 68, 69, 72, 85	517–532	220	CC(I/V)KITDFGTSR[f]	40	4	4/5	3/4	3
B1	5, 12, 43, 76, 86, 91, 95, 100, 101, 103, 104	897–916	135	KLTDFGS(A/S)R[g]	0	1	25	2	9
B2	2, 8, 10, 11, 14, 15, 31, 36, 41, 62, 74, 75, 77, 87, 88, 92, 94, 105	822–1762	133	KLTDFGS(A/S)R[h]	0	0	90	0	25
B3	21, 28, 29, 30, 32, 35, 37, 38, 42, 48, 51, 96	830–2117	145	KLTDFGS(A/S)R	0	0	24	0	7
C	9, 13, 39, 60, 63, 71	547–624	160	C(A/G)KLTDFGTC[i]	59–82	0	33	0	11
D1	3, 18, 40, 56, 70, 79	520–619	234	PITAKVTDFGTS	63	3	5	3	7
D2	19, 27, 44, 46, 50, 57, 64, 67, 82, 97, 98	399–614	233	V(T/V)(C/X)KV(T/S)DFGTS	55	4	5	3	9
E	22, 54, 66	401–412	150	AKLSDFGTSR	60–97	1	0	0	0
F	34, 45, 59, 80	231–368	24	VKVSDFGLS and WXAPE	0	0	0	0	0

[a] Group G transmembrane kinases are diverse and include kinases 1, 6, 16, 24, 47, 49, 73, 79, with no common signature motif or other features within the group or with other groups.
[b] Number of amino acid residues in the extracellular domain.
[c] Amino acids from the C terminus of the transmembrane domain to the N-terminal glycine of the kinase domain.
[d] Amino acids from 10 residues after the conserved arginine to the stop codon.
[e] Number of times each motif is found in the protein sequence. The number of CXC, CXXC, CXXXC, and CXXCXXGYY motifs may vary between individual family members. Values with a slash indicate that some family members have 4 or 5, or 3 or 4, of the motif.
[f] Group A TMKs can additionally be identified by a moderately conserved (K/R)XXXDI(E/N)I(Y/F)KQQQPXYYYYIXGSXXXPXKXX(K/R)Y motif C-terminal to the transmembrane domain.
[g] Group B1 TMKs can be identified by a KRKEKKEREKTTIFKTITQSNI(K/R)FI(S/P)LGDG sequence after their transmembrane domain.
[h] Group B2 TMKs can be identified by a PV(N/G)(Q/E/K)E(S/T)(K/R)DL(L/I)ClGNXXKXXXKVQ sequence after their transmembrane domain.
[i] Group C TMKs can be identified by a RRR(K/R)XXKXXXXXIKPF(H/K)VSSD(L/V)ELXLL sequence following the transmembrane domain.

EUKARYOTIC CELL, Apr. 2005, p. 722–732 Vol. 4, No. 4
1535-9778/05/$08.00_0 doi:10.1128/EC.4.4.722–732.2005
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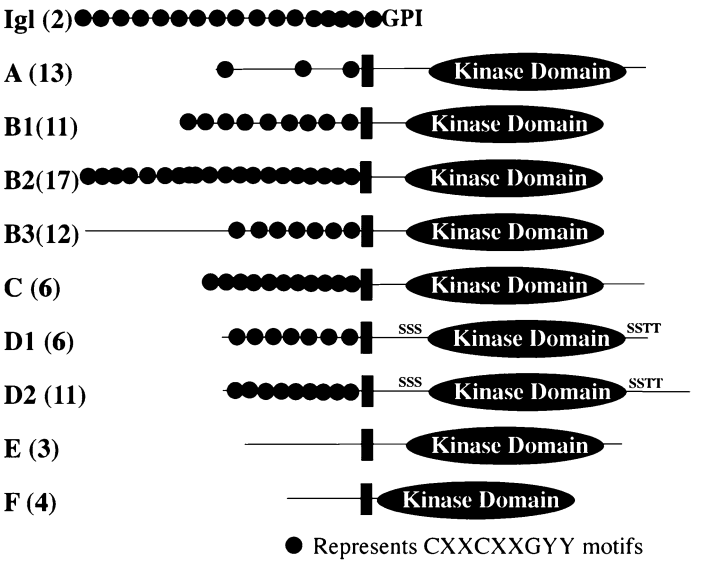
Fig. 14.1 Properties of the domains of the *Entamoeba histolytica* transmembrane kinase groups. (From Beck et al. [16])

However, this tentative grouping as a gene family did not point to functional attributes other than the cysteine-rich regions that indicated a potential to sense the extracellular environment, suggested by the limited similarity of the Igl subunit to the variant surface proteins (VSP) of *Giardia* [11, 15]. Beck et al. subsequently conducted an exhaustive phylogenetic analysis on this gene family and found that many of its members contained a putative kinase domain [16]. Further, these genes were organized into six main families and nine distinct groups or subgroups (groups A, B1, B2, B3, C, D1, D2, E, and F), according to common signature motifs in the kinase domain as the number and composition of CXXC repeats in each gene varied (Fig. 14.1), although differences in the extracellular domains could distinguish between subgroups. Known collectively as the transmembrane kinase family (TMK), the common features of the TMKs included a long extracellular domain, a single-pass transmembrane domain, and an intracellular kinase-containing region (Fig. 14.2). Beck et al. then determined the transcript levels of various TMKs by both oligoarray and reverse transcriptase-polymerase chain reaction (RT-PCR) and found that members of each group were expressed during mid-log-phase growth though expression varied widely for different TMKs. Polyclonal antibodies raised to the kinase domains of TMK members showed localization of multiple kinases to the plasma membrane, confirming surface expression. Also, although many of the newly identified TMKs had all the major conserved features of an active kinase domain, some members were missing one or more, raising the possibility that these TMKs could serve as pseudo-kinases or be implicated in antigenic variation and immune evasion by the parasite [16].



EUKARYOTIC CELL, Apr. 2005, p. 722–732 Vol. 4, No. 4
1535-9778/05/$08.00_0 doi:10.1128/EC.4.4.722–732.2005
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Fig. 14.2 Organization of the transmembrane kinase (TMK) family. Diagram of Igl and the TMKs shows the approximate sizes of the different proteins and the distribution of the CXXCXXGYY motifs in the extracellular domain (*black circle*). The CXXCXXGYY motifs are part of a larger motif, CXXCXXG(Y)(Y/F)(L/V/F/Y/M)-Polar–Polar, which also can begin with CXC instead of CXXC. GPI represents a putative GPI anchor. *Black rectangle* indicates a transmembrane domain, and *black oval* indicates a putative kinase domain. Serine (SSS)- and serine/threonine (SSTT)-rich regions found in groups D1 and D2 are shown. Numbers in *brackets* indicate numbers of known family members. (From Beck et al. [16])

14.2.2 Brief Survey of the E. histolytica *TMKs in Comparison to Other Receptor Kinase Families*

The discovery of the TMK family in the genome of *E. histolytica* was exciting because similar receptor kinase families are well known in a number of other organisms. These other receptor/kinase families point to potential roles for the TMKs. For example, the high number of TMK family members in the amoebic genome is similar to that seen in plant species. More than 400 genes termed plant receptor kinases (PRKs) have been identified in the *Arabidopsis* genome [17]. The PRKs are primarily serine/threonine kinases resembling the transforming growth factor (TGF)-β receptors but do not share similarity in their extracellular domains, indicating that they may have a wide range of ligand recognition specificities and functional differences, including the recognition of ligands from both endogenous plant and exogenous bacterial produced sources [18]. As is discussed further, evidence thus far indicates that different TMK families may also have varying functions and roles.

The human genome encodes another group of receptor kinases, the receptor tyrosine kinases (RTKs) with 58 individual members in 20 subfamilies that have a similar structure composed of an ecto-domain, a single transmembrane domain, and a kinase- or pseudo-kinase-containing intracellular region [19]. One of the most well known metazoan RTK members is the epidermal growth factor receptor (EGFR), which controls growth and proliferation in response to extracellular ligand binding-induced stimuli from circulating growth factors. The cysteine-rich extracellular domains of the RTK family members contain a high number of repeated motifs that promote dimerization and mediate ligand binding. As noted above, the *E. histolytica* TMKs were originally identified and classified by similar cysteine-rich repeats in their ecto-domains; it is reasonable to suspect that these repeats serve a similar function for both ligand binding and dimerization in the TMK family. Following homodimerization or dimerization with other family members, the EGFR autophosphorylates to become enzymatically active on other substrates and to serve as a docking site for adaptor proteins such as the SH2 domain-containing Grb2 [20]. Although no easily identified docking sites have been noted on the intracellular regions of amebic TMKs, it is possible that some docking proteins (e.g. SH2 domain-containing) are present during TMK signaling in *E. histolytica* [14].

Aside from the RTKs, amebic TMK members may also signal similarly to receptor serine/threonine kinases (RSTKs) such as the TGF-β receptor superfamily, the different members of which recruit and phosphorylate each other in sequence as a heteromeric complex after ligand binding [21]. A cursory evaluation of the intracellular regions of one TMK member, TMK39, and the TGF-β receptor I shows a region of alignment of 31 % amino acid identity, with similarity of 48 %, likely caused by the commonality of the kinase regions of these proteins. Such TMK-containing oligomeric complexes may be found to be present in the cell-surface lipid rafts described by Mittal et al. [22].

Finally, several other protozoal species maintain families of cell-surface proteins that may bear functional similarity to the TMK family. Although *Entamoeba* species including *E. histolytica* and *E. dispar* are relatively unique among single-celled protozoa in expressing TMKs with kinase domains, some TMK members may be similar to the kinase-deficient VSPs of *Giardia* species and trypanosomatids, briefly mentioned previously, in their provision for immune evasion by the alternate expression of different cysteine-rich ecto-domain-containing members of the VSP families [15, 23, 24].

14.3 Functional Characterization of the TMKs

14.3.1 Role in Growth Response

The first evidence of a functional role for a transmembrane kinase member emerged during studies by Mehra et al. on the B1 family of TMKs [25]. The Mehra et al. analysis utilized the newly published genome data for *E. histolytica*, which identified a total of 90 TMKs; 28 constituting the B1 family [14]. However, Mehra et al.

showed by RT-PCR that only a few of these were expressed and when antibodies were raised to the B1.I subfamily, these TMKs exhibited localized expression to the amebic surface at uroid and pseudopod regions in a manner similar to that seen by Beck et al. (discussed earlier). *E. histolytica* trophozoites expressing a C-terminus-truncated version of the B1.I TMK (also known as EhTMKB1-2) that lacked its cytoplasmic kinase domain allowed Mehra et al. to study the function of this TMK in a dominant-negative model system, finding that amoebic growth was significantly abrogated; this was the first indication that the intracellular kinase domain was critical in the function of a TMK and that signaling through phosphorylation might be a critical component of receptor activity by the TMKs. Interestingly, the B1.I TMK dominant-negative-expressing amoebae also showed a very poor growth recovery after restoration from a serum starvation state, suggesting that these cells lacked a normal response to a factor in the serum medium component. The studies of Mehra et al. were especially significant in that they showed at least one TMK family member to bear a *functional* as well as structural resemblance to higher metazoan growth factor receptors such as the EGFR family [25].

A later study by Shrimal et al. found that another member of the B1 TMK family, EhTMKB1-9, had higher expression levels than EhTMKB1-2 or any other B1 TMKs and was also localized to the surface of trophozoites [26]. Interestingly, the fraction of this receptor expressed in comparison to other B1 family members was proportional to the restoration of serum following serum starvation of *E. histolytica* trophozoites. The Shrimal et al. studies showed that this was the result of control by an upstream repressive promoter. Further, in a dominant-negative model system, this serum response modulated growth of the parasite and had effects on target cell adhesion and killing, possibly secondarily caused by the growth defect. Finally, EhTMKB1-9 was also shown to be an active kinase with both serine/threonine and tyrosine specificity, the first such instance of a TMK predicted kinase activity being confirmed in vitro, although an amebic substrate has not yet been found. The latest studies by Shrimal et al. have identified unsaturated fatty acid lipids in serum as the regulator of the promoter element controlling EhTMKB1-9 [27].

Work by Abyankar et al. showed that the EhTMKB1-9 dominant-negative model conferred a defect in colitis during *E. histolytica* mouse infection, although liver abscess formation was not changed [28]. This study also found that EhTMKB1-9 showed a small but significant defect or increase in erythrophagocytosis and fluid-phase pinocytosis during dominant-negative and healthy full-length expression, respectively, although these results were not directly linked to a binding/receptor activity by this TMK.

14.3.2 Control of Phagocytosis Mechanisms

E. histolytica has the opportunity to phagocytose extracellular targets in different host environments ranging from bacteria and epithelial host cells in the intestine to erythrocytes and other host cells during dissemination to sites such as the liver [29, 30]. The propensity for ingestion has been shown to be uniquely correlated

with the pathogenicity of the parasite [31, 32]. *E. histolytica* also has recognizable internal signaling machinery for the control of the initiation and maturation of phagocytosis [33–35]. However, only a few amoebic receptors that actually recognize extracellular targets have been identified, and little information exists to link such receptors with known intracellular signaling components. Further, *E. histolytica* trophozoites are likely to maintain a multiplicity of receptors engaging unique signaling pathways for the ingestion of differing targets similar to mechanisms by metazoan professional phagoctyes such as macrophages [36]. To identify new receptor candidates, multiple groups performed proteomic analyses of early timepoint phagosomes utilizing mass spectrometry approaches [37–39]. Boettner et al. found that a TMK B3 family member, christened PATMK, was present at early timepoints (5 and 10 min) of purified phagosomes, indicating it may serve as a cell-surface receptor [40]. Antibodies to the extracellular region of PATMK showed that it was present on the cell surface and confirmed that it internalized with ingested carboxylate-modified beads. Rates of erythrocyte ingestion by trophozoites were inhibited in multiple experiments, including antibody pre-incubation, the expression of a dominant-negative version of PATMK, and reduced native protein expression through an interfering short hairpin RNA strategy. The dominant-negative PATMK-expressing cells also exhibited lower pathogenicity in a mouse intestinal colitis model but were still not deficient in liver abscess formation.

In a comprehensive study of TMK gene expression, Buss et al. used laser capture microdissection in conjunction with microarray analysis to determine the concurrent presence of TMK family member transcripts in a single cell [41]. Multiple TMK genes were expressed at the same time in individual cells, indicating to the authors that the individual TMK members served functional roles versus contributing to antigenic variation by the parasite. Two TMK members, TMK54 (E family) and TMK39 (C family), were then compared in localization, expression, and function. When stained with specific antibodies, TMK54 showed a general distribution on the cell surface whereas TMK39 was present in punctate spots across the plasma membrane. Also, in dominant-negative-expressing cell lines for both TMKs, disruption of TMK54 but not TMK39 led to a deficiency in growth. TMK39 dominant-negative expression singularly led to a defect in phagocytosis. This inhibition was specifically limited to apoptotic Jurkat T lymphocytes and not erythrocytes, indicating that TMK39 might serve as a receptor with recognition for specific targets. Utilizing the same dominant-negative model, further studies by Christy et al. on TMK39 showed that it also mediated the ingestion of bacteria [42]. The ingestion defect of bacteria by the TMK39 dominant-negative cells was matched by a twofold increase in bacterial adhesion, which was the first evidence of direct binding to an extracellular target by a TMK member, further supporting its role as a cell-surface receptor.

14.4 Perspective

From its origins from the sequencing of the genome of *Entamoeba histolytica*, the discovery of the transmembrane kinase family has informed our understanding of how this parasite successfully interacts with and responds to the myriad of differing

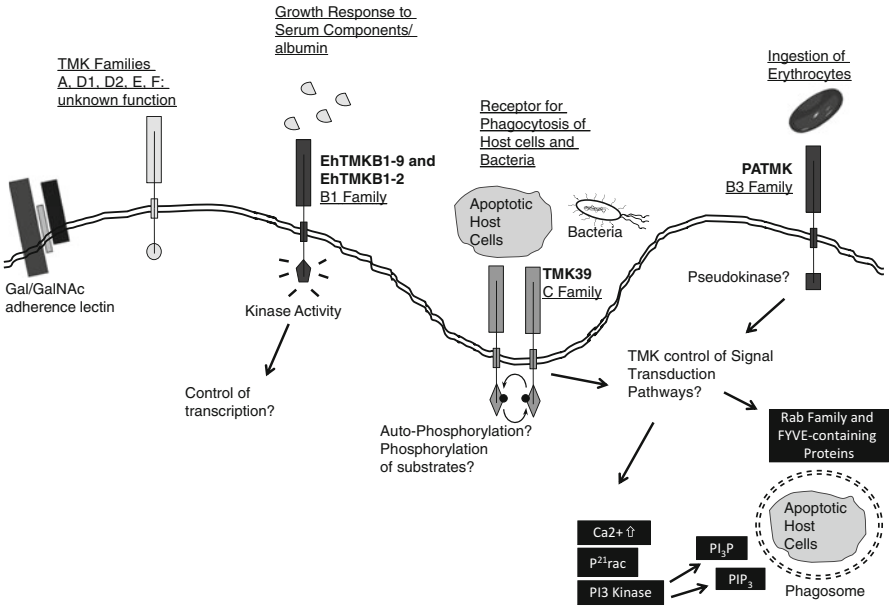

Fig. 14.3 Function and signal Transduction by the transmembrane kinases. Members of the TMK families A, D, E, and F do not have assigned function or ligand recognition. EhTMKB1-9 and EhTMKB1-2 have been implicated in growth response to serum components. EhTMKB1-9 is an active kinase and may phosphorylate amoebic substrates. TMK39 regulates the phagocytosis of apoptotic host cells and bacteria and may autophosphorylate as part of its activation. PATMK does not have all the requisite elements of an active kinase but regulates the internalization of erythrocytes and may function as a pseudokinase. It is likely that these transmembrane kinases interact with known amoebic intracellular signaling networks that control processes such as the development of phagosomesy

environments in its host (Fig. 14.3). The discovery that several TMK members play a key role during growth and response to serum components is not surprising given the structural similarity of the TMK family to metazoan growth factor receptors such as the EGFR [43]. That other TMKs are critical in the regulation of phagocytosis and may serve as direct receptors during this process is also interesting because the extracellular domains of some TMKs bear similarity to that of several macrophage scavenger receptors. The EGFR receptor itself has been shown to modulate phagocytosis, although this is a nonclassical role for this receptor [44]. Studies thus far have used the dominant-negative model of receptor characterization to great effect, which points to the key role that the intracellular kinase domains of TMK members are likely to play in signal transduction, although it is also possible that some TMKs function as pseudo-kinases, in a fashion similar to the metazoan HER3 receptor [45]. In this model, TMKs with and without kinase activity could function as hetero-oligomeric receptors similar to the HER family, with varying signaling outcomes based on combinations of different receptors [46, 47]. Future studies on these

and other remaining questions concerning the function and role of the transmembrane kinase family promise to greatly enhance our understanding of the structure and biological role of the cell-surface receptors of *E. histolytica*.

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Chapter 15
Cell-Surface Molecules as Virulence Determinants in *Entamoeba histolytica*

Daniela M. Faust and Nancy Guillen

Abstract Virulence of the human parasite *Entamoeba histolytica* is related to adherence to, killing, and phagocytosis of host cells, and contact between trophozoites and host cells is necessary for amoebic effectors to act. Virulence also relies on parasite motility in extracellular matrices and human tissues and on the capacity of the parasite to escape the host defense response and to trigger inflammation. Amoebic surface components implicated in all these processes have been identified as important virulence factors. This chapter presents current knowledge on the major surface molecules of *E. histolytica* critically involved in parasite interactions with healthy or apoptotic cells (Gal/GalNAc lectin, LPG/LPPG proteophosphoglycans, KERP1, CPADH, STIRP, proform of CP-A5, ROM1), in recognition of apoptotic cells (calreticulin, M17, C2PK, SREHP, TMK96), host cell phagocytosis (CPADH, SREHP, TMK96, ROM1), attachment to extracellular matrices (β1FNR, β2 integrin-like), and immune evasion or triggering the inflammatory response (Gal/GalNAc lectin, KERP1, proform of CP-A5, LPG/LPPG).

Abbreviations

ALA	Amoebic liver abscess
CC	Coiled-coils
CP	Cysteine protease
ECM	Extracellular matrix
Gal/GalNAc	Galactose- and *N*-acetyl-D-galactosamine
GPI	Glycosylphosphatidylinositol
LPGs	Lipophosphoglycans
PPGs	Proteophosphoglycans
PS	Phosphatidylserine

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© Springer Japan 2015

T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_15

RGD	Arginine-glycine-aspartic acid
TM	Transmembrane
TNF	Tumor necrosis factor

15.1 Introduction

Host–microbe interactions contribute to shape the infection outcome by initiating signaling events in both cells, for example, thus triggering their respective responses. Some of the interactions involve the microbial and host cell surfaces, and knowledge of the nature and the functions of these surfaces is crucial for a proper understanding of microbial pathogenicity. The study of the heterogeneous and chemically complex microbial surfaces is critically important because they display components associated with virulence, such as adhesins, and surface structures are often major targets for immune responses. *Entamoeba histolytica* employs diverse strategies for immune evasion, such as the cysteine protease (CP)-mediated degradation of complement components, the inhibition of the membrane attack complex assembly, and the surface receptor capping. Amoebic surface targets for the host immune system are clustered upon ligand binding and subsequently translocated toward particular membrane structures, called uropods, with which they are then released into the extracellular medium. Surface receptor capping and shedding enables *E. histolytica* to discard bound, harmful substances such as anti-amoeba antibodies and complement components [1, 2].

The major surface components of *E. histolytica* involved in interactions with the extracellular matrix (ECM) and with cells from intestine or liver, in phagocytosis and in immune evasion, have been extensively studied aiming to discover key factors in amoebic virulence. So far, the best-characterized molecule is the galactose- and *N*-acetyl-galactosamine (Gal/GalNAc)-inhibitable lectin that is believed to mediate trophozoite adhesion to intestinal epithelial cells by high-affinity interactions with cell-surface glycoproteins [3]. Glycosylphosphatidylinositol (GPI)-anchored proteophosphoglycans (PPGs) are the most abundant trophozoite surface molecules. As for the Gal/GalNAc lectin, anti-PPGs antibodies reduce parasite adhesion and cytotoxicity, suggesting an important role of PPGs in parasite–host interactions [4]. More recently discovered, the lysine- and glutamic acid-rich protein KERP1 is specific for *E. histolytica*, localizes on the trophozoite plasma membrane, and binds to the host cell surface [5]. Besides Gal/GalNAc lectin, further uropod-associated surface components as the immunodominant antigen M17, the serine- and threonine-rich protein STIRP, and the adhesin ADH112 [2] are of interest as adhesion molecules and for the impact of their elimination on amoebic immune escape.

In addition to cell adhesion molecules (Table 15.1), receptors for the recognition of apoptotic host cells have been identified, including calreticulin [32], C2 domain-containing protein kinase C2PK [33], serine-rich protein SREHP [34], and transmembrane (TM) kinase TMK96 [35]. Apoptotic cells are preferentially phagocytosed by the parasite, and their clearance not only impacts parasite survival (providing nutrients) but also contributes to the modulation of the host immune

Table 15.1 *Entamoeba histolytica* cell-surface components involved in adhesion to extracellular matrix (ECM) and cells

Factor	Subunits/domains	Function in process	References
Gal/GalNAc lectin	Hgl subunit	– Adhesion intestinal mucus	[3, 6]
	– Type 1 TM	– Adhesion healthy host cells	[7]
	– C-rich domain with CD59 homology	– Signaling for amoebic actin cytoskeleton organisation	[8–12]
	– Carbohydrate-binding domain	– Inhibition complement MAC[a] formation	
	– β2-/β7-integrin signalling domain	– Immune escape	
		– Recruitment inflammatory cells during ALA	
		– Host cell killing	
	Lgl subunit	– Adhesion host cells	[7]
	– Signal peptide	– Host cell killing	[13–15]
	– GPI-anchor domain	– Phagocytosis	
		– ALA formation	
		– Capping	
	Igl subunit	– Not identified	[16]
	– GPI-anchor domain		[17]
	– C-rich		
	– CXXC and CXC repeats		
	– EGF-like domain of β1-integrin		
KERP1	– K-, E-rich	– Adhesion host cells	[5, 18, 19]
	– α-helical	– Triggering inflammation	
	– Coiled-coil		
CPADH112	ADH112 subunit	– Adhesion host cells	[20–22]
	– Adhesion domain	– Cytotoxicity	
	– Bro1 domain	– Phagocytosis	
	– Potential Src kinase phosphorylation site	– Potential role in vesicular trafficking	
	CP112 subunit	– Cysteine protease	[20]
	– TM domain		
	– RGD motif		
STIRP	– S-, T-, I-rich	– Adhesion host cells	[23, 24]
	– Single TM domain	– Cytotoxicity	
	– Signal peptide		
β1FNR (140 kDa)	– CXXC repeats	– Adhesion FN	[17, 25–27]
	– EGF-like domain of β1-integrin		
β2-integrin like	– Not Identified	– Potential adhesion endothelial cell ICAM-1	[28]
proCP-A5	– RGD motif	– Binding αvβ3-integrin	[29]
		– Adhesion host cells	
		– Inflammatory response	
ROM1	– Seven-pass TM	– Adhesion healthy host cells	[30, 31]
		– Phagocytosis	
		– Cleavage Hgl in vitro	

[a]*MAC* membrane attack complex

response. Finally, adhesion to host cells [36] as well as to ECM components [37] may involve integrin–ligand motif interactions (β1FNR [25, 38], β2-integrin like [28]), and may be relevant for the induction of the host inflammatory response during invasion (proform of CP-A5 [29]). This chapter provides an overview of the current knowledge concerning these surface-associated factors.

15.2 *Entamoeba histolytica* Surface Components Interacting with Human Cells

15.2.1 The Galactose- and N*-Acetyl-D-Galactosamine-Inhibitable (Gal/GalNAc) Lectin*

Trophozoite adhesion to the intestinal mucus layer and to epithelia as well as interactions with host inflammatory cells are mainly mediated through a major surface component, the Gal/GalNAc lectin [3]. The lectin is an immunodominant antigen recognized by sera from 95 % of patients with liver abscesses [39], and children with stool anti-lectin IgA antibodies appear protected from intestinal infection [40]. Therefore, the lectin is a preferred candidate for the diagnosis of *E. histolytica*/*E. dispar* and vaccination proposals. The Gal/GalNAc lectin of 260 kDa is a heterodimer of a disulfide-linked heavy (the type 1 TM protein Hgl, 170 kDa) and light (the GPI-anchored Lgl, 35/31 kDa) subunit, noncovalently associated with the intermediate subunit (the GPI-anchored Igl, 150 kDa) (reviewed by [3, 6]). The function of Gal/GalNAc lectin was deduced from experiments showing that the purified lectin mixed with [41] galactose or *N*-acetyl-D-galactosamine competitively inhibited trophozoite adherence to the surface of Chinese hamster ovary (CHO) cells, preventing cytotoxicity [8]. Furthermore, deficiency in Hgl [9, 10] or Lgl [13, 14] function caused a significant decrease in trophozoite adherence, target cell killing, and reduced virulence in experimental amoebic liver abscess (ALA) formation in the hamster animal model, and impaired Lgl function impeded lectin capping at uropods [14, 15].

Although the Gal/GalNAc lectin has been identified among the amoebic surface components interacting with the brush border of cultured intestinal epithelial cells [5], its role in intestinal amebiasis is not yet well established. Notably, trophozoites expressing a dominant-negative form of Hgl that inhibits signaling function of the cytoplasmic domain of the subunit (HGL-2) and Lgl-silenced trophozoites penetrate the intestinal barrier [42], indicating that the lectin function does not seem to be critically involved in early stages of intestinal invasion.

A clearer picture concerning the dependence on Gal/GalNAc lectin function was obtained for hepatic pathogenesis caused by amoebae. The contribution of the lectin to inflammatory cell recruitment and cytokine production was examined in the hamster ALA model. Impairment of Hgl-dependent signaling pathways (HGL-2 trophozoites) caused smaller inflammatory foci containing twice as many

trophozoites as the virulent controls. Analysis of the inflammatory loci 24 h after inoculation showed low tissue penetration by HGL-2 trophozoites associated with weak neutrophil and macrophage attraction and the absence of pro-inflammatory tumor necrosis factor (TNF). The Gal/GalNAc lectin could thus be implicated in cytoskeletal and adhesion plaque reorganizations during chemotactic trophozoite migration toward TNF, secreted by mature macrophages and acting as a chemoattractant for the amoeba [11, 43]. The low host inflammatory response in HGL-2 infections correlated with a delay in apoptosis of hepatic cells [12]. Competition with D-galactose for Gal/GalNAc lectin binding to liver sinusoidal endothelial cells in culture diminished trophozoite adhesion and human cell death that correlated with a loss of the capacity to induce perturbations of the actin cytoskeleton and focal adhesion complex organization in endothelial cells in contact with trophozoites [36]. The data are in line with pioneer observations suggesting the implication of the Gal/GalNAc lectin in cytotoxicity and cell death [44]. Interestingly, Gal/GalNAc lectin that localizes at the plasma and vacuolar membranes is transferred to different types of host cells, in culture (Fig. 15.1) and in animal models of amebiasis [41], and, in particular, to the lateral surface of cultured epithelial cells before their death [45].

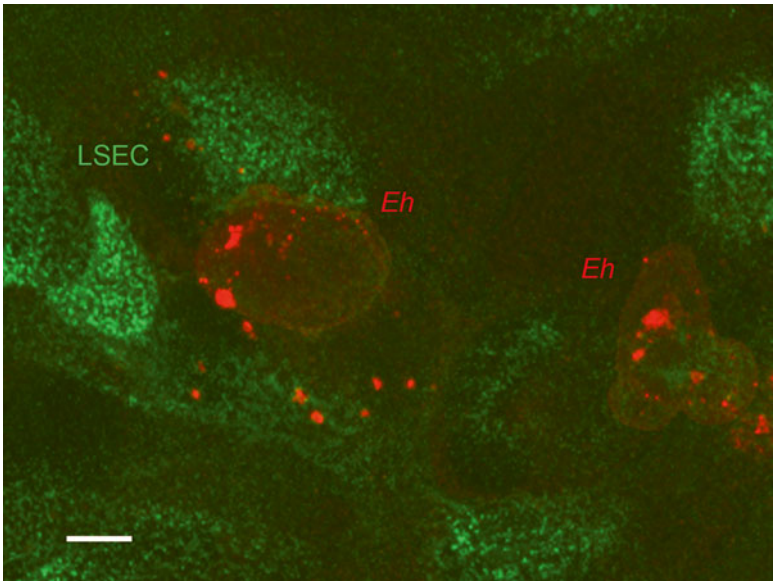

Fig. 15.1 Amoebic Gal/GalNAc lectin clustering at the trophozoite surface and shed clusters adhering to the surface of human cells during *Entamoeba histolytica* interactions with liver sinusoidal endothelial cells. Human liver sinusoidal endothelial cell (LSEC) monolayers were incubated for 60 min with virulent *E. histolytica* HM1:IMSS trophozoites (*Eh*); cells were fixed, and immunofluorescence labeling was carried out with antibodies against human ICAM-1 (*green*) and the Hgl subunit of amoebic Gal/GalNAc lectin (*red*). Micrograph acquired by confocal microscopy: the 23 focal planes (0.38 μm each) were used for the projection presented. *Bar* 10 μm

Moreover, the Gal/GalNAc lectin appears to participate in evasion from the host complement system: the Hgl subunit contains a cysteine-rich domain (CRD) displaying homology with CD59, a human inhibitor of the complement membrane attack complex, and a monoclonal antibody against the Gal/GalNAc lectin abrogated amoebic resistance to complement [3].

15.2.2 The Lysine- and Glutamic Acid-Rich Protein KERP1

Affinity chromatography experiments using *E. histolytica* membrane proteins and purified enterocyte brush border preparations identified two lysine- and glutamic acid-rich proteins designated KERP1 and KERP2 [5]. KERP1 is exclusively present in *E. histolytica* and has no protein homologues, whereas KERP2 is also present in *E. dispar*. KERP1 contains 19 % lysine and 25 % glutamic acid residues; it has an isoelectric point (pI) of 8.95 and a molecular mass of approximately 22 kDa. The high number of charged residues induces α-helical structures that may fold into coiled-coils (CC). Our recent results show that KERP1 is an α-helical trimer that is able to reversibly unfold during thermal denaturation with a thermal melting point (T_m) of 89.6 °C, unprecedented for an *E. histolytica* protein. Bioinformatic analyses predicted CC regions within the KERP1 central segment, and tertiary structure modeling suggested that these regions play a central role in trimer formation. Interestingly, expression of the KERP1 CC domains in living parasites reduced the parasite adhesion to human cells [18].

KERP1 localizes at the trophozoite plasma membrane and is associated with intracellular vesicles. Upon contact with enterocytes, KERP1 has been found associated with the brush border of the human cells, indicating a role in the process of adhesion to host cells [5]. Gene expression studies revealed higher levels of the *kerp1* transcripts in virulent and lower protein levels in nonvirulent *E. histolytica* strains. In vivo studies in the hamster model of amoebic liver infection corroborated the role of KERP1 as a virulence factor. An antisense approach to downregulate *kerp1* expression prevented liver abscess formation, indicating the importance of this protein for amoebic pathogenicity [19]. The exact function of KERP1 during infection is still unknown, but the protein is certainly involved in (1) trophozoite interactions with host cells and adherence, which lead to host cell death and phagocytosis, and (2) the triggering of the inflammation during ALA development [46].

15.2.3 The CPADH Protein Complex

The surface-associated component CPADH (112-kDa adhesin) is a complex composed of cysteine protease CP112 (49 kDa) and adhesin ADH112 (75 kDa) encoded by adjacent genes [20]. The complex has been associated with virulence and is recognized by sera from amebiasis patients [47]. Immunization of hamsters

with a recombinant protein containing a portion of ADH112 led to reduced ALA formation [48], rendering the protein a potential candidate for vaccination trials.

CPADH is localized at the plasma membrane and in intracellular vacuoles, and during phagocytosis it is translocated from the plasma membrane to phagosomes. The inhibitory effects of the recombinant proteins and antibodies directed against the complex revealed its implication in phagocytosis, adherence, and cytotoxicity [20]. Trophozoites overexpressing the *adh112* gene showed enhanced phagocytosis, highlighting the ADH112 contribution to adhesion and phagocytosis [21]. CP112 contains a carboxy-terminal putative TM domain and the mature enzyme contains an arginine-glycine-aspartic acid (RGD) integrin-binding motif [20] that may play a role in binding to RGD receptors (see following).

ADH112 comprises a carboxy-terminal adhesion domain and possesses similarity (36–42 %) to the multifunctional scaffold protein apoptosis-linked gene (ALG) 2-interacting protein X (ALIX)/programmed cell death 6 interacting protein (PDCD6IP) family. ADH112 is structurally related to this family, having an amino-terminal Bro1 domain and a potential Src kinase phosphorylation site, implying roles in signaling or in regulation of protein cargo and vesicular trafficking. Recently, analysis of the phenotype of trophozoites expressing the Bro1 domain of ADH112 and the detection of the in vitro binding of the putative *E. histolytica* ESCRT-III subunit Vps32 to ADH112 suggesting an alternative role of the adhesin, as a novel ESCRT-accessory protein acting in multivesicular body (MVB) formation. Similar to mammalian Alix, ADH112 may be implicated in the vesicular trafficking pathway, transiently present at the cellular surface and in endosomal compartments, probably contributing to MVB formation in *E. histolytica* [22].

15.2.4 The Serine-, Threonine-, and Isoleucine-Rich Protein STIRP

The gene encoding the serine-, threonine-, and isoleucine-rich protein STIRP has been identified among the genes expressed only in pathogenic trophozoites, upon comparison of gene expression profiles between virulent HM1:IMSS and 200:NIH and virulence-attenuated Rahman *E. histolytica* strains and the nonpathogenic species *Entamoeba dispar* [23]. Upon contact with human mucus in the colon explant model, an important increase in the expression level of STIRP genes has been observed in virulent trophozoites [37].

The STIRP gene family contains five members. Genes *STIRP-1*, *-2* and *-3* have a coding sequence of at least 8 kb and are highly expressed in virulent trophozoites. *STIRP-4* and *-5* seem to be truncated genes. The Rahman strain most likely possesses at least one full-length copy gene, whereas none has been found for *E. dispar* [24]. STIRP is a predicted single TM domain protein with a short cytoplasmic tail (34 residues). STIRP is likely present on plasma or vesicular membranes [24] and was found in uropod-enriched fractions [2]. Roles for STIRP in adhesion to and cytotoxicity for cultured mammalian target cells have been revealed with a double-stranded RNA-based approach, targeting all STIRP family members [24].

15.3 Receptors for Apoptotic Host Cell Markers

The Gal/GalNAc lectin is the major adhesion molecule for healthy, but not for apoptotic, cells, implying distinct additional receptors recognizing the latter [49]. Phosphatidylserine (PS) is externalized in early steps of apoptosis. Surface-exposed PS is recognized by phagocytes, leading to engulfment of the apoptotic bodies. The preferential phagocytosis of apoptotic target cells by *E. histolytica* [49] may be of importance for the outcome of the host immune response. PS has been identified as one of the molecules serving in *E. histolytica* recognition and phagocytosis of apoptotic host cells [49, 50] and several amoebic molecules have been identified as receptors for apoptotic cell-surface markers (Table 15.2).

15.3.1 The Immunodominant Antigen M17

The amoebic 125-kDa surface protein M17 is an immunodominant variable antigen recognized by 73 % of the sera from patients with hepatic amebiasis [57]. M17 is an abundant protein in uropod-enriched fractions prepared from *E. histolytica* [2].

Table 15.2 *Entamoeba histolytica* cell-surface components potentially involved in the recognition and phagocytosis of apoptotic host cells

Factor	Subunits/domains	Function in process	References
M17	– Signal peptide	– Potential PS receptor	[2, 51–53]
	– Y-, N-rich	– Potential role in adhesion to bacteria of intestinal flora	
	– F5/8-type C domain		
	– Galactose binding-like domain		
	– C-type lectin domain		
Calreticulin	– KDEL ER-retrieval motif	– ER-resident chaperone, calcium buffer	[32, 35, 54, 55]
		– Surface receptor for collectins (apoptotic cells, bacteria)	[32]
		– Phagocytosis	[32, 53, 55]
C2PK	– C2 domain	– PS binding	[33]
		– Recruitment CaBP1, actin cytoskeleton organization	
		– Phagocytosis	
SREHP	– S-rich	– Phagocytosis	[34]
		– Potential adhesion apoptotic host cells	
TMK96/PATMK	– Type 1 TM	– Phagocytosis healthy and apoptotic host cells	[35, 56]
	– CXXC motif repeats		

The protein is clustering upon cap induction by either concanavalin A or serum of patients with hepatic amebiasis and is translocated and concentrated at uropod-forming regions. These findings suggest M17 as an important candidate for triggering the host immune response. However, genes encoding M17 were also found in nonpathogenic species *Entamoeba dispar* and *Entamoeba invadens* [2].

Functions of M17 have not yet been determined, despite the potentially interesting features of its domain structure, revealed by bioinformatic analysis [51]. The N-terminal signal peptide suggests that the protein is secreted or transported to the trophozoite surface, consistent with its plasma membrane localization [2]. M17 is particularly rich in tyrosine and in asparagine residues, contains 17 potential glycosylation sites, and exhibits similarities with the F5/8-type C domain (residues 857–985) present in coagulation factor V and VIII [51]. This domain mediates factor V and VIII binding to membrane phospholipids, as PS ([58] and references therein). Protein M17 could function as a PS receptor involved in the recognition and clearance of apoptotic host cells. An implication of M17 in phagocytosis has already been inferred from its presence in isolated phagosomes [51, 52].

The sequence spanning residues 874–985 of M17 has also been identified as a galactose binding-like domain [2] that binds to cell surface-attached carbohydrate substrates and phospholipids on the mammalian cell surface. Furthermore, M17 exhibits similarities (residues 240–419) with the C-type lectin domain, found in surface receptors such as DC- (dendritic cell-) or L- (liver-) SIGN, which binds carbohydrates in a calcium-dependent manner [59]. The TM C-type lectins act as cell adhesion molecules and recognize specific carbohydrate structures present on self-antigens and pathogens [60]. They serve as pattern recognition receptors and shape the adaptive immune response by triggering several relevant signaling pathways [61]. Therefore, amoebic M17 may have a role in *E. histolytica* interactions with and adhesion to bacteria of the intestinal flora or, upon host invasion, human cells, and in particular, cells dying by apoptosis.

15.3.2 Calreticulin

Calreticulin is a major endoplasmic reticulum (ER) resident protein with calcium-buffering and chaperone functions. Recently, *E. histolytica* calreticulin has also been localized at the surface during interaction with target cells and at phagocytic cups and implicated as a receptor for human collectins and the related C1q [32]. Collectins are pattern recognition proteins of the innate immune system that bind to apototic cells and bacteria and are recognized by macrophages. They drive engulfment (possibly by macropinocytosis) through interactions with a complex of calreticulin and CD91 at the phagocyte surface [62]. Collectins also stimulate *E. histolytica* phagocytosis and act as trophozoite chemoattractants [63].

The sequence of *E. histolytica* calreticulin is 53 % identical and 70 % similar to the human protein that functions in macrophage phagocytosis of apoptotic cells

opsonized with collectins, but also binds directly to apoptotic cells and enables C1q/collectin-independent phagocytosis. *E. histolytica* calreticulin binds to apoptotic cells and to human C1q and trophozoites overexpressing the protein have increased phagocytic activity for apoptotic cells, without changes in adhesion to or cytotoxicity for cultured cells and unaltered surface expression of Gal/GalNAc and SREHP [32]. These data suggest that cell-surface calreticulin is an *E. histolytica* receptor for C1q that facilitates phagocytosis of apoptotic host cells. Calreticulin was found in the proteome of early phagosomes [35, 53] and localized to the uropod [55]. A function in early stages of ALA was inferred from the increase in calreticulin levels shortly after inoculation [54].

It remains to be investigated how calreticulin reaches the surface and how it is retained (in the absence of a TM domain, presumably by interaction with membrane lipid or protein components). So far, an *E. histolytica* homologue of the endocytic receptor CD91, also known as low-density lipoprotein receptor-related protein 1 (LRP1), that in macrophages retains calreticulin at the surface and has a signaling function that initiates phagocytosis) [62], has not been identified.

15.3.3 The C2-Domain-Containing Protein Kinase C2PK

Binding to PS has been detected for the novel *E. histolytica* C2-domain-containing protein kinase (C2PK) [33]. Binding is specific for PS-containing liposomes (phosphatidylcholine liposomes are not bound) and is dependent on the presence of calcium. C2PK localizes to the trophozoite plasma membrane in a calcium-dependent manner and subsequently leads to the recruitment of the calcium-binding protein CaBP1 and of actin to the membrane. These proteins were found enriched at phagocytic cups but were absent from phagosomes. The role of this process in the initiation of erythrophagocytosis was demonstrated by the phenotype observed upon suppression or enhancement of C2PK activity. Note that experiments were not conducted with apoptotic host cells; the significance of PS binding by C2PK for their recognition remains presently unknown.

15.3.4 The Serine-Rich SREHP

The *E. histolytica* SREHP (approximately 50 kDa) is an abundant immunogenic surface protein of unclear function and an important vaccine candidate that has been identified in a screening for monoclonal antibodies directed against membrane antigens inhibiting phagocytosis of apoptotic target cells ([34] and references therein). One antibody blocked Gal/GalNAc-independent engulfment of apoptotic cells, inhibited adherence to apopotic and viable Jurkat T-lymphoma cells and their killing. The antigen was identified as being SREHP. The data implicate SREHP in phagocytosis and also suggest a role in adherence to apoptotic cells.

15.3.5 The Transmembrane Kinase TMK96 (PATMK)

In a proteomic screen for early phagosomal proteins aiming to discover amoebic proteins involved in the recognition and ingestion of dead cells, a member of the TM kinase family, phagosome-associated TMK96 (PATMK), was identified [35]. TMK96 is a type I integral membrane protein belonging to the big family of TMKs (more than 80 putative members) containing multiple CXXC repeats in their extracellular domain with considerable similarity to the Gal/GalNAc lectin Igl subunit [56]. TMK96 is expressed on the trophozoite surface and colocalized with human erythrocytes at the site of contact, suggesting that it may directly interact with the human cells during engulfment [35]. Its role in phagocytosis of healthy and of apoptotic erythrocytes was deduced from the inhibitory effects observed by using specific antibodies, an expression knockdown approach and the expression of truncated TMK96. Expression of the truncated form also caused a reduction in the ability to establish infection in a mouse intestinal model of amebiasis, without concomitant change in the ability to cause ALA. Currently, it is not known whether TMK96 acts in adhesion as a receptor for apoptotic cells or in the regulation of their ingestion [35].

15.4 Extracellular Matrix and Host Cell Adhesion Through Integrin Receptor–Ligand Interactions and Signaling

In higher eukaryotes, cell-surface receptors of the integrin family mediate cell attachment, spreading, migration on ECM glycoproteins as fibronectin (FN) or collagen, as well as certain cell–cell recognitions [64]. The receptors consist of α-/β-integrin heterodimers that recognize either cellular counterreceptors or an RGD sequence motif. Ligand binding occurs through the extracellular domains and initiates integrin clustering and the subsequent assembly of signaling complexes and actin cytoskeleton organization. The short intracellular domains of integrin thus communicate the adhesion state to downstream signaling cascades that contribute to regulate adhesion properties, cell death, and cell survival.

For tissue invasion by *E. histolytica*, attachment to ECM components and host cell surfaces are crucial for trophozoite motility, host cell killing, and the induction of the inflammatory response, and may involve triggering of integrin-mediated signalling. However, genes encoding bona fide integrins have not been identified in the *E. histolytica* genome [7, 65], but several proteins comprise either structural and functional similarities with integrins or an RGD motif (Table 15.1).

15.4.1 The E. histolytica *Fibronectin Receptor β1FNR*

Trophozoite adhesion to fibronectin, followed by actin rearrangement and formation of adhesion plates, local protease activation, and FN degradation [66], has been described to occur through at least two membrane proteins, a 37-kDa

protein [66, 67] and a 140-kDa protein [68]. The 140-kDa protein was further characterized as a β1-integrin-like molecule, acting as an RGD-dependent amoebic FN receptor (FNR) [26, 27]. An antibody recognizing the 140-kDa protein abrogated trophozoite adhesion to FN [69]. FN binding to β1FNR induces the formation of signaling complexes containing the receptor and key components of mammalian focal adhesions, as activated focal adhesion kinase (FAK), paxillin, or vinculin [25].

A fragment of the 140-kDa protein (336 residues) was identified as being almost identical in sequence to a carboxy-terminal portion of the Igl subunit of Gal/GalNAc lectin (96/99 % with Igl1/2) [17] comprising CXXC and CXC repeats and resembling the EGF-like domain found in β1 integrins [38]. Identity was confirmed by cross-recognition with specific antibodies [17], but direct molecular proof is still pending.

Upon trophozoite adhesion to FN or during ALA development, β1FNR redistributes from intracellular vesicles to the plasma membrane and is enriched in regions of cell contacts [17]. Surface localization implies vesicle trafficking and recycling, independent from de novo protein synthesis [38]. The actin cytoskeleton and Rab7, a protein involved in the regulation of vesicular trafficking, participate in the relocalization of β1FNR upon FN contact [70].

15.4.2 E. histolytica *Molecules with Similarity to β2-Integrin*

The Gal/GalNAc lectin subunit Hgl was reported to share an epitope with human β2-integrin [71], and its carboxy-terminal portion that is involved in the reorganization of the amoebic cytoskeleton presents sequence similarity with the cytoplasmic tail of β2- and β7-integrin implicated in the regulation of integrin-mediated adhesion [10]. The role for the cytoplasmic domain of Hgl in controlling adhesion has been shown and discussed [9, 10, 12].

A β2 integrin-like surface component distinct from the Hgl subunit (though recognized by the same antibody directed against human neutrophil β2 integrin) has been identified [28]. The protein forms clusters at the trophozoite surface but does not colocalize with the Gal/GalNAc lectin. The data allowed the interesting hypothesis that *E. histolytica* may mimic neutrophil–endothelial cell interactions by expressing a β2 integrin-like surface molecule binding to the intercellular adhesion molecule (ICAM)-1 counterreceptor, thus interfering with the inflammatory reaction or profiting from the transmigration route to cross endothelial (and also epithelial) barriers before tissue invasion [28].

Finally, an important role for neutrophil β2-integrin (CD18) in *E. histolytica-induced* neutrophil apoptosis has been demonstrated [72]. Ligands of β2-integrins are cell-surface receptors as ICAM-1 to -4 and binding occurs in an RGD-independent manner, but the amoebic ligand for neutrophil β2-integrin has not yet been identified.

15.4.3 The Proform of Cysteine Protease A5 (proCP-A5)

The secreted and surface-bound *E. histolytica*-specific CP-A5, a major virulence factor, contains an RGD integrin-binding motif in its propeptide region. Among *E. histolytica* CPs, RGD is also present in the CP112 and CP18 mature enzymes. Although a function for the RGD motif of CP112 and CP18 has not been described, the binding of CP-A5 through its RGD to the αvβ3 integrin surface receptor of enterocytes has been demonstrated that triggers PI3 kinase/AKT signalling and induces NFkB proinflammatory responses [29]. The study infers an additional, protease activity-independent mechanism of CP-A5 action, that appears relevant for amoebic pathogenesis, for which the host inflammatory reaction is critically involved. This mechanism can also explain observations indicating the requirement of protease-dependent or -independent CP-A5 activities during invasion, and that have also been suggested for the induction of apoptosis and the killing of liver sinusoidal endothelial cells [36] as well as for the onset of the inflammatory response during human colon explant invasion [37].

15.5 Other Surface Molecules with Roles in Parasite Adhesion or Triggering Host Immune Responses

15.5.1 Protease Activity at the Trophozoite Surface: The Example of Rhomboid Protease ROM1

Rhomboid proteases are intramembrane (seven-pass TM) serine proteases able to cleave single-pass TM proteins. Rhomboid proteases of the apicomplexan parasites *Plasmodium falciparum* or *Toxoplasma gondii* were suggested to mediate cleavage of surface adhesins to facilitate host cell entry [73, 74].

The single functional rhomboid protease of *E. histolytica*, ROM1, has been localized at the trophozoite surface, associated with internal vesicles during erythrophagocytosis and accumulated in patches at the base of uropods during surface receptor capping [30]. Colocalization with Gal/GalNAc lectin at the parasite surface and in vesicles upon phagocytosis and the ability to cleave the lectin Hgl subunit in vitro suggested an implication of ROM1 in immune evasion and an activity in the cleavage of adhesion proteins, notably the Gal/GalNAc lectin [30].

A role for ROM1 in adhesion to host cells and phagocytosis has been deduced from the adhesion defect observed for trophozoites in which protease expression has been epigenetically silenced [31]. Diminished adhesion was observed with healthy host cells, but adhesion to apoptotic cells was normal. The altered adhesion properties were also obtained upon rhomboid protease inhibitor treatment of normal trophozoites. In contrast, phagocytosis was reduced without distinction of the host

cell state, indicating that the reduced phagocytic activity was independent of the changes in adhesion. The authors concluded two distinct roles of ROM1 in the regulation of (1) adhesion to healthy cells and (2) phagocytosis [31].

15.5.2 The Role of LPPG in Virulence and Immune Responses to Entamoeba histolytica

The most abundant surface molecules of *E. histolytica* trophozoites are GPI-anchored lipophosphoglycans (LPGs) that form a densely packed glycocalyx on the entire trophozoite surface, ensuring a protective physical barrier. Virulent *E. histolytica* synthesize two types of LPG, forming a family of GPI-anchored proteophosphoglycans (PPGs) with unusual properties, named LPG (or PPG1) and LPPG (or PPG2). PPGs are associated with extensively modified polypeptides with glucan side chains of various lengths. The attenuated *E. histolytica* strain Rahman synthesizes only one class of PPGs [75]. Anti-PPG antibodies reduce parasite adhesion and cytotoxicity suggesting an important role of PPGs in parasite-host interactions [4]. A role for LPPG in the induction of host immune responses has been deduced from the following observations: Reduction of surface PPG (by interference with the expression of GPI-biosynthetic enzyme) provokes a drastic decrease in trophozoite survival in the presence of complement, and these parasites are avirulent in the hamster ALA model [76]. LPPG released from lysed trophozoites is recognized through toll-like receptors TLR2 and TLR4/CD14 and induces the production of interleukins IL-8, IL-10, IL-12p40, and TNF by monocytes ([77] and references therein). Finally, LPPG is implicated in the protection against invasive amebiasis and specific activation of natural killer (NK) T cells by LPPG conferred significant protection [78].

15.6 Concluding Remarks and Perspectives

From the vast amount of data accumulated concerning *E. histolytica* surface components, it has become clear that virulence results from the concomitant action of a number of molecules and that these are involved in different cellular processes. Moreover, it has turned out that molecules may be implicated in several of these processes; for example, they may function in adhesion, cell killing, and phagocytosis. In addition, evidence has been obtained that amoebic surface components may interact and may require these interactions for function (e.g., as suggested for ROM1, that may either cleave Hgl or another substrate interfering with Gal/GalNAc activity). In addition to the activity of surface components, recent evidence indicates a possible role for the membrane composition, the plasma membrane organization in microdomains (lipid rafts) appearing required for the efficiency of Gal/GalNAc lectin function [79].

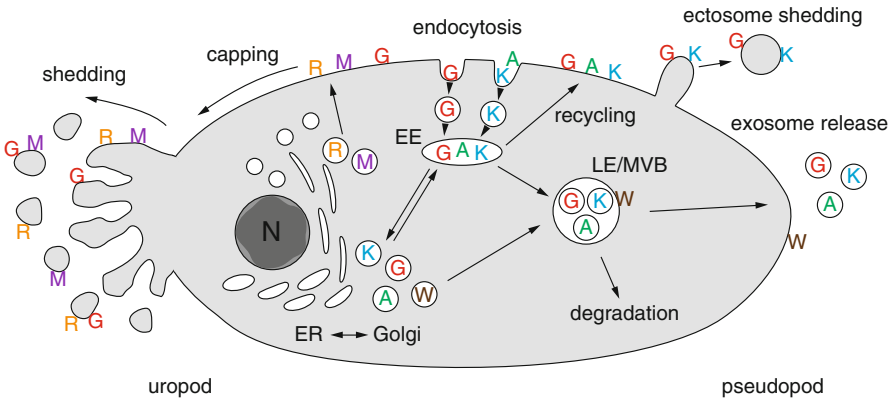

Fig. 15.2 Routing of *E. histolytica* surface proteins from sites of synthesis to destination and recycling. Schematic presentation of main steps potentially involved in surface protein trafficking from the sites of synthesis and modification [endoplasmic reticulum (ER) and Golgi] to their different destinations (vesicle exocytosis, ectosome shedding, association with the trophozoite surface). Elimination of surface proteins by capping and uropod shedding in trophozoites polarized by surface receptor activation upon binding of anti-amoeba antibodies or carbohydrate structures, for example. Endocytosis and recycling of surface components. *A* proCP-A5, *G* Gal/GalNAc lectin, *K* KERP1, *M* M17, *R* calreticulin, *W* CPADH, *EE* early endosome, *LE* late endosome, *MVB* multivesicluar bodies, *N* nucleus

The important aspect of the transport of surface-associated molecules from intracellular sites of synthesis/modification to their membrane destination (schematically represented in Fig. 15.2), that is, their trafficking and its regulation, will require intense investigation. Major tasks remaining to be accomplished are the identification of ligand–binding sites and counterreceptors on target cells and the elucidation of the molecular mechanisms by which the amoebic factors act and ultimately the relevance for pathogenesis in amebiasis.

Acknowledgments The work of the BCP unit is supported by grants from the French Parasitology consortium ParaFrap (ANR-11-LABX0024), the French National Agency for Research (ANR-MIE08, ANR-Genomics, and ANR SVE3-Paractin), the French Ministère de la Recherche et la Technologie (MRT), the Pasteur-Weizmann research council, ECOS-NORD, and from Fondation pour la Recherche Médicale (FRM).

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Chapter 16
Mechanism of Cell Division in *Entamoeba histolytica*

Jaspreet Singh Grewal and Anuradha Lohia

Abstract The cell division cycle of *Entamoeba histolytica* shows important differences from that of unicellular and higher eukaryotes. We have observed that *E. histolytica* cultures are made up of a heterogeneous population of cells that contain one or many nuclei and varying DNA content in each nucleus. Chromosome segregation occurs on a variety of atypical microtubular assemblies, and daughter cells are formed from mechanical rupture of cytoplasmic extensions that may need "helper cells" to complete the separation. Our observations suggest that whole genome copies are lost when cells shift from axenic to xenic cultures or from trophozoites to cysts. Gain or loss of whole genome copies during changes in growth conditions is possibly sustained by the inherent plasticity of the amoeba genome. Molecular studies have shown that orthologues of conserved checkpoint proteins that regulate the eukaryotic cell cycle are absent in this organism. Absence of checkpoint control leads to unregulated DNA synthesis, asymmetrical chromosome segregation, and aberrant cytokinesis in eukaryotes. In spite of the perceived lack of control and atypical mode of genome multiplication and partitioning, these cells survive in a foreign host, to multiply and cause disease or remain dormant for long periods of time, followed by active growth. Absence of known regulatory mechanisms coupled to a unique form of cell division and propagation makes the events leading to formation of *Entamoeba* daughter cells an interesting and challenging study. This chapter summarizes our recent attempts in understanding the cell division process of *Entamoeba histolytica*.

16.1 Introduction

Most eukaryotic cells have stringent regulatory mechanisms to coordinate DNA duplication with chromosome segregation followed by cytokinesis so that daughter cells are formed with exact copies of their parental genetic material. In mitotic cells, the genome is copied just once during the S phase [1]. DNA-repair mechanisms

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_16

ensure that errors generated during synthesis are corrected. The cell then prepares to segregate the two copies of the genome (M phase). Duplicate copies of the genome are aligned on a bipolar microtubular spindle and separated into two daughter cells [2].

Our studies during the past two decades showed that this paradigm was not followed in trophozoites of *Entamoeba* spp. We focused most of our studies on *Entamoeba histolytica* HM1:IMSS strain and also compared the data with other strains such as 200:NIH and more recent isolates that were maintained in axenic culture [3]. Analyzing the progression of the cell cycle in *E. histolytica* posed several challenges because routine microbiological methods and genetic manipulations could not be carried out in this organism. Thus, our understanding of the cell-cycle processes has largely relied on the use of multiparameter flow cytometry, immunofluorescence, scanning cytometry, and other molecular biological techniques.

16.1.1 The Axenic Cell Cycle of E. histolytica

In contrast to other eukaryotic cells, the different phases of the cell division cycle (G_1, S, G_2/M) were not clearly demarcated in flow cytometric analysis of the axenic cultures of *E. histolytica* [4, 5]. Mathematical modeling analyzed the G_1, S, and G_2/M phases of the *E. histolytica* cell cycle as a series of overlapping Gaussian curves, differing from the discrete peaks of these phases in other organisms (Fig. 16.1a, b) [4]. These results suggested that *E. histolytica* cultures were made up of cells containing heterogeneous amounts of DNA. Cells with polyploid nuclei were found in axenically growing populations of HMI:IMSS cells (Fig. 16.1c). Thus, heterogeneity of DNA content could be caused by differences in nuclear DNA content or the number of nuclei or both. The average DNA content of cells and the number of cells with DNA content >2*n* was found to increase with time in axenic culture [6], which indicates that *E. histolytica* trophozoites possibly reduplicated their genome several times without nuclear division or cytokinesis. We confirmed that this heterogeneity is not specific to the laboratory strain *E. histolytica* HMI:IMSS and that other isolates of *E. histolytica* showed a similar phenotype [3]. Thus, typical checkpoint mechanisms that ensure the progression of chromosome segregation and cytokinesis (immediately after the completion of genome duplication) were either absent or altered in this parasite.

It has been difficult to synchronize *E. histolytica* cells in any one phase of the cell cycle. Mitotic blockers such as colchicine, nocodazole, and thiabenzadole were not active against *E. histolytica* cells. Our best results were obtained by using serum starvation for 12 h followed by addition of serum [5, 6]. During serum starvation, cells were arrested at different phases. Using this method in our subsequent studies we could obtain synchronization for one mitotic cycle [7, 8]. Using BrdU (5′-bromo-2′-deoxyuridine) incorporation, we showed that after addition of serum, DNA synthesis was initiated after a lag phase of 2 h. More than 80 % of the cells showed a

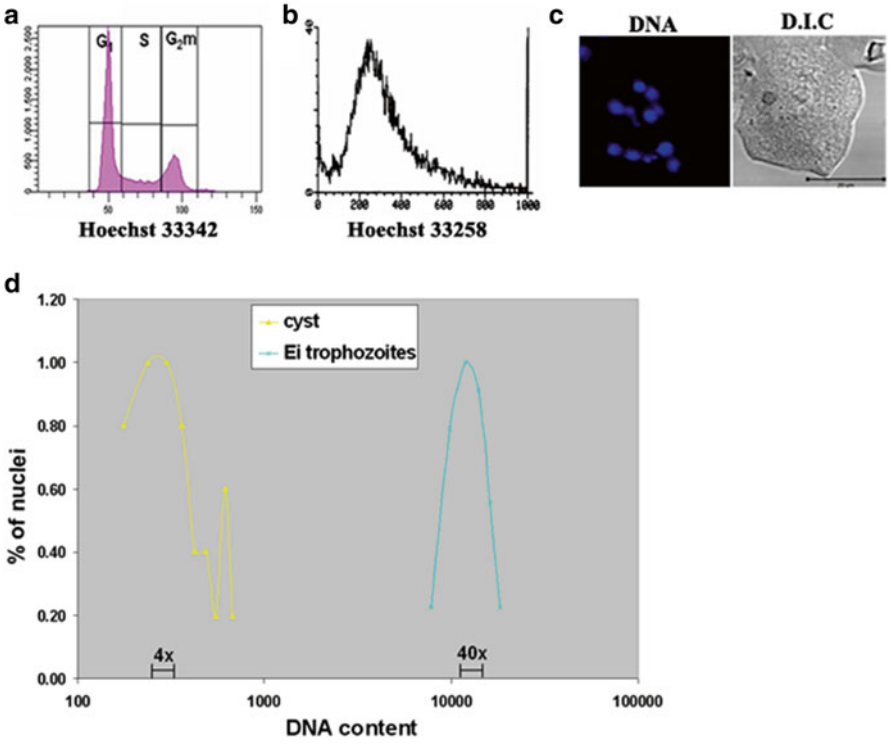

Fig. 16.1 Comparison of the cell-cycle phases as analyzed in a flow cytometer. **a** Flow cytometric analysis of a typical eukaryotic cell cycle (adapted from Louise Russel, Cutaneous Research, Institute of Cell and Molecular Science, Queens Mary College, London) showing distinct G_1, S, and G_2/M phases. **b** Flow cytometric analysis of the *Entamoeba histolytica* cell cycle (adapted from Gangopadhyay et al. [5]) shows the absence of discrete cell-cycle phases. **c** A multinucleated *E. histolytica* cell in an axenically growing culture. DNA has been stained with DAPI. *Bar* 20 μM. **d** The DNA content of *Entamoeba invadens* cyst and trophozoites (~40 Mb) shown in the logarithmic scale. 4× and 40× indicate the average genome content corresponding to the major peaks in each cell type [3]

uniform DNA content at this time [6]. Cells continued to accumulate multiple genome contents subsequently in the next several hours [3, 6]. These data were confirmed both by BrdU incorporation and by scanning cytometry, clearly showing that nuclear and cell division are not temporally linked to genome duplication and segregation. In addition, the number of duplication cycles was not fixed as the nuclei accumulated heterogeneous amounts of DNA [3, 6]. Thus, genome segregation did not necessarily occur immediately after genome duplication. In serum synchronized cells, we observed that the number of microtubular assemblies increased 4 h after addition of serum and were highest after 8 h of serum addition [8]. The number of binucleated cells was highest at 10 h after addition of serum. These observations

suggested that the majority of cells in this synchronized population underwent several rounds of genome multiplication followed by chromosome segregation, possibly between 4 and 8 h after initiation of DNA synthesis, followed by nuclear division between 8 and 10 h [8].

16.2 The *E. histolytica* Genome Segregates on Atypical Microtubular Assemblies

Chromosome segregation is carried out on the mitotic spindle in eukaryotic cells. The sister chromatids are pulled apart on these spindle fibers: complete sets of chromosomes are moved to each pole of the cell where they are packaged into daughter nuclei. The mitotic spindle is composed of dynamic microtubular fibers that are polymers of α- and β-tubulin subunits. These subunits are nucleated at the microtubule organizing center (MTOC) [9]. One of the key proteins of the MTOC is the γ-tubulin that regulates the nucleation of microtubules in higher eukaryotes [10]. The homologues of α-, β-, and γ-proteins have been identified in *E. histolytica* [11–13]. The amino acid sequences of Ehαβγ-tubulins are significantly divergent from their eukaryotic homologues, and this difference has been attributed to their resistance to antimitotic drugs such as colchicines and benomyl [11, 13], although sensitivity to the microtubule stabilizing drug, taxol was predicted from the conserved taxol-binding site in the modeled tertiary structure of Eh γ tubulin [14].

Despite the presence of essential spindle and MTOC forming proteins i.e alpha, beta and gamma subunits [12, 15], metaphase-like equitorial alignment of condensed chromosomes could not be identified in E.histolytica cells [16, 17]. Anaphase and telophase were identified on the basis of nuclear shape [18]. Moreover, nuclear microtubular assemblies with fibers radiating from a central region in most *E. histolytica* cells were shown by indirect immunofluorescence [15].

In one of our studies, serum synchronized cells were fixed and stained with anti-β tubulin antibody, and a time-course study was done to observe the microtubular structures in *E. histolytica* cells. Several novel microtubular (MT) assemblies including monopolar, bipolar, and multipolar spindles for the segregation of chromosomal DNA were identified [8]. These unusual MT structures suggested that genome segregation occurred on different kinds of MT structures in *E. histolytica* cells, likely required for the heterogeneous number of genome copies in a single nucleus. A model was based on these observations (Fig. 16.2) from Mukherjee et al. [8]. This model highlights the possible modes of genome segregation on MT assemblies ranging from radial, bipolar, or fan-shaped structures to bundles of multiple MTs in assorted shapes. Real-time data with fluorescence-tagged MTs to validate this exciting hypothesis remains to be validated with live cell imaging using fluorescently labelled microtubules.

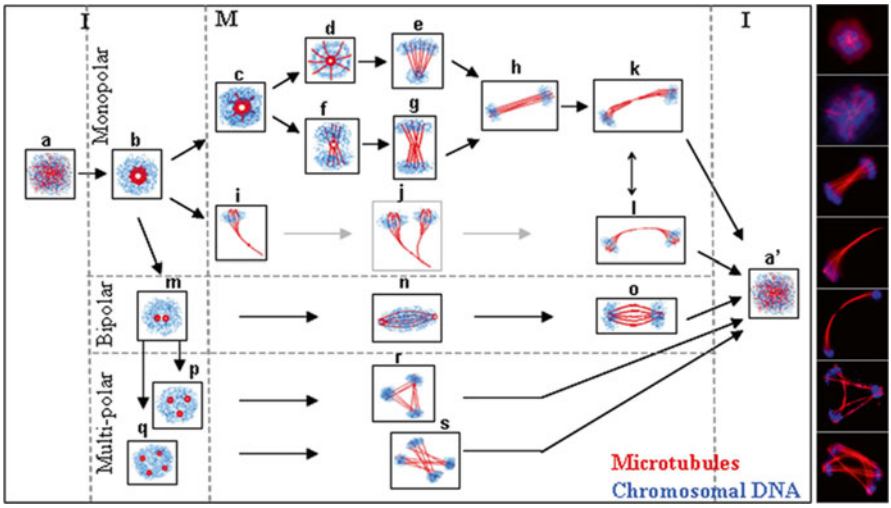

Fig. 16.2 Model showing chromosome segregation on different types of microtubular assemblies seen in *Entamoeba histolytica*. The schematic diagrams are adapted from the microscopic images shown alongside (on *right*) [8]. The cells were stained with anti-beta tubulin antibody and DAPI for staining the microtubules and DNA, respectively. Images were taken in a Zeiss LSM 510 META confocal microscope under 63× oil objective. The different MT assemblies suggest multiple modes of genome segregation

16.3 Cytokinesis in *Entamoeba histolytica*

Cytokinesis is the process of physical separation of a mother cell that gives rise to two daughter cells. One of the earliest modes of cell division used might have been the motility and consequent mechanical force driven by actin polymerization in a polymorphic cell [19]. Cytokinesis in *E. histolytica* can occur in three different ways. In the first mode, the intercellular bridge between dividing *E. histolytica* trophozoites is formed at random sites. The extension and rupture of this cytoplasmic bridge leads to formation of two daughter cells. In the second mode of cytokinesis the severing of the intercellular bridge is assisted by helper cells. In such cases the helper cells migrate to the intercellular bridge and rupture the bridge either mechanically or by unidentified mechanisms. Helper cell-assisted cytokinesis was estimated to occur in 45 % of the cases (Fig. 16.3a). Interestingly, the microtubular structure was found to extend through the entire length of the intercellular bridge (Fig. 16.3b). Finally, failure of cytokinesis is quite common where (Fig. 16.3c) the two halves of a cell are separated by an extremely long bridge that ultimately failed to separate and cytokinesis was aborted in approximately 20 % of the cells [8]. This event may well lead to cells with multiple nuclei in which the mitotic cycle continues for many rounds without successful cell division. Unequal or aberrant

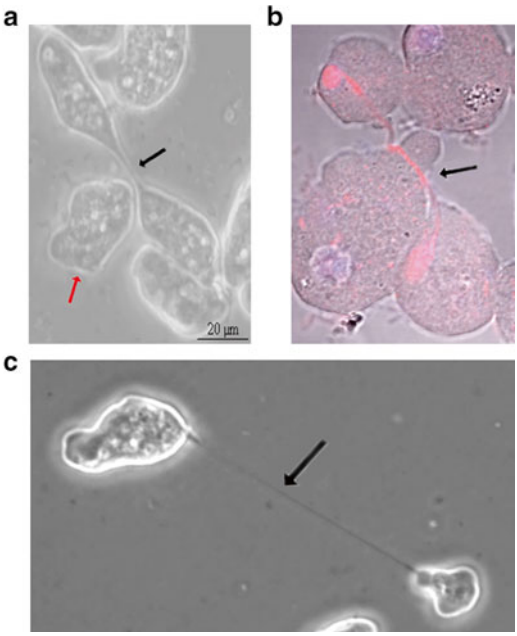

Fig. 16.3 Various events leading to cytokinesis in *Entamoeba histolytica*. **a** Helper cell (*red arrow*) approaches the intercellular bridge (*black arrow*) between two daughter *E. histolytica* cells. **b** The microtubular assembly stretches across the intercellular bridge, a part of which was seen on top of a helper cell as the latter moves beneath the bridge. The cells were stained with anti-**β**-tubulin antibody and DAPI to visualize microtubules and DNA, respectively. **c** A still from live-cell imaging of cytokinesis in *E. histolytica* cells shows a long and thin intercellular bridge (*black arrow*) connecting the two daughter cells

division was frequent and gave rise to "anucleate" cells [8]. Altogether, the data suggested that the heterogeneous modes of cytokinesis contributed to the genetic heterogeneity in the population of *E. histolytica* cells.

16.4 Checkpoint Genes Are Absent in *Entamoeba histolytica*

The regulation of a typical eukaryotic cell division cycle depends upon a set of proteins known as the "checkpoint" proteins [20–22]. Cell-cycle progression is closely monitored by these checkpoint proteins and occurs only if the preceding phase has been completed correctly. Analysis of the *E. histolytica* genome shows that most of the conserved checkpoint proteins are absent. A large amount of information regarding the cell-cycle regulation in eukaryotes has been obtained from genetic and biochemical studies on the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [23]. Additionally, it was seen that proteins controlling the cell cycle in yeasts were well conserved in other eukaryotes [24]. The complete genome sequence of *E. histolytica*, published in 2005, consists of about 8,201 genes with an average size of 1.17 kb. No homologues could be identified for one-third of the predicted proteins (32 %) from public databases. Even before the completion of the genome sequence few cell-cycle-related genes had been identified, such as p^{34}Cdc2 [25], Mcm2-3-5 [26], α-, β-, and γ-tubulin [11–13], and Diaphanous1 [27] in *E. histolytica*. An in silico analysis of the *E. histolytica* genome for homologues of cell-cycle genes in *S. cerevisiae* was conducted for a better understanding of the amoeba cell cycle.

1. Genes involved in DNA replication initiation and entry into S phase.

   Replication initiation in eukaryotes is characterized by the formation of a pre-replication complex (Pre-RC) with subsequent firing of the replication origins. In *S. cerevisiae* Orc1-6 (origin recognition complex) proteins bind at the pre-RC site and subsequently Cdc6, Cdt1, and Mcm2-7 are sequentially recruited at the site to form the pre-RC [28]. The helicase complex of Mcm2-7 remains inactive until it is activated by Cdc45 protein. The kinases Cdc7 and Cdk2 are involved in the loading of Cdc45 on origins. Cdc7 phosphorylates the subunits of Mcm2-7, which, through an unknown mechanism, changes the conformation of Mcm2-7 to facilitate the loading of subsequent factors such as Mcm10 and Cdc45. Our studies showed that sequence homologues of several proteins required for DNA replication initiation such as Mcm2-7, Cdc45, and subunits of GINS complex were present in the *E. histolytica* genome (Table 16.1). Except for a single protein that shared homology with both Cdc6 and Orc1p, none of the Orc2–6 proteins were identified in this organism. Studies with Eh Cdc6/Orc1 are currently in progress to understand the role of this protein in regulating DNA synthesis initiation.
2. G_1–S-phase checkpoint proteins in *E. histolytica.*

   Regulation of DNA replication in *S. cerevisiae* involves four checkpoint proteins: Mec1, Mrc1, Tof1, and Dpb11. No homologs of these 4 proteins are found in *E. histolytica*. Several crucial genes required for the G_1–S transitions in

Table 16.1 *Entamoeba histolytica* genes encoding homologues of *Saccharomyces cerevisiae* genes involved in DNA replication initiation

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
MCM2 (868)	Replication helicase	XP_656059 (883)	EHI_117970
MCM3 (972)	Replication helicase	XP_653372 (601)	EHI_103600
MCM4 (934)	Replication helicase	XP_649080 (608)	EHI_187720
MCM5 (775)	Replication helicase	XP_648784 (639)	EHI_069980
MCM6 (1,018)	Replication helicase	XP_654108 (682)	EHI_118870
MCM7 (846)	Replication helicase	XP_650807 (690)	EHI_158110
Cdc28 (298)	Cdk2 like kinase	XP_652398 (291)	EHI_065280
Pol I (1,468)	DNA pol I	XP_657373 (1,132)	EHI_151520
Cdc45 (651)	Recruited to pre-RC	XP_657172 (543)	EHI_049900
Psf1 (208)	Subunit of GINS	XP_652581 (189)	EHI_136990
Psf2 (213)	Subunit of GINS	XP_655297 (199)	EHI_069340
Psf3 (194)	Subunit of GINS	XP_648782 (216)	EHI_069960
Genes sought but not found: MCM1, MCM10, Cdc7, DBF4, Orc2, Orc3, Orc4, Orc5, Orc6, Cdt1, geminin, DNA pol ε			

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

Table 16.2 *E. histolytica* genes encoding homologues of *S. cerevisiae* checkpoint genes for G_1–S phase transition

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
Cdc28 (298)	CDK	XP_652398 (291)	EHI_065280
Skp1 (195)	Part of SCF ubiquitin ligase complex	XP_654128 (162)	EHI_118670
Cdc34 (295)	Ubiquitin-conjugating enzyme	XP_652735 (165)	EHI_048700
Cln1 (547)	G (sub)1 cyclin	XP_651788 (311)	EHI_121830
Cln2 (545)	Cyclin, role in START	XP_648667 (307)	EHI_030880
RecQL4 helicase (1,208)	Role in cell cycle START	XP_653505 (509)	EHI_023090

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

yeast and humans such as the homologues of p21, p27, p53, and retinoblastoma (RB) genes are absent in the genome of *E. histolytica*. The conserved intra-S-phase checkpoint proteins Chk1 and Chk2 (which act downstream of ATM and ATR kinases) are also absent in the *E. histolytica* genome; however, a homologue of human Chk2 has been identified in this organism [29] (Table 16.2).

3. Chromosome segregation and spindle checkpoint proteins in *E. histolytica.*

   Chromosome segregation occurs in the M phase and is the process of separating the two sister chromatids formed as a result of DNA replication in the S phase. The key players regulating this process are the kinetochore proteins. An assembly of the kinetochore proteins is organized around the centromeric nucleosomes. The outer, central, and inner kinetochore assemblies form the bridge between DNA and microtubule in budding yeast [30]. Strikingly, except five kinesin-like proteins (Eh KlpA1–5), no other components of the kinetochore are found in *E. histolytica* (Table 16.3). One of these Eh Klps (Eh KlpA1/A2) is homologous to CENP-A. Eh Klp5, a BimC kinesin homologue, was localized with both radial and bipolar spindle assemblies and required for regulating genome content in *E. histolytica* [31]. However, the absence of other sequence homologues required for kinetochore formation suggests a typical kinetochore may not be present in *E. histolytica*.

4. Several G_2/M or spindle checkpoint proteins are absent from *E. histolytica*.

   Spindle checkpoint proteins are activated when there is a perturbation in the alignment of chromosomes to the spindle. Monopolar, bipolar, and multipolar spindles have been observed in E. histolytica [8, 32]. However, the mechanism of chromosome alignment and partitioning on either of these spindles is not well

Table 16.3 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes required for chromosome segregation

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	Amoeba DB Accession number of *E. histolytica* homologue (length in aa)
Cse4 (230)	Hs CENP-A homologue	XP_653386 (147)	EHI_051840
HRR25 (495)	Protein kinase	XP_657385 (335)	EHI_151950
Smc2 (1,171)	Component of the condensin complex	XP_657185 (1,151)	EHI_049770
Ipl1 (368)	Aurora kinase	XP_649507 (317)	EHI_193840
Tub1 (448)	α-tubulin	XP_653419 (455)	EHI_005950
Tub2 (458)	β-tubulin	XP_657170 (459)	EHI_049920
Mck1 (376)	Ser/Thr kinase	XP_657520 (370)	EHI_148220
Apc11 (166)	Catalytic core of APC	XP_651657 (87)	EHI_135110
Cin8 (1,001)	Kinesin motor protein	XP_649446 (863)	EHI_124890
Smc4 (1,449)	Condensin subunit	XP_654748 (1,226)	EHI_199700
Kin3 (436)	Protein kinase	XP_648115 (484)	EHI_048410
Cdc20 (611)	Cell-cycle regulated activator of APC	XP_657064 (377)	EHI_051010
Smc1 (1,226)	Chromosome segregation	XP_656581 (1,197)	EHI_050790
Cdc16 (840)	Component of APC	XP_656055 (497)	EHI_118010
Esp1 (1,631)	Separase, cysteine protease, sister chromatid separation	XP_651118 (1,450)	EHI_120330
Genes sought but not found: Bir1, Cbf1, Cbf2, Chl4, Ctf19, Ctf3, Ctf13, Cep3, Ctr9, Dsn1, Dam1, MCM16, MCM21, MCM22, MTw1, NNf1-2, APC1-9, Brn1, Bur2, Cik1, Cse2, Doc1, Dyn1, Pds1, Src1			

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

understood. Sequence analysis shows absence of several spindle checkpoint proteins. Interestingly, homologues of two checkpoint proteins, Bub2, which blocks the mitotic exit by inhibiting Cdc14 in response to checkpoint activation, and Mps2, a dual specificity protein kinase required for spindle pole body duplication and spindle checkpoint activation, were found to be present in *E. histolytica* (Tables 16.4, 16.5, and 16.6). *E. histolytica* accumulates multinucleated cells, suggesting uncoupling of nuclear and cell division. Sequence analyses revealed that the homologues of all proteins required for cytokinesis in budding yeast are present in this organism (Table 16.7).

Table 16.4 *E. histolytica* genes encoding homologs of *S. cerevisiae* checkpoint genes required for spindle formation

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
Glc7 (312)	Mitosis	XP_651611 (302)	EHI_176170
Pph21 (370)	Catalytic subunit of protein phosphatase	XP_656214 (309)	EHI_011950
Cdc55 (527)	Protein phosphatase	XP_657525 (440)	EHI_148170
Mps1 (764)	Dual-specificity kinase, spindle pole body duplication	XP_653959 (352)	EHI_073650
Bub2 (307)	Spindle checkpoint protein	XP_649751 (289)	EHI_135170
Tpd3 (635)	Regulatory subunit A of the heterotrimeric protein phosphatase 2A, role in mitosis	XP_649444 (549)	EHI_124870
Genes sought but not found: Mad1-3, Bub1, Bub3, Cep3, Dma1, Bim1, Gac1			

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

Table 16.5 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in mitosis

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
Kip3 (806)	Kinesin-related protein, mitotic spindle positioning	XP_656748 (629)	EHI_140230
HRR25 (495)	Protein kinase, DNA repair, chromosome segregation	XP_657385 (335)	EHI_151950
BNI1 (1,954)	Formin	XP_653884 (1,212)	EHI_192460
SAC3 (1,302)	Transcription, mRNA export from nucleus	XP_655223 (670)	EHI_136450
TOR1 (2,471)	Cell-cycle signaling, meiosis	XP_648644 (2,416)	EHI_104570
Ark1 (638)	Control of endocytosis	XP_652314 (519)	EHI_127410
Genes sought but not found: Cik1, Dma2, Dad3, Dad4, Dyn1			

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

Table 16.6 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in exit from mitosis

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
Tem1 (246)	Termination of M-phase	XP_657549 (189)	EHI_027640
Cdc15 (975)	Promotes mitotic exit	XP_654672 (1,760)	EHI_009590
Mob1 (314)	Transcriptional regulator	XP_650241 (211)	EHI_159570
Lte1 (1,435)	Putative GDP/GTP exchange factor	XP_651306 (491)	EHI_139450
Genes sought but not found: Spo12, Net1, Cdc14, Rio1			

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

Table 16.7 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in cytokinesis

Bait protein (length in aa)	Function	GenBank Accession number of *E. histolytica* homologue (length in aa)	AmoebaDB Accession number of *E. histolytica* homologue (length in aa)
Actin (375)	Cytoskeletal functions	XP_648054 (376)	EHI_107290
Ark1 (638)	Ser/Thr kinase	XP_652314 (519)	EHI_127410
Bir1 (954)	Baculovirus inhibitor of apoptosis repeat	XP_657286 (1,387)	EHI_093850
Cdc15 (975)	Protein kinase of the mitotic exit network	XP_654672 (1,760)	EHI_009590
Chs2 (964)	Chitin synthase II	XP_651026 (642)	EHI_170480
Cla4 (843)	Involved in localizing cell growth	XP_657512 (467)	EHI_148240
Exo70 (623)	70-kDa subunit of the exocyst complex	XP_650775 (765)	EHI_142040
Ipl1 (368)	Aurora kinase	XP_649507 (317)	EHI_193840

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org/]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum "*e*" value obtained was 10^{-5} or less

16.5 Novel Proteins That Regulate the Cell Cycle of *Entamoeba histolytica*

Analysis of the genome of *E. histolytica* showed that a large number of proteins involved in the cell division process of this parasite were either absent or significantly divergent. Given the fact that a large number of proteins encoded by the parasite are hypothetical opens up a possibility the *E. histolytica* may contain novel regulatory mechanisms to ensure that the cell cycle proceeds even in the absence of the conventional regulatory proteins. Functional analysis of a kinesin-like protein, *E. histolytica* Klp5 (EhKlp5), showed that increased expression of this protein, whereas promoting microtubular spindles leads to homogenization of the average DNA content in growing cells [31]. In addition, EhKlp2 was also found to alter the frequency of bipolar spindles and genome content in this parasite. EhKlp2-4 were found to associate with both microtubules and actin cytoskeletal networks although none of these proteins has any actin-binding domains [33]. Yeast two-hybrid analysis with the nonmotor domains of these proteins identified several actin-binding proteins as interactors (Grewal and Lohia, unpublished observations). This observation suggests that these kinesin proteins might associate with the actin cytoskeleton with the help of these interactors and might be involved in regulating motility and cell division.

A novel group of formin proteins was also discovered in *E. histolytica* of which EhFormin-1 and -2 led to delay in cell division [34]. EhFormins-5 and -8 were phylogenetically distinct from their other eukaryotic counterparts [34]. The characteristic FH1 domain, which is crucial in initiating actin polymerization, is absent in these formin proteins. An InterProScan (EMBL-EBI) of EhFormin-5 and -8 identified a GTPase-binding domain at the N-terminal of these proteins, indicating that this region was unique and may respond to unknown signaling mechanisms or protein networks that subsequently may effect actin remodeling. EhFormin-5 was a nucleocytoplasmic protein, which localized as a ring inside the nucleus of *E. histolytica*. Furthermore, EhFormin-8 also colocalized on microtubular structures, suggesting that both these proteins might be involved in the regulation of essential nuclear function such as assembly and disassembly of microtubules. Ectopically expressed EhFormin-5 and -8 decreased the genome content in the stable transformants, suggesting that both these proteins are involved in the process of chromosome segregation. To identify the downstream targets of EhFormin-5 and -8, the N-terminal regions of these two formins used as a bait to screen the Eh cDNA library by yeast-two-hybrid assay. EhFormin-5 interacted with a p21 Ras family GTPase, EhRas family GTPase, and five hypothetical proteins. The identification of EhRas family GTPase as an interactor of EhFormin-5 was a significant and novel finding, because, among the members of the p21 Ras family GTPases, only Rho and Rac proteins have been implicated in binding to formin proteins and assisting actin polymerization in other eukaryotic systems. EhFormin-8 interacted with a zinc-finger domain-containing protein, a helicase domain-containing protein, and two hypothetical proteins. Our data suggest that EhFormin proteins have evolved to perform cell cycle-specific functions in addition to their role as actin nucleators Grewal and

Lohia unpublished observations. Previous studies have shown that expression of a constitutively active mutant of $RacG_{Eh}$ ($RacG^{Gly12Val}{}_{Eh}$), a dominant-negative mutant of RabA, and the kinase domain of $PAK2_{Eh}$ led to cytokinetic defects and consequently to the accumulation of multinucleated cells [35–37]. Taken together, these observations suggest that cell division in *E. histolytica* requires the combined activity of several signaling molecules and proteins.

Calcium signaling plays a major role in the cell cycle of eukaryotes. *E. histolytica* encodes a large repertoire of novel multi-EF hand CaBPs [38]. The calcium-binding proteins characterized thus far have been implicated in having a crucial role in phagocytosis and cell proliferation [39, 40]. EhCaBP6 on the other hand was found to localize at the end of microtubular structures and on the intracellular bridge during cytokinesis [41]. Immunolocalization data suggest that EhCaBP6 is functionally similar to mitotic CaM proteins in other organisms. A large number of cellular processes are regulated by protein–protein interactions. Most proteins require physical interactions with other proteins to execute their biological function. In most eukaryotic cells, calmodulin initiates various signaling cascades by binding to target proteins. Immunofluorescence studies show that EhCaBP6 in mitotic cells may be interacting with the microtubules or with microtubule-associated proteins. In an effort to identify the proteins interacting with EhCaBP6, the latter was used as the bait in a yeast two-hybrid genetic screen against the *E. histolytica* cDNA library. Among the interactors obtained from the screen EhCaBP6 was also found to interact with a zinc-finger domain protein and ribosomal protein P2. The zinc-finger protein obtained in this screen was the same as obtained as an interactor for EhFormin-8. The interaction of both EhFormin-8 and CaBP6 with a zinc domain protein suggests that EhFormin-8 and EhCaBP6 are part of the same protein complex that affects genome segregation in *E. histolytica*. Thus, novel proteins such as EhFormins, kinesins, and EhCaBP6 have evolved to regulate crucial processes of microtubule assembly and chromosome segregation in the protozoan parasite *E. histolytica*.

16.6 Genome Content of Cysts and Trophozoites

Axenic cultures of *E. histolytica* have been used since 1961 after the introduction of the TYI-S-33 medium and are indispensable for molecular biology and cell biology studies of this parasite. It was observed that heterogeneity of DNA content was a common feature of all the *E. histolytica* strains growing under axenic conditions. *E. histolytica* normally grows in the presence of the microbial flora of the large intestine. We compared the DNA content of two recent isolates, *E. histolytica* 2592100 and DS4-868, that could be cultured both under xenic and axenic growth conditions. *E. histolytica* HM1:IMSS has been cultured axenically for more than 40 years and is difficult to revert to xenic culture. The nuclear DNA content of both strains was found to be tenfold lower when grown under xenic conditions compared the corresponding axenically grown cultures. Furthermore, a comparison of the size

of the nuclei of these two populations of *E. histolytica* revealed that the nuclear size in the xenically growing *E. histolytica* cells was less than that of axenically growing cells. Additionally, the number of multinucleated cells was also greater in the axenic cultures [3]. These results were corroborated by histological sections obtained from patient's large intestine [3]. In addition to xenic and axenic growth we compared the DNA content of trophozoites and cysts. *Entamoeba* cells are found in two major forms in nature, that is, cysts and trophozoites. Because in vitro encystation is difficult for *E. histolytica*, we analyzed the genome content of the cyst and trophozoites in *E. invadens*. During excystation a cyst gives rise to single amoeba. Scanning cytometry showed that excysted trophozoites had 40 times more DNA content than the cyst nucleus (Fig. 16.1d). Molecular data [3] supported our interpretation that several rounds of whole genome duplication occur during the conversion of a cyst into a viable trophozoite.

16.7 Concluding Remarks

Taken together, the data suggest that the *Entamoeba* genome is able to lose or gain multiple copies that are partitioned into cysts or daughter cells which are actively multiplying. Dynamic plasticity of the genome allows the cells to adapt to different growth conditions both inside and outside the human host. Absence of conserved regulatory mechanisms is therefore a likely necessity for these parasites to constantly adapt to challenging environmental changes. Over and above all these mechanistic differences, genome duplication, segregation, cell division, and conversion to different forms of the protist are unique among all eukaryotes, reminding us again that there is always a deviation from paradigms and rules.

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Chapter 17
Molecular Basis of the Trafficking of Cysteine Proteases and Other Soluble Lysosomal Proteins in *Entamoeba histolytica*

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Abstract Cysteine proteases (CPs) are the essential virulent factor of *Entamoeba histolytica*. Although the physiological and pathological roles of CPs have been demonstrated, the molecular basis of intracellular trafficking of CPs has only begun to be unveiled. Recent work has revealed the mechanisms of intra- and extracellular transport of CPs and other soluble lysosomal proteins in *E. histolytica*. Such proteins involved in the mechanisms include Rab small GTPases, their effectors, the intrinsic inhibitor of CPs, and a unique family of receptors responsible for lysosomal transport. In this chapter, we give an overview of the current understanding of molecules and mechanisms involved in the transport of CPs and other soluble lysosomal proteins in *E. histolytica*.

17.1 Introduction

Cysteine proteases (CPs) play a pivotal role in the pathogenesis of *Entamoeba histolytica*. Both the invasive and cyto (and histo-)lytic capacity of this parasite is mainly attributed to CPs, as demonstrated by numerous in vitro and in vivo studies [1–4]. The central importance of CPs to pathogenesis prompted a wide range of investigations, from basic studies such as analysis of the substrate specificity, activation mechanisms, transcriptional regulation, to applied studies such as drug development [5–12]. Although CPs per se have been extensively studied, the regulation

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_17

of their intracellular processing and transport is poorly understood, partly because of the lack of an available system to trace the trafficking of CP in the cell. Recently, ectopic expression of the epitope-tagged CP-A5 in *E. histolytica* trophozoites under the tetracycline-inducible promoter by episomal plasmid-based transfection [13–16] made it possible to track the transport of CP in the cell [17]. CP-A5, but neither CP-A1 nor CP-A2, tagged with the influenza hemagglutinin (HA) peptide at the carboxyl-terminus, was shown to be transported in the trophozoite in a manner indistinguishable from the endogenous protein.

Similar to cathepsins in mammals, *Entamoeba* CPs are primarily transported to lysosomes and phagosomes, where they are utilized to degrade endocytosed fluid, engulfed bacteria and host cells [18–21]. The organelles involved in protein biosynthesis such as the endoplasmic reticulum (ER) and the Golgi apparatus in *E. histolytica* do not retain typical morphology as in other eukaryotic organisms. For instance, in trophozoites the ER is not placed at the perinuclear region as in mammalian cells but distributed like a network throughout the cytoplasm [22, 23]. The Golgi apparatus is also observed as vesicles, instead of the stacks as seen in other eukaryotes. These unusual characteristics make it difficult to discern the intracellular organelles and dissect the trafficking pathways of lysosomal proteins. These technical problems also delayed analyses of the intracellular trafficking of CPs.

Other than CPs, there are many soluble lysosomal proteins known or considered to be involved in the pathogenesis of *E. histolytica*. Amoebapores are the cytolytic peptides homologous to granulysin, which is present in human cytotoxic lymphocytes, displays potent cytolytic activity toward bacterial and human cells, and forms ion channels in artificial membranes [24]. Amoebapores are targeted to lysosomes and mainly involved in degradation of ingested bacteria. Inhibition of expression of the *amoebapore A* gene by antisense or gene silencing caused reduction in virulence, suggesting that this protein plays a key role in pathogenesis [25, 26]. β-N-Acetylhexosaminidase is involved in the hydrolysis of terminal *N*-acetyl-D-hexosamine residues in hexosaminides. When *E. histolytica* trophozoites propagate extraintestinally, they take a route similar to that during metastasis of cancer cells [27], which requires both proteases and glycosidases during the passage of the basement membrane [28, 29]. The importance of hexosaminidase activity in the amebic virulence has been was reported [30]. Lysozymes are well-known glycosidases that degrade the bacterial cell wall [31]. It was reported that *lysozyme* gene expression is low in the avirulent *E. histolytica* Rahman strain and in *E. dispar* [10, 32]. Furthermore, expression of the *lysozyme* gene was also repressed in *E. histolytica* trophozoites that were treated with 5-azacytidine (5-AzaC), a potent inhibitor of DNA methyltransferase, and the repression of *lysozyme* gene correlated with the reduction in virulence [33].

It was also demonstrated that some soluble lysosomal proteins are excreted and function in the extracellular milieu, but the molecular mechanisms underling the process remained unsolved [34, 35]. As two major lysosomal targeting receptors, that is, mannose 6-phosphate receptor (MPR) and Vps10p/sortilin, are not conserved in *E. histolytica*, unique sorting receptors and molecular mechanisms of the targeting

of lysosomal proteins were presumed [36–41]. In this chapter, we summarize the current understanding of the molecular mechanisms of the CP transport, particularly unique transport receptors/carriers for CPs and other lysosomal proteins, exclusively present in *Entamoeba*.

17.2 Rab Small GTPases and Their Effectors

17.2.1 Rab Small GTPases

Small GTP-binding proteins (GTPase) are the molecular switches involved in various important cellular processes including cell proliferation, cytoskeletal assembly, and membrane traffic. Small GTPases are classified, based on their primary sequences, into five families: Ras, Rho/Rac, Rab, Sar/Arf, and Ran [42]. Rab small GTPases constitute the largest group of this superfamily and are essential regulators of vesicular transport [43–45]. Lower eukaryotes generally have a smaller repertoire of *Rab* genes compared to the more complex multicellular eukaryotes (e.g., *Saccharomyces cerevisiae* has only 11 Rabs). However, *E. histolytica* harbors more than 100 *Rab* genes, which are more than in humans (60), although *E. histolytica* is unicellular throughout its life cycle [46, 47]. Thus, *E. histolytica* shows the notable complexity of Rab proteins, suggesting their diverse and differential roles in membrane traffic. Among all Rabs, only 8 of them have been studied: Rab5, 7A, 7B, 8A, 11A, 11B, A, and B [47]. Among them, Rab7A, 7B, and 11B have been shown to be involved in the CP transport and secretion.

17.2.1.1 General Mechanisms of Rab-Mediated Regulation of Vesicular Traffic

Rabs are present in two conformational forms: active GTP-bound and inactive GDP-bound states. The transition between the two states is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which activate and inactivate Rabs, respectively [48, 49]. GTP-bound active Rab binds to specific proteins (called “effectors”), which define the downstream functions of individual Rab proteins. Rabs are partitioned to both the cytosol and membranes, and its membrane association is negatively regulated through interactions with the cytosolic chaperone GDP dissociation inhibitor [49]. The membrane association as well as the interaction with downstream effectors of Rab proteins are mediated by the mostly carboxyl- or occasionally amino-terminal posttranslational modifications with geranylgeranyl, farnesyl, palmitoyl, and methyl moieties [50–52].

A variety of effectors elicit Rab type-specific and organism- or tissue-specific downstream effects in a spatially and temporally regulated manner. However, although several small GTPases such as Rab5, 7, and 11 are almost ubiquitously

conserved among all eukaryotes, the effector proteins are not at all conserved. For instance, the Rab5 effector, early endosome-associated protein 1 (EEA1), and the Rab7 effector, Rab-interacting lysosomal protein (RILP), are conserved only in limited phylogenetically related lineages [53, 54].

17.2.1.2 Rab7

Rab7 is one of the most investigated Rabs in general. Rab7 is associated with both endosomes and lysosomes and is involved in endosomal maturation, transport from late endosomes to lysosomes, and positioning of endosomes and lysosomes via regulating their movement along the cytoskeleton [53, 55]. Thus, Rab7 is involved in the regulation of multiple trafficking processes including the biogenesis of lyososomes, phagosomes, autophagosomes, and other lysosome-related organelles [56–59].

Many uni- and multicellular eukaryotes including *S. cerevisiae*, *Trypanosoma brucei*, *Plasmodium falciparum*, *Caenorhabditis elegans*, and *Drosophila melanogaster* encode a single Rab7 [60–63], *E. histolytica* and *Trichomonas vaginalis* have nine and three Rab7 isotypes, respectively [46, 64], which is similar to the plants *Lotus japonicus* and *Arabidopsis thaliana*, which have four and eight Rab7 isotypes, respectively [60, 65]. In plants, some Rab7 isotypes are expressed in a tissue-specific and stress-inducible manner [65, 66]. However, the functional differences between Rab7 isotypes have been only partially demonstrated in *E. histolytica*. The redundancy of Rab7 isotypes implies the complexity of lysosome biogenesis in *E. histolytica*, but its biological significance needs to be better elucidated.

It was shown that *E. histolytica* Rab7A is involved in the transport of CP and amoebapore to lysosomes, but localized only partially in the lysosomes [67]. Interestingly, overexpression of Rab7A reduced intracellular CP activity, suggesting that Rab7A directly or indirectly mediates excretion of CPs [68]. It was also suggested that Rab7A regulates retrograde transport or recycling of a putative CP receptor (see Sect. 17.3) from phagosomes to the *trans*-Golgi network via interaction with the retromer (or retromer-like; retromer is used in this chapter hereinafter) complex ([68, 69]; see Sect. 17.2.2). Rab7A is also involved in the formation of the unique vacuolar compartment, named prephagosomal vacuoles (PPV), which are formed during phagocytosis, and likely serves to process, activate, and store these lysosomal proteins prior to targeting to phagosomes [67, 70]. Rab7B is localized to late endosomes and lysosomes and involved in the formation of or fusion to lysosomes [70]. Thus, the function and localization of these two isotypes, Rab7A and Rab7B, appear to be different. There is no precedent in which two Rab7 isotypes are involved in the trafficking of lysosomal proteins from the post-Golgi to lysosomes and phagosomes in a sequential and coordinated fashion. To fully understand the specific roles of Rab7B, as well as other Rab7 isotypes and Rab11B (Sect. 17.2.1.3), in CP transport, it is essential to identify and characterize their unique effectors, as shown for Rab7A (Sect. 17.2.2).

17.2.1.3 Rab11B

Rab11 is one of the most conserved and almost ubiquitous Rabs; it mediates protein recycling by regulating membrane transport from recycling endosomes in general [71]. Present in the trans-Golgi network and post-Golgi vesicles, it is required for membrane transport to the plasma membrane [72]. It also contributes to apical recycling and basolateral to apical trans-cytosis of immunoglobulin receptors in polarized cells [73–75] and is required for the transport of integrin to the leading edge in motile cells [76]. Rab11 has been shown to bind to a subunit of the exocyst complex, Sec15, which regulates polarized vesicle transport in epithelial cells and neurons [77–79]. It is well known that the Rab11-dependent vesicle recycling is regulated by the effector molecules named Rab11-family interacting proteins (Rab11-FIPs) [80], which are restricted to the Metazoa [81]. However, a novel Rab11-interacting protein with a coiled-coil structure named TbRBP74 was recently identified in trypanosomes [82].

E. histolytica possesses four Rab11 isotypes: Rab11A [35, 83], Rab11B [84], Rab11C [46, 85], and Rab11D [46]. It was shown that Rab11B plays a crucial role in the regulation of transport and secretion of CP-A1, CP-A2, and CP-A5. Overexpression of Rab11B caused a dramatic increase in the activity of secreted and, to a lesser degree, intracellular CP, and thereby enhanced in vitro cytolytic activity [84]. This observation also indicates that Rab11B and its effectors have a pivotal role in the regulation of CP secretion.

17.2.2 Retromer Complex

Rab7 effectors such as RILP in other organisms are not conserved in *E. histolytica*. It was previously demonstrated, for the first time in the eukaryotes, that *E. histolytica* Rab7A interacts with the retromer complex in a GTP-dependent manner [68]. Rab7A regulates retrograde transport of the presumed CP receptor. It should be noted that the identity of this presumed CP receptor to the novel CP carrier/receptor, described next (Sect. 17.3), has not been confirmed. It is not known whether the retromer complex also functions as a downstream effector of Rab7A that elicits signals for further cellular response or is primarily a vehicle for recycling of the receptor.

The retromer complex is implicated for the retrograde transport of the hydrolase receptor from late endosomes to the trans-Golgi network [86–88]. In general, the retromer complex is composed of four or five components: Vps5p, Vps17p, Vps26p, Vps29p, and Vps35p in yeast [89], and Vps5p homologue sorting nexin1 (SNX1), Vps26, Vps29, and Vps35 in mammals [90]. The retromer is composed of two subcomplexes: a stable trimer of Vps26, Vps29, and Vps35, and a membrane-bound heterodimer of the SNX proteins Vps5 and Vps17 [91]. The Vps26–Vps29–Vps35 trimer is termed the cargo selective adaptor, as Vps35 directly binds to sorting motifs

present in the cytoplasmic domains of cargo receptor proteins, whereas the SNX heterodimer is called the membrane-deforming subcomplex, as it induces or stabilizes the formation of membrane tubules. Interwoven with these events, retromer recruits additional proteins that aid further cargo capture and packaging [92, 93], as well as accessory proteins which regulate maturation and scission of the retromer tubules [94–98]. Retromer therefore shares a number of similarities with archetypal coat complexes such as COPI, COPII, and clathrin coats [99]. The Vps26–Vps29–Vps35 subcomplex is a highly conserved protein assembly that originated before the last eukaryotic common ancestor [100]. In *E. histolytica*, Vps26, Vps29, and Vps35, but not Vps5, SNX1, or Vps17, are present in the genome [68, 101].

The binding of the *E. histolytica* retromer to Rab7A is direct and mediated via the carboxyl-terminal extension of Vps26. The interaction is also dependent on the GTP status of Rab7A [68]. Overexpression of Vps26 rescued the reduced intracellular CP activity caused by Rab7A overexpression, which reinforces physical interaction between retromer and Rab7A and its involvement in CP transport. In the Vps26–Vps29–Vps35 trimeric cargo selective adaptor, Vps35 is known to interact with the hydrolase receptor, Vps10p, sortilin, or MPR in yeast and mammals [41, 102–104], and to also be involved in vacuolar sorting receptor (VSR) trafficking in plants [105]. Interestingly, despite the conservation of Vps35 in *E. histolytica*, none of MPR, Vps10p/sortilin, or VSR is conserved. As already described, it remains unknown whether the functional homologue of MPR, Vps10p/sortilin, or VSR in *E. histolytica* is a group of the newly identified cysteine protease-binding proteins, described next (Sect. 17.3). It is important to identify the receptors that interact with the retromer or Rab7A and all the components of the retromer complex. Size fractionation by gel filtration revealed that the amoebic retromer complex has an apparent molecular weight of 300–500 kDa [68], which is comparable to the retromer complex in mammalian cells [90]. Two subunits that are present in the retromer complex from mammals and yeast, but missing in *E. histolytica*, that is, sorting nexins and Vps5/17 contain the PX and BAR domains for the interaction with phosphatidylinositol 3-phosphate (PI3P) and the curved membrane [86, 106, 107]. These domains are involved in the membrane association of the retromer complex in the model organisms, and in *E. histolytica* other not yet identified proteins possessing these domains may be involved in the membrane association. Alternatively, the functional homologues of sorting nexins and Vps5/17 are dispensable as Rab7A mediates membrane association of retromer. There is no clear evidence suggesting that the retromer–Rab7A interaction exclusively regulates retrograde transport, because the phenotypes of overexpression of Rab7A or Vps26 may also be well explained by the hypothesis that Rab7A–retromer interaction is involved in the forward transport of CPs in the post-Golgi pathway. Active GTP-bound Rab7A on the post-Golgi vesicles recruits the retromer complex to the membrane, where the retromer or Rab7A may interact with a not yet identified CP receptor or cysteine protease-binding protein family (CPBF)1 to regulate the intracellular CP traffic. However, the overall mechanisms of how Rab7A, retromer, and CP receptor(s) regulate CP transport are likely complex and require further investigation.

17.2.3 Inhibitor of Cysteine Proteases (ICPs)

Lysosomal cysteine proteases in mammalian and plant cells are also negatively regulated by intrinsic proteinaceous cysteine protease inhibitors that belong to the cystatin superfamily [108–111]. For example, cystatin C represses cell-surface expression of MHC class II molecules in dendritic cells [112], and consequently decreases the risk of Alzheimer's disease, by a direct binding to and an inhibition of cathepsin B, leading to reduction of the generation of amyloid β-peptide [113]. There is also a report showing the involvement of cystatin C in the control of viral replication [114]. In plants, cystatin modulates host defense against fungal pathogens [115].

In protozoan parasites, a novel class of proteinaceous CP inhibitors, named chagasin or inhibitors for cysteine peptidases (ICP), were discovered in *Trypanosoma cruzi* [116]. The inhibitory activity of this approximately 12-kDa inhibitor against CP, that is, cruzipain, in epimastigote (insect stage) lysates was heat stable. Orthologues of ICP are widely conserved in the protozoa including *E. histolytica* [117, 118], *Leishmania mexicana* [119], *Leishmania major*, *Trypanosoma brucei* [120], *Plasmodium falciparum* [121], *Plasmodium berghei* [122], *Cryptosporidium parvum* [123], as well as Bacteria [120] and Archaea [124], but absent in mammals. It was shown that ICP inhibited papain family CPs with an inhibition constant (K_i) of a nano- to picomolar range [116], similar to cystatin [125]. Structural analyses by nuclear magnetic resonance (NMR) revealed that chagasin and *L. mexicana* ICP consisted of eight β-strands and one small α-helix, and three mobile loops in the same side bound to CP [126, 127]. The structure of ICP showed no similarity to CP inhibitors of other families at the level of the overall fold. However, there are suggestive similarities between the CP-interacting regions of ICP and the cystatin family of inhibitors. The three conserved ICP loops may form a tripartite wedge similar to that of the cystatin family. Both ICP and cystatin appear to have been adapted to fit the active site cleft of clan CA, family C1 CPs.

The role of ICP and its homologues in protozoan parasites were well studied. Chagasin has been shown to regulate differentiation and invasion of mammalian cells [128]. In *L. mexicana*, ICP has been assumed to play a role in the protection against a hydrolytic environment in insect vectors or hosts [119]. In *P. falciparum*, falstatin was shown to be involved in red cell invasion. In *P. berghei*, ICP is expressed throughout the life cycle and has a role in the sporozoite invasion of host cells. *P. berghei* ICP was also implicated for survival during liver stage development by inhibiting host proteases involved in programmed cell death [121, 122].

Dissimilar to other organisms, *E. histolytica* have two isotypes of ICP, ICP1 and ICP2. ICP2 has a signal sequence and localized in the vacuolar lumen, whereas ICP1 lacks the signal peptide and is localized to the cytosol [117, 118, 129]. There are several reports showing the extracellular secretion of ICP and cystatins in other organisms. However, it is not the case for *E. histolytica* ICPs; neither ICP1 nor ICP2 appears to be secreted in axenic culture conditions [118, 129]. The inhibitory constants of recombinant ICPs against three major CPs in *E. histolytica*, CP-A1, -A2,

and -A5, are intriguing. The K_i values of the ICPs were comparable for CP-A1 and -A2: 132–166 pM (CP-A1) and 809–950 pM (CP-A2), respectively. However, for CP-A5, ICP2 showed a 12-fold-lower K_i value than that of ICP1 (ICP1, 590 pM; ICP2, 50 pM) [118], suggesting that ICP2 may primarily inhibit CP-A5 in the secretory pathway (from the ER to lysosomes/phagosomes). Recombinant ICPs showed binding activity to CP-A2 and CP-A5. The crystal structure of ICP2 suggested that the 10-fold-lower K_i value of ICP2 for papain, compared to EhICP1 or chagasin, is likely attributed to be the existence of phenylalanine at a.a. 80 and the electrostatic potential on BC and FG loops, two of three loops involved in substrate binding [130]. Although the high substrate specificity of ICP2 toward CP-A5 was not explained by structure, analysis of surface electrostatic distribution of three CPs showed that only CP-A5 has a neutral surface charge, whereas the other two CPs have an acidic charge distribution. These differences in the surface charge distribution of EhICP2 may account for an initial electrostatic binding to a battery of cysteine proteases, although the regions surrounding the active site should be responsible for the relative selectivity.

Overexpression of HA- or GFP-tagged *E. histolytica* ICPs in trophozoites caused remarkable inhibition of CP activity in both lysates and culture supernatant, and the inhibition was more pronounced for the secreted CPs. The amount of intracellular CP-A2 and CP-A5 did not change, and processing of these CPs was not affected. These results clearly indicate the role of ICPs as the negative regulator of CP activity and secretion. Interestingly, overexpression of ICP1, which is the cytosolic form of ICP, also caused inhibition of CP secretion [118]; this was puzzling because ICP1 does not appear to be transported through the secretory pathway as it lacks the signal peptide and is indeed observed in the cytosol but not in endosomes/lysosomes. There are no examples in other organisms in which ICP or cystatins are involved in the regulation of CP secretion.

17.3 Novel Receptors That Mediate Lysosomal Transport of Cysteine Proteases and Other Hydrolases

17.3.1 General Mechanisms of the Transport of Lysosomal Proteins in Eukaryotes

In general, the transport of soluble lysosomal proteins is mediated by specific receptors. There are three major classes of soluble lysosomal protein transport receptors: MPR, sortilin or Vps10p, and plant-specific VSR. Sortilin/Vps10p is conserved in a wide range of eukaryotes, whereas MPR is mainly conserved among the Opisthokonta and the Amoebozoa and VSR is specific to the Planta and the Chloroplastida. MPRs consist of two classes of proteins, cation-independent MPR (CI-MPR) and cation-dependent MPR (CD-MPR) and recognize the mannose 6-phosphate moiety on the soluble lysosomal proteins via its carbohydrate recognition domain (CRD). CI-MPR

is approximately 400 kDa in size and has 15 CRDs, among which three domains are responsible for the recognition of the ligands [131]. CD-MPR is a homodimer with a 40-kDa monomeric size with a single CRD. In contrast to MPRs, sortilin/Vps10p recognizes peptides, not carbohydrates, on the ligands [132]. Sortilin/Vps10p has a β-propeller structure with ten blades, named the Vps10p domain, and recognizes a variety of molecules via the cavity between the blades [133]. VSRs recognize short peptide sequences, as there are no sugar modifications on vacuolar proteins in plants [134, 135]. It was shown that the epidermal growth factor (EGF)-like repeat at the carboxylterminus of the luminal VSR domain is responsible for binding to the cargo, as the deletion of the EGF-like repeats from soluble VSRs caused decrease in the affinity to the cargo [136, 137]. Despite the lack of similarity at the amino acid levels, these receptors share the mechanistic features such as binding to the retromer complex and adaptor proteins. It was also demonstrated that neutral pH and high calcium concentrations enhance the ligand binding of VSRs [136–140]. In *E. histolytica*, there are two CD-MPR homologues, one of which was detected in phagosomes and occasionally localized in lysosomes ([20, 21]; Nakada-Tsukui, unpublished data). However, their functions remain unknown.

17.3.2 Identification of Entamoeba*-Specific Receptors for Lysosomal Enzymes*

Rab small GTPases, the retromer, and ICP were shown to be involved in the intracellular traffic of CPs in *E. histolytica*, but the enzymes that catalyze mannose 6-phosphate modifications on lysosomal proteins and sortilin/Vps10p are missing in *E. histolytica*. Genes encoding GlcNAc-1-phosphotransferase and *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase are not present in the *E. histolytica* genome [141]. There are two genes encoding putative CD-MPR in *E. histolytica*. Although it is not known whether these CD-MPRs are involved in the binding and transport of lysosomal proteins, immunoprecipitation of HA-tagged CD-MPRs demonstrated no interaction with soluble lysosomal proteins (Nakada-Tsukui et al., unpublished data). These data indicate that MPRs in *E. histolytica* are unlikely to function as a lysosomal targeting receptor.

The *Entamoeba*-specific receptor that is involved in the binding to CPs has recently been identified [17]. The putative CP receptor was first identified by immunoprecipitation of CP-A5 with the HA tag at the carboxyl-terminus with anti-HA antibody. CP-A5 is believed to play a pivotal role in the pathogenesis of *E. histolytica*, as CP-A5 is exclusively expressed in *E. histolytica* but not in the non-virulent sibling species *E. dispar* [142, 143], and the repression of CP-A5 expression by antisense inhibition caused loss of virulence [144]. Temporal and spatial information on the trafficking of CP-A5 was demonstrated with confocal micrographic imaging and immunoblot analyses of the transformant in which CP-A5-HA was expressed under the tetracycline-inducible promoter. Unprocessed Pre-pro

CP-A5-HA was newly synthesized and localized to the ER, as demonstrated by colocalization with the marker protein Sec61α. CP-A5-HA, as processed into the mature form, was further transported to lysosomes. It was demonstrated that the processing and maturation of CP-A5-HA closely correlates with its trafficking.

Using the CP-A5-HA-expressing *E. histolytica* cell line, the putative CP-A5 receptor was immunoprecipitated and identified by mass spectrometry to be XP_655218, a 903-a.a. protein, which has a 16-a.a. signal sequence, a single transmembrane domain (a.a. 863–885), and a 19-a.a. cytoplasmic tail containing the YGKL tetrapeptide at the carboxyl-terminus. The YxxΦ motif (x is any amino acid and Φ is any aliphatic amino acid) is present in the cytoplasmic portion of numerous receptors, and shown to be responsible for the binding to the adaptor protein (AP) complex [145].

In the *E. histolytica* genome database, ten additional proteins with a similar structural organization (16–23 % identity at the amino acid level; *E* value <6.30 E^{-21} with BLASTP) to the CP-A5-binding protein were identified. These proteins have no identifiable homologues in other prokaryotic and eukaryotic organisms. This *Entamoeba*-specific protein family was designated as cysteine protease-binding protein family (CPBF), and the identified putative CP-A5-binding protein was named CPBF protein 1 (CPBF1), and other members as CPBF2–11 based on their similarity to CPBF1 in a descending order of the percentage identified to CPBF1.

17.3.3 Cysteine Protease-Binding Protein Family 1 (CPBF1): A Novel Transport Receptor of CPs

CPBF1 binds to CP-A1, A2, A4, and A6 (Table 17.1), as well as CP-A5, as demonstrated by the affinity pull-down assay ([17]; Nakada-Tsukui et al., unpublished data). It was demonstrated that interaction of CPBF1 and CP-A5 is independent of asparagine-linked sugar modifications of CP-A5 [17], because a mutant CP-A5 lacking this modified asparagine binds to CPBF1 as efficiently as does wild-type CP-A5, and tunicamycin treatment of trophozoites does not interfere with the binding of CP-A5 and CPBF1. CP-A1 and A2 as well as CP-A4 and A6 have no apparent site for N-linked carbohydrate modifications [7, 17]. Furthermore, *E. histolytica* has no genes for enzymes involved in M6P modifications, as already described.

When CPBF1 was repressed by gene silencing, transport of CP-A5-HA to lysosomes was abolished and CP-A5-HA was retained in the ER. Furthermore, processing of CP-A5-HA was also inhibited by CPBF1 gene silencing. Thus, CPBF1 is essential for the maturation and lysosomal transport of CP-A5. It was also observed that secretion of collagenogenic activity was increased in a CPFB1-repressed strain (Nakada-Tsukui et al., unpublished data), similar to the phenotype caused by repression of other lysosomal targeting receptors in other organisms. These data also suggest that proper targeting of CP-A5 to lysosomes negatively regulate the secretion of CP-A5. However, it remains unknown whether the inactivation or displacement of CPBF1 is the principal mechanism in wild-type trophozoites to initiate secretion of CP-A5 to the extracellular milieu.

Table 17.1 Cysteine protease-binding protein family (CPBF)-interacting proteins

CPBF	Protein name	MW[a] (kDa)	GenBank	AmoebaDB	Quantitative value[b]	
CPBF1[c]	EhCP-A5	35	XP_650937	EHI_168240	76.26	0
	EhCP-A2	35	XP_650642	EHI_033710	28.43	0
	EhCP-A4	34	XP_656602	EHI_050570	25.85	0
	EhCP-A1	35	CPP1_ENTHI	UniProtKB/Swiss-Prot: Q01957.1	12.92	0
	EhCP-A6	35	XP_657364	EHI_151440	9.05	0
CPBF2[d]	α-Amylase	69	XP_655699	EHI_152880	122.46	0
CPBF6[e]	α-Amylase family protein	57	XP_655636	EHI_023360		
	γ-Amylase	75	XP_652381	EHI_044370		
CPBF7[d]	β-*N*-Acetylhexosaminidase	64	XP_656208	EHI_012010	17.95	0
	β-*N*-Acetylhexosaminidase, beta subunit	64	XP_650273	EHI_007330	16.32	0
	Pore-forming peptide ameobapore B precursor	10	XP_001913632	EHI_194540	9.79	0
CPBF8[b]	β-Hexosaminidase alpha-subunit	60	AJ582954[f]	N/A		
	Lysozyme1	23	XP_653294	EHI_199110		
	Lysozyme2	23	XP_656933	EHI_096570		
CPBF10[d]	α-Amylase	53/50	XP_656406/XP_651479[g]	EHI_153100/EHI_119580[g]	49.05/14.83	0/0
	α-Amylase family protein	57	XP_655636	EHI_023360	27.38	5.76
	β-Amylase	47	XP_653896	EHI_192590	17.11	2.31

Ligands for CPBFs were identified by immunoprecipitation of HA-tagged CPBFs followed by LC-MS/MS analysis (CPBF1, 2, 6–8, and 10). HA-expressing cell line was used as control

[a]Molecular weight

[b]Furukawa et al. [147]

[c]Nakada-Tsukui et al. [17] and Nakada-Tsukui et al., unpublished

[d]Nakada-Tsukui et al., unpublished

[e]Furukawa et al. [146]

[f]Closest to EHI_148130

[g]These two α-amylases were not differentiated because of the high identity (87 %)

17.3.4 Roles of Other CPBF Proteins

17.3.4.1 Determinants of the Ligand Specificity

As shown, CPBF1 apparently binds only a limited members of CPs. Thus, compared to MPR or sortilin/Vps10p, CPBF1 has narrow ligand specificity. However, it needs to be further investigated whether CPBF1 also binds to other CPs or other lysosomal hydrolases. However, the fact that no other soluble lysosomal protein was detected by affinity pull-down of CPBF1 suggests that binding of CPBF1 is specific to several CPs. Among CPBF members so far examined (CPBF1, 2, 6, 7, 8, and 10), CPBF1 is the sole receptor that has affinity to CPs ([146, 147]; Nakada-Tsukui et al., unpublished data).

Affinity copurification of the ligands from the transformant that expresses CPBF6 and CPBF8 with the carboxyl-terminal HA tag demonstrated that CPBF6 binds to α-amylase (XP_655636) and γ-amylase (XP_652381), whereas CPBF8 binds to β-hexosaminidase (AJ582954) and lysozymes (XP_653294 and XP_656933) [146, 147] (Table 17.1). Gene silencing of CPBF6 and CPBF8 resulted in the decrease of the transport of their respective cargos to phagosomes [146, 147]. These data are consistent with the previous phagosome proteome studies showing that CPBF1, CPBF6, and CPBF8 are localized on phagosomes [20, 21] and indicate the involvement of these CPBFs in the transport of the lysosomal hydrolases to phagosomes. Similar to CPBF6 and CPBF8, CPBF2 and CPBF10 also appear to be involved in the transport of other isotypes of α-amylases [148]. In contrast, CPBF7 binds to β-hexosaminidases and amoebapore B (Table 17.1). Although the cargos of individual CPBFs mostly appear to be mutually exclusive, there are some exceptions, including CPBF6 and CPBF10 (see Sect. 17.3.4.2).

17.3.4.2 Multiple Isotypes of Carbohydrate Hydrolases Transported by CPBFs

Several CPBF members bind to carbohydrate hydrolases such as amylases and are involved in the transport of a variety of carbohydrate-degradative or sugar-modifying enzymes. There are at least five independent (non-allelic) genes (XP_656406, XP_655636, XP_655699, XP_649162, XP_652044) that potentially encode α-amylases. They are annotated as α-amylase, oligo-1,6-glucosidase, or hypothetical protein, and show significant similarity to α-amylase from *Homo sapiens* and/or *Dictyostelium discoideum*. CPBF2, 6, and 10 bind to three of them (XP_656406, XP_655636, and XP_655699), all of which possess the signal peptide, while the remaining two, XP_649162 and XP_652044, lack it, suggesting that the latter two α-amylase-like proteins may not be localized to the ER/Golgi/vesicles/vacuoles, but present in the cytoplasm. Thus, it is reasonable to speculate that they are not CPBF ligands. Among α-amylases that interact with CPBFs, XP_655699 and XP_656406 specifically interact with CPBF2 and CPBF10, respectively, whereas XP_655636 interacts with both CPBF6 and CPBF10. XP_655636 is the most highly transcribed

among all putative α-amylase genes, as demonstrated by microarray analysis [149]. This is the only example that more than one CPBFs are assigned to one ligand known today. It is possible that two CPBFs are involved in the transport of this α-amylase because of the biological importance of this enzyme.

There are three independent β-hexosaminidase genes in the *E. histolytica* genome. All three β-hexosaminidases bind to CPBFs. XP_656208 and XP_650273 bind to CPBF7, and AJ582954 binds to CPBF8. XP_650273 and AJ582954 were previously reported as HEXA and HEXB, respectively [150]. Lysozymes are encoded by six independent genes and annotated as lysozymes or *N*-acetylmuraminidase. Among them, only two lysozymes, XP_653294 and XP_656933, bind to CPBF8. Phylogenic analysis of 11 CPBFs indicates that CPBF7 and CPBF8 forms monophyly, and a clade consisting of CPBF7 and CPBF8 forms monophyly with CPBF10 and CPBF6 [17], suggesting that these CPBF are more closely related to one another than to other CPBF members. There must be distinct structural determinants in these CPBF proteins to distinguish α-amylase, β-hexosaminidase, and lysozymes, but they have not been demonstrated.

17.3.5 Role of the Serine-Rich Region and Its Carbohydrate Modifications in CPBF6–8

Only three members of CPBFs, CPBF6, -7, and -8, contain a serine-rich region at close proximity to the transmembrane domain of the luminal part. It was demonstrated that deletion of the 20- to 30-a.a.-long serine-rich region of CPBF8 diminished ligand binding without changing its localization [147]. Moreover, the removal of the 23-a.a.-long serine-rich region significantly reduced the apparent molecular weight of CPBF8 by approximately 50 kDa, which is larger than 2.4 kDa calculated from the loss of 23 amino acids. These data suggest that the serine-rich region harbors carbohydrate modifications and is responsible for the ligand recognition. It is puzzling that a deletion of the serine-rich region of CPBF8 abolished interaction with β-hexosaminidase although the binding to lysozymes was not affected [147]. It is also worth noting that α-amylase binds to both CPBF6 and CPBF10, but the latter lacks the serine-rich region. These data suggest that these ligands may be recognized by different mechanisms.

17.3.6 Regulation of Ligand Binding and Dissociation of CPBF

The two fundamental, rational functions of a carrier/receptor of lysosomal proteins are the binding to the newly synthesized and unprocessed proteins in the prelysosomal compartment such as the ER, the Golgi, and endosomes, and the release of the

ligands when they are to be fully processed and activated in the lysosomes. In general, soluble lysosomal protein receptors such as MPRs show high affinity for ligands at neutral pH whereas they have low affinity at acidic pH [151]. In contrast, in the case of the mammalian KDEL receptor, ligand binding is believed to be strong in the Golgi apparatus at acidic pH, although the neutral pH of the ER lumen would stimulate ligand release [152, 153]. CPBF1 also showed the pH-dependent ligand binding, similar to MPRs [17]. At neutral pH (6.5–7.0), CPBF1 interacts with CP-A5, whereas CPBF1 is dissociated from CP-A5 at pH 6.0 [17]. These data suggest that the interaction between CPBF1 and CP-A5 is, at least in part, regulated by pH.

It is unknown whether the ligand binding of CPBFs involves calcium. It is known that CD-MPR and VSRs require calcium ion for the ligand binding and that these receptors reside in the calcium storage organelle [136, 137]. The compartment of calcium storage in *E. histolytica* has not been clearly demonstrated, but inositol-1,4,5-trisphosphate (IP_3) has been reported to elicit calcium release from intracellular stores, most likely the ER [154]. If the ER indeed functions as calcium storage, there is possibility that ligand binding of CPBFs is also regulated by the calcium levels.

17.3.7 Regulation of Intracellular Traffic of CPBF

Molecular mechanisms that define the localization of CPBF are not well understood. Nine of 11 CPBFs have the YxxΦ motif at the carboxyl-terminus of the protein. The YxxΦ motif is considered as the binding site for the adaptor protein complex, which recruits clathrin to generate a transporting vesicle [145]. Although it was expected that deletion or mutation of the motif causes mislocalization of CPBF, neither mislocalization of CPBF1 nor a defect in the CP-A5 processing or trafficking was observed by point mutations or deletion of the motif (tyrosine and leucine to alanine) in CPBF1 [17]. There is, however, a possibility that the defect was not evident because the mutants did not cause a dominant-negative phenotype in the amoeba possessing wild-type CPBF1. As the AP complexes and clathrin are conserved in *E. histolytica*, it is reasonable to assume that they are involved in the trafficking of CPBFs.

In contrast, deletion of the entire cytoplasmic region, but not the 8-a.a. carboxyl-terminal region containing the YxxΦ motif, of CPBF6 abolished translocation of CPBF6 to phagosomes but did not affect ligand binding [146]. However, as already mentioned, deletion of the serine-rich region or site-directed mutagenesis of the sugar modification residues of CPBF8 did not affect its localization [147]. These data indicate that the cytoplasmic region contains the localization and transport signal involved in the traffic of CPBFs. The interaction between CPBF and possible interacting proteins such as the μ-subunit of the AP complex needs to be confirmed, but the association may only transiently occur.

17.3.8 Intracellular Localization of CPBFs

CPBF1 is mainly localized to the ER and occasionally observed in phagosomes, although it is hardly observed in lysosomes [17]. This localization fits the premise that CPBF1 is a transporting receptor of CPs from the ER to phagosomes/lysosomes. On the other hand, localization of CPBF6 and CPBF8 is slightly different from that of CPBF1. They are often colocalized with pyridine nucleotide transhydrogenase (PNT) and phagosomes [146, 147]. PNT was detected as the most abundant protein in phagosomes by phagosome proteomic studies [20, 21]. Although PNT is localized to the mitochondria in most eukaryotes, it is localized on phagosomes as well as undefined vesicles and vacuoles in *E. histolytica* [155], and its transhydrogenase activity depends on acidic conditions [156]. The PNT-associated vesicles were partially colocalized with LysoTracker red, suggesting that PNT and thus, CPBF6 and CPBF8, are involved in the later step, that is, closer to lysosomes than CPBF1, of the lysosomal transport. Although the physiological role of PNT in *E. histolytica* has not been elucidated, PNT may be involved in the detoxification of reactive oxygen and nitrogen species by supplying NADPH as a reducing power by using a proton gradient across the lysosomal and phagosomal membranes. The similar distribution of PNT and CPBFs may suggest that these molecules share the same molecular mechanisms for targeting to lysosomes/phagosomes.

17.3.9 Structural Analysis of CPBFs

Structural information is needed to unequivocally demonstrate the molecular basis of ligand specificities and structural determinants of the binding between individual CPBFs and their ligands. Structural prediction by bioinformatics indicates that CPBF contains tandem repeats of globular domains exclusively consisting of β-strands, called prepeptidase carboxyl-terminal domain [148]. The structure of CPBF shows no similarity to other proteins including lysosomal protein receptors. Structural information of CPBF should assist structure-based drug designing to provide new drug candidates that hamper the trafficking of the major virulence factors of this parasite.

17.4 Conservation and Evolution of the Transport Receptors of Lysosomal Proteins

The distribution and origin of lysosomal targeting receptors discussed in this chapter, that is, CD-MPR, CI-MPR, Vps10/sortilin, and CPBF, in eukaryotes were examined (Table 17.2). It was previously indicated that the M6P/MPR-dependent lysosomal targeting system is widely conserved in the Metazoa, from Mollusca

(*Unio*) to *Homo sapiens*, including zebrafish, crustaceans, amphibians, and birds [141]. MPRs are widely conserved in the Amoebozoa, and only in a free-living amoeba *Naegleria gruberi* from the Excavata, as well as Opisthokonta, including *H. sapiens*, *D. melanogaster*, and *S. cerevisiae*. As the Amoebozoa are known to be evolutionary close to the Opisthokonta, the last common ancestral protein containing a primitive CRD may have occurred in the common ancestor of the Amoebozoa and the Opisthokonta. Vps10p/sortilin homologues are found in all five eukaryotic subgroups, but only in limited lineages in each subgroup, except for the Chromalveolata, in which it is especially well conserved (Table 17.2). Although Vps10p/sortilin is also well conserved in the Amoebozoa, it is absent in *Entamoeba* and *Mastigamoeba balamuthi*, suggesting a possible secondary loss of the gene after speciation within the Amoebozoa.

Most of the CI- and CD-MPRs found in unicellular eukaryotes have a single CRD, with *D. discoideum* being an exception (Table 17.2). However, the carbohydrate-binding activities of the CRDs appear to be promiscuous. Although in *D. discoideum* CI-MPR with 3 CRDs, CD-MPR, and Vps10 are present, and carbohydrate modifications of lysosomal hydrolases and mannose:GlcNAc phosphotransferase activity were demonstrated, neither CI-MPR nor CD-MPR is involved in lysosomal targeting [157–160]. The *D. melanogaster* CI-MPR homologue, LERP, has five recognizable CRD domains, but it does not depend on either M6P or other carbohydrates for ligand binding [161]. It is of note that in *S. cerevisiae* Vps10p and a single CRD domain-containing protein, Mrl1p [162], cooperatively function in the traffic of lysosomal (vacuole in yeast) proteins. Vps10p is primarily responsible for the targeting of carboxypeptidase Y or proteinase A to the vacuole, a lysosome-equivalent organelle in yeast. Although a specific role of Mrl1p is not yet clear, Mrl1p colocalizes with Vps10p, and deletion of both Mrl1p and Vps10p, in contrast to Vps10p alone, interferes with the sorting of proteinase A to the vacuole, suggesting that Mrl1p and Vps10p cooperatively function in the sorting of vacuolar proteins. Mrl1p is not a lectin as *N*-glycan structures on the ligand are not required for the binding. In addition, only three of four residues known to be involved in M6P recognition are not conserved in Mrl1p [141]. It has been proposed that an ancestral protein of Mrl1p with a single primitive CRD, involved in lysosomal transport, lacked carbohydrate-binding activity and led to the duplication of the domain and generation of proteins containing multiple CRDs, which gained carbohydrate binding. By analogy to Mrl1p and Vps10p, it is also possible that CD-MPRs and CPBFs also genetically or physically interact in *Entamoeba*.

CPBFs have been discovered only in *Entamoeba*. However, there is a single CPBF-like gene in the *M. balamuthi* genome (Spears and Roger, unpublished data), which may represent a prototype of CPBFs. *M. balamuthi* CPBF also has the YxxΦ motif at the carboxyl-terminal end, which indicates the functional conservation of the motif. It is worth investigating whether the ligand specificity and the mechanisms of recognition of *M. balamuthi* CPBF. CPBF may have occurred in the common ancestor of *Entamoeba* and *Mastigamoeba* and expanded only in *Entamoeba* by gene duplication and functional diversification.

Table 17.2 Distribution of MPRs and Vps10p/Sortilin homologues among eukaryotes

Superfamily	Parasitic	Species	CI-MPR	CD-MPR	Vps10/sortilin
Amoebozoa		*Dictyostelium discoideum*	3		
	+	*Entamoeba histolytica*			
	+	*Entamoeba invadens*	1		
		Mastigamoeba balamuthi	1		
		Polysphondylium pallidum	1		
		Acanthamoeba castellanii	1		
Opisthokonta		*Homo sapiens*	15		
		Drosophila melanogaster	5		
		Caenorhabditis elegans			
		Saccharomyces cerevisiae	1		
	+	*Encephalitozoon cuniculi*			
Archaeplastida		*Cyanidioschyzon merolae*			
		Chlamydomonas reinhardtii			
		Ostreococcus tauri			
		Ostreococcus lucimarinus			
		Arabidopsis thaliana			
Chromalveolata		*Paramecium tetraurelia*			
		Tetrahymena thermophila			
	+	*Cryptosporidium hominis*			
	+	*Plasmodium falciparum*			
	+	*Theileria species*			
	+	*Toxoplasma gondii*			
	+	*Phytophthora species*			
		Thalassiosira pseudonana			
Excavata	+	*Giardia lamblia*			
	+	*Trichomonas vaginalis.*			
	+	*Trypanosoma brucei*			
	+	*Leishmania major*			
		Naegleria gruberi	1		
		Andalucia incarcerata			
		Sawyeria marylandensis			
		Psalteriomonas lanterna			

Homo sapiens CI-MPR, CD-MPR, *Saccharomyces cerevisiae* Vps10p, and *H. sapiens* Sortilin were used as a query to search for homologues against the selected taxa in the NCBI BLASTP nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

■: *E*-val $<10^{-28}$

▒: *E*-val is between 10^{-6} and 10^{-3}. CRD or Vps10p domains were assigned by BLAST, Pfam, or Superfamily

The numbers of the CRD domains are shown

It remains totally unknown what sorting receptors are involved in the trafficking of lysosomal enzymes in the organisms that apparently lack MPR, Vps10, VSR, and CPBF. Recently, a novel receptor-containing WD40 domain, which is presumed to be involved in ligand binding, was demonstrated in *Giardia lamblia* named GlVps [163]. The cytoplasmic YxxΦ motif of GlVps, which is also shared by CPBFs from *Entamoeba* and *Mastigamoeba*, is involved, in cooperation with the adaptor AP-1 complex, in its targeting. It needs to be further determined what functional advantages or constraints drive the conservation of the motif and its interaction with the adaptor complex in limited species of receptors.

Acknowledgments We thank Courtney Spears and Andrew J. Roger, Centre for Comparative Genomics and Evolutionary Bioinformatics, Department of Biochemistry and Molecular Biology at Dalhousie University for the information on *Mastigamoeba* genome information, and Konomi Marumo for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to T.N. (23117001, 23117005, 23390099) and K.N.-T. (24590513), a Grant-in-Aid on Bilateral Programs of Joint Research Projects and Seminars from Japan Society for the Promotion of Science, a Grant-in-Aid on Strategic International Research Cooperative Program from Japan Science and Technology Agency, a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan (H23-Shinko-ippan-014) to T.N., a grant for research to promote the development of anti-AIDS pharmaceuticals from the Japan Health Sciences Foundation (KHA1101) to T.N., Strategic International Research Cooperative Program from Japan Science and Technology Agency to T.N., and by Global COE Program (Global COE for Human Metabolomic Systems Biology) from MEXT, Japan to T.N

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Chapter 18
Mitosomes in *Entamoeba histolytica*

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Abstract Mitosomes are highly divergent, often reduced, mitochondrion-derived organelles. Mitosomes in *Entamoeba histolytica* possess a unique metabolic role that has not been discovered in other eukaryotes. Sulfate activation, in which sulfate is activated with two ATP molecules to form 3′-phosphoadenosine-5′-phosphosulfate, is compartmentalized in *Entamoeba* mitosomes. Sulfate activation is essential for the production of sulfur-containing polar lipids and proliferation of trophozoites. Besides its unique metabolic role, the mechanisms of protein and solute transport across mitosomal double membranes are also highly divergent from other eukaryotes. For instance, the translocator of the outer membrane consists of the β-barrel pore component Tom40 and the unique peripheral membrane component Tom60, the latter of which is localized to both the mitosomal outer membrane and the cytoplasm and functions as a shuttle carrier of mitosomal proteins. In this chapter, we summarize the discovery, functions, and protein transport of *Entamoeba* mitosomes, and also discuss remaining important biochemical and biological riddles of mitosomes, including other metabolic functions, redox control, regulation of gene expression, solute/metabolite transport, replication, and degradation.

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_18

18.1 History of the Identification of the Mitochondrion-Related Organelle in *Entamoeba*

Until the early 1990s, *Entamoeba histolytica* was classified, together with diplomonads, parabasalids, and microsporidia, into the Archezoa [1]. The Archezoa were considered to be the most primitive eukaryotes, which had diverged before the mitochondria arose as a consequence of the endosymbiosis of an ancestral α-proteobacterium ("the archezoan hypothesis"). The archezoan organisms had atypical eukaryotic features including a lack of morphologically discernible organelles such as mitochondria, peroxisomes, and well-developed Golgi dictyosomes [1]. Microsporidia were positioned near the root of the Eukaryota by phylogenetic analysis of small subunit ribosomal RNA sequences (SSU-rRNA) and translational elongation factor 1α (EF1α), and the presence of 70S prokaryote-like ribosomes [2]. In contrast, *Entamoeba* was positioned among mitochondrion-possessing eukaryotes in phylogenetic reconstructions [2–4]. Despite the inference obtained by phylogenetic analysis, *E. histolytica* apparently lacked, based on biochemical assays, the tricarboxylic acid cycle, the cytochrome-mediated electron transport chain, and oxidative phosphorylation, suggesting a lack of functional mitochondria [5].

In late 1990s, Clark and Roger reported that genes encoding chaperonin 60 (Cpn60) and pyridine nucleotide transhydrogenase (PNT) are conserved in the *Entamoeba histolytica* genome [6], both proteins believed to be localized in canonical mitochondria. Furthermore, phylogenetic analysis showed that *Entamoeba* Cpn60 forms a monophyletic clade with Cpn60 from mitochondriate eukaryotes. These data are consistent with the hypothesis that *E. histolytica* possessed mitochondria in the past and has secondarily lost them. The discovery of the mitochondrion-related *Cpn60* and *PNT* genes in *E. histolytica* prompted a survey of several mitochondrion-related genes in other archezoan organisms. In the next few years, genes encoding a 70-kDa mitochondrial molecular chaperon (mt Hsp70), Cpn10, and Cpn60 were identified from the genome of diplomonads (*Giardia intestinalis*), parabasalids (*Trichomonas vaginalis*), and microsporidia (*Encephalitozoon cuniculi*, *Nosema locustae*, and *Varimorpha necatrix*) [7–14]. These findings suggested that all extant archezoan organisms possessed the mitochondrion or a related organelle in the past, and that it has subsequently undergone reductive evolution.

Electron microscopic examination of archezoan organisms, including *E. histolytica*, revealed that the existence of homogeneous double membrane-bound organelles that lack cristae, which are protrusions of inner membranes toward the matrix, but contain the aforementioned mitochondrial-type chaperones [15–20]. These organelles in the Archezoa are often called mitochondrion-related organelles (MROs) or mitochondrion-like organelles (MLOs), also crypton [21, 22], hydrogenosomes, and mitosomes [23]. Currently available data provided by biochemical, morphological, and phylogenetic investigations in principle support the notion that all extant anaerobic/microaerophilic eukaryotes possess a form of α-proteobacterium-derived MROs, including the highly divergent form found in *E. histolytica.*

18.2 Variation and Classification of Mitochondrion-Related Organelles

It is widely accepted that canonical aerobic mitochondria have been generated from the endosymbiosis of an ancestral α-proteobacterium with a methane-producing archae [24–26]. Mitochondria are thus surrounded by a double membrane of lipid bilayers and possess a separate circular or linear form of genome ranging from 6 kbp (in *Plasmodium*) to more than 2,000 kbp (in cucurbit plants) [27]. Aerobic mitochondria from humans contain about 1,500 proteins [28]. The main function of aerobic mitochondria is the production of ATP by oxidative phosphorylation, which is driven by a proton gradient across the inner membrane [29, 30]. Aerobic mitochondria are also involved in heme and phospholipid synthesis, calcium homeostasis, programmed cell death [31, 32], and biosynthesis of iron–sulfur (Fe–S) clusters [33].

During anaerobic or microaerophilic adaptation of organisms, the content and function of mitochondria have been highly modified. The reductive changes in mitochondria leading to the generation of MROs may be in principle caused by the independence of ATP generation from oxidative phosphorylation in mitochondria. MROs are found in a wide range of anaerobic/microaerophilic lineages that belong to all distinct eukaryotic subgroups, except for the Planta (Archaeplastida). These MROs have common features shared with authentic mitochondria, including a double membrane structure [34], specific mitochondrial-type chaperones [10, 16, 21, 35, 36], and the compartmentalization of Fe–S-cluster biosynthesis (namely, the ISC system) [19, 37–40]. MROs have reduced or no organellar DNA and have lost the majority of proteins involved in energy generation via the tricarboxylic acid cycle and oxidative phosphorylation, β-oxidation of fatty acids, and heme biosynthesis. However, some anaerobic mitochondria can still produce ATP via some components of the respiratory chain and ATPase [23]. Although some common features are shared among MROs from different anaerobic/microaerophilic eukaryotes, the morphology, components, and functions of MROs vary substantially among lineages.

MROs are classified into hydrogenosomes, mitosomes, and unclassified (intermediate) MROs (or MLOs); the latter do not resemble either the typical hydrogenosomes, as in *Trichomonas*, or highly reduced mitosomes, as in *Giardia.* In parasitic protists, MROs have been found in diplomonads, *G. intestinalis* [37], parabasalids, *T. vaginalis* [38], microsporidia, *Encephalitozoon cuniculi* [39], amoebozoa, *Entamoeba histolytica* [35], alveolata, *Cryptosporidium paruvum* [17], and stramenopiles, *Blastocystis hominis* [41]. MROs were also demonstrated in free-living anaerobic unicellular eukaryotes such as a heterolobosean, *Sawyeria marylandensis* [42], an anaerobic rumen fungus, *Neocallimastix patriciarum* [43], and a free-living amoebozoan, *Mastigamoeba balamuthi* [44]. The number of organisms that are known to possess MROs is expected to increase as the genomes of additional anaerobic/microaerophilic unicellular eukaryotes are sequenced.

Hydrogenosomes were named after their metabolic characteristic of producing hydrogen gas and ATP as a consequence of reduction of protons. The best known hydrogenosome is that of *T. vaginalis. T. vaginalis* hydrogenosomes contain malic enzyme, ferredoxin, acetate:succinate CoA transferase, succinyl-CoA synthetase, pyruvate:ferredoxin oxidoreductase, and hydrogenase. These enzymes carry out substrate-level phosphorylation to generate ATP, acetate, CO_2, and H_2 from pyruvate or malate. In addition to the ATP generation, *T. vaginalis* hydrogenosomes also generate Fe–S clusters and metabolize amino acids [45, 46]. In contrast to hydrogenosomes, mitosomes produce no hydrogen gas, and are found in *Giardia*, *Encephalitozoon*, *Cryptosporidium*, and *Entamoeba*. Mitosomes are not involved in energy production. It is generally presumed that the minimal common function of mitosomes in these organisms, as well as hydrogenosomes, is the Fe–S cluster biosynthesis by the iron–sulfur cluster biosynthesis (ISC) machinery (see Sect. 18.4.2). The composition and function of mitosomes are best studied in *G. intestinalis* [47], *Cryptosporidium* [48], and *E. histolytica.* In *Blastocystis,* the organelle is known as the MLO, as it has unique features and functions distinct from those found in hydrogenosomes, mitosomes, and MROs, such as the retention of a 28- to 29-kbp circular DNA [41, 49]. The *Blastocystis* MRO also contains the ISC system [40], and proteins involved in respiratory complexes I and II, but lacks components of complexes III, IV, and ATP synthase [50].

18.3 The Key Metabolic Functions of *E. histolytica* Mitosomes

18.3.1 Sulfate Activation

A core function of MROs in *Giardia*, *Trichomonas*, and microsporidia is the biogenesis of Fe–S clusters, but the function of *Entamoeba* mitosomes remained totally uncharacterized. Establishment of a protocol for mitosome purification using two rounds of discontinuous Percoll gradient ultra centrifugation allowed proteomic analysis of purified mitosomes by mass spectrometric analysis [51]. Of 95 putative mitosomal proteins, approximately 50 % of proteins identified in this study were annotated as hypothetical proteins with neither identifiable functional domains nor homology to proteins in other organisms.

The proteome did, however, revealed several key proteins involved in the core metabolism and biogenesis of mitosomes. The most unexpected finding of the study was the discovery of three key enzymes involved in sulfate activation: ATP sulfurylase (AS; XP_653570, EC 2.7.7.4), APS kinase (APSK; XP_656278, EC 2.7.1.25), and soluble inorganic pyrophosphatase (IPP; XP_649445, EC 3.6.1.1) (Fig. 18.1). In bacteria, the pathway generally exists in the cytosol, whereas in plastid-containing eukaryotes, the enzymes in the pathway are distributed to the cytosol or the plastid depending on the organism (*Arabidopsis, Oryza*, *Chlamydomonas*, *Cyanidioschyzon*, and *Toxoplasma*) [52]. It was previously reported that the enzymatic activities of

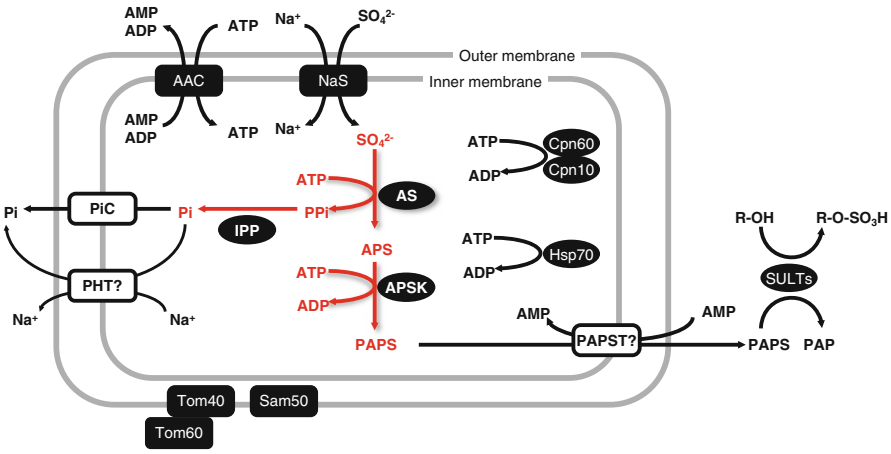

Fig. 18.1 Proteins localized in the mitosome of *E. histolytica*. Metabolites and reactions in the sulfate activation pathway are shown in *red*. Proteins shown in *boxes* and *ovals* indicate membrane and soluble proteins, respectively. Proteins whose localization is or is not confirmed by the immunofluorescence assay are shown as *black* or *white objects*, respectively. *AS* ATP sulfurylase, *APSK* APS kinase, *IPP* soluble inorganic pyrophosphatase, *Cpn60* chaperonin 60, *Cpn10* chaperonin 10, *Hsp70* mitochondrial-type heat-shock protein 70, *AAC* ADP/ATP carrier, *NaS* sodium/sulfate symporter, *PiC* phosphate carrier, *PHT* phosphate transporter, *PAPST* PAPS transporter, *Tom40* and *Tom60* 40- and 60-kDa subunit of the TOM complex, respectively, Sam50 50-kDa subunit of the SAM complex, *SULT* sulfotransferase

sulfate activation in *Euglena gracilis* are present in the mitochondria [52–54], but the proteins involved in the pathway have not been demonstrated. Therefore, compartmentalization of the sulfate activation pathway in mitochondria and MROs seems to be unprecedented in the Eukaryota.

Inorganic sulfate is an inert substance [55] and must be converted to its biologically active form to be transferred onto amino acids, peptides, proteins, carbohydrates, lipids, and small organic molecules. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) is an activated sulfate produced from inorganic sulfate and two molecules of ATP by the sulfate activation pathway, which consists of a two-step enzymatic process. AS first transfers the AMP moiety from ATP to sulfate and produces adenosine-5′-phosphosulfate (APS) and inorganic pyrophosphate (PPi) [56, 57]. APSK subsequently catalyzes the transfer of the gamma phosphoryl group of ATP to the 3′-hydroxyl group of APS. PPi, which is a by-product in the reaction of AS, is converted to phosphate by IPP, as PPi inhibits the forward reaction of AS, and also other PPi-related metabolism, by product inhibition at high concentrations [58].

Sulfate activated in *Entamoeba* mitosomes is mainly used to synthesize sulfur-containing polar lipids including cholesteryl sulfate [51, 59]. It is not fully understood whether the transfer of the sulfate moiety from PAPS to lipids occurs in mitosomes and/or the cytoplasm. However, several major sulfotransferases have been localized in the cytoplasm [51]. The physiological significance of the compartmentalization of the sulfate activation pathway in mitosomes remains unknown [51]. The premise that the pathway is indispensable for the parasite has

been demonstrated [20]: the repression of AS, APSK, or IPP by gene silencing caused a reduction in the incorporation of sulfate into lipids and produced a concomitant growth retardation.

The sulfate activation pathway is highly conserved in Bacteria, Archaea, and eukaryotes. In some bacteria including δ-proteobacteria, fungi, and alveolata, AS is fused with APSK as an "AS-APSK" fusion protein [51, 60, 61]. In contrast, an inversely linked fusion protein "APSK-AS" is present as PAPS synthase in Metazoa, and an "APSK-AS-IPP" fusion protein exists in stramenopiles. In *Entamoeba* species, three independent proteins are present for AS, APSK, and IPP activity. *Entamoeba* APSK apparently contains the amino-terminal region with sequence similarity to AS as seen in "AS-APSK" fusion proteins. However, its AS-like region has highly degenerated and lacks the AS activity. *Entamoeba* AS has a strong affinity by phylogenetic analysis with bacterial homologues, especially those in sulfate-reducing δ-proteobacteria such as *Desulfovibrio vulgaris*, suggesting that AS was acquired by lateral gene transfer (LGT) from a bacterium similar to extant δ-proteobacteria to the *Entamoeba* ancestor [51]. Moreover, *Entamoeba* APSK may also have a δ-proteobacteria origin, although we can not exclude the possibility that *Entamoeba* APSK has been acquired from anchestral α-proteobacteria [51]. In global phylogenetic analyses of IPP [51, 62], almost all eukaryotic lineages including *Entamoeba* form a monophyletic clade independent of the prokaryotic cluster with strong bootstrap support, suggesting that eukaryotic IPPs have a single evolutionary origin that was established in the common ancestor of eukaryotes. However, *Entamoeba* IPP does not seem to have significant affinity with mitochondrial IPP from Metazoa. Therefore, it is possible that the common ancestor of *Entamoeba* species has undergone the second endosymbiotic event involving a δ-proteobacterium after the establishment of the aerobic mitochondrion derived from initial endosymbiosis with an α-proteobacterium, followed by fusion of the two endosymbiosis-derived organelles and elimination of other δ-proteobacterium proteins from the fused organelles (destined to become mitosomes). Subsequently, eukaryotic cytoplasmic IPP replaced the IPP in the organelle to yield the chimeric mitosomes. The detailed evolutionary analyses of additional proteins and pathways are required to clarify the processes leading to *Entamoeba* mitosomes.

18.3.2 Transporters for Ions and Metabolites

To better understand the metabolism of mitosomes, it is essential to identify and characterize transporters and channels involved in the transfer of ions and intermediary metabolites. As the mitochondrial matrix is enclosed by the two membranes, machinery that allows directional translocation of molecules across both inner and outer membranes is needed. The mitochondrial inner membrane, in particular, is equipped with a number of selective transporters for ions and metabolites [63]. It was shown that the *Entamoeba* mitosome inner membrane is also equipped with an ADP/ATP carrier (AAC; XP_649800), which belongs to the mitochondrial carrier family (MCF) [20, 64] (Fig. 18.1). Recently, a homologue of the phosphate

carrier (PiC; XP_656350), which also belongs to the MCF and is usually found on the inner membranes of canonical mitochondria, was demonstrated in *E. histolytica* [65]. In addition, the *E. histolytica* genome has five potential genes encoding sodium sulfate symporters with 12 transmembrane-spanning regions (NaS; XP_649603, XP_654527, XP_654503, XP_657578, and XP_655929). Although XP_649800 was identified in the mitosome proteome analysis, it has not been localized to mitosomes by immunofluorescence assays, whereas XP_655929 has been demonstrated to be targeted to mitosomes [51]. Moreover, the *Entamoeba* genome also contains a gene for a potential PAPS transporter (PAPST; XP_654175), although its localization on the inner membrane of *E. histolytica* mitosomes has not been demonstrated. When new metabolic functions are implicated in *Entamoeba* mitosomes, transporters of the metabolites involved in the metabolic pathway need to be identified and their functions must be demonstrated. Protein transport into mitosomes is discussed in Sect. 18.5.

18.4 Other Possible Metabolic Roles of *Entamoeba* Mitosomes

18.4.1 Fatty Acid and Lipid Metabolism

Acyl-CoA synthetases (ACSs) have roles in lipid synthesis and lipid degradation [66, 67]. ACSs in general convert fatty acids and coenzyme A (CoA) to acyl-CoA, which is in turn oxidized to acetyl-CoA by β-oxidation. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA cycle) and is utilized to generate ATP. Two ACSs (XP_656410 and XP_649712) were detected in the proteome analysis of *E. histolytica* mitosomes [51]. *Saccharomyces cerevisiae* acyl-CoA synthetase Faa1p (NP_014962) is the closest homologue of these putative mitosomal ACSs with 27 % and 33 % amino acid identity (*E*-value = 3e−55 and 1e−93), respectively. Faa1p was detected in yeast mitochondria by proteome analysis [68]. One should note, however, that Faa1p was also detected in the plasma membrane [69], lipid particles [70], and endoplasmic reticulum [71] by proteomic analyses of the purified organellar fractions. One of the two potential *E. histolytica* homologues, XP_656410, shows 31 % amino acid identity (*E*-value = 2e−91) to ACSL1 (NP_036952) from rat, localized in mitochondria of 3T3-L1 adipocytes [72]. These data suggest that XP_656410 and XP_649712 proteins may be *Entamoeba* mitosomal homologues of ACS. However, one should be cautious in the interpretation of the mitosomal proteome [51] because proteins such as ER-localized calreticulin (XP_655241) and lysosomal-localized CPBF1 (*c*ysteine *p*rotease-*b*inding protein *f*amily *1*: XP_655218) [73] were also found in the mitosome-enriched fraction. Therefore, further investigation into the potential mitosomal ACSs of *Entamoeba* is needed. Moreover, because the *Entamoeba* genome apparently lacks components of the TCA cycle and β-oxidation, and the corresponding enzymatic activities are undetectable, the biological significance of ACS in the mitosome remains puzzling.

It is well known that the mitochondrial inner membrane contains cardiolipin, which is produced from CDP-diacylglycerol and phosphatidyl glycerol by cardiolipin synthase (CLS) in mitochondria. A putative *E. histolytica* CLS (XP_656112) was also detected in the proteome analysis, and its mitosomal localization was confirmed by immunofluorescence using an *E. histolytica* cell line expressing CLS tagged with hemagglutinin (HA) at the carboxyl-terminus [51]. However, the CDP-OH-P motif [D(X)2DG(X)2AR(X)8-9G(X)3D(X)3D(X)2L], which is largely conserved in CLS, is not conserved in the putative *E. histolytica* CLS. Therefore, there is a possibility that the putative *E. histolytica* CLS may have a function other than in the biogenesis of cardiolipin. In addition, cardiolipin was not detected in trophozoite lysates (Mi-ichi et al., unpublished data).

18.4.2 Iron–Sulfur Cluster Biogenesis

It is generally accepted that all forms of mitochondrion-related organelles, ranging from the aerobic mitochondria to highly reduced organelles such as hydrogenosomes and mitosomes, contain the ISC system for Fe–S cluster biogenesis. However, because *E. histolytica* lacks the ISC system, it remains an open question whether the NIF system, which substitutes for the ISC system in this organism, is also compartmentalized in mitosomes. Recent immunoelectron microscopic and biochemical studies suggest that NifS (BAD15366) and NifU (AAK85709), which were acquired from ε-proteobacteria via LGT [74], are predominantly present in the cytoplasm, but that a relatively small amount may also be present in mitosomes [19]. This conclusion was based on (1) the quantitation of gold particles per unit area on immunoelectron micrographic images, showing approximately tenfold concentration of NifS and NifU within mitosomes compared to the cytosol; and (2) the quantitation of NifS and NifU in the soluble and pellet fractions of lysates in immunoblot analysis. However, as most of the NifS and NifU proteins are localized in the cytoplasm, it has not been demonstrated unequivocally that the NIF system is concentrated in mitosomes. Moreover, NifS and NifU were not found in the proteome analysis of *Entamoeba* mitosomes [51]. Finally, a recent study by Tachezy and colleagues (unpublished data) clearly demonstrated that neither NifS nor NifU is enriched in the organellar fraction, suggesting that the previous conclusion may be erroneous. Therefore, the minimal common function(s), if any, of MROs is still in debate.

18.4.3 Redox System

Because *E. histolytica* lacks catalase and glutathione peroxidase, cytosolic peroxiredoxins and the thioredoxin/thioredoxin reductase system play an important role in the removal of hydroperoxides produced in the cell [75–77]. In contrast to the cytosolic system, proteins that participate in mitosomal redox regulation are largely unknown.

However, the genome project revealed that *E. histolytica* genome contains a gene encoding rubrerythrin (Rbr; XP_652131) [78], which is a non-heme, sulfur- and iron-containing (but not iron–sulfur cluster-containing) protein, and involved in oxygen detoxification. Rbr catalyzes the reaction [$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$], and is mainly found in strictly anaerobic or microaerophilic bacteria and Archaea [79–84]. The mitosomal localization of Rbr in *E. histolytica* was confirmed by immunoelectron microscopy [19], which is also supported by the similar localization of Rbr in the *T. vaginalis* hydrogenosome [85]. However, similar to NifS and NifU, Rbr was not detected in the proteome analysis of *Entamoeba* mitosomes [51]. Thus, the mitosomal localization of Rbr needs to be further verified by independent methods.

E. histolytica also encodes iron-containing superoxide dismutase (Fe-SOD; XP_648827) [86]. SOD catalyzes the dismutation of superoxide (O_2^-) radical into oxygen and hydrogen peroxide. SODs can be classified into three isoforms based on their associated metal cofactors: Cu, Zn-SODs; Fe-SODs, Mn-SODs or Fe/Mn-SODs; and Ni-SODs. Generally, mammals have Cu, Zn-SODs in the cytosol and Mn-SOD in mitochondria [87]. It has been reported that Fe-SOD of *Cryptosporidium parvum* apparently possesses a potential amino-terminal mitochondrial targeting signal, suggesting a mitosomal localization [88]. Although *E. histolytica* Fe-SOD, similar to other mitosomal proteins in *E. histolytica*, lacks an amino-terminal mitochondrial targeting signal, further study to demonstrate the localization of *E. histolytica* Fe-SOD is needed to understand the redox control of *Entamoeba* mitosomes. It is also important to note that mitosomal proteins that contain intra- or intermolecular disulfide bonds need to be oxidized before entering the transport channel of the mitosomal outer and inner membranes, and reduced once the protein is translocated to the matrix. Thus, the redox system regulating this oxidation and reduction of thiol/disulfide of mitosomal proteins is necessary (see Sect. 18.5).

18.5 Protein Transport into Aerobic Mitochondria and *Entamoeba* Mitosomes

Except for those encoded by the organellar genome, mitochondrial proteins are encoded by the nuclear genome, synthesized in the cytosol as precursor proteins, and transported into mitochondria. Mitochondria have at least four subcompartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix. Multiple independent machineries are necessary for subcompartment-dependent translocation of mitochondrial precursor proteins. Protein translocation into mitochondria is mediated by four main complexes: TOM (*T*ranslocase of *O*uter *M*itochondrial membrane) complex, SAM (*S*orting and *A*ssembly *M*achinery) complex, and two TIM (*T*ranslocase of *I*nner *M*itochondrial membrane) complexes (TIM23 and TIM22 complexes), together with small TIMs and the MIA (*M*itochondrial *I*ntermembrane space *A*ssembly) pathway [89]. Mitochondrial precursor proteins translated in the cytosol are first recognized by the TOM complex

and pass through the mitochondrial outer membrane. Membrane precursor proteins such as β-barrel proteins composed of antiparallel β-strands are delivered to the SAM complex, which functions to sort and assemble β-barrel membrane proteins. In the translocation across the inner membrane, the TIM23 complex transports mitochondrial matrix precursor proteins into the matrix. The presequence translocase-associated motor (PAM), which localizes to the matrix and contains the mitochondrial-type heat-shock protein 70 (mtHsp70) as a central component, assists precursor proteins to pass through the TIM23 complex. The TIM22 complex is required for assembly of the inner membrane proteins. The MIA pathway functions in the biogenesis of proteins localized to the intermembrane space.

As *E. histolytica* mitosomes lack organelle DNA, all mitosomal proteins are synthesized in the cytosol and imported into mitosomes. The demonstration of mitosomal proteins normally found in each of the four subcompartments of mitochondria predicts the need for all four of these aforementioned complexes for translocation of mitosomal proteins [20, 51, 64, 65]. However, very few candidates orthologous to the proteins known to be involved in mitochondrial protein import were discovered by hidden Markov model-based similarity search in *E. histolytica* [51, 65], namely, Tom40 (XP_655014), Sam50 (XP_651988), mtHsp70 (XP_649403), Cpn60 (XP_656268), and Cpn10 (XP_656663) [51, 65, 90]. Therefore, protein import into *Entamoeba* mitosomes seems highly divergent compared to mitochondrial import in yeast and mammals.

18.5.1 Mitochondrial Targeting Sequences

The tag for the delivery of proteins to mitochondria, commonly called the mitochondrial targeting signal, is often found at the amino-terminal end of precursor proteins. The presequences are generally 10–80 residues long, rich in basic, hydrophobic, and hydroxylated amino acids, and form an amphipathic α-helix. Some mitochondrial proteins do not possess the amino-terminal targeting sequence and, instead, possess an internal targeting signal. The nature of the internal targeting signal remains largely unknown, but is best characterized in MCFs. The precursor proteins with presequences are mainly sorted to the matrix, the inner membrane, or the intermembrane space, whereas the precursor proteins with internal targeting signals are directed to the inner membrane, the intermembrane space, or the outer membrane [89, 91].

In *E. histolytica*, none of the predicted mitosomal proteins has the amino-terminal transit peptide. Prediction programs such as Mitoprot and PSORT II did not predict the mitosomal localization of the confirmed mitosomal proteins, whose localization has been demonstrated by proteome and immunofluorescence analyses, including Cpn60, Cpn10, mtHsp70, AS, APSK, IPP, NaS, CLS, and Rbr [19, 51]. The 15-amino-acid long amino-terminal stretch of Cpn60 particularly resembles the mitochondrial targeting signal, and removal of this region abolished mitosomal targeting and caused mislocalization of Cpn60 in the cytosol [35]. However, this region

was not sufficient per se for mitosomal targeting of luciferase. Moreover, although the PNT (XP_001913541) has an amino-terminal extension that slightly resembles known mitochondrial and hydrogenosomal targeting sequences [6], it was not discovered in either the mitosome proteome [51] or colocalized to mitosomes by immunofluorescence assays [92].

In general, the amino-terminal presequence of the mitochondrial precursor proteins is cleaved off after passing through the TIM complex by the mitochondrial processing peptidase (MPP) complex, which is a heterodimer consisting of an α-subunit (MPPα) involved in substrate capture and a β-subunit (MPPβ) involved in peptide cleavage. MPP orthologues were recently identified in the *T. vaginalis* and *Giardia intestinalis* genomes, and their hydrogenosomal and mitosomal localization, respectively, has been verified [93, 94]. In *E. histolytica* only the MPPβ homologue (XP_656023) appears to be present, and this protein tagged with the HA epitope at the carboxyl-terminus was localized in the cytosol (Makiuchi et al., unpublished data). Together with the lack of amino-terminal transit peptides on *E. histolytica* mitosomal proteins, these data indicate that the *E. histolytica* MPPβ homologue may not function as the presequence peptidase in the mitosome. Instead, these data strongly suggest the protein targeting system dependent on canonical amino-terminal transit peptides may not be conserved in *E. histolytica.*

The cryptic internal targeting signal necessary for protein import to *Entamoeba* mitosomes is not well understood. However, the mechanism appears to be conserved and interchangeable between *Entamoeba* and yeast, as demonstrated for two *Entamoeba* MCFs, AAC (XP_649800) and PiC (XP_656350), which were shown to be targeted to the mitochondria in *S. cerevisiae* [63, 64]. The mechanisms of import of mitosomal matrix proteins, such as AS, APSK, and Cpn60, remain totally unknown. Similarly, cryptic mitochondrial targeting signals have also been suggested in *G. intestinalis*, *T. vaginalis*, and microsporidian species [16, 95, 96]. In *G. intestinalis*, IscS, which is localized to mitosomes, lacks any recognizable mitochondrial targeting presequence [95, 97]. However, when *G. intestinalis* IscS was expressed in *T. vaginalis*, it was transported into *T. vaginalis* hydrogenosomes, suggesting conservation of the transport system between mitosomes and hydrogenosomes [95]. In contrast, *G. intestinalis* IscS, when divided into halves and expressed in *T. vaginalis*, was not transported into hydrogenosomes [95]. These results suggest that *G. intestinalis* IscS possesses cryptic signals for the protein import into MROs.

18.5.2 Conserved Canonical Components of the Transport System

E. histolytica has only Tom40 and Sam50 homologues among the components known to make up the TOM and SAM complexes, respectively [51, 65]. *E. histolytica* Tom40 and Sam50 were demonstrated on mitosomes [65, 98]. Moreover, repression of the *Tom40* gene by gene silencing caused growth retardation of *E. histolytica* trophozoites [98], reinforcing the indispensable role of Tom40 in

proliferation, as in yeast [99–101]. In the *Tom40*-gene silenced strain, mRNA from matrix protein genes such as *AS*, *APSK*, *IPP,* and *Cpn60* genes as well as *Sam50* increased ([98] and unpublished data), suggesting that the defect in mitosomal transport caused compensatory upregulation of expression of genes encoding matrix proteins and the SAM core component, but that this compensation was not sufficient to overcome the negative effects.

Sam50 is one of the β-barrel mitochondrial outer membrane proteins and is involved in the assembly of other β-barrel membrane proteins, including Tom40. Repression of the *Sam50* gene also caused growth retardation and a slight accumulation of *Tom40* mRNA, which is likely a compensatory way to overcome a defect in the SAM machinery (Makiuchi et al., unpublished data). Further studies are needed to identify all components involved in the biogenesis of β-barrel membrane proteins on the *Entamoeba* mitosomal outer membrane.

18.5.3 Unique Features of the Outer Membrane Transport System

In yeast, the TOM complex exists as a complex of approximately 400 kDa, composed of Tom40, Tom22, Tom5, Tom6, and Tom7 [102]. In *G. intestinalis,* among the components in TOM complex only Tom40 was identified by in silico analysis; however, biochemical analysis reveals that Tom40 is located in an approximately 200-kDa complex composed of 32-, 65-, and 18-kDa partner proteins [103]. These data suggest that *E. histolytica* Tom40 and Sam50 may also be a part of unknown complexes consisting of novel components that cannot be detected by current searching tools.

The *E. histolytica* TOM complex was recently purified to homogeneity and demonstrated to be a 600-kDa complex that contains Tom40 and a novel 60-kDa subunit named Tom60 (XP_657124) [97] (Fig. 18.2). Tom60 orthologues were also found in *E. dispar* and *E. invadens* (EDI_218540 and EIN_149090, respectively), but they were not identified in Bacteria, Archaea, or other eukaryotes, including other amoebozoan species. *Entamoeba* Tom60s are predicted to possess tetra-tricopeptide repeats (TPRs) [104]. TPRs are implicated in protein–protein interactions and are also present in Tom20 and Tom70, which are membrane-spanning receptors for mitochondrial import [105]. Although both Tom20 and Tom70 are involved in the recognition of mitochondrial preproteins, they have different specificity for precursor proteins. Tom20 directly recognizes the amino-terminal presequence of soluble matrix proteins, whereas Tom70 interacts directly with membrane preproteins, or indirectly via cytosolic heat-shock protein 70 (Hsp70) and Hsp90 chaperones. In the latter case, Hsp70 and Hsp90 that are bound to mitochondrial membrane preproteins [89] further bind to the TPR domains of Tom70 via their conserved tetrapeptide "EEVD" motif at the carboxyl-terminus [106]. It was demonstrated by in vitro binding experiments using recombinant proteins that *E. histolytica* Tom60 binds to the

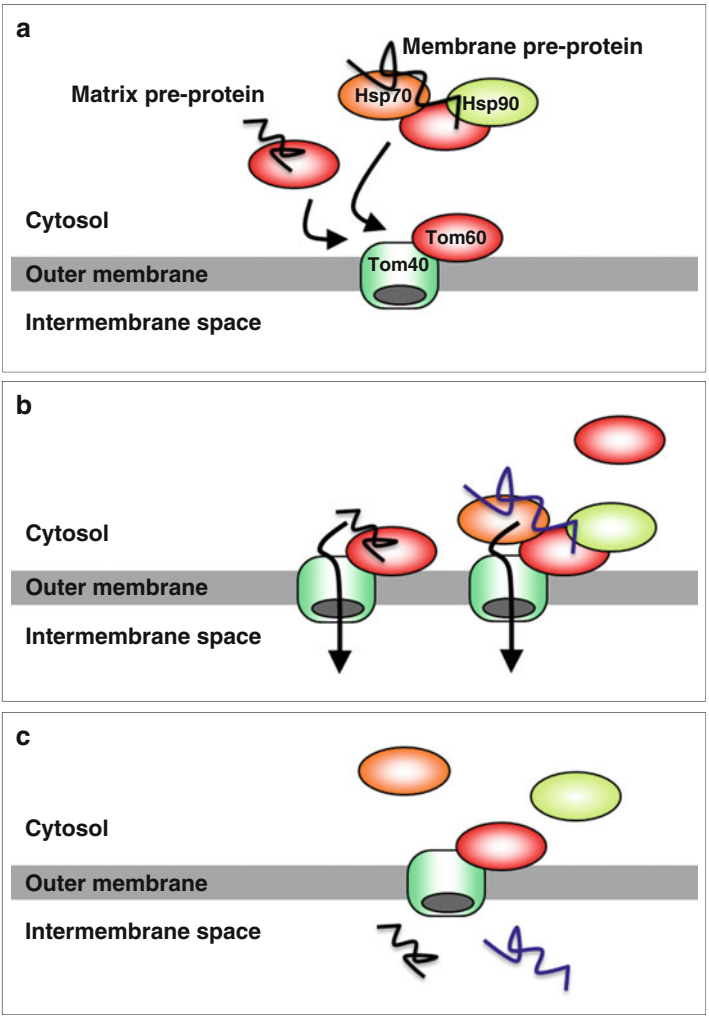

Fig. 18.2 A model of protein import on the *Entamoeba* mitosomal outer membrane. The process occurs in the order of **a**–**c. a** Cytosolic Tom60 captures mitosomal soluble and membrane precursor proteins. Cytosolic Hsp70 and Hsp 90 chaperons assist with binding to Tom60s of the membrane precursor proteins. After binding to precursor proteins, Tom60 is recruited by Tom40 on the outer membrane. **b** After Tom60 binds to Tom40, the precursor proteins are transferred from Tom60 to Tom40. The mechanism of this step is still unknown. **c** After the precursor proteins pass through Tom40 and reach the intermembrane space, the TOM complex returns to inactive status and waits for another round of transport of precursor proteins

representative mitosomal matrix protein AS. Moreover, *E. histolytica* Tom60 also bound to the carboxyl-terminal EEVD motif fused to an irrelevant cytosolic protein. Therefore, it appears that *Entamoeba* has uniquely acquired a single receptor with the dual roles of Tom20 and Tom70 (Fig. 18.2).

Entamoeba Tom60 also showed a unique bipartite localization, the mitosomal outer membrane and the cytoplasm [98]. The significance of the bipartite localization of *Entamoeba* Tom60 is not clear. However, the mobilization of Tom60 to the cytoplasm in theory maximizes the chance of its interaction with mitosomal preproteins and thus serves to increase the efficiency of mitosomal targeting via Tom60. This arrangement is similar to the case of Tom20 and Tom70, which are loosely associated with other components of the TOM complex, mobilized on the entire mitochondrial surface, and capable of interacting with preproteins [107]. The mechanism of recruitment of Tom60 to the mitosomal outer membrane remains unsolved. One possibility is that Tom60 loaded with a precursor protein docks to the TOM complex, whereas free unloaded Tom60 remains dissociated from the TOM complex in the cytosol. Another possibility is posttranslational modifications. It has been recently reported that the binding of mammalian Tom20 and Tom70 to preproteins is regulated by phosphorylation [108].

18.6 Future Perspectives

Genome-wide surveys for known mitochondrial proteins in the *E. histolytica* genome [65, 78, 90], and proteomic analysis of isolated mitosomes [20, 51], have greatly contributed to our understanding of the components and functions of *Entamoeba* mitosomes. Despite the great amount of knowledge accumulated by previous investigations, many fundamental questions still remain to be addressed.

18.6.1 Energy Flux of IPP-Derived PPi and Potential Fatty Acid Metabolism

Entamoeba IPP (EC 3.6.1.1) is localized in the mitosomal matrix [20] and catalyzes the hydrolysis of one molecule of PPi to two Pi ions [109]. This process is highly exergonic (accounting for an approximately −19kJ change in free energy), and therefore greatly increases the energetic favorability of a reaction system when coupled with a typically less favorable reaction. In plants, a large amount of PPi, produced as a by-product by several metabolic processes, is utilized as a source of energy for active transport of protons into vacuoles by membrane-bound inorganic pyrophosphatases (H^+-PPases) [110]. Although *Entamoeba* IPP is a soluble protein, in contrast to H^+-PPases, the reaction catalyzed by IPP and H^+-PPases is identical. It remains unknown how energy generated by PPi hydrolysis in *Entamoeba* mitosomes is utilized. The energy may be utilized in a coupled reaction such as the transport of metabolites, ions, or proteins. It is also worth noting that two acyl-CoA synthetases have been identified in the mitosome proteome. Although β-oxidation

of fatty acids is likely absent in *Entamoeba* [111], in other organisms fatty acids must first be activated via a thioester linkage to coenzyme A in β-oxidation. This process is catalyzed by acyl-CoA synthetase and occurs on the outer mitochondrial membrane. This activation is accomplished in two reactions: (1) the fatty acid reacts with a molecule of ATP to form an enzyme-bound acyl adenylate and PPi, and (2) the sulfhydryl group of CoA attacks the acyl adenylate, forming acyl-CoA and a molecule of AMP. As both these two steps are reversible under biological conditions, the forward direction is favored by the additional hydrolysis of PPi by IPP [112]. This coupled hydrolysis provides the driving force for the overall forward activation reaction, as in sulfate activation. The role of acyl-CoA generation, if present at all in mitosomes, remains to be demonstrated.

18.6.2 Novel Outer Membrane Metabolite Channels and Sorting and Assembly Machinery for Outer Membrane Proteins

It is known that, in general, the transport of molecules across the outer membrane is mediated by voltage-dependent anion channels (VDACs) [113]. Their core function is to form a diffusion pore for small molecules entering or leaving the mitochondria [114]. As sulfate activation is compartmentalized in *Entamoeba* mitosomes, the mitosomal outer membrane must be equipped with transport channels for sulfate, adenine nucleotides, PAPS, and Pi. However, the *Entamoeba* genome lacks genes for VDACs, suggesting that this parasite may possess an alternative channel(s) on the mitosomal outer membrane. Yeast Tom40 is known to transport some metabolites, such as NADH and superoxide anion [115, 116]. Similarly, *Entamoeba* Tom40 may also be involved in the transport of metabolites/ions between the mitosome and the cytosol.

Tom40 and Sam50 are β-barrel membrane proteins composed of antiparallel β-strands. Four β-barrel membrane proteins are well characterized in eukaryotes: Tom40, Sam50, Mdm1, and VDAC. A genome-wide survey for β-barrel membrane proteins in *E. histolytica* indicates the presence of novel β-barrel membrane proteins in *Entamoeba.* The proteins of this type possess the β-signal near the carboxyl-terminus, which is essential for recognition by the SAM complex for proper sorting and assembly on the mitochondrial outer membrane. *Entamoeba* Tom40 actually possesses the putative β-signal [64]. Sam35, which is an essential subunit of the SAM complex in other organisms, recognizes and binds to this signal [89]. However, the gene encoding Sam35 is missing from *Entamoeba* genomes, suggesting that *Entamoeba* possesses a novel receptor for the β-signal involved in sorting and assembly of β-barrel membrane proteins. Alternatively, β-signal-dependent recognition, sorting, and assembly of outer membrane proteins may not be present in *Entamoeba* species.

18.6.3 Missing TIM Complexes

The genome of *Dictyostelium discoideum*, which belongs to the phylum Amoebozoa together with *Entamoeba* species, encodes Tim23 and Tim22 as core subunits of the inner membrane complexes [117]. However, genes encoding Tim23 or Tim22 cannot be detected in *Entamoeba* genomes, indicating that the inner membrane complexes of *Entamoeba* mitosomes are likely divergent. Thus, homology-based gene surveys for the inner membrane complexes may not lead to the identification of the components, and biochemical approaches such as affinity-based isolation of components using an epitope-tagged precursor protein as a translocation intermediate [118, 119], will be required to identify unknown components.

18.6.4 Regulation of Gene Expression of the Mitosomal Proteins

It has been demonstrated that the expression of genes for mitosomal proteins is regulated in *E. histolytica* [98], indirectly shown by the fact that repression of gene expression of the subunits of the TOM complex (Tom40 and Tom60), or Cpn60 caused a compensatory upregulation of mitosomal matrix enzymes (AS, APSK, and IPP). Although a full cascade of this regulatory system remains unknown, transcriptome and metabolome analyses of *E. histolytica* strains in which Tom40, Tom60, or Sam50 genes are silenced may give clues to the mechanisms for the observed increase of transcripts for mitosomal matrix enzymes, including key metabolites for the sensing of stress, transcription factors, or regulators of mRNA stability and decay.

18.6.5 Localization, Membrane Association, and Function of Mitosomal Proteins

None of the currently available prediction tools is able to predict the mitosomal localization of the *E. histolytica* mitosome proteome. However, in silico programs such as OCTOPUS allow us to predict transmembrane regions (TMs) in putative mitosomal proteins. Such analysis suggested that 32 of 96 potential mitosomal proteins have at least one TM, and 3 and 9 proteins have two and more than two TMs, respectively [51]. However, the cellular localization of individual potential mitosomal proteins needs to be verified by immunofluorescence assays and immunoelectron micrography as well as physical separation of organelles. The number of the mitosomal proteins identified by physical purification and mass spectrometry in the previous proteomic study was limited, and information on their intraorganellar localization, that is, the outer and inner membrane, the intermembrane space, or the matrix, is not available. Thus, determining the outer and inner membrane proteomes of highly purified mitosomal membrane fractions with better sensitivity is needed.

18.6.6 Redox Regulation in Mitosomes

Mechanisms for redox regulation of the mitosomal proteins remain uncharacterized. The list of mitosomal proteins includes three proteins (XP_656645, *E*-value of Pfam = 4.6e−23; XP_654476, *E*-value = 3.2e−12; and XP_651237, *E*-value = 0.14) [51] that belong to the thioredoxin superfamily, which includes thioredoxin, glutaredoxin, peroxiredoxin, and protein disulfide isomerase [120]. Protein disulfide isomerases were also detected in the mitosome-enriched fraction (Mi-ichi et al., unpublished data), but were excluded from the tentative mitosome proteome as they were initially presumed to be localized to the ER. However, they also possess the thioredoxin-like domain involved in the formation and cleavage of disulfide bonds in protein folding and may be involved in the quality control of mitosomal proteins. Further studies are needed to understand whether these proteins are involved in the quality control of *Entamoeba* mitosomal proteins.

18.6.7 Replication, Division, Segregation, Fusion, and Decomposition of Mitosomes

The mechanisms controlling the replication, division, segregation, and fusion of *Entamoeba* mitosomes are totally unknown. Dynamin superfamily proteins are known to participate in the processes in other organisms [121], and the *E. histolytica* genome encodes several dynamin-related proteins (DRP) [78]. However, none of the *Entamoeba* DRPs investigated thus far has been shown to participate in the fission/fusion of mitosomes [122]. The mechanisms of decomposition of damaged mitosomes also remain unknown, but may involve a mitophagy (mitochondrial autophagy)-like degradation system [123, 124], because *Entamoeba* has the ability to perform autophagy [125, 126] and mitosomes are occasionally observed in the vacuoles. However, a homologue of the receptor on damaged mitochondria, Atg32 [127], has not been found in *Entamoeba* genomes.

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Part IV
Metabolism

Chapter 19
Metabolomic Analysis of *Entamoeba* Biology

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Abstract Whole genome or transcriptome information provides the annotation of genes and proteins and predicts metabolic pathways, but unequivocal demonstration of the functionalities of the enzymes and metabolic pathways remains challenging. Because nearly 56 % of the *Entamoeba histolytica* genes remain unannotated, correlative "omics" analyses of genomics, transcriptomics, proteomics, and biochemical metabolic profiling can be useful in uncovering new, or poorly understood, metabolisms and metabolic pathways. Current understanding of metabolic pathways constructed by genes and pathway predictions are based on homology search of the genome, transcriptome, and proteome databases and conventional biochemical demonstration of enzymatic activities. However, it is well known that there are large disparities between the pathways predicted in silico and the pathways actually operating in vivo. Thus, it is important to demonstrate the presence and kinetics (flow or flux) of the metabolites involved in the pathways. To this end, a variety of analytical methods and platforms for metabolomics and metabolite profiling has been developed, in which intracellular and extracellular metabolites can be selectively or globally analyzed. Global metabolomics analysis of *Entamoeba histolytica* under environmental stress conditions, in different life-cycle stages, and heterogenic (i.e., clinical) isolates, should potentially uncover unpredictable metabolic

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_19

pathways, interaction and regulation of pathways, and also directly demonstrate the role of individual genes on metabolic pathways, and thus helps our understanding of the physiological and biological roles of metabolic pathways and a network of regulatory interactions between them. Metabolomics of *Entamoeba* is still in its infancy and only a handful of studies have been reported thus far. In this chapter, we summarize a few examples of the application of metabolomics, combined with transcriptomic analysis, to the analysis of global changes in metabolism in response to three representative physiological conditions: encystation, oxidative stress, and cysteine deprivation. We also discuss future applications of metabolomics to understand the biology and pathogenesis of *E. histolytica*. Furthermore, because major metabolic differences between the parasite and its host provide rational drug targets, which are either selectively present in pathogens or highly divergent from humans, multi-"omics" approaches, including metabolomics, should lead to important discoveries of unique exploitable metabolic networks crucial to develop new effective drugs against amebiasis.

19.1 Basics of Metabolomics

19.1.1 Terminology

The metabolome is defined as the quantitative profile of all, or a large number of, either targeted or untargeted metabolites in a given biological system. Metabolomics is defined as the simultaneous qualitative and quantitative measurement of all cellular and extracellular metabolites in an organism [1]. Metabolomics combines strategies using sophisticated metabolite separation platforms such as gas and liquid chromatography (GC, LC), capillary electrophoresis (CE), and analytical technologies such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) with the application of statistical and multivariant methods for information extraction and data interpretation. Fluxomics is the metabolomic mass isotopomer analysis using specific labeled (usually ^{13}C or ^{15}N) substrates that are fed to microorganisms, cells, or tissues to follow the destination of carbon or nitrogen within metabolic pathways. To study the metabolome and the fluxome, and to decipher the biological roles of metabolites, it is needless to say that the identification and quantification of the metabolites should be unequivocal.

Target analysis denotes the identification and quantitation of predefined metabolites, whereas metabolite-profiling deals with both predefined metabolites generally related to a specific pathway or all nontargeted (detectable by a selected analytical technique) metabolites. Metabolic fingerprinting usually indicates a rapid high-throughput screening to discriminate between samples from different biological origin, status, and process. Metabolic footprinting is analysis of the metabolites secreted or excreted by an organism and is also known as an exometabolome.

19.1.2 Significance and General Applications of Metabolomics

The significance of the metabolome in the discoveries of novel aspects of cellular metabolism was originally reported nearly a decade ago [2]. Because metabolites act as spoken language and broadcast signals from the genetic architecture and the environment, metabolomics is considered to provide a direct functional readout of the physiological state of an organism under the given conditions. Although the current technologies cannot yet be used for the comprehensive analysis of metabolites in living cells, the metabolome is a potential pool of the substrates and products of metabolic enzymes, and thus contains information about the intracellular biochemical reactions and regulatory processes of enzymes.

For understanding the dynamic behavior of a complex biological system, it is essential to measure its response to environmental stimuli and genetic perturbations at the genome, transcriptome, proteome, and metabolome levels. Interpreting metabolism in the organism in light of the genome alone can be misleading, and it is important to remember that in silico metabolic networks predicted by the genome only reveal the metabolic potential of the organism. The actualization of this potential is restricted by regulation at numerous levels, including gene expression, post-translational regulation such as transport, stabilization, compartmentalization, and degradation of the enzymes, as well as concentrations, transport, distribution, and turnover of the metabolites, that is, substrates, products, and cofactors. Although transcriptional and proteomic analyses potentially help to understand temporal and spatial coexistence of the enzymes—what is expressed when and where—they cannot directly demonstrate the actual status and flow of metabolites, which metabolomics can. Hence, integrating the conventional enzymatic assays, quantitation of all metabolites, and simulation of metabolic fluxes, combined with transcriptomic and proteomic approaches, is essential to refine the genome scale reconstruction process of biochemical networks [3, 4]. Metabolomic analysis has been widely applied to study the systems biology of numerous model organisms across the tree of life, including Archea [5], Eubacteria [6], Fungi [7], plants [8], animals [9, 10], and human cell tissue culture [11]. Metabolomics have been applied to a variety of purposes such as determination of gene function; phenotyping of strains and variants, such as genetically modified microorganisms and plants; monitoring stress responses, for example, temperature, drought, toxins, and biomarker discovery; disease markers, crops, target discovery for drug development; and cancer, cardiovascular disease, and obesity [12].

19.1.3 Methods for Metabolomics and Fluxomics

Currently, MS is the most sensitive method enabling the detection of hundreds of compounds within single extracts. MS varies in the method of ionization [e.g., atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI),

and thermospray] and the kind of mass analyzers [triple-quadrupole, ion trap, and time-of-flight (TOF)]. A variety of chromatography platforms coupled to MS, such as LC-MS, GC-MS, and CE-MS, can be used to identify and quantify the metabolites [13]. However, it is important to note that no single analytical method is capable of extracting and detecting all metabolites at once because of the enormous chemical heterogeneity of metabolites and the large range of concentrations at which metabolites can be present. Therefore, the characterization of a complete metabolome requires different complementary analytical technologies.

Properties of metabolic networks cannot be fully determined based on interconnectivity and coexpression alone, but depend critically on the reaction rates of each step [14]. Metabolite flux through the network can be probed by feeding isotopically labeled metabolic substrates or intermediates and following label incorporation into downstream metabolites by successive metabolomic analyses (fluxome and fluxomics) [15]. Isotopic labeling can reveal nonintuitive network behavior and network redundancy [16], highlighting potential drug targets [17] and mechanisms of action [18] that are not apparent based on interconnectivity alone.

19.1.4 Advantages and Expected Outcomes of Metabolomic Approaches

Metabolomic approaches are particularly valuable to identify missing enzymes or gaps in an otherwise complete metabolic map or orphan biochemical activities. They are also advantageous for high-throughput screening to discover differences in metabolism between a pathogen and its host that may be exploited for drug development. In addition, the identification of previously uncharacterized metabolites will lead to an initiation of search for the enzymes involved in their synthesis [19].

The sequenced genome of *Entamoeba histolytica* has already been used to predict the metabolic potential of the organism [20, 21]. An inherent shortcoming of homology-based approaches for predicting metabolic networks is that they cannot predict the function of novel or highly divergent metabolic genes. Such hypothetical genes of unknown function represent a sizable fraction of predicted genes in the available *Entamoeba* genomes [22]. Depending on their activity, unidentified metabolic genes among them could alter the structure of metabolic networks considerably. Integrating metabolomics with other omics data already in place will lead to a deeper biological understanding and is likely to unveil additional targets for prevention and therapy.

19.2 Application of Metabolomics to *Entamoeba* Biology

Entamoeba histolytica trophozoites are microaerophilic and have been shown to consume oxygen and tolerate low levels of oxygen pressure [23]. *E. histolytica* lacks the general form of mitochondria as found in the aerobic eukaryotes, but instead

possesses a highly divergent mitochondrion-related organelle, named mitosomes [24]. *Entamoeba* mitosomes lack features of aerobic energy metabolism including the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and energy generation is primarily dependent on substrate-level phosphorylation in glycolysis and fermentation [25]. In *E. histolytica*, pyruvate is converted to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR), and acetyl-CoA is either converted to acetate with a concomitant ATP generation or reduced to ethanol with regeneration of NAD [25, 26]. Amino acid metabolism is also unique in *E. histolytica*. Despite the fact that amino acid metabolism is largely reduced in majority of parasitic protozoa, sulfur-containing amino acid metabolism is highly operational and unique in *Entamoeba*, including the presence of de novo L-cysteine/*S-methylcysteine* (SMC) biosynthesis and methione/homocysteine/cysteine degradation by methionine γ-lyase (MGL) [27]. Metabolomics of *Entamoeba* is still in its infancy and only a handful of studies have been reported thus far [28–31]. Some of the metabolomic studies discussed in this chapter marked the first step toward systems biology-based understanding of *Entamoeba* metabolisms and biology. A few studies demonstrated global changes in metabolite levels in *Entamoeba* in response to changes in environmental stress and stage transition by target metabolome analysis. The observed metabolomic profiles were compared to those based on transcriptomic profiling. Such comparisons showed large disparities in changes between gene expression at the transcriptional and protein (or activity) levels, and between the transcriptional and metabolite levels. They also demonstrated how metabolites are involved in the feedback regulation of gene expression of metabolic enzymes (see following).

19.2.1 Toward Understanding of Central Energy Metabolism in Entamoeba

19.2.1.1 Conventional Biochemical Studies to Demonstrate the Metabolite Profile and Metabolic Flux

Glycolysis and fermentation serve mostly for ATP generation in *E. histolytica*. The kinetic properties of recombinant proteins of major glycolytic and fermentation enzymes have been demonstrated by Saavedra et al. [32] (also see Chap. 20). It was shown that four enzymes, that is, fructose-1,6-bisphosphate aldolase, phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate phosphate dikinase likely regulate the glycolytic flux. Kinetic modeling and pathway reconstitution have also been used to identify the flux-controlling steps in *E. histolytica* glycolysis [33, 34].

Metabolic labeling studies using ^{14}C-labeled glucose in the 1970s showed that more than 95 % of labeled glucose was converted to glycogen, carbon dioxide, ethanol, and acetate [35]. Only a trace amount of label was found in protein, lipid, and nucleic acids. A more recent study by Bakker-Grunwald et al. [36]

using ^{13}C-NMR spectroscopy identified various abundant metabolites in *E. histolytica*. They measured both the intracellular and extracellular concentrations of individual amino acids (ranging from 0.2 to 22 mM), glucose (2.1 mM), and glycogen (30 mM) in axenic cultures. *E. histolytica* contains large amounts of glycogen as the main carbohydrate storage. Glycogen is a polymer of α-1,4-linked glucose chains with α-1,6 branch points, which has a compact structure as suggested by branch points every five to six glucose residues in *E. histolytica* [36]. Apart from glycolysis, a number of enzymes also secondarily yield ATP through the catabolism of amino acids. It was demonstrated using HPLC analysis that *E. histolytica* and *E. invadens* preferentially consume several amino acids (asparagine, arginine, leucine, threonine, and serine), particularly in the absence of glucose [37].

19.2.2 Metabolomic Analysis of Encystation

19.2.2.1 Differentiation (Stage Conversion) of *Entamoeba*

The life cycle of *Entamoeba* species consists of two developmental stages: the dividing trophozoite stage present within the infected host and the transmissible, environmentally resistant cyst stage. Most studies on *E. histolytica* have focused on the trophozoite stage, because *E. histolytica* trophozoites, particularly in axenic cultures, do not encyst, that is, differentiate from trophozoites to cysts, for reasons so far unknown. The *Entamoeba* model organism for studying encystation or excystation is *Entamoeba invadens*, a reptilian parasite that has a life cycle similar to *E. histolytica* but efficiently forms cysts in vitro [38].

Recently, Jeelani et al. carried out metabolomic analysis of encystation using *E. invadens* [29]. The in vitro encystation process was initiated by transferring the trophozoites from the regular medium to 47 % LG medium having approximately half osmolarity and no glucose. Under these conditions, approximately 80 % of the trophozoites differentiated into the Sarkosyl-resistant cysts within 72–120 h. Using the capillary electrophoresis time-of-flight mass spectrometry (CE-ToFMS)-based metabolomics approach, more than 100 intermediary metabolites were identified and quantitated, including amino acids, nucleotides, biosynthetic precursors, and central carbon metabolism intermediates.

19.2.2.2 Redirection of Glucose from Glycolysis to Chitin Biosynthesis

It was shown that during encystation the utilization of glucose is redirected from glycolysis to the pathway for the synthesis of chitin by an inducible chitin biosynthetic pathway. Chitin, a linear polymer of β-1-4-linked *N*-acetylglucosamine, had been shown to be a major component of the cyst walls of both *E. invadens* and *E. histolytica* by X-ray diffraction [39] and lectin-binding studies [40]. During encystation, it

was found the levels of various glycolytic pathway intermediates, including glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-biphosphate, and phosphoenolpyruvate were significantly depleted, whereas the chitin biosynthesis intermediates glucosamine-6-phosphate and *N*-acetylglucosamine-1-phosphate were increased, suggesting that the flux of glycolysis was redirected toward the chitin biosynthetic pathway. Furthermore, transcriptomic analysis also showed that the expression of all the enzymes involved in chitin wall biosynthesis increased, whereas expression of enzymes involved in the glycolytic pathway decreased. As chitin does not occur in vertebrates, its synthetic pathway represents an excellent parasite-specific target to develop new chemotherapeutics, as proposed [41].

19.2.2.3 Amino Acid and Nucleotide Phosphate Depletion During Encystation

Interestingly, CE-ToFMS analysis demonstrated decrease in most of the amino acids except for alanine during encystation. These data suggest that amino acids were used as an alternative energy source during encystation when all the glucose is used for chitin synthesis [42]. Furthermore, transcriptomic analysis showed that the expression of one of two aspartate aminotransferases, which is involved in the conversion of aspartate to oxaloacetate, increased more than 400 fold during encystation. This finding, together with metabolome analysis, suggests that amino acids such as aspartate and asparagine serve as an energy source in the absence of glucose in *E. histolytica* [37]. Consistent with the interruption of the flux through the glycolytic pathway, CE-ToFMS analysis further demonstrated that the levels of most nucleotides such as ATP, GTP, UTP, and CTP were, after a transient increase in the precyst stage, drastically decreased.

19.2.2.4 Discovery of Unpredicted Pathways: Polyamine and GABA Metabolism

It was totally unexpected that intermediates of polyamine metabolism showed remarkable changes during encystation. Polyamines such as putrescine, spermidine, and spermine are small organic compounds containing two or more amino groups. Polyamines are ubiquitous components of all living cells and have important functions in the stabilization of cell membranes, macromolecular synthesis, cell growth, and differentiation, as well as adaptation to osmotic, ionic, pH, and thermal stress [43, 44]. The absence of *S*-adenosyl-L-methionine decarboxylase, which converts *S*-adenosylmethionine into decarboxylated *S*-adenosylmethionine, spermidine synthase, and spermine synthase, suggests a lack of polyamine metabolism in this parasite [20]. However, metabolomic analysis revealed that, beside putrescine, other polyamines including spermidine, spermine, and N^8-acetylspermidine were present in the proliferating trophozoites, and the level of these metabolites dramatically

decreased as encystation proceeded. These findings indicate that the amoeba may consume or utilize these polyamines during encystation and that biosynthetic or scavenging pathways exist in this organism.

It was also shown that the level of γ-aminobutyric acid (GABA) increased at the later stage of encystation. GABA is made from L-glutamate in a single reaction catalyzed by glutamate decarboxylase, which is missing in the *Entamoeba* genome. However, a number of amino acid decarboxylases are encoded in the genome, and some of these decarboxylases may convert glutamate to GABA. Interestingly, the time-dependent kinetics of the changes in the concentrations of *N*-acetylputrescine during encystation was similar to that of GABA; the increase of *N*-acetylputrescine slightly preceded that of GABA. These data are consistent with the premise that GABA is synthesized from *N*-acetylputrescine by removal of the acetyl moiety. However, further experiments using isotopic labeling should unveil the exact pathway and enzymes involved in the formation of GABA.

19.2.2.5 Synthesis of Biogenic Amines

Another important and totally unexpected finding by metabolomics was the discovery of a few biogenic amines and their transient increase during encystation. Biogenic amines are mainly nitrogenous low molecular weight compounds with biological activity that are formed or metabolized in organisms [45]. Biogenic amines are derived mainly from amino acids catalyzed by substrate-specific decarboxylases [46]. Biogenic amines are known to have an important role in cell proliferation and differentiation in mammals [47]. Metabolomic analysis of encysting *E. invadens* trophozoites revealed remarkable transient accumulation of biogenic amines, that is, cadaverine, isoamylamine, and isobutylamine, all of which increased in the early period (0.5–8 h) of encystation, when the trophozoites formed large multicellular aggregates (pre-cyst), and then decreased when the precyst differentiated to the cyst. Interestingly, these three biogenic amines showed distinct kinetics of upregulation during encystation. Cadaverin peaked at 0.5–2 h, isobutylamine at 2 h, and isoamylamine at 8 h post induction. These data suggest that these biogenic amines may play distinct and specific roles in encystation. However, further investigation is warranted to prove the premise. Biogenic amines have been previously shown to play an important role in induction of encystation in *Hartmannella vermiformis*, a nonpathogenic free-living amoeba, which is the natural reservoir of *Legionella pneumophila*, the causative agent of legionellosis [48]. These biogenic amines, that is, cadaverine, isoamylamine, and isobutylamine, are the decarboxylated products of amino acids (lysine, leucine, and valine, respectively). It was shown that the formation of a cyst-like structure in *E. invadens* and *E. histolytica* was induced by carbon dioxide [49]. Thus, carbon dioxide, which is released during the formation of biogenic amines from corresponding amino acids, per se may be involved in the induction of the encystation process.

The finding of the time-dependent production of biogenic amines and γ-butyric acid during encystation was totally unexpected. Such unprecedented metabolomic

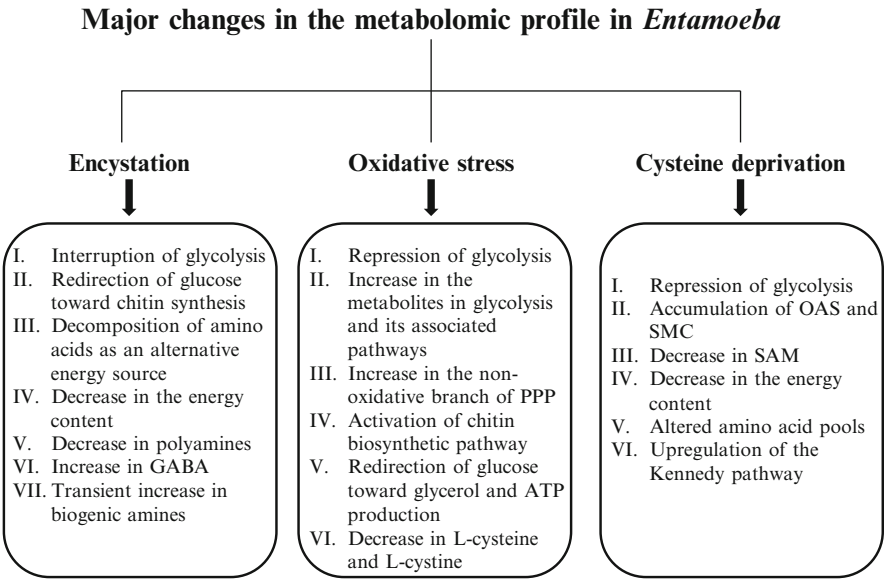

Fig. 19.1 Summary of major metabolomic findings in three representative conditions: encystation, oxidative stress, and cysteine deprivation. *PPP pentose phosphate pathway*, *OAS O*-acetylserine, *SMC S*-methylcysteine, *SAM S*-adenosylmethionine

findings during encystation can potentially expand new areas of *Entamoeba* metabolism and biochemistry (Fig. 19.1). Future work is needed to understand how the production and degradation of these metabolites are regulated and how they are involved in encystation.

19.2.3 Metabolomic Analysis Under Oxidative Stress

19.2.3.1 Known Antioxidative Mechanisms of *E. histolytica*

E. histolytica trophozoites are microaerophilic and can tolerate low levels of oxygen pressure [23]. In addition, they are also exposed to various reactive oxygen or nitrogen species (ROS and RNS) during tissue invasion, colonization, and extraintestinal propagation [50, 51]. *E. histolytica* lacks most of the components, such as catalase, glutathione, and the glutathione-recycling enzymes [52, 53], that are involved in the usual antioxidant defense mechanisms in aerobic organisms. *E. histolytica* also lacks glucose-6-phosphate dehydrogenase (G6PD), and thus the functional pentose phosphate pathway is absent [20]. However, the genome of *E. histolytica* encodes several proteins such as peroxiredoxin, superoxide dismutase, rubrerythrin, hybrid-cluster protein, and flavo-di-iron proteins for detoxification of ROS and RNS [54, 55]. In addition, *E. histolytica* also possesses pyridine nucleotide transhydrogenase, which

utilizes the electrochemical proton gradient across the membrane to drive NADPH synthesis from NADH [56, 57]. NAPDH is in turn engaged in the detoxification of ROS and RNS.

19.2.3.2 Metabolomic Analysis of Oxidative Stress Mechanisms

The response to oxidative stress in *E. histolytica* is only partially understood at the molecular level. Several genes have been shown to be differentially expressed in *E. histolytica* subjected to oxidative or nitrosative stress [58]. Genes encoding proteins involved in the oxidative stress response in eukaryotes, namely heat-shock proteins, ubiquitin-conjugating enzymes, protein kinases, and small GTPases, were modulated by both oxidative or nitrosative stress. However, a significant fraction of the genes modulated by oxidative stress were not annotated ("hypothetical proteins") [58]. Metabolomic analysis of *E. histolytica* trophozoites upon hydrogen peroxide (H_2O_2) or paraquat (PQ) treatment using CE-ToFMS shed light on the dynamics of metabolic changes involved in glycolysis and its associated pathways, and amino acid, nucleotide, and phospholipid metabolism (Fig. 19.1), employed either to bypass damaged enzymes or to support adaptive responses to cope with oxidative stress [30].

19.2.3.3 Oxidative Stress Inhibits the Flux of Glycolysis

The pattern of metabolomic changes in response to PQ and H_2O_2 treatment was similar in terms of the species of metabolites that changed. It was previously shown [59, 60] that oxidative stress caused changes in the amount of metabolites involved in glycolysis and its associated pathways in central energy metabolism [30]. Oxidative stress caused increase in most of the glycolytic intermediates from glucose-6-phosphate to pyruvate. The level of produced ethanol, which is the major end product of glucose catabolism in *E. histolytica* [61], decreased, supporting the premise that the glycolytic flux downstream of pyruvate was indeed repressed. In contrast, no significant change was found in the acetate production. It was further demonstrated that the accumulated glycolytic metabolites were rerouted toward the pathways associated with glycolysis upon oxidative stress. For example, the metabolites were redirected towards the chitin biosynthetic pathway, glycerol production via glycerol-3-phosphate (Gly 3-P), and the serine biosynthetic pathway via *O*-phosphoserine [62]. Accumulation of the glycolytic intermediates hints repression of glycolysis as result of free radical-mediated inactivation of key glycolytic enzymes downstream. Activities of several key enzymes of glycolysis and its associated pathways such as PFOR, phosphoglycerate mutase, and NAD^+-dependent alcohol dehydrogenase were decreased by 40–70 % upon PQ-mediated oxidative stress, whereas the activities of glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, enolase, and pyruvate phosphate dikinase were decreased by only 16–22 %, and those of the other enzymes remained unchanged.

19.2.3.4 Redirection of Glycolysis Toward the Nonoxidative Branch of the Pentose Phosphate Pathway

E. histolytica lacks the first two enzymes of the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase and transaldolases [63], and thus a hexose monophosphate shunt pathway is unlikely to exist. Metabolome analysis under oxidative stress revealed that most of the intermediates of the nonoxidative branch of the pentose phosphate pathway, that is, erythrose-4-phosphate (E4-P), ribulose-5-phosphate (ribulose 5-P), and ribose-5-phosphate were increased. However, sedoheptulose-7-phosphate showed a maximum increment upon PQ or H_2O_2 treatment. In yeast, it was shown that the blockade or the increment in the production of sedoheptulose-7-phosphate and other intermediates of the nonoxidative branch of the pentose phosphate pathway decreased or increased, respectively, tolerance against oxidants [64]. In addition, ribose-5-phosphate, which may be synthesized from sedoheptulose-7-phosphate, is also a precursor of NAD^+, which is used by NAD kinase to synthesize NADP [65].

19.2.3.5 Oxidative Stress Affects the Pool of Nucleotides and Amino Acids

The levels of the nucleoside triphosphates ATP, GTP, UTP, and CTP significantly decreased upon oxidative stress, whereas the levels of the nucleoside monophosphates AMP, CMP, GMP, and IMP increased upon H_2O_2 stress, in a manner opposite to the decrement in their corresponding triphosphate counterparts. The levels of nucleoside monophosphates upon PQ-mediated oxidative stress remain unchanged, except IMP. Energy charge was decreased upon oxidative stress; however, the decrement was more prominent by H_2O_2 stress compared to PQ-mediated oxidative stress. The decrement in nucleoside triphosphates and energy charge is consistent with the repression of glycolysis, which is also evident by the accumulation of glycolytic intermediates and decreased ethanol production. Both PQ and H_2O_2-mediated oxidative stress led to a decrement in L-cysteine and L-cystine, and a slight increment in cysteine *S*-sulfinate, in a time-dependent manner. Based upon these findings, it was suggested that L-cysteine is likely involved in scavenging of oxygen free radicals in *E. histolytica*.

19.2.3.6 Redirection of Glycolysis Toward Glycerol Production

Interestingly, this study further demonstrated that Gly 3-P is one of the most highly modulated (10- to 14-fold) metabolites upon oxidative stress. The drastic accumulation of Gly 3-P, together with the increase in other upstream glycolytic intermediates, upon oxidative stress clearly suggests the presence of functional Gly 3-P dehydrogenase (G3PDH) in this parasite. The level of intracellular glycerol was also dramatically increased upon PQ-mediated oxidative stress. As the fold changes

in the levels of Gly 3-P and glycerol were similar, most of the Gly 3-P produced upon oxidative stress is likely converted to glycerol. Altogether, metabolomic analysis during oxidative stress demonstrated that *E. histolytica* is capable of glycerol biosynthesis from glucose, similar to other protozoa such as *Trichomonas vaginalis*, *Trypanosoma brucei*, and *Plasmodium falciparam* [66–68]. This observation conflicts with the previous finding that G3PDH activity was not detected in the soluble fraction of *E. histolytica* by conventional enzymatic methods [69]. It was speculated in this study [69] that dihydroxyacetone phosphate, but not Gly 3-P, is mainly used for triglyceride synthesis. It is important to demonstrate the fate and physiological significance of the increased production of Gly 3-P and glycerol upon oxidative stress; it is plausible if ATP production by glycerate kinase is the primary reason of the redirection of the glycolytic pathway upon oxidative stress.

19.2.4 Metabolomic Analysis Under L-Cysteine Deprivation

19.2.4.1 Role of L-Cysteine in *E. histolytica*

L-Cysteine is the major low molecular weight thiol in *E. histolytica* and has been implicated in the survival, growth, attachment, elongation, motility, gene regulation, and antioxidative stress defense of this organism [53, 70–73]. In vitro cultivation of amebic trophozoites requires high concentrations of L-cysteine, which can be replaced only with D-cysteine, L-cystine, or L-ascorbic acid, indicating that the extracellular cysteine/cystine or thiols have an indispensable role in parasite growth [72]. To better understand the role of extracellular L-cysteine, the metabolome was examined under cysteine-deprived conditions to understand their roles on the central metabolism in *E. histolytica* trophozoites [28].

19.2.4.2 An Unexpected Role of the De Novo L-Cysteine Biosynthetic Pathway

Comparative CE-ToFMS-based metabolome analysis under cysteine-deprived and normal conditions revealed that L-cysteine regulates various metabolic pathways and influences the concentrations of amino acids, phospholipids, and intermediary metabolites involved in central energy metabolism in *E. histolytica* (Fig. 19.1). Under L-cysteine deprivation, two metabolites that had never been demonstrated in *E. histolytica*, SMC and *O*-acetylserine (OAS), were accumulated. SMC is a sulfur-containing amino acid that was never detected in protozoa, but is widely present in relatively large amounts in several legumes, where it is considered to serve as sulfur storage [74]. Stable isotopic labeling with L-serine and L-methionine demonstrated that SMC is synthesized from these amino acids in *E. histolytica* via OAS and methanethiol, respectively. OAS was presumed to be present in *E. histolytica* as it is a substrate for the de novo synthesis of L-cysteine.

However, it was never demonstrated because its steady-state concentrations are very low as a result of the product inhibition of serine acetyltransferase by L-cysteine (see following) [75], or OAS has a short half-life, because of immediate conversion to *N*-acetylserine.

Surprisingly, the metabolomic study using labeled L-serine did not confirm the premise that L-cysteine is formed from L-serine and sulfide by the sequential action of serine acetyltransferase and cysteine synthase. It was further demonstrated that the formation of SMC from OAS and methanethiol is catalyzed by cysteine synthase. OAS is exclusively directed toward the synthesis of SMC, but not L-cysteine, even in the presence of high concentrations of substrates. Therefore, these findings denied the commonly accepted notion on the primary role of the L-cysteine biosynthetic pathway and indicate that it plays a major role in the SMC production. However, the apparent lack of the L-cysteine production by this pathway remains to be tested in vivo. SMC may serve as storage of sulfur in other organisms. However, downstream metabolites of isotopic labeled SMC were not determined in the metabolomic analysis [28]. Thus, the exact role and fate of SMC in *E. histolytica* are not yet understood.

It is also important to note that despite the dramatic changes of metabolites involved in the SMC production, mRNA expression of the genes involved in sulfur-containing amino acid metabolism was not significantly affected under L-cysteine deprivation, as revealed by transcriptome analysis [76]. This general lack of correlation between metabolome and transcriptome appears to be a general characteristic in various organisms, indicating that they have more complex mechanisms of expression regulation.

19.2.4.3 Interruption of SAM Synthesis, Glycolysis, and Nucleotides by L-Cysteine Deprivation

L-Cysteine depletion also resulted in reduced levels of *S*-adenosylmethionine (SAM), a precursor for polyamine biosynthesis and the essential methyl donor for numerous trans-methylation reactions, including DNA methylation, in general. It is important to note, however, in *E. histolytica* the conventional polyamine synthetic pathway is likely absent (see earlier). L-Cysteine deprivation also affected the levels of the majority of metabolites involved in glycolysis and its associated pathways. Upon L-cysteine depletion, pyruvate and other upstream glycolytic intermediates accumulated and were apparently rerouted toward the associated pathways. For example, the metabolites linked to pyruvate and phosphoenolpyruvate (i.e., alanine, malate, and fumarate), 3-phosphoglycerate (i.e., *O*-phosphoserine), and dihydroxyacetone phosphate (i.e., Gly 3-P) increased in response to L-cysteine depletion. In contrast, the levels of acetyl-CoA, ethanol, and the major nucleotide triphosphates significantly decreased. The reduced glycolytic flux caused a significant decrement in the levels of the nucleotide triphosphates ATP, GTP, UTP, and CTP in the L-cysteine depleted cells.

L-Cysteine also regulates the Kennedy pathway, the major pathway for phospholipid biosynthesis. L-Cysteine deprivation resulted in the accumulation of an unusual phospholipid, phosphatidylisopropanolamine, and also affected the composition and ratio of the major phospholipids. Under L-cysteine-depleted conditions, the synthesis of isopropanolamine, isopropanolamine phosphate, ethanolamine phosphate, and choline phosphate was elevated, whereas phosphatidylethanolamine synthesis was downregulated, and the levels of ethanolamine, choline, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid were unaffected. Further investigation is needed to understand the physiological role of phosphatidylisopropanolamine, its derivatives, and related pathways, which are potentially a new attractive drug target for the development of new chemotherapeutics against amebiasis [77].

19.3 Remaining Questions and Future Perspectives

Metabolomics has caused a paradigm shift in metabolic research, shifting from the analyses that focus on a limited number of enzymatic reactions or single pathways to approaches to capture a bird's view of the complex metabolic networks. The metabolomics-based studies have solved long-standing questions on central energy metabolism. For instance, metabolomics studies of oxidative stress, discussed in Sect. 19.2.3, clearly indicated the redirection of glycolytic metabolic flux toward glycerol production, and thus the presence of the functionality of G3PDH and the glycerol biosynthetic pathway in *E. histolytica* [30]. Further studies are needed to understand whether activation of the glycerol pathway leads to generation of ATP and reducing powers, which in turn help the parasite to cope with oxidative stress.

Encystation metabolomics revealed a number of metabolites that were not predicted to be present in this parasite by either genomic or proteomic approaches. Although GABA, a compound more commonly found as a neurotransmitter in the synapses of the central nervous system, and biogenic amines were modulated during encystation in *Entamoeba*, their exact roles in trophozoites and in encystation per se remain unclear. Further studies are needed to clarify the synthetic and degradative pathways of GABA and biogenic amines and to identify their biological roles in proliferation, differentiation, parasitism, and virulence of the parasite, as well as in pathophysiology and defense against the host immune system. The existence of polyamine metabolism in *Entamoeba* has also suggested a possibility that extant and new inhibitors for polyamine metabolism [29, 78] may be worth testing for amebicidal activities, although conventional enzymes involved in polyamine biosynthesis, *S*-adenosyl-L-methionine decarboxylase, spermidine synthase, and spermine synthase, are apparently lacking in *Entamoeba* genome [20]. Probing the polyamine biosynthetic pathway by fluxomics using stable isotopic labeled amino acids (arginine and methionine) should help to further identify intermediates in the pathways and also to discover and design appropriate inhibitors.

Metabolomic analysis under cysteine deprivation has solved one enigma concerning the biological roles of the sulfur assimilatory de novo L-cysteine biosynthetic pathway in *E. histolytica*. It has been clearly demonstrated, at least in axenic cultures, that the L-cysteine biosynthetic pathway plays a major role in the production of SMC, not L-cysteine. The fate and role of SMC and its derivatives need to be demonstrated to fully understand the significance of the cysteine/SMC biosynthetic pathway, not only in *Entamoeba*, but also in bacteria and plants, which also possess homologous pathways. Further investigation of the cellular distribution of SMC, possible excretion to the environmental milieu, and the biological effects of its deprivation to the parasites and their hosts is needed. The significance of the remarkable alterations of phospholipid metabolism caused by L-cysteine deprivation also remains to be further investigated. These biochemical questions are ubiquitously important for the better understanding of the general metabolic pathways.

A number of fundamental and critical questions related to *Entamoeba* biology and pathogenesis are expected to emerge through more elaborate metabolomic analyses in the future. One such example is the identification of the enzymatic reaction of "hypothetical proteins," which constitute nearly 56 % of the *E. histolytica* genome [22]. A CE-ToFMS-based in vitro assay in which a recombinant "hypothetical protein," produced by in vitro transcription and translation systems, is mixed with a pool of hundreds or thousands of known (targeted) and unknown (untargeted) physiological metabolites (or artificial compounds), and all the metabolites are measured before and after the reaction, should in theory identify the potential substrates and products of the reaction catalyzed by the hypothetical protein. When combined with high-throughput protein production and automated enzymological assays, such approaches should greatly help with annotation of the genome and construction of an actual metabolic map.

Metabolic profiling of the organelles such as mitosomes, endosomes, and lysosomes is another new line of applications of metabolomics. Metabolomic analysis of the mitosomes is of particular interest because of the unique unprecedented function of *Entamoeba* mitosomes, sulfate activation (see Chap. 18) [79–82], and shall further reveal novel metabolic roles of the highly divergent mitochondrion-related organelles in anaerobic eukaryotes in general. Mitosomes have been successfully purified from *E. histolytica* trophozoites, free of contamination by other organelles [79]. Needless to say, similar approaches may be applied to other vesicular structures such as endosomes, lysosomes, and the endoplasmic reticulum.

Other potential applications of metabolomics include the investigation of metabolic communication between the parasite and the host. Important questions are how do *Entamoeba* trophozoites steal metabolites from the host? How is the host metabolism affected by infection? How does the host modify its metabolism to combat infections? Finally, because *E. histolytica* is capable of acquiring resistance in vitro easily against the widely used and newly developed drugs, new rational targets for the development of chemotherapeutics with distinct mode of action are always needed. Thus, metabolomics with recent advances in current technologies should provide us with a better understanding of crucial parasite-specific pathways exploitable for drug development.

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Chapter 20
Glucose Metabolism and Its Controlling Mechanisms in *Entamoeba histolytica*

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Abstract *Entamoeba histolytica* lacks the genes encoding the enzymes of the Krebs cycle and oxidative phosphorylation; therefore, glycolysis is the main pathway for ATP supply and for providing carbon skeleton precursors for the synthesis of macromolecules. External glucose is metabolized through a fermentative glycolysis producing mainly ethanol and, to a lower extent, acetate as end products. The pathway in the parasite deviates in several aspects from the typical glycolysis present in mammals and yeasts, for instance, (1) the use of pyrophosphate as high-energy phosphate donor in several reactions; (2) the feasibility of thermodynamic reversibility of all pathway reactions under physiological conditions; and (3) the presence of fermentative enzymes similar to those of anaerobic bacteria. These and other enzyme peculiarities impose different mechanisms of control of the glycolytic fermentative flux in the parasite compared to the highly allosterically regulated glycolysis in other eukaryotic cells. In this chapter, we summarize the previous and current knowledge of the carbohydrate metabolism in *E. histolytica* and analyze its underlying controlling mechanisms by applying the fundamentals of metabolic control analysis (MCA).

20.1 Introduction

Entamoeba histolytica and other members of the same genus live in microaerophilic environments of less than 5 % O_2 atmospheric in in vitro culture [1, 2], which is approximately 30 μM dissolved O_2. Such O_2 concentration is close to that found in the human colon (0.1–4.4 %) [3, 4]. Therefore, *E. histolytica* is adapted to catabolyze external glucose through fermentative glycolysis because it has neither mitochondria nor the enzymes of oxidative phosphorylation typical of aerobic cells.

The glycolytic enzymes and the anaerobic and microaerophilic route of glucose degradation in *E. histolytica* (Fig. 20.1) were mostly characterized in the late 1960s

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_20

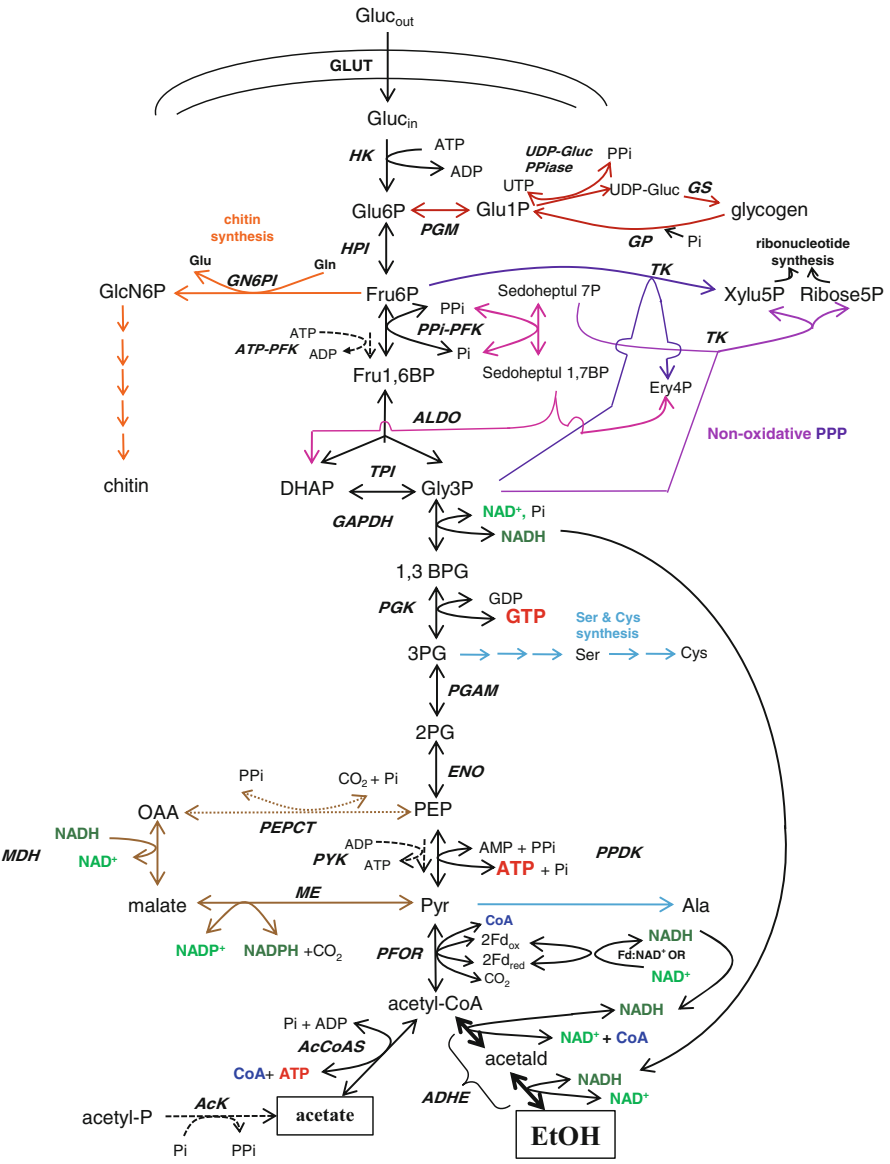

Fig. 20.1 Glucose metabolism in *Entamoeba histolytica*. The main end product of glucose metabolism is EtOH, indicated with a *thick arrow*; acetate flux represents only 10 % of the EtOH flux (*thin arrow*). Main branches of the fermentative pathway are marked in *different colors*. *AcCoAS* acetyl-CoA synthetase (ADP-forming), *AcK* acetate kinase (PPi-forming), *ADHE* bifunctional aldehyde:alcohol dehydrogenase, *ALDO* fructose-1,6 bisphosphate aldolase, *ATP-PFK* ATP-dependent phosphofructokinase, *ENO* enolase, *Fd:NAD*$^+$*OR* ferredoxin:NAD$^+$ oxidoreductase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *Gln* glutamine, *Glu* glutamate, *GN6PI* glucosamine-6-phosphate isomerase, *GS* glycogen synthase, *GP* glycogen phosphorylase, *HK*

to early 1980s by Richard E. Reeves, who depicted the intermediary metabolism in the parasite [5]. Most recently, a comprehensive compendium of the carbohydrate metabolism genes annotated in the *E. histolytica* genome was published [6]. The recombinant amoebal glycolytic enzymes have been recently kinetically characterized under near-physiological conditions [7]. Relevant kinetic parameters are summarized in Table 20.1.

An integral analysis of the controlling mechanisms of the metabolic pathway has been performed by our group through metabolic modeling of the entire pathway and by theoretically and experimentally applying the fundamentals of metabolic control analysis (MCA) [7, 10, 31]. This approach allows the quantitative determination of the degree of control that each enzyme/transporter has on the pathway flux and the understanding of the molecular basis of the controlling and regulatory mechanisms of metabolic pathways (reviewed in [32]).

20.2 Biochemical Properties of the *E. histolytica* Glycolytic Enzymes

Glucose is taken up from the extracellular environment by a low-activity and low-affinity glucose transport (GLUT) mechanism [8]; to date, its molecular identity has not been associated to any gene [6] (Table 20.1). Amoebal GLUT activity is not inhibited by compounds that affect mammalian GLUTs such as colchicine and phloretin [8], which suggests a highly divergent molecular nature from typical GLUT transporters. However, similar to mammalian GLUTs, amoebal GLUT is also potently inhibited by cytochalasin B (K_i = 3 μM; Rusely Encalada and Emma Saavedra, unpublished results). In his pioneering studies, Reeves proposed that as the amoebal glucose transport and pathway flux exhibited similar rates, GLUT could represent a rate-limiting step of the glycolytic flux [9].

Once internalized, glucose is immediately phosphorylated because no free intracellular glucose has been detected [9]. This reaction is catalyzed by hexokinase (HK; EC 2.7.1.1), which consumes 1 mole of ATP to produce glucose-6-phosphate (Glu6P) and ADP (Fig. 20.1). In the *E. histolytica* genome, there are two genes coding for isoforms HKI and HKII, which have 89 % identity at the amino acid sequence

Fig. 20.1 (continued) hexokinase, *HPI* hexose-6-phosphate isomerase, *MDH* malate dehydrogenase, *ME* malic enzyme, *PEPCT* phosphoenolpyruvate carboxytransphosphorylase, *PFOR* pyruvate:ferredoxin oxidoreductase, *PGK* 3-phosphoglycerate kinase, *PGAM* 3-phosphoglycerate mutase, *PGM* phosphoglucomutase, *PPDK* pyruvate phosphate dikinase, *PPi-PFK* PPi-dependent phosphofructokinase, *PYK* pyruvate kinase, *UDP-GlucPPiase* UDP-glucose pyrophosphorylase, *TK* transketolase, *TPI* triosephosphate isomerase, *UDP-Gluc* UDP-glucose, *Xylu5P* xylulose-5-phosphate. *Dashed arrows* indicate enzymes present in amebas whose contributions to the pathway fluxes are unknown. *Dotted arrow* indicates that the gene encoding PEPCT has not been identified

Table 20.1 Kinetic properties of carbohydrate metabolism enzymes from *Entamoeba histolytica*

Enzyme	V_{max} recombinant enzymes[a]	V_{max} in cell extracts[b]	Kinetic parameters[c]	$C^{Jetoh}{}_{enzyme}$[d]	References
GLUT	ND	27–67	$K_{m\ gluc}$: 1,600–2,500	ND	Serrano and Reeves [8, 9]
			$K_{m\ 2\text{-}DOG}$: 40,000		
HK	FW: 86–158	95	$K_{m\ gluc}$: 25–40	0.73	Saavedra et al. [7, 10], Reeves et al. [11], Kroschewski et al. [12]
	RW: NA		$K_{m\ ATP}$: 77–290		
			$K_{i\ AMP\ vs\ ATP}$: 0.6–36		
			$K_{i\ ADP\ versus\ ATP}$: 36–235		
			$K_{a\ PPi}$: 68–150		
HPI	FW: 392–608	233	$K_{m\ Glu6P}$: 610–750	0.08	Saavedra et al. [7, 10], Marín-Hernández et al. [13]
	RW: 182–620		$K_{m\ Fru6P}$:130–480		
			$K_{i\ Ery4P}$: 5.9		
PPi-PFK	FW: 112–298	213	$K_{m\ Fru6P}$: 100–695	0.13	Saavedra et al. [7, 10], Reeves et al. [14, 15], Deng et al. [16]
	RW: 338–392		$K_{m\ PPi}$: 50–380		
			$K_{m\ Fru1,6BP}$: 109–124		
			$K_{m\ Pi}$: 1,140–2,300		
ATP-PFK	ND	1.4	$K_{m\ Fru6P}$: 3,800	ND	Saavedra et al. [10], Chi et al. [17]
			$K_{m\ ATP}$: 120		
ALDO	FW: 15–31	160	$K_{m\ Fru1,6BP}$: 4–28	0.09	Saavedra et al. [7, 10], Kalra et al. [18]
	RW: 29–34		$K_{m\ Gly3P}$:108–210		
			$K_{m\ DHAP}$: 105–265		
			$K_{i\ PPi}$: 210–420		
TPI	FW: 199–284	4,366	$K_{m\ DHAP}$: 445–1,655	0.003	Saavedra et al. [7, 10], Landa et al. [19]
	RW: 1,096–3,364		$K_{m\ Gly3P}$: 320–830		
GAPDH	FW: 13–27	405	$K_{m\ Gly3P}$: 59–86	0.08	Saavedra et al. [7, 10]
	RW: 36–40		$K_{m\ NAD+}$: 59–83		
PGK	FW: 279–628	3,182	$K_{m\ 1,3\ BPG}$: 125–127	0.04	Saavedra et al. [7, 10], Reeves and South [20], Encalada et al. [21]
	RW: 62–87		$K_{m\ GDP}$: 40–292		
			$K_{m\ ADP}$: 600–3,400		
			$K_{m\ 3PG}$: 505–570		
			$K_{m\ GTP}$: 61–75		
			$K_{m\ ATP}$: 1,840–3,000		
PGAM	FW: 53–42	116	$K_{m\ 3PG}$: 500–840	0.65	Saavedra et al. [7, 10]
	RW: 6–13		$K_{m\ 2PG}$: 66–106		
			$K_{i\ PPi}$: 173–660		
ENO	FW: 89–103	508	$K_{m\ 2PG}$: 55–60	0.08	Saavedra et al. [7, 10]
	RW: 26–33		$K_{m\ PEP}$:63–102		
			$K_{i\ PPi}$: 137–280		
			$K_{i\ 3PG}$: 460–610		

(continued)

Table 20.1 (continued)

Enzyme	V_{max} recombinant enzymes[a]	V_{max} in cell extracts[b]	Kinetic parameters[c]	C^{Jetoh}_{enzyme}[d]	References
PPDK	FW: 8–12	304	$K_{m\ PEP}$: 20–30	0.0009	Saavedra et al. [7, 10], Reeves [22], Saavedra-Lira et al. [23]
	RW: 1.5–2.3		$K_{m\ AMP}$: 2–20		
			$K_{m\ PPi}$: 91–470		
			$K_{m\ Pyr}$:68–305		
			$K_{m\ ATP}$: 284		
PYK	ND	28	$K_{m\ PEP}$: 18–25	ND	Saavedra et al. [10, 24]
			$K_{m\ ADP}$: 210–1,050		
PFOR	ND	1,080–1,145	$K_{m\ Pyr}$:1,500–3,500	0.09	Pineda et al. [25, 26]
			$K_{m\ CoA}$: 6–13		
			$K_{i\ acetyl\text{-}CoA}$: 24–36		
			$IC_{50\ oxygen}$: 34		
			$IC_{50\ H2O2}$: 6–35		
ADHE	FwALDH: 230 Fw:ADH: 500	ALDH: 75–93	$K_{m\ acetyl\text{-}CoA}$: 15–40	0.33	Pineda et al. [25, 26], Espinosa et al. [27], Bruchhaus and Tannich [28], Yong et al. [29]
		ADH: 469	$K_{m\ acetaldehyde}$: 150–230		
			$K_{m\ NADH}$: 150–280		
			$K_{m\ EtOH}$: 85,000		
			$K_{m\ NAD+}$: 150, 550		
Ac-CoAS	ND	176–254	$K_{m\ acetyl\text{-}CoA}$: 120	<0.05	Pineda et al. [25, 26]
AcK	FW: 800	60	$K_{m\ acetyl\text{-}P}$: 20–70	ND	Reeves and Guthrie [30], this group
			$K_{m\ Pi}$: 2,200–2,600		

Full names of enzymes are given in legend to Fig. 20.1

[a] V_{max} in the forward (FW) and reverse (RW) reaction in μmol/min × mg protein

[b] V_{max} of the FW reaction in nmol/min × mg cell protein where determined at pH 6.0 in cytosol-enriched cell fractions [10], except for GLUT, which was determined in whole cells [8, 9]

[c] Values in μM

[d] Flux control coefficient (C^{J}_{ai}) over etOH flux reported in Saavedra et al. [10], Moreno-Sánchez et al. [31], and Pineda et al. [26]

ND not determined, *NA* not applicable

level [6, 33]. The differences in the isoelectric points of the HK isoforms caused by these changes have been used to distinguish *E. histolytica* from *Entamoeba dispar* [34, 35]. As do most HKs, the amoebal enzymes function as dimers and catalyze a reaction far from equilibrium (K_{eq} ~844), which precludes its reversibility under physiological conditions. Kinetically, both *E. histolytica* HK isoforms have similar high affinities for glucose [7, 11, 12] (Table 20.1), but they differ in their affinity for ATP, which is higher in the HKI isoform [12]. None of them use fructose or galactose as substrate, although they can use mannose but with low affinity ($K_{m\ mannose}$ HKI = 1 mM; HKII >2 mM) [12]. In addition, PPi is a potent, nonessential mixed-type activator of HKI (Table 20.1) [31]. Remarkably, Glu6P does not inhibit

amoebal HKs as occurs for the mammalian enzymes in which serves as a regulatory mechanism [36]; instead, amoebal HKs are potently and competitively inhibited by AMP and ADP against ATP with K_i of 24–36 and 120–235 μM, respectively [7, 11, 12]. These K_i values are well within the physiological concentrations found in trophozoites (1.6 mM AMP, 3.3 mM ADP, 5 mM ATP) [10]. Therefore, the ratios of the adenine nucleotide pools (i.e., ATP/ADP and ATP/AMP) may serve as regulatory mechanisms of this reaction.

Glu6P is isomerized to fructose-6-phosphate (Fru6P) by hexose phosphate isomerase (HPI; EC 5.3.1.9) (Fig. 20.1). The *E. histolytica* genome contains two genes encoding HPIs that differ in the insertion/deletion of seven amino acids [6], and its isoforms have been used to distinguish different isolates from *E. histolytica* or *E. dispar* [34, 37]. The enzyme is functional as a dimer [7] and catalyzes a reaction near its thermodynamic equilibrium (K_{eq} ~0.3) under physiological conditions, which may preclude a relevant controlling role on glycolytic flux [10]. However, it has been recently demonstrated that the amoebal enzyme can be potently inhibited by erythrose-4-phosphate (Ery4P), an intermediary of the nonoxidative branch of pentose phosphate pathway (PPP), with a K_i value of 5.9 μM [13]. Therefore, the PPP branch flux may indirectly exert control on the first reactions of the glycolytic flux.

The next step of glycolysis is phosphorylation of Fru6P to fructose-1,6-bisphosphate (Fru1,6BP). In most cells, this reaction is catalyzed by an allosterically modulated, tetrameric ATP-dependent phosphofructokinase (ATP-PFK; EC 2.7.1.11) [38], which catalyzes an irreversible reaction under physiological conditions because of thermodynamic constraints (K_{eq} ~2,300). In contrast, in *E. histolytica*, 90 % of PFK activity is accounted by a pyrophosphate-dependent phosphofructokinase (PPi-PFK; EC 2.7.1.90), which catalyzes a fully reversible reaction (K_{eq} ~3.2) under physiological conditions [7, 10, 14, 15]. In the amoebal genome there is only one gene for PPi-PFK, coding for a protein of 60 kDa [6]. This enzyme has been kinetically characterized [7, 14–16, 39]. It functions as a dimer showing moderate affinity for PPi and Fru6P at physiological pH [7] (Table 20.1). The physiological concentrations of Fru6P (1.1 mM) and PPi (0.45 mM) [10] represent only one- to twofold the K_m values (Table 20.1), although to circumvent this limitation, the cells express relatively high PPi-PFK content/activity [10]. It is tempting to speculate that the use of PPi instead of ATP at the level of PFK prevents the negative effect of the turbo design of glycolysis, in which at high glucose concentration, the first ATP-consuming reactions of glycolysis may exhaust the ATP before being resynthesized in the late stages of the pathway [40].

Interestingly, the specificity for PPi of amoebal PPi-PFK can be changed to ATP by introducing the single mutation Asn105Gly; this change in the PPi-PFK gene might have phylogenetic implications [41]. Besides Fru6P, PPi-PFK can use sedoheptulose-7-phosphate (sedoheptul-7P) to produce sedoheptulose-1,7 bisphosphate (sedoheptul-1,7BP) [15]; hence, the complete nonoxidative branch of PPP can be catalyzed by the combined action of PPi-PFK, transketolase (TK), and aldolase [42] (Fig. 20.1).

In addition, the *E. histolytica* genome also contains three genes encoding ATP-dependent PFKs of 48 kDa [6], which have only 17 % identity with the PPi-PFK sequence [17]. The ATP-PFK is an allosterically modulated enzyme with low affinity for Fru6P ($K_{m\ Fru6P} = 3.6$ mM). Considering that (1) the physiological Fru6P concentration of 1.1 mM [10] is less than its K_m value and (2) within the cell, there is one order of magnitude lower ATP-PFK activity than PPi-PFK activity [10], it seems that the contribution of ATP-PFK to the glycolytic flux in *E. histolytica* is negligible. Therefore, the physiological function of this enzyme remains to be elucidated.

Fru1,6BP is cleaved to glyceraldehyde-3-phosphate (Gly3P) and dihydroxyacetone phosphate (DHAP) (Fig. 20.1) by a type II metal-dependent Fru1,6BP aldolase (ALDO; EC 4.1.2.13) that diverges from type I metal-independent aldolases present in plants and animals [7, 43]. Two genes are present in the amoebal genome, differing in an insertion of 28 amino acids [6]. The *E. histolytica* enzyme is a tetramer that shows high affinity for Fru1,6BP (Table 20.1) [7, 18] and can be saturated in *in vivo* conditions (Fru1,6BP concentration of 0.43 mM) [7, 10]. The enzyme prefers Co^{2+} as an essential cofactor, but other divalent metals can also be used [7, 18]. PPi is a competitive inhibitor of the enzyme [31] (Table 20.1). As already mentioned, ALDO may participate in the nonoxidative branch of the PPP, by cleaving sedoheptul-1,7BP to DHAP and Ery4P [42]. Hence, Fru6P, DHAP, and Gly3P are precursors of the non-oxidative PPP to produce ribose-5-P (R5P) for the synthesis of nucleotides (Fig. 20.1).

Interconversion of DHAP to Gly3P is catalyzed by triosephosphate isomerase (TPI; E.C. 5.3.1.1). Only one gene is found in the amoebal genome [6, 19], and the enzyme has been crystallized [44]. In most cells, the enzyme is the most catalytically efficient of the glycolytic enzymes despite its low affinity for substrates (Table 20.1) [7], and it catalyzes a reaction close to equilibrium (K_{eq} ~0.05); therefore, its control over the glycolytic flux is negligible [10].

Next, Gly3P is phosphorylated and oxidized to 1,3 bisphosphoglycerate (1,3BPG) by glyceraldehyde-3-phosphate dehydrogenase (GAPDH; E.C.1.2.1.12), transferring two electrons to NAD^+. In the *E. histolytica* genome there are five annotated GAPDH genes [6]. The enzyme is a tetramer that shows high affinity for its ligands and specificity for NAD^+ [7] (Table 20.1). Recently it was reported that the amoebal enzyme is ADP-ribosylated and excreted to the extracellular milieu [45]. This posttranslational modification suggests that other functions besides its metabolic role might be operating in amoebas; indeed, GAPDH is a typical example of a moonlighting enzyme, that is, an enzyme that has other physiological functions besides its main and essential metabolic role [46]. In this last regard, it has been described that GAPDH is involved in gene regulation, cell signaling, and DNA integrity [47].

The first substrate-level phosphorylation reaction of glycolysis is catalyzed by 3-phosphoglycerate kinase (PGK; E.C. 2.7.2.3). There is only one gene coding for the amoebal dimeric enzyme [6]. In general, PGKs of most cells transfer the phosphate from position 1 of 1,3 BPG to ADP, producing 3-phosphoglycerate (3PG) and ATP. However, the amoebal PGK has two orders of magnitude higher preference for

guanine nucleotides [7, 20, 21], although by means of a nucleotide transphosphorylase ATP can be readily synthesized from GTP. By site-directed mutagenesis analysis we demonstrated that tyrosine 239 and glutamic acid 309 are involved in the higher affinity for guanine nucleotides [21].

3-Phosphoglycerate mutase (PGAM; E.C. 5.4.2.1) isomerizes 3PG to 2-phosphoglycerate (2PG). Five genes are annotated in the amoebal genome as PGAMs [6], from which two correspond to bacterial-like 2,3 bisphosphoglycerate (2,3BPG)-independent PGAMs that are not related to the 2,3BPG-dependent PGAMs present in mammalian cells. Accordingly, the recombinant and the enzyme present in clarified amoebal extract do not require the cofactor for activity [7, 10]. The enzyme is a monomer that has low affinity for 3PG [7], and it is present in low content/activity in the cell [10] (Table 20.1); these data suggested that the enzyme represents a constraint in the pathway flux, which was further demonstrated by metabolic modeling [10, 31]. The enzyme is competitively inhibited by PPi (Table 20.1) [31] and it is also inhibited in amoebas incubated with reactive oxygen species [48].

Enolase (ENO; E.C.4.2.1.11) converts 2PG into phosphoenolpyruvate (PEP). There is only one gene in the amoebal genome coding for this enzyme [6]. The amoebal enzyme is a tetramer and shows high affinity for its ligands [7], whereas 3PG and PPi are weak competitive inhibitors (Table 20.1) [31]. It was demonstrated recently that amoebal ENO may translocate to the nucleus where it inhibits cytosine-5-methyltransferase 2 (Dnmt2), which participates in DNA and tRNA methylation [49]. Moreover, ENO protein from *Entamoeba invadens*, *E. histolytica*, and *Naegleria fowleri* has also been found in vesicle and cyst walls [50]. Thus, the ENO non-glycolytic functions described for other cells [46] can be also extended to the amoebal protein.

In most cells, PEP is dephosphorylated to pyruvate (Pyr), producing ATP in the second reaction of glycolytic substrate-level phosphorylation catalyzed by pyruvate kinase (PYK; E.C. 2.7.1.40). Thermodynamically, this reaction is irreversible under physiological conditions (K_{eq} ~3.2×10^5). In contrast, amoebas have the PPi-dependent enzyme pyruvate phosphate dikinase (PPDK; E.C. 2.7.9.1), which catalyzes a reversible reaction at physiological pH (K_{eq} ~0.005) and is also present in anaerobic bacteria and other protists such as trypanosomatids and plants. The tetrameric enzyme transfers high-energy phosphate groups from PEP and PPi to AMP to produce ATP, Pi, and Pyr [7, 22, 23]. The enzyme kinetic parameters have been determined [7, 23]. Despite being the slowest enzyme in its pure form, this inconvenient is circumvented by its high content/activity in the cell and its high affinity for substrates (Table 20.1).

In addition, the amoebal genome contains three open reading frames for putative PYK genes [6], and the kinetic characterization of a PYK activity in cytosolic parasite extracts was reported by our group [24]. The amoebal PYK activity represents only 10 % of the PPDK activity within the cell (Table 20.1) [10] and shows nonessential activation by Fru1,6BP, similar to many other PYKs [24]. However, the recombinant or native enzyme has not been purified and studied, nor has its physiological function been elucidated.

20.2.1 Alternative Routes of PEP to Pyr Transformation

Alternatively to PPDK (and to a minor extent PYK), amoebas can transform PEP to Pyr by three sequential reactions (Fig. 20.1). First, a PEP carboxytransphosphorylase (PEPCT; E.C. 4.1.1.38) [51] catalyzes carboxylation of PEP to oxaloacetate (OAA) using Pi as cosubstrate instead of GDP as the typical PEP carboxykinase (E.C. 4.1.1.32). Hence, the amoebal enzyme produces PPi, thus representing another source of PPi for glycolytic enzymes, whereas in bacteria, PEPCT serves for anaplerotic supply of OAA. Further, OAA is reduced to malate by a NADH-dependent malate dehydrogenase (MDH; E.C.1.1.1.37), and malate is in turn decarboxylated and oxidized by a $NADP^+$-dependent and decarboxylating malate dehydrogenase (malic enzyme; ME, E.C. 1.1.1.40) to produce Pyr [51]. The resulting effect of this PEP-Pyr loop is transhydrogenation of NADH to NADPH, making available NAD^+ for glycolysis and PPi and dicarboxylic acids for anaplerotic purposes. Unfortunately, the gene encoding a PEPCT has not been identified [6], and to date, no kinetic information has been reported for these enzymes. ME has been used to distinguish *E. histolytica*/*E. dispar* from other amoebas [34].

20.3 Pyruvate Metabolism

20.3.1 Pyruvate Decarboxylation and Oxidation

Pyruvate is at a crossroad of glucose catabolism (Fig. 20.1). *E. histolytica* lacks pyruvate dehydrogenase and pyruvate decarboxylase; hence, decarboxylation and oxidation of Pyr to acetyl-coenzyme A (acetyl-CoA) and CO_2 is catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR; E.C. 1.2.7.1), which transfers two electrons to two ferredoxin (Fd) molecules [52, 53]. Two genes for PFOR and seven for Fd are annotated in the amoebal genome [6]. Reduced Fd (Fd_{red}) is involved in metronidazole activation, the current drug used to treat amebiasis. It has been demonstrated that PFOR downregulation appears not to be a mechanism for resistance against this drug; instead, superoxide dismutase and peroxiredoxin overexpression and Fd downregulation were observed in metronidazole-resistant strains [54]. The kinetic properties of the amoebal PFOR have been determined [25, 52, 53]. PFOR is the third most abundant enzyme activity from the amoebal fermentative pathway, preceded only by PGK and TPI [25] (Table 20.1). At physiological pH, the enzyme has low affinity for Pyr and high for coenzyme A (CoA); high acetyl-CoA concentration is a potent inhibitor [25]. The enzyme can use other 2-oxoacids such as oxaloacetate and α-ketobutyrate but with extremely low affinities (K_m >10 mM) and cannot use α-ketoglutarate [25]. Consequently, these carbon skeletons derived from amino acid degradation appear not to be physiologically used by PFOR for acetyl-CoA formation as previously suggested [6]. Therefore, other 2-oxoacid oxidoreductases are very likely involved in amino acid utilization as fuels.

PFOR contains three iron–sulfur clusters that are highly sensitive to oxidation and inactivation by ROS [55]. In agreement, the amoebal PFOR is inhibited at low micromolar concentrations of O_2 and H_2O_2 (Table 20.1) [25, 56]. Our group reported that *E. histolytica* subjected to an acute supraphysiological O_2 concentration (0.63 mM) promoted accumulation of Glu6P, Fru6P, and Pyr and diminished EtOH flux and ATP content. These changes were the result of strong inhibition of PFOR and the NADH-dependent bifunctional aldehyde-alcohol dehydrogenase (ADHE; E.C. 1.2.1.10 1.1.1.1) [25, 56]. Interestingly, virulent *E. histolytica* was able to rapidly restore PFOR activity after the insult whereas nonvirulent amebas were not, suggesting a more robust antioxidant protection in the former [56].

20.3.2 Ethanol Formation

Acetyl-CoA produced by PFOR is further transformed to EtOH, which is the main product of glucose catabolism in amoebas. In *E. histolytica*, EtOH fluxes under aerobic conditions are 15–39 nmol EtOH/min × mg cell protein, values that are one order of magnitude higher than those for acetate formation of 2.8–3.6 nmol acetate/min × mg cell protein, determined under the same condition [10, 25, 26]. Under anaerobic conditions (N_2 atmosphere), axenic amoebas do not produce acetate [52]. Hence, in contrast to the common belief, *E. histolytica* mainly produce EtOH under both anaerobic and aerobic conditions.

Ethanol production from acetyl-CoA is catalyzed by ADHE [53]. This enzyme is similar to the bifunctional enzyme present in bacteria in which the N-terminal domain contains aldehyde dehydrogenase activity whereas the C-terminal domain has alcohol dehydrogenase activity containing an Fe^{2+}-binding site [27]. The enzyme transforms acetyl-CoA to EtOH through a thiohemiacetal intermediary bound to the enzyme that is further reduced to EtOH (Fig. 20.1). Two NADH molecules are oxidized per EtOH produced in the overall ADHE reaction; because one NADH per pyruvate formed is synthesized by GAPDH in glycolysis, then, the extra NADH molecule required to synthesize EtOH can be provided by Fd_{red} produced in the PFOR reaction. Fd_{red} can be oxidized by a ferredoxin:NAD^+ oxidoreductase (Fd:NAD^+ OR) producing NADH and regenerating oxidized Fd (Fd_{ox}) [53, 57] (Fig. 20.1).

Recombinant ADHE has been kinetically characterized [27–29, 53]. The enzyme has almost two to three orders of magnitude higher affinity for acetyl-CoA and acetaldehyde than for EtOH (Table 20.1). This finding suggests that ADHE *in vivo* mostly functions in the direction of EtOH production. The enzyme is also highly sensitive to inhibition by oxidative stress [25].

Interestingly, ADHE can form helical rod-like ultrastructures called spirosomes that serve for its stabilization [58]. Moreover, this enzyme seems to be associated with Fe^{2+} transport from the extracellular milieu [59], which suggests additional functional roles for this enzyme.

At least 17 other genes coding for alcohol dehydrogenases and 1 for aldehyde dehydrogenase have been annotated in the *E. histolytica* genome [6]. Two NADPH-dependent alcohol dehydrogenases (ADH1 and ADH3) and one $NADP^+$-dependent aldehyde dehydrogenase have been kinetically characterized [6]. These enzymes can use free medium-chain and aromatic aldehydes but not acetyl-CoA as substrates; therefore, they seem not to be involved in fermentative glycolysis. It has been suggested that a NADPH-dependent alcohol dehydrogenase might function as a sink for NADPH in microaerophilic parasites at low oxygen tension [60]. Therefore, ADHE is the main enzyme involved in EtOH formation in amoebas.

20.3.3 Acetate Metabolism

Under aerobic conditions, part of the acetyl-CoA can be alternatively transformed to acetate (Fig. 20.1) by an ADP-forming acetyl-CoA synthetase (AcCoAS; E.C. 6.2.1.13) named after the reverse reaction and also known as acetate thiokinase [52]. In amoebas it catalyzes hydrolysis of the high-energy thioester bond of acetyl-CoA to synthesize ATP from ADP and Pi [52]. In contrast to the most common acetate thiokinases, which use AMP and PPi as substrates, the amoebal, giardial, and some anaerobic bacterial enzymes use ADP and Pi [52, 61]. Recently we reported the properties of the enzyme in *E. histolytica* cytosolic fractions [25]. The enzyme has lower affinity for acetyl-CoA compared to ADHE (Table 20.1); hence, the latter represents an important competitor of AcCoAS for the metabolite (Fig. 20.1).

There is controversy on the functional role of acetate formation in amoebas. It was early described by Richard Reeves that amoebas subjected to aerobic conditions can produce 75 % acetate and 25 % EtOH whereas under anaerobic conditions this ratio was reversed and EtOH production was favored [52]. As ATP is synthesized during production of acetate, it has been thought that amebas are able to support aerobic conditions because they have an extra provision of ATP. However, we have consistently found that after a challenge of hyperoxia (0.63 mM O_2) or under mild aerobic conditions (air-equilibrated saline buffer, 0.18 mM O_2), trophozoites can produce a maximum of just 20 % acetate from glucose catabolism [25, 26]. After the supraphysiological O_2 challenge, *E. histolytica* trophozoites show decreased ADHE activity and decreased EtOH formation, whereas AcCoAS activity remains unaltered and acetate formation slightly increases [25]. ADHE inhibition induced by ROS can bring about a buildup of acetyl-CoA that can stimulate the flux through AcCoAS, resulting in increased acetate production. However, under conditions in which ADHE is 50 % inhibited by disulfiram, the low flux through the acetate branch is not sufficient to maintain the steady ATP concentration in the cell [26]. These results indicate that contribution of AcCoAS to the ATP supply seems negligible. Hence, we have hypothesized that under prolonged aerobic conditions, an increased acetate flux may transiently drain the acetyl-CoA accumulation caused by ADHE inhibition regenerating CoA (Fig. 20.1). It may be also possible that

buildup of acetyl-CoA could serve for the synthesis of other biomolecules. All these hypotheses remain to be tested.

Amebas also have a PPi-dependent acetate kinase (AcK) that synthesizes PPi and acetate starting from acetyl-phosphate (acetyl-P) and Pi [30] (Fig. 20.1). It has been demonstrated that the enzyme can only catalyze the reaction in the direction of acetate formation [30, 62]. In contrast, in other microorganisms that use acetate as carbon fuel, AcK catalyzes acetyl-P production using acetate as substrate and ATP as high-energy P donor; acetyl-P is further transformed to acetyl-CoA by phosphotransacetylase, an enzyme that is absent in amoebas. The native [30] and recombinant ([62]; Citlali Vázquez and Emma Saavedra, unpublished results) amoebal AcKs have been characterized. The enzyme reported by Fowler et al. showed lower affinities for acetyl-P (0.5 mM) and Pi (48.9 mM) compared to those determined for the native [30] and our recombinant enzyme (0.02–0.07 and 2.2–2.6 mM, respectively). This discrepancy may be caused by the low sensitivity of the enzymatic assay that they used. In *E. histolytica*, Pi concentration is near 5.4 mM [10] whereas acetyl-P has not been detected (Citlali Vázquez and Emma Saavedra, unpublished results). It is unknown whether other acyl-phosphates can be used by the amoebal enzyme. Further studies are required to determine the physiological role of this AcK in *E. histolytica*.

20.4 Main Glycolytic Branches

20.4.1 Glycogen Metabolism

Glu6P is another glycolytic metabolite localized at an important crossroad. Amoebas lack the genes coding for the enzymes of the PPP oxidative branch: glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, and 6-phosphogluconate dehydrogenase [5, 6]. Hence, Glu6P can continue through its fermentative route and serve as precursor for glycogen synthesis, or is a glycogen degradation product (Fig. 20.1). Glu6P is interconverted to glucose-1P (Glu1P) by the action of phosphoglucomutase (PGM) [63]. This enzyme together with HK have been used as the gold standards to distinguish *E. histolytica* from *E. dispar* [34, 64]. Glycogen is abundant in the parasite, accounting for up to 3 M of stored glucose equivalents [10]; therefore, glycogen synthesis is highly active in amoebas grown on high external glucose concentrations. Glycogen synthesis (Fig. 20.1) is performed by the sequential action of UDP-glucose pyrophosphorylase (UDP-Gluc PPiase) and glycogen synthase (GS) [5, 63]. Then, glycogen synthesis may be an important source of PPi [5, 10]] (Fig. 20.1). Interestingly, UDP-Gluc PPiase activity is affected by redox modification of thiol groups, which opens the possibility of redox modulation of glycogen synthesis [65]. On the other hand, glycogen degradation is catalyzed by the action of glycogen phosphorylase (GP) [5, 66]. No information is available on the mechanisms that regulate degradation of glycogen in amoebas such as that in mammalian cells.

20.4.2 Chitin Synthesis

It has been recently demonstrated in *E. invadens* that glycogen degradation mainly provides the carbon skeletons for chitin synthesis [67, 68]. Glu6P from glycogen degradation, or from external glucose, isomerizes to Fru6P by HPI (Fig. 20.1). Fru6P is the glycolytic precursor for chitin synthesis. This metabolite is in turn isomerized and aminated by glucosamine-6-phosphate isomerase (GN6PI) to produce glucosamine-6-phosphate (GlcN6P), the first intermediate of chitin synthesis (Fig. 20.1). Increase in the level of this metabolite during encystation of *E. invadens* [67, 68] and in *E. histolytica* subjected to oxidative stress [48] has been observed. It has been recently demonstrated that *E. histolytica* can form cyst-type structures induced by ROS, which stimulate chitin synthesis [69].

20.4.3 Amino Acid Synthesis

Glycolytic triose-phosphates are precursors for amino acid synthesis: 3PG for serine, glycine, and cysteine [70] and Pyr for alanine (Fig. 20.1). Genes encoding the enzymes in the respective routes have been identified in the *E. histolytica* genome [6]. Ser and Cys synthesis are relevant for amoebas because they lack glutathione [71], the main antioxidant molecule in most cells; therefore, Cys replaces GSH as the antioxidant metabolite in amebas. In comparison, other amino acid synthesis pathways are not present or are reduced, because these metabolites can be abundant in the extracellular medium by the action of a great variety of amoebal proteases. In this regard, amebas are able to actively take up and consume a wide range of amino acids [72].

20.5 Controlling Mechanisms of Amoebal Fermentative Glycolysis

The amoebal HK, PPi-PFK, and PPDK markedly diverge in their enzymatic properties from those of the tightly regulated mammalian HK, ATP-PFK, and PYK, which are, together with GLUT, the main controlling and regulatory steps of glycolysis in these last cells [32]. Moreover, with the exception of HK, in amoebas all the reactions from HPI to ADHE are thermodynamically reversible under physiological conditions (Fig. 20.1). Thus, it seems that neither allosteric regulatory mechanisms nor thermodynamic constraints can be applied to identify and determine what controls the fermentative glycolysis in *E. histolytica*. Therefore, it becomes apparent that only an integral analysis of all the parameters and variables of the pathway, that is, enzyme/transporter kinetic properties and levels of enzyme/transporter expression, metabolite concentrations, and metabolic fluxes in the cell,

and using the fundamentals of metabolic control analysis (MCA) [32, 73] for analysis, it is possible to determine the control structure and understand the underlying controlling mechanisms of the pathway in this parasite.

20.5.1 Metabolic Control Analysis

Metabolic control analysis (MCA) is a systems biology approach that analyzes metabolic networks with the goal of elucidating their underlying mechanism of control and regulation [32, 73, 74]. In this regard, MCA makes a clear distinction between control and regulation of metabolism. "Control" indicates the extent to which the flux or the concentration of an intermediary of a metabolic pathway is altered when the activity of one enzyme (or group of enzymes) is changed. These values are quantitatively represented by the *flux-* and *concentration-control coefficients*, respectively. "Regulation" refers to how the flux of a pathway or a metabolite level is modified when the rate of an individual step is changed by cellular factors other than their substrates/products (e.g., enzyme activity modulators such as hormones and ions), and is quantitatively represented by the *response coefficient* [73]. Regulation is related more to the homeostatic control of metabolic pathways.

MCA studies have demonstrated that, in contrast to the common concept of rate-limiting step used in biochemistry textbooks and scientific literature outside metabolic regulation, the control of a pathway is distributed in different degrees among all the pathway components, with two or three steps displaying the highest control [32, 73]. Thus, using MCA it is possible to determine the control distribution and controlling mechanisms of a pathway flux in any cell model.

The flux control coefficient is symbolized as C^J_{ai}, in which J is the pathway flux and a is the activity of an enzyme/transporter i in the cell. To determine the C^J_{ai}, the enzyme/transporter within the cell has to be gradually titrated above and below the wild-type (WT) level (the reference point), and the pathway flux has to be assessed in parallel. From plots of normalized pathway flux versus enzyme activity, the C^J_{ai} is calculated from the slope of the tangent of the fitting of the closest points to the WT level (Fig. 20.2a). Performing this experiment for each pathway enzyme, the whole set of C^J_{ai} can be calculated and hence the pathway control distribution. The sum of all C^J_{ai} in the pathway must add up to 1 (summation theorem of MCA). In concordance, an enzyme with a C^J_{ai} equal to 1 (enzyme E1 in Fig. 20.2a) means that it is the one and only controlling step of the pathway flux, that is, it is a true rate-limiting step. In contrast, an enzyme with a C^J_{ai} approaching 0 (enzyme E2 in Fig. 20.2a) means that it has negligible control of the pathway flux.

Experiments similar to those described in Fig. 20.2a to determine the control distribution of a metabolic pathway, which seems simple at first glance, has demanded the design of several different experimental strategies to change the enzyme rate in the cells of only one enzyme at a time, without affecting the rest of the pathway enzymes/transporters. MCA has been successfully applied to several pathways such as oxidative phosphorylation in normal and tumor cells and glycolysis in several biological models [32].

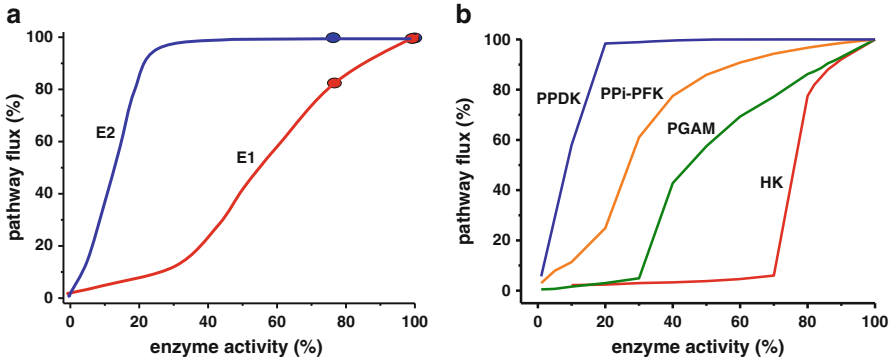

Fig. 20.2 Metabolic control analysis (MCA) and drug targeting. **a** Flux control coefficient distribution for a two-step pathway. The plots represent how the pathway flux can change by varying the activity of the pathway enzymes. The C^{J}_{ai} for each enzyme is equal to the slope of the tangent of the experimental points closer to the reference value of 100 %. In this example, enzyme 1 has a C^{J}_{ai} close to 1 whereas enzyme 2 has negligible control. **b** In silico titration of amebal glycolytic enzymes and its effect on the pathway flux. Inhibition of the most controlling pathway enzymes (HK and PGAM) have more effects on the flux than inhibition of low controlling enzymes (PPi-PFK and PPDK)

20.5.2 Kinetic Modeling of Metabolic Pathways

Bioinformatics tools have been incorporated into systems biology analyses and MCA studies to construct kinetic models of several pathways to determine their control distribution and controlling mechanisms (for a kinetic model database, see the JWS Online Cellular Systems Modeling at http://jjj.biochem.sun.ac.za/index.html and [75]). This approach is now called bottom-up systems biology to distinguish it from the top-down systems biology strategies that are fueled from data obtained from high-throughput transcriptomic, proteomic, and metabolomic analyses [74]. Only a few kinetic models of glycolysis in pathological cells have been reported, in particular for tumor cells [13] and for the human parasites *Trypanosoma brucei* [76] and *E. histolytica* by our research group [10, 31]. The reason for this scarcity of studies is that kinetic metabolic modeling requires an increased amount of experimental data for their construction and validation [75].

The aim of kinetic modeling is to build a computational representation that may reproduce the physiological behavior of a metabolic/signaling pathway using the knowledge of the kinetic properties of the individual enzymes and transporters [77]. A validated model should predict with the highest possible accuracy the pathway behavior in the cell regarding flux rates, metabolite concentrations, and control structure, and discover not so apparent properties [77]. Kinetic models that apply MCA also help to identify the kinetic mechanisms of why an enzyme controls or does not control a pathway flux or a metabolite concentration.

20.5.3 Kinetic Modeling of Amoebal Glucose Fermentation

To construct the kinetic model of amoebal glycolysis [10] it was necessary to determine the kinetic properties of all the pathway enzymes from HK to PPDK determined under the same experimental conditions and close to the physiological pH, temperature, and ionic composition values as described by Saavedra et al. [7]. Such dataset included the affinity for substrates, products, activators, and inhibitors for each pathway reaction. Moreover, it was also necessary to determine the enzyme maximal activities (V_{max}) in the forward and reverse direction of each reaction, metabolite concentrations, and pathway fluxes within amoebal trophozoites, employing the same strain and under identical incubation conditions. Using the enzyme kinetic data, appropriate rate equations for each reaction and concentrations of some precursors that supply the pathway such as glucose, ATP, Pi, and NAD^+ were assembled in the software GEPASI (later renamed COPASI, complex pathway simulator), both of which allow MCA [78]) used as a bioinformatics platform. After several rounds of refinement, in which *in vivo* experimentation was required, a kinetic model emerged that could predict the metabolite concentrations and pathway fluxes to values acceptably close to those determined *in vivo* in amoebas incubated in the presence of 10 mM glucose [10]. The agreement between modeling and experimentation allowed validation of the kinetic model as a tool to analyze the pathway control distribution.

The model predicted that the highest flux control steps corresponded to HK, PGAM, glycogen synthesis, and PPi synthesis, showing C^J_{ai} *values* of 0.73, 0.65, −0.32, and −0.28, respectively (Table 20.1); the latter two reactions with negative signs mean that they represent branches that drain metabolites from the main pathway [10]. The main reason for the high HK control is that it is one of the less abundant glycolytic activities/contents in the cell (Table 20.1); the contribution of AMP and ADP competitive inhibition to the HK controlling role was lower because of the high ATP concentration present in amoebas. However, it is possible that these metabolites may have homeostatic control on the metabolic pathway. PGAM also exhibited significant flux control because it showed low activity/content in the cell (Table 20.1). These main controlling roles of HK and PGAM were further validated by in vitro pathway reconstitution of the two pathway sections and kinetic modeling [31]. This last study allowed identification of new interactions of metabolites different to substrates and products for several of the enzymes; for example, the inhibitory effect of PPi over ALDO, PGAM, and ENO, and its activation effect over HK.

Some limitations of the amoebal kinetic model [10] were that it did not explicitly include kinetics of GLUT nor those of the fermentative enzymes PFOR, ADHE, and AcCoAS because, at that time, few or no kinetic parameters were available for these enzymes. For GLUT, the values reported by Reeves [8, 9] were recalculated and used in the model, which however was unable to reproduce the metabolite concentrations and fluxes found in the cells [10]. This limitation was an important inconvenience because it has been demonstrated by kinetic modeling that GLUT has significant control of glycolysis of several biological models [32].

20.5.4 Flux Control Distribution of Amoebal Glucose Fermentation in Intact Cells

The C^{J}_{ai} of PFOR and ADHE have been recently determined in live amoebal trophozoites by inhibitor titration, another MCA strategy, using diphenyleniodonium and disulfiram as specific inhibitors, respectively. The analysis was performed in amoebas subjected to aerobic conditions because PFOR and ADHE are susceptible to inhibition by oxidative stress [25, 26, 56]. The results showed that PFOR has low control on the fluxes to EtOH and acetate ($C^{JEtOH/Jacetate}_{PFOR} < 0.09$), whereas ADHE emerged as the main controlling step of both fluxes ($C^{JEtOH}_{ADHE} = 0.33$ and $C^{Jacetate}_{ADHE} = -0.19$). The analysis also indicated negligible AcCoAS control of both fluxes [26].

Another MCA strategy called elasticity analysis [32] can be used to determine the flux control exerted by groups of enzymes/transporters of a given pathway in intact cells. This strategy involves determination of the sensitivity (elasticity coefficient) of a group of enzymes to change its rate when a pathway metabolite (M) changes its concentration; M can be any measurable pathway intermediary in the cell. To determine the elasticity coefficients, variations in the steady-state concentration of M are attained, for instance, by gradually increasing the pathway precursor supply and by inhibiting the last pathway reactions. In each experimental setting, the M concentration and pathway flux are in parallel determined in the cells to calculate the elasticity coefficients of the producer and consumer group of enzymes connected through M. From the elasticity coefficients, the C^{J}_{ai} is calculated through the connectivity theorem of MCA [32]. Following this strategy and moving along the intermediaries Glu6P, Fru6P, and Pyr, it was determined that the group constituted by GLUT, HK, and glycogen degradation controls approximately 0.86 of the glycolytic flux, whereas the group formed by PFOR, ADHE, and AcCoAs controls 0.18 (Erika Pineda and Emma Saavedra, submitted). These results are in agreement with those obtained by both kinetic modeling and inhibitor titration and highlight the relevance of the first segment of the glycolytic pathway, along with ADHE, in controlling the glucose catabolism in the parasite.

20.6 MCA and Drug Targeting

Metabolic control analysis can be used to identify and validate drug targets in metabolic pathways by identifying the most controlling steps as additional criteria to the essentiality determined by genetic methods [10, 32, 75, 79–81]. The plot shown in Fig. 20.2a helps us to understand why the majority of gene-silencing experiments identify as essential their targets. Decreasing by more than 80–90 % the activity/content of both controlling and noncontrolling steps in a metabolic or signaling pathway results in a significant decrease in the pathway flux or function. Therefore, a convenient drug target should be an enzyme or protein that needs to be just slightly

inhibited to significantly decrease the pathway flux (enzyme E1 in Fig. 20.2a); that is, appropriate drug targets should be the components that are essential and also which mainly control the pathway function.

Based on the marked kinetic/genetic differences, the PPi-dependent enzymes have long been proposed as targets for drug development to tackle the energy metabolism pathway of amoebas. The results described on the control distribution of *E. histolytica* fermentative glycolysis using different MCA strategies established that PPi-PFK and PPDK did not exert significant control on the pathway flux and metabolite concentrations. By using kinetic modeling, it was predicted the degree of enzyme inhibition required to decrease the amoebal glycolytic flux (Fig. 20.2b) [10] or the ATP concentration (data not shown). As expected from its low control (Table 20.1), PPi-PFK and PPDK have to be inhibited by 70 % and 92 %, respectively, to decrease the pathway flux by 50 %. In contrast, a similar pathway flux inhibition can be attained by inhibiting HK and PGAM by only 24 % and 55 %, respectively (Fig. 20.2b) [10]. Therefore, after determining the essentiality of an enzyme/protein by genetic methods, kinetic modeling and MCA can help to prioritize drug targets by identifying the main controlling steps in metabolic pathways of parasitic and pathological cells [10, 13, 32, 79–81]. In this regard, inhibitors against amoebal HK have been identified [82].

20.7 Conclusions

The divergent kinetic properties of the amoebal glycolytic fermentative enzymes compared to their human counterparts did not allow envisioning the controlling and regulatory mechanisms of this pathway in the parasite. By applying MCA-designed experiments and *in silico* metabolic modeling, the main controlling steps in amoebal glycolysis were identified. HK, glycogen metabolism, (and very probably glucose transport) are important controlling reactions of the first pathway section whereas PGAM and ADHE control the latest stages. By increasing knowledge of the kinetic parameters of the enzymes from the most important glycolytic branches together with metabolomic studies will advance our understanding of the dynamic of control and regulation of the intermediary metabolism in *Entamoeba histolytica*.

Acknowledgments Research in the authors' laboratory received financial support from CONACyT-México (grants No. 83084 and 178638 to E.S.).

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Chapter 21
Structural Biology of Cysteine Biosynthetic Pathway Enzymes

Isha Raj, Sudhir Kumar, Mohit Mazumder, and S. Gourinath

Abstract The cysteine biosynthetic pathway is of central importance for the growth, survival, and pathogenicity of the anaerobic protozoan parasite *Entamoeba histolytica*. This pathway is present across all species but is absent in mammals. Cysteine, the product of this pathway, is the only antioxidative thiol responsible for fighting oxidative stress in *E. histolytica*. Serine acetyl transferase (SAT) and *O*-acetyl serine sulfhydrylase (OASS) are the two enzymes catalyzing the de novo cysteine biosynthetic pathway. In all organisms in which so far this pathway is known to exist, both these enzymes associate to form a regulatory complex, but in *E. histolytica* this complex is not formed. The cysteine biosynthetic pathway has been optimized in this organism to adapt to and fulfill its cysteine requirements. Here we describe recent studies of the structure, function, and complex formation of cysteine biosynthetic enzymes in *E. histolytica*. The findings reveal subtle modifications that lend both cysteine biosynthetic enzymes their unique characteristics to escape inhibitory regulation; allowing *E. histolytica* to maintain high levels of cysteine at all times.

21.1 Cysteine Biosynthetic Pathway: An Overview

The de novo cysteine biosynthetic pathway is of primary importance in anaerobic microorganisms as it incorporates inorganic sulfur into an organic skeleton to produce cysteine. Cysteine serves important roles both as an antioxidative agent and as a source of sulfur for biomolecules such as thiamine, Fe–S clusters, biotin, Co-A, methionine, and various antioxidative thiols (glutathione, mycothiol, trypanothione) [1, 2]. In *Entamoeba histolytica*, the antioxidative role of cysteine is critical as it is the sole thiol responsible for maintaining the redox state in this catalase- and peroxidase-deficient parasitic protozoan [3, 4]. Cysteine deprivation has far-reaching effects in *E. histolytica*. Gene expression analysis has shown that it alters the

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_21

expression of the genes implicated in metabolism, signaling, oxidative defense, and DNA/RNA regulation and transport, resulting in growth defects in this organism [5].

E. histolytica is unique in lacking the forward trans-sulfuration pathway and an interrupted reverse trans-sulfuration pathway for cysteine biosynthesis: it depends entirely on the cysteine biosynthetic pathway for its cysteine requirements [6]. The de novo cysteine biosynthetic pathway is a two-step process (Fig. 21.1) that begins with the acetylation of serine by the enzyme serine acetyltransferase (SAT) to form the activated sulfide acceptor, *O*-acetyl serine (OAS). A β-replacement reaction, which inserts sulfide into OAS, is then catalyzed by the second enzyme of the pathway, *O*-acetyl serine sulfhydrylase (OASS), generating cysteine with the release of acetate. In general, in most known organisms and plants, the pathway is regulated both by feedback inhibition of SAT by end-product cysteine and by the interaction of SAT and OASS to form the cysteine synthase complex (CSC) under sulfur-sufficient conditions [7]. In the CSC, SAT activity is enhanced whereas OASS activity is downregulated. Accumulation of OAS under low sulfur conditions dissociates the CSC. In *E. histolytica*, however, OASS and SAT do not interact. Therefore, in this organism, there is no CSC formed to inhibit regulation [4, 8].

E. histolytica has three isoforms each of SAT and of OASS [9]. The three different isoforms of SAT are feedback inhibited to different extents by the end product cysteine [10], which helps to maintain high cysteine levels in *E. histolytica* at all times, highlighting the importance of cysteine in this organism. It is characteristic of eukaryotic cells to localize the different isoforms of the enzymes of a pathway in different subcellular compartments. In *E. histolytica*, however, the cysteine biosynthetic pathway exists exclusively in the cytosol [4]. Recent metabolome analysis

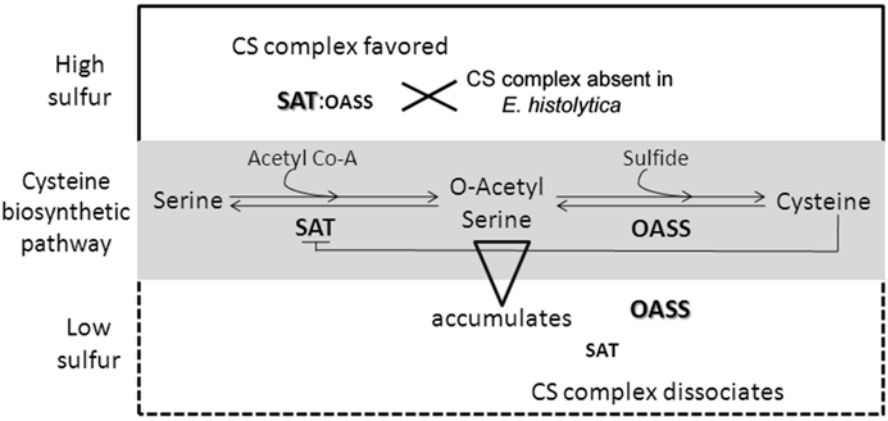

Fig. 21.1 Schematic representation of the cysteine biosynthetic pathway and its regulation. The *grey panel* outlines the general pathway. Feedback inhibition of serine acetyl transferase (SAT) by cysteine is shown. The cysteine synthase (CS) complex forms under high sulfur conditions and dissociates under low sulfur conditions. *Shaded* and *larger text* indicates active forms of SAT and *O*-acetyl serine sulfhydrylase (OASS). *Smaller text* indicates downregulated forms of the enzymes. The cysteine synthase complex is absent in *Entamoeba histolytica*

revealed the role of OASS in synthesizing *S*-methylcysteine (SMC) from OAS and methanol under cysteine-deprived conditions [6] and also suggested that the cysteine biosynthetic pathway could be primarily involved in SMC synthesis, thus suggesting alternate roles of the cysteine biosynthetic enzymes. Moreover, although the sulfur assimilatory cysteine biosynthetic pathway is present in bacteria, protozoa, and plants, it is absent in mammals in which cysteine is produced from methionine by a reverse trans-sulfuration pathway. This difference can be exploited by using the de novo cysteine biosynthetic pathway in pathogenic microorganisms as a drug target.

In this chapter, we examine the structural and functional aspects of both enzymes of the cysteine biosynthetic pathway from *E. histolytica* in detail, explain their characteristic behavior, and also discuss the reasons for the loss of CSC formation in this organism.

21.1.1 Serine Acetyltransferase from Entamoeba histolytica *(EhSAT)*

EhSAT exists in three isoforms: EhSAT1, EhSAT2, and EhSAT3. EhSAT2 and EhSAT3 are about 73 % and 48 % identical, respectively, with EhSAT1. EhSAT1 is a protein of 305 amino acid residues, EhSAT2 consists of 311 amino acid residues, and EhSAT3 is 336 residues long. EhSAT1 was characterized by Nozaki and coworkers [4], who showed that the end product cysteine is a competitive inhibitor of serine, which is one of the substrates of this enzyme, but has no effect on the binding of acetyl CoA, which is another substrate. These findings are in contrast to other SATs from *Escherichia coli* and *Haemophilus influenzae*, where cysteine inhibits both serine and acetyl CoA binding [11, 12]. Of the three isoforms, EhSAT1 has been reported to be feedback inhibited by cysteine almost completely although the other two isoforms are relatively insensitive to this inhibition [10]. Although all SAT isoforms in *E. histolytica* are localized in the cytoplasm, they all show differential regulation [10]. All SATs that form a CSC have an Ile at the C-terminal end. Among EhSATs, only EhSAT1 has the Ile residue at its C-terminal end. However, a GST pull-down assay and yeast two-hybrid experiments showed that EhSAT and EhOASS do not interact, and hence cysteine synthase complex (CSC) formation is absent in *Entamoeba histolytica* [4].

21.1.1.1 Structure of EhSAT1

The crystal structure determination of EhSAT1 revealed its unique trimeric arrangement and loss of inhibition kinetics to acetyl CoA by Cys [8]. Each protomer of EhSAT1 has two distinct domains: a globular N-terminal domain containing 8 α-helices and a C-terminal domain containing 14 small β-sheets arranged as a left-handed parallel helix (LβH) (Fig. 21.2a), which is a signature fold of all acetyltransferases [8, 13]. The active site of EhSAT1, which is located between the two adjacent

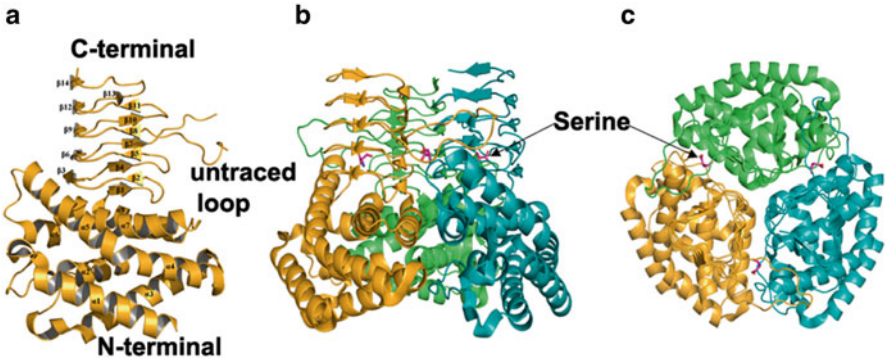

Fig. 21.2 Crystal structure of EhSAT1. **a** Monomer shows the N-terminal α-helix-rich domain and the C-terminal β-sheet LβH domain. **b** Trimer of EhSAT shows serine bound at active site. **c** "*Top*" view of the EhSAT1 trimer shows three serine-binding sites

monomers (Fig. 21.2b, c), consists of three histidine residues (His208A, His223A, His180B), one arginine residue (Arg222A), and two aspartate residues (Asp114B, Asp179B). Cysteine binds at exactly the same site at which serine is bound and engages the same residues, albeit with reoriented side chains of His 223A and Arg222A, supporting the observation that cysteine is a competitive inhibitor of serine. SATs follow the random order ternary complex mechanism in which the breakdown of the enzyme–CoA and the enzyme–serine complexes is partially rate determining for the forward and reverse directions, respectively [14].

21.1.1.2 EhSAT1 Is a Trimer

Structural and biochemical studies have shown that EhSAT1 self associates to form a trimer [8]. Hexamers are also formed in some SATs, stabilized by hydrophobic interactions between N-terminal domains of two trimers [15]. The sequence of the N-terminal domain of EhSAT1, however, differs from that in *E. coli* SAT (EcSAT) and HiSAT (Fig. 21.3). A kink at Pro 94 in EhSAT1 causes a diversion of about 55° between helix 4 and 5, leading to the reorganization of the N-terminal domain and loss of the hexameric arrangement (Fig. 21.3b). Upon analysis of the amino acid distribution of the surface of the N-terminal domain, it was observed that in EhSAT1 the charged residues are more evenly distributed than the hydrophobic ones as compared to EcSAT where the clustering of hydrophobic patches results in the hexamerization of EcSAT (Fig. 21.4). This observation is relevant to the lack of formation of the decameric synthase complex in *E. histolytica* [4]. About 40 amino acid residues at the C-terminal end lacked traceable electron density in all three reported structures of EhSAT1, and these residues thus appear to be forming a flexible random coil structure. This region had been implicated in the formation of the complex between SAT and OASS in other organisms. Cysteine binding stabilizes the C-terminal end and the extended loop and blocks the acetyl CoA-binding site in *E. coli* SAT and HiSAT [16]. In EhSAT1–Cys complex structures, this region remains disordered,

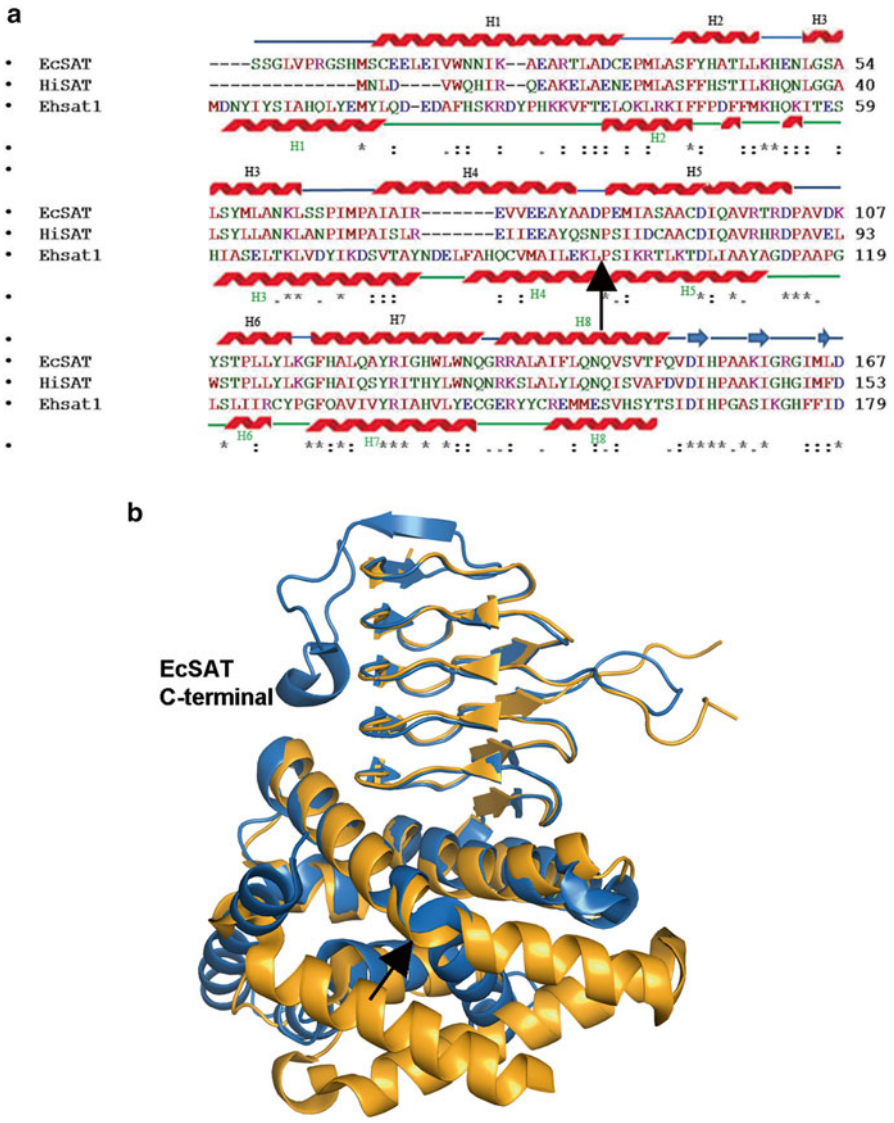

Fig. 21.3 Comparison of EhSAT1 with EcSAT. **a** Sequence alignment of EhSAT with EcSAT and HiSAT. N-terminal part of the sequence is not conserved and has insertions. Position 94 is marked by an *arrow*. **b** Superposition of EcSAT (*blue*) with EhSAT1 (*orange*) shows reorientation of the N-terminal domain. A *black arrow* identifies the position at which the major diversion starts in the EhSAT1 structure and leads to the reorganization of the N-terminal domain as compared to the *Escherichia coli* SAT

supporting the fact that acetyl CoA binding is not inhibited by cysteine binding. Deletion mutants of EhSAT1 in which 36 and 40 residues were deleted from the C-terminal end showed that this region is nevertheless important for the stability and solubility of the protein [8].

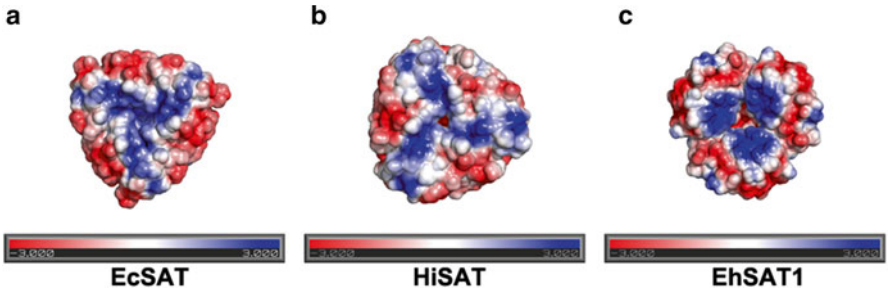

Fig. 21.4 EhSAT1 is a trimer. Image shows overall charge distribution over the N-terminal surface of EcSAT (**a**), HiSAT (**b**), and EhSAT1 (**c**). The N-terminal surface of the EcSAT shows a clear streak of the hydrophobic charges responsible for the hexamerization of the molecule. Similar distribution is also visible in the HiSAT N-terminal structure. Both these structures are hexameric in nature, as proved by crystal structure and biophysical studies. EhSAT1 on the other hand has a trimeric structure, and the N-terminal hexamerization domain has major charged residues that cover the surface and hence avoid hexamer formation

21.1.1.3 Differential Regulation of EhSAT Isoforms

The three SAT isoforms of *E. histolytica* are inhibited to different extents by cysteine. EhSAT1 is fully inhibited at micromolar concentrations of cysteine although EhSAT3 is not inhibited by even millimolar concentrations [10]. The inhibition of EhSAT2 by cysteine lies midway between the two other EhSATS; the K_i value of EhSAT2 by cysteine was found to be about sixfold higher than that of EhSAT1 [10]. While comparing the sequences of EhSAT1 and EhSAT3, it was revealed that all the residues of the active site in EhSAT1 are the same as in EhSAT3 except for His208, which is replaced by a serine residue in EhSAT3. A three-dimensional model of EhSAT3 and molecular dynamics simulations suggest that His208 could be crucial in distinguishing serine and cysteine binding. The Ser208His-EhSAT3 and His208Ser-EhSAT1 mutants were prepared by site-directed mutagenesis, and the feedback inhibition of all the constructs was determined spectrophotometrically. Experimental results suggested that His208 is indeed one of the residues involved in the selective binding of serine and cysteine, but the effect is not absolute. Ser208His-EhSAT3 regained inhibition by about 36 % (Fig. 21.5), whereas the results with the EhSAT1 mutant showed an increase in IC_{50} from 9.59 μM (for native) to 169.88 μM [17].

The reaction kinetics is not only dependent on the final binding energies of substrate and inhibitor at the active site, but also on the transfer rate of substrate and inhibitor to the active site. His 208 is located in the long loop between coil 2 and coil 3 of the left-handed β-helix domain, where there are significant differences in the sequence of this long loop in EhSAT1 compared to EhSAT3. Double mutation of M201V along with E166G in *E. coli* SAT renders the enzyme insensitive to cysteine inhibition [18] In EhSAT3, the position equivalent to Met201 is already occupied by valine, which may account for its lack of sensitivity for cysteine inhibition.

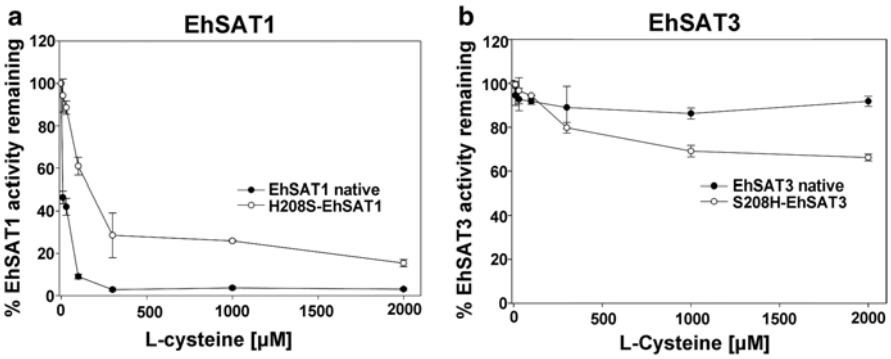

Fig. 21.5 Mutation of Ser208 to His results in partial recovery of the cysteine feedback inhibition in EhSAT3. **a** The effect of cysteine on native EhSAT1 and His208Ser-EhSAT1. **b** The effect on native EhSAT3 and Ser208His-EhSAT3. EhSAT1 is inhibited by the micromolar concentration of the cysteine; EhSAT3 is resistant to inhibition even at millimolar cysteine concentrations. The mutation of His 208 has altered the cysteine inhibition efficiency of both the isoforms. The His208Ser-EhSAT1 mutant showed the increased resistance to cysteine inhibition and an increase in the IC50; the Ser208His-EhSAT3 mutant gained partial inhibition signifying the important of the said residue

There are several studies indicating the importance of the C-terminal residues in Cys feedback inhibition [19, 20]. The differences between SAT1 and SAT3 around the active site involve the loop connecting β-coil 2 and β-coil 3 and C-terminal end, which is close to the acetyl Co-A-binding site. These regions may not be playing a direct role in the binding of the substrate or inhibitor, but appears to be affecting the path of the substrate or inhibitor before reaching the active site. The presence of SAT isoforms with different inhibition properties could allow the *E. histolytica* parasite to survive during infection.

21.1.2 O-*Acetyl Serine Sulfhydrylase from* Entamoeba histolytica *(EhOASS)*

There are three OASS isotypes present in *E. histolytica* (referred as EhOASS herein), of which EhOASS1 and EhOASS2 are identical except for two conservative amino acid changes [9]. EhOASS3 has 83 % sequence identity with EhOASS1 and EhOASS2 [21]. Both EhOASS1 and EhOASS2 are 337 amino acid residues long whereas EhOASS3 contains 336 amino acids. In prokaryotes, two differentially expressed isozymes of OASS exist, namely, OASS-A and OASS-B [22]. Isozyme A can interact with SAT and is more substrate specific, whereas isozyme B does not interact with SAT and is able to accept larger sulfide donors [22, 23]. Based on sequence similarity and substrate specificity, all EhOASSs resemble the prokaryotic OASS A isozyme.

21.1.2.1 Structure of EhOASS1

Of the three isotypes of OASS found in *E. histolytica*, only the structure of EhOASS1 has been reported [24]. EhOASS1 exists as a dimer, and each monomer of 37 kDa has two domains: a relatively small and flexible N-terminal domain and a relatively large and rigid C-terminal domain (Fig. 21.6a). The cofactor PLP is in an internal aldimine linkage with Lys 58 and is positioned between the two domains. The N-terminal domain (residues 57–164) consists of four parallel β-sheets surrounded by four α-helices, and the C-terminal domain (residues 1–56 and 165–336) consists of six mixed β-sheets surrounded by four α-helices. This arrangement is typical of fold type II of PLP-dependent enzymes.

In the dimeric form, each monomer of EhOASS1 is arranged in a head-to-tail manner with respect to the other; that is, the N-terminal domain of one of the monomers is packed close to the C-terminal domain of the other. This arrangement causes the entrance to the active site of each monomer to face the same side (Fig. 21.6b). EhOASS1 has an extended N-terminal region consisting of about ten extra residues compared to OASS from other sources; these residues participate in domain-swapping dimeric interactions and contribute to making the EhOASS1 dimer interface one of the largest buried dimeric interfaces for any OASS (5,905 $Å^2$; 19.1 % of total surface area buried). Ile4 of one monomer makes a "knob-into hole"-like interaction in the hydrophobic pocket formed by Leu18, Phe63, Tyr67, and Phe100 of the other monomer. Similarly, Ile6 of one molecule interacts with Ile21, Phe63, and Tyr171 of the other molecule in the dimer. The N-terminal region also makes various hydrophilic interactions, such as hydrogen bonds between the carbonyl groups of

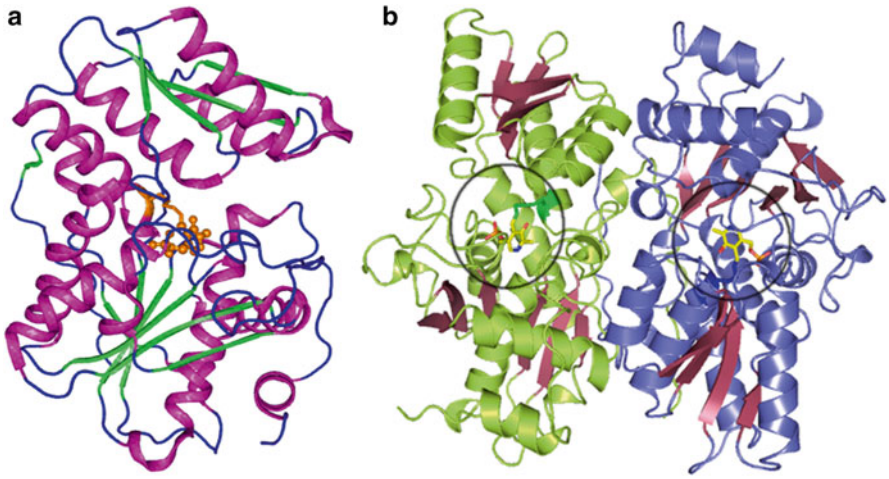

Fig. 21.6 Structure of EhOASS1. **a** The monomer has a large C-terminal domain and a small N-terminal domain, separated by the active site cleft. Cofactor PLP is seen at the active site cleft. **b** The dimer, which shows the active site clefts on the same side (i.e., toward the reader)

Ile6 and Ser5 from one molecule and Arg120 of the other molecule, that also help stabilize the EhOASS dimer. EhOASS also has an extended C-terminal end helix that is oriented differently than in other OASS structures; it occupies the small hydrophobic pocket formed between α7, α8, and N-terminal loop. This groove is free in other OASS structures.

21.1.2.2 Substrate Binding and Active Site Cleft Closure

The biochemical reaction at the active site proceeds through the formation of a number of aldimine intermediates with the cofactor PLP at the active site. The position of PLP results in the formation of an active site cleft lined on one side by the residues of the N-terminal domain and on the other by C-terminal domain residues. Insights into the structural changes occurring on the binding of the substrate came from methionine (substrate analogue)-bound and cysteine (product)-bound EhOASS1 structures [24, 25]. Both structures show the ligand bound at the active site directly over PLP, and even though there is a difference in the external aldimine linkage formation in the two cases (cysteine is seen in an aldimine linkage with PLP but methionine is not), the active site is still closed upon binding of both these ligands [26].

The binding of ligand is accompanied by a conformational change in the enzyme where the N-terminal domain moves by about 7 Å to close the catalytic cleft (Fig. 21.7a, b), rendering the active site inaccessible to larger molecules but allowing the passage of small molecules, for example, acetate (the first by-product) and sulfide (the second substrate). The twist in the β-sheet 3 of the N-terminal domain accommodates this movement, and to complement this movement β-sheets 4 and 5 also undergo twisting, which causes the movement of helix 4. Overall the twist in the β-sheets 3, 4, and 5, as well as the shift in α-helix 4, causes the closure of the active site. The closure of the active site also helps in positioning the reactants close to each other to form the product. In the closed conformation, N-terminal domain residues of the conserved Asn loop (85TSGNT89) form a network of hydrogen bonds with the substrate. From the C-terminal domain, Gly192 and the Gly loop (236GIGA239) are the major contributors. Molecular dynamics studies based on ligand-bound structures performed by our group reveal that Asn88 on the N-terminal domain, PLP attached to Lys58 at the catalytic center, and Gly192 from the C-terminal domain are the hotspot residues that form a triad of energy contributors to effect the closure of the active site (Fig. 21.7c) [26]. The carboxylate moiety of the incoming ligand acts as an anchor and interacts with the residues of the Asn loop to stabilize itself at the active site by additional hydrogen bonding with the residues of the Asn loop and Gly loop. This phase is followed by the reorientation of the α-amino group of the ligand toward the internal aldimine linkage of Lys58 with PLP. The correct orientation is critical for the attack and subsequent displacement of the ε-amino group of Lys58 from the internal Schiff linkage and the formation of an external aldimine linkage with the ligand. In fact, this interaction could be the decisive factor providing the trigger for the closure of the active site.

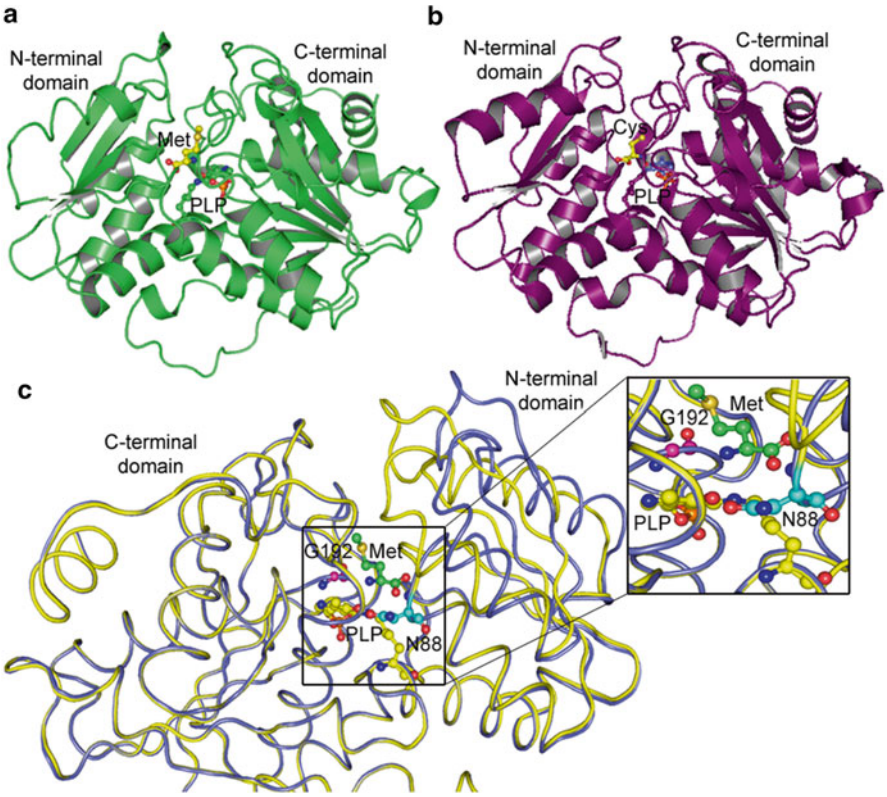

Fig. 21.7 Ligand bound to EhOASS1. **a** EhOASS1 with methionine bound at the active site. **b** EhOASS with cysteine bound at the active site. **c** The hotspot residues are represented in the *ball and stick* model and labeled. The methionine-bound structure is represented by a *yellow ribbon* and the EhOASS1 native structure is represented by a *blue ribbon*. Methionine is shown at the active site as a *green ball and stick* model. *Inset* shows close-up view of the active site with the hotspot residues and ligand

21.1.2.3 Inhibition

Because SAT is already known to interact with and inhibit OASS, it provides a good model to study the mechanism of inhibition for OASS. The conserved C-terminal Ile was found to be important for the SAT–OASS interaction [27]. Multiple studies in various organisms with designed inhibitory peptides have all contained an Ile at their C-terminal end. The structure of EhOASS1 in complex with isoleucine is available to shed light on its inhibitory mode. It is bound at exactly the same site as methionine and cysteine in the active site cleft (Fig. 21.8a), but in contrast does not show a closure of the catalytic cleft. Isoleucine seems to lock the enzyme in an open conformation. As is methionine, isoleucine also is anchored at the active site through

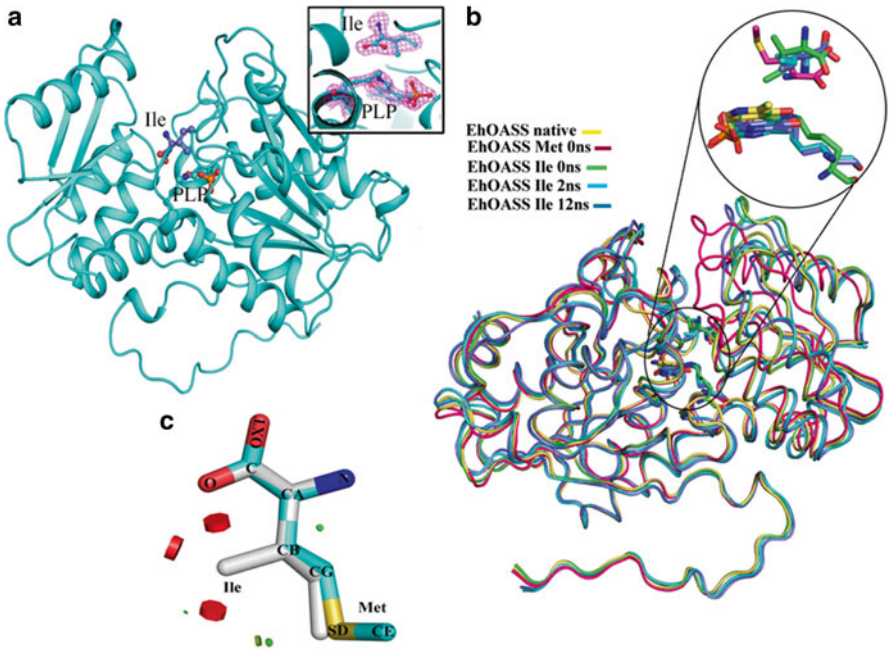

Fig. 21.8 Isoleucine interaction at EhOASS1 active site. **a** EhOASS1 with isoleucine bound at the active site. *Inset* shows the difference Fourier electron density map (2Fo-Fc) at 1.5σ cutoff for isoleucine and 2σ cutoff for cofactor PLP and catalytic lysine. **b** Comparison of structural snapshots at various time intervals in the MD trajectories of EhOASS1-isoleucine complex. *Inset* shows variations in ligand orientation at equivalent time points. **c** Bad contacts would be generated between the terminal oxygen of the carboxyl group and the γ-methyl group of the β-branched isoleucine if it were to adopt a similar conformation as methionine at the EhOASS1 active site

interactions with the Asn loop; however, the α-amino group faces the opposite direction to that of the internal Schiff bond of PLP (distance between the isoleucine N and Schiff base N, ~6.2 Å) with catalytic Lys58. Hence, the α-amino group is not in a favorable conformation to interact with PLP. Without the trigger to close the active site; the catalytic cleft is locked in an open conformation and thus isoleucine exerts its competitive inhibitory effect. Our molecular dynamics studies reveal that among various reorientations that isoleucine undergoes after binding to the active site, those in which the α-amino group comes close to PLP (~3.5 Å) are accompanied by the movement of the N-terminal domain by about 4 Å (Fig. 21.8b), but further movement to close the active site is disabled as the β-branched isoleucine cannot achieve a suitable conformation to interact with PLP because of a geometric conflict arising between the terminal oxygen of the isoleucine carboxyl group and its γ-methyl group (Fig. 21.8c). Thus, the N-terminal domain relaxes back to the open conformation.

21.1.2.4 Wide Active Site Cleft of EhOASS1 and Small C-Terminal Tail Residues of EhSAT1 Lead to Loss of CSC Formation in *E. histolytica*

Crystal structures of OASS in complex with the C-terminal peptide of SAT, from organisms where SAT and OASS interact to form the CSC, reveal that the peptide occupies the active site cleft of OASS. It is the terminal Ile that anchors the peptide in the active site cleft while the rest of the residues form hydrogen-bonding interactions with the residues of the enzyme which line the active site cleft, thus blocking and competitively inhibiting OASS. Of the three SAT isoforms present in *E. histolytica*, only EhSAT1 has an Ile at its C-terminal end. A comparison of SAT C-terminal ends from various organisms to EhSAT1 showed that although all interacting SATs have bulky residues at their C-terminal end, EhSAT1 has relatively small residues, with the terminal tetrapeptide sequence being SPSI (Fig. 21.9a). In the structure of EhOASS1 with this peptide, interpretable electron density could be seen only until the third amino acid from the C-terminal end (Fig. 21.9b). The terminal Ile makes extensive H-bonding interactions with the Asn loop and the remaining two resolved amino acid residues contribute only three extra H-bonding interactions, whereas in all other cases, the residues following Ile make substantial interactions to bind to the active site. The small size of the EhSAT1 C-terminal residues, coupled with the fact that Pro at the third position from the C-terminal end might not allow the peptide to achieve the proper conformation for stronger binding, could be the reasons behind the lack of interaction of OASS with SAT in *E. histolytica*. A comparison of the size of the active site cleft opening from various organisms showed that EhOASS1 has the widest opening, with a length of 13.3 Å, whereas all others showed an opening about 11–12.5 Å wide (Fig. 21.9c). This difference might be another contributing factor for loss of CSC formation in *E. histolytica* [25].

21.1.2.5 EhOASS1 Can Interact and Form a CSC with SATs Having Bulkier Peptides

The possibility of CSC formation in *E. histolytica* has been investigated using bioinformatics by screening a SAT C-terminal-mimicking tetrapeptide library against the EhOASS1 structure. Docking and in silico studies followed by biochemical experiments on EhOASS1 with various SAT C-terminal mimicking peptides resulted in identification of three peptides (DWSI, DFSI, DYSI) with binding affinities in the micromolar (μM) range [8]. DXSI, where X is a large hydrophobic residue, seemed to be the best possible C-terminal sequence for EhSAT to interact with EhOASS. When the EhSAT1 C-terminal end was mutated from SPSI to one of these strong binding peptide sequences (DWSI), it was found that the modified EhSAT1 (DWSI-EhSAT1) could interact with and inhibit EhOASS1 [8]. Biomolecular interaction analysis using surface plasmon resonance between EhOASS1 and DWSI-EhSAT1 confirmed a 1:1 interaction between the two, with a K_d of about 4 μM, confirming that EhOASS1 is capable of forming the CSC, albeit with a SAT that has a bulkier peptide at its C-terminus.

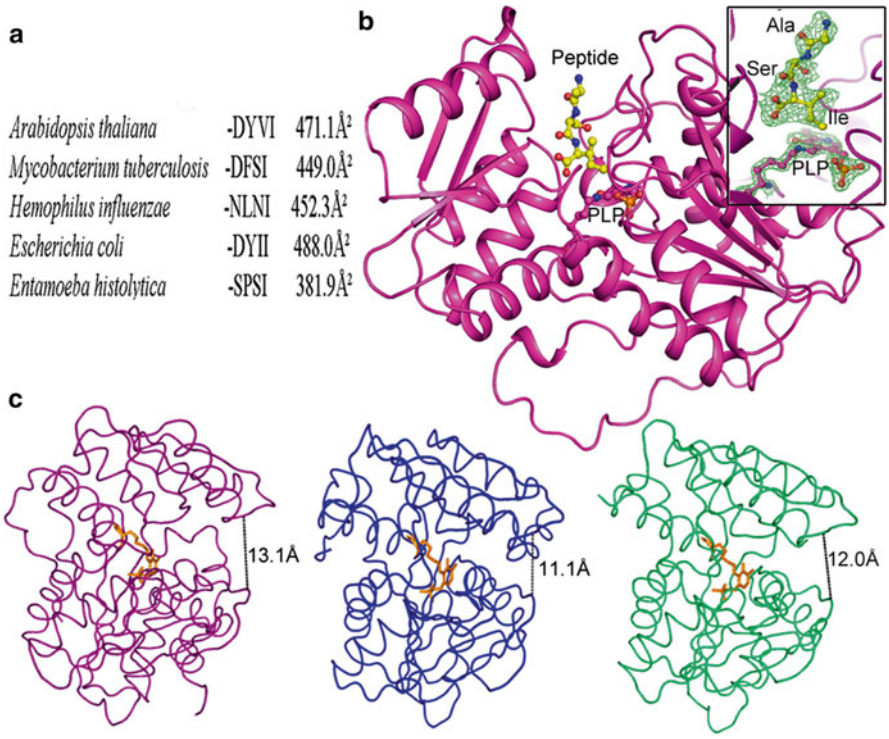

Fig. 21.9 Comparison of SAT C-terminal peptides and OASS active site cleft dimensions from various sources. **a** SAT C-terminal tetrapeptide sequences from various sources and their corresponding surface area. **b** SPSI bound to EhOASS1 at the active site. *Inset* shows the difference Fourier electron density map (2Fo-Fc) at 1.5σ cutoff for the peptide, and 2σ cutoff for cofactor PLP and catalytic lysine. **c** Widths of the openings of the active site clefts of OASS from *E. histolytica* (*magenta*), *Leishmania donovani* (*blue*), and *Mycobacterium tuberculosis* (*green*) are shown

21.1.3 Model of the Cysteine Synthase Complex in E. histolytica

Enzymes that act sequentially in a metabolic pathway frequently form macromolecular complexes to regulate the activity of the interacting enzymes and respond to changes in metabolic fluxes. One of the most prominent examples of this type of regulation is the cysteine synthase complex (CSC) [28], which is formed by the association of the two cysteine biosynthetic enzymes SAT and OASS. The reversible association and dissociation of SAT and OASS forming the CSC is dependent on the cellular level of sulfur. At optimum sulfur concentrations SAT and OASS form the CSC complex, in which SAT activity is upregulated while OASS activity is inhibited [7, 29]. When the sulfur levels are low, OAS accumulates and leads to dissociation of the CSC. This mechanism coordinates sulfate assimilation and cysteine production in plants and bacteria (Fig. 21.1) [30].

OASS is known to exist as a dimer across all species of bacteria, plants, and protozoa [27, 28, 31], but the oligomeric state of SAT has been reported to be trimeric [8, 32] or hexameric [15, 16, 33]. Hexameric SAT is in fact formed as a dimer of trimers. The interaction between OASS and SAT in plants and bacteria has been analyzed by multiple approaches, including size-exclusion chromatography, yeast two-hybrid analysis, surface plasmon resonance (SPR), and fluorescence spectroscopy [34–41]. Earlier efforts to identify and characterize the protein–protein interaction regions in CSC highlighted the role of the SAT C-terminus in the association process with OASS [34–37, 41]. Later, determination of the structure of OASS from various sources with the C-terminal peptides of their cognate SATs showed that the C-terminal peptide of SAT with the conserved Ile at its end is important for CSC formation and that this interaction occurs at the OASS active site. This finding also explains the inhibition of OASS when it forms the CSC complex [27, 41].

Information from the foregoing studies raises the question about the stoichiometry of the interacting partners in the CSC, as each SAT protomer is capable of interacting with each OASS monomer. So far, ultracentrifugation and size-exclusion chromatography results have indicated the SAT-OASS stoichiometry to be one OASS dimer per SAT trimer [36, 40]. Recently, simulations of complex formation suggested a model for the structure of CSC in a 1:2 interaction with a SAT hexamer and OASS dimer [42]. However, results from a recent SPR experiment on *Glycine max* enzymes has questioned this view again and proposed that the three C-terminal tails of each SAT trimer can interact with the active site of three OASS dimers and form a multimeric complex [32]. In the absence of a crystal structure of the CSC, the mode of complex formation and architecture of CSC remain unknown.

Entamoeba histolytica seems to be unique in this aspect as the cysteine biosynthetic pathway in this organism does not involve the formation of CSC at any stage [4, 8]. This adaptation could be in place so as to maintain a ready supply of the important thiol, cysteine, under all conditions in this glutathione-deficient, amitochondriate pathogenic protist. However, a modification of two amino acid residues at the C-terminus of EhSAT1 was shown to reestablish protein–protein interaction (see Sect. 21.1.2.5). In an attempt to understand complex formation, a hypothetical model of the CSC was constructed employing docking techniques using EhOASS1 and DWSI-EhSAT1 structures. The C-termini of SAT were modeled de novo because they are missing in the template structures but are crucial for CS formation. This construct was energy minimized using molecular dynamics simulations; the resultant model is shown in Fig. 21.10a. The modeling of the full-length DWSI-EhSAT1trimer and EhOASS1 in the complex revealed that the C-terminal tail of the molecule depicted by protomer A in the SAT trimer interacts with one of the OASS molecules in the dimer at the active site, whereas the other two SAT molecules, protomer B and protomer C, interact with the same OASS molecule at different symmetrically located sites. An analysis of the charge distribution on the interacting surfaces of EhOASS1 and DWSI-EhSAT1 (Fig. 21.10b) showed charge complementarities favorable for this interaction. The active site of EhOASS1 is positively charged whereas the C-terminal tails of the DWSI-EhSAT1 trimer are negatively charged. Therefore, the negatively charged C-terminal end of a protomer

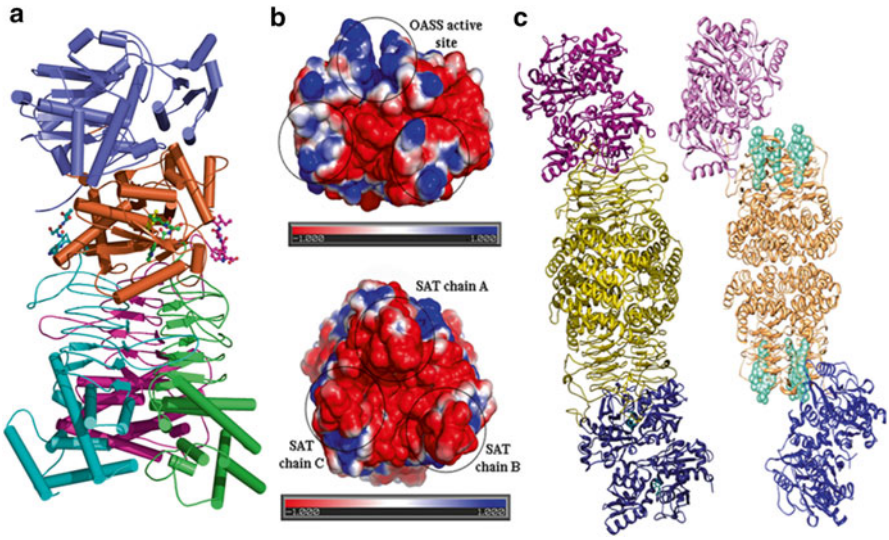

Fig. 21.10 The cysteine synthase complex (CSC) model from *E. histolytica.* **a** Protomer A of the SAT trimer is depicted in *green*; protomers B and C are colored *cyan* and *magenta*, respectively. The C-terminal of only protomer A interacts with the PLP (*yellow balls* and *sticks*) at the active site of one of the molecules in the OASS dimer. The C-terminal DWSI of each SAT protomer is represented by *balls* and *sticks.* **b** Charge distribution on interacting surfaces of EhOASS1 and DWSI-EhSAT1 in the cysteine synthase complex. The negatively charged residues at the SAT C-terminus are complementary to the positively charged OASS active site. **c** Comparison of the CSC model from *E. histolytica* (*left*) with mechanistic model proposed by Feldman-Salit et al. (*right*). The CSC model from *E. histolytica* shows nearly symmetrical head-to-head interactions of each SAT trimer with an OASS dimer. In the Feldman–Salit model, only one of the molecules of the SAT trimer interacts with an OASS dimer

of DWSI-EhSAT1 can be comfortably accommodated at the active site; there are two more patches of positive charges on EhOASS1, and they correspond to contact sites with the negative C-terminal ends from the other two protomers of DWSI-EhSAT1. This arrangement of EhSAT1 and EhOASS1 is in agreement with our SPR results and reiterates the consensus view that one OASS dimer is capable of interacting with only one SAT trimer [28, 40]. This model also explains the observations of partial catalytic activity of OASS in the CSC of bacteria and plants [7, 28, 32, 37], indicating that one of the active sites of OASS remains free whereas the other is blocked by the SAT C-terminal tail. Our model also supports the recent observation of a real-time fluorescence experiment that reported a three-stage assembly of the CSC in which the first step was fast and optically active whereas the later two were slow and optically silent [43]. The first step would correspond to the C-terminal end of the first protomer of SAT interacting with an OASS active site, for which PLP gives the optical signal, and the next two events would correspond to interactions with other positively charged patches on the OASS surface by the C-terminal ends of the other two SAT protomers.

Because hexameric SAT is a dimer of trimers, the foregoing EhSAT1-EhOASS1 model was used to generate a CSC complex through superposition of a hexameric *E. coli* SAT structure [15], resulting in a CSC model similar to the simulated *Arabidopsis thaliana* model [42] (Fig. 21.10c). However, the point of departure from the mechanistic model generated by Feldman-Salit et al. [42] is that in their model the OASS dimer interacts at an angle, in an asymmetrical manner to the SAT trimer, with only one SAT-C terminal end interacting with an OASS active site, whereas our model suggests a symmetrical interaction. Our results show the involvement of all three molecules in the SAT trimer to form the CSC complex, hence making the interaction of more than one OASS dimer with the SAT hexamer geometrically impossible.

21.2 Concluding Remarks

These findings highlight the detailed structural and functional aspects of enzymes involved in the de novo cysteine biosynthetic pathway. EhSAT1 exists as a trimer both in solution and in crystals. The differences in the N-terminal domain and charge distribution over its surface area may be responsible for the absence of hexamer formation as seen in EcSAT and HiSAT. Structural and functional studies of SAT isoforms have revealed that *Entamoeba* has adopted survival strategies that bypass the regulation mechanism of cysteine biosynthesis. The differential regulation of EhSATs could be traced to a single residue (His208), and it was shown that a single site mutation at this position in the EhSAT3 active site resulted in partial recovery of feedback inhibition by cysteine. Thus, a basic level of the cysteine biosynthetic pathway is always operational in *E. histolytica* because of the presence of different isoforms of SATs that are inhibited by cysteine to different extents.

EhOASS1-binding studies with SAT C-terminal-mimicking peptides lead to the conclusion that there are two important reasons for the loss of interactions between these proteins: the EhOASS1 active site cleft is more open than in other OASS structures and the EhSAT1 C-terminal residues are too small for adequate interactions at the EhOASS1 active site. When the EhSAT1 C-terminal residues are mutated by two residues, from SPSI to DWSI, both proteins interact to form a CSC complex. Thus, *E. histolytica* has employed slight modifications in its SAT C-terminal tail and OASS1 active site to escape regulation of the cysteine biosynthetic pathway to maintain the optimum cysteine concentration.

Recent information garnered from metabolome analysis under cysteine deprivation indicates that cysteine biosynthetic enzymes could be involved in formation of SMC [6]. Further work is required to shed light on this aspect and investigate the roles of SAT and OASS with respect to their altered functions.

As seen in many crystal structures, the activity of OASS involves the movement of the N-terminal domain upon substrate binding. However, the SAT C-terminal peptide acts as inhibitor, locking the enzyme in an open conformation. Based on structural and molecular dynamics studies on EhOASS1, a detailed mechanism for

how the ligand causes active site cleft closure can be visualized. Studies into CS complex formation with EhOASS1 and modified EhSAT1 offer insights into a novel mode of interaction where all the three SAT molecules are involved in the formation of the complex.

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Chapter 22
Archetypical and Specialized DNA Replication Proteins in *Entamoeba histolytica*

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Abstract Accurate DNA replication and repair are essential tasks for survival. In eukaryotes, DNA polymerases replicate genomes that can be composed of billions of base pairs. These genomes can be chemically damaged or modified, jeopardizing its integrity, and cells have evolved mechanisms to ameliorate the mutagenic effect of DNA damage. DNA replication and DNA lesion bypass in bacteria, yeast, and humans have been widely studied; however, little is known about these processes in other organisms. *Entamoeba histolytica* is a parasitic protozoan responsible for amebic dysentery and hepatic abscess. Herein, we define the DNA replication apparatus of *Entamoeba histolytica* and review the biochemical peculiarities of family A and family B2 DNA polymerases involved in DNA lesion bypass. Our data indicate that *E. histolytica* is a mosaic of archetypical family B DNA polymerases (α, ε, and δ) present at the replication fork and specialized DNA polymerases with novel lesion bypass properties.

22.1 Archetypical and Specialized DNA Polymerases

The number of DNA polymerases in eukaryotes is highly variable: for instance, *Homo sapiens* contains 15, *Arabidopsis thaliana* 12, and *Saccharomyces cerevisiae* 8 [1]. Eukaryotic DNA polymerases are grouped into A, B, X, and Y families

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_22

according to their evolutionary origin [2, 3]. Family A DNA polymerases are ubiquitous and are present in phages, bacteria, and eukaryotes. The first DNA polymerase characterized by Kornberg and coworkers was a family A DNA polymerase from *Escherichia coli* [4]. These proteins are modular proteins with three separated enzymatic activities: 5′–3′ polymerization, and 3′–5′ and 5′–3′ exonuclease. Crystal structures of family A DNA polymerases revealed their structural organization and division of their polymerization domain into three subdomains dubbed thumb, palm, and fingers [5, 6]. Family B of DNA polymerases includes DNA polymerases from phages such as φ29 and RB69, and all the eukaryotic nuclear replicating DNA polymerases α, δ, and ε. Family B polymerases are modular enzymes with an associated 3′–5′ exonuclease domain, and their polymerization domain also resembles a cupped right hand [7]. The family X is represented by DNA polymerases β, λ, and μ in humans. Family X contain a 5′–3′ polymerase activity and dRP lyase activity but not editing activities. They have a role in DNA repair, during base excision repair and nonhomologous end-joining, but not in replication [8]. Because the genetic code only consists of four bases, the large number of DNA polymerases in eukaryotes is counterintuitive. However, these bases can be chemically modified in more than a dozen variants; from these modifications, the most common are the oxidation of pyrimidines, thymidine photoproducts, abasic sites, and bulky substituent groups. Family Y DNA polymerases include DNA polymerase ι, κ, ν, and Rev1 in humans. These polymerase are low-fidelity enzymes but are specialized in bypassing DNA lesions such as thymidine dimers and benzopyrenes. The last family of DNA polymerases, DNA family Y, is associated with the classification of "translesion synthesis DNA polymerases" that depicts a broader classification which includes all DNA polymerases involved in bypassing DNA lesions independently of their family classification. For instance, DNA polymerases θ and ν belong to family A and are capable of lesion bypass opposite abasic sites and thymine glycol, respectively.

22.2 The Replisome of *Entamoeba histolytica* Is Archetypical

The genome of *Entamoeba histolytica* is AT rich (approximately 76 %), containing at least 14 linear chromosomes and a large number of circular plasmid-like molecules involved in ribosomal synthesis [9]. Some strains of *E. histolytica*, such as HK-9, contain one ribosomal transcriptional unit whereas other strains, such as HM1-IMSS, contain two copies of the ribosomal unit ordered as a palindrome. In strain HM1-IMSS is estimated that 200 copies of the circular DNA dubbed EhR1 are present for haploid genome. Little is known about the mechanism of DNA replication of these circular molecules; however, it is known that replication starts at multiple replication origins [10–12]. The chromosomes of *E. histolytica* do not condense, and the extensive length variability observed between

homologous chromosomes from different isolates makes the exact chromosome number difficult to determine. The genome of 20.8 Mb is twice the size of genomes from other organisms such as the fission yeast [13]. The genome of *E. histolytica* is expected to be replicated by DNA polymerases α, δ, and ε, as suggested by its inhibition by aphidicolin [14] (Table 22.1).

22.2.1 DNA Polymerase-α

DNA polymerase-α is known as the replicative primase because it can initiate DNA synthesis by a self-synthesized RNA primer and use this primer for DNA synthesis. This RNA/DNA hybrid is used for replicative DNA polymerases in lagging and leading strands synthesis. In humans, DNA polymerase-α consists of four subunits of 180, 86, 58, and 48 kDa. The p180 subunit is a family B DNA polymerase and the p48 subunit contains primase activity. The genome of *E. histolytica* contains an archetypical DNA polymerase-α catalytic subunit of 1,135 amino acids (XP_657373.1), also identified by proteomic analysis [15] . The primase subunit consists of a catalytic and an associated subunit; orthologues of the p58 and p48 subunits are present in *E. histolytica* (XP_654525.1 and XP_657092.1). Interestingly, no orthologues of the p86 subunit are present in the genome. The p86 (also dubbed B) subunit of yeast is essential for cell growth and chromosomal replication, and in humans this subunit is phosphorylated by cyclinA-Cdk2 [16, 17]. Orthologues of the p86 are found in Archaea, indicating its conservation and suggesting that this subunit is involved in numerous protein–protein interactions (reviewed in [18]).

22.2.2 DNA Polymerase δ and DNA Polymerase ε

DNA polymerase-δ and -ε are the replicative polymerases. Recent studies using fidelity-hampered DNA polymerases indicate that DNA polymerase-δ is responsible for synthesizing the lagging-strand chain and DNA polymerase-ε is responsible for synthesizing the leading-strand chain [19]. Both DNA polymerases are required for DNA synthesis in nucleotide excision repair [20, 21]. DNA polymerase-δ has been purified from several species and shows some variation in its subunit composition. *Saccharomyces cerevisiae* DNA polymerase-δ contains three subunits [22], whereas in humans DNA polymerase-δ consists of four subunits: A (p125), B (p51), C (p68), and D (p12). The p125 subunit or catalytic subunit is a family B DNA polymerase with 5′–3′ polymerase and 3′–5′ exonuclease activities. The catalytic and p50 subunits of DNA polymerase-δ associates with proliferating cell nuclear antigen (PCNA) [23, 24]. The initial draft of the genome of *E. histolytica* did not contain open reading frames (ORFs) for DNA polymerases δ and ε; however, a new assembly and reannotation of the genome indicated that *E. histolytica* contains a

Table 22.1 Comparison of replication fork proteins in humans and *Entamoeba histolytica*

Protein	Component	HGB_ID	Length (aa)	*E. histolytica*_ID	Length (aa)	% Identity	*E* value
RFC	RFC1	NP_001191676.1	1,148	XP_649703.1	718	18.2	6e-45
	RFC2	NP_002905.2	320	XP_651156.1	315	33.3	2e-61
	RFC3	NP_002906.1	356	XP_649322.1	345	30.8	1e-47
	RFC4	NP_002907.1	363	XP_654775.1	329	38.5	3e-84
	RFC5	NP_001123584.1	255	XP_651283.1	325	31.3	1e-61
PCNA		NP_002583.1	261	XP_651510.1	262	33.5	2e-49
PolD	A	NP_001243778.1	1,107	XP_654477.1	1,077	42.5	0.0
	B	NP_001120690.1	469	XP_001913482.1	395	22.7	3e-26
	C	NP_006582.1	466				
	D	NP_001243799.1	79				
PolE	A	NP_006222.2	2,286				
	B	NP_002683.2	527				
	C	NP_059139.3	147	XP_654674.1	119	21.8	7e-04
	D	NP_063949.2	117	XP_654925.1	212	14.0	1e-05
PolA	A	NP_058633.2	1,462	XP_657373.1	1,135	26	6e-100
	B	NP_002680.2	598	XP_652187.1	457		
	C	NP_000938.2	509	XP_654525.1	523	18	3e-27
	D	NP_000937.1	420	XP_657092.1	372	29	4e-63

MCM	MCM1	NP_079110.1	642				
	MCM2	NP_001257401.1	818	XP_656059.2	881	19.8	8e-83
	MCM3	P25205.3	719	XP_653372.1	601	48	1e-46
	MCM4	NP_005905.2	863	XP_649080.1	608	32.6	0.0
	MCM5	NP_006730.2	734	XP_648784.1	639	38.4	1e-165
	MCM6	NP_005906.2	821	XP_654108.1	682	36.4	0.0
	MCM7	NP_005907.3	719	XP_650807.1	690	38.4	2e-159
	MCM8	NP_115874.3	840	EAL48818.1			
	MCM9	NP_060166.2	1,143	XP_654939.1	810		
	MCM10	AAI01728.1	875				
RPA	RPA1	NP_002936.1	616	XP_650233.1	588	28	4e-33
	RPA2	NP_002937.1	270	XP_649713.1	150	23	0.078
	RPA3	NP_002938.1	121				
	DNA Ligase	NP_000225.1	919	XP_657595.1	685	40	4e-142

putative DNA polymerase-δ catalytic subunit of 124 kDa (XP_654477.1) and a subunit B of 45 kDa (XP_001913482.1) [25].

In humans, DNA polymerase ε consists of four subunits: p262, p60, p17, and p12. The p262 subunit or catalytic subunit is family B DNA polymerase with 5′–3′ polymerase and 3′–5′ exonuclease activities. DNA polymerase-ε is implicated in replication of the leading-strand chain and DNA repair. We and other authors have failed to identify by bioinformatic analysis the catalytic subunit of DNA polymerase-ε in *E. histolytica* [26, 27]. Our search had included functional protein domain analysis through hidden Markov models (HMM) of orthologous Amoebozoa members from *Dictyostelium* as query. Although we have not identified the catalytic subunit of DNA polymerase-ε, orthologous proteins of the p17 and p12 subunits are present in the genome of *E. histolytica* (Table 22.1). DNA polymerase-δ and -ε interact with proteins involved in replication such as PCNA, but also interact with proteins implicated in DNA repair, recombination, and cell-cycle control that are present in *E. histolytica* [28].

22.2.3 Proliferating Cellular Nuclear Antigen (PCNA)

The proteins that coordinate DNA replication between leading- and lagging-strand synthesis are circular sliding clamps; in eukaryotes this clamp is PCNA, and besides its requirement as a processivity factor this protein is known as "the maestro of DNA replication" [29]. In eukaryotes, three identical PCNA monomers, each comprising two similar domains, are joined in a head-to-tail arrangement through an extended loop called the interdomain connector loop (IDCL) to form a homotrimer. The genome of *E. histolytica* contains a putative PCNA in locus XP_651510.1 with 33 % amino acid identity to human PCNA. The purified protein from this locus assembles as a homotrimer that is able to interact and stimulate the activity of a family A DNA ligase from *E. histolytica* that contains a canonical PCNA-binding motif or PIP box [30]. The crystal structure of EhPCNA indicates that it consists of two structural domains with a β-α-β-β-β-β-α-β-β-β architecture joined by an IDCL (Fig. 22.1). EhPCNA has an isoelectric point of 4.63; however, the central PCNA channel contains 27 positively charged amino acids (arginines and lysines) that are important for nonspecific interactions between EhPCNA and the sugar–phosphate backbone of DNA [30].

22.2.4 Replication Factor C (RFC) Clamp Loader

The RFC clamp loader couples ATP hydrolysis to open and close the homotrimeric PCNA processivity factor at the replication fork. The RFC clamp loader consist of five associated polypeptide subunits dubbed RFC1 to RFC5. Human

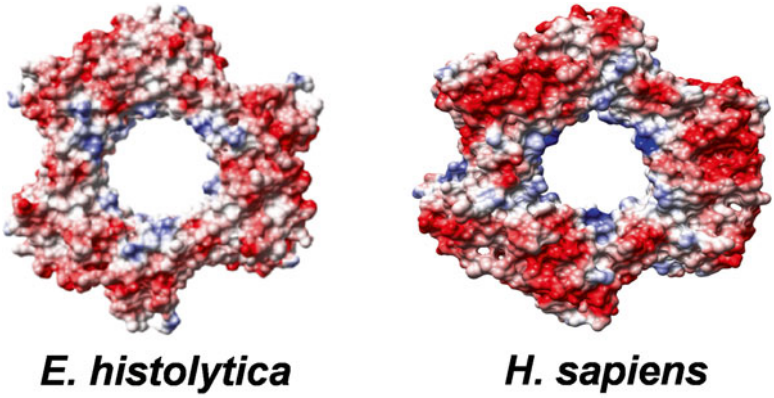

Fig. 22.1 Structural comparison of proliferating cell nuclear antigens (PCNAs) from *Entamoeba (E.) histolytica* and *Homo (H.) sapiens*. Crystal structure of PCNAs depicting the three identical subunits assembled head-to-tail to form a trimeric ring with a pseudo sixfold symmetry that is positively charged at the center of the ring

RFC1 has 128 kDa and the other subunits have a molecular mass between 38 and 41 kDa. All five subunits share a region that is used to define a set of proteins known as ATPases associated with a variety of cellular functions (AAA+ proteins) [31]. Five orthologous proteins of the RFC are present in *E. histolytica* (Table 22.1). As in other organisms, the four small subunits have a molecular weight between 36 and 41 kDa, and the RFC1 subunit is 30 kDa smaller than its human counterpart.

22.2.5 Minichromosome Maintenance (MCM) Proteins

MCM proteins are a group of ten proteins first identified in yeast that form numerous interactions at the replisome [32]. MCM proteins share a common ATPase active site (AAA+ domain) of approximately 250 amino acids. MCM2-7 complex is a hexameric assembly with helicase activity that is loaded onto the origin recognition complex (ORC) to form the pre-replication complex for DNA unwinding during DNA synthesis. MCM2 to -9 are present in *Entamoeba histolytica*, but MCM10 is not present [33]. Our current understanding regarding MCM in *E. histolytica* is thanks to the Lohia laboratory. They have cloned and purified MCM proteins demonstrating that MCM2-3-5 are bound to chromatin in cycling cells, suggesting an involvement of the MCM2-7 complex in the phenomena of genome reduplication in *Entamoeba* [34, 35]. The MCM2-7 complex is the only hexameric helicase that is not formed by a repetition of identical subunits. MCM8 and MCM9 are present in Metazoa, but not in yeast. Interestingly, *E. histolytica* and *Dictyostelium discoideum* contain orthologues of these genes [33, 36]. MCM8 and MCM9 are absent in

Trichomonas vaginalis and *Giardia lambia*. Recent studies indicate that MCM8 and -9 form a complex that promotes homologous recombination repair in response to DNA interstrand crosslinking [20].

22.2.6 Replication Protein A (RPA)

During lagging-strand DNA synthesis, single-stranded DNA is exposed. This single-stranded DNA can be degraded by nucleases and interfere with replication, so replisomes contain proteins that specifically bind to single-stranded DNA. Human RPA is a heterotrimeric protein that consists of 70-, 32-, and 14 kDa subunits, dubbed RPA1, RPA2, and RPA3, respectively [37]. The central region of RPA1, 2, and 3 has an oligonucleotide/oligosaccharide-binding (OB) fold and binds single-stranded DNA with a specific polarity. RPA stimulates the activities of polymerase alpha, delta, and epsilon and has a role in processing Okazaki fragments. The recent crystal structure of heterotrimeric RPA with single-stranded DNA indicates that RPA suffer associated conformational change that may increase its binding partners [21]. Orthologues of RPA1 and RPA2 are found in *E. histolytica* with 28 % and 23 % amino acid identity, respectively, to the human orthologues.

22.2.7 DNA Ligase I

The genome of *E. histolytica* only codes for one DNA ligase (EhDNAligI), located in locus EHI_111060 (XP_657595.1), with 40 % amino acid identity to human DNA ligase I (HsDNAligI). The cloned and purified ORF from this locus is an ATP-dependent ligase that performs nick sealing with an higher efficiency to human DNA ligase [30]. The gene product of this DNA ligase is transcribed in basal conditions and contains a PCNA interacting peptide and a nuclear localization sequence. EhDNAligI is able to ligate an RNA substrate located at the strand upstream of a nick, but not in the downstream or the template position [38]. Thus, it is proposed that EhDNAligI is involved in sealing DNA nicks during lagging-strand synthesis and may have a role in repair as this ligase is overexpressed upon UV insult (Cardona et al., in preparation). Structural and biochemical data indicates that EhDNAligI interacts with EhPCNA through a specific interaction with the PIP-box, which has the consensus sequence Q-xx-(h)-x-x-(a)-(a), where ‘h’ represents residues with moderately hydrophobic side chains (e.g., L, I, M) and ‘a’ represents residues with highly hydrophobic aromatic side chains (e.g., F, Y), and ‘x’ is any residue. This interaction stimulates the activity of DNA ligase in the order of four- to fivefold [39]. Biochemical data indicate that the DNA ligase–PCNA complex in *E. histolytica* functionally and structurally resembles the human complex.

22.3 The Genome of *E. histolytica* Encodes Specialized DNA Polymerases

22.3.1 Family A of DNA Polymerase

Higher eukaryotes such as *Homo sapiens* contain three family A DNA polymerases in their genome: DNA polymerase-γ, DNA polymerase-ν, and DNA polymerase-θ. DNA polymerase-γ is the mitochondrial DNA polymerase whereas DNA polymerases ν and θ are nuclear enzymes capable of translesion DNA synthesis and DNA repair. *Arabidopsis thaliana* contains two family A DNA polymerases involved in DNA repair. Family A DNA polymerases are present at the apicoplast as a multicomplex enzyme that contains domains for DNA primase, DNA helicase, and exonuclease activities [40, 41]. The mitochondria of kinetoplastids contain at least four DNA polymerases not related to DNA polymerase γ, involved in its DNA replication [42, 43]. The mitochondria of *E. histolytica* degenerated to an organelle with no detectable DNA called a mitosome [44, 45]. In contrast to mitochondria, the mitosome does not contain genetic material. *E. histolytica* contains only one family A DNA polymerase. A phylogenetic analysis of family A DNA polymerase indicated that this polymerase is located in a separated branch with respect to mitochondrial DNA polymerase-γ and nuclear DNA polymerases ν and θ [27]. This DNA polymerase is a protein of 657 amino acids and 25 % amino acid identity to the Klenow fragment. The biochemical characterization of recombinant EhDNApolA indicates that this protein is an active DNA polymerase able to primer extension and moderated strand displacement (Fig. 22.2). The biochemical characterization indicates that EhDNApolA bypasses thymine glycol with high fidelity (Fig. 22.2). Confocal microscopy data show that EhDNApolA is localized in the nucleus. EhDNApolA shares biochemical properties with DNA polymerase-ν and is perhaps an example of convergent evolution because DNA polymerases of subfamily N are only present in vertebrates [19]. *E. histolytica* is subject to reactive oxygen species produced at the colonic tissue and by phagocyte release, and thymine glycol is formed by chemical oxidation and ionizing radiation [46]. In eukaryotic organisms, thymine glycol can be bypassed by DNA polymerases κ and η [47]. However, *E. histolytica* lacks those DNA polymerases. *E. histolytica* contains genes for base excision repair including 8-oxoguanosine and thymine glycol glycosylases. Although the in vivo function of EhDNApolA is unknown, its ability to bypass thymine glycol and nuclear localization suggests a possible role of this enzyme in translesion DNA synthesis (Table 22.2). This role is reminiscent of family A DNA polymerases of *Arabidopsis thaliana* postulated to be involved in DNA repair at the chloroplast [48] and eukaryotic family A DNA polymerases ν and θ [19, 24].

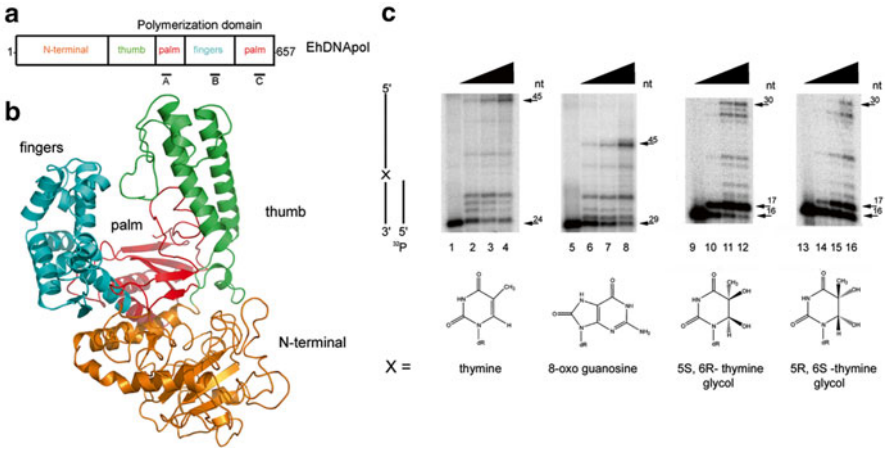

Fig. 22.2 EhDNApolA of *E. histolytica*. **a** EhDNApolA is a specialized DNA polymerase of 657 amino acids that contains a polymerization domain with motif for the thumb, palm, and fingers subdomains. Its N-terminal domain lacks the motifs necessary for DNA editing. **b** A structural model suggests that EhDNApolA adopts a polymerase fold in which the polymerization subdomains form a crevice in which a double-stranded DNA can bind. **c** EhDNApol A is able to efficiently bypass a lesion generated by oxidative stress such as 8-oxoguanosine and thymine glycol as observed in a denaturing polyacrylamide gel electrophoresis. The chemical structure of each DNA lesion and thymine is present for comparison. The *bottom arrow* indicates the substrate length and the *top arrow* indicates the expected full-length products. The reactions were incubated with increased amounts of EhDNApolA and fixed amount of each substrate: thymine (*lanes 1–4*); 8-oxo guanosine (*lanes 5–8*); 5S-6R thymine glycol (*lanes 9–12*); 5R-6S thymine glycol (*lanes 13–16*)

22.3.2 Lesion Bypass DNA Polymerases

22.3.2.1 REV1, REV3, and DNA Polymerase ζ

Rev1 and Rev3, family Y and family B DNA polymerases, respectively, are error-prone DNA polymerases. These DNA polymerases are involved in translesion DNA synthesis (TLS). In humans and yeast, Rev3 and Rev7 assemble to form DNA polymerase-ζ. Rev3 contains the catalytic amino acids whereas Rev7 is the regulatory subunit that interacts with the central region of Rev3 and stimulates its activity [10, 11]. During TLS, specialized DNA polymerases incorporate one nucleotide across from a specific DNA lesion, and in a second step DNA polymerase-ζ extends from the latter nucleotide incorporation. Rev1 is a family Y DNA polymerase that incorporates cytosine across from abasic sites and efficiently bypasses bulky adducts such as N2 methyl benzopyrene. Because Rev1 interacts with TLS polymerases, it is suggested that it functions as a scaffold during translesion DNA synthesis.

E. histolytica contains orthologues of Rev 1 and Rev 3, but a protein with identity to Rev 7 is not found in the genome. *E. histolytica* contains a Y-family DNA polymerase of 708 amino acids (XP_654241.1) that share 34 % amino acid similitude

Table 22.2 Lesion bypass DNA polymerases in *E. histolytica*

Gene	Length (aa)	Exonuclease domain	GenBank ID	Pathema locus	Polymerase family	Related DNA polymerase	GenBank ID
EhREV1	709	–	XP_654241.1	EHI_053480	Y	HsREV1	NP_057400.1
EhREV3	1,386	–	XP_656768	EHI_068010	B	HsREV3	NP_002903
EhDNAPOLB1	813	+	XP_001914292	EHI_018010	B2	DNA pol φ 29	YP_002004529
EhDNAPOLB2	1,080	+	XP_649845	EHI _132860	B2	DNA pol φ 29	YP_002004529
EhDNAPOLB3	1,279	+	XP_001913700	EHI_164190	B2	DNA pol φ 29	YP_002004529
EhDNAPOLB4	1,231	+	XP_648196	EHI_196700	B2	DNA pol φ 29	YP_002004529

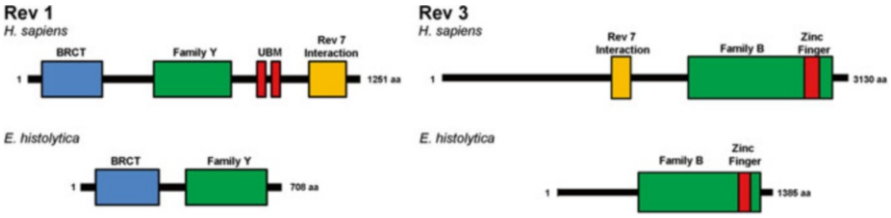

Fig. 22.3 Cartoon representation of Rev1 and Rev3 from *H. sapiens* in comparison to *E. histolytica*

with human Rev1 in the polymerization domain. Human Rev1 is composed of 1,251 amino acids. Both amino acids share a conserved BRCT interaction module at their N-terminal, but the Rev7 interacting module of human Rev1 is not conserved in Rev1 from *Entamoeba*. The genome of *E. histolytica* also contains the catalytic subunit of Rev3 (XP_656768) as a protein of 1,385 amino acids. The amino acid length of Rev3 in humans is 3,130 amino acids, and the last 685 amino acids of both HsRev3 and the putative Rev3 from *E. histolytica* share 33 % amino acid identity. The first 700 amino acids of the putative Rev3 from *E. histolytica* only are present in putative Rev1 proteins from *Entamoeba*. The reduced amino acid length of proteins from protozoan parasites is common [38]. As the interacting domains of Rev7 are not present in Rev1 and Rev3, it is possible that this protein has been lost during evolution (Fig. 22.3 and Table 22.2).

22.3.3 Transposon-Derived Family B2 DNA Polymerases

The genomes of protozoan parasites are abundant in transposable elements [49]. For instance, several families of transposable elements (TEs) have been described in *Entamoeba*, and EhLINEs/SINEs account for 11 % of its genome [49, 50]. A new class of transposable elements of approximately 20 kb dubbed Maverick/Polinton are highly abundant in protozoan parasites; for instance, it is estimated that 5 % of the genome of *T. vaginalis* consists of Maverick/Polinton derived DNA [46]. The genome of Maverick/Polinton contains open reading frames for retroviral-like integrase, adenoviral-like protease, ATPase, and a family B2 DNA polymerase. Although a complete Maverick/Polinton has not been identified in *E. histolytica*, the genome of *Entamoeba invadens* contains a Maverick/Polinton sequence of 16,504 bp that contains all canonical proteins for self-replication. The genome of *E. histolytica* contains four family B2 DNA polymerases in its genome; these DNA polymerases rank from 813 to 1,279 amino acids and contain a conserved C-terminal domain with a 3′–5′ exonuclease and 5′–3′ polymerization motifs (Table 22.2) [51]. As φ29 DNA polymerase, they contain two elements dubbed terminal protein

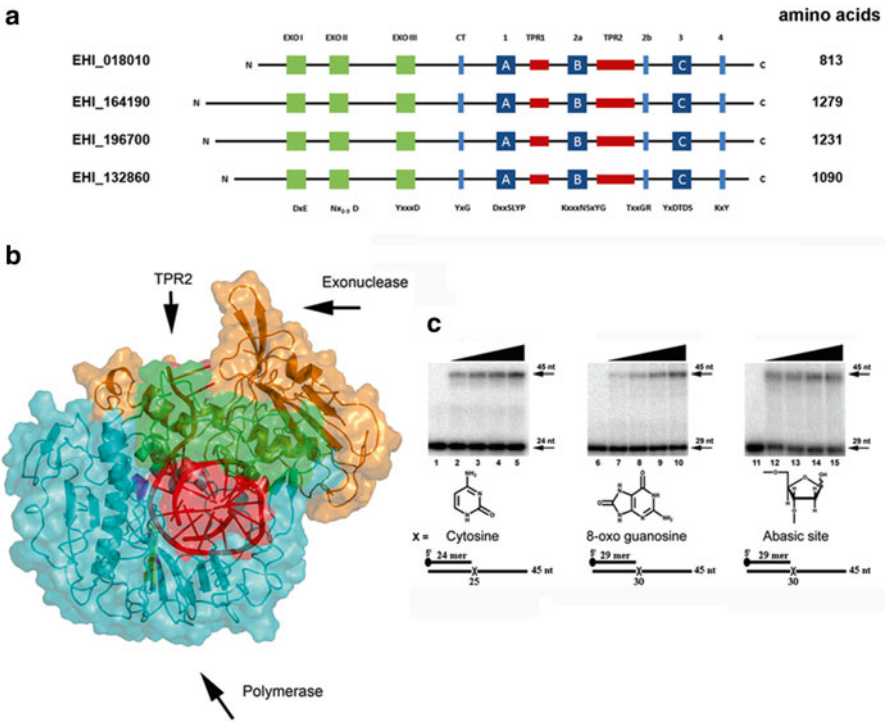

Fig. 22.4 Family B2 DNA polymerases present in *E. histolytica*. **a** The genome of *E. histolytica* contains four family B2 DNA polymerases that rank from 813 to 1,279 amino acids. These polymerases contain conserved motifs for editing and polymerization activities. **b** The structural model of EhDNApolB2 predicts a similar fold to DNA polymerase from bacteriophage ϕ29. **c** EhDNApolB2 is able to efficiently bypass 8-oxoguanosine and abasic sites. The *bottom arrow* indicates the substrate length and the *top arrow* indicates the expected full-length products. The reactions were incubated with increased amounts of EhDNApolB2 and fixed amount of each substrate: cytosine (*lanes 1–5*); 8-oxo guanosine (*lanes 6–10*); abasic site (*lanes 11–15*)

regions 1 and 2 in comparison to other family B DNA polymerases.. The cloning of ORF located in locus EHI_018010 resulted in an active polymerase with high strand displacement, processivity, and DNA lesion bypass (Fig. 22.4). The character of the terminal region protein 2 (TPR2) motif in family B2 DNA polymerases from *E. histolytica* is twice the length of the character of TPR2 motifs from other DNA polymerases. Mutagenesis studies indicated that the extended TPR2 motif is responsible for strand displacement, processivity, and DNA lesion bypass. EhDNApolB2 is one of the most efficient enzymes in bypassing an abasic site, indicating that this motif may influence the active site architecture so this polymerase can accommodate and extend across from an abasic site.

22.4 Perspectives

The majority of antibiotics in clinical use target RNA polymerase and the ribosome [47, 48]. However, there is an increasing interest in targeting enzymes and protein–protein interaction of the DNA replication machinery [52–54]. The general process of DNA replication is conserved among all organisms; however, the interactions observed at the replisome between species are different. Although no single antibiotic in clinical use is targeted against replisomes [53], the differences between DNA polymerases of *E. histolytica* and humans indicate the potential of DNA polymerases as drug targets [27, 51]. Consequently, the crystal structure of PCNA from *E. histolytica* indicates that the protein–protein interactions between PCNA and protein replication partners are different in *E. histolytica*, making possible to design small drugs to abolish specific protein–protein interactions at the replisome [30].

Acknowledgments Research in the LGB laboratory is supported by CONACYT-grant 128647. We thank the Howard Hughes Medical Institute for support.

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Part V
Pathogenesis and Immunity

Chapter 23
Pathology, Pathogenesis, and Experimental Amebiasis

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Abstract The most important feature of the pathology of human amebiasis is the greatly destructive nature of the anatomical lesions produced by the protozoan *Entamoeba histolytica*. Recent advances on the knowledge of biochemistry, immunology, cellular and molecular biology, and genetics of this parasite, added to the use of different in vitro, in vivo, and ex vivo models to analyze host–parasite interactions or the production of intestinal and extraintestinal amebic lesions, all have given a better perception of the mechanisms of pathogenesis in amebiasis. The present chapter is divided into three parts: first, a general review of the pathology of human amebiasis; second, a short review of the mechanisms of invasion and production of damage in the host, and third, a review of the different in vivo experimental models currently available to study the mechanisms involved in amebic infection. In reference to pathogenesis, each factor, molecule or gene, or mechanism of target cell damage is reviewed individually in other chapters of this section on "Pathogenesis and Immunity" in the present book. Therefore, the meticulous or probing aspects of the studies are mentioned by each responsible and expert group of researchers. In this review, we mention in general each of the different factors of pathogenesis in amebiasis. The contributions obtained using different techniques and methodologies of experimental models are emphasized, and the subjects that still need to be unraveled to understand how this microscopic parasite has earned its well-deserved "histolytic" name are discussed.

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_23

Abbreviations

ALA	Amebic liver abscess
CP	Cysteine proteinase
E-64	L-*trans*-Epoxisuccinil-leucylamido-(4-guanidino butane)
Gal	Galactose
GalNAc	Acetylgalacosamine
GM-CSF	Granulocyte macrophage-colony stimulating factor
GTP	Guanosine triphosphate
IL-1β	Interleukin-1-beta
INF-γ	Interferon-gamma
Muc2	Mucin 2
NF-κb	Nuclear factor kappa-light-chain-enhancer of activated B cells
SCID	Severe combined immunodeficiency
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-dUTP nick-end labeling

23.1 Introduction

Although it has been known for more than 100 years that *Entamoeba histolytica* is the causative agent of human amebiasis, and in spite of its great impact as a public health problem, especially in developing countries [1], there are still many aspects of the host–parasite relationship that are not clearly understood. However, recent advances in mounting in vitro, in vivo, and ex vivo models for producing experimental intestinal and hepatic amebiasis, added to the interesting reports on the genetic analysis of the parasite with determination of several virulence factors and advances in the understanding of host response to infection, all have given a better perception of the multiple mechanisms that are taking place during the pathogenesis of the disease.

Amebiasis is an intestinal infectious disease, affecting mainly the large intestine; however, the parasite is capable of damaging practically any human tissue [2]. Moreover, it is known that the highly potent cytotoxic and cytolytic activity of the parasite constitutes the major factors of tissue damage. The classical description of the pathology of human amebiasis appeared less than 20 years after Lesh's discovery of amoebas in the stool of a dysenteric patient in 1875 [3]. The monograph by Councilman and LaFleur [4] based on 14 autopsy cases contains many careful descriptions of both intestinal and hepatic lesions that are still valid today.

Intestinal amebiasis may be associated with a wide variety of anatomical changes. When it corresponds to a fairly well-defined clinical condition, it is known as amebic ulcerative colitis, although other less commonly seen conditions can be present, such as fulminating amebic dysentery, amebic granuloma or ameboma, and amebic appendicitis [2, 5]. Hepatic amebiasis, on the other hand, constitutes the most frequent extraintestinal condition and is usually distinguished by the production of one or more large areas of necrosis or abscesses in the organ [5].

Considering that the human being is eventually the main target suffering from this important infectious disease, it is fundamental to review, as a first step, the essential pathology of human amebiasis, which will be the basis for better interpretation, correlation, and analysis of the pathogenesis when using different experimental models.

23.2 Pathology of Amebiasis

The first and most important characteristic of the pathology of human amebiasis is the greatly destructive nature of the anatomical lesions [2, 6, 7]. Thus, the pathology of invasive amebiasis, either intestinal or extraintestinal, is characterized by some peculiar features: (a) highly destructive damage causing a rapid and extensive necrosis, with common liquefaction of all affected tissues; (b) a relatively small inflammatory reaction, particularly at later stages; (c) as seen in other parasitic infections, eosinophilia is not a frequent finding; (d) although tissue destruction is extensive, hemorrhagic areas are scarce; and (e) when anti-amebic treatment is appropriate, even in very large lesions, tissue regeneration is optimal with practically no fibrosis.

23.2.1 Amebic Ulcerative Colitis

Amebic intestinal lesions appear most commonly in the colon (primarily in the cecum), the sigmoid colon, and the rectum. Initial lesions are small (0.1–0.5 cm), round, slightly elevated areas of the mucosa with irregular necrotic centers surrounded by a rim of edematous tissue (nodular ulcers). The necrotic center may appear depressed and is often filled with mucoid-like material (Fig. 23.1). These ulcers, when few, are sometimes difficult to detect because of the mucosal normal folds, but when they are numerous and cover almost all the mucosa of the colon these can be easily observed, and the unaffected segments appear congested and edematous. Identification of the ulcers suggestive of intestinal amebiasis can be reported when colonoscopy is undertaken by an experienced eye [8].

Amebic ulcerative colitis may also appear as one or more irregular lesions, measuring several centimeters in length. These ulcers are usually found in the cecum and the ascending colon. They are shallow, broad with elevated margins, and filled with fibrin; sometimes they resemble pseudomembranous colitis.

Both types of anatomical lesions described, nodular and irregular, can be present in the same patient. In specimens obtained during surgery prompted by signs of intestinal perforation, the usual findings are extensive superficial denudation of the mucosa.

The earliest microscopic changes have been studied in rectal biopsies (Fig. 23.2). Different types of lesions depending on the severity of damage can be observed [8]. The nonspecific lesion without ulceration, but thickening of mucosa caused by

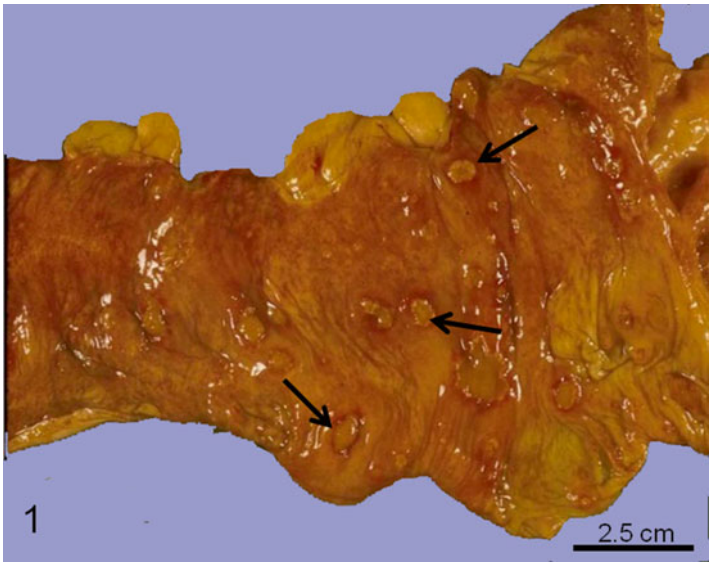

Fig. 23.1 Gross aspect of human intestinal amebiasis. Several irregularly round ulcers (*arrows*) with necrotic center with a rim of edematous tissue (nodular ulcers) are seen in an autopsy case

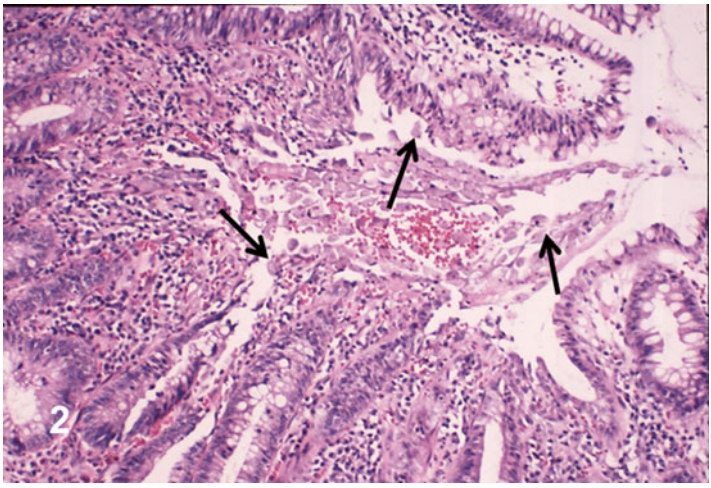

Fig. 23.2 Light microscopy of a rectal amebic ulcer obtained by an endoscopic biopsy shows superficial necrosis of the mucosa, with *Entamoeba histolytica* trophozoites (*arrows*) associated with mucus, hemorrhage, and inflammatory infiltrate. Hematoxylin and eosin (H&E) stain. ×20

glandular hyperplasia and stromal edema, can be considered as one of the earliest changes. Exudates or mucus may rarely contain amoebas. As the lesion progresses, superficial ulcerations can be seen with small interglandular foci of microinvasion with minimal tissue necrosis and acute inflammatory response. Larger areas of superficial

ulceration may contain amebic aggregates associated to zones of necrosis and surviving tissue. Muscularis mucosa is usually intact.

A deeper ulceration observed in the late invasive lesion has been described as "flask ulcer," reported by Councilman and LaFleur [4], or the "buttonhole ulcer" of Brandt and Pérez-Tamayo [2]. These lesions are characterized by an undermining of the submucosa that extends over an area greater than the ulcer itself in the overlying mucosa. The areas of necrosis are connected through the lamina propria by a narrow bridge, also of necrotic tissue. The external muscular layers are usually intact.

When the amebic intestinal lesions are fully developed, a mixture of necrosis and inflammation is seen and, although far from specific, a careful search for the parasites is necessary when they are not readily apparent. However, in fact, it is rare to encounter extensive foci of necrosis without finding at least some recognizable amoebas. Frequently, the parasites present in an area of necrosis also show signs of disintegration, and when absent it is assumed that they have been lysed. Necrosis often involves the mucosal surface, lamina propria, and submucosa, stopping at the external muscular layer. In most severe cases, necrosis can involve the whole intestinal wall and may result in perforation. Inflammatory reaction is usually localized in the same layers of the intestinal wall. In these severe cases with necrosis of the muscular layers, inflammation is usually present, and inflammatory cells surround the parasites and infiltrate in all the damaged structures.

Cases of amebic appendicitis are usually related to extension of cecum or colonic infection by the parasite. This appendicitis is characterized by proliferation of *E. histolytica* trophozoites in the lumen and appendiceal wall with acute inflammatory reaction and necrosis, which, starting in the mucosa, may extend to the serosa and cause perforation of the organ. Other anatomical types of intestinal amebiasis such as fulminating amebic colitis or toxic megacolon and ameboma are not described herein, as these are clinically less commonly seen cases.

23.2.2 Amebic Liver Abscess

The most common extraintestinal complication of invasive amebiasis is the amebic liver abscess (ALA); however, one third of the cases of ALA do not show evidences of invasive intestinal amebiasis when they are clinically diagnosed for the first time [5, 9].

Macroscopically, lesions of the liver by *E. histolytica* trophozoites are formed by areas of hepatic parenchyma substituted by necrotic material, which is yellowish, creamy in consistency, and outlined by congestive hepatic tissue. When the damage is large, the center of the necrotic material could be substituted by liquid, giving a cavitary aspect (Fig. 23.3).

When the tissues are examined microscopically, amoebas are localized mainly at the periphery of the necrotic material close to apparently normal hepatic tissue and occasionally mixed with the necrotic parenchyma. Trophozoites may contain ingested red blood cells or cell debris. Inflammatory reaction is variable, although periportal areas show abundant chronic inflammatory reaction.

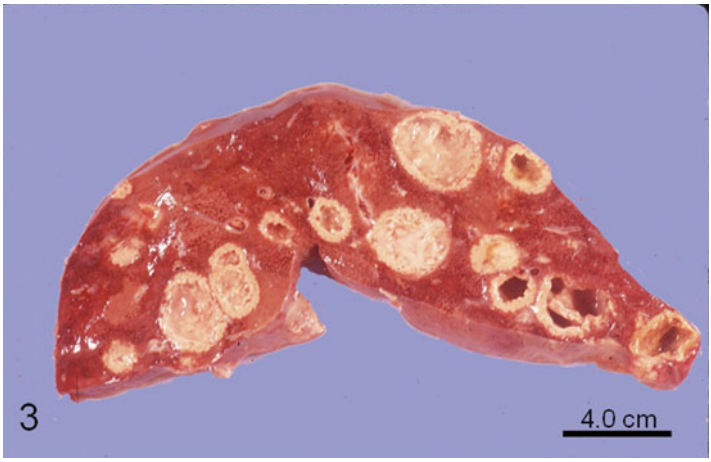

Fig. 23.3 Gross aspect of human hepatic amebiasis shows multiple round abscesses of different sizes and some cavitary necrosis at *right side* in an autopsy case

Information regarding early lesions before the development of a well-established ALA in humans is scarce. Much of these data refer to experimental studies performed in susceptible animals inoculated with the amoebas and analyzed at early stages of infection.

23.3 Pathogenesis of Amebiasis

Based on basic and fundamental studies related to pathogenesis reported in the past, in this section we analyze some of the recent biochemical, immunological, and molecular studies related to the mechanisms of invasion and damage of target cells by *E. histolytica*. The outcome of *E. histolytica* infections depends not only on the parasite genotypes and phenotypes, but also on individual gender, genetic loci, environmental factors, including microbiota, and the availability of food.

There are many reports supporting pathogenesis of *E. histolytica* as a multifactorial process that takes place at several levels, in which many molecules have been implicated and identified, such as lectins present on the parasite surface, pore-forming proteins, and multiple enzymes with proteolytic activities.

23.3.1 Entamoeba histolytica *and Intestinal Environment*

Role of Bacteria In the large intestine, environmental elements interacting with *E. histolytica* trophozoites constitute important factors to initiate the process of invasion. Contact between *E. histolytica* and bacterial flora is one of the signals that

starts the process of infection. Besides having a feeding role to the amoebas, bacteria also provide anaerobic conditions, or redox potential that favors the growing and proliferation of the trophozoites or the process of cyst formation through the Gal/GalNAc lectin [10].

E. histolytica trophozoites have shown selectiveness to interact with different species of bacteria, and only those that are better recognized by the amoebas are ingested by them [11]. It has been also suggested that bacteria of the intestinal flora may induce an important increase in the virulence; moreover, mixed infections with *Shigella dysenteriae* and enteropathogenic *Escherichia coli* may increase the clinical signs of the disease [12]. These findings are relevant to analyze the pathogenicity of the parasite in endemic areas and countries where intestinal amebiasis is commonly associated with infections with enteropathogenic bacteria. When trophozoites of *E. histolytica* are axenically cultured in vitro for long periods, their virulence is progressively lost, making them incapable of producing experimental amebic lesions; however, the virulence can be recovered by adding pathogenic bacteria such as *Escherichia coli*, *Salmonella typhi*, or *Salmonella parathyphi* to the culture medium [13]. Therefore, we may deduce that the intestinal bacterial flora has an important role in the virulence of the amoeba; however, the biological and molecular bases of these interactions are still poorly understood.

Intestinal Mucus Another element with which amoebas have environmental interaction, previous to invasion of the intestinal mucosa, is the presence of mucus secreted by epithelial goblet cells. The initial increase and posterior degradation of this material caused by the amoeba is apparently the result of proteolytic enzymes produced by *E. histolytica*. So, besides interaction with the bacterial flora, the first evidence of amebic histopathology of the intestinal mucosa is the local increase of mucus and changes in the epithelial barrier, which are followed by a reduction of mucus that alters in turn the epithelial barrier, facilitating parasite invasion. Further phenomena related to the activity of proteolytic enzymes to degrade multiple extracellular matrix components constitute a very important factor in tissue invasion and destruction. A recent work published by Kissoon-Singh et al. [14] has shown, using Muc2$^{-/-}$ mice, that *E. histolytica* increases the permeability of epithelial cell tight junction and proinflammatory responses. All these aspects of the mucin and epithelial cell tight junctions are amply reviewed by the respective authors elsewhere.

23.3.2 E. histolytica *Tissue Invasion*

Adherence Contact and adherence constitute the first steps during the invasion to target cells by the parasites. Several specific molecules called adhesins participate during the pathogenesis by the amoeba. One of these molecules that has been amply characterized is the galactose/*N*-acetyl D-galactosamine lectin (Gal/GalNAc). The native protein is composed of a 260-kDa heterodimer with a heavy subunit of 170 kDa bound to a light subunit of 35/31 kDa by disulfide bridges.

This lectin was characterized by Petri et al. [10], who first demonstrated the adhesion of *E. histolytica* trophozoites to cultured cells using the sugar galactose *N*-acetyl D-galactosamine as a competitor molecule of adhesion. By using the same sugar, amebic adherence can also be inhibited in other cell types, such as epithelial cells, erythrocytes, and neutrophils. Moreover, using a monoclonal anti-170-kDa lectin antibody, it was established that amebic adhesion to target cells is a requirement for damaging cells, because antibodies bond to lectin inhibit adhesion and in turn damage to the cells. Also, it has been shown that this amebic lectin binds to C8 and C9 proteins of the complement, impeding amebic destruction and protecting against the action of the antibodies. Because this lectin has demonstrated its importance in the pathogenesis of amebiasis, the molecule has been tested as a possible candidate for vaccine production.

Other molecules associated to adherence that have been reported; although less studied, are also important. Among these, a 220-kDa cell-surface protein was characterized by inhibiting parasite adhesion using a polymer of the sugar *N*-acetyl glucosamine [15]. Another molecule, the 112-kDa adhesion, discovered by using adhesion-deficient mutants, has also been shown to have a cysteine proteinase activity and may participate during tissue invasion [16].

Previously mentioned molecules also have the property of binding to the surface of red blood cells and consequently participate in the phenomenon of erythrophagocytosis. Therefore, the initial mechanisms of mucosal penetration are related to the adhesion of amoebas to epithelial cells, and during this process may ingest erythrocytes that provide iron to the parasites, which is an important factor for adherence and cytotoxicity [17].

Another critical factor for target cell death is related to the dramatic influx of the calcium soon after parasite contact. The important increase of calcium in the target cell is irreversible and precedes death. It has been suggested that this activity could be attributable to the Gal/GalNAc lectin, because the addition of the affinity-purified lectin to target cells also results in calcium elevation [18]. Calcium influx in the parasite seems to be also occurring, as pretreatment of amoebas with calcium channel blockers inhibits killing [19]. Recently, a calcium-regulated transcription factor, URE3-BP, was reported that apparently modulates the expression of known virulence factors and URE3-BP mutants that constitutively bind DNA, resulting in an enhanced parasite invasion in vivo [20].

Many events that follow adhesion are still enigmatic, although some evidence has suggested that, during the amoeba-to-amoeba contact, induction of the apoptosis or programmed cell death of the target cell could be occurring [21]. This phenomenon may also be related to the possible role of amoebapores (see following).

As mentioned, adherence to the target cell is one of the necessary requirements that take place during cytotoxicity. Bos [22] proposed that *E. histolytica* can damage tissues in two ways. One is a rapid process, which occurs during a close cell-to-cell contact, and the second is a slower process that is produced by soluble substances secreted by the amoebas or molecules present in the intestinal microenvironment.

Apoptosis There are several kinds of evidence that suggest that *E. histolytica* induces apoptosis in the target cell. Stanley [23] suggested that although apoptosis

is a significant phenomenon, it is not exclusive during the formation of ALA, because mice treated with inhibitors of caspases (lytic enzymes related to apoptosis) also showed hepatic lesions. The authors suggested that cytolysis and apoptosis induced by *E. histolytica* in these conditions may contribute to ALA development and that direct contact of the trophozoites could be the first step in cell death. However, it is important to mention that hepatocytes with signs of apoptosis (pycnotic and positive to TUNEL) have been seen at some distance from the site of trophozoites, suggesting that apoptosis may be originated by the action of molecules secreted by the parasites, such as amoebapores or proteolytic enzymes, including other molecules as inflammatory cytokines produced by host cells after contact with the amoebas. It is also known that ischemia caused by obstruction of blood vessels is another cause of parenchymal cell death. On the other side, the rapid elimination of apoptotic cells by amoebic phagocytosis diminishes the inflammatory infiltrate, producing a prolonged infection or progressive, because no adequate immune response is acting. In vivo studies, using the hamster model of ALA independent of contact, have shown that the amoebas displayed typical signs of cell death [24]. The authors demonstrated that changes were caused by the activation of inflammatory cells of the peritoneum and the production of highly reactive and toxic metabolites, such as nitric oxide. In more recent studies, a bacterial antibiotic (aminoglycoside G418) was shown to produce a programmed cell death in amoebas. The apoptosis phenomenon was evaluated by ultrastructural analysis and diverse biochemical and pharmacological techniques, including the determination of the expression of genes involved in this process [25]. These studies sustained that programmed cell death can be induced in amoebas. At present, however, the mechanisms involved in apoptosis, which eventually may control the advancement of amoebic infection, are not clearly understood.

Amoebapores Another mechanism of cell death in which amoebas appear to participate belongs to a family of peptides that are called amoebapores. These molecules have similar functions to those proteins of the immune system that participate in forming pores. This family of amoebapores is constituted of small peptides (5–30 kDa) that are contained in the vesicles of *E. histolytica* and have a maximum activity at an acidic pH [26]. These amoebapores cleavage in the membrane, causing cell lysis. Trophozoites that do not express amoebapores are incapable of killing target cells or ALA [27]. Affected hepatocytes showed nuclear chromatin condensation, bubbling of the cell membrane, and DNA fragmentation. All these processes are markers of direct activation of the apoptosis machinery and suggest that apoptosis can be induced during the amoeba–target cell contact through the initial action of amoebapores.

Proteolytic Activity A variety of proteinases are secreted by *E. histolytica*. As mentioned, initially these proteins were implicated with the invasion of trophozoites in the intestine by degrading the luminal mucus. To perform an active process of invasion, amoebas need to degrade different components of the extracellular matrix, which occurs by the action of proteinases secreted by *E. histolytica*. These enzymes degrade collagen, elastin, fibrinogen, fibronectin, and laminin [28]. Levels of expression of proteinases have been correlated with the virulence involved in the

destruction of human collagen by *E. histolytica*, having a major effect on collagen type I [29, 30]. Amebic collagenase was localized in the electron-dense granules and showed to be actively secreted when contacted with the substrate [31]. Cysteine proteinases are the main agent responsible for monolayer degradation in culture. These proteinases are known to interfere with the immune system function, participate in cyst formation and desencystment, and also have been related to induction of apoptosis. Presently is known that more than 50 genes can codify for cysteine proteinases, which are expressed in different forms depending on amoeba strain and culture conditions [32, 33].

One of the most commonly studied proteinases is the cysteine proteinase 5, which is localized at the membrane of the trophozoites. Recent studies using RNA (antisense) against cysteine proteinase 5, reported lesser mucin degradation and greater destruction of various cell lines [34]. Moreover, trophozoites deficient in this cysteine proteinase showed minor phagocytic activity, and studies of experimental ALA in hamsters showed smaller abscesses. Specific inhibitors of these peptides, such as E-64, when administrated in infected experimental animals, reduced ALA size [35, 36].

Another factor known to contribute to tissue invasion is the pro-inflammatory reaction produced by the presence of amoebas. Coculture of trophozoites with epithelial cell lines in vitro has been shown to induce the production of pro-inflammatory cytokines [37]. Amoebic cysteine proteinases also stimulate pro-inflammatory signaling; they possess interleukin (IL)-1β-converting activity and a surface-localized protease that can activate the alternative pathway of the complement [38]. A recent report also suggests that EhCP5 binds integrin and stimulates NF-κB dependent pro-inflammatory response [39].

Phagocytosis The process of phagocytosis is one of the biological characteristics of the amoeba that has been amply studied. Most of these studies have been done using different in vitro models for phagocytosis, interacting different amoebic strains with a variety of cells, including primary cell line cultures and, most commonly, erythrocytes (Fig. 23.4). This later phenomenon, called erythrophagocytosis, has been considered as an important feature of invasive amoebas, and in the past, the cytoplasmic presence of erythrocytes in the trophozoites was a biological criterion used, in human feces, to determine amebic dysentery [5]. Posterior in vitro studies of interactions and tested with the capacity to produce ALA experimentally have shown that amoebas with great capacity to produce liver lesions do not necessarily have high erythrophagocytic capacity [40].

The process of phagocytosis is in general a complex and dynamic phenomenon in which diverse molecules, including adhesins, cytoskeleton, and digestive enzymes, are participating. The process is a highly ordered and synchronized phenomenon in which the parasite contacts and adheres to the target cell, causing rapid death and consequent ingestion of the cells, and processing by specific vacuoles called lysosomes and phagosomes, in which the damaged cells are eventually degraded. This process is accomplished by a sequential fusion of endocytic compartments to form phagolysosomes, where GTPases and Rab proteins act as modulators of vesicular fusion as well as fusion to the target cell membranes. All these data suggest that

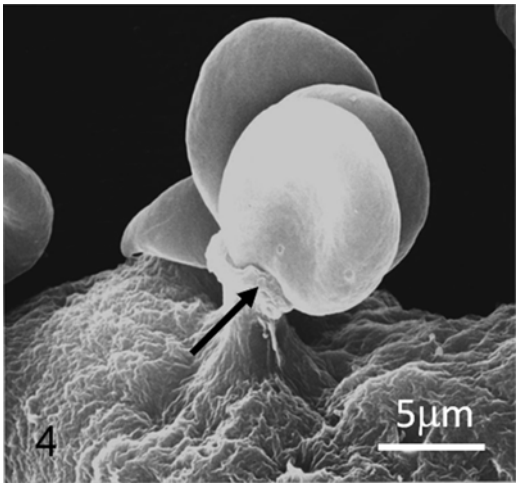

Fig. 23.4 Erytrophagocytosis by *Entamoeba histolytica*. Scanning electron microscopy of 5 min of interaction of amoebas with erythrocytes shows the single phagocytic mouth of an amoeba (*arrow*) with four erythrocytes in the process of endocytosis

amoeba virulence can be correlated with intracellular activities, such as membrane traffic between lysosomes and phagosomes, and the activity of diverse proteases present in the amebas during the cell contact or target substrates or secreted to alter or to modify the immune response of the host.

Genetic Aspects of Pathogenesis Completion of the *E. histolytica* genome and subsequent analysis have unveiled novel aspects of the amoeba genome organization [32]. Progress in the transcriptional profiling of *E. histolytica* to study diverse aspects of amoebic biology, including genes involved in amoebic pathogenesis, has provided interesting data [41, 42]. An important characteristic of the *E. histolytica* genome is the structure and organization of the tRNA genes that are organized into distinct arrays. Consequently, differences between virulent and nonvirulent amoebic strains, changes during stress response, differences in pharmacological responses, and changes during stage conversion are, among others, interesting molecular data that are related to the process of pathogenesis.

23.4 Animal Models to Study Amebiasis

Although there is no animal model that mimics the whole cycle of human amebiasis, the use of different susceptible or resistant laboratory animals and the accessibility, already available for many years, of techniques to culture trophozoites of *E. histolytica* in axenic conditions [43], have improved significantly our understanding of the parasite and the host–parasite relationship. Moreover, the recent introduction of frontier methodologies in biology has increased our comprehension of this parasite. New information on the cellular and molecular biology and genetics of this organism has been extensively reported, and much of this information has constantly required the more frequent necessity of testing in animal models to verify or validate specific molecular or genetic facts.

Based on experimental animals characterized previously, the introduction of new models with genetic, immunological, or surgical modifications has allowed a more adequate analysis of the mechanisms of pathogenesis. Multiple factors have been considered in the promotion of invasiveness and virulence of *E. histolytica*. Presently, the involvement of various substances or molecules in the development of lesions, including lectins, proteases, amoebapores, promoters of apoptosis, cytokines, nitric oxide, etc., are being examined using different in vivo models.

As mentioned, in the absence of an experimental model reproducing the whole parasite cycle, the two most importantly affected organs, intestine and liver, are investigated by separate approaches, and in general using different animal models.

Historically, it can be considered that experimental amebiasis using in vivo models began when Lesh [3] produced intestinal lesions in one of the dogs inoculated with feces from patient suffering from dysentery. Subsequently, numerous studies have been performed using dogs, cats, rabbits, and monkeys; however, their use is limited at present for several reasons including lack of uniformity, difficulty in handling, and insufficient number of animals. From the middle of the last century, easy access to many species of rodents for laboratory research, along with uniform conditions and acceptable susceptibility, has helped to improve our knowledge on the pathogenesis of amebiasis.

23.4.1 Experimental Intestinal Amebiasis

Development of a rodent model for studying intestinal amebiasis has been much more difficult and complex than the hepatic model. Previous reports for intestinal amebiasis in rodents (rats, mice, guinea pigs) have been performed with poorly characterized amoebic inoculum and mixed with other protozoan or multiple bacteria. This deficiency of the exact characterization of inocula, together with the lack of knowledge on the intestinal content of the rodent, are factors that influence the great heterogeneity and poor confidentiality of results, especially when these are related to the analysis of mechanisms of pathogenicity. In spite of these limitations, we believe that there are some useful laboratory animals for intestinal amebiasis, the selection depending on the specific objective sought. Therefore, interesting information regarding the early stages of intestinal pathology and host immune response have been obtained, and much of the current knowledge about different aspects of the disease has come from the integration of data obtained using particular animal models.

The availability of different strains of rodents with variable susceptibility, especially in mice and rats, has provided more information on the mechanisms of pathogenicity for intestinal amebiasis.

Rats have been used for many years for experimental amebiasis, but features reported for intestinal infections have been unclear. Jones [44] reevaluated the use of rats in studies for amebiasis and showed that weaning rats are more susceptible to amebic intestinal infection. A description of intestinal amebic lesions in this

rodent was reported by Neal [45] who proposed the use of the Wistar strain and a scoring system of the amoebic damage based on the macroscopic appearance of the intestinal wall and the characteristics of the luminal content. However, as there was great variability in results, this scoring system is no longer in use.

Guinea pigs have been also used to study intestinal amebiasis, but there are only a few reports concerning the successful production of amebic ulcers when using amoebas cultured in axenic conditions. Anaya-Velazquez et al. [46] described typical intestinal ulcers in guinea pigs and hamsters by producing an artificial cecal loop inoculated with axenic or monoxenic *E. histolytica* trophozoites. Using this model, the authors showed the early stages of amoeba invasion of the mucosal and submucosal layers with substantial inflammatory reaction.

The use of gerbils for experimental amebiasis was introduced by Diamond et al. [47]. However, the application of this rodent in the study of intestinal amebiasis was reported by Chadee and Meerovitch [48], who analyzed the sequence of cecal infection from 1 to 10 days post inoculation. Shibayama et al. [49], using axenic and monoxenic cultures, showed during the first hours of interaction an increase in mucous production mixed with trophozoites in the lumen. Micro-ulcerative mucosal lesions appeared 24 to 72 h post inoculation, characterized by inflammatory infiltrate and edema of the lamina propria associated with the necrotic ulcer. However, as in most ulcerative intestinal lesions observed in other rodents, lesions healed spontaneously after 96 h with complete absence of trophozoites.

In the past, mice were not commonly used as models for amebiasis, the main reason being that these rodents were always considered as naturally resistant to *E. histolytica* infection because a substantial number of attempts to produce intestinal or hepatic lesions were practically unsuccessful. After using multiple strains of mice, Ghadirian and Kongshavn [50] reported different susceptibilities to develop intestinal amebiasis, depending on the strain of mouse employed. In our group we were also able to produce temporal intestinal ulcers using C57BL/6 and C3H/HeJ strains (Fig. 23.5). the authors concluded that genetic factors may be involved in the differences in susceptibility to intestinal infection. Similarly, Owen [51] and Ghosh et al. [52] reported differences in susceptibilities depending on the strain under study. However, it is important to consider, besides the mouse strain under study, other factors such as a diet rich in cholesterol [53], association with bacteria, and the presence of other protozoans [54, 55] that could be influencing the results.

An in vivo neutropenic model using BALB/c mice has been reported, suggesting the role of neutrophils in the innate intestinal resistance to *E. histolytica* infection. Animals treated with anti-neutrophil monoclonal antibody did not show any significant difference in the development of intestinal lesions when compared with control untreated mice. This work reports for the first time the production of granulomatous inflammatory reaction in the intestinal wall of mice when axenically cultured amoebas were inoculated. The presence of this type of chronic inflammatory reaction in an acute stage of amebic intestinal infection is still unknown [56].

Using C3H mice, Houpt et al. [57], inoculated *E. histolytica* trophozoites grown in a bacterial flora of the xenic strain CDC:0784 intracecally. Histopathological changes were evident as early as 4 days after challenge, including crypt hyperplasia,

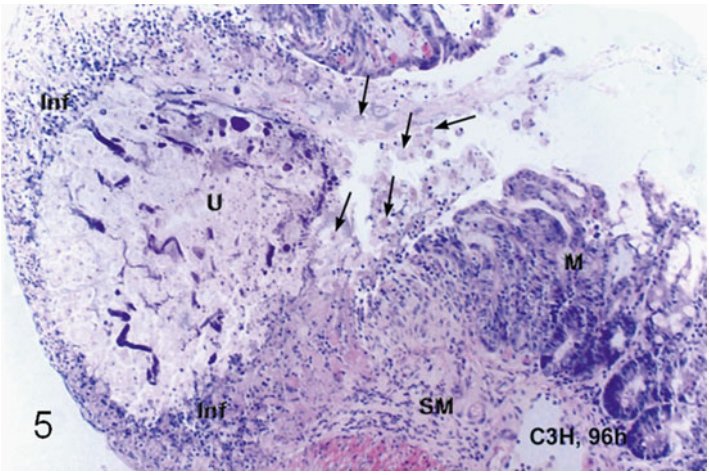

Fig. 23.5 Experimental intestinal amebiasis in C3H/HeJ mouse. After 96 h of intracecal inoculation of *Entamoeba histolytica* trophozoites, intestinal wall shows a typical "flask ulcer" (*U*) containing amorphous and basophilic material and abundant trophozoites (*arrows*). Inflammatory cells (*Inf*) in the mucosa (*M*) and submucosa (SM) are associated with the ulcer. H&E stain. ×20

epithelial ulceration, and submucosal infiltration. At that time, viable amoebas were usually seen in areas of epithelial ulceration, and they were still seen in the intestinal lumen 3 weeks post inoculation. Also, they determined the presence of different cytokines and a depletion of $CD4^+$ T cells. One of the interesting findings is that when $CD4^+$ cells were depleted, both parasite burden and inflammation diminished significantly, correlating with the decrease in IL-4 and IL-13 production and loss of mast cell infiltration. This model revealed important immune factors that may influence susceptibility to the infection and the physiopathology of intestinal amebiasis. Asgharpour et al. [58] used the same mouse model and compared the findings with those with C57BL/6 (a resistant mouse). They examined the role of neutrophils in the course of amebic colitis (C3H/HeJ) and found that in neutrophil depletion, using an anti-Gr-1 monoclonal antibody and dexamethasone treatment, the innate resistance diminished in certain mouse strains (e.g., CBA). However, there was no effect on the high level of resistance of C57BL/6 mice, suggesting that the mechanisms of innate immunity to intestinal *E. histolytica* infection vary depending on the host genetic background.

An interesting model for amebic colitis has been introduced for experimental studies [59]: using SCID mice, a fragment of human intestine was implanted into the subcapsular region (SCID-HU-INT). This human intestinal xenograft was allowed to mature into a morphologically, mucin-secreting normal segment, which was then infected with *E. histolytica* trophozoites. They found that after 12 h post inoculation, the intestinal xenograph developed focal ulcerations with invasion of the submucosa similar to that seen in humans with amebic colitis. Using this xenograph model, the same authors suggested that cysteine proteinase (ehcp5) of *E. histolytica* trophozoites affects the mucosa with a neutrophil-predominant inflammation.

An ex vivo human intestinal model to study *E. histolytica* pathogenesis was introduced by Bansal et al. [60]. To analyze the molecular mechanisms underlying human colon invasion, the authors acquired segments of human colon, which were dissected under a stereomicroscope to remove the fat and muscle and retain the mucus, the mucosa, and the submucosa. The explants were pinned onto a 4 % agarosa layer in tissue culture Petri dishes. Trophozoites from different strains were added to the luminal face of the colon and incubated in KREBS medium at 37 °C for different times. Besides analyzing the histology, authors studied multiple molecules, including lactate dehydrogenase, and cytokines such as IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF-γ, and TNF. Interesting data related to molecular mechanisms during colon invasion have been reported and are discussed by the authors in a respective section.

Another experimental model that uses porcine colon explants as an alternative to human tissue [61] or a surgical modification of the pig intestine by producing a washed closed jejunal loop [62] has been reported. The authors have considered these models as new potential tools to study intestinal amebiasis and inclusive amebic liver abscess, which was produced after injecting amoebas in the portal vein and directly into the liver parenchyma.

23.4.2 Experimental Hepatic Amebiasis

The first laboratory animal used successfully to produce amebic liver abscess (ALA) was the hamster. Reinertson and Thompson [63] injected *E. histolytica* trophozoites cultured with three species of bacteria either directly into the hepatic lobe or through the portal vein, producing large amebic abscesses. Posterior studies using the same rodent, but inoculated with axenic amebas, confirmed the high susceptibility and the uniformity of lesions [64]. From these initial in vivo studies, different authors have obtained multiple data regarding pathogenesis of amebic damage, including aspects of the host immune response and many other factors involved in in vivo host–parasite interactions.

A sequential morphological study of hepatic infection from the very early stages of portal inoculation with *E. histolytica* trophozoites in hamsters was reported almost 30 years ago [65]. Based on this work and posterior ultrastructural studies [66], the authors suggested, for the first time, the role of host inflammatory cells in the process of tissue damage (Fig. 23.6). They suggested that *E. histolytica* trophozoites do not produce ALA through direct lysis of hepatocytes; instead, tissue damage is mediated by lysosomal enzymes released from disintegrating inflammatory cells that accumulate around the amoebas and are killed by the parasite. This original finding broke the old-time paradigm of considering that amebic liver (and other tissues) lesions produced by *E. histolyitca* do not practically produce inflammatory reaction. These studies have been the bases for numerous subsequent studies investigating the role of inflammation as an important factor of host tissue destruction.

Gerbils are another rodent highly susceptible to hepatic amebiasis [67]. Lesions can be obtained similarly as those produced in hamsters, although *E. histolytica*

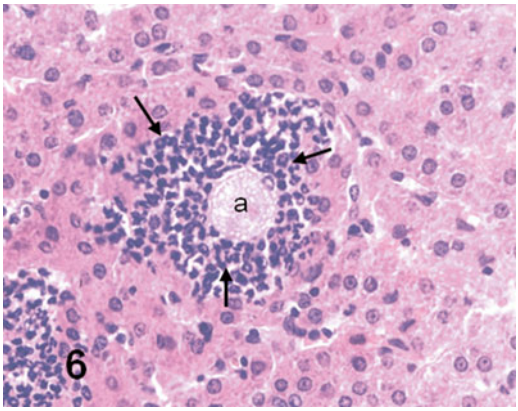

Fig. 23.6 Light microscopy shows a focus of inflammatory reaction (*arrows*) surrounding an *Entamoeba histolytica* trophozoite (*a*). Hepatocytes closely associated to the infiltrate appear slender with more eosinophilic cytoplasm. H&E stain. ×40

trophozoites apparently show a less virulent behavior in gerbils than in hamsters. In addition to the association of inflammatory cells with trophozoites as shown in hamster liver, gerbils also showed trophozoites in direct contact with hepatocytes. Studies in host immune response and the effect of various vaccine candidates purified from the parasites and administrated parenterally or orally have been tested [68]. Production of ALA by inoculating *E. histolytica* trophozoites by the intraperitoneal route has also given the possibility of using this model when a large amount of animals with ALA are necessary for pharmacological testing or inclusive also for immunoprophylactic purposes [69].

The use of mice in hepatic amebiasis has provided some interesting data. Using SCID mice Cieslak et al. [70] developed liver abscesses when animals were challenged intrahepatically with virulent *E. histolytica*. In this study, only one of seven similarly challenged immunocompetent congenic CB-17 mice (control) developed abscess.

To determine whether inflammatory cells play a similar role in mice as observed in hamster and gerbil livers, BALB/c mice were treated with anti-neutrophil RB6-8C5 monoclonal antibodies and then challenged intrahepatically with *E. histolytica* trophozoites. Animals were killed at different time points, and hepatic analysis showed that neutrophil-depleted mice presented abscesses larger than in untreated animals. Based on previous data, the authors suggested that neutrophils may play a role in resistance mechanisms in mice, dissimilar to hamsters or gerbils; however, it is important to mention that mice are, in general, resistant to amoebic infection. In both RB6-8C5-treated and -untreated mice challenged similarly, animals survived the infection, although the treated animals required a longer time to heal completely from the hepatic lesion [71].

Using immunohistochemistry in BALB/c and C3H/HeJ strain mice, it has been suggested that mouse resistance to develop liver abscesses depended on the activation of neutrophils, and this in turn was related to abundant nitric oxide production to kill the amoebas. However, contradictory roles of nitric oxide have been mentioned, mainly related to whether this molecule is participating in resistance or susceptibility

caused by the inflammatory reaction. A different study, using hamsters, has shown that this molecule plays no role in arresting the development of amebic liver abscesses. The levels of nitric oxide measured by the presence of nitrites and nitrates in serum were directly proportional to the size of abscesses, suggesting that nitric oxide does not have a lytic effect on *E. histolytica* and is, therefore, incapable of providing protection against the hepatic lesion [72].

Regarding the role of apoptosis in hepatic damage, Velazquez et al. [71] found areas of TUNEL-positive dead hepatocytes in neutrophil-depleted mice. These apoptotic liver cells were found either close to inflammatory infiltrates or at some distance free of amoeba or inflammatory cells. It is known that inflammatory conditions may cause apoptosis or that amoebas may release apoptosis-inducing factors that affect hepatocytes at a distance [73]. Ischemic status, activation of caspase, and amoebapores, among other factors, have been considered as necessary for both lytic and apoptotic pathways of cell death. Moreover, it is suggested that sublytic concentrations of pore-forming proteins can induce apoptosis in target cells [73].

Other important molecules, as already mentioned, are the proteinases. Studies reported by Olivos-García et al. [74] suggested that cysteine proteinase has either a minor role or no role in the damage of liver parenchyma during the development of ALA.

In reference to the hepatic changes that may produce *E. dispar* trophozoites, the initial study performed using the strain SAW760 of *E. dispar* in axenic conditions showed early inflammatory foci around trophozoites, including inflammatory cell damage [75]. However, lesions did not progress to ALA, and only small areas of inflammatory infiltrates were observed after 24 h. Other recent studies, however, using different strains of *E. dispar* in xenic or monoxenic conditions, have reported the production of ALA [76, 77]. The possible effect of bacteria in the virulence of these isolates has been suggested, although experimental animals were treated with antibiotics to eliminate the effect of bacteria in the virulence as discussed previously in "Pathogenesis" in this same chapter.

23.5 Conclusions

In spite of the abundant information obtained using different in vitro and in vivo experimental procedures, many aspects related to the mechanisms of invasion by *E. histolytica* trophozoites are still unknown. To damage the host's target tissues, amoebas have to adapt, inhibit, modify, or evade the multiple factors present in the host intestinal microenvironment, such as intestinal microbiota, including commensal and pathogenic bacterial flora, cytokines, reactive oxygen species, and availability of oxygen, glucose, and iron, among other factors. Moreover, virulent amoebas activate and express genes in response to the stress produced by the inflammatory reaction of the host that are essential for the invasive process.

Recent information regarding ALA production by *E. histolytica* has been focused on the programmed cell death of hepatocytes, which is probably regulated by pro-inflammatory cytokines and other molecules, such as ROS and nitric oxide;

on the other hand, it has also been shown that the parasite itself may also suffer a programmed cell death, both at the intestine and liver, triggered by different environmental factors.

Better knowledge of these mechanisms is necessary to have a clearer picture of the basic mechanisms taking place in the pathogenesis of amebiasis and to begin developing new therapeutics and efficient prophylactic procedures.

Acknowledgments The authors gratefully acknowledge Ms. Silvia Galindo-Gómez, Angélica Silva-Olivares, Karla Gil-Becerril, and Claudia Pérez-Galindo for their technical assistance. The present work was partially supported by SEP-CONACyT (México), grant No. 128317 to MS.

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Chapter 24
Innate Host Defenses in the Gut

Leanne Mortimer and Kris Chadee

Abstract In the human intestine, evolutionary pressures have selected host and parasite mechanisms that maintain spatial separation of *Entamoeba histolytica* on the luminal side of the mucus–epithelial barrier. The function of the barrier is conferred by many systems acting on multiple levels. Mechanisms that strengthen and maintain stability of the epithelial barrier are critical for preventing disease and keeping *E. histolytica* infections asymptomatic. It is unknown why invasion happens. Intestinal epithelial cells are in close and continuous proximity to the parasite, and abnormal responses by epithelial cells are suspected to instigate disease. This interaction, however, is poorly understood. When invasion occurs the gut has a second line of innate defenses that rapidly eliminate the parasite: sensing of invasion by resident cells, innate humoral immunity, and recruitment of competent immune cells to sites of invasion. The pathology that arises during invasion, which culminates as amebic dysentery or colitis, is a combined effect of direct damage by trophozoites and collateral damage from host defenses.

24.1 Intestinal Barrier Integrity

Humans normally have a high capacity to tolerate colonization by *Entamoeba histolytica* without disease. Pathological *E. histolytica* infections are rare events that occur less than 10 % of the time [1]. Trophozoites are normally sequestered on the luminal side of the colon where they remain attached to the mucous layer via binding of the *E. histolytica* galactose/*N*-acetyl galactosamine-inhibitable lectin (Gal-lectin) to mucus oligosaccharides [2]. In this scenario, the host perceives the level of threat from *E. histolytica* to be low and does not wage a costly immune response to eliminate the parasite. The effectiveness of this strategy depends on the integrity of the epithelium and overlying mucous layer to create a physical barrier that separates *E. histolytica* from direct contact with host tissue (Fig. 24.1).

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_24

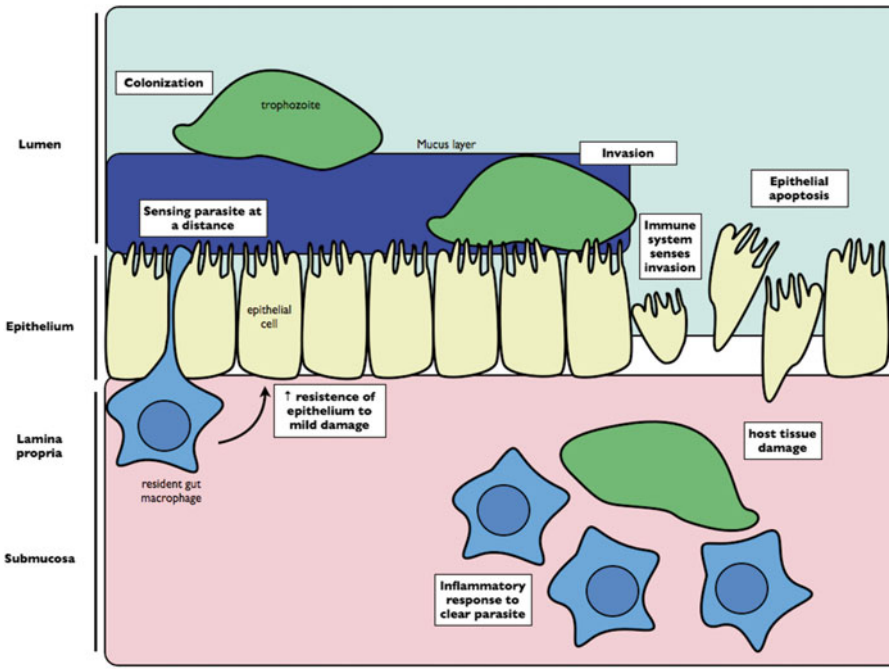

Fig. 24.1 Trophozoites are normally sequestered on the luminal side of the colon where they remain attached to the mucous layer. In this scenario, host cells sense the parasite at a distance. Mechanisms that strengthen and maintain stability of the epithelial barrier are critical for preventing disease and keeping *Entamoeba histolytica* infections asymptomatic. Trophozoites secreted components precondition the barrier and bolster it against noxious stimuli via an interaction with resident gut macrophages and epithelial cells. The trigger for invasion is unknown. Upon direct contact with the parasite, epithelial cells rapidly lose mitochondrial outer membrane integrity, and a program of caspase-3 dependent apoptosis is initiated. The immune system mounts an intense antimicrobial response when it senses disruption of the mucus–epithelial barrier and direct contact between the parasite and host tissue. The pathology that arises during invasion, which culminates as amebic dysentery or colitis, is a combined effect of direct damage by trophozoites and collateral damage from host defenses

The mucus layer, composed of highly glycosylated proteins, forms the first major physical barrier between host cells and the parasite. The colonic mucus is thick and striated into a dense inner layer, which few microbes penetrate, and a heavily colonized loose outer layer [3]. *E. histolytica* presumably resides in this outer layer under normal conditions. It is unclear how the mucus layer is maintained during colonization, as trophozoites secrete glycosidases that cleave mucous oligosaccharides, leaving the mucin protein exposed to *E. histolytica* cysteine proteases that break covalent bonds between mucin monomers which hold the mucous gel intact [4, 5].

A single layer of epithelial cells forms the cellular component of the intestinal barrier. In experimental amebic colitis, genetic ablation of pathways that regulate normal tissue architecture, homeostasis, and function of the intestinal epithelium renders naturally resistant mice highly susceptible to amebic colitis [6–10]. Provided the epithelium is preserved, resistance precludes innate mechanisms of microbe

detection and killing that are protective in invasive situations against *E. histolytica* [6, 11, 12]. In the opposite scenario, mouse strains that succumb to intestinal *E. histolytica* infection develop extensive epithelial apoptosis at sites of invasion [7–10]. On direct contact with the parasite, epithelial cells rapidly lose mitochondrial outer membrane integrity and a program of caspase-3-dependent apoptosis is initiated. Inhibition of this cell death pathway by caspase-3 deletion or overexpression of the antiapoptotic factor Bcl-2, which prevents outer mitochondrial membrane permeablization, preserves the epithelium and prevents *E. histolytica* from causing invasive disease [7].

In humans a common polymorphism in the leptin receptor gene, *LEPR*, confers susceptibility to intestinal amebiasis in children likely because of diminished pro-survival and proliferative signaling in the intestinal epithelium [8, 9, 13]. In mammals, leptin plays an important role in regulating food intake by acting on the central nervous system (CNS), but also functions in host defense by promoting inflammatory responses and maintaining integrity of the intestinal mucosal barrier [14–17]. The susceptibility allele for intestinal amebiasis substitutes an arginine for a glutamine and produces a dramatic phenotype in the intestinal epithelium in vivo. In experimental amebic colitis, homozygosity for the arginine allele enhances epithelial apoptosis and impaired epithelial replication that results in severe damage to the mucosal barrier upon challenge with *E. histolytica* [9].

Normally, *E. histolytica* lives in the gut in close proximity with the intestinal epithelium. To maximize its own survival, the parasite has probably evolved strategies to promote epithelial tolerance to avoid triggering unwanted immune responses. A way to achieve this would be to precondition the epithelial barrier and bolster it against noxious stimuli. In vitro parasite-secreted components suppress activation of NFκB p65 and increase resistivity to apoptosis in the colonic epithelium [18], which occurs via a heat-shock protein (Hsp)-mediated stress response that is indirectly mediated by macrophages. Preconditioning of the epithelium may be important for raising the threshold required to trip a full immune attack against *E. histolytica* by dampening pro-inflammatory signals and rendering epithelial cells more resistant to either parasite- or host-inflicted damage (Fig. 24.1). In this regard, prostaglandin E_2 secreted by trophozoites may also confer protection on the epithelium. *E. histolytica* constitutively expresses a functional cyclooygenase that synthesizes prostaglandin E_2 (PGE_2) from the precursor arachidonic acid, which is usually present at high concentrations in the gut [19, 20]. The functional significance of PGE_2 production by *E. histolytica* may vary under different conditions, but PGE_2 is well known to enhance survival and resistance of intestinal epithelial cells to various forms of stress [21].

24.2 Intestinal Barrier Disruption

It is not known whether intestinal amebiasis is initiated by a failure of host resistance or a failure of host tolerance toward *E. histolytica*. Nevertheless, the immune system perceives invasion and turns on an intense antimicrobial response when it

senses disruption of the mucus–epithelial barrier and direct contact between the parasite and host tissue (Fig. 24.1).

Acute amebic dysentery accounts for more than 90 % of the disease caused by *E. histolytica* [22]. An episode of dysentery is, however, usually sufficient to eliminate the offending parasites. Provided dehydration and electrolyte imbalance are endured, secretory diarrhea and acute inflammatory responses are important secondary host defenses when the intestinal barrier fails. In rare instances amebic colitis develops gradually over a period of weeks. At these stages, there is infiltration of neutrophils, macrophages, leukocytes, and plasma cells surrounding the infection site [23–25]. For reasons that are not entirely clear, the host does not mount a protective immune response. Patients with amebic colitis often have multiple discrete lesions of varying stages. Symptoms are variable, but most commonly manifest as mild to moderate abdominal discomfort/pain and frequent loose watery stools containing variable amounts of blood and mucus. Pathology ranges from superficial erosion of the colonic mucosa to deep flask-shaped ulcers with edges that undermine the mucosa and extend deep into the muscularis and serosa [23, 24]. The pathologies of both amebic dysentery and amebic colitis arise from the combined effect of direct damage inflicted by the parasite and collateral damage from the immune response (immunopathology).

24.3 Parasite-Inflicted Damage

Entamoeba histolytica received its Latin name for the ability of the trophozoites to destroy tissue (*histo-lytic*) [24]. Its capacity to directly damage tissue and to engulf large amounts of cell debris underlies the parasite's potential to invade, achieved by causing mass cell death, breakdown of the extracellular matrix and phagocytosis. When live trophozoites directly contact host cells, many succumb to rapid death. The parasite then immediately proceeds to phagocytose the cellular corpses. During bacterial infections of the gut that cause massive epithelial cell apoptosis, engulfment of infected cells undergoing apoptosis by host phagocytes has an important role in promoting inflammatory responses [26]. Thus, rapid clearance of cellular debris by trophozoites may actually dampen inflammation and could facilitate immune evasion at later stages of disease.

The cellular mechanism of death induced by *E. histolytica* has been studied in a number of cell types and indicates the parasite triggers contact-dependent apoptosis as evidenced by exposure of phosphatidylserine on the outer cell membrane, chromatin condensation, DNA fragmentation, and membrane blebbing [27–33]. Within 30 s of contact, *E. histolytica* induces a rapid and irreversible rise of intracellular calcium concentration in target cells [34]. The surface adhesin, Gal-lectin, which binds *E. histolytica* to the mucous layer and the surface of host cells, elevates intracellular calcium and inhibition of the Gal-lectin blocks cytotoxicity, suggesting Gal-lectin engages an unknown host cell receptor as part of this cell death pathway [35]. The mechanism does not signal through Fas or tumor necrosis factor (TNF) death receptors nor does it activate the upstream caspases of apoptosis, caspases 8 and 9 [32, 36].

In some systems overexpression of the antiapoptotic factor Bcl-2 does not affect apoptosis, whereas in vivo it prevents apoptosis of the intestinal epithelium during intestinal challenge with *E. histolytica* and consequently severe disease does not develop [7, 27]. In vivo, it is unresolved whether Bcl-2 protects from host inflammation-mediated apoptosis or apoptosis triggered directly by the parasite. Thus, it is not possible to conclude whether different apoptotic programs are triggered in different cell types by *E. histolytica*. It is, however, likely that cells have different sensitivities to death upon contact with the parasite. In neutrophils, inhibition of $\alpha_M\beta_2$ (CD11b/CD18) integrin and generation of reactive oxygen species has some effect blocking apoptosis [37]. The pathway seems to be specific for $\alpha_M\beta_2$, as inhibition of other β_2 integrin pairs or β_1-containing integrins that are present on neutrophils has no effect. At the downstream end, direct *E. histolytica-induced* apoptosis converges on loss of outer mitochondrial membrane integrity and activation of caspase-3 [7, 27, 36]. In addition to apoptosis, some host cells die by necrosis upon contact with *E. histolytica*, as one study found parasites triggered necrosis in human myeloid cells [38]. However, the mechanism has not been further investigated.

The enteric nervous system (ENS) is particularly sensitive to destruction by *E. histolytica*. Enteric neurons are the only cell types known to die of exposure to *E. histolytica*-secreted components and preparations of dead parasites, as all other cells require direct contact with live trophozoites to trigger death [39]. In enteric neuron cocultures, secreted components and lysed trophozoites cause axonal disintegration and necrotic-like death of neurons [39]. Other cell types in these cocultures, such as glia and smooth muscle cells, survive. The cytotoxic factor remains an unidentified cysteine protease of *E. histolytica* other than the major virulence factor *Eh*CP5, which is the most highly expressed cysteine protease of in vitro cultured trophozoites. In vivo intestinal invasion by the parasite causes axonal damage and de-innervation in inflamed regions [39]. Thus, parasite-inflicted damage of the ENS may be an important aspect of pathology in invasive amebiasis of the gut.

In addition to causing massive cell death, *E. histolytica* directly damages the gut by secreting proteases that degrade the extracellular matrix onto which cells adhere, and whose function is to maintain structure and organization of the tissue. Purified cysteine proteases from the parasite cleave the major components of the extracellular matrix, including collagen, fibrinogen, laminin, and elastin [40, 41]. In cell cultures *E. histolytica* cysteine protease activity causes detachment of cell monolayers from the substratum, and in the gut, parasites that are deficient for cysteine proteases are unable to cause tissue destruction and invade [42–44].

24.4 Sensing Invasion, Inflammation, and Host Immunopathology

Acute inflammation usually forms part of a protective host defense in response to microbial invasion or tissue trauma. In the case of microbial invasion, the goal is to act quickly and effectively to prevent the spread of infection. Inflammatory mediators released by resident cells at the affected site trigger recruitment of immunocompetent

cells and plasma proteins from the blood. These components, which are normally restricted in blood vessels, gain access to the infected extravascular tissue, and if successful, help eliminate the infectious agent and initiate tissue repair processes that restore normal tissue function. Many actions of acute inflammation do not discriminate between microbes and host tissue, so collateral damage is unavoidable and contributes to the pathology of disease. Immunopathology is a significant component of the pathology during invasive *E. histolytica* infections. It is debatable whether robust immune responses toward *E. histolytica* inflict more harm than good, or whether immunopathology is in fact the main cause of disease.

24.4.1 Epithelial Cells

The luminal face of the intestinal epithelium is exposed to high levels of microbes and their products, and epithelial cells are adapted to be somewhat anergic to noxious stimuli. Under homeostatic conditions, epithelial cell responses to gut microbes are regulatory in nature and tend to limit microbe growth by noninflammatory means, such as release of antimicrobial peptides into the lumen. Yet, epithelial cells still distinguish between colonization and invasion and actively participate in generating inflammatory responses toward pathogens. *E. histolytica* elicits proinflammatory responses in epithelial cells, and in vivo activation of innate immune pathways in intestinal epithelial cells can either help eliminate the parasite and restore homeostasis or exacerbate disease by destabilizing the barrier.

Similar to immune cells, intestinal epithelial cells express innate immune receptors, although their repertoire is less diverse. Invasion sensing by these innate receptors converges on the NFκB system to drive proinflammatory responses. NFκB is a pleiotropic transcription factor that regulates many cellular functions, including key aspects of inflammation. It forms homo- and heterodimeric transcription factors consisting of five possible subunits, p65, p50, p52 RelB, and c-Rel, which are sequestered in the cytoplasm when inactive. Activation of canonical p65–p50 heterodimers leads to their nuclear translocation and transcription of genes that turn on inflammatory responses. Epithelial recognition of the parasite involves binding of a three-amino-acid RGD sequence contained in the virulence factor, *Eh*CP5, to integrins on the surface of colonic epithelial cells [45], thus activating integrin-Akt signaling and leading to NFκB induction of pro-inflammatory genes. In a human intestinal graft model, epithelial cell p65 initiates innate proinflammatory responses that cause intestinal damage and permeabilize the epithelial barrier upon challenge with *E. histolytica*. Inhibition of epithelial p65 expression prevents *E. histolytica* from eliciting a robust inflammatory response, as measured by IL-1β, IL-8, and neutrophil infiltration [32]. When epithelial p65 expression is blocked, intestinal pathology and parasite invasion are prevented, suggesting an overly robust proinflammatory response arising from epithelial cells can be injurious and permit parasite invasion.

Although the NFκB system initiates inflammatory responses toward *E. histolytica*, some of its functions are probably more restrained. In experimental amebic colitis both susceptible and resistant strains of mice challenged with *E. histolytica* have robust nuclear translocation of the NFκB p50 subunit. When p50 is deleted, epithelial integrity is rapidly lost upon exposure to the parasite, and as a result the gut becomes highly susceptible to invasion [10]. p50 possibly activates a noninflammatory restorative processes in the epithelium. This concept, however, is only speculation.

24.4.2 Complement

When *E. histolytica* penetrates the epithelial barrier it is instantly exposed to humoral components of host defense. Complement is a central part of innate humoral immunity. Complement is a system composed of soluble plasma proteins and cell-surface receptors whose main function is to bind the surfaces of pathogens and mediate direct pathogen destruction by formation of a membrane attack complex, thus to clear pathogens and their products by opsonization with host proteins that target them to receptors on phagocytic cells [46]. Several cleaved protein fragments of the complement cascade, termed anaphylatoxins, are potent activators of inflammation. They trigger release of histamine from mast cells, lysosomal enzymes from leukocytes, and proinflammatory cytokines including IL-6 and tumor necrosis factor (TNF)-α [47–49]. They also increase vascular permeability and attract neutrophils, monocytes, eosinophils, mast cells, basophils, and activated B cells and T cells to sites of activation [48, 49]. The complement cascade can be activated by either the alternative pathway or the classical/mannose-binding lectin pathway. In vitro *E. histolytica* activates both these pathways and, interestingly, parasite cysteine proteases directly activate the alternative pathway of complement by cleaving C3 into functional C3b [50]. Therefore, it can be assumed that complement mediates some host detection and inflammatory events during *E. histolytica* invasion of the gut.

In turn, the ability of *E. histolytica* to avoid complement may underlie part of its success as a pathogen. Parasites recovered from amebic colitis are resistant to complement-mediated killing by the C5b-9 membrane attack complex [50], and in vitro this resistance can be induced by culturing *E. histolytica* in media containing serum [51]. *E. histolytica* Gal-lectin has epitopes of the complement inhibitory protein, CD59, which in humans prevents self-lysis by inhibiting the formation of the membrane attack complex [52]. The thick glycolax composed of lipophosphoglycans (LPG) and lipophosphopeptidogylcans (LPPG) on the parasite surface may also afford parasites protection by creating a layer impenetrable by complement components. When GPI anchor synthesis is blocked, resulting in disrupted localization of Gal-lectin and LPG/LPPG, *E. histolytica* becomes highly susceptible to complement-mediated killing [53]. *E. histolytica* also attenuates anaphylatoxin-triggered inflammation by cleaving and inactivating C5a and C3a via cysteine proteases [54], which may have a role in immune evasion in chronic amebic colitis.

24.4.3 Neutrophils

Neutrophils are the most abundant immune cells in the blood and are the first cells dispatched to sites of infection. The activated endothelium of postcapillary venules interacts selectively with neutrophils through selectins, integrins, and chemokine receptors to promote their extravasation. When they reach infected sites they become activated by contact with pathogens or cytokines released from tissue-resident cells. Their primary function is to isolate, kill and engulf invading microbes [55]. For extracellular pathogens such as *E. histolytica*, neutrophils do this by releasing reactive oxygen and nitrogen species and the toxic contents of their granules toward the pathogen. Their actions are further concentrated by releasing neutrophil extracellular traps, which are sticky webs of chromatin covered in antimicrobial molecules that ensnare invading microbes [55]. Neutrophils will phagocytose and eliminate microbes and debris whose surfaces are opsonized with molecules such as complement and IgG via CR3 (complement receptor 3) and FcγR receptors.

When *E. histolytica* invades the gut, neutrophils are critical for limiting infections. In amebic colitis, neutrophil depletion causes partially resistant strains of mice to become highly susceptible to invasion and develop severe intestinal disease when challenged with *E. histolytica*. In vitro, parasites are susceptible to killing by reactive oxygen species produced by activated neutrophils [56, 57]. If neutrophils are activated with interferon (IFN)-γ and TNF-α, they can kill parasites primarily by H_2O_2-dependent mechanisms [58] and they become highly resistant to *E. histolytica-induced* contact-dependent cell death [58]. If neutrophils are not activated, however, they rapidly succumb to *E. histolytica* at an extremely low ratio of parasites to neutrophils [59]. Highly virulent strains of *E. histolytica* are less susceptible to reactive oxygen [56, 57]. For defense, the parasite expresses several enzymes that detoxify the oxidative burst, including a superoxide dismutase, a bifunctional NADPH:flavin oxidoreductase that converts superoxide anions to H_2O_2, and a surface perioxiredoxin that localizes to the point of contact with host cells and converts H_2O_2 into water [60–64].

Early studies examining neutrophil–parasite interactions suggest neutrophil granule contents are ineffective at killing trophozoites. It has been suggested that if the invading parasites can resist oxidative killing, neutrophil degranulation may cause significant bystander damage that facilitates invasion [65, 66]. This concept is based on in vitro studies showing that neutrophils elevate destruction of cell monolayers incubated with *E. histolytica* and that monolayer destruction is not reduced by adding catalase (H_2O_2 scavenger) or neutrophils from patients with granulomatous disease, which are unable to produce an oxidative burst but are still capable of releasing granules [66]. Other nonoxidative mechanisms of neutrophil-mediated host defense may contribute to host defense but these have not been studied.

24.4.4 Macrophages

The intestinal lamina propria harbors the largest reservoir of macrophages in the body [67]. As an adaptation to the sea of microbes and the antigen-rich environment, resident gut macrophages fulfill host defense functions by engulfing and degrading microbes and debris that have crossed the epithelial barrier without inciting inflammatory responses. Normal gut macrophages lack respiratory burst activity and the mechanisms by which they kill microbes that penetrate the epithelial barrier appear to be independent of reactive oxygen [67]. *E. histolytica* is about two to three times larger than a macrophage and thus probably too large to be phagocytosed while still alive by the gut resident macrophages. It is not known whether this population of macrophages can kill *E. histolytica*, but it seems unlikely given the importance of an extracellular respiratory burst to kill trophozoites. When the epithelial barrier is breached during intestinal infection, however, fresh monocytes are recruited from the blood to the lamina propria and differentiate into macrophages that produce reactive oxygen and mount inflammatory responses [67]. It is more likely that monocytes recruited in response to *E. histolytica* invasion participate with neutrophils to eliminate the parasite.

Activated inflammatory macrophages that are stimulated with IFN-γ and TNF-α produce large amounts of reactive nitrogen in addition to oxygen radicals. Reactive nitrogen is toxic to trophozoites and inhibits activity of the highly destructive cysteine proteases of the parasite [68, 69]. In vivo, high-output production of nitric oxide, via the gene encoding inducible nitric oxide synthase (iNOS), is required to control invasive infections [70]. However, in chronic amebiasis there are defects in cell-mediated immunity [71, 72], especially impaired macrophage functions. Macrophages from chronic lesions are locally refractive to IFN-γ activation and inflammatory stimuli, do not produce inflammatory cytokines, and are unable to kill trophozoites [71–74]. Trophozoites secrete proteins that decrease macrophage expression of iNOS, which reduces high-output production of reactive nitrogen and impairs trophozoite killing [73]. The parasite also consumes the metabolic precursor of nitric oxide, L-arginine, via an arginase that hydrolyzes L-arginine to L-ornithine and urea [75]. *E. histolytica* also suppresses an inflammatory phenotype in macrophages by inducing biosynthesis and signaling of PGE_2 [73].

During an invasive infection, neutrophils and macrophages will each produce reactive oxygen and nitrogen species. Given the fact that killing of *E. histolytica* is extracellular, this type of defense, although critical to eliminate the parasite and ultimately protect the host, will cause a significant amount of oxidative stress. Many host cells die of free radical damage, which will contribute significantly to the immunopathology of amebic colitis.

24.5 Summary

Finding a common factor underlying invasion by *E. histolytica*, and therefore understanding the genesis of disease, is difficult. Colonization remains virtually unknown because there is no experimental model of colonization for the parasite. Studies of the intestinal microbiota reveal commensalism results from complex systems interacting on many levels. For *E. histolytica*, we know that protecting and maintaining the epithelial barrier is a requirement for host resistance and that both host and parasite employ mechanisms to adapt the epithelium to parasite challenge. During invasion the parasite directly damages host tissue, but can avoid host defenses by suppressing inflammatory responses. Host inflammatory responses in the gut are generally sufficient to eliminate invading parasites but also cause significant tissue damage that may escalate disease and promote invasion in some situations.

Acknowledgments The research presented in this chapter was supported by grants from the Natural Science and Engineering Research Council of Canada (NSERC) and the Canadian Institute for Health Research (CIHR). L.M. is supported by studentships from NSERC and Alberta Innovates Health Solutions. K.C. holds a Tier 1 Canada Research Chair supported by CIHR.

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Chapter 25
Cysteine Peptidases in Pathogenesis

Iris Bruchhaus and Jenny Matthiesen

Abstract *Entamoeba histolytica* is characterized by its extraordinary capacity to invade and destroy human tissues. The main lytic activity has been attributed to cysteine peptidases, and a number of studies have shown that cysteine peptidases constitute major pathogenicity factors in *E. histolytica*. Interestingly, although most of the classes of peptidases are present in *E. histolytica*, only cysteine peptidases, and on a lesser scale, metallo-peptidases and serine peptidases, have been adequately studied. In this chapter, the peptidase families of *E. histolytica* are introduced, and their involvement in colonic invasion and in liver abscess formation are discussed.

25.1 Introduction

For several protozoan parasites, it is known that peptidases, and especially cysteine peptidases, play an important role in their life cycle or for their virulence.

Entamoeba histolytica is characterized by its enormous capacity to destroy and invade human tissues. This ability is mainly attributed to their cysteine peptidases, which have been shown by various in vitro and in vivo studies [1–9]:

1. Fibroblast monolayers are disrupted by purified cysteine peptidases [3], likely because of their ability to degrade extracellular matrix components [5, 8, 10, 11];
2. A direct correlation between cysteine peptidase activity and pathogenicity was observed [2, 3, 6, 12];
3. The abscess formation can be inhibited by the use of specific cysteine peptidase inhibitors [4, 9];
4. It has been postulated that secreted cysteine peptidases interfere with the host immune system by cleaving immune molecules such as IgG and IgA [11, 13, 14], processing of complement C3 [15, 16], inactivation of complement C3a and C5a [17], inactivation of pro-IL-18 [18], and generation of mature IL-1β from its pro-form [19];

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_25

5. In addition, *Entamoeba* transfectants overexpressing or silencing individual cysteine peptidase genes are greatly altered in their ability to induce amoebic disease [1, 19–21].

25.2 History

McLaughlin and Faubert [22] gave the first description of cysteine peptidase activity as a neutral sulfhydryl protease [22]. After that, a similar activity was characterized in additional studies and named as cytotoxic protease [23], cathepsin B [6], neutral proteinase [24]; histolysin/histolysain [5, 25], and amoebapain [8, 26, 27]. Although the corresponding proteins differ slightly in their molecular weight and enzymatic properties, it was postulated that all these are related [28].

Subsequent molecular cloning revealed a large number of cysteine peptidase genes in the *E. histolytica* genome [29–35]. Until 2003, in total 20 genes coding for cysteine peptidases of *E. histolytica* were described, all of which belonged to the C1 papain superfamily of cysteine peptidases [30].

In 2005 the genome of *E. histolytica* was sequenced [36]. Surprisingly, in silico analyses indicated the existence of approximately 86 genes coding for cysteine-, serine-, metallo-, and aspartic peptidases [37, 38]. In contrast to the cysteine peptidases, very little is known about the other peptidase families of *E. histolytica*.

25.3 Peptidase Families

25.3.1 Serine Peptidases

The genome analyses indicated 11 genes belonging to the serine peptidase family, which can be grouped into clan SC including the family S9 (five members) and S28 (three members), clan SF with the family S26 (two members), and clan ST with the family S54 (EhROM1) [38, 39].

Only three of the corresponding serine peptidases (EhSP28-1, EhSP28-2, EhROM1) had been characterized previously [39, 40].

The two serine peptidases EhSP28-1 and -2 are able to degrade the substrate Suc-AFF-AMC. They can be inhibited by EDTA, they are present as homodimers and homotetramers, and are found associated with the trophozoite membrane [40].

In the *Entamoeba* genome only one functional rhomboid peptidase was identified. EhROM1 is an intramembrane peptidase, which is localized at the trophozoite surface, to vesicles during phagocytosis and the uroid during cap formation. Transcriptional silencing of *ehrom1* implies an involvement in phagocytosis and adhesion. Furthermore, it was shown that the rhomboid peptidase can cleave the surface-associated amoebic Gal/GalNac lectin and thus may be involved in receptor shedding. In summary, it is postulated that EhROM1 is involved in immune evasion [39, 41].

25.3.2 Aspartic Peptidases

Only four genes coding for aspartic peptidases were found in the *Entamoeba* genome, all belonging to clan AD and family A22. They are homologous to intramembrane-cleaving peptidases such as presenilins [38]. Nevertheless, a respective activity has not been described for *E. histolytica* so far.

25.3.3 Metallo-Peptidases

A large number of genes encode putative metallo-peptidases, which can be assigned to seven clans in total including 11 families: clan MA with the families M1 (one member), M3 (two members), M8 (two members), and M48 (one member); clan ME containing one member of the family M16, clan MG containing six members of the family M24, clan MH with the families M18 (two members) and M20 (four members), clan MK containing one member of the family M22, clan M containing one member of the family M49, and clan U containing one member of the family U48.

The only metallo-peptidase characterized so far is the leishmanolysin-like peptidase EhMP8-1. Leishmanolysin (also called gp63) is essential for the virulence of *Leishmania major*. It degrades extracellular matrix proteins during tissue invasion, prevents complement mediated lysis of *Leishmania* promastigotes by inactivating C3b deposited on the cell membrane and was shown to defend the parasite against antimicrobial peptides [42–45].

The homologous amoebic peptidase EhMP8-1 is localized on the trophozoite surface. Transfectants deficient in EhMP8-1 show greater adherence to cell monolayers as well as to viable and apoptotic Jurkat lymphocytes, reduced cytopathic activity (destruction of cell monolayer), reduced mobility on cell monolayers, and increased phagocytosis. In contrast to leishmanolysin, EhMP8-1 does not seem to be involved in complement inactivation [46]. Interestingly, quantitative real-time polymerase chain reaction (PCR) indicated that the EhMP8-1 homologue EhMP8-2 is approximately 70 fold higher expressed in a nonpathogenic amoeba strain in comparison to a pathogenic strain, although both have the same genetic background [47].

25.3.4 Cysteine Peptidases

25.3.4.1 C1 Papain Superfamily

The majority of all identified peptidases belong to the cysteine peptidases. The largest group (35 members) is structurally related to the C1 papain superfamily. The members of the C1 family can be assigned to three distinct clades (A, B, C) with 13 (EhCP-A1-A13), 11 (EhCPB1-B11), and 11 (EhCP-C1-C6, -C8. -C9, -C11-C13) members, respectively. Members of clade A and B are organized as classical pre-pro enzymes.

They have a cathepsin L-like structure that is indicated by an ERFNIN motif in the pro-region of nearly all EhCP-A/-B enzymes [48]. Nevertheless, biochemical studies with purified EhCP-A1, -A2, and -A5 indicated a cathepsin B-like substrate specificity with a preference for arginine in the P2 position [11, 27, 31], likely the result of substitution of an alanine residue by acidic or charged amino acids in the postulated S2 pocket, corresponding to residue 209 of the papain sequence [48]. In contrast, EhCP-A4 has a preference for valine and isoleucine at P2, presumably the result of the hydrophobic nature of the S-pocket with valine at the base [49].

A peculiarity of the amoebic cysteine peptidases is their characteristic migration behavior in sodium dodecyl sulfate (SDS)-gelatin gels [5, 24]. The calculated molecular weight of the mature form of all family A members is approximately 29 kDa. Nevertheless, within the gelatin gel distinct prominent bands between 20 and 50 kDa are observed that can be attributed to specific cysteine peptidases [50, 51].

A major difference of members of clade A and clade B is their length of the pro-domain as well as catalytic domain. Furthermore, they have different conserved sequence motifs in the N-terminal region of the mature enzymes. In addition, the majority of the members of clade B, but none of the members of clade A, contain a hydrophobic domain, a transmembrane domain, or a GPI-attachment domain at the C-terminus [37, 38].

From all clade B family members, only EhCP-B9 (EhCP112) has been characterized. EhCP-B9 forms a complex with an adherence domain protein (EhADH112). As a complex, both proteins bind to target cells and are translocated during phagocytosis from the plasma membrane to phagocytic vacuoles [32, 52]. Furthermore, EhCP-B9 has a putative transmembrane domain and contains an RGD (Arg-Gly-Asp) motif at positions 249–251 between the active site cysteine-167 and histidine-328 [32]. This RDG motif is also found in EhCP-B8 at a similar position (245–247), as well as in the pro-region of EhCP-A5 (positions 92–94). For EhCP-A5 it was shown that this molecules binds via its RGD motif to $\alpha_V\beta_3$ integrin on colonic cells and stimulates NFκB-mediated pro-inflammatory responses in the pathogenesis of intestinal amebiasis [53].

In contrast to the members of clade A and B, proteins belonging to clade C are not organized as classical papain-like cysteine peptidases. A predicted transmembrane domain can be found close to the N-terminus, indicating a membrane association. All EhCP-C molecules contain the active site residues of the C1-family members [37]. Nevertheless, as no structurally related cysteine peptidases is known from other organisms, the specific functions of these molecules as well as their substrate activities remain completely unknown.

Various localizations have been described for the cysteine peptidases, one being lysosome-like vesicles [16, 26, 28]. These vesicles seem to consist of early and late endosomes [54, 55]. In addition, a punctate cytoplasmic localization has been observed and during phagocytosis a translocation of the cysteine peptidases to phagocytotic vesicles occurs [56, 57]. Furthermore, cysteine peptidases have been found to be membrane associated [31, 32, 56, 58] and secreted [16, 52, 59, 60]. Recently, an individual cysteine peptidase (EhCP-A4) was localized not only in cytoplasmic vesicles and was released extracellularly but localized also in the nuclear

region and the perinuclear endoplasmic reticulum [49]. In a proteomic approach analyzing the composition of *E. histolytica* uropods, the CPs EhCP-A1, -A2, -A4, -C4, -C5, -C6, and -C13 were identified [61]. Interestingly, this was the first time that proteins belonging to the EhCP-C family were detected in *E. histolytica*.

25.3.4.2 Additional Cysteine Peptidase Families

In addition, eight genes have been identified that putatively code for cysteine endopeptidases belonging to families C2 (calpain-like, two members), C19 (ubiquintinyl hydrolase-like, one member), C54 (autophagin-like, four members), and C65 (otubain-like, one member) [37]. No functional information is available for these putative peptidases.

25.4 Involvement of Cysteine Peptidases in Colonic Invasion

A first step for *E. histolytica* during invasion of the colonic mucosa is to colonize the mucin substrate. Here, it has been shown that *E. histolytica* is able to degrade the secreted mucin, which allows the amoebae to traverse the mucous layer. Secreted cysteine peptidases cleave the less glycosylated cysteine-rich domains of the MUC2 polymer, the major glycoprotein component of the colonic mucous gel layer. This cleavage is believed to depolymerize the MUC2 polymers, which leads to disruption of the mucous gel, consequently allowing the amoebae to reach the epithelial surface [62, 63]. After attachment, the secreted cysteine peptidases induce villin lysis and microvilli disruption, allowing the amoebae to come into direct contact with enterocytes [64]. One consequence of the crosstalk between enterocytes and amoebae is the release of pro-inflammatory cytokines by apoptotic enterocytes, which cause leukocyte recruitment [65]. These results were confirmed by the use of transgenic amoebae with a reduced cysteine peptidase expression that are not able to penetrate the colonic lamina propria or induce pro-inflammatory cytokine secretion [21]. However, as already mentioned, it was shown that cysteine peptidases are able to cleave IL-18, a pro-inflammatory cytokine, which induces γ-interferon and activates macrophages, one of the major effector cells against *Entamoeba* [18].

25.5 Involvement of Cysteine Peptidases in Liver Abscess Formation

A direct correlation between the amount of cysteine peptidase activity and liver abscess formation in animal models was observed in several studies [2, 6, 12, 66]. Furthermore, the inhibition of cysteine peptidases using specific cysteine peptidase inhibitors leads to a reduction in abscess formation [9, 67]. It was shown that

incubation of trophozoites with laminin, which bind cysteine peptidases, leads to a reduction in liver abscess formation, which confirmed the assumption that cysteine peptidases play an important role in liver abscess formation [4].

Transgenic amoebae with reduced cysteine peptidase activity are also greatly impaired in their ability to induce amoebic liver abscesses in animals [1]. On the other hand, transgenic amoebae with increased cysteine peptidase activity induce larger abscesses [20].

Currently, it can only be speculated about the specific role of the cysteine peptidases during liver abscess formation. It is possible that the secretion or the activity of membrane-associated peptidases leads to the initiation of inflammation and to tissue damage. Alternatively, cysteine peptidases may be necessary for the amoebae to survive within the liver tissue. It is known that only live amoebae and neither amoebic extracts nor dead amoebae can induce abscess formation and therefore only vital amoebae can lead to tissue damage.

25.6 Cysteine Peptidase Gene Expression

As mentioned, 35 papain-like *ehcp* genes are present in the *E. histolytica* genome. To date, only 4 of the 35 papain-like (*ehcp-a1*, *-a2*, *-a5*, and *-a7*) peptidases have been shown to be highly expressed in amoeba trophozoites under standard axenic culture conditions [30, 37, 38, 51]. In contrast, very little is known about the function and regulation of the majority of cysteine peptidases that are expressed at low levels in axenic culture or about their involvement in excystation and encystation, colon invasion, and ALA formation.

For some of those cysteine peptidase genes (*ehcp-a3*, *-a4*, *-a8*, *-b1*, *-b3*, *-b8*, *-b9*, and *-b10*) that are expressed at low levels in the trophozoite stage, a cyst-specific expression was detected [38, 68]. In this context it was shown for the reptile pathogen *Entamoeba invadens* that cysteine peptidase inhibitors block excystation [69]. Therefore, it can be postulated that at least some of the cyst-specific peptidases are involved in the process of excystation.

Intestinal invasion resulted in a change in the expression of *ehcp* genes in *E. histolytica* isolated from mouse ceca with four *ehcp* genes (*ehcp-a1*, *-a4*, *-a6*, *-a8*), showing increased expression and one (*ehcp-a7*) showing decreased expression. Thus, it was proposed that this individual regulation indicates nonredundant functions for these peptidases [70].

In contrast, very little information is available about CP expression during ALA formation. Freitas and colleagues compared the expression levels of *ehcp-a5* in ALA samples and axenic cultivated strains and found a higher level of *ehcp-a5* mRNA in amoebae derived directly from ALAs [71].

Expression of the cysteine peptidase genes *ehcp-a4*, *ehcp-a6*, and *ehcp-a8* was statistically significantly higher (approximately 3 fold) in the pathogenic HM-1:IMSS strain than in the nonpathogenic Rahman strain. The most striking difference was seen in the expression of *ehcp-a3*, which was approximately 100 fold higher in Rahman than in HM-1:IMSS [72].

25.7 Generation of Transgenic Amoebae to Study the Influence of Cysteine Peptidases on Pathogenicity

The generation of transgenic amoebae allows studying the influence of specific peptidases on pathogenicity. In summary, these investigations conclude that EhCP-A5 is a major pathogenicity factor, with possible involvement in colon invasion, induction of host inflammatory responses, and ALA formation [1, 20, 21, 73].

Ankri and colleagues generated an *E. histolytica* transfectant that produces *ehcp5* antisense RNA. This antisense inhibition resulted not only in the inhibition of *ehcp5* expression but also in repression of the expression of the other *ehcp*s. The overall cysteine peptidase activity of these transfectants was reduced by approximately 90 %. Growth and also cytopathic and hemolytic activity of the transfectants were not altered. Nevertheless, an inhibition of phagocytosis was observed [74]. Most importantly, it was shown that these transfectants characterized by only low levels of cysteine peptidase activity were nonpathogenic as they were could not induce liver abscess formation in hamsters [1] and they also failed to induce intestinal epithelial cell inflammation [19]. In addition, the reduction of cysteine peptidase activity led to an ineffective degradation of colonic mucin [75].

A few years later, Mirelman and colleagues were able to transcriptional silence *ehcp-a5* [76]. Unfortunately, this silencing is only possible in trophozoites silenced for the gene encoding for the pathogenicity factor amoebapore A (G3 strain). Therefore, these amoebae are greatly impaired in their ability to induce abscess formation [77]. In contrast to the *ehcp5* antisense transfectants, which showed a reduced expression of several *ehcps*, transfectants transcriptionally silenced specifically for *ehcp5* are able to overcome the mucous barrier [21]. Nevertheless, it was shown that these transfectants could not penetrate the colonic lamina propria or induce the host pro-inflammatory cytokine secretion [21]. Further experiments indicate the importance of EhCP5 for cleavage of the collagen network, resulting in collagen network disorganization [73]. In addition, these transfectants killed significantly fewer liver sinusoidal endothelial cells compared to HM-1:IMSS or G3 strains [78] and showed a significant reduction in their hemolytic activity [51]. The digestion of erythrocytes was impaired in transfectants silenced for *ehcp-a1* and *ehcp-a7*, but also in transfectants silenced for the majority of the family A members [51]. This change could also be observed during the inhibition of cysteine peptidases using peptidase inhibitors, which similarly leads to a blockade in erythrocyte digestion [79].

To analyze the involvement in abscess formation, transfectants were generated that overexpress EhCP-A1, -A2-, and -A5, respectively. Interestingly, overexpression of *ehcp-a1* and *ehcp-a2* increased the activity of the corresponding enzyme only, whereas overexpression of *ehcp-a5* increased the activity of all three enzymes. The use of animal models indicated that increased amounts of EhCP-A1 and EhCP-A2 have no influence on liver abscess size. Overexpression of *ehcp-a5* led to significantly increased amebic liver abscess formation and was also able to compensate the reduction of in vivo pathogenicity in G3 trophozoites, which have been silenced for the gene encoding amoebapore A [20, 80].

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Chapter 26
Host Immunity and Tissue Destruction During Liver Abscess Formation

Elena Helk, Hannah Bernin, and Hanna Lotter

Abstract Amebic liver abscess (ALA) is a severe focal destruction of liver tissue caused by infection with the parasite *Entamoeba histolytica* (*E. histolytica*). In the past, tissue damage has been mainly attributed to pathogenicity factors of the parasite. However, the massive presence of innate immune cells raises the question whether host cells contribute to the destruction of the liver tissue as well. In this chapter, we discuss the role of neutrophils, monocytes, and macrophages during ALA in animal models for the disease. In brief, neutrophils contribute only partially to the observed pathology, whereas inflammatory monocytes and resident liver macrophages are substantially involved in tissue damage seen during *E. histolytica* infection. Therefore, we conclude beyond parasite-specific effector molecules, immune pathological mechanisms of the host substantially contribute to the development of ALA.

26.1 Background

Entamoeba histolytica is a parasite that usually colonizes the human gut without harming its host. Most infected individuals do not develop any disease; however, in approximately 10 % of patients the parasite progresses from its asymptomatic stage in the lumen of the intestine and invades the mucosal wall. This process leads to one of the major symptoms of invasive amebiasis, amebic colitis, which is characterized by ulcerations and severe bleeding. Under conditions so far unknown and independent of amebic colitis, trophozoites may spread via the bloodstream and become trapped within the sinusoids of the liver, which are similar to a coarse-mesh sieve. The presence of *E. histolytica* trophozoites in the liver provokes the second major symptom of invasive amebiasis, amebic liver abscess (ALA), which is characterized by the focal, progressive destruction of liver tissue and may be fatal without treatment. It is unclear whether the tissue damage observed in patients with ALA is caused primarily by parasite

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_26

effector molecules or by host factors. Because of the lack of an immunocompetent animal model for ALA, most studies have focused on pathogenicity factors associated with the parasite, including amoebapores and cysteine peptidases, with little assessment of the contribution made by host effector mechanisms.

In vitro studies show that amoebapores and cysteine peptidases can kill and lyse host cells [1, 2]. Furthermore, early in vivo experiments showed that inhibition of *E. histolytica* cysteine peptidases by the specific inhibitor, E64, decreases the pathogenicity of the parasite, resulting in smaller abscesses [3, 4]. The ability to overexpress single genes in *E. histolytica* allowed investigation of the contribution of individual molecules, such as members of the cysteine peptidase family, to the pathogenicity of *E. histolytica* trophozoites in animal models [5–7]. The findings show that *E. histolytica* expresses effector molecules that can damage host cells and may contribute to liver destruction during ALA. It is unclear, however, whether the targets of parasite effector molecules are environmental gut bacteria or host cells. In addition, homologues of most of the genes thought to be essential for pathogenicity have been detected in nonpathogenic amebic strains, including *Entamoeba dispar*, which is genetically very closely related to *E. histolytica* but is incapable of inducing clinical symptoms (for review, see AmoebaDB, Amoeba Genomics Resource, version 2.0 [8, 9]).

There is considerable evidence that host factors contribute to the development of ALA. The disease in humans is age- and gender dependent, being sevenfold more common in adult men than in women and children, although the latter are more frequently infected [10, 11]. Additionally, the abscess area is infiltrated by a vast number of immune cells, especially neutrophils, suggesting that these cells may cause as much tissue destruction as the parasite itself.

26.2 ALA Histology

Although careful analyses of histological sections from abscessed liver tissue are necessary to understand the role of host immune cells in the pathology of ALA, there have been few histological investigations of patients with ALA. Several reasons have been suggested, including the lack of detection or misdiagnosis of early phases of ALA development. In addition, the ability to successfully treat the disease with drugs abolished the need to obtain biopsy samples from diseased patients. Nevertheless, histological investigation of samples obtained from patients with ALA (subsequent to the initial detection of trophozoites in the liver) show extensive necrosis of the abscess areas, with amebic trophozoites widely distributed in these regions of liver tissue. In addition to $CD8^+$ and $CD4^+$ T cells, activated $CD68^+$ macrophages and $CD15^+$ neutrophils are present in the liver during ALA [12]. Because the performance of periodic histological assays that follow the abscess course is almost impossible in humans, animal models of ALA are required. Since humans are the only natural hosts for *E. histolytica*, sophisticated and artificial infection routes are needed to induce amebic liver abscesses in other species. In rodent models, axenically cultured trophozoites are injected into the peritoneum [13], the portal

vein [14], or directly into the liver tissue [15]; however, most of these models entail complex surgical procedures. The liver lesions that develop in most rodent models differ from those in humans. For example, infections in highly susceptible hamsters resemble those in human patients on the one hand in that both are characterized by the host's inability to control *E. histolytica*-induced liver damage. However, in contrast to abscess formation in humans, the ALA lesions in hamsters are not focal and restricted to one liver lobe but comprise multiple granulomas that spread throughout the liver lobe. The gerbil model resembles ALA in humans more closely, as the lesions appear more focal and do not involve the whole liver; however, in contrast to hamsters, gerbils are able to recover from an infection [15]. ALA has also been induced in severe combined immunodeficiency mice (SCID mice), but these mice lack essential components of the immune response, complicating their use for investigating host contribution to ALA development [16]. Most conventional immunocompetent laboratory mice strains are resistant to ALA. However, we recently generated a highly virulent amebic strain from HM1:IMSS, cell line B [17], which stably induces abscesses in C57BL/6 mice [18]. In contrast to other amoebic strains used to induce ALA, the virulence of this strain has remained unaltered over the years and its maintenance does not require serial liver passages [19]. The ALA lesions induced by this strain are focal and show organized abscess structures, but, as in gerbils, they are self-restricted. In addition, and similar to humans, male mice are more susceptible to ALA than female mice. In addition, and similar to humans, male mice are more susceptible to ALA than female mice in that they develop larger abscesses, are less able to recover from abscess development and less able to control the parasite survival within the liver [18].

Classical histology shows that the majority of immune cells that infiltrate the abscesses in all these rodent models are neutrophils which concentrate in close proximity to *E. histolytica* trophozoites. The parasites are located in the abscess center and close to healthy or lysed hepatocytes in the early stage of infection.

This consideration might suggest that hepatocytes, as well as ingested leukocytes and red blood cells, may serve as nourishment for the parasites. The abscesses in immunocompetent mice become more organized during the later stages of infection, forming a necrotic center surrounded by multiple margins formed by different infiltrating immune cells. Until a capsule begins to form and the abscess resolves completely, these immune cells are mainly neutrophils (Figs. 26.1 and 26.2). More recent investigations show that, following neutrophil lysis, macrophages concentrate around the necrotic areas and define the margins of the abscess; thus, macrophages may be involved in removing debris from the necrotic center [55].

26.3 Contribution of Neutrophils to ALA Development

Neutrophils are part of the innate immune system and comprise up to 70 % of leukocytes in humans and up to 50 % in mice. Neutrophils mature in the bone marrow and circulate in the bloodstream. Therefore, they are able to arrive at the site of infection and migrate into the infected tissue by crossing the endothelial barrier.

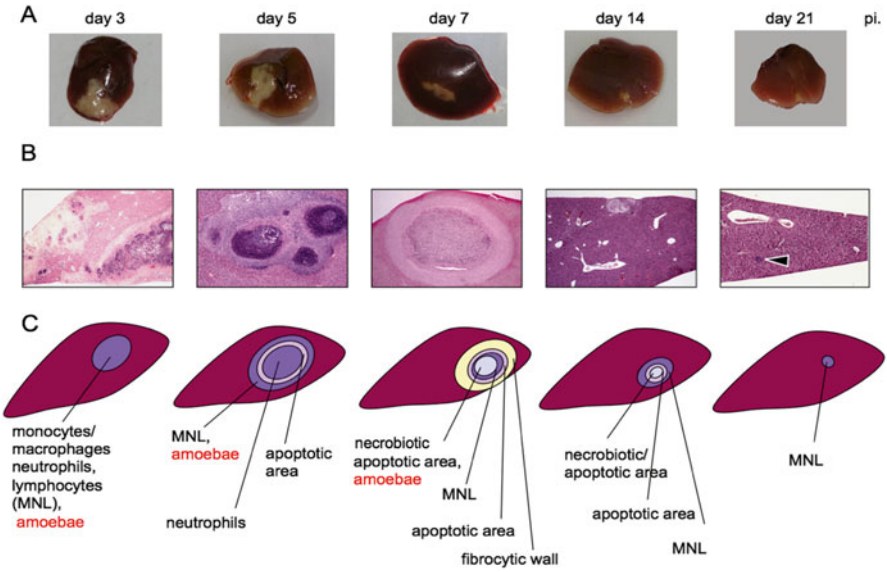

Fig. 26.1 Representative course of amebic liver abscess (ALA) in male C57BL/6 mice. **a** Course of ALA lesions in the left liver lobe documented by light photography. **b** Histological sections taken at indicated time point post infection (*pi.*) and stained by periodic acid–Schiff (PAS). **c** Scheme of localization of amebic trophozoites and infiltrating immune cells during abscess consolidation

Upon activation, neutrophils are not only able to phagocytose pathogens but can also release granules containing mediators such as reactive oxygen species (ROS) and hydrolytic enzymes, a process called the "respiratory burst." During disease progression, a collection of enzymes is released from azurophilic or primary granules, specific or secondary granules, and tertiary granules. Some of the most effective enzymes include myeloperoxidases, defensins and cathepsins, neutrophil elastase, and other molecules with antimicrobial properties; these molecules help to combat infection [20, 21]. Many in vitro studies show that human and murine neutrophils activated by tumor necrosis factor (TNF)-α or interferon (IFN)-γ induce amebicidal activity via H_2O_2-dependent mechanisms [22, 23]. By contrast, unstimulated neutrophils are rapidly killed by *E. histolytica* [22]. The half-life of activated neutrophils in the tissues is extremely short (~12–24 h). When they undergo programmed cell death, neutrophils release mediators that kill microorganisms; however, these mediators can also damage host tissues. For example, neutrophil elastase kills *E. histolytica* but also disrupts collagen [24, 25]; however, this is not necessarily detrimental to the host because it initiates wound healing [26]. In all animal models of ALA, neutrophils arrive soon after infection and may contribute to ALA development. Thus, tissue destruction during ALA results from the accumulation and subsequent lysis of the neutrophils and macrophages surrounding the amoeba, rather than from the action of the parasite itself [14]. In vitro studies also indicate

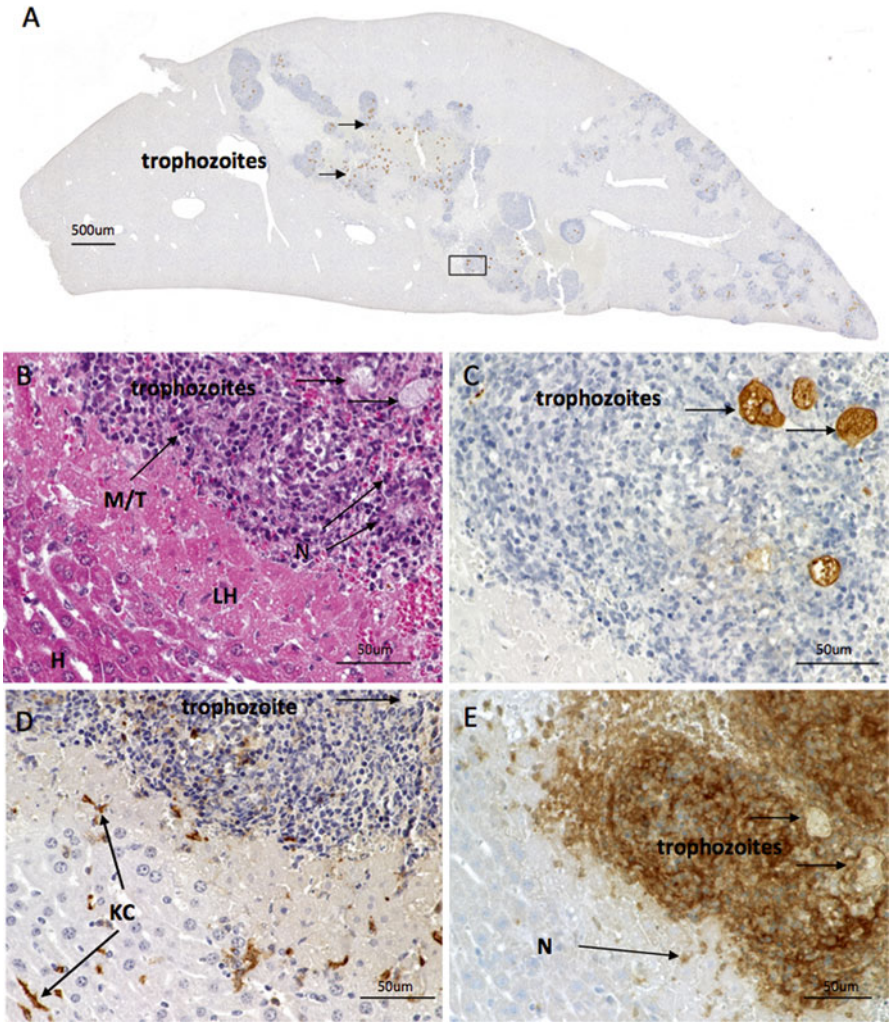

Fig. 26.2 Immunohistology of a liver section from a mouse intrahepatically infected with *Entamoeba histolytica*. **a** Representative overview of liver section day 2 post infection developed with a pool of antisera raised against selected recombinant *E. histolytica* antigens: the *rectangle* encompasses the area shown in more detail in **b** and **c**. *E. histolytica* trophozoites (*arrow*) are surrounded by infiltrating immune cells. **b** Hematoxylin and eosin (H&E) stain of a selected area shows healthy hepatocytes (*H*), a zone of lysed hepatocytes (*LH*), demarcating an area of infiltrating neutrophils (*N*) and monocytes and T cells (*M/T*). **c** Same area developed with a pool of antisera raised against recombinant *E. histolytica* proteins. **d** Identical area stained for F4/80 liver resistant macrophages (Kupffer cells, *KC*). **e** Same area developed with antineutrophil antibody (mAb7-4)

that lysis of neutrophils increases the destruction of host cells by *E. histolytica* trophozoites [27].

The contribution of neutrophils to liver tissue destruction during ALA can be examined by immune depletion experiments using defined immunological tools. The murine monoclonal antibody (mAb) used most frequently to deplete neutrophils is anti-GR1 (clRB6-8C5). Anti-GR1 recognizes the Ly6G epitope, which is expressed exclusively by neutrophils. The antibody also recognizes Ly6C-expressing monocytes, meaning that these cells are removed along with neutrophils. By contrast, antibodies specific for Ly6G only deplete neutrophils [28, 29]. Immune depletion experiments in SCID mice using anti-GR1 mAbs suggest that neutrophils play a protective role in ALA. Abscesses in SCID mice depleted for $GR1^+$ cells were significantly larger and contained fewer immune cells and more amoebic trophozoites than those in control mice. However, SCID mice are unable to mount an appropriate immune response because they lack T and B lymphocytes, suggesting that neutrophils may play a more prominent role in the control of ALA in SCID mice than in immunocompetent mice; thus, studies in SCID mice using anti-GR1 must be carefully interpreted [30]. By contrast, more recent investigations of ALA in immunocompetent mice show opposite results. In these studies, neutrophils were selectively depleted by injecting a monoclonal antibody directed against Ly6G. Abscesses in these mice were smaller than those in control immunoglobulin-treated mice. Thus, neutrophils appear not to be involved in the control of amoebic trophozoites in immunocompetent mice, as was suggested by previous in vitro studies, rather, they appear to contribute to liver damage during amebic infection.

26.4 Kupffer Cells and ALA

Kupffer cells (KCs), or liver resident macrophages, represent 15 % of the total liver cell population. Because of their sinusoidal localization, they have been studied mainly in terms of their role as scavenger cells that remove particulate material from the portal circulation and contribute to host antimicrobial defenses in the liver [31]. Similar to neutrophils, activated KCs release a variety of products, including cytokines, nitric oxide, and ROS, all of which affect the phenotype of adjacent cells in the liver, including hepatocytes, stellate cells, and liver-trafficking immune cells [32]. Recent results (mainly derived from animal models) suggested that KCs are involved in the pathogenesis of several inflammatory liver diseases, including viral hepatitis, steatohepatitis, and chemically induced hepatitis (such as that induced by acetaminophen) [33]. Because of the heterogeneity and plasticity of liver macrophages, identifying the KC population responsible for liver injury (and the type of liver injury caused) is very challenging. Recent reports suggest that the liver contains two subpopulations of KCs that are distinguished according to phenotype and function [34]. All KCs express the macrophage-restricted glycoprotein, F4/80, on their surface [35]; however, the subsets can be further characterized by their expression of CD11b (a C3b receptor present on the surface of monocytes, macrophages,

and neutrophils) [36] and CD68, also known as macrosialin [37]. $CD11b^+$ cells mainly produce cytokines and show weak cytolytic activity whereas $CD68^+$ cells are highly phagocytic and mediate cytotoxicity via the production of ROS [38] and superoxide [39]. Similar to neutrophils, KCs cannot discriminate between host tissue and amoebic trophozoites. Thus, they can cause collateral damage by secreting reactive oxygen species and proinflammatory cytokines such as interleukin (IL)-6, IL-1-β, and TNF-α.

The removal of KCs using gadolinium chloride ($GdCl_3$), clodronate or ethylpyrovate inhibits the immune pathology observed in various toxicity-induced hepatitis models [38–40]. For a long time, attempts to identify the roles played by macrophages and KCs in ALA were hampered by the lack of an immunocompetent animal model, which meant that the immunological tools required to precisely analyze KC function were not developed. One early strategy to determine the role of macrophages in controlling ALA involved injecting hamsters with rabbit anti-macrophage serum followed by infection with *E. histolytica* trophozoites. The effectiveness of this depletion therapy could not be determined; however, the authors noted larger abscess loads and the spread of abscesses to other organs [41]. In vitro experiments also suggest a protective role for macrophages and KCs during amebiasis. For example, preactivating macrophages with IFN-γ and lipopolysaccharide enhances their ability to kill trophozoites [42–44]; a further study identified reactive nitrogen intermediates as the cytotoxic effectors [45]. Recent studies, which used more modern approaches (such as clodronate treatment) to increase the efficacy of KC depletion, suggest another role for these cells in amebic liver pathology. Surprisingly, clodronate treatment led to a marked and unexpected reduction in abscess size. Moreover, histological examination of the residual abscess tissue showed the complete absence of otherwise typical immune cell infiltrates. However, amebic trophozoites were not affected by clodronate treatment and could be reisolated from the liver tissue up to 5 days after the first treatment [55]. Taken together, these findings indicate that KCs contribute to tissue destruction during ALA, whereas amebic trophozoites can survive within the liver for several days without inducing substantial tissue damage.

26.5 Monocytes and Liver Tissue Destruction

Monocytes are a population of circulating white blood cells that develop in the bone marrow. Because they constitute a reservoir of myeloid cells, both in the bone marrow and the bloodstream, they are rapidly recruited to infected or injured tissues, where they differentiate into macrophages and dendritic cells [46–48]. Monocytes can mount a direct attack on microorganisms but can also exacerbate inflammatory diseases. Notably, $CD11b^+$ murine monocytes express C-C chemokine receptor 2 (CCR2) and express high levels of Ly6C ($Ly6C^{hi}CCR2^+$). These monocytes, also referred to as inflammatory monocytes, represent about 2–5 % of peripheral blood leukocytes circulating in mice. Secretion of CCL2 by injured or inflamed epithelial

cells induces Ly6C^{hi}CCR2^{+} monocytes to migrate from their reservoir in the bone marrow to the bloodstream and, finally, to the site of infection. Once there, they take part in immune responses directed against pathogenic microorganisms [49]. Activated Ly6C^{hi}CCR2^{+} monocytes exhibit strong antimicrobial activity and promote proinflammatory responses [29]. In particular, Ly6C^{hi}CCR2^{+} monocytes in the liver give rise to TNF-α- and iNOS-producing dendritic cells (TipDCs), inflammatory macrophages, and inflammatory dendritic cells (DCs) [50]. CCR2-mediated recruitment of inflammatory monocytes is essential for resistance to bacteria, fungi, viruses, and protozoans. However, under certain circumstances (e.g., infection by influenza or trypanosomes) the recruitment of inflammatory monocytes can be detrimental to the host and exacerbate disease pathology [29]. For example, mice harboring monocytes unable to express CCR2 show impaired monocyte recruitment to the lungs during influenza virus infections; this results in reduced pathology and mortality, but clearance of the virus is delayed [51]. The lack of CCR2 expression by monocytes increases resistance and decreases parasitemia during *Trypanosoma bruzi* infections; however, this is not the case upon infection by intracellular protozoans such as *Toxoplasma gondii*, *Leishmania donovani*, and *Plasmodium chabaudi* [50]. Moreover, monocyte recruitment contributes to the pathology observed in sterile inflammatory conditions such as atherosclerosis [52] and in models of acute and chronic hepatic injury [53, 54]. The mechanisms underlying the transition of monocytes from a cell that is primarily protective to one that damages tissues have not yet been identified. Ly6C^{hi} inflammatory monocytes contribute to ALA pathology in mice. The onset of ALA is associated with an increase in CCL2 mRNA expression. Mice lacking CCR2 develop less severe ALA symptoms; however, the adoptive transfer of CD115^{+} bone marrow-derived monocytes into these mice restores the ALA phenotype [55]. Monocytes recruited to sites of infection or inflammation most often differentiate into major producers of iNOS and TNF-α, with the production of both being reduced in mice that lack CCR2 or CCL2 [56]. Although iNOS expression generally leads to the production of the antimicrobial product NO, TNF-α functions as a broadly acting proinflammatory cytokine because its receptor is expressed on a variety of immune cells and on endothelial cells. Ligation of TNF-α activates and promotes the survival of these target cells and induces the production of mediators such as IL-6, IL-8, and iNOS [57, 58]. However, the immunopathology of ALA is independent of iNOS synthesis because ALA development is unaffected in mice lacking iNOS and in mice previously treated with an iNOS inhibitor. By contrast, ALA development is prevented by a monoclonal antibody that neutralizes TNF-α, suggesting that TNF-α is involved in the tissue destruction observed during ALA [55].

Taken together (Fig. 26.3), these results strongly indicate that immunopathological processes are crucially involved in the destruction of liver tissue during ALA. In addition to the mouse model, these findings have been observed in the hamster model of ALA, because treatment of these animals with the immunosuppressant dexamethasone reduces the number of immune cells and prevents abscess development [59]. However, it is still unclear whether the immune mechanisms responsible for liver pathology in these rodent models of ALA are actually involved in human invasive amebiasis.

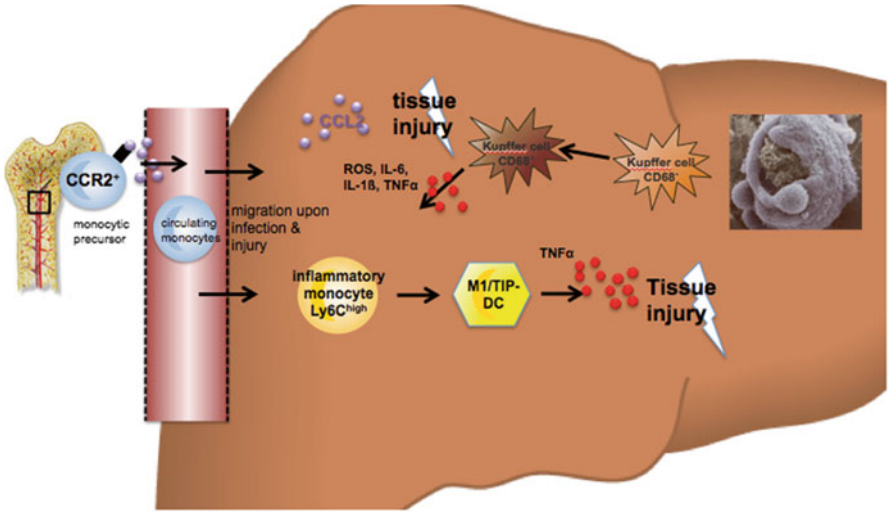

Fig. 26.3 Scheme of immunopathology leading to development of amebic liver abscess. Upon activation by *E. histolytica* trophozoites, Kupffer cells secrete tissue inflammatory effector molecules, leading to the expression of CCL2 by hepatocytes and other liver cells. CCL2 expression mediates the recruitment of $CCR2^+$ monocyte precursors from the bone marrow. $CCR2^+$ $Ly6C^{hi}$ monocytes are recruited from the circulation into the infected liver where they differentiate into TNF-α-producing classically activated M1 macrophages or TIP-DCs (TNF-α- and iNOS-producing dendritic cells). *ROS* reactive oxygen species, *IL* interleukin

Acknowledgments The research presented in this chapter was supported by grants from the Deutsche Forschungsgemeinschaft SFB 841 ("Liver inflammation: Infection, Immune Regulation and Consequences"). E.H. is supported by studentships from the SFB 841, Hamburg, Germany. H.B. is supported by the Werner-Otto Stiftung, Hamburg, Germany. H.L. is supported by and holds a group leadership in the Department of Molecular Parasitology from the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. We thank Claudia Marggraff for the excellent immunohistology of ALA.

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Chapter 27
The Effect of *Entamoeba histolytica* on Muc2 Mucin and Intestinal Permeability

V. Kissoon-Singh, E. Trusevych, and K. Chadee

Abstract The intestinal parasite *Entamoeba histolytica* (*Eh*) is a significant health risk in the developing world where infection occurs via ingestion of contaminated food or water. The resulting disease, amebiasis, claims 100,000 lives each year. In the colon, the parasite colonizes the mucous layer and interacts with the underlying mucosa to induce a variety of symptoms. The intestinal barrier has three main components: bacteria and host proteins found within the lumen, a protective layer of mucus, and a single layer of intestinal epithelial cells (IEC) connected by tight junctions (TJ). *Eh* possesses a variety of virulence components that target the intestinal barrier to cause increased gut permeability, resulting in diarrhea. In the colon *Eh* can phagocytose pathogenic bacteria, which may increase the virulence of the parasite. Moreover, *Eh* binds colonic mucins via the parasite surface Gal/GalNAc lectin to facilitate colonization of the mucous layer. Subsequently, the virulence factor, *Eh*CP-A5, degrades the MUC2 polymer, allowing the parasite access to the epithelia where it binds to and destroys IEC by apoptosis or alters the expression of TJ proteins, causing secretory diarrhea. The sequence of events in intestinal amebiasis is not well understood and is based largely on in vitro and in vivo models of the disease.

27.1 Introduction

The protozoan parasite *Entamoeba histolytica* (*Eh*) is the causative agent of intestinal amebiasis. *Eh* infects an estimated 10 % of the world's population [1], and 12 % to 25 % of infections are associated with invasive diarrheal disease [2]. Amebiasis is a leading cause of morbidity and mortality in the developing world [2, 3], resulting in more than 100,000 deaths per year [4]. Because transmission of the parasite is through the fecal–oral route, recurrent infections are common in areas of

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_27

poor sanitation where approximately 50 % of children with a first diarrheal episode have a second [2]. Amebic colitis is characterized by abdominal cramping, pain, mild to moderate dehydration, watery or bloody diarrhea, and weight loss [5]. In this chapter we describe the mechanisms by which *Eh* overcomes the intestinal mucosal barrier to cause disease.

27.2 Intestinal Mucosal Barrier

The intestinal barrier is critical for protecting the host from a variety of pathogens and antigenic stimuli present in the gut while at the same time facilitating nutrient and water absorption. This multilayered barrier consists of protective components within the lumen, a layer of mucus, and a single layer of intestinal epithelial cells (IEC) joined by tight junctions (TJ) (Fig. 27.1a) [6, 7]. Disturbance in any one component of the intestinal barrier could facilitate disruption of the other components, leading to unrestrained movement from the lumen into the interstitial spaces, which may occur before the development of overt colonic disease [7]. Here, we examine the multiple layers of the mucosal barrier and how *Eh* virulence factors interact with these to promote increased permeability and disease.

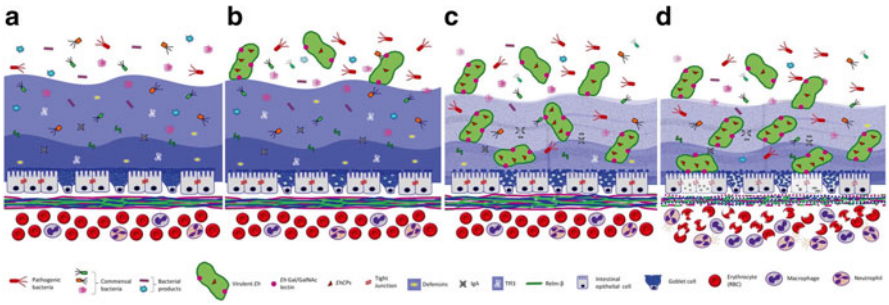

Fig. 27.1 *Entamoeba histolytica* (*Eh*) overcomes the intestinal mucosal barrier to cause invasive disease. **a** The intact intestinal barrier is formed by host proteins and commensal and pathogenic bacteria present in the intestinal lumen, a mucous layer with embedded host and bacterial proteins, and the intestinal epithelial cells (IEC) and tight junctions (TJ) between them. *Eh* compromises intestinal barrier integrity by disrupting every level of the barrier. **b** *Eh* alters the luminal composition by changing the balance of commensal and pathogenic bacteria, and *Eh* virulence is modified by phagocytosis of pathogenic bacterial species. **c** *Eh* colonizes the mucous layer by binding via the Gal/GalNAc lectin, and *Eh*CPs degrade colonic mucins. **d** *Eh* binds to epithelial cells, disrupts the microvilli and the TJ proteins, destroys the extracellular matrix, and induces an acute inflammatory cellular infiltrate

27.2.1 The Luminal Barrier and E. histolytica

Commensal bacteria contribute considerably to mucosal barrier function, having major roles in digestion and nutrient absorption, and by outcompeting pathogenic organisms to prevent their colonization [7]. Importantly, in areas of poor sanitation the balance between commensal and pathogenic bacteria can be lost in individuals with intestinal infections. Interestingly, if pathogenic bacteria are present in the lumen, *Eh* can become more virulent [8, 9]. A recent study showed that *Eh* trophozoites that phagocytosed pathogenic bacteria increased the expression of key virulence factors including the Gal/GalNAc lectin and cysteine protease activity [10], resulting in increased adhesion to and damage of IEC. This enhanced *Eh* virulence was also observed when IEC were exposed to pathogenic bacteria before *Eh* exposure [10]. Although further studies are necessary to determine if these relationships hold true in vivo, there is evidence to suggest that the bacterial composition in the intestinal lumen impacts *Eh* virulence (Fig. 27.1).

27.2.2 Intestinal Mucous Layer

In the intestine, the mucous layer is the first line of innate host defense against potential pathogens [11]. It acts as a physical barrier against noxious agents and is colonized by a number of commensal species that occupy valuable niches which prevent binding of pathogenic organisms [12]. Embedded in this layer are a number of nonmucin glycoproteins, antimicrobial compounds, and components of the microbiota (Fig. 27.1) [12–14]. Additionally,, a variety of antimicrobial peptides together with secretory IgA interact with bacteria to prevent or limit bacterial overgrowth and facilitate the killing of various microorganisms [12].

Intestinal mucin MUC2 (Muc2 in mice) secreted by intestinal goblet cells is the main component of the mucous layer [15, 16]. On its release, the mucin polymer is rapidly hydrated and forms two distinct layers [13, 17]. The inner, dense mucous layer, which is thin and water insoluble, is adherent to the mucosal surface and impermeable to commensal bacteria [18]. In contrast, the outer layer is a soluble, viscous solution that acts as a lubricant, preventing erosion caused by mechanical forces during movement through the colon [12]; it is colonized by a variety of commensal bacterial species [19].

The MUC2 monomer consists of a protein backbone with oligosaccharide side chains [12]. The N- and C-terminal regions are rich in cysteine residues, which contribute to mucin stability through the formation of disulfide bonds [15, 20]. These terminal regions also contain von Willebrand D-domains involved in mucin polymerization [11], where dimerization of MUC2 monomers occurs at the C-termini, and trimerization mediated by disulfide bonds occurs at the N-terminal ends, rendering them resistant to proteolytic cleavage [21]. The oligosaccharide side chains attach to the variable number tandem repeat regions (VNTR), which are rich in proline, threonine, and serine residues (PTS domain) (Fig. 27.2a) [11, 15, 20]. In MUC2,

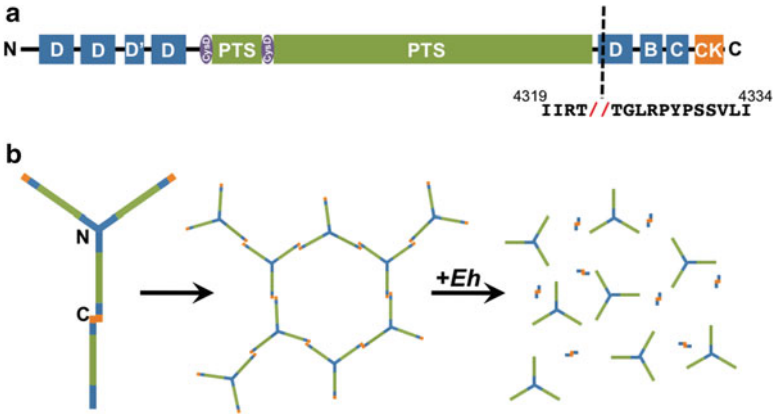

Fig. 27.2 *Eh* cleaves the MUC2 monomer and degrades the mucous gel. **a** Schematic representation of a MUC2 monomer with location of the principal *Eh* cleavage site. MUC2 (5,179 amino acids) contains four complete and one incomplete von Willebrand factor type D domains, as well as type B and C domains. The center of MUC2 contains two variable number tandem repeat regions rich in proline, threonine, and serine (*PTS*) that become heavily *O*-glycosylated in the Golgi apparatus. Surrounding the first PTS domain are two cysteine-rich domains (CysD), important for mucous gel stability. The C-terminus contains a cysteine knot domain important for dimerization. The main cleavage site of *Eh* is between amino acids 4,322 and 4,323, consistent with cysteine protease specificity for arginine in the P2 position. **b** Degradation of the mucous gel by *Eh*. MUC2 forms N-terminal trimers (*N*) and C-terminal dimers (*C*). When multiple units come together, MUC2 forms a net-like structure as depicted. *Eh* disrupts the mucous gel by cleaving MUC2 near the C-terminus, depolarizing the mucous gel

these side chains are heavily glycosylated, consisting of glucose (Glu), galactose (Gal), *N*-acetyl-glucosamine (GluNAc), *N*-acetyl-D-galactosamine (GalNAc), and fucose as well as sialic acid and sulfate residues [12, 22]. The glycosylation of mucins is critical for their physical properties, including rigidity and protease resistance [23], and the negative charge on the MUC2 monomer is essential for hydration, giving the mucous layer its gel-like structure [15, 20, 24]. Additionally, these side chains are a major food source for commensal species, which colonize by lectin binding of fimbriae/pili to sugar moieties on colonic mucins [12]. Consequently, the sugar side chains are crucial to mucin biology in terms of both structure and function.

27.2.2.1 *E. histolytica* Interacts with Colonic Mucus

When *Eh* trophozoites come in contact with the host mucous layer, one of three things may occur [13]. The parasite may bind the mucous layer without invading, resulting in transient colonization [16]. A second alternative is that the parasite interacts with the mucous layer to deplete mucin stores, at the same time binding to oligosaccharide side chains and cleaving the MUC2 monomer [11, 21].

In some cases, the host is able to expel the parasite either through increased mucin secretion or parasite-specific antibody responses [12]. In the third scenario, the parasite colonizes the mucous layer and the host is unable to expel the parasite.

The interaction between *Eh* and colonic cells has been derived from both in vitro and in vivo models of the disease. In vitro, CHO cell monolayers that do not secrete a protective mucous layer were completely destroyed by *Eh* as early as 1 h, whereas MUC2 mucin-producing LS 174T cells with a protective mucous blanket were only slightly disrupted with high concentrations of amoebae [25]. The protective effects of mucin were also demonstrated in CHO cell adherence assays where high molecular weight purified mucins bound with high affinity to the Gal/GalNAc lectin and inhibited *Eh* adherence to CHO cells [26]. Interestingly, proteolytically degraded or undegraded mucins that were glycosylated were also protective [26], although the unglycosylated regions of mucin were not. The mucin moiety that mediated adherence to *Eh* was identified as the Gal and GalNAc residues that bound the Gal/GalNAc adherence lectin [27, 28], clearly demonstrating that mucin glycosylation was critical in mediating cell protection.

In the gut, *Eh* is a potent mucin secretagogue and induces the secretion of both preformed and newly synthesized mucins and increases mucin glycoprotein biosynthesis [25]. The earliest response of the colonic mucosa to trophozoites was derived using the gerbil model of intestinal amebiasis in which secretion and subsequent depletion of goblet cell mucin preceded IEC disruption [29]. In colonic loop studies in mice and rats, *Eh* induced a dose-dependent increase in mucin and non-mucin glycoprotein secretion [30, 31]. Not surprisingly, in acute human amebic colitis, enhanced mucous secretion and subsequent depletion was also evident from histological sections, reinforcing the notion that *Eh* mucous secretagogue activity causes exhaustion and depletion of mucus, resulting in an altered mucous blanket [29] that facilitates the pathogenesis of invasive amebiasis (Fig. 27.1) [30, 32].

27.2.2.2 *E. histolytica* Virulence Factors That Interact with the Mucous Layer

The Gal/GalNAc inhibitable lectin is a key virulence factor of *Eh* and serves a number of functions associated with parasite survival and cytotoxicity. It is a 260-kDa protein consisting of a heavy chain (170 kDa) covalently linked to a light chain (31–35 kDa), and a noncovalently linked intermediate chain (150 kDa) [22]. The first step crucial for *Eh* colonization is the high affinity binding of the Gal/GalNAc lectin to Gal and GalNAc residues on colonic mucins [28, 33]. *Eh* binding to CHO cells and mucin was inhibited with Gal/GalNAc blocking antibodies [28], clearly demonstrating a role for the lectin in parasite adhesion. Curiously, although the heavy and light chains of the Gal/GalNAc lectin are not directly required for the parasite to penetrate the colonic mucosa [34], they facilitate colonization in/on the mucous layer and binding to epithelial cells, leading to cytolysis [35]. Dissolution or penetration of the mucous layer and cytolysis of IEC is mediated primarily by another virulence factor, *Eh* cysteine proteases (*Eh*CPs).

*Eh*CPs are cathepsin-like enzymes that have cathepsin-L-like structure and cathepsin-B-like substrate specificity [14]. A large portion of the *Eh* genome encodes *Eh*CPs, and more than 20 CP genes have been identified to date, although only a few are expressed in vitro [36]. *Eh*CPs manipulate and destroy host defenses to facilitate parasite colonization, invasion, and nutrient acquisition [14, 37]. They have been shown to promote degradation of the host mucous layer [21], and cleavage of secretory IgA and IgG [38, 39]. Furthermore, *Eh*CPs destroy villin, a key component of IEC, and compromise barrier integrity by acting on host cell TJ barrier proteins as well as degrading proteins of the extracellular matrix (ECM). thereby promoting cell death through loss of contact with the cell membrane [40, 41]. Indeed, trophozoites that are deficient in *Eh*CP activity are unable to traverse the mucous layer, induce an inflammatory response from IEC, or cause amebic liver abscesses [42–44]. It is estimated that 90 % of all CP activity in vitro can be attributed to *Eh*CP-A1, *Eh*CP-A2, and *Eh*CP-A5 [45], and within this subset, *Eh*CP-A5 is the best characterized [46–48]. It is a soluble membrane-bound protein [48], secreted by trophozoites, but is also found in punctate patches bound to the amoeba surface [49] through hydrophobic RGD domains [36]. *Eh*CP-A5 has been shown to induce a number of cytopathic effects in cultured intestinal monolayers and is absent in the nonpathogenic *Entamoeba dispar* [48]. Additionally, amoebae deficient in *Eh*CP5 were shown to have reduced mucinase and cysteine protease activity [44]. Importantly, studies to identify the specific proteases involved in disrupting the intestinal mucosal barrier have shown that *Eh* silenced for CP5 could not penetrate the colonic lamina propria or induce host pro-inflammatory cytokine secretion [31, 34].

We have shown that *Eh*CPs specifically cleave the MUC2 monomer near the C-terminus in a dose- and time-dependent manner [21, 50], resulting in the liberation of A- and B-subunits, cleaved products of 170 and 75 kDa, respectively [21], which destabilized the MUC2 polymer (Fig. 27.2b). Moreover, the degraded mucin fragments were 38 % less effective than native mucins at inhibiting amoebic adherence to IEC [13]. This finding is not surprising because the MUC2 C-terminus is critical for linking MUC2 monomers together into polymeric structures that confer many of the protective functions of the mucus layer. As predicted, pharmacological inhibition of *Eh*CPs with the selective, irreversible CP inhibitor E-64 (L-trans-epoxysuccinyl-leucyclamido-4-guanidino-butane) abrogated mucin degradation in vitro [44, 50]. In addition, trophozoites that were knocked down for *Eh*CP5 activity were ineffective at degrading ^{35}S-cysteine-labeled mucins [44], and they could not overcome the mucous barrier to disrupt high mucin-producing cell monolayers (LS-174T or HT29Cl.16E), but readily destroyed CHO cells in vitro [44].

27.2.3 E. histolytica *Destroys Intestinal Epithelial Cells and Tight Junction Proteins*

The epithelial barrier in the gastrointestinal (GI) tract offers a complex challenge for *Eh* to overcome. This single layer of epithelial cells functions to prevent the passage of harmful and noxious components, such as *Eh*, into the body while facilitating the

absorption of nutrients and maintaining physiological balance in the gut. The two main routes of movement across the epithelium, transcellular and paracellular transport, are tightly regulated and allow the barrier to be extremely selective. Intestinal permeability and barrier integrity depend on the structure and function of the IEC as well as the TJ.

27.2.3.1 Intestinal Epithelial Cells (IEC)

IECs are essential for nutrient absorption, electrolyte transport, and production of mucus glycoproteins, antimicrobial peptides, and bacteriolytic enzymes [51–54]. In addition, they prevent luminal antigens, solutes, and other microorganisms from making direct contact with the immune cells below, while at the same time allowing some nonpathogenic bacteria to communicate with the immune system to facilitate maturation, immune tolerance, and intestinal homeostasis [51, 55–57]. *Eh* binds to IEC via the Gal/GalNAc lectin and cytolyses these cells in a contact-dependent manner [33, 58, 59]. In addition, *Eh*CPs have been shown to degrade intestinal microvilli by villin proteolysis [60, 61] as well as components of the ECM including fibronectin, laminin, and collagen [41, 62, 63], which leads to detachment of IECs [64] and cell death [65, 66]. In this fashion, *Eh* may directly or indirectly cause cytolysis, thereby introducing a route for invasion (Fig. 27.1d; [67, 68]).

27.2.3.2 Epithelial Tight Junctions

The paracellular space between adjacent cells contains the apical junctional complex (AJC) composed of the TJ and the adherens junction (AJ), and, below this, the desmosome (Fig. 27.3). At the TJ, the membranes are joined by dynamic protein complexes responsible for determining the rate of paracellular passage. In contrast, the AJ and desmosome serve important roles in cell–cell communication and attachment [69]. The TJ is composed of membrane-spanning proteins such as claudins (CLDN) and occludin (OCLN), and scaffolding proteins including the zonula occludens (ZO), which anchor the transmembrane proteins to the cellular cytoskeleton [70–72]. Importantly, there are at least 24 members of the CLDN family of proteins, each with unique permeability properties and tissue expression (reviewed in [73]), which allows for tissue-specific permeability. Regulation of the TJ is accomplished by a variety of factors [74], but mechanistically through two general methods: (1) a change in the type and amount of protein at the TJ, or (2) contraction of the actomyosin ring through the activation of myosin light chain kinase (MLCK).

Disruptions of TJ by *Eh* have been shown both in vitro and, more recently, in vivo. The first studies into TJ disruption found that trophozoites were able to abolish the trans-epithelial electrical resistance (TER) of T84 cells and this was accompanied by increased paracellular permeability [75]. Although imaging of the cells was not done to show immunocytochemical changes in the levels of TJ proteins, the study found that disruption of TJ structure occurred through dissociation

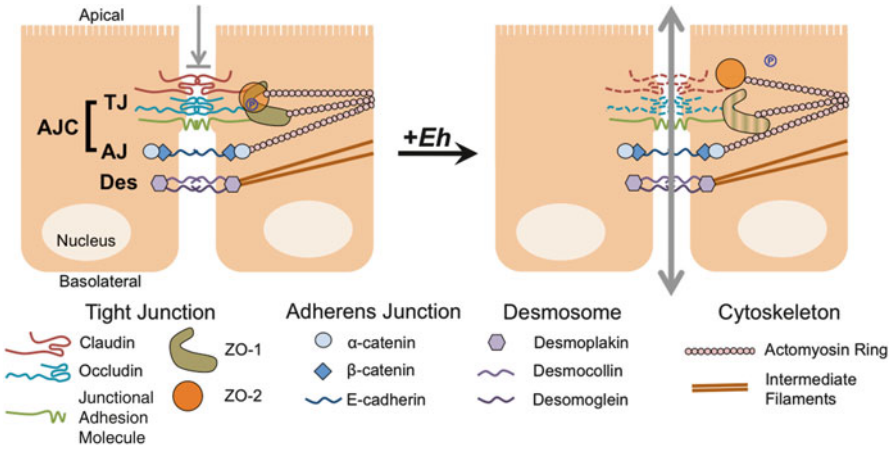

Fig. 27.3 *Eh* disrupts tight junction (*TJ*) proteins. Studies in vitro and in vivo demonstrate that *Eh* disrupts TJs preceding cell lysis by dissociating the scaffolding proteins ZO-1 and ZO-2, dephosphorylating ZO-2, increasing levels of pore-forming claudin-2, and decreasing levels of barrier-forming occludin and ZO-1. TJ disruption results in increased paracellular permeability. *AJC* apical junctional complex, *AJ* adherens junction, *Des* desmosome

of the scaffolding proteins that anchor the transmembrane components to the cytoskeleton. Specifically, the release of ZO-1 from ZO-2, dephosphorylation of ZO-2, and decreased levels of ZO-1 protein were shown although no effect was observed on OCLN [75]. Curiously, this was observed within 15 to 30 min of coculture and preceded lysis of the IEC monolayer [75]. Thus, the decrease in TER was not caused by dead cells but rather by *Eh*-induced changes in paracellular permeability. Importantly, this change was only witnessed in the presence of live virulent *Eh* in a dose- and time-dependent manner [76]. The potential virulence factor causing these changes in the host was unknown until 4 years later, when it was discovered that the drop in T84 TER following incubation with *Eh* could be prevented by pretreating the ameba with protease inhibitors [60], suggesting that an *Eh*-derived protease was responsible for the dysregulation of the TJ protein complex.

27.2.4 E. histolytica *and the Inflammatory Milieu*

The TJ protein complex is extremely sensitive to changes in physiological conditions and inflammation [55, 77], and pro-inflammatory cytokines have been shown to induce redistribution/endocytosis of TJ proteins [78, 79] or disassembly of the AJC. This disturbance could lead to increased intestinal permeability and exposure of intestinal immune cells to luminal antigens and pathogens [55, 80], further propagating the inflammatory response.

Early in an infection, *Eh* interacts with the host epithelium to induce activation of NF-κB to elicit pro-inflammatory responses [5]. In vitro, *Eh* induced an immune

response in IEC characterized by the secretion of various pro-inflammatory mediators [42, 81], causing the epithelial cells to behave much like antigen-presenting cells [43]. In studies with human colonic explants, *Eh* induced inflammation characterized by IL-1β, IFN-γ, TNF-α, IL-6, and IL-8, and cell lysis occurred as early as 4 h post incubation. Curiously, none of these responses was observed with the nonpathogenic *E. dispar* [34], and blockade of IL-1β and IL-8 released by intestinal cells reduced gut inflammation and tissue damage, as assessed by histology, neutrophilic influx, and changes in intestinal barrier permeability [82]. Furthermore, higher levels of IFN-γ and TNF-α were observed in patients with an active *Eh* infection who were passing amoebae in stool, , and the frequency of mRNA expression of IFN-γ, IL-4, IL-10, and transforming growth factor (TGF)-β was also significantly higher [83]. Interestingly, elevated levels of TNF-α production were associated with *Eh* diarrhea and recurrent diarrheal episodes, and significantly higher TNF-α protein levels were observed in children with diarrhea than those with asymptomatic infections [84]. These studies clearly demonstrate an association between the *Eh*-induced inflammatory responses and altered intestinal permeability, and this may be mediated by changes in the TJ. We have previously shown that the 170-kDa subunit of the Gal/GalNAc lectin induces robust TNF-α mRNA expression and protein release in macrophages [85]. More recently, the pro-mature form of *Eh*CP5, PCP5, induced an NF-κB-mediated pro-inflammatory response in colonic cells by binding of the RGD motif to $\alpha_v\beta_3$ integrin, resulting in the expression of TNF-α, IL-1β, COX-2, and IL-6 [86], demonstrating a role for these virulence factors in the induction of inflammation. Clearly, a pro-inflammatory response is an important feature of *Eh* infection, but what is not known is whether this response is beneficial to the host or not.

IL-1β, IFN-γ, and TNF-α are key factors in the inflammatory response induced by *Eh*, and treatment with these cytokines in vitro has been shown to increase intestinal permeability by altering the arrangement of TJ proteins [87, 88]. At physiologically relevant concentrations, IL-1β is known to increase intestinal TJ permeability [80, 87, 89]. Similarly, TNF-α increases cell permeability, leading to impairment of intestinal barrier function [90–92]. Numerous reports have also shown that IFN-γ and TNF-α act synergistically to increase intestinal permeability by altering the expression and localization of various TJ proteins, including CLDN-2, OCLN, and JAM [93–96]. Taken together, these data suggest that not only is *Eh* directly altering the expression of TJ proteins to increase intestinal permeability, but it may also induce a pro-inflammatory response that further exacerbates this loss of intestinal barrier function.

Two different routes of paracellular transport regulated by the TJ complex have been described, termed the “pore” and “leak” pathways [78]. Using colonic cells in vitro, the two pathways were defined based on size selectivity: a pore pathway is permeable to molecules with radii of 4 Å or less, and a leak pathway is permeable to larger uncharged molecules [97]. The current paradigm describes CLDN as the regulators of the pore pathway, where protein expression dictates permeability to smaller molecules, and the leak pathway, which is regulated by ZO-1 and OCLN [78]. Interestingly, distinct cytokines are associated with each pathway, where IL-13 is

well studied in regard to the pore pathway, increasing permeability by upregulating CLDN-2 [98, 99], and TNF-α is prominently featured when describing increased permeability associated with the leak pathway [96, 100]. Thus, it is possible that *Eh* induces a robust pro-inflammatory response with IL-1β and IL-13 to alter the expression of CLDN-2, thereby increasing intestinal permeability to ions, while at the same time inducing expression of IFN-γ and TNF-α, which modify the expression of ZO-1 and OCLN, thus facilitating the leak pathway. Together, these responses could lead to diarrhea, thereby facilitating the spread of infection.

Recently, using a murine colonic-loop model of acute infection in Muc2-deficient animals, virulent *Eh*-induced a robust pro-inflammatory response characterized by increased IL-1β, IFN-γ, TNF-α, and IL-13 [31]. Concomitantly, permeability changes were observed with increased expression of pore-forming Cldn-2 and loss of barrier-forming Ocln and Zo-1 [31]. Furthermore, these changes were reversed both in the presence of the *Eh*CP inhibitor E-64 and in amoebae deficient in *Eh*CP-A5 [31], further implicating a role for *Eh*CPs in the pathogenesis of intestinal amebiasis. Taken together, this suggests that in the absence of an intact mucin barrier, *Eh* are able to directly or indirectly disrupt TJ proteins leading to opening of the pore and leak pathways, thereby increasing intestinal permeability.

27.2.4.1 Prostaglandin E_2 (PGE_2) Produced by *E. histolytica* and Disruption of TJ

Prostaglandin E_2 (PGE_2) is a host lipid mediator that regulates a number of physiological functions in the gut, including GI secretion and motility as well as mucosal protection [101]. However, it is also involved in the pathophysiology associated with IBD and diarrheal disease [102]. PGE_2 exerts its physiological functions by binding to and signaling through one of four G protein-coupled E-prostanoid (EP1-4) receptors but has the highest affinity for EP4 [103, 104]. In the healthy mucosa, EP4 receptors are localized to the apical cell surface and the tips of mucosal folds, but under inflammatory conditions, this receptor is diffusely overexpressed throughout the mucosa [102]. In addition to host production, *Eh* also produces and secretes PGE_2 and it is considered a major virulence factor. Indeed, amoebic nuclear proteins that are enriched for the COX-like enzyme were shown to dose-dependently increase the production of PGE_2 in vitro [105], and during infection PGE_2 levels rise tenfold [106]. Studies in our laboratory have shown that *Eh* possesses a COX-like enzyme that allows live trophozoites to synthesize PGE_2 in the presence of an arachidonic acid substrate, and this can act to modulate host responses to infection in the gut where arachidonic acid is abundant [105, 107]. We have recently shown that PGE_2 signaling through the EP4 receptor reduced barrier integrity in a dose- and time-dependent manner [102], which was associated with increased chloride (Cl^-) secretion. Furthermore, PGE_2-induced spatial alteration of CLDN-4 and movement of sodium (Na^+) ions into the intestinal lumen establishing a NaCl gradient that could trigger water flow, leading to diarrhea [102].

27.3 Conclusions

Based on the data presented herein, it is likely that the *Eh* Gal/GalNAc lectin facilitates colonization of the mucous layer. However, during invasive disease, PGE_2 released by the parasite induces robust mucous secretory response that depletes mucin stores. Together with *Eh*CP-A5, this compromises the integrity of the mucous layer to facilitate invasion. Once the parasite penetrates this innate first line of host defense, it binds to and destroys IEC in a contact-dependent manner, disrupts the TJ, and induces a robust pro-inflammatory response. Consequently, intestinal barrier integrity is compromised, leading to the movement of proteins, water, and ions into the lumen, which causes diarrhea, one of the hallmark symptoms of *Eh* infection.

Acknowledgments Research presented in this chapter was supported by grants from the Canadian Institute for Health Research (CIHR), Natural Science and Engineering Research Council of Canada, Crohn's and Colitis Foundation of Canada (CCFC), and the Alberta Inflammatory Bowel Disease Consortium. V.K.S. and E.T. are supported by Alberta Innovates Health Solutions Studentships. K.C. holds a Tier 1 Canada Research Chair supported by CIHR.

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Chapter 28
Human Genetic Susceptibility to Amebiasis

Shannon N. Moonah, Nona M. Jiang, and William A. Petri Jr.

Abstract One important unanswered question in amebic research is why only a proportion of individuals exposed to *Entamoeba histolytica* develop clinical disease. Variation in host genetic makeup is a key to explaining interindividual differences in susceptibility to amebiasis. In this chapter, we discuss the effects of a nonsynonymous polymorphism of the leptin receptor and HLA class II alleles in mediating resistance to amebiasis. In a prospective study on a cohort of preschool children living in Dhaka, Bangladesh, children with two copies of the ancestral Q223 allele in the leptin receptor were resistant to amebiasis. Children and mice with at least one copy of the leptin receptor 223R mutation were more susceptible to amebic colitis. HLA class II allele DQB1*0601 and the heterozygous haplotype DQB1*0601/DRB1*1501 were found to be protective against human intestinal amebiasis. An understanding of the human genes that underlie susceptibility to amebiasis could open new potential therapeutic and prophylactic pathways to the management of amebiasis.

28.1 Introduction

A common feature of many infectious diseases is that only a proportion of exposed individuals develop symptomatic disease. Heritable factors have long been thought to play a significant role in individual susceptibility to infectious disease [1]. A breakthrough study in the 1980s reported that adopted children had a 5.8-fold increased relative risk of death from infection if his or her biological parent died prematurely from infection. In contrast, premature death of the adoptive parent from infection carried no increased relative risk of death from infection for the child [2]. The heritability of resistance to infection is supported by studies of monozygotic and dizygotic twins, in whom susceptibility to infectious disease is more similar in genetically identical monozygotic twins [3, 4].

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_28

Our understanding of how human genes influence susceptibility to amebiasis is still limited, but the recent findings are noteworthy. Millions of people worldwide are colonized with *Entamoeba histolytica*, yet only 20 % of individuals develop symptomatic disease, and with highly variable outcomes [5]. Why only a minority of individuals infected with *E. histolytica* develop disease is one of the most important unanswered questions in amebic research. Variation in host genetic makeup explains, in part, these interindividual differences in susceptibility to amebic infection. In this chapter, we focus on the role of leptin signaling and HLA class II alleles in mediating resistance to amebiasis in children.

28.2 Leptin and Its Receptor (LepR)

28.2.1 Leptin

Leptin is a 16-kDa adipocytokine that has important functions in both nutrition and immunity. Leptin was first characterized for its metabolic effects but is now recognized as an important immunomodulator [6–10]. Adipocytes are the primary source of leptin, but leptin is also produced by other cell types, including gastric and colonic epithelial cells and T cells [9, 11–13]. Leptin acts centrally to regulate appetite by signaling satiety, thereby inhibiting food intake and increasing energy expenditure [14]. In the periphery, leptin promotes the survival and function of a variety of immune cells [15]. Leptin also acts as a pro-inflammatory cytokine and skews the inflammatory response toward a Th1 phenotype while suppressing Th2 and regulatory T-cell functions [6, 10].

The immunomodulatory effects of leptin are of particular interest with regard to *E. histolytica* infection, as a Th1 response [through interferon (IFN)-γ] is a critical component of protective immunity against amebiasis [16–18]. Leptin also helps maintain intestinal mucosal integrity, stimulates mucin secretion upon muscosal damage, and modulates intestinal barrier function via its pro-proliferative and anti-apoptotic effects. Thus, normal leptin signaling may mediate resistance to amebiasis via several mechanisms such as stimulating a Th1 response, inducing antiapoptotic pathways, and promoting tissue repair [15, 19].

28.2.2 Leptin Receptor Signaling

The LepR gene directs the production of three alternatively spliced LepR isoforms: secreted, short, and long. The secreted forms only contain extracellular domains that bind circulating leptin, perhaps regulating the concentration of free leptin. Short-form LepRs lacks much of the intracellular domain, and the capacity to signal. The long form of the receptor (LepRb) contains all canonical signaling sites and is the crucial receptor isoform for leptin action (Fig. 28.1) [14, 20, 21].

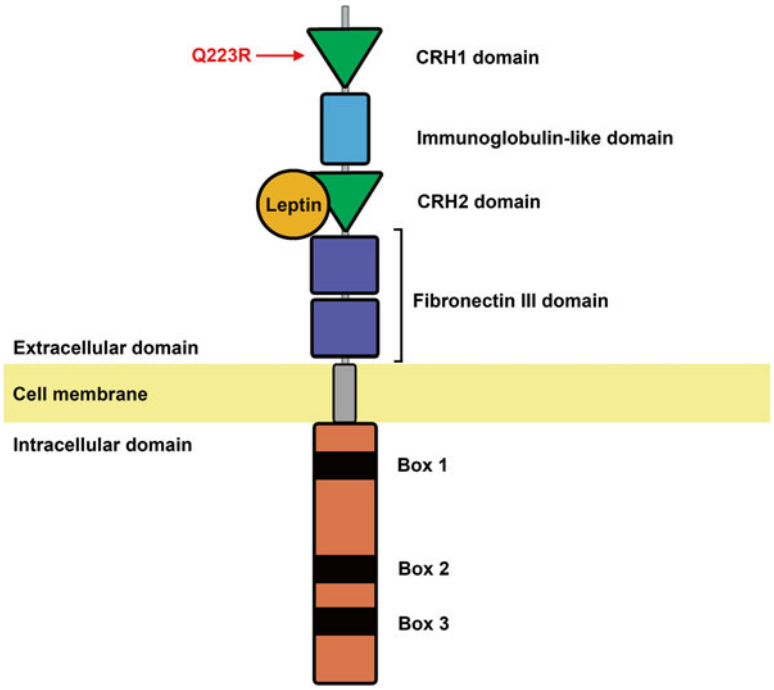

Fig. 28.1 Human leptin receptor and location of Q223R allele. Schematic diagram of the domain structure of the long-form leptin receptor and the location of the Q223R allele. Leptin binds to the cytokine receptor homology 1 (CRH1) domain. (Figure adapted from Kimber et al. [22])

LepRb belongs to the interleukin (IL)-6 receptor family of type 1 cytokine receptors [14]. LepRb contains an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic signaling domain. The extracellular domain is composed of two canonical cytokine receptor homology domains (CRH1 and CRH2), which are separated by an Ig-like domain, and followed by two membrane proximal fibronectin III domains [22]. LepRb is expressed in numerous cell types, including intestinal epithelial cells (IECs) and immune cells [11, 12, 15, 23–31].

The binding of leptin to the homodimeric LepRb activates Janus kinase 2 (JAK2), which phosphorylates three conversed tyrosine residues on the intracellular tail of LepRb (Tyr 985, -1077, and -1138) (Fig. 28.2). Phosphorylated Tyr 1138 binds and recruits signal transducer and activator of transcription 3 (STAT3), which upon phosphorylation induces effector functions along with the expression of suppressor of cytokine signaling 3 (SOCS3). During prolonged stimulation, SOCS3 binds to Tyr985 and attenuates LepRb signaling through a negative feedback inhibition. Phosphorylated Tyr985 recruits the SH2-containing tyrosine phosphatase (SHP2), which mediates activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK). Phosphorylated Tyr1077 recruits STAT5 to induce STAT5-dependent transcription activation. The functional effect of STAT5, however, is less clear [15, 19].

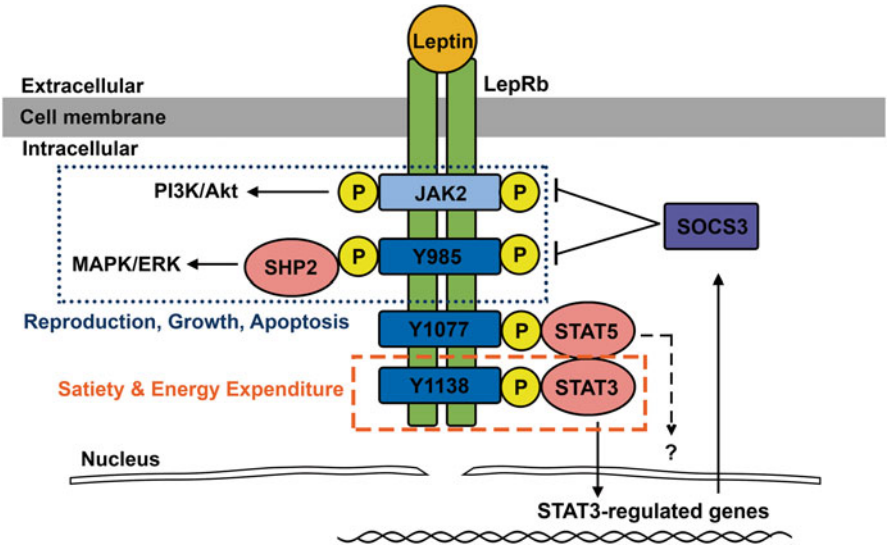

Fig. 28.2 Long-form leptin receptor (LRb) signaling. Leptin binding to the homodimeric LRb results in the activation and autophosphorylation of Janus kinase 2 (JAK2), subsequently inducing phosphorylation of downstream tyrosine residues (Tyr985, Tyr1077, and Tyr1138) in the intracellular tail of LRb. Phosphorylated Tyr1138 mediates the phosphorylation-dependent activation of signal transduced and activator of transcription 3 (STAT3), resulting in the transcription of SOCS3 and STAT3-dependent genes. During prolonged stimulation, SOCS3 inhibits leptin signaling via binding to phosphorylated Tyr985. Phosphorylated Tyr985 recruits SHP2, which activates the signaling pathway that culminates in ERK activation. Phosphorylated Tyr1077 recruits STAT5 to induce STAT5-dependent transcription; the functional effect of STAT5 is unclear. The phosphoinositide 3-kinase (PI3K)/Akt pathway emanates directly from phosphorylated JAK2. PI3K/Akt signaling is important for the LRb-mediated antiapoptotic and proproliferative effects via downregulating pro-apoptotic factors while upregulating anti-apoptotic/survival factors. *ERK* extracellular signal-regulated kinase, *JAK* Janus kinase, *MAPK* mitogen-activated protein kinase, *SHP2* Src homology phosphatase 2, *SOCS3* suppressor of cytokine signaling 3, *STAT* signal transducer and activator of transcription, *PI3K* phosphoinositide 3-kinase. (Figure adapted from Guo et al. [15])

In addition to the signal pathways coupled to tyrosine residues, some signals emanate directly from JAK2, including the phosphoinositide 3-kinase (PI3K)/Akt pathway and a minor component of the ERK pathway. It has been reported that PI3K/Akt or SHIP2/MAPK/ERK signaling contributes to LRb-mediated antiapoptotic and pro-proliferative effects. LRb Tyr1138/STAT3 and Tyr985/SHP2 signaling in the hypothalamus is crucial for the regulation of neuroendocrine functions such as food intake, energy balance, and reproduction [15, 21].

Individuals with congenital leptin or leptin receptor deficiency display immune dysfunction and are at increased risk for recurrent infection [32–34]. This observation is supported by studies in murine models where leptin and leptin signaling were found to be important in the host response against human pathogens such as *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae* [35–37].

28.3 Leptin Mediates Resistance to Amebiasis

28.3.1 Leptin and Amebiasis in Humans

In a cohort of 221 preschool children in Dhaka, Bangladesh, it was found that malnourished children were more likely to suffer from *E. histolytica*-associated diarrhea. Children with *E. histolytica*-associated diarrhea were 2.9 times more likely to be underweight and 4.7 times more likely to be stunted [38, 39]. Malnourished children in this study also had significantly lower levels of circulating leptin levels in their blood [23]. Because leptin is known to have immunomodulatory functions, low levels of leptin were hypothesized to account for the increased susceptibility of malnourished children to amebiasis. Murine models of amebic colitis supported a key role of leptin in *E. histolytica* infection, where leptin-deficient (*ob*/*ob*) and leptin receptor-deficient (*db*/*db*) mice were highly susceptible to *E. histolytica* infection, whereas wild-type C57BL/6 mice were resistant [15, 23].

In a 9-year prospective study in a cohort of preschool children in Dhaka, Bangladesh, a single amino acid polymorphism (Q223R) located on the CRH1 domain of the leptin receptor was associated with increased susceptibility to *E. histolytica* infection (Fig. 28.3a). Children with at least one arginine allele (223R) were nearly four times more likely to suffer *E. histolytica* infection as compared to those homozygous for glutamine (223Q) [23]. The Q223R association persisted after controlling for the effects of malnutrition, sex, and age. In a cohort of 86 males with amebic liver abscess (ALA), the Q223R association was also present [23]. Although congenital leptin or leptin receptor deficiency was shown to increase the risk of recurrent infection, these studies were the first to show a direct association of a leptin

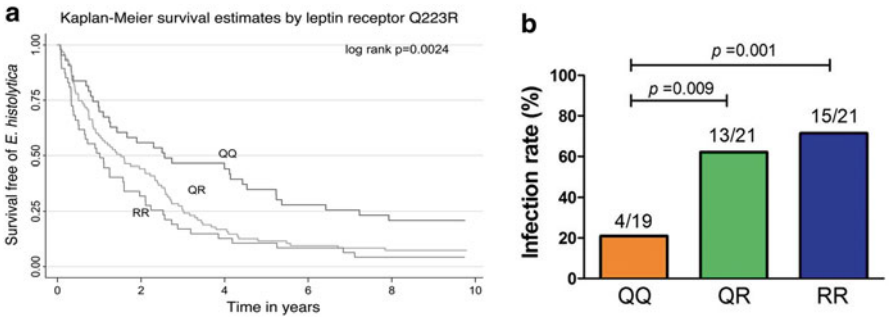

Fig. 28.3 The 223R allele of the leptin receptor increases susceptibility to intestinal amebic infection. **a** Graph shows how leptin receptor polymorphisms at position 223 influence susceptibility to amebic infection. Kaplan–Meier analysis of time to *Entamoeba histolytica* (*E. histolytica*) infection of children ($n = 185$) with Gln/Gln (QQ), Gln/Arg (QR), or Arg/Arg (RR) genotypes. **b** Mice carrying one or two copies of the arginine (QR or RR) allele at the 223 codon were more susceptible to intestinal infection with *E. histolytica* than those homozygous for the glutamine (QQ) allele (QQ vs. QR, $p = 0.009$; QQ vs. RR, $p = 0.001$; by χ^2 test). (Figure adapted from Duggal et al. [23])

receptor mutation with human infection [19]. These findings were recapitulated in murine models; mice with at least one 223R allele were significantly more susceptible to amebic infection and exhibited greater levels of intestinal epithelial apoptosis and mucosal destruction following infection (Fig. 28.3b) [23, 40].

28.3.2 Site and Mechanism of LepR Signaling During Amebiasis

To define the target site on which leptin acts to protect, bone marrow chimeras were created from wild-type and *db*/*db* mice, demonstrating that LepRb signaling in hematopoietic cells was not responsible for the susceptibility observed in *db*/*db* mice. Because LepR is expressed in IECs during infection, mice with IEC-specific deletion of LepRb (vil-cre LepR) were created. These IEC-specific LepR knockout mice were significantly more susceptible to infection with *E. histolytica* when compared with wild-type littermate controls [15, 23]. Thus, the site of leptin-mediated resistance was localized to the intestinal epithelium.

The study of downstream signaling pathways important in LepRb-mediated resistance identified that signaling through both LepRb/Tyr985 and -1138 was crucial for protection from *E. histolytica* challenge in mice. Although both Tyr985 and Tyr1138 knockout mice were more susceptible to *E. histolytica* challenge, there were notable phenotypic differences. S1138 mice exhibited greater mucosal damage whereas the L985 mice had minimal mucosal damage with higher levels of mucosal hyperplasia [15]. These observations suggest that leptin signaling activates more than one pathway to mediate resistance to amebiasis. An *in vitro* model that utilized knock-in mutants showed that STAT3 signaling via Tyr1138 was essential for leptin-mediated cellular resistance to *E. histolytica* killing in vitro. This study also showed that the 223R LepRb is significantly impaired in activation of STAT3 following leptin binding [41].

28.4 Human Leukocyte Antigen Class II Alleles and Amebiasis

Human leukocyte antigens (HLAs) have a crucial role in the initiation and regulation of immune responses and are also highly polymorphic, which has led to numerous studies of the influence of HLAs on susceptibility to infectious disease [1]. As mentioned previously, $CD4^+$ cells are important in conferring protective immunity against amebic infection through an interferon (IFN)-γ-mediated response [16]. Thus, the ability of specific HLA II alleles to present amebic antigens to $CD4^+$ T cells may alter the response to an infection and may be responsible for susceptibility to *E. histolytica* [42].

The HLA class II genotype frequencies were determined for a cohort of 185 preschool Bangladeshi children who were actively monitored for *E. histolytica* infection over a 3-year period. A protective association of the DQB1*0601 HLA allele and the heterozygous haplotype DQB1*0601/DRB1*1501 with *E. histolytica* infection was observed. Children who were heterozygous for the DQB1*0601/DRB1*1501 haplotype were 10.1 times more likely to not have suffered from *E. histolytica* infection. These findings support a role for HLA class II antigen processing and $CD4^+$ T cells in resistance to amebiasis. It remains unknown whether the DQB1*0601 allele directly protects the host or whether the allele is in linkage disequilibrium with another gene controlling resistance to *E. histolytica* [42].

28.5 Future Directions and Conclusions

These studies on the leptin receptor and HLA class II alleles demonstrate a clear role for human genetics in explaining differences in susceptibility to amebiasis, but there remain several outstanding questions. Although there is a protective association between the homozygous Q233 genotype in the leptin receptor with *E. histolytica* infection, the exact mechanism by which leptin signaling protects against mucosal tissue destruction remains unknown. Additionally, the emerging field of microbiome science yields new avenues for amebic research. How host genetics influences the microbiota and how the microbiome, in turn, affects susceptibility to amebiasis remains to discovered. A thorough understanding of the mechanisms by which host genetic factors influence susceptibility to amebic infection would increase our understanding of the pathogenesis of *E. histolytica* and also open new potential therapeutic and prophylactic pathways to the management of amebiasis.

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Chapter 29
Immune Response in Human Amebiasis: A Protective Response?

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Abstract Infection with *Entamoeba histolytica* provokes human diseases that range from amebic colitis to the life-threatening amebic liver abscess; nonetheless, about 90 % of infected people do not develop any symptom. What circumstances or which events in the dynamics of the host–parasite relationship define the outcome of infection? What are the causes that allow the apparently spontaneous clearance of the infection? How protective is the immune response against *E. histolytica*? Why do some infected people remain asymptomatic and why do others develop clinical symptoms? What is the role of immunogenetic polymorphism in the final outcome of the infection? These questions are the major aims of many research groups around the world. In the present review, we analyze the potential participation of the immune system and the genetic variants of genes associated with immune response in the human host with its susceptibility or resistance to develop amebic invasive disease.

Abbreviations

ALA	Amebic liver abscess
APC	Antigen-presenting cell
COX	Cyclooxygenase
CRD	Carbohydrate recognition domain
DC	Dendritic cell
Eh/CP5	*Entamoeba histolytica* cysteine proteinase 5
Gal/GalNAc	Galactose/*N*-acetylgalactosamine
HLA	Human leukocyte antigen

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_29

iTreg	Induced regulatory T cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
LFA-1	Leukocyte function-associated antigen-1 integrin
LPPG	Lipophosphopeptidoglican
LRR	Leucine-rich repeats
LTi	Lymphoid tissue inducer
MHC	Major histocompatibility complex
NFκB	Nuclear factor κB
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SFB	Segmented filamentous bacteria
SNP	Single-nucleotide polymorphism
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

29.1 Introduction

The World Health Organization has reported an indirect estimation of prevalence of human infection caused by the protozoan *Entamoeba histolytica* in 1997. The invasive disease causes 100,000 deaths per year around the world, ranking in second place after malaria as cause of death from parasites [1–3]. Every year, as many as 50 million people will develop intestinal amebiasis and about 10 % of individuals with amebic colitis will progress to an amebic liver abscess [1, 2, 4]. The *E. histolytica* resistance and infective form are the cysts that are ingested with fecal-contaminated food or water. During their gastrointestinal transit, the cyst wall is removed in a process known as excystation, giving origin to eight trophozoites per cyst, the trophozoites colonize the large bowel mucosa. Colonization can develop two different outcomes of the host–parasite relationship: one, the asymptomatic colonization of the intestinal mucosa creating a commensal association with the host, and second, the invasion of the deeper structures of the intestinal tissues producing the invasive intestinal colitis and/or dysentery including development of the bottle flask ulcers

characteristics of intestinal amebiasis. As a consequence of intestinal invasion, *E. histolytica* can be responsible for extraintestinal clinical forms of the disease, the most frequent being the amebic liver abscess. What circumstances or which events in the dynamics of host–parasite relationship define the outcome of infection? Why do some infected people remain asymptomatic and others develop clinical symptoms?

The host immune system interacts with the environment and its potential harmful foreign factors through two evolutionarily conserved systems in mammalians: one, the innate host defense mechanism that responds to external invaders in an unspecific schedule, and the other, the highly specific acquired or adaptative immune response.

In the first case, there are the so-called pattern recognition receptors (PRRs) that identify generic molecules of pathogen. The major PRRs are transmembrane glycoproteins with leucine-rich repeats called Toll-like receptors (TLRs). TLR binding provokes a series of intracellular signaling that leads to the activation of the innate immune system toward the target cells. TLRs can bind the pathogen-associated molecular patterns (PAMPs), the sensing of PAMPs being the first step in inflammatory response to pathogens. The second step in the immune response is the one induced by different inmmunogens of invaders: this is antigen specific and long lasting. Effector molecules (antibodies) and sensitized T lymphocytes (Th-CD4 and Tc-CD8) are the major components of this response. However, molecules encoded in the major histocompatibility system (MHC) genes restrict almost all adaptive responses to foreign invaders. The immune response-associated genes are the most polymorphic family genes in nature, and human genes are not an exception. The characteristic of resistance or susceptibility to pathogens displayed by mammalians is based on the genotype of TLRs and MHC genes of a particular host within different species. Human–parasite interaction in amoebic infection induces an immune response producing specific antibodies and sensitized T lymphocytes, particularly when this interaction is deleterious to the host. Is this response effective and protective? Epidemiological studies of infection and disease face two potential scenarios for morbidity caused by amebiasis: Are the characteristics of genetic variants of the parasite to which the host is exposed what determines the incidence of disease? Or is it the genetically defined type of the host immune response? These questions are of the outmost interest to many research groups around the world.

In the present review, we intend to analyze the recent data about the potential participation of polymorphism of genes associated with immune response in the susceptibility or resistance of human host to develop amoebic invasive disease.

29.2 The First Encounter and Infection Progress

The cysts of *E. histolytica* are protected from anti-microbe peptides and digestive enzymes in the mouth, and from stomach gastric acid by the outer chitinous wall. It is at the small intestine, where the cyst breaks down giving eight trophozoites,

where *E. histolytica* faces the immune system. The first parasite–host encounter is with mucins at the epithelial layer of the intestine. One major function of mucins in the colon is to stratify and compartmentalize microorganisms. In the colon, there are two structurally distinct mucous layers: the outer layer, which is normally full of bacteria, and the inner layer that is resistant to penetration [5]. Both layers are composed of basically MUC2 mucin. However, the inner layer has a higher concentration of this mucin, making it a thicker barrier. At the outer layer *E. histolytica* may live and move without many complications. The amoeba can adhere to mucin oligosaccharides of the mucous layer by its 170-kDa galactose/*N*-acetylgalactosamine (Gal/GalNAc) lectin [6]. In most cases (around 90 %), *E. histolytica* remains at the outer mucous layer where it obtains nutrients from cellular debris and bacteria, causing an asymptomatic infection [3].

An important factor for the stratification of the microorganisms in the mucous layer is the production of immunoglobulin A (IgA) [7]. The major antigen-presenting cells (APCs), the dendritic cells (DCs), may sample *E. histolytica* antigens at the luminal side of the epithelium [8]; they then migrate to the lymph nodes where they interact with B lymphocytes, inducing them to produce IgA. The IgA^+ B cells migrate to the lamina propria and secrete specific anti-*E. histolytica* IgA, which is translocated through the epithelial cells into the intestinal lumen. Once in the lumen, the IgA helps to maintain the stratification of microorganisms in the mucous layers [7]. Thus, the *E. histolytica*-specific IgAs may contribute to keeping a balance between the epithelium integrity and the trophozoites. Furthermore, in a study made on Bangladeshi children, Haque and coworkers [9] showed that children with anti GalNAc lectin IgA found in their stool were free of *E. histolytica* colonization in the intestine, and they were less susceptible to new infections, supporting evidence of the protective role of IgA in amebiasis.

Normal intestinal anaerobic bacteria produce extracellular glycosidases and proteases that can degrade mucin glycoproteins, cell-surface glycoproteins, and glycolipids [10]. Although amebic Gal/GalNAc lectin is resistant to many proteases, the bacterial glycosidases and proteases degrade the lectin on *E. histolytica* trophozoites and make them less capable to adhere to target cells [10]. However, in a small percentage of infected individuals, as a result of unclear mechanisms, the parasite can express an invasive phenotype, allowing the crossing of the mucous barrier to invade the underlying epithelium. The cysteine-dependent proteases of *E. histolytica* digest the MUC2 mucin [11] degrading the mucous layer; thus the trophozoites, by taking advantage of their high motility, move across the mucous layer to contact the epithelial cells. It has also been proposed that mucous depletion is caused by the continuous irritation by *E. histolytica* that causes collapse of the mucous cells, which are responsible for the major feature of intestinal amebiasis, that is, bloody diarrhea with mucus.

Once the parasite contacts the enterocytes through the attachment of Gal-GalNAc residues by its Gal-GalNAc lectin [12], its activity can cause the erosion of the epithelium. The cytolytic capacity of *E. histolytica* is a consequence of amoebapores, pore-forming proteins produced by the parasite. Epithelial and stromal cells are stimulated to increase the expression of chemokines such as IL-8, GROα, GM-CSF [13, 14], and

MCP that provoke the infiltration of polymorphonuclear leukocytes (PMNs), which surround invading trophozoites. In addition, these host cells produce pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-6 [13]. At this stage, not only are neutrophils recruited to the site of tissue lesions but also other cells such as plasma cells, lymphocytes, eosinophils, and macrophages [15]. The influence of *Entamoeba* over acute inflammation facilitates its crossing through the intestinal epithelium; this notion is supported by the capacity of *E. histolytica* cysteine proteinase 5 (*Eh*CP5) to cleavage secreted pre-IL-1β into its active form [16], promoting a persistent inflammatory state. Moreover, *Eh*CP5 activates inflammatory responses without proteolysis. It can bind integrin αvβ3, leading to the activation of nuclear factor κB (NFκB) that results in the expression of pro-inflammatory genes [17].

For unclear reasons, some trophozoites colonizing and invading the colon reach the mesenteric and portal circulation, and by following the bloodstream arrive at the liver sinusoids.

29.3 Innate Immune Response to *E. histolytica*

At the intestine, the first immune response cells to arrive are the neutrophils. There is evidence of their importance in resistance to *E. histolytica* infection. In the liver of neutropenic mice, the inoculation of trophozoites generates larger lesions than those observed in normal mice [18]. Furthermore, using a monoclonal antibody against the neutrophil marker Gr-1, the suppression of neutrophils in CBA mice increased not only the susceptibility but also the intensity of the lesions [19].

Normally, neutrophil response would be able to eliminate an invading parasite; nevertheless, *E. histolytica* not only survives to neutrophil response but in addition it massively lyses the neutrophils. It can destroy them by the engagement of β2-integrin CD18, promoting a reactive oxygen species (ROS)-dependent apoptosis of neutrophils [20], and inhibiting the enhancement of inflammatory response through phagocytosis of the remaining neutrophil [21]. Even low-virulence strains of *E. histolytica* survive neutrophil attack [22]. Neutrophil activation is a double-edged sword; there are also detrimental effects of neutrophils over the intestinal epithelium. Neutrophils not only do not succeed in killing trophozoites, but they increase the damage to the surrounding tissue by the liberation of their granules during their lysis. Activated neutrophils release ROS such as nitric oxide (NO), hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-). Trophozoites are able to survive neutrophil attack by the expression of reducing enzymes that detoxify ROS; moreover, their superoxide dismutase [23], peroxiredoxin, thioredoxin, and p34 thioredoxin reductase [24] help *Entamoeba* to survive the oxidative burst. Furthermore, it has been shown that *E. histolytica*, through its arginase gene, is able to affect directly the NO production competing for arginine, hydrolyzing it to urea and L-ornithine, impairing its use by the NO synthases of host cells [25].

In vitro experiments of interaction between epithelial monolayers of a human colonic carcinoma cell line [26] and in monolayers (or in suspension) of liver cells [27]

with trophozoites showed that the colonic cells and the hepatocytes were more affected when the interaction was in the presence of neutrophils. On in vivo models of infection it is likely that neutrophils make a major contribution to the tissue damage.

Macrophages are key participants in the intestinal innate immune system. They are highly abundant in the lamina propria and also can be found in the mucosa, submucosa, and muscularis mucosae [28]. However, they show low proliferation and chemoattractant activity in response to microbial ligands, cytokines, and chemokines, despite possessing the molecular mechanisms to elaborate strong phagocytic and microbicidal responses [29]. Resident intestinal macrophages express a high level of human leukocyte antigen (HLA)-DR and are highly phagocytic, just like blood monocytes [30], and express CD36, which is an important molecule for phagocytosis of apoptotic cells [31]. In contrast to circulating monocytes, intestinal macrophages do not express important innate immune receptors such as CD14, growth factors receptors for IL-2 and IL-3, IgA, IgG, and CR3 receptors, and the integrin leukocyte function-associated antigen-1 (LFA-1) [30, 31].

In general, normal intestinal mucosa does not display inflammation, which suggests that the intestine must have an immune-tolerant environment resulting from the exposure to food antigens. Thus, the intestinal immune system is in a constant status of hyporesponsiveness. This semi-anergy state is in part caused by the presence of transforming growth factor-β (TGF-β) in the stroma of the intestinal lamina propria, produced by intestinal epithelial cells and mast cells [31, 32].

Another important mediator for this condition is the production of interleukin-10 (IL-10). The principal sources of this cytokine are the neutrophils and intestinal macrophages; the importance of IL-10 is related to the antiinflammatory properties of this cytokine. Individuals with normal production of IL-10 develop a hyporesponsive state in the presence of *E. histolytica*, generating an asymptomatic infection. The disruption of the epithelium layer integrity is a prerequisite for the trophozoite invasion of the mucosa and submucosa [15, 33, 34]. Interestingly, the appropriate production of IL-10 has been shown to contribute to the maintenance of the integrity of the mucosal barrier by deregulating the pro-inflammatory signal of NFκB in epithelial cells, stimulating MUC2 production, suppressing antigen-presenting cell activation, inducing $CD4^+$ regulatory T cells (Treg), promoting B-cell class switching to IgA, and maintaining an antiapoptotic effect on the intestinal epithelium [35–37].

E. histolytica takes advantage of the previous immune-tolerant scenario and progress through the lamina propria to the submucosa. As the invasion continues, the ulcer formation and its extension form the typical flask shape lesion. In contrast to intestinal macrophages, blood monocytes are responsive to the microbial stimulus. Thus, their recruitment is necessary for initiating a macrophage response against the parasite. Macrophage responses are directed toward PAMPs on infectious agents. The major PRRs that recognize these PAMPs are the TLRs. They comprehend a family of type I membrane glycoproteins that possess domains with extracellular leucine-rich repeats (LRRs) responsible for PAMP recognition and a Toll/interleukin-1 receptor (TIR) domain required for signal transduction. *E. histolytica*

PAMPs are recognized in vitro by TLRs. Murine TLR 2 and 4 identify the *Entamoeba* lipophosphopeptidoglycan (LPPG) [38, 39], TLR 2 and 4 in macrophages recognize the Gal-GalNAc lectin [40], and *E. histolytica* DNA can be recognized by TLR9 [41]. Activated macrophages in vitro kill trophozoites through cytolytic proteases and by the production of ROS [42, 43]. However, in vivo there is a deficient response to *Entamoeba* trophozoites, which may be caused by the attenuated immune response observed in local intestinal macrophages. Interestingly, the binding of LPPG by TLR does not activate a pro-inflammatory response. Instead, it induces a tolerance-like response through production of interleukin-10 and downregulation of *tlr*-2 gene expression [38]. This action is consistent with the complete panorama of immune tolerance at the intestinal mucosa, maintained by the macrophages in response to the presence of *E. histolytica*.

Intestinal macrophages can be viewed as very important effector cells in the presence of intestinal flora that may affect *E. histolytica* to some extent without generating an inflammatory response which could be potentially hazardous for the host. Macrophages have little effect over immune regulation because they do not perform key activities characteristic of mononuclear phagocytes, including chemotaxis and accessory cell function. They fail to express the costimulatory signals such as molecules CD80 (B7.1), CD86 (B7.2), and CD40, and the cytokines IL-1, IL-10, and IL-12 [29], essential components for priming and expansion of T-cell clones.

At the lamina propria the key antigen-presenting cells are the intestinal DCs. DCs may sample *E. histolytica* antigens at the luminal side of the epithelium, and then interact with B lymphocytes in lymph nodes for the activation of the adaptive immune response. It has been shown that DCs can recognize the Gal/GalNAc lectin of *E. histolytica*, increasing the expression of CD80, CD86, CD40, and MHC class II molecules, inducing a Th1-type cytokine production of IL-12 and tumor necrosis factor-α (TNF-α) [44].

In its way through blood circulation toward the liver, *E. histolytica* has to face the complement system. The parasite can subvert the complement system because the Gal/GalNAc lectin impairs the assembly of membrane attack complex [45] and by amoeba cysteine proteases that degrade C3a [46] and C5a [47]. Recently, the calreticulin gene of *E. histolytica* has been cloned [48, 49]. As other calreticulins in mammals, the protein is a reticulum endoplasmic-associated protein and is probable that in *E. histolytica* and *E. dispar* may display the same ubiquitous functions as in other parasites. One interesting property of *Eh*CRT, both the native and recombinant forms, is its immunogenicity for the human host [50]. Recombinant *Eh*CRT displays one of the functions described in other protozoa as immune evasion mechanisms, that is, the capacity of *Eh*CRT to bind the human C1q component of serum complement, inhibiting the activation of the cascade of classical complement pathway (González et al., in preparation).

Once the trophozoites reach the liver sinusoids, they cross the endothelium and enter the hepatic parenchyma. Liver parenchyma includes endothelial cells, hepatocytes, and stellate cells. In addition, there are immune cells such as Kupffer cells, DCs, polymorphonuclear leukocytes, and lymphocytes. Kupffer cells are monocytes with cytotoxicity, phagocytic, and antigen presentation functions [51].

They also produce ROS, NO, TNF, and other pro-inflammatory mediators. The liver lymphocytes consist of T cells, natural killer T (NKT) cells, natural killer (NK) cells, and B cells. NKT and NK cells can recognize trophozoites directly by the sensing of PAMPs by their PRRs. NK cells produce interferon-γ (IFN-γ) that activates macrophage for producing TNF and NO [52]. TNF production has been considered as an important feature of amebic liver abscess (ALA).

Although DCs can be observed in sinusoidal spaces [53], there is debate about if they are or are not permanent residents of the liver sinusoids. They participate in trophozoite recognition and antigen presentation functions. DCs interact closely with NK cells, producing pro-inflammatory cytokines from both cell types [54]. DCs exert their effects on NK cells through the expression of IL-12, TNF-α, and type I IFNs, inducing secretion of IFN-γ, expression of CD69, cell proliferation, and enhanced cytotoxicity. In return, NK cells express NKp30, TNF-α, and IFN-γ to promote the maturation of DCs, the production of pro-inflammatory cytokines, and the increase of their capacity to stimulate T-cell responses [54].

Lotter and coworkers [52, 55] report that Jα18$^{-/-}$ mice without invariant natural killer T cells (iNKT) develop more severe liver abscess from *E. histolytica* infection. They also showed that when iNKT cells are specifically activated with α-galactosylceramide (α-GalCer), there is a significant reduction of ALA lesions, whereas CD1d$^{-/-}$ mice develop more severe abscesses. The authors mentioned that the purified phosphoinositol moiety (PI) of LPPG from *E. histolytica* membranes (*Eh*LPPG) is the potential NKT cell ligand. NKT recognition of this moiety induces protective production of IFN-γ. They demonstrate that IFN-γ production depends on the presence of CD1d, a simultaneous signaling of TLR2/6, and the secretion of IL-12. Because the production of IFN-γ has been considered to be important in ALA development [56], the role of iNKT cells in the outcome of liver infection is crucial.

Another component of the innate immune system that protects against *Entamoeba* infection is the leptin molecule and its receptor. Better known as an appetite-regulating hormone with effects on appetite, energy expenditure, behavior, and glucose metabolism, leptin is also related to pro-inflammatory signals toward a Th1 phenotype, inducing proliferation and activation of T lymphocytes, dendritic cells, and NK cells. The leptin receptor also expresses in the intestinal mucosa, where it has a protective role in amebic infection. Guo et al. [57] reported, in a series of experiments using a murine model with mutations in leptin and leptin receptor genes, that they were more susceptible to *Entamoeba histolytica* infection, displaying severe intestinal lesions as a consequence of the impairments of signaling pathways.

29.4 Adaptive Immune Response to *E. histolytica*

The infection of *E. histolytica* could persist for several weeks or months, time enough to form an adaptive immune response. Trophozoite antigens such as Gal/GalNAc lectin can be presented to lymphocytes by macrophages or dendritic cells.

E. histolytica antigenic peptides are presented to $CD4^+$ T cells through HLA class II molecules encoded by the MHC genes. It has been suggested that effective immunity against *E. histolytica* must include a strong cellular response via the production of Th1 cytokines, for example, IFN-γ produced via peripheral blood mononucleated cells (PBMCs) stimulated by *E. histolytica*. This event has been associated with protection from future episodes of diarrhea caused by *E. histolytica* infection [56]. In addition, there are reports of *E. histolytica*-infected patients treated with steroids or splenectomized whose cell-mediated immunity is suppressed, which increase their susceptibility to develop invasive amebiasis [58, 59]. Moreover, during amoebic infection T cells appear to be hyporesponsive to mitogens or antigen-stimulated proliferation [60], promoting Th2 cytokine production and suppression of the Th1 cytokines, resulting in a retarded T-cell response directed to amoebic antigens [61].

Approximately 80 % of ALA patients have an IgG systemic response. However, until today there is controversy related to the protective role of these antibodies against infection. At the experimental level, the explanation has been the capability of trophozoites to concentrate surface-bound antibodies to the posterior edge of trophozoites (capping) to thereafter release this cap [62]. Capping formation has been considered an immune evasion mechanism displayed by *E. histolytica* trophozoites; nevertheless, this phenomenon has been only observed in in vitro experiments. In our experience, in human amebic liver abscesses biopsies and in slides of ALA in animal models, capping formation has not been observed up to the present.

Haque et al. in a prospective study [63, 64] showed that Bangladeshi children with IgA antibodies against the CRD domain of Gal/GalNAc lectin had diminished frequency (17 %) of new *E. histolytica* infection in comparison with the frequency of children who did not produce IgA anti-CRD domain antibody (57 %). However, in the same population, there was a paradoxical increase of the frequency of new infections in children who expressed serum anti-trophozoite IgG. Furthermore, infection was not only more frequent in these children but also more severe.

Another explanation of the transient antibody response to *Entamoeba* is that the amoeba cysteine proteinases are able to degrade the secretory IgA [65] and the serum IgG [66], which might be a mechanism of evasion of antibody response that may influence the invasion outcome.

29.5 Immune Response-Associated Genes: The Role of Polymorphisms in Host Susceptibility or Resistance

In nature, the host–parasite relationship is highly dynamic and its spectrum goes from the harmful interspecies interaction named parasitism, in which the parasite can damage the host tissues or alter their function, causing infectious disease, to the commensal interspecies relationship where one or both species take advantage of the association; this is the type of interspecies relationship characteristic of asymptomatic infection in intestinal amebiasis (asymptomatic cysts passers). What kind of mechanism between human host and *Entamoeba* protozoa defines the outcome of

E. histolytica infection? We do not now know. Much more work has been developed on the virulent characteristics of *E. histolytica* and in the genetic variability of different strains of both *E. histolytica* and *E. dispar*. Studies in the polymorphism of encoding genes or nonencoding sequences of DNA of both *Entamoeba* species have been performed. A clear association of particular *Entamoeba* genotypes with the outcome of the infection (luminal, noninvasive; amebic colitis, intestinal invasive; extraintestinal, amebic liver abscess) is at least controversial [67, 68]. Furthermore, at the opposite edge of the host–parasite relationship is the host response [69], in this case the human host. Soon after the discovery of the high polymorphism of TLRs genes [70], several genetic variations had been associated with susceptibility to develop a variety of diseases [71–73], some of which are parasitic diseases [74–76]. Recently, we studied single-nucleotide polymorphisms (SNPs) of TLRs in ALA patients to estimate the associations between genetic variants of TLRs and susceptibility to ALA in Mexican populations. We have found some interesting results (article in progress) that suggest susceptibility relationships of TLR SNPs and ALA in Mexican populations. The immunogenetic predisposition of the host is a research field that had been deeply studied for many infectious diseases [77], but in the case of *E. histolytica* infection it needs further exploration. Besides TLRs, there are other polymorphisms within candidate innate immune genes that may have participation in immune recognition, apoptosis, and inflammation influencing the disease outcome. One of such genes is the leptin receptor gene, whose polymorphism at Q223R position has been associated with susceptibility to *Entamoeba histolytica* infection and epithelial ulceration after infection in Bangladeshi children [78].

During evolution, mammals have evolved a family of genes associated to the adaptive immune response; in fact, molecules encoded by these genes are responsible for the successful triggering of the adaptive immune response in all its ubiquity. The MHC that in humans is represented by the HLA encodes one of the most polymorphic genes in mammals. These genes encode the class I, class II, and class III molecules. The extensive polymorphism of HLA class I and II genes that are responsible for present antigen peptides in the context of the mentioned molecules, class I molecules, are engaged in the presentation of antigenic peptides to T-cell receptors (TCR) from endogenous antigens, intracellular pathogens, or self-antigens; in contrast, class II molecules are engaged in presentation of antigens peptides from external origins, non-self-antigens, or extracellular antigens. In all cases the polymorphisms may be maintained by genetic pressure that results from the need to present many different foreign peptides to the immune system to effectively combat multiple different and potentially lethal infectious diseases.

Previous studies have shown associations of HLA class II alleles with susceptibility or protection to particular infectious diseases. The HLA class II genetic polymorphism may be associated with infectious pathogens, such as protozoan parasites; for example, the association of the HLA class II haplotype *DRB1*1302/DQB1*0501* with *Plasmodium falciparum* in Gambia [79], and HLA-DR4 with more severe malaria in Gabon [80]. Because most of individuals infected with *E. histolytica* do not develop a symptomatic disease, it is likely that genetic factors of the host are responsible for that resistance. There have been some reports about genetic predisposition

to develop ALA [78, 81]. For example, in a Mexican population Arellano et al. [82] described for the first time a susceptibility association of HLA-DR3 antigen with the predisposition to develop ALA in Mexican mestizos; moreover, the authors detected an extended haplotype in these patients related to the expression of complotype SC01 (HLA Class III molecule) [83–85]. However, this association was not observed in individuals with amoebic colitis or asymptomatic infections [85, 86]. Furthermore, in Dhaka, Bangladesh, a protection association has been reported against intestinal amebiasis with the HLA class II allele *DQB1*0601* and *DQB1*0601/DRB1*1501* haplotype [81]. After many years of Dr. Kretschmer's and Arellano's work in Mexico the interest in approaching this part of the host–parasite relationship has reawoken. In our laboratory, we have studied the allele frequencies of *HLA-DRB1* and *HLA-DQB1* by PCR-SSO in patients with ALA and healthy blood donors. Results showed *HLA-DRB1*04* alleles as a potential susceptibility factor in ALA patients from the State of Sonora and Mexico City (Hernández et al., in preparation).

29.6 Immune Response in Experimental Models of Amebiasis

Despite the abundance of reports about *E. histolytica* pathogenesis and innate and adaptive immune response, most of them come from studies made on animal models, the major models being those developed in gerbils, hamsters, rats, and mice [87]. Recently, the use of pigs has provided a valuable large animal model. The immune system and mechanisms of immune responses in pigs are closer to human hosts than rodents, based on genetic, anatomical, and physiological similarities. Currently, pigs represent an alternative and better model for human amebiasis [88]. Furthermore, there is also the option of using pig colon explants [89] that seem to resemble what happens on human intestine explants [90]. Although the pig model is promising, there are some inconveniences to solve in the management of this animal in many research laboratories; nevertheless, a number of *E. histolytica* pathogenesis key features have been unveiled in this experimental model [88].

Nowadays, the in vivo animal model closest to human amoebic infection is that previously described by Seydel et al. [91]; in this experimental model the human intestine is xenografted in the back of SCID mice. The *E. histolytica* infection of these human intestines had shown the local production of pro-inflammatory cytokines such as TNF-α, interleukin-1B, interleukin-8, and cyclooxygenase (COX)-2 [91, 92]. The role of intestinal inflammation is highlighted by impairing NF-κB, activation on intestinal epithelial cells, which provokes the inhibition of IL-1β and IL-8 and neutrophil influx into the *E. histolytica*-infected human intestinal xenografts [93]; in addition, secreted components from trophozoites induce a protective response in human colonic epithelial cells through an interaction with macrophages that suppresses activation of NF-κB and increases resistance of the epithelium to apoptosis [94]. Moreover, when human intestinal xenografts are depleted

from neutrophils, reduction of tissue damage occurs in the first stage of infection with *E. histolytica* [93].

There are other tools such as ex vivo experiments using segments of human colon [90]. These studies showed the kinetics of parasite colonization into the mucous layers and the mucosa; structural changes in the mucosa as well as the development of the inflammatory response from the first encounter of host cells and *E. histolytica* strains. For the ex vivo research of the amoeba interactions in the liver, slices taken from hamster livers have been used [95]. This promising approach could be, in the near future, the answer for proteomic and genomic research in human amebiasis.

The contribution of these studies is the demonstration of the obvious role of inflammation in tissue damage in the infection of *E. histolytica* and that innate immunity might ultimately be the key factor for the restraint, resolution, or susceptibility to the infection outcome, not only for amebic colitis but also for ALA (Fig. 29.1).

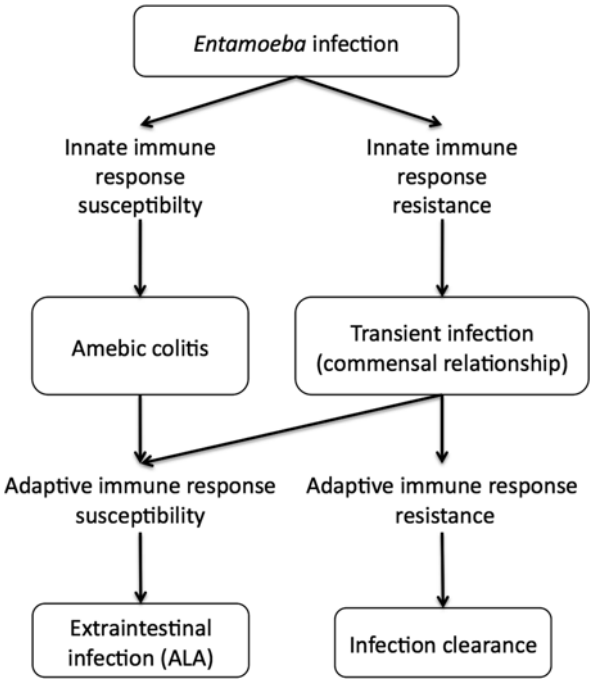

Fig. 29.1 Influence of immune response on *Entamoeba* infection outcome. The result of *Entamoeba histolytica* infection depends highly on the susceptibility or resistance provided by the immune system. In the intestine, a resistant innate immune response provides protection from most *E. histolytica* strains, leading to either a transient infection or even a commensal relationship, whereas a susceptible response would lead to the development of an amebic colitis. Individuals with amebic colitis depend on the ability of the adaptive immune system to clear the infection. Otherwise, in a susceptible adaptive response, an extraintestinal invasive infection such as amebic liver abscess (ALA) would result

29.7 Human Intestinal Microbiota and the Modulation of the Immune Response to *Entamoeba* Infection

An emerging issue related to the development of disease caused by pathogenic microorganisms is their relationship with the host microbial communities such as bacteria, fungi, and viruses, known as microbiota, and the capability of these communities to modulate the immune response against pathogens. In the human intestine there is a very large quantity of commensal microorganisms. The immense amount of bacteria at the intestine comprise $10^{12}/cm^3$ or more of the intestinal contents [7], and include more than 1,000 species based on the diversity of sequences of the small subunit ribosomal RNA genes (16S), and the metagenome of the intestinal microbiota has more than 100 fold the number of genes at the human genome. The coevolution of host and microbiota had created a mutualistic status that avoids invasion of the microbes to host tissues and the generation of an intense inflammation while preventing the elimination of important microbial constituents through the activation of innate and adaptive immune components. In fact, gut microbiota contribute to host nutrition [96], to protection against enteropathogens [97], and to the development of robust innate and adaptive immune systems [98]; thus, the alteration of the complex and dynamic host–microbiota relationships (often referred to as dysbiosis) is involved in the development of a broad range of diseases from obesity and malnutrition to inflammatory intestinal disease, cancer, and infectious diseases [99].

The mucosa at the gastrointestinal tract possesses more cells of the immune system that all combined secondary lymphatic tissues [100]. Besides the mesenteric lymph nodes and the Peyer's patches, there are tertiary lymphoid tissues, developed postnatally, including isolated lymphoid follicles (ILFs) and crypto-patches in the small intestine and colonic patches in the large intestine [101]. These lymphoid tissues are stimulated by innate lymphoid cells (ILCs) such as lymphoid tissue inducer (LTi) cells, ILFs, both sites of secretion of IgA. Cryptopatches consist of $ROR\gamma t^+$ LTi cells and $CD11c^+$ dendritic cells underlying the intestinal epithelium. In addition to organized tissues, there are throughout the intestinal lamina propria ILCs, myeloid cells such as $CD103^+$ and $CD103^-$ $CX3CR1^{hi}$ dendritic cells, and different types of T lymphocytes. $CD103^-$ $CX3CR1^{hi}$ dendritic cells sample luminal antigens by extending dendrites through the tight junctions of the epithelium [8], a process considered important for the development of immunological tolerance to orally ingested antigens [102] and that may be related to the tolerance to commensal microbial products. The T lymphocytes comprise a variety of cells that express either the αβ- or γδ-TCR and exhibit a memory-like phenotype. The αβ CD4 T cells include Th17 cells and regulatory T cells (Treg). Th17 cells produce IL-17 and IL-22, cytokines that have influence in maintaining the mucosal barrier preventing inflammation by microbial products that cross the epithelium. However, when not properly regulated, Th17 cells can be potent mediators of inflammation. Treg cells express Foxp3 and IL-10 and are an important regulator of inflammation. Many $Foxp3^+$ cells acquire their regulatory phenotype in the lamina propria and are known as induced regulatory T cells (iTreg) [103].

Guo et al. [57] demonstrated in an *Entamoeba histolytica* vaccination model that IL-17 provides protection to mice vaccinated with the recombinant LecA fragment of the Gal/GalNAc lectin. Interestingly, the major source of IL-17 in these mice was the CD8 T cells, whereas CD4 T cells express high levels of IFN-γ. The authors suggest that IL-17 may enhance the protective functions of Th1 immune response. The major role of the intestinal immune system is the control of the exposure of host tissue to microorganisms. Both the innate and the adaptive immune systems exert control over the intestinal microbiota [7, 100, 104–106]: first, by stratification made through the production of antibacterial proteins by epithelial cells to the mucous layer such as the Reg IIIγ lectin [107] and the α-defensins [108], or through IgA by B cells activated by dendritic cells that sample bacteria at the epithelium; and second, by compartmentalization of the microbes, confining the intestinal microorganisms that invade intestinal tissue. Usually, commensal bacteria that penetrate the intestinal barrier are engulfed and eliminated by macrophages at the lamina propria. Some of them are phagocytosed by dendritic cells and are taken alive to the mesenteric lymph nodes to be presented to T and B cells, which recirculate and exert a protective specific IgA response all through the intestine surface [109]. Both stratification and compartmentalization mediate control the intestinal microbiota composition.

In return, the intestinal microbiota influences the development of lymphoid tissue and the regulation of several cells of the immune system. For example, in the innate immune response, the reduction in Myd88-TLR signals induced by bacterial products in mice augment the permeability of the mucosa, allowing commensal bacteria invasion and diminishing its postinjury reparation [110]. Furthermore, TLR signaling by LPS can replace gut bacteria, protecting the epithelium integrity [111]. Thus, activation of TLRs by commensal microbiota is a key factor to protect the intestinal mucosa against injury and to promote its regeneration.

The microbiota also shapes the development and regulation of the adaptive immune system, for example, driving the accumulation of either Th17 or Treg cells. In germ-free mice the small intestine is not developed and there are not isolated lymphoid follicles, or production of secretory IgA and CD8αβ intraepithelial lymphocytes [7]. Also, the colonization of mice with segmented filamentous bacteria (SFB) provokes a marked increase of Th17 and Th1 cells [112]. The colonization of gnotobiotic mice with a cocktail of members of the cluster IV and XIVa of the *Clostridium* genus stimulates the proliferation of lamina propria and systemic Tregs cells; many of these iTregs express IL-10 [113].

The intestinal microbiota forms a complex ecological community that not only interacts with the host immune system but also has collective interactions [114]. Some commensal microbiota act as barriers that protect the intestinal epithelium against pathogen invasion [115], representing a potential obstacle for invasion. Such is the case of the parasite *Cryptosporidium parvum*, in which normal microbiota protects the host from invasion [116]. In contrast, early reports regarding *E. histolytica* intestinal infection underline the need of gut microbiota for the development of pathology [117]. More recently, it was shown that pathogenic bacteria *Escherichia coli* (EPEC) and *Shigella dysenteriae* augmented the cytopathic effect of *E. histolytica* in vitro [118].

When human colonic cells are exposed to pathogenic bacteria *Staphylococcus aureus* (Gram$^+$) or *Shigella dysenteriae* (Gram$^-$) and then to the carbohydrate recognition domain (CRD) of *E. histolytica* Gal/GalNAc lectin, an inflammatory microenvironment that facilitates cell destruction by trophozoites is established, suggesting that CRD acts as a PRR-like molecule inducing the innate immune response through the recognition by TLR-2 and TLR-4, which may be a key factor for development of invasive disease [119]. One possible explanation for this is the increase of pathogenicity of *Entamoeba* surface polysaccharide ligands of the superficial membrane that are altered by the presence of intracellular bacterial symbionts [120]. This factor opens the door to new potential therapies against *E. histolytica* extraintestinal diseases by changing the microbiota structure of the intestine, but to achieve this goal it is necessary to know first the composition and the interactions of gut microbiota with *E. histolytica* and the human intestinal cells.

In summary, we have an incredible mass of new knowledge about the intestinal immune response and the regulatory interactions between the intestinal ecosystem and the host immune response, notwithstanding, in the case of *E. histolytica* human infection, the combination of the host genotype and the composition of the intestinal microbiota, which combined with the influence of environmental factors such as diet and antibiotics are the key determinants of the outcome of *E. histolytica* infection (Fig. 29.2). Taking the epidemiological evidence of the high prevalence of human asymptomatic infection (90 % of infected individuals) and the low rate of invasive disease (10 % of infected people), in our opinion it is likely that the asymptomatic infection is the expression of an effective innate immune response capable of maintaining the balance between the host–parasite relationship during short or long periods, allowing the spontaneous clearance of infection, although individuals are not exempted of new infections.

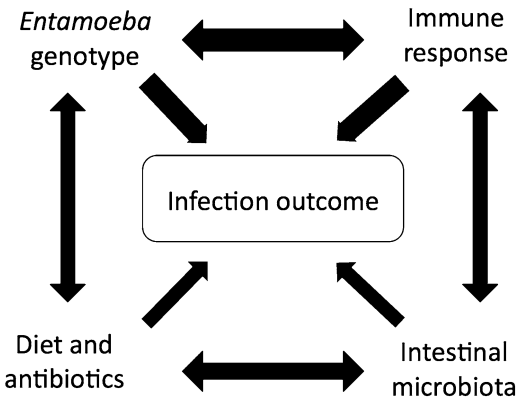

Fig. 29.2 *Entamoeba* infection outcome is a multifactor phenomenon. Results of *E. histolytica* infection depend not only on the genotypes of the parasite strains but also on the diet and antibiotics taken, as well as relationships with the intestinal microbiota. A major factor that determines infection outcome is the relationship that the parasite and the intestinal microbiota have with the host immune system, mainly with the innate response, which is important to establish if the infection is either transient or commensal or begins its progression to amebic colitis

Acknowledgments The authors thank, for financial support, the National Council for Science and Technology (CONACyT): Grant 2010-1-140990, and SEP-CONACyT 79220; and by the PAPIIT program for Scientific Research Development (DGAPA) UNAM: Grants PAPIIT IN204208, IN206408, IN206405, IN226511 and PAPIME 200105.

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Part VI
Drug Resistance and Drug Discovery

Chapter 30
Metronidazole and the Redox Biochemistry of *Entamoeba histolytica*

Michael Duchêne

Abstract Metronidazole remains the gold standard drug for treatment for *Entamoeba histolytica* infections. In the introduction of this chapter, the drug is described: its history, its major uses, and some concerns raised about its genotoxicity in the past. Even a short introduction shows that the activity of metronidazole is closely linked with the redox metabolism of its target organisms. Thus, in the first section, the metabolism of *E. histolytica* under microaerophilic conditions is described. The second section reviews the thiol-containing antioxidant systems of *E. histolytica* in comparison to other organisms. The third part considers how *E. histolytica* can handle and inactivate molecular oxygen and more reactive oxygen and nitrogen species. In the fourth part, the activity of metronidazole is discussed in the light of the redox metabolism of *E. histolytica*. The fifth part addresses the prospects of metronidazole resistance, and the chapter ends with a final section on perspectives, where we stand, and some of the unresolved questions.

Abbreviations

DPI	Diphenyleneiodonium
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
FDP	Flavin diiron protein
PFOR	Pyruvate ferredoxin oxidoreductase
SAT	Serine acetyl transferase

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_30

30.1 Introduction

Metronidazole is the gold standard drug for the treatment of amebic dysentery and liver abscess. Since 1966 this nitroimidazole drug has been used successfully to treat *Entamoeba histolytica* infections. Metronidazole is also used against *Trichomonas vaginalis*, *Giardia lamblia*, and bacteria such as *Helicobacter pylori*, *Clostridium difficile*, and *Bacteroides fragilis* [1, 2]. Consequently, this important antibiotic agent is found in the WHO "Model List of Essential Medicines" [3]. All the target organisms of metronidazole have in common that they live in microaerophilic or anaerobic environments, which makes them more susceptible to oxidative damage.

Historically, the parent compound of metronidazole is azomycin (2-nitroimidazole). This compound (Fig. 30.1) was first isolated in the Tokyo region from a soil bacterium designated as a new *Streptomyces* or possibly *Nocardia* species [4]. The manuscript describes the activity of the compound against various bacteria such as *Bacillus subtilis*, *Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, and a *Mycobacterium* species with least activity against *E. coli* and the *Mycobacterium*. No antifungal activity was detected, and no protist parasites were included in the study. Independently, a team led by R. Despois in the Research Centre of Rhône Poulenc in Vitry-sur-Saine discovered the same compound. They had extracted it from another streptomycete found on Réunion Island, an island in the Indian Ocean not far from Mauritius. The main focus of the French team was on *Trichomonas vaginalis*: they prepared and tested several derivatives of azomycin for their activity against this parasite, and their favorite became compound R. P. 8823, today known as metronidazole [5] (Fig. 30.1). In the Rhône Poulenc patent, filed in 1957 in France, *E. histolytica* is mentioned as another target without providing detailed data. The work by Cosar and Julou on *T. vaginalis* was finally published in the French language in 1959 [6]. The next of the microaerophilic protist parasites to be treated with metronidazole was *Giardia lamblia* [7]. Around the same time metronidazole

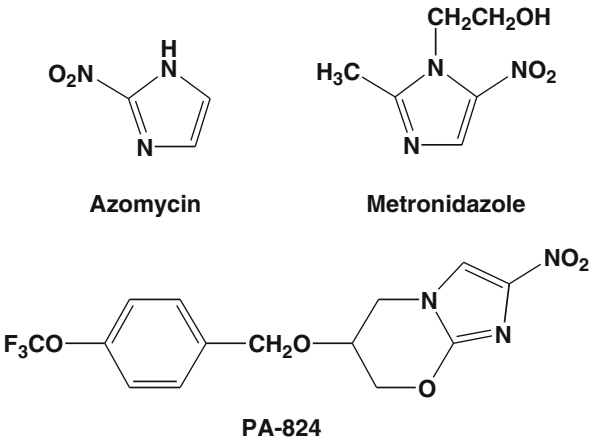

Fig. 30.1 Important nitroimidazoles: azomycin (2-nitroimidazole); metronidazole (2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethanol); PA-824 ((6*S*)-2-nitro-6-{[4-(trifluoromethoxy)benzyl] oxy}-6,7-dihydro-5H-imidazo[2,1-*b*][1, 3]oxazine)

was used to treat bacterial gingivitis [8], the first time to the author's knowledge that metronidazole was used against a bacterial infection. Finally, in 1966 the landmark paper by Powell and coworkers in the *Lancet* [9] demonstrated that *E. histolytica* was the most serious parasite that could be treated with metronidazole. Since then, this inexpensive and inconspicuous molecule has certainly saved many millions of human lives: it might have saved the life of Fritz Schaudinn, the discoverer of *E. histolytica* who died at an early age of invasive amebiasis. Interestingly, this does not appear to be the end of the story on anti-infectious nitroimidazoles; rather recently, a new member, PA-824 (Fig. 30.1), with activity against *Mycobacterium tuberculosis* has been introduced [10], which is currently being evaluated in clinical studies.

Metronidazole has very favorable pharmacokinetic properties. It can be administered by the oral route, and it is readily absorbed. In one study on trichomoniasis patients, peak serum levels of 115 ± 22 μM between 1 and 3 h after administration of 1 g of drug were obtained [11]. To this date, to the author's knowledge there are no published reports on clinically resistant *E. histolytica* strains able to cope with this drug concentration.

Although metronidazole is the gold standard to treat amebiasis, it is not without adverse effects. It may cause a metallic taste in the mouth, diarrhea, or nausea, or rarely more severe neurological symptoms. The most troubling property is its genotoxicity. Clearly, DNA damage has been observed in bacteria, laboratory animals, and even humans, but the question has not been settled whether metronidazole is really teratogenic or really causes cancers in humans [12], and the genotoxic effects will probably play a minor role as usually metronidazole treatment will be short term.

30.2 *Entamoeba histolytica*: Redox Twists of Glucose Fermentation

E. histolytica, as a microaerophilic organism, lacks conventional mitochondria. It does not possess the enzymes of the Krebs cycle and is unable to carry out oxidative phosphorylation [13]. The glycolytic pathway from one glucose molecule to two molecules of pyruvate invests one ATP for the hexokinase reaction and one molecule of pyrophosphate for the phosphofructokinase reaction [14], and gains two molecules of GTP in the unusual phosphoglycerate kinase reaction [15] and two molecules of ATP in the pyruvate orthophosphate dikinase reaction [16]. In the process, two molecules of NADH are generated. Similar to other microaerophilic parasites, *E. histolytica* oxidizes pyruvate by a pyruvate:ferredoxin oxidoreductase (PFOR) [17–19] generating acetyl-CoA, carbon dioxide, and reduced ferredoxin. *E. histolytica* possesses an acetate thiokinase [18] carrying out the important reaction acetyl-CoA + Pi + ADP → acetate + CoA + ATP, allowing the gain of one molecule of ATP per acetyl-CoA.

When *E. histolytica* trophozoites are grown axenically under microaerophilic conditions, the electrons from the reduced ferredoxin and the NADH generated can be transferred to oxygen, regenerating oxidized ferredoxin for the pyruvate:ferredoxin oxidoreductase and NAD^+ for the glyceraldehyde phosphate dehydrogenase. Then, acetyl-CoA can be used to generate an extra ATP, and acetate is excreted. Under anaerobic conditions, the alcohol dehydrogenase reduces acetyl-CoA to ethanol using NADH, the extra ATP is lost, and ethanol is excreted instead. This phenomenon has been known for a long time [20], and it shows although high concentrations of oxygen are toxic to *E. histolytica* trophozoites, they are able to profit from a limited aerobic metabolism. A more detailed study showed that glucose and galactose could best stimulate oxygen consumption in *E. histolytica*, whereas amino acids or lipids were unable to do so. The study also confirmed that the inhibitors of oxidative phosphorylation such as cyanide or rotenone and inhibitors of Krebs cycle such as fluorocitrate had no or only minor effects on the oxygen consumption [21]. Although this limited aerobic metabolism has some benefits for energy balance, the reduction of oxygen may produce reactive products such as hydrogen peroxide that have to be processed, as described next.

Definitively, hydrogen peroxide is able to kill *E. histolytica* in a process resembling apoptotic cell death [22, 23]. It is able to oxidize free protein cysteine thiol groups, leading to incorrect disulfide bridges, but it can also form cysteine sulfenic and sulfinic acids [24]. This is a complicated matter: some proteins are very sensitive, others are more stable, so definitively *E. histolytica* needs good protection mechanisms for its protein thiols. In addition, hydrogen peroxide can damage iron–sulfur cluster proteins such as PFOR and ferredoxin.

30.3 *Entamoeba histolytica*: Protecting and Fixing Reduced Biomolecules with Large and Small Thiols

Protecting biomolecules from oxidative damage or repairing oxidatively damaged biomolecules is important in aerobic and anaerobic organisms from man to bacteria. Most organisms possess two major systems, a thioredoxin system and a glutathione system. Thioredoxin was initially named as the factor providing electrons for ribonucleotide reductase, an enzyme involved in the biosynthesis of deoxynucleotides [25]. The thioredoxin system uses the electrons from the two cysteine residues in the active site of the small protein to act on incorrect disulfide bridges in a target protein to reduce the disulfide, allowing the target protein to fold correctly. In the process, thioredoxin is oxidized and has to be regenerated by the flavin enzyme thioredoxin reductase. Thioredoxin reductase itself is reduced by NADPH. Although thioredoxins are ubiquitous [26], in plants an especially complex thioredoxin system appears to be operative [27]. Plant thioredoxins cannot only repair damaged proteins, but they can also act as sensors that can fine tune enzymatic activities.

In *E. histolytica*, the thioredoxin system is a major target of metronidazole, as is described next, and is highly complex. Recently we used a method from the plant

field [28], and in analogy generated a mutant *E. histolytica* thioredoxin with one active site cysteine (C34) replaced by serine. This protein can interact and form intermolecular disulfide bonds with its target proteins but is unable to complete the reaction and release its reduced targets. The mutant thioredoxin with bound target proteins was then isolated, the proteins were released by dithiothreitol (DTT) and analyzed by two-dimensional gel electrophoresis and mass spectrometry. In total, 234 target protein spots were resolved: a very large thioredoxin interactome, among these important proteins such as peroxiredoxin, serine acetyl transferase, and purine nucleoside phosphorylase, were found after analyzing an initial set of 20 spots [29].

The second ubiquitous redox protection system is based on glutathione (γ-glutamylcysteinylglycine) and glutathione reductase, the NADPH-dependent enzyme that regenerates oxidized glutathione. Currently PubMed lists more than 109,000 papers on glutathione, more than tenfold the number of thioredoxin papers. This system is essential in plants [30]; in newborn animals such as guinea pigs and rats, pharmacologically induced glutathione deficiency is lethal within a few days [31], in *Plasmodium falciparum*, the glutathione and thioredoxin systems form a complex network as in higher organisms [32], but surprisingly, in *E. histolytica*, glutathione is absent [33], and also the enzymes involved in glutathione metabolism such as glutathione reductase, glutathione peroxidase, and glutathione-*S*-transferase are absent from the genome.

In trypasomatids, another redox protective thiol is found, trypanothione. This molecule is a spermidine linking two glutathiones, which was originally isolated from the nonpathogenic trypanosomatid *Crithidia fasciculata* [34]. Its synthesis has been studied extensively as a drug target in the trypanosomatids [35, 36]. There is ongoing unresolved controversy whether this molecule is present in *E. histolytica*. Originally, Ondarza and coworkers had reported its isolation from *E. histolytica* [37, 38], and this finding was strongly disputed by Ariyanayagam and Fairlamb [39]. Later the group of Ondarza reported the cloning of a trypanothione reductase from *E. histolytica* [40]. An agreement has not been reached. Two arguments weaken the view of the presence of trypanothione in *E. histolytica*: on the one hand, even in trypanosomatids, glutathione, the precursor of trypanothione, is present at about tenfold the concentration of trypanothione [34], but glutathione was not found in *E. histolytica*; it is strange that this would be missed. The other problem is that the reported sequence of the putative trypanothione reductase is not found in the *E. histolytica* genome databases. The matter needs to be investigated by independent colleagues.

So, in the absence of glutathione, the major small thiol in *E. histolytica* is cysteine [33]. Cysteine is also essential for the culture of *E. histolytica*. Definitively its main purpose is not only to provide reducing power, as it can be replaced by cystine but not by DTT in culture media [41]. Recently, it was shown that *E. histolytica* can be kept in protein-free media for a short period of time only if the media are supplemented with cysteine and ascorbate [42]. Cysteine depletion of the medium was recently demonstrated to cause extensive changes in the metabolite composition in *E. histolytica* [43]. In vivo, it is assumed that *E. histolytica* trophozoites import cysteine from their host; however, they are also able to synthesize cysteine from serine.

The first step is catalyzed by serine acetyltransferase (SAT) [44]. There are three isoforms of this enzyme [45]: SAT1 is most sensitive to feedback inhibition by L-cysteine, SAT3 is almost insensitive, and SAT2 is intermediate in this respect. Oxidatively damaged SAT1 can be repaired by thioredoxin [29]. The second step is catalyzed by cysteine synthase, two isoforms of which have been characterized [46]. Dissimilar to other organisms, serine acetyl transferase and cysteine synthase do not form a cystein synthase complex [47]. The described route is the only one found in *E. histolytica*; the other route via homocysteine and cystathionine is absent in this parasite. Treatment of *E. histolytica* trophozoites with inhibitors of *E. coli* SAT inhibited their proliferation [48].

Besides cysteine, there could be other minor thiols in *E. histolytica* such as methanethiol, 1- and 2-propanethiol, and hydrogen sulfide, but these have not yet been studied well.

30.4 *Entamoeba histolytica*: Managing Small Stressful Oxidants

As already indicated, *E. histolytica* is able to make use of small amounts of oxygen for its energy metabolism [21]. This process appears to be normal so long as the oxygen concentration remains limited: one estimation was less than about 5 % [49].

Although molecular oxygen is only mildly toxic to *E. histolytica*, sometimes it must be removed. The best solution is the full reduction to water, which can be achieved by flavodiiron proteins (FDPs). These proteins, which contain a flavin mononucleotide-binding site and a di-iron cluster, are usually found in anaerobic bacteria where they reduce oxygen or nitric oxides [50]. *E. histolytica* possesses four isoforms. The FDPs were probably acquired by lateral gene transfer from prokaryotes. Homologous proteins were also found in *T. vaginalis* and *G. lamblia* [51]. *E. histolytica* FDP1 is able to reduce molecular oxygen to water, but surprisingly has only marginal activity toward nitric oxide [51]. Thioredoxin reductase is another flavin protein that can reduce oxygen [52, 53], but it generates hydrogen peroxide, which has to be removed separately (see following). The same is true for two recently discovered atypical NADPH-dependent flavin- and iron-sulfur cluster-containing oxidoreductases termed EhNO1 and EhNO2, which among others reduced molecular oxygen to hydrogen peroxide [54].

A classical reactive oxygen species, many times more toxic than molecular oxygen, is the superoxide radical anion O_2^-; this can be produced by attacking phagocytes [55] or it can be generated when the nitroradical anion formed from metronidazole reacts with molecular oxygen (see following). Superoxide anions are managed by superoxide dismutases (SODs) carrying out the reaction $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$. There are iron-, copper/zinc-, and manganese-dependent superoxide dismutases, but *E. histolytica* has only a single gene, coding for an iron-dependent SOD [56].

Hydrogen peroxide is quite toxic for *E. histolytica*, as already described, and therefore it is essential for the parasite to be able to reduce this compound. In contrast to higher organisms, *E. histolytica* lacks a catalase; its main system for this job are the peroxiredoxins. In the molecular cloning papers of the first member of the group [57, 58] the protein was described as a cysteine-rich surface antigen by its high cysteine content and presumed surface location. The protein possesses an unusual sequence, KECCK KECQE KECQE KECCC, close to its amino-terminus. In the genome database, seven highly similar isoforms are found. Their function as peroxidases was discovered in 1997 by Bruchhaus et al. [59],and independently by Poole et al. [60]. It was also shown that the peroxidase is also found throughout the cytoplasm of the trophozoites as well as on the surface [59] and that its activity depends on thiols. Later Arias et al. [61] demonstrated that peroxiredoxin obtains its electrons from thioredoxin. Not surprisingly, a search for interaction partners of thoredoxin in *E. histolytica* revealed peroxiredoxins as major targets [29]. So, taken together, the electrons flow from NADPH via thioredoxin reductase (TrxR) to thioredoxin (Trx) to hydrogen peroxide, producing water as the final product (Fig. 30.2). When *E. histolytica* trophozoites invade the host tissues, they encounter increased oxidative stress, so it comes as no surprise that virulent strains express more peroxiredoxin mRNAs [62]. This expression is also reflected on the level of the proteome [63] so that peroxiredoxins can even be regarded as a virulence factor. Treatment of trophozoites with trichostatin A increased the expression of the peroxiredoxins and made the trophozoites more resistant to oxidative stress [64].

A second *E. histolytica* protein that can reduce hydrogen peroxide is rubrerythrin, a 21-kDa protein encoded by a single gene and containing an iron–sulfur cluster that is present in the mitosomes as well as the cytoplasm [65]. So far the electron donor for this protein is not known, nor its exact contribution to the detoxification of hydrogen peroxide in comparison to the major peroxiredoxins.

Without any doubt, nitric oxides are toxic to *E. histolytica*. They are part of the human defense against the parasite [66, 67]. Exogenously added nitric oxides kill *E. histolytica* in a process resembling apoptotic death [68], and they lead to numerous and drastic changes in the amoebae: upregulation of heat-shock proteins, redox proteins, and glycolysis enzymes as well as fragmentation of the endoplasmic reticulum [69]. Treatment of amoebae with an NO-releasing metronidazole derivative NCX-972 was more effective than treatment with metronidazole [70].

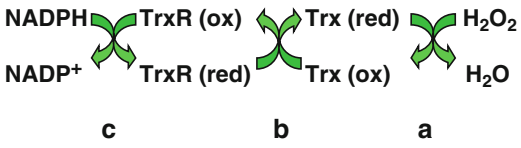

Fig. 30.2 The peroxiredoxin reaction (**a**) transfers electrons from thioredoxin (Trx) to hydrogen peroxide. Thioredoxin is regenerated (**b**) by thioredoxin reductase (TrxR), and thioredoxin reductase by NADPH (**c**)

So far it is not known which enzymes detoxify nitric oxides in *E. histolytica*. When the parasites were exposed to nitrosative stress, a microarray study showed numerous upregulated genes [71], but many of them are hypothetical proteins. Flavodiiron proteins (FDPs) would have been good candidates, but the first study found only a marginal activity of FDP1 against nitric oxide and only activity toward molecular oxygen [51]. The other FDPs from *E. histolytica* have not been characterized in this study, so one might have higher nitroreductase activity. It was also argued, as FDP1 is an abundant protein, that the small activity against NO could still be sufficient.

30.5 Metronidazole: How Does It Work in *E. histolytica*?

Metronidazole and other nitroimidazoles are pro-drugs that need to be reduced at the nitro group to become cytotoxic [72]. The possible fate of metronidazole is depicted in Fig. 30.3. The transfer of one electron results in the formation of a nitro radical anion (Fig. 30.3b); further reduction produces a nitroso group (Fig. 30.3c), a hydroxylamine group (Fig. 30.3d), and finally an amine group (Fig. 30.3e). Although there is general agreement on this sequence of reduction steps, even today dispute remains about the relative contribution of each intermediate to the cytotoxic activity. The main problem is that the intermediates form as a mixture, are all very reactive and unstable, and cannot be investigated individually. The nitro radical anion was discovered in *Tritrichomonas foetus* [73] and it was seen as the main damaging agent derived from metronidazole, destroying biomolecules such as DNA [74] and thus leading to cell death. Compared to other radicals,

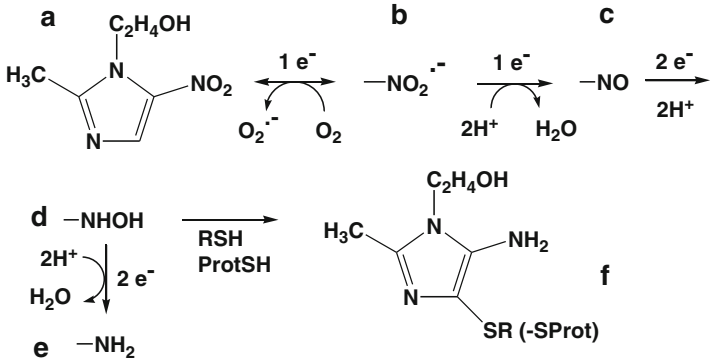

Fig. 30.3 The possible fates of metronidazole (**a**) during reductive activation. The first step is the nitro radical anion (**b**) followed by the nitroso compound (**c**) and a hydroxylamine derivative (**d**). The final reduction product is the amine (**e**). The hydroxylamine (or nitroso) derivatives can react with thiol groups in proteins (ProtSH) or with small thiols (RSH) such as cysteine, leading to covalent adducts (**f**)

however, the metronidazole-derived nitro radical anion is rather stable and less reactive [75]. Therefore, it has been questioned whether it is able at all to cleave DNA. An undisputed property of the nitro radical anions is their reactivity with molecular oxygen-forming superoxide radical anions and reconstituting the original nitroimidazole (Fig. 30.3a, b). This process is called "futile cycling" [76]. This name is used frequently, but strictly speaking it is not a cycling as superoxide anions are continuously generated. In aerobic organisms, these can be managed efficiently. In microaerophilic organisms such as *E. histolytica*, however, the process continues to generate more and more oxidative stress. This cannot be the full story, however, as metronidazole is also active under anaerobic conditions.

When metronidazole is further reduced to the nitroso- and hydroxylamine derivatives (Fig. 30.3c, d), these then become reactive with free protein thiols (Fig. 30.3f). This process was discovered for another nitroimidazole, ronidazole [77]; these reduced nitroimidazole adducts can also react with DNA [78]. When we investigated the effects of the treatment of *E. histolytica* trophozoites with 50 μM metronidazole for 2 h by two-dimensional electrophoresis, we detected a few new protein spots, which turned out to correspond to modified proteins with covalently bound metronidazole [79]. Because the modification generates a basic amino group (Fig. 30.3f), the modified proteins migrated at a similar molecular weight but shifted to a more basic isoelectric point: these proteins were thioredoxin, thioredoxin reductase, superoxide dismutase, purine nucleoside phosphorylase, and a small hypothetical protein with a carbohydrate-binding domain that we called MTP1 (metronidazole target protein-1). It was also possible to covalently modify thioredoxin and thioredoxin reductase expressed in *E. coli*, and their disulfide reductase activity was diminished significantly, putting a heavy load on the metabolism of *E. histolytica*. As described [29], we found 234 proteins interacting with *E. histolytica* thioredoxin, which means that interfering with thioredoxin function will have far-reaching consequences, particularly if the parasite has no glutathione backup system. If metronidazole treatment in the presence of molecular oxygen produces superoxide anions that are reduced to hydrogen peroxide, it is detrimental if this cannot be further reduced because of lack of thioredoxin activity.

Regarding the damage done by the reduced metabolites of metronidazole, an important question is which factors are responsible for the reduction, and extensive research has been carried out to address this question. In rat liver cells, the nitroimidazole ronidazole can be reduced and activated by the microsomal enzyme NADPH-cytochrome P450 reductase [80]. In *Tritrichomonas foetus*, active enriched hydrogenosomes generated nitro radical anions from metronidazole [81], which could be detected by erythrocyte sedimentation rate (ESR) spectroscopy. Addition of purified plant or bacterial ferredoxins significantly stimulated this reaction. Not long before the publication of this work, hydrogenosomes, which are known to harbor the metabolism of pyruvate [82], had been discovered. In *T. vaginalis*, purified hydrogenosomes were also shown to produce nitro radical anions [83], so the reduced ferredoxin was seen as the major metronidazole-reducing agent. In *E. histolytica*, there are no hydrogenosomes, and although *E. histolytica* ferredoxin was isolated as early as 1980 [84], the metronidazole-reducing activity

of the ferredoxin/PFOR system has not yet been demonstrated: this is certainly not a simple experiment because both ferredoxin and PFOR contain iron sulfur clusters and must be handled under anaerobic conditions. More recently, it was shown, however, that isolated *T. vaginalis* ferredoxin can reduce nitroimidazoles better than ferredoxin from the cyanobacterium *Anabaena* sp. strain 7120 [85].

When we analyzed the *E. histolytica* proteins that were covalently modified by reduced metronidazole, we noted that most of them were associated with the thioredoxin/thioredoxin reductase system, which led to the hypothesis that thioredoxin reductase itself might be able to reduce metronidazole, and indeed recombinant thioredoxin reductase had this activity [79]. So this enzyme has ambivalent properties: as Dr. Jekyll it helps to repair misfolded proteins and as Mr. Hyde it turns metronidazole into a toxic metabolite. Recombinant *T. vaginalis* thioredoxin reductase was also found to reduce metronidazole [86], as well as thioredoxin reductase from *G. lamblia* [87].

Taken together, the question arises whether the thioredoxin reductase or PFOR/ferredoxin systems are more important for metronidazole activation. One way to address this question is to look at the spatial distribution of the systems. As in *T. vaginalis* the PFOR is located in the hydrogenosomes, one could argue that metronidazole may encounter first the thioredoxin reductase before reaching the ferredoxin in the hydrogenosomes. This is not a strong argument, however, as metronidazole diffuses quickly throughout the cell [88]. In *E. histolytica*, this argument is even weaker, as both the thioredoxin reductase [89] and the PFOR [90] are located close to the plasma membrane, so the relative activity will depend on the local concentrations of all components involved and the activities of reduced thioredoxin reductase or ferredoxin at the site.

What makes the matter even more complicated is that even more *E. histolytica* factors were found that were able to reduce metronidazole, such as the atypical NADPH-dependent flavin-containing oxidoreductases EhNO1 and EhNO2 [54].

30.6 Metronidazole Resistance: *T. vaginalis* Can Do It, *G. lamblia* Can Do It, but Not *E. histolytica*

Drug resistance in protozoan parasites is a serious problem, especially against malaria parasites, trypanosomes, and leishmania. Multiple mechanisms have been described, including lack of uptake, export of the drug, lack of activation, or changes in the drug target [91]. For unknown reasons, the discovery of the activity of metronidazole against *E. histolytica* was a lucky punch in drug development; to the author's knowledge, there is no report on clinically relevant metronidazole resistance of *E. histolytica*. Definitively, *E. histolytica* can become resistant; as an example, it can be resistant to emetine by upregulating the expression of P glycoproteins, which can export the drug [92].

Bacteria such as *Bacteroides fragilis* can become resistant to metronidazole by generating Nim proteins such as NimA, reductases that are able to reduce the drug

directly to the aminoimidazole (Fig. 30.3e) of low toxicity [93]. *E. histolytica* possesses one gene coding for a homologue with 41 % amino acid identity to the NimA protein from *B. fragilis*. This inclusion conferred strong (30-fold) metronidazole resistance when expressed in *E. coli* JM109, but surprisingly it does not make *E. histolytica* resistant [94].

In *T. vaginalis*, the main mechanisms to achieve resistance to metronidazole are aimed at preventing its activation of metronidazole. Both putative mechanisms of activation, via the PFOR/ferredoxin system and via thioredoxin reductase, are supported by studies on resistance. Historically, it first appeared that there would not be much difficulties with resistance in clinical practice [95], but in several years resistant strains emerged and could be isolated from patients [96, 97]. Comparison of metronidazole-resistant and -susceptible strains showed a downregulation of ferredoxin [98]. In 1993, Kulda and coworkers managed to grow resistant *T. vaginalis* in the laboratory under aerobic [99] and anaerobic [100] conditions by slowly and stepwise increasing the drug pressure in the cultures. The first stage is aerobic resistance, which resembles the clinically resistant isolates. The highest resistance was found in the anaerobically resistant laboratory strains that had lost PFOR and hydrogenase activity. Instead, pyruvate was reduced to lactate, which was excreted. Integrating these results it was concluded that metronidazole resistance is caused by the shutdown of PFOR activity and lack of reduced ferredoxin leading to diminished activation of metronidazole [101]. This lack of hydrogenosomal activity also led to shrinkage of these organelles to about one-third of those in the susceptible strain [102]. Manipulation of the ferredoxin levels by genetic manipulation gave inconsistent results. Upregulation of ferredoxin in a metronidazole-resistant strain indeed increased its susceptibility [103], but targeted disruption of a ferredoxin gene in a susceptible strain resulted in a complete loss of ferredoxin protein and mRNA and a 95 % decrease of PFOR activity but did not lead to metronidazole resistance [104].

Work in our laboratory pointed in the direction of metronidazole activation via the thioredoxin reductase pathway. As already described, the *T. vaginalis* enzyme was able to reduce metronidazole, and a strain made resistant in the laboratory lost more than 95 % of its thioredoxin reductase activity as measured by the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) in hydrogenosome-free extracts [86]. Surprisingly the protein levels of thioredoxin reductase were the same as determined by two-dimensional gel chromatography; however, the resistant strain lacked the flavin cofactor of the enzyme, and addition of FAD could reconstitute the activity. In a further study, treatment of metronidazole-susceptible *T. vaginalis* with the flavin inhibitor diphenyleneiodonium (DPI) within hours rendered the parasite resistant to up to 1,000 μM metronidazole [105]. This treatment also abolished the covalent modification of proteins by reduced metronidazole, but it also abolished the activity of PFOR and increased lactate production. Compared to *T. vaginalis*, *E. histolytica* was highly susceptible to DPI, so it was impossible to perform the same experiment with *E. histolytica*.

In *G. lamblia*, metronidazole resistance problems appear to be more severe than in *T. vaginalis* or in *E. histolytica*. The parasite can activate metronidazole both via thioredoxin reductase and via the PFOR/ferredoxin system, so the examination of

various resistant strains revealed downregulation of either of the two systems [87]. For the treatment of giardiasis, it is proposed to combine metronidazole with other drugs [106].

So—one may say fortunately—there is only little work on metronidazole resistance in *E. histolytica*. The first attempt to grow resistant parasites in the laboratory [107] managed to obtain cultures growing stably at 10 μM metronidazole. The partially resistant trophozoites did not show any decrease of PFOR expression, neither in the immunoflorescence staining nor in the activity assay. The activity of SOD was increased threefold, however. The landmark paper by Wassmann and colleagues [108] reported the adaptation of *E. histolytica* trophozoites to a metronidazole concentration of 40 μM within 200 days. As measured by Northern blots, resistant amoebae did not contain significantly less PFOR or increased P-glycoprotein mRNA. Ferredoxin-1 mRNA was lower, whereas ferredoxin-2 mRNA was unchanged, suggesting different functions of the ferredoxins. The superoxide dismutase and peroxiredoxin expression was significantly higher in the resistant amoebae. Stable episomal overexpression of superoxide dismutase in the wild-type amoebae reduced their susceptibility, and the amoebae upregulated also their peroxiredoxin gene expression. When peroxiredoxin was overexpressed episomally together with the superoxide dismutase, the effect was augmented, although the episomal expression of peroxiredoxin alone was inactive. Interestingly, the enzyme the authors called "flavin reductase" is the thioredoxin reductase. The mRNA coding for this enzyme was downregulated in the resistant amoebae, and overexpression led to an increase of the susceptibility. In the discussion, the authors hypothesized correctly that this enzyme might reduce metronidazole itself, which we could show later [79]. In 2000, Wassmann and Bruchhaus [109] extended their work to show that overexpression of superoxide dismutase increased the resistance only if amoebae were grown under microaerophilic conditions. In the absence of oxygen, there was no difference in susceptibility. In the light of what is now known about the redox protection systems in *E. histolytica*, all this makes sense. In the presence of oxygen, the nitro radical anions quickly react to form superoxide anions, which SOD reduces to hydrogen peroxide. Then, the peroxiredoxins are needed to handle the hydrogen peroxide. Both enzymes are needed together and so it is not surprising that there is a coordinated induction. The large problem for the amoebae is that they need the thioredoxin system for the functioning of the peroxiredoxins, but this is damaged by the modification with the activated metronidazole. Interestingly, the amoebae when treated with metronidazole quickly upregulated superoxide dismutase and peroxiredoxins on the level of mRNA but also ferredoxin and thioredoxin reductase [110].

30.7 Perspectives and More Open Questions

Certainly the development of metronidazole against *E. histolytica* was a lucky punch in the world of antibiotics. Looking at the situation of malaria chemotherapy, with resistance emerging even against the gold standard artemisinins [111], the

situation is much more threatening. In a way it is extremely interesting to study why exactly metronidazole is so effective. Such studies can lead to new nitroimidazoles against pathogenic microorganisms that are not susceptible to metronidazole, such as PA-842 against *M. tuberculosis* [10].

In recent years it has become clear that the thioredoxin system has a central role in metronidazole toxicity [79, 86]; for microaerophilic protozoan parasites, this is probably aided by the absence of the glutathione system. In one molecular pathway in the presence of oxygen, cycling between the nitro radical anions and unreduced metronidazole continuously produces superoxide anions and hydrogen peroxide, two reactive oxygen species. The situation is aggravated by the chemical modification of thioredoxin reductase and thioredoxin, which prevent their disulfide-reducing activity, needed for the peroxiredoxin reaction. So what happens is that the reactive oxygen species are damaging many components of the cells and the thioredoxin system cannot act as the fire brigade because it is damaged. A recent paper independently showed that the anti-rheumatic drug auranofin is active against *E. histolytica* and its target is likely to be the thioredoxin reductase [112]. Auranofin is also active against metronidazole-resistant *G. lamblia*, where it inhibits thioredoxin reductase at nanomolar concentrations [113].

In *E. histolytica*, under anaerobic conditions, as the thioredoxin system is also damaged, it is difficult to say what parts of the thioredoxin interactome [29] are the most relevant targets. Thus, it will be interesting to identify more proteins in the thioredoxin interactome. Moreover, in *E. histolytica*, there is only one thioredoxin reductase, but the amoebaDB database (http://amoebadb.org/amoeba/, Accessed 29 Aug 2013) lists 22 thioredoxins, 13 entries with up to 150 amino acid residues, of which 4 contain the classical thioredoxin consensus sequence WCGPC and 9 nine larger thioredoxins. So there is a world of unexplored interactions.

Further aspects of metronidazole action need more attention, such as the role of small thiols. Definitively, cysteine in the cytoplasm is reacting with reduced metronidazole [79], but it is not known if other small thiols are targets, such as coenzyme A. Another open question is which enzymes could reduce nitric oxides: could it be another isoform of the flavodiiron proteins [50], or one or more of the many unannotated proteins?

Definitively, both thioredoxin reductase and ferredoxins are the main candidates able to reduce metronidazole, but what is their relative contribution, which other proteins and small molecules could also reduce metronidazole? Further, while they reduce the drug, which intermediates from the nitro radical anion to the hydroxylamine derivative are made in which proportion?

Finally, resistance. So far the question has not been answered why *T. vaginalis* can become metronidazole resistant and *E. histolytica* cannot. Definitively, *T. vaginalis* can live without thioredoxin reductase activity [86] and without the PFOR/ferredoxin system [101], whereas *E. histolytica* needs one or the other or both. Unfortunately, the methods to disrupt *E. histolytica* gene expression are not completely reliable, so experiments to disrupt thioredoxin reductase, PFOR, or the ferredoxins have not been performed. When PFOR is disrupted in *T. vaginalis*, lactate dehydrogenase can reduce pyruvate. *E. histolytica* does not have a lactate dehydrogenase, so if the PFOR

reaction were decisive, expressing a lactate dehydrogenase might help *E. histolytica* to gain some resistance. However, this is a far shot.

So, taken together, quite a bit of work remains to really understand metronidazole activity and resistance in *E. histolytica* and other pathogenic microorganisms, However, previous research gives us a glimpse behind the curtain and allows us to ask better and more specific questions.

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Chapter 31
Thioredoxin Reductase and Its Role as a New Drug Target

Rosa M. Andrade and Sharon L. Reed

Abstract Amebiasis causes approximately 70,000 deaths annually and is the fourth cause of death from protozoan parasites in the world. It is treated primarily with metronidazole, which has adverse side effects and is mutagenic and carcinogenic. Also, emergence of metronidazole resistance is an increasing concern. Unfortunately, better therapeutic alternatives are lacking. Drug discovery efforts have led to reprofiling of older FDA-approved drugs. This approach is advantageous because safety and pharmacokinetic effects in humans have already been confirmed clinically and approved for use.

In high-throughput screening studies, we have recently demonstrated that auranofin, a gold-containing compound that was originally approved to treat rheumatoid arthritis, has activity against trophozoites of *Entamoeba histolytica*, the causative agent of amebiasis. The antiparasitic activity of auranofin [1, 2] likely stems from the monovalent gold that readily dissociates and inhibits *E. histolytica* thioredoxin reductase, which is the only thiol-dependent flavoreductase present in this protozoan parasite [3–5]. Auranofin has also shown promising in vitro activity against metronidazole-resistant *Giardia* (Lars Eckmann, personal communication), *Plasmodium falciparum* [6], and *Schistosoma mansoni* [1]. Altogether, this evidence suggests that auranofin has the potential to become a broad-spectrum alternative therapeutic agent for diseases that represent a huge global burden.

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_31

31.1 Thioredoxin Reductase and Its Diversity Among Protozoan Parasites

Protozoan parasites are constantly exposed to reactive oxygen species (ROS) from their own metabolism and those generated by host defense mechanisms. As most forms of life, their defense mechanisms include antioxidant systems to handle these environmental challenges. Among these anti-oxidant systems, free radicals and radical-free systems are pivotal to maintain the oxidation–reduction homeostasis and prevent oxidative stress in these organisms. Radical-free systems that include thiol-oxidoreductases generally account for 0.5–1 % of the cell proteome, establishing them as essential and abundant enzymatic systems within the cells [7]. Many of these thiol-oxidoreductases form protein complexes, in which reducing equivalents are transferred from one thiol oxidoreductase to another, both containing conserved catalytic residues, frequently cysteine (Cys) (i.e., thioredoxin reductase provides reducing equivalents to thioredoxin, which in turn reduces other substrates) [7]. Among them, thioredoxin-thioredoxin reductase (Trx/TrxR) and glutathione-glutathione reductase (GSH/GR) are the two main independent reactive oxygen species detoxifying systems.

Significant attention has been devoted to the Trx/TrxR system because of its unusually broad substrate specificity, allowing it to have important roles in regulating DNA synthesis, gene transcription, cell growth, and apoptosis [4, 8]. Thioredoxin reductases (TrxRs) are enzymes that belong to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases [9]. Members of this family are homodimeric proteins in which each monomer contains a FAD domain, a NADPH-binding domain, and an active site containing a redox active disulfide [9]. Cys is present in the catalytic redox active center, which is highly conserved in thiol-reductases. The majority of thiol-oxidoreductases have a single catalytic Cys, but some of these enzymes are composed of two or more thiol-oxidoreductase domains, each having the catalytic redox Cys [7].

Two main types of TrxRs [10] are recognized:

- High molecular weight (H-TrxR) enzymes contain a redox active center (motif CXXXXC) in the FAD-binding domain. H-TrxR is closely related to glutathione reductase (GR), thypanothione reductase (TryR), mercuric reductase (MerR), and lipoamide dehydrogenase (LipD) [4]. There are two varieties of H-TrxR: (a) one that contains a selenocysteine at the penultimate position in the C-terminal interface domain, the mammalian form; and (b) another wherein selenocysteine has been replaced by cysteine in the interface domain, the apicomplexan parasite form.
- Low molecular weight (L-TrxR) enzymes contain a redox active disulfide (motif CXXC) in the NADPH domain. L-TrxRs are related to alkyl hydroperoxide reductase F52A (AhpF), present in bacteria, fungi, plants, and some protozoan parasites including *Trichomonas vaginalis* [4, 11, 12], *Gardia lamblia*, and *Entamoeba histolytica*.

Despite about 20 % primary sequence identity between H-TrxR and L-TrxR [4], they appear to have evolved from a common ancestor but developed independently. L-TrxR and H-TrxR are mutually exclusive, suggesting that they do not act synergistically [4].

Electrons are transferred from NADPH via FAD to the active site disulfide of TrxR, which then reduces the substrate (thioredoxin). H-TrxR has three redox active centers, whereas the L-TrxR have two redox centers and the transfer of reducing equivalents requires a conformational change, in contrast to H-TrxR [12].

Trx/TrxR systems vary according to parasite subgroups. Among aerolerant protozoans, the absence of GSH/GR is most relevant, making Trx/TrxR a main member of their antioxidant systems. In fact, *T. vaginalis* possesses a full thioredoxin system, consisting of thioredoxin (Trx), Trx peroxidase, and a L-TrxR [11, 13]. Similarly, *G. lamblia* has an active Trx/L-TrxR system and encodes a gene for a putative Trx peroxidase [11]. In the apicomplexan group, *Plasmodium* possesses both GSH/GR and Trx/TrxR redox systems. Its complete thioredoxin system comprises thioredoxin reductase (TrxR), different thioredoxins, thioredoxin-like proteins, and thioredoxin-dependent peroxidases (TPx) [14–16].

Genome sequencing of *Trypanosoma brucei* [17], *Trypanosoma cruzi* [18], and *Leishmania major* [19] revealed that trypanosomatids lack genes for GSH/GR and Trx/TrxR. Although in most eukaryotic organisms these latter systems maintain the intracellular thiol redox homeostasis, trypanosomatids depend exclusively on trypanothione [*N*1,*N*8-bis(glutathionyl)spermidine, T(SH)2] [20, 21] and trypanothione reductase (TryR) to keep the dithiol in the reduced form [21, 22]. TryR is the only enzyme that connects the NADPH- and the thiol-based redox systems in these parasites; it is related to H-TrxR. As such, it shares many physical and chemical properties with GR. TryR has been biochemically characterized in *T. cruzi* [23], *Leishmania* [24], and *T. brucei* [25, 26].

31.2 *Entamoeba histolytica* Thioredoxin Reductase

The *Entamoeba histolytica* invasive form, the trophozoites, are considered aerotolerant organisms. They can survive in an anaerobic environment such as that of the human gut. During tissue invasion, they are exposed to high levels of reactive oxygen species (ROS). Earlier studies showed that the parasite can tolerate up to 5 % oxygen in the gas phase [27–29]. Hence, the parasite must have means to minimize damage caused by ROS produced by the host immune assault. In contrast to most organisms that have two largely independent antioxidant systems (one based on glutathione and the other based on thioredoxin), *E. histolytica* lacks both glutathione reductase activity and glutathione synthetic enzymes; therefore, it relies on TrxR to prevent, regulate, and repair the damage caused by oxidative stress.

The *E. histolytica* genome has a single TrxR-encoding gene (23.m00296), which belongs to the low molecular weight TrxR family (L-TrxR), and is similar to bacterial

and yeast enzymes, including the TrxR from *Escherichia coli*. This single gene is 964 bp in length, lacks introns, and encodes a 314-amino-acid protein (EhTrxR) with a molecular mass of 33.7 kDa and a calculated pI of 6.34.

E. histolytica TrxR (EhTrxR) is of very similar size and domain topology as *E. coli* TrxR, a well-studied enzyme. Both proteins have an active site dithiol/disulfide center (Cys-Ala-Thr-Cys for EcTrxR, Cys-Ala-Ile-Cys for EhTrxR). In EhTrxR, the Cys residues in the catalytic center correspond to Cys 140 and Cys 143. Sequence homology to other TrxRs is depicted by its 21 % identity to *E. coli* TrxR (L-TrxR), *Trichomonas vaginalis* (23 %), *Trypanosoma cruzi* (27 %), and *Homo sapiens* (36 %). The *Eh* TrxR has been crystallized (PDB A5L and 4A65) and the dimer structure with interface formed by the FAD domains similar to the *E. coli* enzyme confirmed (manuscript under preparation). Gold molecules do bind EhTrxR when donated by auranofin or AuCN, but the exact site of binding is under investigation.

The catalytic mechanism of L-TrxR has been extensively characterized in *E. coli*. Spatially, the NADPH and FAD domains of *E. coli* TrxR do not make close contact with the isoalloxazine ring of FAD. Its NADPH domain rotates 66° while the FAD domain remains fixed. Then, the bound NADPH moves into close contact with the FAD isoallaxazine ring that allows electron transfer to FAD and the active site disulfide [30–35].

EhTrxR enzymatic activity demonstrates an unusually high level of NADPH oxidase activity, which is protective against molecular oxygen required for the survival of these aerotolerant, anaerobic organisms [36]. EhTrxR exhibited NADPH oxidase activity with hyperbolic saturation kinetics for NADPH, and its estimated K_m and V_{max} values are 3.6 μM and 0.37 U/mg, respectively [37]. It is also unusual in that it does not require Trx to transfer reducing equivalents to the substrate peroxidases but is able to perform this catalytic step by itself just like the AhpF protein [36]. On the other hand, high intracellular levels of cysteine compensate for the lack of glutathione, preventing auto-oxidation in highly reducing environments [4]. Thus, the ultimate concentration of reactive oxygen species in the microenvironment of trophozoites in the presence of auranofin depends on both the level of gold from the auranofin, which can bind either the EhTrxR or cysteines, and the resulting level of reduced cysteine. Interestingly, despite the fact that neither thioredoxin or thioredoxin reductase configurations include a transmembrane hydrophobic domain, the *E. histolytica* thioredoxin-thioredoxin reductase system is primarily located to the plasma membrane [38] without evidence of intracytoplasmic presence.

Besides ROS, *E. histolytica* is exposed to high concentrations of reactive nitrogen species (RNS) such as nitric oxide (NO) or *S*-nitrosothiols (such as GSNO and CySNO) during tissue invasion. Although high levels of these RNS might inhibit *E. histolytica* growth in vitro [39], the parasite is able to survive and multiply during tissue invasion. This finding suggests that *E. histolytica* detoxification system is versatile enough to tolerate hostile environments. This versatility was recently demonstrated by the ability of the *E. histolytica* Trx-TrxR system to reduce RNS and use an alternative electron donor such as NADH [39].

As *E. histolytica* lacks glutathione, Cys is its major intracellular low molecular mass thiol [4] that can also react with NO to generate CysSNO. This metabolite is thought to be critical for *S*-nitrosylation (addition of NO to the thiol group in Cys) or *S*-thiolation (addition of Cys to another Cys thiol group) of cellular proteins [40] These metabolites can be reduced by *E. histolytica* TrxR, as was demonstrated by in vitro assays in which NADPH and EhTrxR were exposed to different concentrations of CySNO and GSNO. The rates of NADPH oxidation were increased proportionally, suggesting that these compounds (CySNO or GSNO) can be reduced in a reaction catalyzed by EhTrxR. Additionally, EhTrxR and EhTrx seem to synergistically reduce *S*-nitrosothiols [39].

In contrast to other thioredoxin reductases, *E. histolytica* TrxR does not exhibit high specificity for NADPH. Arias et al. [39] evaluated the reduction of DTNB by EhTrxR using NADPH or NADH as electron donors. Although *E. histolytica* TrxR affinity for NADH is ten times lower than that for NADPH, the enzyme activity with NADH is not negligible when compared to other thioredoxin reductases that exhibit high specificity toward NADPH [41]. These results suggest that EhTrxR can use either NADPH or NADH as its reduced cofactor. This evidence strongly shows the versatility of EhTrx and its ability to protect the parasite from the ROS and RNI byproducts during host invasion. Altogether, these characteristics make EhTrxR a desirable drug target.

31.3 Thioredoxin Reductase–Gold Interactions: An Unexploited Drug Target

31.3.1 Summary

Despite the huge public health burden of parasites worldwide, the paucity of available antiparasitic drugs is striking. Reprofiling of old FDA-approved drugs has become an alternative expeditious approach to drug discovery. Its advantage relies on already known drug safety and pharmacokinetic effects in humans that make them readily available for off-label indications.

Auranofin, a gold-containing compound that was originally approved to treat rheumatoid arthritis, has become the center of attention in recent efforts to develop new more efficacious therapies against parasites. Indeed, we have recently demonstrated that auranofin has activity against trophozoites of *Entamoeba histolytica*. Because of its activity as shown in high-throughput screening studies, in in vitro and in vivo rodent models of colitis and liver abscesses, auranofin was granted Orphan Drug Status. Auranofin has also shown promising in vitro activity against metronidazole-resistant *Giardia lamblia* (Lars Eckmann, personal communication), *Plasmodium falciparum* [6], *Schistosoma mansoni* [1], and *Leishmania infantum* [2].

Thus, auranofin has the potential to become an alternative broad-spectrum antiparasitic agent.

31.3.2 Auranofin as an Antiparasitic Agent

Auranofin is an FDA-approved, oral, gold-containing drug used to treat rheumatoid arthritis for more than 25 years. Following an oral dose, its elemental gold molecule is absorbed into the blood (25 %) whereas 60 % is bound by plasma proteins [42]. Despite its clinical use, the mechanism of action of auranofin is poorly understood. Efforts to elucidate its activity have shown that gold(I) compounds are known to be highly thiophilic [3]; hence, the auranofin antiparasitic activity likely stems from the gold molecule that readily dissociates and inhibits thiol-dependent flavoreductases [3].

Kuntz et al. [43] were the first to describe the effect of auranofin in *S. mansoni*. In in vitro assays, they demonstrated that auranofin rapidly killed juvenile and adult forms of *S. mansoni* [43]. Their preliminary in vivo data correlated with their in vitro findings [43]: mice infected with *S. mansoni* and treated with auranofin showed a 59 % and 63 % decrease in worm burden compared to control mice [43].

Additional in vitro evidence shows that auranofin strongly inhibits the growth of the malarial parasite *Plasmodium falciparum* [6], the promastigote stage of *Leishmania infantum* [2], the bloodstream and procyclic stages of *Trypanosoma brucei* [44], and the larval worms of *Echinococcus granulosus* [45]. All these in vitro data were achieved with auranofin doses in the nanomolar range, which are achievable in patients with rheumatoid arthritis (~5 μM serum blood levels) [5].

Angelucci et al. further analyzed *S. mansoni* thioredoxin-glutathione reductase (TGR) crystal structure in the presence of auranofin [1]. The structure revealed gold (I) rather than auranofin as an adduct between pairs of cysteines (Cys-Au-Cys) in two different sites and also bound to the proposed NADPH-binding site of the reductase in a third location.

Similarly, the crystal structure of reduced *Leishmania infantum* trypanothione reductase complexed with NADPH and auranofin also demonstrated that gold binds two active cysteine residues of trypanothione reductase (TryR) [2], that is, Cys52 and Cys57, although the thiol sugar moiety of auranofin binds to the trypanothione-binding site; thus, auranofin appears to inhibit TryR through a dual mechanism.

We recently demonstrated that auranofin has activity against *E. histolytica* trophozoites. Although the target of auranofin is largely unknown, our studies support the hypothesis that *E. histolytica* TrxR likely is the target for auranofin. Our qualitative in vitro assays showed that *E. histolytica* trophozoites treated with auranofin were more vulnerable to oxidative stress, although auranofin effect was time dependent (Fig. 31.1a). Staining *E. histolytica* trophozoites with dichloro-dihydrofluorescein, which develops into green fluorescence upon contact with ROS, showed that trophozoites treated with auranofin accumulated more ROS [46] (Fig. 31.1b). This effect was counteracted by Cys, the major reductant in *E. histolytica*, which protected *E. histolytica* trophozoites that were treated with auranofin (Fig. 31.1a). Our in vitro findings were corroborated in animal models of colitis and liver abscesses in which auranofin not only decreased the parasite tissue burden but also decreased the local inflammatory response as measured by activity of myeloperoxidase in cecal tissue [46] (Fig. 31.2).

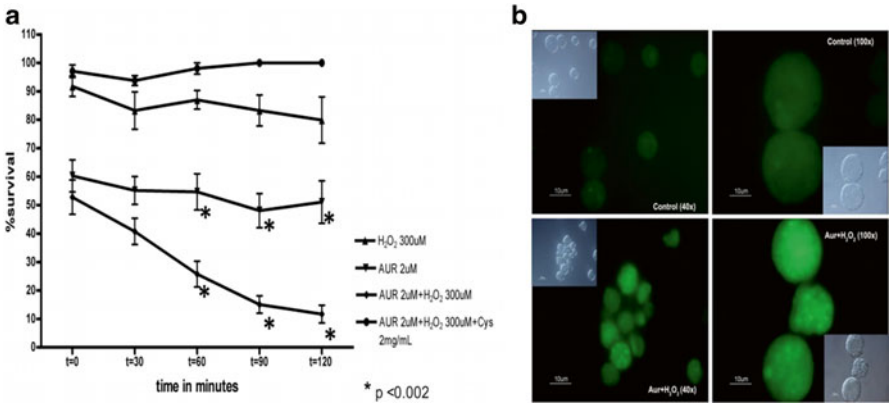

Fig. 31.1 **a** In vitro susceptibility of trophozoites (control and treated with auranofin) to reactive oxygen species (H_2O_2) and the effect of added cysteine. Time points with mean ± SEM % survival. Experiments are shown in triplicates. *$p < 0.002$ by Student's *t* test. **b** Immunofluorescence microscopy. Detection of reactive oxygen species within *Entamoeba histolytica* trophozoites following treatment with auranofin or auranofin plus H_2O_2. Control trophozoites were treated with ethanol alone (auranofin carrier). *Insets* are differential interference contrast images. *Bars* 10 μm

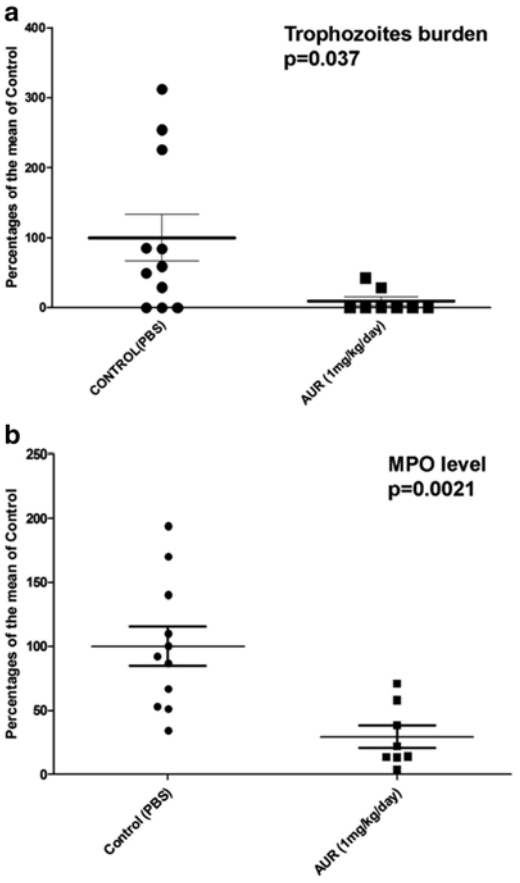

Fig. 31.2 Experimental mouse model of amebic colitis. Control and treatment with auranofin groups ($n = 8$) are presented as (**a**) the percentage of trophozoites per gram of tissue, or (**b**) myeloperoxidase (MPO) units per gram of tissue compared with the means of infected controls (as 100 %)

These findings suggest that auranofin may be an entirely new class of drugs to treat amebiasis and, potentially, other parasitic infections. On the basis of these results, the FDA has approved an orphan-drug designation of auranofin for treatment of amebiasis, and clinical trials are in the planning stages.

Acknowledgments We thank Larissa Podust, Derek Parsonage, Anjan Debnath, and James McKerrow for their critical reading of this manuscript

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Chapter 32
Drug Development: Old Drugs and New Lead

Anjan Debnath

Abstract Amebiasis is a global disease caused by the protozoan parasite *Entamoeba histolytica*. It affects 50 million people, leading to 70,000 deaths each year, and about 500 million people are at risk. Current treatment requires both metronidazole and paromomycin, but metronidazole has several adverse effects and resistance is an increasing concern. Moreover, treatment with two drugs for 20 days makes compliance difficult. Although amebiasis affects millions people, it is a disease primarily of the poor, and therefore new drug development for this disease is not a priority for major pharmaceutical companies. Yet finding a better drug for such a significant cause of morbidity and mortality would have an impact on millions of people suffering from this diarrheal disease. To facilitate the identification of new drug leads for this anaerobic parasite, we developed and validated a novel automated, high-throughput screening methodology. Screening identified an FDA-approved drug, auranofin, used therapeutically for rheumatoid arthritis, as active against *E. histolytica* in culture. Auranofin was ten times more potent against *E. histolytica* than metronidazole. The discovery of auranofin offers a promising drug repositioning opportunity for the treatment of amebiasis.

32.1 Introduction

Each year 50 million people contract amebiasis through contaminated food and water, making it the third leading cause of illness and fourth leading cause of death from protozoan infections worldwide [1]. Despite the prevalence of this disease only two drugs are available to treat amebiasis. Emetine, the drug first used to treat amebiasis, was scientifically determined 100 years back in 1912 [2], but later it was replaced by a better tolerated drug, metronidazole. Current therapy for amebiasis, mainly amebic colitis and amebic liver abscess, relies on metronidazole. Potential resistance of *Entamoeba histolytica* to metronidazole is an increasing concern,

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_32

and clinical failures have been linked to other metronidazole-resistant protozoa. Standard treatment with metronidazole requires at least 10 days at high dosage (750 mg t.i.d) to eradicate intestinal amoebae [3–5] and is accompanied by frequent side effects. In addition, follow-up treatment with a second drug such as paromomycin is recommended to prevent prolonged carriage and excretion. Therefore, a shorter, less toxic, and more effective therapy is needed. To accelerate the identification of better drugs, we developed a high-throughput screen that could be conducted in an oxygen-free environment to mimic the natural environment of the amoeba [6]. The screen was performed with a collection of bioactive compounds donated by Iconix Biosciences, Inc. These compound collections contain molecules that are already approved for human use. The use of drugs already approved for human use opens the possibility to rapidly and cost-effectively reprofile or repurpose [7] drugs to treat amebiasis. A notable discovery from this screen was the amebicidal activity of auranofin, an FDA-approved anti-arthritic gold(I) phosphine compound [6].

32.2 Emetine: Historical Perspective

Emetine, an alkaloid from ipecacuanha, is one of the oldest drugs in the formulary. It has a long and fascinating history. The naturalist Georg Marggraf and the physician Willem Pison first reported the use of ipecacuanha in a 1648 collection of papers and notes entitled *De Indiae utriusque re naturali et medica*, based on their travels to Brazil in 1638. Later, Le Gras imported ipecacuanha from South America to Paris in 1672 as a treatment for flux (dysentery) and the ague (fever) [8]. This Brazilian root was successfully used by Helvetius in the treatment of the Dauphin, son of Louis XIV, and he sold the secret of the remedy to the French Government, which in 1688 made its composition public. It was given for dysentery, chiefly in small doses, but it was not until 1858, that an army surgeon, E.S. Docker, introduced the use of large doses (60 grains two or three times a day) of powdered ipecacuanha in the treatment of severe dysentery in Mauritius, with the remarkable result of reducing the death rate of the disease from a former annual rate of 10–18 % to only 2 %. His excellent results to treat amebic dysentery were rapidly confirmed by numerous physicians in India.

In 1911, Captain Edward B. Vedder, Medical Corps, U.S. Army, demonstrated experimentally the powerful amebicidal action of emetine [9] and this paved the way for Leonard Rogers' clinical use of emetine salt hypodermically in cases of acute amebic dysentery in Calcutta. In a paper published in 1912, Rogers gave details of 12 cases of amebiasis who were all treated by emetine injections and concluded that in his method of the subcutaneous injection of soluble salts of emetine a specific treatment of amebic hepatitis and amebic dysentery had been discovered [2].

Emetine was extremely effective in eradicating amoebae, but there were several practical difficulties in its use: the patient needed bedrest for the duration of treatment, the drug was administered by subcutaneous injection, and the treatment required close observation to detect potentially fatal cardiovascular complications,

including hypotension and tachycardia [10]. In spite of rigorous precautions taken during treatment, sudden death occasionally occurred [11]. If given orally, the drug usually causes vomiting and very little is absorbed. It is dangerously cardiotoxic when given intravenously.

The synthetic derivative racemic 2-dehydroemetine was introduced in 1959 and had equal efficacy and lower toxicity than emetine. Pharmacodynamic studies in guinea pigs showed that this compound is released and excreted more rapidly than emetine from most body organs, in particular the heart [12]. Because of its more rapid excretion dehydroemetine is given intramuscularly at a daily dosage of 1.25 mg/kg (maximum, 90 mg daily), slightly higher than the dosage for emetine [13].

Recently, when emetine was screened for similarity to target classes using the chemoinformatic Similarity Ensemble Approach, it showed similarities to the adrenergic α2-blocker ligand set and, consistent with that similarity, emetine antagonized α2 receptors in the micromolar and possibly submicromolar range. This activity is consistent with the known side effects of emetine, such as hypotension, tachycardia, dyspnea, myocarditis, and congestive heart failure [14]. The toxicity of emetine led to the search for alternative drugs that could be taken orally and would be free from adverse effects.

32.3 Metronidazole: The Current Drug of Choice

With the discovery of antibiotics it was natural to test those for activity against *E. histolytica*. According to Hargreaves [15], penicillin and sulfonamide were useful adjuvants but nonspecific for amebiasis. Aureomycin had no lasting therapeutic effect, and significant numbers of relapses occurred with terramycin, although it showed some efficacy in acute amoebic infections. The efficacy of fumagillin was reported by Anderson et al. [16] in 1952 [17].

Extracts of *Streptomyces* spp. were screened for activity against *Trichomonas vaginalis*, a cause of vaginal itching. Azomycin, a nitroimidazole, was identified, and metronidazole, a synthetic derivative, was used to treat chronic trichomonad infections, beginning in 1959 [18, 19]. Metronidazole was found efficacious against *E. histolytica* in 1966 [20].

Since this discovery, metronidazole has been considered as the drug of choice for treating invasive amebiasis [21–23]. The standard dose of metronidazole for the treatment of amebic colitis is 500–750 mg three times daily in adults and 30–50 mg/kg/day in children for 5–10 days [21–23]. Metronidazole is absorbed rapidly and almost completely after oral administration; it penetrates body tissues and secretions such as saliva, breast milk, semen, and vaginal secretions [24]. Binding to plasma proteins is very low. The drug is oxidized and glucuronidated in the liver. The hydroxymethyl metabolite is also antibiotically effective. The metabolites are excreted in the urine. The half-life is approximately 8 h [25]. Metronidazole has variable efficacy in eliminating cysts in the colonic lumen [26–28].

Adverse effects of metronidazole treatment include headache, vertigo, loss of appetite, nausea, metallic taste and furred tongue, and vomiting [29, 30]. Consumption of alcohol while taking this drug should be avoided because of the inhibition of aldehyde dehydrogenase. When alcohol is taken metronidazole can cause disulfiram-like action such as severe vomiting, headache, flushing, and abdominal pain. Dizziness, convulsions, poor coordination, and numbness of the extremities are less common, but discontinuation of metronidazole is required when serious adverse effects are seen [30, 31]. Prolonged use of this drug, as in Crohn's disease, may cause chromosomal abnormalities in circulating lymphocytes [32], and occasionally peripheral neuropathy [13, 33]. A transient leukopenia is sometimes noted, and this may be significant when other drugs such as cotrimoxazole are used concomitantly [13]. Other nitroimidazole drugs with longer half-lives, such as tinidazole, ornidazole, and secnidazole, are better tolerated and allow shorter periods of treatment.

Although metronidazole has been used for decades, it has been shown to be both mutagenic and carcinogenic in animals [34]. As cross-resistance exists among the nitroimidazoles [35], reliance on a single drug to treat a population of 50 million infected worldwide seems particularly perilous because of the specter of resistance to metronidazole. Therefore, there is a high priority for discovery of equally effective and better tolerated classes of antiamebic drugs.

32.4 Paromomycin and Its Cysticidal Effect

Although millions of people are infected by *E. histolytica* each year, most individuals remain asymptomatic but may transmit amebiasis through fecal excretion of infective cysts [36]. Therefore, asymptomatic *E. histolytica* intestinal carriage should also be treated because of its potential for causing invasive disease and public health risks [37–39]. Because metronidazole is readily and almost completely absorbed from the gastrointestinal tract [40], it lacks cysticidal activity against *E. histolytica*. Parasites persist in the intestine in as many as 40–60 % of patients who receive metronidazole. Therefore, an additional drug is recommended to treat patient with intestinal and asymptomatic infections following metronidazole [41]. Paromomycin, an antibiotic produced from cultures of *Streptomyces ramosos*, and a member of the aminoglycoside family, was first isolated in 1956 [42] and was first used in amebiasis in 1959 [43]. It is poorly absorbed from the gastrointestinal tract, excreted almost 100 % unchanged in the feces, and is used as a luminal amebicide. The usual dosage of paromomycin for adults and children is 25–35 mg/kg daily in three doses for 5–10 days [13]. Metronidazole and paromomycin should not be given at the same time, because the diarrhea that is a common side effect of paromomycin may make it difficult to assess the patient's response to therapy [41].

32.5 Development of a High-Throughput Screen (HTS) for *Entamoeba histolytica*

Earlier, research on drug development in *E. histolytica* employed two approaches: (1) identification of molecular targets and (2) measurement of compound efficacy by phenotypic screening in in vitro or animal models of disease. The latter approach is usually without specific knowledge of the target or mechanism of action, or for which bioactivity has been characterized in other parasitological or biomedical settings [44]. Both approaches are of proven value, but the pace of discovery with these techniques is somewhat slow, relying on discrete, focused compound sets or a small number of compounds with known pharmacological actions [45]. Screening large chemical libraries to identify amebicidals has been hindered by the throughput of traditional assays that were labor intensive, relying on microscopic visualization [46], radioisotopes [47, 48], and extensive staining methods [49]. To accelerate the identification of better drugs, we developed a novel HTS. The challenge of developing the HTS for *E. histolytica* included the fact that it is an anaerobe and cannot survive in the oxygenated environment present in the microtiter plate, and no rapid robotic-driven readout assay was available. We overcame this challenge by developing a screen that could be conducted in an oxygen-free environment in microtiter plates, mimicking the amoeba's natural habitat. This assay measures ATP bioluminescence generated when luciferase catalyzes the transformation of luciferin into oxyluciferin, yielding PPi, AMP, and light in the presence of cellular ATP and oxygen [6], using a variation of the CellTiter-Glo Luminescent Cell Viability Assay (Promega) technology; the assay is compatible with workstation-based automation and represents a rapid, sensitive, and more efficient assay to identify active compounds against the pathogen [50]. The assay development was performed with exponentially growing *E. histolytica* trophozoites with 50,000 parasites/ml in 96-well or 30,000/ml in 384-well microtiter plates. This inoculum was chosen so that confluent but not excessive growth took place. The relationship between numbers of parasites seeded into 96- and 384-well plates and relative luminescence from CellTiter-Glo of parasites showed a linear correlation ($R^2 = 0.86$ and $R^2 = 0.9$) (Fig. 32.1a, b). Trophozoites readily tolerated up to 0.5 % DMSO with no reduction of growth rate [6].

32.6 High-Throughput Compound Screening Protocol for *E. histolytica*

The HTS technology was intended to streamline and accelerate the identification of lead compounds and subsequent SAR determination in vitro. Briefly, compounds were diluted using a Biomek FX^P Laboratory Automation Workstation (Beckman Coulter) and the Matrix WellMate bulk dispenser (Thermo Fisher Scientific) to

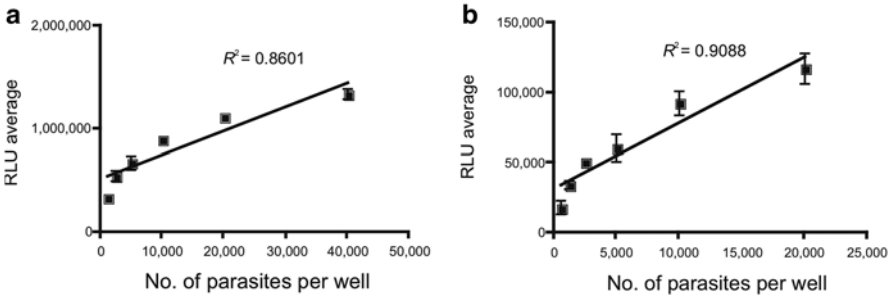

Fig. 32.1 Assay development for high-throughput screening (HTS) and scatter plot of percentage inhibition of each well from plates of compound library. **a** Correlation between the number of viable *Entamoeba histolytica* trophozoites and ATP bioluminescence in 96-well microtiter plate. **b** Correlation between the number of viable *E. histolytica* trophozoites and ATP bioluminescence in 384-well microtiter plate. *Values* plotted (**a**, **b**) are the means and standard deviations of triplicate wells. *Line* (**a**, **b**) represents the linear regression for plotted data [6]

yield 125 μM compound in 12.5 % DMSO. Finally, FX^p transferred 4 or 2 μl diluted compound to the 96- and 384-well screen plates, respectively, followed by addition of 96 μl (5,000 parasites) or 48 μl (1,500 parasites) of *E. histolytica* trophozoites in TYI-S-33 complete medium to the 96- and 384-well plates, respectively, by the WellMate. Final concentrations of test compound and DMSO per well were 5 μM and 0.5 %, respectively. Negative controls in the screen plates contained 0.5 % DMSO and positive controls contained 30 μM metronidazole. Assay plates were incubated for 48 h at 37 °C in the GasPak to maintain an anaerobic condition throughout the incubation period. At the end of incubation, the assay plates were equilibrated to room temperature for 30 min; 50 μl and 25 μl of CellTiter-Glo were added in each well of the 96-well and 384-well plates, respectively, using the WellMate. The plates were then placed on an orbital shaker at room temperature for 10 min to induce cell lysis. After lysis, the plates were again equilibrated at room temperature for 10 min to stabilize the luminescent signal. The resulting ATP bioluminescence of the trophozoites was measured at room temperature using an Analyst HT plate reader (Molecular Devices) [6] (Fig. 32.2).

For EC_{50} determination of hits, defined as that concentration of compound necessary to reduce the culture density to 50 % of that of a DMSO-treated culture, we diluted 5 mM stock compounds to yield a 625 μM working concentration of library compounds. A threefold serial dilution of compounds was then performed, yielding a concentration range of 0.25 to 625 μM. From this dilution plate, 4 μl was transferred into the 96-well screen plates followed by addition of 96 μl of trophozoites (5,000 parasites) to yield a final 8-point concentration range spanning from 0.01 to 25 μM in final 0.5 % DMSO. The assays were performed in triplicate using CellTiter-Glo. Visualization and statistical analysis of secondary screening were

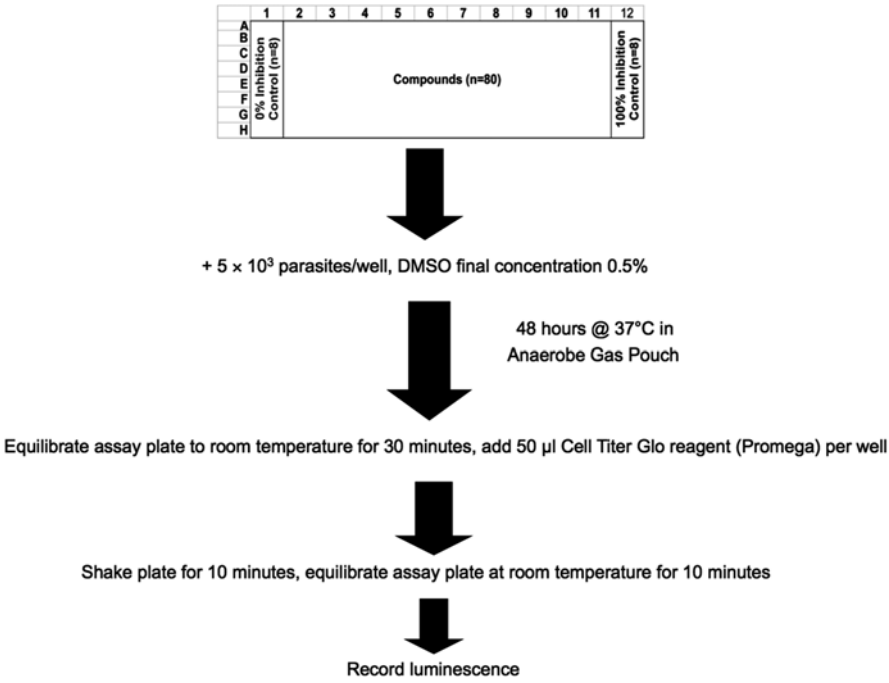

Fig. 32.2 High-throughput compound screening protocol for *E. histolytica* trophozoites in 96-well microtiter plate [44]

performed using GraphPad Prism software 4.0. Percent inhibition relative to maximum and minimum reference signal controls was calculated using the formula:

% Inhibition = [(mean of Maximum Signal Reference Control − Experimental Value)/(mean of Maximum Signal Reference Control − mean of Minimum Signal Reference Control)] × 100

The cutoff was selected to determine actives from the primary screen, which was at least 50 % inhibition and three standard deviations above the mean of the population of compounds tested [6].

32.7 Identification of a New Lead

Once we were able to design a high-throughput assay for drug screening with *E. histolytica*, our next step was to screen large compound libraries, including drugs that were already approved for other uses by the FDA. The screening of existing drugs for new purposes has several advantages. First, these drugs have an established safety record that can offer decreased risk and can save significant time in the

development process. Second, the combination of an off-patent drug, known clinical safety, and possible low production costs can potentially bring down drug pricing to make a reprofiled drug affordable throughout the world; this is particularly important with neglected tropical diseases, which are not a priority for drug companies [44]. The screening of a chemical library of 910 compounds, consisting of 746 approved drugs and 164 bioactive compounds, identified 11 compounds as "active," causing statistically significant growth inhibition (>50 %; Fig. 32.3, Table 32.1). Among these 11 compounds, auranofin was the most potent of all against *E. histolytica* with an EC_{50} tenfold better in vitro than metronidazole (0.5 versus 5 μM). The assay showed excellent discrimination between active and inactive compounds with a Z' (dimensionless calculation used to assess the quality of a high-throughput assay) of 0.96 ± 0.13 in the screening experiment using 12 different plates [6].

We selected auranofin for follow-up studies because auranofin is an FDA-approved drug and has been in clinical use to treat rheumatoid arthritis since 1985; second, this drug is taken orally, and finally it is relatively cheap. In the animal model of amebic colitis a single oral dose of 1 mg/kg/day for 7 days significantly reduced the number of parasites and the detrimental host inflammatory response but not by a similar dose of metronidazole. Similarly, 3 mg/kg/day of oral auranofin

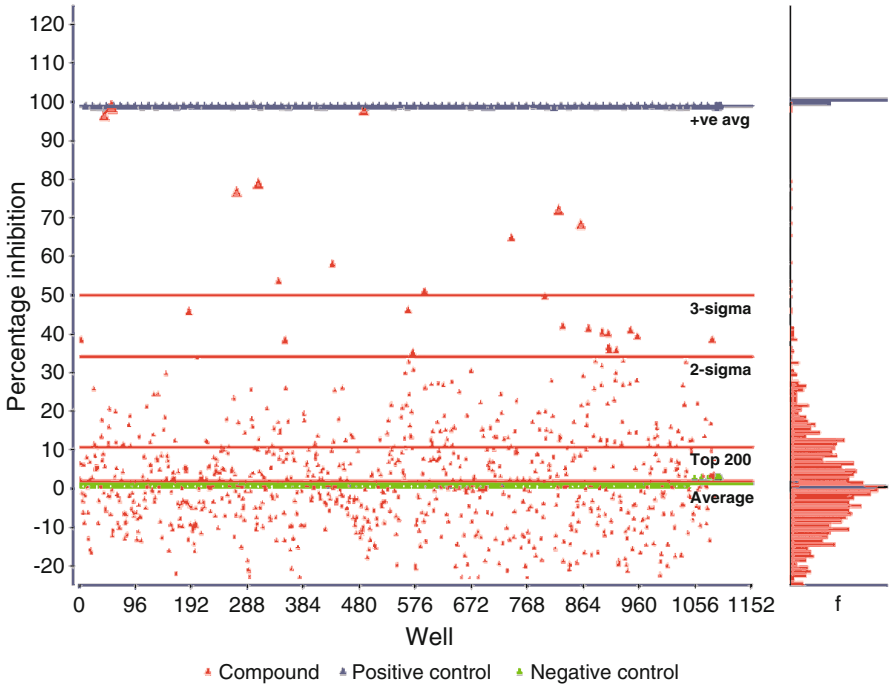

Fig. 32.3 Scatter plot of percentage inhibition of each well from twelve 96-well plates of the Iconix library. Eleven compounds yielded both 50 % inhibition and three standard deviations above the mean of the population of compounds tested in the primary screen at 5 μM [6]

Table 32.1 Hits obtained after screening the Iconix library [6]

Compound	Inhibition (%) (5 μM)
Auranofin	100
Sporidesmin A	99
Cycloheximide	98
Cladribine	79
Fludarabine	77
Homochlorcyclizine	73
Trifluoperazine	69
Idarubicin	65
4,4′-Diethylaminoethoxyhexestrol	58
Clomiphene	54
Amiodarone	51

for 7 days significantly reduced the liver damage in the animal model of amebic liver abscess, but the same dose of metronidazole had no effect on liver abscess progression [6].

32.8 Auranofin: An Oral, Gold-Containing Drug

Auranofin (Ridaura) was the first oral gold-containing compound, which was approved in 1985 for the treatment of rheumatoid arthritis (RA) in adults, and is off patent. It is the only oral formulation of a gold salt available for the treatment of RA for patients who have not responded adequately to one or more nonsteroidal antiinflammatory drugs. Auranofin was not approved for use in children, as it was not effective in the treatment of juvenile RA, but no additional safety issues were found in children [51].

The published pharmacokinetic data of auranofin come from studies in rheumatoid arthritis patients following long-term therapy. Auranofin is rapidly metabolized so no intact drug can be detected, but gold levels have been measured. Following an oral dose, 25 % of auranofin is absorbed, 60 % is plasma protein bound, and 85 % excreted in feces [52]. Auranofin was approved for the long-term treatment of patients with unresponsive rheumatoid arthritis with courses for a minimum of 6 months at doses of 3 mg once or twice a day. Steady-state mean blood gold levels are 0.68 ± 0.45 μg/ml (package insert) or approximately 3.5 μM, more than seven times the EC_{50} for *E. histolytica* [6]. Steady-state levels were achieved in 8–12 weeks with an elimination half-life of 26 days [52]. Auranofin is contraindicated in patients with gold allergy, and is not recommended during pregnancy or severe hepatic or renal insufficiency. The only reported drug interaction is a single patient with elevated phenytoin blood levels [44]. The complications listed in the package insert for long-term (>1 year) auranofin therapy include dermatological (rash, 26 %), gastrointestinal (loose stools, 42 %), abdominal pain (14 %), nausea (10 %); hematological (anemia, leukopenia, thrombocytopenia in up to 3 %); hepatic

(elevated liver enzymes, 2 %); mucous membranes (stomatitis, 13 %); and renal (proteinuria, 1 %). Because treatment for amebiasis is only 5–10 days, the likelihood of gold toxicity should be extremely small [6].

Auranofin has received Orphan Drug status for treatment of amebiasis from the FDA [6]. Orphan drugs in the United States are meant to develop the products for orphan or rare diseases for which drug development costs are unlikely to be recovered through sale in the United States [53, 54]. Amebiasis is a rare disease in the US, and its Orphan Drug status may bring several incentives such as tax credits totaling half of development costs, research and development grants, fast-track development and approval, access to Investigational New Drug (IND) Program and pre-approval, waived drug application fees, and 7-year market exclusivity [53–55].

32.9 Future of Auranofin for Amebiasis

We developed the first HTS that could be performed in an anaerobic environment and showed that it was feasible to screen large numbers of compounds versus *E. histolytica* and that robust and reproducible results could be generated from this HTS. By screening a rational chemical library, we discovered a new drug lead, auranofin, for the treatment of amebiasis. Our future steps involve conducting the human clinical trial of auranofin for amebiasis. Because the drug is off-patent and has been in clinical use for more than 25 years, the cost and development time for this "repurposed drug" can be significantly reduced. This cost savings is important in the context of the disease afflicting the poor.

Because auranofin has received the Orphan Drug status, there will be future incentives for pharmaceutical companies to develop a drug for an orphan disease [44]. Clinical trials of auranofin for amebiasis are being planned in adults, and ultimately will be important in children as malnourished children are more susceptible to amebiasis [56]. If the clinical trial is successful, auranofin could be the first new drug with a defined target for the treatment of amebiasis in more than 50 years.

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Chapter 33
Heterocyclic Lead Compounds Against Amebiasis

Amir Azam and Subhash Mohan Agarwal

Abstract Amebiasis is a widespread parasitic disease caused by *Entamoeba histolytica*. This protozoan organism is the third leading parasitic cause of death in the developing world. Nitroimidazole-based drugs such as metronidazole, tinidazole, and ornidazole are the only agents for treating invasive amebiasis, followed by administration with luminal agents. Several side effects in addition to clinical resistance and carcinogenicity to this class of compounds, especially metronidazole, have been observed. Therefore, to combat this neglected protozoan disease it has become essential to discover new leads for the development of novel drugs that are superior or equally effective against *E. histolytica* but less toxic for humans. Keeping this in mind our group has been making sustained efforts in developing heterocyclic compounds as noble leads because these molecules act as highly functionalized scaffolds. Over a period of a decade hundreds of compounds corresponding to several classes (thiosemicarbazone, metronidazole analogues, azoles, pyrimidines, hydrazones, and triazines) have been synthesized, screened, and evaluated for their antiamoebic activity. In this chapter we discuss the compounds that exhibit new structural scaffolds having antiamoebic activity and elaborate on molecular fragments that play an essential role in improving the activity.

33.1 Introduction

Amebiasis is the most aggressive gastrointestinal disease resulting from infection by the anaerobic protozoan parasite *Entamoeba histolytica*, which causes amebic colitis and amebic liver abscess. Occurring in a number of countries of the world, it results in 50 million cases of invasive disease and up to 100,000 fatalities per year [1, 2]. Brain abscess is another dreadful complication reported from this disease [3].

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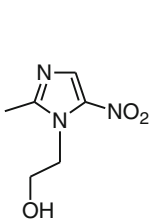
T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_33

It has been reported that the parasite can be transmitted by homosexual as well as heterosexual activity [4]. Amebiasis is an emerging parasitic complication in human immunodeficiency virus (HIV)-infected patients [5, 6].

Modern chemotherapy offers a range of antiamoebic agents, but there is no ideal treatment for amebiasis because many of the available drugs elicit serious adverse reactions [7]. Moreover, relapses of intestinal and hepatic amebiasis have also been reported [8] Nitroimidazoles, such as metronidazole (**1**), ornidazole (**2**), and tinidazole (**3**), are the main drugs used for the treatment of this protozoan disease, but long-term use of these medicaments produces several side effects in patients [9].



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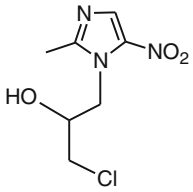

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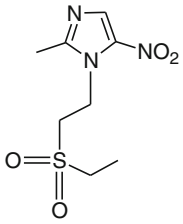

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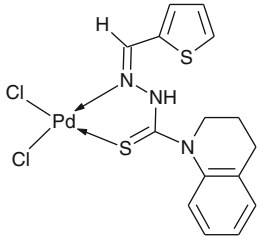
Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), the first line medicament against amebiasis, is potentially carcinogenic to humans because it is genotoxic to human cells [10]. Moreover, resistance of *E. histolytica* to metronidazole has been reported [11], and the treatment failure may emerge as a major public health issue. Therefore, it is essential to investigate new compounds for the development of novel drugs with better amebicidal activity and lesser toxicity for the host. In the present chapter, we summarize the important lead molecules screened in vitro against the HM1:IMSS strain of *E. histolytica* that have better IC_{50} value than metronidazole (reference: amoebicidal drug).

33.2 Thiosemicarbazone Derivatives and Their Metal Complexes

Thiosemicarbazones have been found to exhibit a broad spectrum of pharmacological applications as antitumor, antiviral, antimicrobial, and antiparasitic agents [12]. Studies of thiosemicarbazone-based libraries proved that they are useful ligands for the building of metal complexes with a wide variety of biological targets including protozoan parasites [13]. Their biological activities are considered to be related to their ability to form chelates with metals by binding through sulfur and hydrazinic nitrogen atoms [14, 15]. Pd(II) and Pt(II) complexes of phenyl acetaldehyde thiosemicarbazones are able to bind to DNA and enhance the capacity to form interstrand crosslinks by comparison with *cis*-platinum [16, 17]. Over the years we have synthesized several thiosemicarbazone derivatives that exhibited potent in vitro antiamoebic activity [18]. It is thus reasonable to believe that thiosemicarbazones are authentic

privileged structures [19]. Transition metal complexes of Pd(II) with N^4-substituted thiosemicarbazones of thiophene-2-carboxaldehyde, indol-3-carboxaldehyde, and furan-2-carboxaldehyde were synthesized and screened against the HM1:IMSS strain of *E. histolytica* [20–23]. The most promising antiamoebic activity of Pd(II) complexes was observed in compounds that have 1,2,3,4-tetrahydroquinoline (**4**), adamantine amine (**5**), (2-chlorophenyl) methanamine (**6**), and piperidine (**7**) at the N^4 position of the thiosemicarbazones moiety. It has been observed that the presence of certain bulky groups at position N^4 of the thiosemicarbazone moiety greatly enhances antiamoebic activity. It was also noted that the incorporation of metal ions enhanced the antiamoebic activity of the basic molecule.



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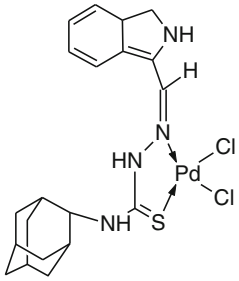

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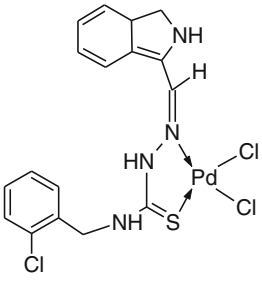

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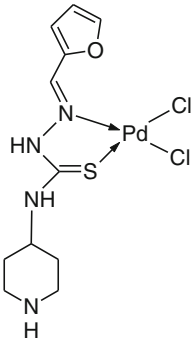

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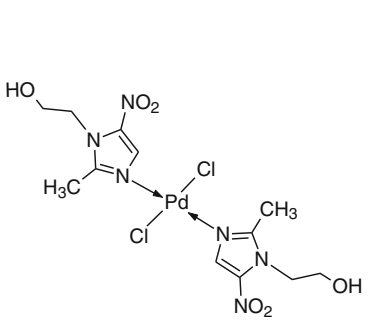
33.3 Metronidazole Analogues

Metronidazole and its derivatives have a wide range of biological activity [24–27]. The coordination ability of the imidazole ring has profound effects on the stereochemistry and the functions of biological systems. This ligand when coordinated to a metal ion can modify factors such as geometry, lipid solubility, and charge of the

complex and therefore enhance or lower their growth inhibitory properties [28]. This observation encouraged us to synthesize complexes by coordinating metronidazole with various metals such as Pd(II), Pt(II), Cu(II), Au(I), and Ru(II) [29, 30]. The in vitro antiamoebic studies suggested that the metronidazole complexes *trans*-$[PdCl_2(mnz)_2]$ (**8**) and $[Cu(mnz)_2(\mu\text{-}Cl)(H_2O)]_2Cl_2$ (**9**) displayed the highest activity. Furthermore, in vivo studies on male golden hamsters showed that the complex (**8**) at 5 mg kg^{-1} produced a reduction in experimental amebic hepatic abscess (EAHA) that was slightly higher (4 %) than the effect of 30 mg kg^{-1} metronidazole. The metronidazole thiosemicarbazone analogues were synthesized wherein the thioamide moiety was substituted by different cyclic and aromatic amines. While considering the substitution, it was observed that the highest antiamoebic activity was found in those derivatives that were substituted by cyclooctylamine and *p*-toluidine (**10**) at position N^4 of the thiosemicarbazide group. Metronidazole thiosalicylate conjugates were synthesized that could act on *E. histolytica* thioredoxin reductase (EhTHRase), a target for nitroimidazole-bearing drugs. It was found that 2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethylsulfinyl) benzoate (**11**) shows promising in vitro antiamoebic activity as well as low cytotoxicity. Further, docking studies indicated that the enhanced antiamoebic activity could be caused by additional hydrophobic interactions in the binding site of *E. histolytica* thioredoxin reductase. These findings provide us lead and encourage us to continue the efforts toward the optimization of the efficacy profile of this structural moiety for treatment of amebiasis [31, 32].



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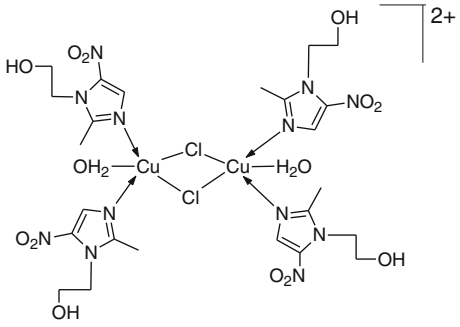

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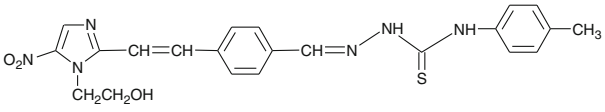

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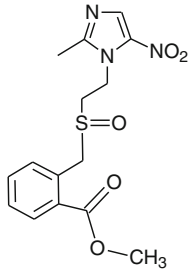

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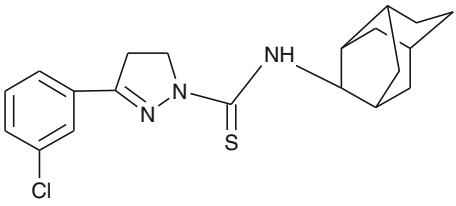
33.4 Azoles

Azoles have long been targeted for synthetic investigation because of their known biological properties [33–35]. The antifungal agents of azoles are useful drugs and are widely used for the treatment of topic or inner mycoses, in particular, acquired immunodeficiency syndrome (AIDS)-related mycotic pathologies [36–39]. Considering that nearly all the classes of the azole family are biologically active, we synthesized and screened variously substituted pyrazolines, bis-pyrazolines, dioxazoles, bis-dioxazoles, oxadizoles, thiadiazoles, and triazoles against *E. histolytica*.

33.4.1 Pyrazolines

Pyrazoline, the reduced form of pyrazole, has drawn the attention of medicinal chemists because of the diverse biological properties [40]. 2-Pyrazoline derivatives have been reported to exhibit various pharmacological activities such as antibacterial, antifungal, antimicrobial, and antidepressant [41]. Two different synthetic strategies have been applied for the synthesis of 3-phenyl- and 3, 5-diphenyl-substituted pyrazoline derivatives. 1-*N*-substituted 3-phenyl 2-pyrazoline derivatives were synthesized by cyclization of the Mannich base with various N^4-substitued thiosemicarbazides [42–44]; 1-*N*-substituted 3,5-diphenyl-2-pyrazolines were prepared by cyclization of chalcone with various *N4-substituted* thiosemicarbazides [45–47] or with 2-(quinoline-8-yloxy acetohydrazide) under basic conditions [48]. The antiamoebic activity of all the pyrazoline derivatives was investigated, and the study led us to conclude that the 3-chloro-substituted phenyl 2-pyrazoline (**12**) derivative was found more active than their respective analogues. The studies suggest that substitution of bromo or chloro on the phenyl ring at position 3 of the pyrazoline ring and the presence of bulky groups at position 1-*N* of the thiocarbamoyl group greatly enhances the antiamoebic activity.



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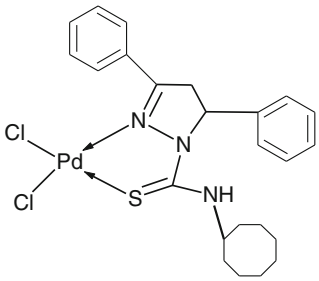
The complexation of pyrazoline derivatives with Pd(II) results in complexes that were found more active than their respective ligands. Among all the Pd(II)

complexes of pyrazoline derivatives, the most active compounds in this class were the compounds that have cyclooctyl amine (**13**) and adamantyl amine (**14**) as the 1-*N* substitution [46].



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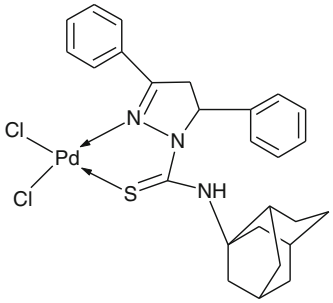

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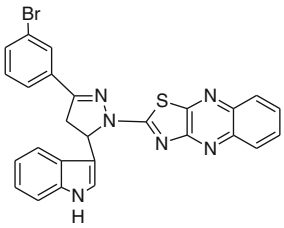
2-Pyrazolines prepared from Mannich base or chalcone precursors on reacting with 2,3-dichloroquinoxaline gave thiazolo[4,5-*b*] quinoxaline-2-yl-2-pyrazoline derivatives [49, 50]. On comparing the antiamoebic potential, it was observed that the quinoxaline derivative (**15**) was the better inhibitor of *E. histolytica* trophozoites. The pyrazoline derivatives bearing a quinoline tail having the substitution at the C-4 position of the phenyl ring with methyl, chloro, or methoxy groups (**16**) showed excellent antiamoebic activity [48].



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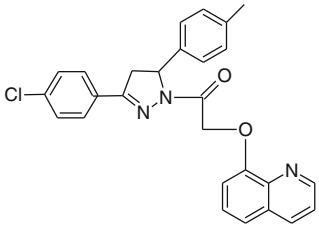

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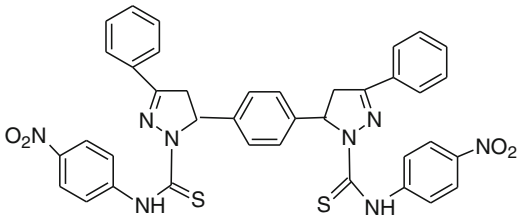
33.4.2 Bis-Pyrazolines

The bis-chalcone precursor prepared by treating acetophenone with terephthaldehyde under basic conditions followed by cyclization with N^4-substituted thiosemicarbazides gave bis-pyrazolines [51]. It has been observed that the compounds with aromatic substituents at the thiocarbamoyl group were more active than those with the cyclic groups (**17**).



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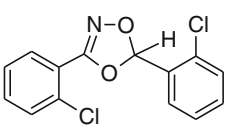
33.4.3 Dioxazole and Bis-Dioxazoles

Oxazole, an important member of the azole family, also contains a number of biologically active molecules that have an important role in the drug chemistry. A number of compounds were screened for anti-tuberculosis agents including dihydrophenazines [52], indoles, and ureas [53]. Oxybenzylglycine, possessing an oxazoline group, is currently in clinical development for the treatment of type II diabetes and dyslipidemia [54]. The synthesis of 5-*o*-chloro, 5-*m*-chloro, and 5-*p*-chloro phenyl dioxazole derivatives having the same substituents at 3-position of the dioxazole ring showed that their antiamoebic activity was structure dependent [55]. It was found that 2-chloro phenyl group (**18**) or 4-chloro phenyl group (**19**) present at the 5-position of the dioxazole ring were more active than the compounds with a 3-chloro phenyl group (**20**). The activity further depends on the nature of the substituents at carbon-5 of the dioxazole ring. Compounds with only phenyl groups attached at carbon-5 were found to be more active than those with additional methyl or ethyl groups present at the same carbon. The structure–activity relationship further revealed that the phenyl rings with electron withdrawing groups present at carbon-5 of the dioxazole ring were more active than those with electron-donating groups.



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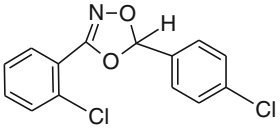

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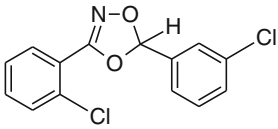

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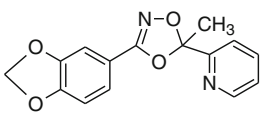
Some new terpene-based dioxazole derivatives were synthesized and screened for antiamoebic activity and cytotoxicity [56]. Their pK_a and log P values have been determined to understand the chemical interactions between the compound of interest and its pharmacological target. The results showed that the compound (**21**) exhibited better antiamoebic activity than the standard drug, metronidazole.

It was inferred that most of the new compounds having the piperonal ring skeleton in conjugation with the pyridine ring or furan ring in the same compound showed significant antiamoebic activity, and the modification of functionality produced a significant change of activity. It is worthy to mention that the position of the CH_3 group at position 5 of the dioxazole ring had marked effects on the activity and toxicity of the synthesized compounds in addition to the presence and position of the pyridine ring. Cyclization of benzene-1,4-dicarboxaldehyde dioxime with different aromatic aldehydes yielded the corresponding new bisdioxazoles. The antiamoebic results showed that those derivatives substituted with the *m*-chloro, *o*-chloro, *p*-methyl, or *p*-methoxy phenyl group (**22**) exhibit better antiamoebic activity than metronidazole [57].



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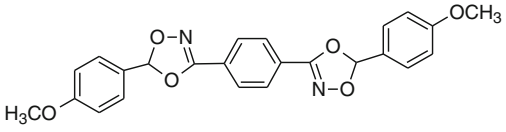

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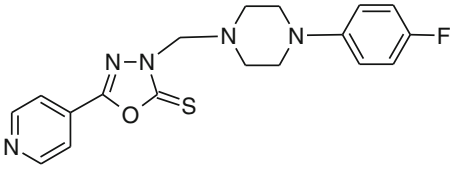
33.4.4 Oxadiazoles

Compounds having an oxadiazole ring have been reported to exhibit remarkable antiamoebic activities. For instance, BTI 2286^{E} (±)-E-3-(4-methylsulfinylstyryl)-1, 2, 4-oxadiazole has been found to show potent amoebicidal activity in a single-dose treatment against *E. histolytica* infection in the liver of golden hamsters and the ceca of mice, hamsters, and rats [58]. A series of Mannich base derivatives of 5-(pyridine-4-yl)-1,3,4-oxadiazole-2-(3*H*)-thione incorporating a piperazine ring were synthesized [59]. The compound (**23**), with a 4-fluorophenyl group attached with a piperazine ring, showed better inhibitory effect. The replacement of the 4-fluorophenyl group with the 2-methoxyphenyl group drastically reduced the antiamoebic activity. This study suggested the possibility of developing 1,3,4-oxadiazole as a potential drug candidate.



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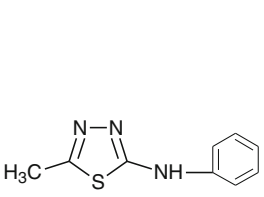
33.4.5 Thiadiazoles

Sulfur–nitrogen heterocycles are important compounds because of their significant and versatile biological activity [60]. The thiadiazole nucleus exhibit multifaceted biological activities, possibly because of the presence of the –N=C=S moiety [61, 62]. 1, 3, 4-Thiadiazole based drugs such as acetazolamide, desaglybuzole, and furidiazine are commercially available [63]. Several derivatives of 1, 3, 4-thiadiazole have been found active against parasitic diseases such as leishmaniasis and trypanosomiasis [64–66]. Therefore we expected that the 1, 3, 4-thiadiazole derivatives might be effective against amebiasis. Among all synthesized 2,5-disubstituted-1,3,4-thiadiazole derivatives, biological behavior revealed that compound (**24**) having the methyl group at the C-5 position of the thiadiazole ring, exhibited an excellent activity in terms of IC_{50} value and was the most active compound in the series [67]. Replacement of this methyl with a phenyl group reduces antiamoebic activity drastically. These results indicated that the antiamoebic activity was influenced by the type of substituent present at the C-5 position of the thiadiazole ring as well as the side chain attached on the C-2 position of the thiadiazole ring. The 1,2,3-thiadiazole derivatives having 4-chloro phenyl (**25**), 4-bromo phenyl (**26**), and a furan ring (**27**) substituted at the C-4 position of thiadiazole rings were also found active against *E. histolytica* [68].



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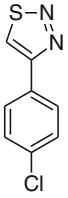

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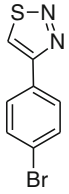

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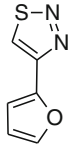

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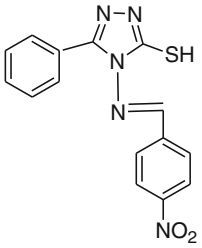
33.4.6 Triazole Conjugates

It was observed that incorporation of a thiosemicarbazone fragment within the triazole ring enhances the antiamoebic activity; especially, substitution at the *para* position of the phenyl ring with methyl, methoxy, nitro, and dimethylamino group. Biological behavior revealed that the compound having a nitro group (**28**) at the *para* position of phenyl ring showed better antiamoebic activity than metronidazole [69].



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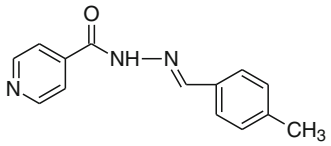
33.5 Hydrazone Derivatives Bearing Pyridyl Moiety

The synthesis of different azole and hydrazone derivatives in earlier years provided enough clue that the combination of these derivatives with pyridyl ring does exhibit antiamoebic activities. The isonicotinyl hydrazone derivatives bearing a pyridyl moiety having methyl group at *para*-position of phenyl ring (**29**) exhibited most promising antiamoebic activity [70]. The incorporation of a nitro or methoxy group at the same position significantly induced inhibitory activity whereas the dimethylamino and isopropyl group resulted in loss of antiamoebic activity. Hence, it can be concluded that the pyridyl and azomethine groups (CH=N) do play the crucial role in modulating activity against HM1:IMSS strain of *E. histolytica*.



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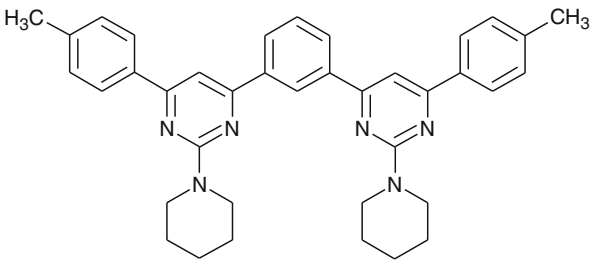
33.6 Pyrimidine Derivatives

Pyrimidines are of great importance in fundamental metabolism, being an integral part of DNA and RNA and found as core structure in a large verity of compounds that exhibited important biological activities such as anticancer, antimicrobial, antioxidant, and antiviral activities [71–75]. Therefore, we synthesized organic molecules containing two pyrimidine rings. The results showed that 2,4,6-trisubstituted bis-pyrimidine derivatives having methoxy, thiomethyl, methyl, and dimethyl substituents on the phenyl ring at C-6 exhibited higher antiamoebic activity (**30**),

whereas substitution of the phenyl ring with a chloro, dimethoxy, or trimethoxy group did not affect the activity [76].



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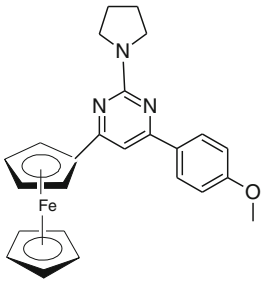
The 2,4,6-trisubstituted pyrimidines were synthesized by the cyclization of ferrocenyl chalcones with pyrrolidine-1-carboxamidine hydrochloride and piperidine-1-carboxamidine hydrochloride, respectively [77]. The in vitro antiamoebic activity results showed that the compounds having methoxy substitution (**31**) and a chloro group (**32**) exhibited higher antiamoebic activity than the reference drug metronidazole.



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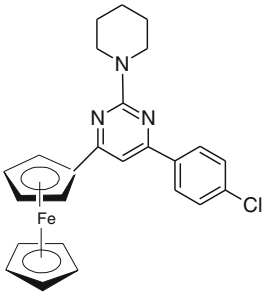

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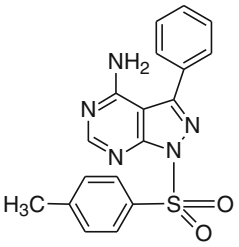
Pyrazole and pyrimidine are crucial nitrogen-containing heterocyclic systems that can provide privileged scaffolds for the development of antiamoebic compounds. Considering this perspective, it was planned to integrate pyrazole and pyrimidine rings together in a single molecular frame to form pyrazolo-[3,4-d] pyrimidine pharmacophores [78, 79]. The introduction of tosyl group (**33**) at –NH of the pyrazole ring greatly enhanced antiamoebic activity. A series of pyrimidine-6-one derivatives were prepared by Biginelli multi-component one-pot cyclocondensation reaction, and compounds substituted by *p*-chloro phenyl, 2-chloroquinoline, and 2-chloro-7-methylquinoline (**34**) showed better inhibition of *E. histolytica*



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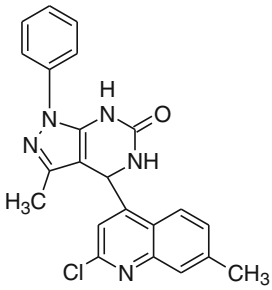

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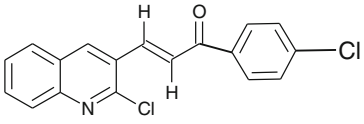
33.7 Chloroquinoline-Based Chalcones

Many natural products bearing a quinoline nucleus such as quinidine, quinidinone, and quinine from the plant *Cinchona ledgeriana* possess antiamoebic activity [80]. The chloroquinoline-based chalcones synthesized by the condensation of substituted 2-chloro-3-formylquinolines with *p*-chloroacetophenone (**35**) and 2-acetyl pyridine (**36**) showed excellent antiamoebic activity [81].



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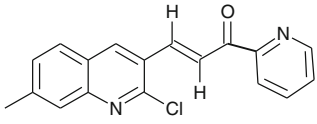

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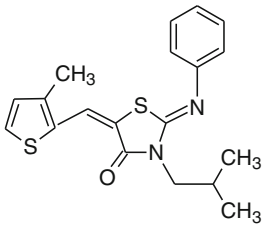
33.8 Thiazolidinone Derivatives

A new series of thiazolidinone derivatives was synthesized in view of their versatile pharmacological significance [82]. The nature of the substituents plays a dominant role in antiamoebic activity. The compounds having methyl thiophene as the substitution on the thiazolidinone ring (**37**) showed better activity than other synthesized compounds.



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33.9 Conclusion

Amebiasis is a major health problem of developing countries. The occurrence and spread of this disease could be controlled by the provision of adequate sanitation worldwide, but this situation is unlikely in the foreseeable future. Therefore, development of effective chemotherapy is required. Tremendous progress has been made in synthetic and natural product chemistry, and scientists from divergent fields have screened a large number of compounds hoping to find antiamoebic activity. Keeping this is in mind, our group has made continuous efforts to synthesize newer and better molecules that could guide development of a molecule with similar beneficial therapeutic activity but with fewer side effects than the known drugs. In this attempt we have been successful to some extent, as over the years we have designed and developed newer molecules which on in vitro evaluation have shown to possess better activity than the standard drug metronidazole. We believe that if in vitro positive compounds are screened through in vivo evaluation models, some of these molecules may pave the way for better treatment.

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