Acanthamoeba

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Martina Köhsler, Martin Mrva, and Julia Walochnik

Abstract

Acanthamoebae are ubiquitous free-living amoebae that occur abundantly in water and soil worldwide, being among the most versatile protozoan organisms. They generally do not need a host, but when they accidentally have contact to the human eye, lung or skin, they can cause severe disease. They are the causative agents of *Acanthamoeba* keratitis (AK), on the one hand, and of several disseminating infections in the immunocompromised host eventually leading to granulomatous amoebic encephalitis (GAE), on the other hand. The infective and invasive form of *Acanthamoeba* is the trophozoite; nevertheless, the cyst plays an important role in the distribution of the amoebae as well as in the course of disease. Acanthamoebae can form cysts within the host tissue, and these cysts are resistant against treatment and also often lead to reinfections.

Altogether, around 25 different species divided into three morphological groups have been described; however, the validity of many species has been challenged by molecular analyses. Currently, the genus is divided into 20 genotypes based on 18S rDNA sequencing, but the number of genotypes is growing constantly. Genotype T4 seems to be the most abundant one in most habitats and also the most common genotype in human infections; however, a classification into virulent and non-virulent genotypes is not possible. Acanthamoebae pathogenicity depends on cell-cell contact, the cytolytic event being triggered by an

M. Köhsler • J. Walochnik (✉)
Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna,
Kinderspitalgasse 15, Vienna A-1090, Austria
e-mail: martina.koehsler@meduniwien.ac.at; julia.walochnik@meduniwien.ac.at

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

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intimate contact between the amoebae with the target cells, established primarily via lectin-like amoebic adherence molecules. The ability of acanthamoebae to lyse cells is mainly based on lysosomal hydrolases and phospholipases.

In 2013, a first genome has become available revealing a significant number of genes presumably acquired by lateral gene transfer and a rather complex cell communication repertoire. However, the genetics of *Acanthamoeba* spp. is far from being fully elucidated. Fact is that acanthamoebae have unusually broad metabolic and biosynthetic capabilities, being able to synthesise most amino acids (even multiple steps), co-factors and vitamins and nucleotides de novo. Moreover, they can digest a wide range of nutrients, they are among the few protozoa with a cellulase, and they are the only protozoans known to date with an alginate lyase. Although acanthamoebae generally divide by binary fission and there is no convincing evidence for genetic recombination, even their asexual nature has been challenged recently.

Abbreviations

AA	Arachidonic acid (AA)
AcAtg	*Acanthamoeba* autophagy-related proteins
AIF	Apoptosis-inducing factor
AK	*Acanthamoeba* keratitis
AOX	Alternative oxidase
aPA	*Acanthamoeba* plasminogen activator
cAMP	Cyclic adenosine monophosphate
CPE	Cytopathic effect
CRD	Carbohydrate recognition domains
CSCP	Cyst-specific cysteine protease
CXCL2	Chemokine (C-X-C motif) ligand 2
cPLA	Cytosolic phospholipase A
DAG	Diacylglycerol
DH	Dehydrogenase
ECM	Extracellular matrix
ERMES	ER-mitochondria encounter structure
EST	Expressed sequence tag
ETC	Electron transport chain
FFA	Free fatty acid
GAE	Granulomatous amoebic encephalitis
GMP	Guanosine monophosphate
GPCR	G protein-coupled receptors
HBMEC	Human brain microvascular endothelial cells
HCE	Human corneal epithelial cells
HNE	4-Hydroxy-2-nonenal
ICL	Isocitrate lyase
LBP	Laminin-binding protein

LGT	Lateral gene transfer
MalS	Malate synthase
MAPK	Mitogen-activated protein kinase
MBP	Mannose-binding protein
MIP	Mannose-induced protein
MMP	Matrix metalloprotease
ORF	Open reading frame
PAR	Protease-activated receptor
PDH	Pyruvate dehydrogenase
PHB	Polyhydroxybutyrate
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C-like gene
PMN	Polymorphonuclear leukocyte
PN	Purine nucleotide
pTyr	Phosphotyrosine
ROS	Reactive oxygen species
SAPLIP	Saposin-like protein
TCA	Tricarboxylic acid
TLR	Toll-like receptors (TLR)
UCP	Uncoupling protein

10.1 Introductory Remarks

Acanthamoebae are ubiquitous free-living amoebae; they occur worldwide in natural habitats such as soil, fresh water or marine water but also in man-made habitats such as swimming pools and airconditioning systems. With their low nutritional requirements and their highly resistant cysts, acanthamoebae can exist almost anywhere and are thus among the most frequently isolated amoebal species worldwide. The life cycle of *Acanthamoeba* spp. consists of two stages, a vegetative trophozoite stage, in which the organism feeds and multiplies, and a metabolically inactive cyst stage, which enables the amoeba not only to survive without nutrients and withstand desiccation and heat but also to resist disinfection and treatment. Generally, acanthamoebae feed on bacteria, algae and yeasts; they are, however, among the most versatile protozoans being able to feed on almost anything and to occur under aerobic and anaerobic conditions and also under extreme conditions concerning pH, salinity and temperature. They do not need a host, but they can cause serious disease upon contact, being the causative agents of very different disease entities, including *Acanthamoeba* keratitis (AK), a sight-threatening infection of the eye, and several disseminating infections, such as *Acanthamoeba* skin lesions and granulomatous amoebic encephalitis (GAE) (Khan 2006; Visvesvara 2010). While AK, particularly in the industrialised countries, occurs mainly in contact lens wearers, *Acanthamoeba* GAE seems to be restricted to immunodeficient patients. All *Acanthamoeba* infections are rare, but they usually show a severe progression, which is mainly due to the

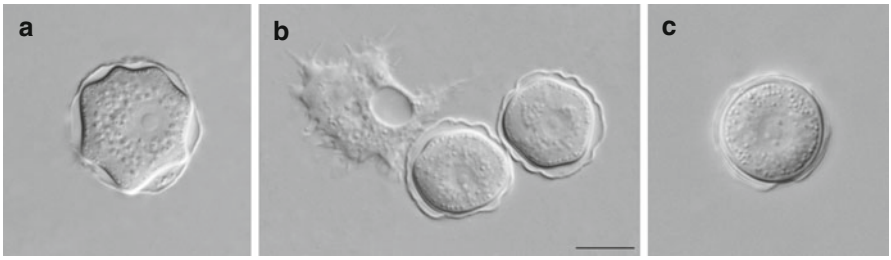

Fig. 10.1 *Acanthamoeba*. Morphological group I, cyst (**a**); morphological group II, trophozoite and cysts (**b**); morphological group III, cyst (**c**). Group I shows typical large trophozoites and cysts of more than 18 µm in diameter. Cysts have a rounded outer wall clearly separated from the inner wall, to which it is joined by radiations, forming a star-shaped structure. The opercula are not immersed into the outer cyst wall. Group II cysts are smaller with a variable endocyst shape, mostly smaller than 18 µm. Ecto- and endocyst may be separated or not. The ectocysts are thick or thin but usually wrinkled. The endocyst may be polygonal, triangular, or even round or oval. Normally no clear radiations are visible. The operculum is usually immersed into the ectocyst. In group III, the cysts show less than 18 µm in diameter and have poorly separated walls. The ectocyst is rather thin and very often ripped. The endocyst is usually round but sometimes slightly triangular or rectangular. Scale bar: 10 µm. Orig

unavailability of effective and easily manageable treatment. The dormant cysts pose a particular problem, residing within the tissue and leading to reinfection after termination of treatment, often even after keratoplasty.

Acanthamoeba is one of the most common and most robust genera of free-living amoebae, functioning also as host, vehicle and training ground for bacteria. Several bacteria, including important human pathogens such as legionellae, have been shown to be 'primed' for virulence by passage through amoebae. Indeed, acanthamoebae are phagocytic cells and behave similarly to the phagocytic cells of the mammalian immune system in many aspects (Siddiqui and Khan 2012).

Acanthamoebae were discovered in 1930 in a culture of *Cryptococcus pararoseus* (Castellani 1930) and placed into the genus *Acanthamoeba* one year later by Volkonsky (Volkonsky 1931). The genus *Acanthamoeba* comprises three morphological groups (Pussard and Pons 1977) based on the rather distinct, polygonal morphologies of the cysts (Fig. 10.1), the number of opercula considered to display species variation. But cysts, although deriving from one clone, may have varying morphologies, and intraspecific polymorphism generally is rather common in *Acanthamoeba*. Around 25 species have been described; however, the validity of most species has been challenged by molecular analyses (Moura et al. 1992; Nerad et al. 1995; Page 1987, 1991). Today, isolates can be assigned to genotypes based on their 18S rDNA sequences (Gast et al. 1996; Stothard et al. 1998). When this chapter was written, 20 *Acanthamoeba* genotypes (T1-T20) had been established. The most common genotype in human *Acanthamoeba* infections is T4; however, this generally seems to be the most abundant genotype. Other genotypes rather frequently isolated are T3 and T11 from AK patients, and T1, T10 and T12 from GAE, but a classification into virulent and non-virulent genotypes is not possible (Booton et al. 2005; Fuerst et al. 2015; Walochnik et al. 2015).

The first cases of AK were recorded in the 1970s in patients who had contact to contaminated water after experiencing a minor trauma in the eye (Jones et al. 1975; Nagington et al. 1974). In Central Europe, the first case of AK was reported in 1984 from Germany and was one of the very first cases of contact lens-associated AK (Witschel et al. 1984). Soon, the association between *Acanthamoeba* keratitis and contact lens wear was established (Moore et al. 1985). Today, the annual incidence of AK is estimated to lie between 1 and 10 cases per million inhabitants in the industrialised countries, the most important risk factor being low contact lens hygiene (Page and Mathers 2013; Walochnik et al. 2015). One of the first human cases of GAE was described in the USA in an AIDS patient (Wiley 1987). In Europe, the first GAE case occurred in an AIDS patient in Italy (Di Gregorio et al. 1992). Altogether, around 300 cases of GAE have been described around the world.

The infective and invasive form of *Acanthamoeba* is the trophozoite; nevertheless, the cysts are spread easily through the air and can transform into trophozoites rapidly under appropriate conditions. Moreover, the cysts play a significant role for the progression of disease, as they may survive treatment within the tissue and lead to reinfection. Importantly, the vast majority of healthy individuals do not develop disease in spite of regular contact to *Acanthamoeba*. Besides mechanical prerequisites, such as microlesions in the cornea and the skin, respectively, also immunologic features seem to play an important role for the establishment of disease.

A very important virulence factor is the ability of the acanthamoebae to adhere to surfaces including the corneal epithelium with the help of their acanthopodia. Trophozoites of *Acanthamoeba* do not only adhere to, but they also can hardly be removed by physical means from contact lenses. Moreover, the amoeboid locomotion enables the amoebae to pass through spaces as narrow as 2 μm (Bamforth 1985), which is of importance for the penetration of host tissue. It has been shown that not all species of free-living amoebae are equally adapted to tissue migration (Thong and Ferrante 1986). *Acanthamoeba* pathogenicity seems to depend on intimate cell-cell contact established primarily via a lectin-like amoebic adherence molecule. Also type IV collagen, laminin and fibronectin have been reported to function as binding sites (Gordon et al. 1993). The ability of amoebae to lyse cells is based on an enzymatic action induced by lysosomal hydrolases and phospholipases. Acanthamoebae were also shown to have an amoebapore protein being released into the intercellular space following cell-cell contact. This protein inserts into the cell membrane and forms ion channels through it. As a result, the target cell probably becomes permeable and lyses (Michalek et al. 2013). Table 10.1 gives an overview of important molecules involved in *Acanthamoeba* pathogenicity.

In 2013, a first genome has become available; several further genome projects are currently on the way (see Chap. 1, Tab. 2.0). The genome has a significant number of genes presumably acquired by lateral gene transfer, and it has a rather complex cell communication repertoire; however, the genetics of *Acanthamoeba* spp. is far from being fully elucidated. Acanthamoebae generally multiply by binary fission, and there still is no convincing evidence for genetic recombination, but *Acanthamoeba* has several genes implicated in meiosis in eukaryotes with sexual reproduction (Khan and Siddiqui 2015; Speijer et al. 2015).

Table 10.1 *Acanthamoeba* molecules involved in pathogenesis.

Process	Type	Characteristics	Known function	Citation
Attachment	Adhesins and lectins (carbohydrate/ glycan-binding proteins)	Mannose-binding protein (*MBP*, 400 kDa; composed of 130 kDa subunits)	Cell surface receptor binding to mannose glycoproteins, mediates adhesion and induces cytopathic effect	Yang et al. (1997)
		Laminin-binding proteins (28.2, 54, 55 kDa)	Adhesion	Gordon et al. (1993), Wang et al. (1994), Hong et al. (2004), Rocha-Azevedo et al. (2009, 2010)
		Surface glycoproteins (8 with mannose and 8 with N-acetyl glucosamine residues)	Adhesion	Soto-Arredondo et al. (2014)
		Acanthamoeba adhesin (207 kDa)	Adhesion	Kennett et al. (1999)
	Glycans	Oligomannosidic, paucimannosidic, hybrid/ complex type	Adhesion	Schiller et al. (2012)
	ecto-ATPases	62, 100, 218, 272, >300 kDa	Hydrolysing extracellular ATP, involved in adhesion	Mattana et al. (2002), Sissons et al. (2004)
Cell lysis	Serine proteases	*MIP-133* (133 kDa); 33, 35, 130, 133 kDa etc.	Hydrolysis of proteins (contact independent)	e.g. Hadas and Mazur (1993), Kong et al. (2000), Sissons et al. (2006)
		Elastases (70–130 kDa)	Degradation of connective tissue (degrade elastin, fibrinogen, collagen, proteoglycans)	Ferreira et al. (2009)
		Acanthamoeba plasminogen activator (aPA)	Facilitates penetration through membranes	Alizadeh et al. (2007), Mitra et al. (1995)
	Metalloproteases	P3; 80 kDa, 150 kDa	Contact dependent, degrade basement membranes and components of extracellular matrix (type I and type II collagens, fibronectins, laminin)	Sissons et al. (2006)

	Cysteine proteases	43 kDa, 65 kDa, 130 kDa etc.	Protein degradation	Hadas and Mazur (1993)
	Phospholipases	A1, A2, B, C, D	Hydrolysis of phospholipids, membrane disruption	Matin and Jung (2011)
	Neuraminidases	Membrane associated and released; optimal activity in acidic range	Degradation of sialylated glycoconjugates (colonisation of the sialic acid-rich corneal epithelium)	Pellegrin et al. (1991)
	Pore-forming proteins	Acanthaporin (probably amphipathic)	Permeabilises membranes	Michalek et al. (2013)
Interaction with host defence	Superoxide dismutases (SODs)	Iron SOD (~50 kDa) and copper-zinc SOD (~38 kDa)	Anti-oxidant and anti-inflammatory	Choi et al. (2000)
Encystment	G protein-coupled receptors (GPCRs)		Quorum sensing, induction of encystment (activation of Ras, activation of adenylate cyclase)	Aqeel et al. (2015), Fouque et al. (2012), Krishna Murti and Shukla (1984)
	Glycosidases and glycogen phosphorylase		Glycogen breakdown	Lorenzo-Morales et al. (2008)
	Cellulose synthase		Cyst wall formation	Moon et al. (2014)
	Xylose isomerase		Cyst wall formation	Aqeel et al. (2015)
	Rho kinase		Regulation of actin polymerisation	Dudley et al. (2009)
	Gelation factor		Actin cross-linking	Dudley et al. (2009)
	Proteases	Serine proteases (33 kDa)	Autolysis	Moon et al. (2008)
		Cysteine proteases, including a metacaspase type I (478 amino acids)	Autolysis	Leitsch et al. (2010), Moon et al. (2012), Trzyna et al. (2008)
	Autophagy-related proteins (AcAtgs)	AcAtg3, AcAtg8, AcAtg16	Autolysis	Moon et al. (2009; 2011)

10.2 Well-Established Facts

10.2.1 Genome

The genus *Acanthamoeba* is currently placed into the taxon Discosea within the phylum Amoebozoa. The Amoebozoa form a valid clade with the Obazoa, including the animals and fungi (see Chap. 12).

Although all available data support the monophyly of the Amoebozoa, the genomic divergence between, e.g. *Dictyostelium* and *Entamoeba* is higher than the one between animals and fungi. Amoebozoan genomes completed so far have a high (A + T) content and a relatively high percentage of horizontal gene transfer and transposal elements. *Dictyostelium* as well as *Acanthamoeba* have essential tRNAs in their mitochondrial genomes, which is not yet known from other organisms. Most studies on the genome of *Acanthamoeba* have been undertaken with the Neff strain of *Acanthamoeba castellanii*, genotype T4, the strain also used in the *Acanthamoeba* genome project. This strain has the advantage that it is very well characterised concerning also its cell biology; however, this strain is a non-pathogenic environmental isolate that has been grown under laboratory conditions since 1957 and, moreover, seems to be rather exceptional, as similar strains have rarely been isolated. Early studies estimated a total cellular DNA content of uni-nucleate acanthamoebae during log phase of 1–2 pg (Byers 1986) and a haploid genome size of 40–50 Mb (Bohnert and Herrmann 1974; Jantzen et al. 1988). The genome project revealed a 45 Mb genome encoding 15,455 compact genes with an average of 6.2 introns per gene, which is among the highest known in eukaryotes (Clarke et al. 2013). The ploidy level and also the number of chromosomes of *Acanthamoeba* spp. are still uncertain. Most probably, acanthamoebae are at least diploid, and the number of chromosomes, previously assumed to be in the range of several dozens, has more recently been estimated to lie between 9 and 21 (Byers 1986; Matsunaga et al. 1998).

10.2.1.1 Nuclear Genome

A first detailed sequence survey of parts of the genome of *A. castellanii* performed by Anderson et al. (Anderson et al. 2005) gave insights into the extensive metabolic capacities and environmental adaptabilities of this organism. In contrast to the parasitic entamoebae, *Acanthamoeba* has very broad biosynthetic potentials; it can synthesise most amino acids (even multiple steps), synthesise co-factors and vitamins and synthesise nucleotides (RNA & DNA) de novo. The biosynthetic machinery of *Acanthamoeba* includes a chorismate synthesis pathway for the synthesis of aromatic amino acids and folate as well as complete purine and pyrimidine biosynthetic pathways and thymidylate synthase and ribonucleotide reductase for the synthesis of ribonucleotides and deoxyribonucleotides.

Moreover, *Acanthamoeba* can utilise complex organic nutrients; as one of very few eukaryotes, it can utilise the bacterial storage compound polyhydroxybutyrate (PHB) as an energy source. It putatively possesses an enzyme for depolymerising PHB, which in bacteria is degraded to acetyl-CoA for the TCA cycle for energy production. *Acanthamoeba* also has a cellulase; it thus can also use ‘plant’ material as food, and unlike most eukaryonts, it also has a β-glucosidase, so it can degrade

cellulose to cellobiose and then to glucose. It can also feed on fungi and also has a putative chitinase. Further, *Acanthamoeba* is the only protozoan known to date with an alginate lyase. Alginate lyases are usually only found in marine invertebrates and bacteria. *Acanthamoeba* probably utilises alginate as a food source, breaking down biofilms and feeding on the enclosed bacteria.

Important enzymes involved in *Acanthamoeba* phagocytosis are a superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and a lysozyme to depolymerise bacterial peptidoglycan. Proteases have been shown to be essential for various functions, including pathogenicity, digestion and encystment. Five cysteine proteases of the papain family, three serine proteases of the subtilisin family and a metalloprotease were detected in the genome.

In contrast to several obligate parasites, *Acanthamoeba* has all standard asparagine-linked glycosylation (alg) genes in its genome and is capable of synthesising the complete lipid-linked oligosaccharide precursor dolichol-PP-$Glc_3Man_9GlcNAc_2$ (Schiller et al. 2012).

For signal transduction, several serine/threonine protein kinases, including putative receptor kinases as well as tyrosine kinases, belonging to the animal tyrosine kinase family, were found (Clarke et al. 2013). The identification of four proteins with predicted SH2 domains and several tyrosine phosphatases gave first evidence for the existence of primitive phosphotyrosine (pTyr) signalling. A group of 21 histidine kinases and 11 response regulator receiver domains were identified, potentially allowing the amoebae to sense and respond to environmental changes and stress conditions. The detection of a trehalose-6-phosphate synthase homolog indicates that one component of *A. castellanii*'s stress tolerance system appears to be the production of the disaccharide trehalose, which is involved in protection from desiccation, osmotic stress and extremes of temperature, suggesting that trehalose plays a role in stress adaptation and might be associated with the induction of encystment (Anderson et al. 2005).

10.2.1.2 Non-coding RNA (ncRNA)

The ribosomal gene repeat unit of *Acanthamoeba* is a typical eukaryotic one and includes one set of 18S, 5.8S and 28S rDNA plus spacer regions located between the genes and between neighbouring sets. As in other lower eukaryotes, the 5S rRNA gene is an integral part of the ribosomal repeat unit. The complete repeat unit is around 12 kb long and probably of a single kind as there is no evidence for sequence or size heterogeneity. The number of rRNA repeats in *Acanthamoeba* was estimated to be 24 per haploid genome; however, because of suspected common polyploidy, each cell might contain up to 600 rRNA genes (Yang et al. 1994). The 18S rRNA gene coding region is exceptionally long, namely, around 2,300 bp in most genotypes and even around 3,000 bp in genotypes having an intron (Stothard et al. 1998). Several species of *Acanthamoeba*, as, e.g. genotype T5, are known to have group I introns in the nuclear 18S rDNA (Gast et al. 1994; Schroeder-Diedrich et al. 1998). The 5.8S ribosomal RNA of *A. castellanii* is approximately 162 bp long and the 5S RNA gene is 119 bp long, and there may be up to 480 genes encoding the 5S RNA in each *A. castellanii* cell. The 5S rRNA genes are dispersed, which is highly unusual, since the majority of eukaryotic organisms contain 5S genes

clustered in tandem repeats (Zwick et al. 1991). The 28S coding sequence is subdivided into two segments of 2,400 and 2,000 bp separated by an internal transcribed spacer of about 200 bp (Byers et al. 1990). The intergenic spacer is 2,330 bp long and contains repeated sequence elements, which exhibit characteristics similar to polymerase I enhancers found in higher eukaryotes (Yang et al. 1994).

10.2.1.3 Mitochondrial Genome

The mitochondrial genome of *Acanthamoeba* spp. consists of circular molecules and is around 41 kb long and thus in the midrange of sizes for other protozoans (Burger et al. 1995; Fučíková and Lahr 2016). It encodes two rRNAs (small subunit and large subunit), 16 tRNAs and 41 proteins (Burger et al. 1995). The interstrain mtDNA sequence diversity in *Acanthamoeba* seems to be rather high (Byers et al. 1990). The mitochondrial genome encodes fewer than the minimal number of rRNA species required to support mitochondrial protein synthesis, suggesting that additional tRNAs are imported from the cytosol into the mitochondria (Burger et al. 1995). Another prominent feature of the mitochondrial genome of *Acanthamoeba* is the number of overlapping reading frames (Burger et al. 1995). Moreover, *Acanthamoeba*, similar to *Dictyostelium*, carries essential tRNAs in its mitochondrial genome, which is not known from any other organism so far.

Interestingly, the translation system in *A. castellanii* mitochondria does not use the standard genetic code (Gawryluk et al. 2014). The 41 proteins specified by the mitochondrial genome are all encoded on the 'plus' strand and are tightly packed with only 6.8 % of the total DNA sequence not having an evident coding function (Burger et al. 1995). There are only 40 predicted open reading frames (ORF), since subunits 1 and 2 of the cytochrome C oxidase (COX1 and COX2) are specified by a single continuous ORF (Lonergan and Gray 1996). Most proteins have assigned functions associated with respiration and translation. Additionally, there are three intron-encoded ORFs, predicted to specify LAGLIDADG homing endonucleases, and three appear to be unique to *A. castellanii* (Gawryluk et al. 2014). The encoded proteins associated with respiration include NADH dehydrogenase, apocytochrome b, cytochrome oxidase and the ATP synthase complex. Interestingly, compared to other (animal or fungal) mtDNAs, the *A. castellanii* mtDNA encodes extra NADH dehydrogenase and ATP synthase genes. The *A. castellanii* genome encodes an unusually high number of mitochondrion-targeted pentatricopeptide repeat proteins (PPR), organellar RNA metabolism factors, thought to be involved in RNA editing, intron splicing, transcript stabilisation and translational control. Altogether, the mitochondrial proteome of *Acanthamoeba* is highly complex in composition and function, not unlike that of multicellular eukaryotes (Gawryluk et al. 2014).

10.2.2 Lateral Gene Transfer

Lateral gene transfer (LGT) is considered a very important constituent of genome evolution. In *Acanthamoeba,* LGT is suggested to reflect trophic strategies driven by the selective pressure of new ecological niches. As mainly bacteriovorus

organisms, which tend to harbour endosymbionts, acanthamoebae are prone to pick up foreign DNA and hence typical candidates for LGT.

Clarke et al. (2013) performed a phylogenomic analysis determining cases of predicted interdomain LGT in the *Acanthamoeba* genome and identified 450 genes in the genome of *A. castellanii* which may have arisen through LGT. Being a host of various and phylogenetically different endocytobionts, *Acanthamoeba* might as well facilitate genetic exchange between phylogenetically disparate organisms, since miniature transposable elements occurring both, in cyanobacteria and DNA viruses, have been detected.

Acanthamoeba encodes 35 G protein-coupled receptors (GPCRs), sensors for extracellular stimuli that intracellularly activate signal transduction pathways and eventually cellular responses (see Sect. 10.2.4.1). In this context, three fungal-associated glucose-sensing Git3 GPCRs and an expansion in the number of frizzled/smoothened GPCRs have been identified. Moreover, seven G protein alpha subunits and a single putative target, phospholipase C, for GPCR-mediated signalling were detected. The exact function of the GPCRs in *Acanthamoeba* is not yet established; however, a role in detecting molecules secreted by their bacterial food source was suggested and an involvement in the induction of encystment seems possible. Additionally, 48 sensor histidine kinases of which 17 may function as receptors were found, which might also be involved in environmental sensing processes of *Acanthamoeba*. Two rhodopsins with homology to sensory rhodopsins in green algae might represent possible candidates for light sensors in *Acanthamoeba*.

Altogether, 377 protein kinases were identified in the genome of *Acanthamoeba*. Protein kinases are involved in the modulation of cellular responses to environmental stimuli, and this is the largest number of protein kinases predicted to date for any amoebozoan. Moreover, two homologues of mitogen-activated protein kinases have been identified.

Phosphotyrosine (pTyr) signalling mediated through tyrosine kinases for transmitting cellular regulatory information has been associated with intercellular communication and was considered absent from the amoebozoan lineage. pTyr signalling is mediated by a triad of signalling molecules; tyrosine kinase (writers) (PTKs), tyrosine phosphatase (erasers) (PTPs) and Src homology 2 (SH2) domains (readers). In *Acanthamoeba*, 22 PTKS, 12 PTPs and 48 SH2 domain-containing proteins were identified, representing a primitive, but already functional pTyr system (Clarke et al. 2013).

A considerable amount of exogenous DNA might derive from the numerous obligate and facultative endosymbionts or endocytobionts of *Acanthamoeba.* Acanthamoebae are known to (partially permanently) harbour viruses, bacteria and also fungi. Obligate bacterial endosymbionts are found from different lineages within the Proteobacteria, the Bacteroidetes and the Chlamydiae, e.g. *Parachlamydia acanthamoebae* (Horn and Wagner 2004). One of the most prominent viral examples is the Mimivirus, a giant virus with 1,181,404 bp linear dsDNA, more than 1,000 genes, many proteins having tandem IP22 repeats (as in *Dictyostelium*) and interestingly showing genome reduction after intra-amoebal culture (Boyer et al. 2011).

10.2.3 Cellular Architecture

10.2.3.1 Trophozoites

The trophozoites (Fig. 10.2a) are 15–45 μm in size and represent typical amoeboid cells, with a granuloplasma containing the cell organelles and a hyaloplasma producing the (sub)-pseudopodia. When the trophozoites attach to surfaces, they are rounded and flat, but they also have a so-called floating form, which has a more spatial shape with radiate pseudopodia. Acanthamoebae are typical eukaryotes, with a Golgi complex, smooth and rough endoplasmic reticula, free ribosomes, digestive vacuoles (performing pinocytosis or phagocytosis), lysosomes, mitochondria, microtubules and a nucleus with a large central nucleolus.

Generally, acanthamoebae are uni-nucleate; although grown under laboratory conditions, multinucleate cells are common (James and Byers 1967). The nucleus has approximately one-sixth the size of the trophozoite and is enclosed by a nuclear envelope, consisting of two membranes with nuclear pores for flow-through of RNA and proteins (Bowers and Korn 1969). The nuclear chromatin is finely granular, and acanthamoebae are characterised by a large, dense, centrally located nucleolus. Usually two Golgi complexes are found on opposite sides of the nucleus in one plane. Acanthamoebae normally reveal numerous oval- or round-shaped mitochondria with typically tubular cristae and intra-crystal inclusions, and they possess a full complement of 'ER-mitochondria encounter structure' (ERMES) proteins, initially thought to be restricted to fungi and so far only known from a few other amoebozoans and some excavates. The ERMES fixes the ER membrane to the mitochondrial outer membrane and thus allows the integration of mitochondria with non-endosymbiotically derived cell organelles (Wideman et al. 2013). The cytoplasm contains lipid droplets and polysaccharide reserves (Bowers and Korn 1969). *Acanthamoeba* trophozoites are to a large extent (approximately 30 %) composed of carbohydrates, of which most is glucose, most likely stored as glycogen. The main phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphoinositide and diphosphatidylglycerol. Oleic acids and longer polyunsaturated fatty acids are the predominant fatty acids (Ulsamer et al. 1971). Interestingly, only growing trophozoites are covered with a carbohydrate coat, while trophozoites in the stationary phase are not (Schiller et al. 2012). The thickness of the plasma membrane is approximately 100 Å, and it consists of proteins (33 %), phospholipids (25 %), sterols (13 %) and lipophosphonoglycan (29 %) (Dearborn and Korn 1974). Acanthamoebae exhibit microtubule-associated movement of mitochondria and small particles (Baumann and Murphy 1995). Microtubules originate from the Golgi complex and are found throughout the cytoplasm (Bowers and Korn 1969; Preston 1985). Another characteristic of *Acanthamoeba* is the prominent contractile vacuole that functions in osmoregulation of the cell. Water expulsion of the vacuole has been shown to be associated with alkaline phosphatase activity in the membrane (Bowers and Korn 1973), myosin-IC (Baines et al. 1992) and metacaspase activity (Saheb et al. 2013).

Acanthamoeba's movement is sluggish and involves the formation of usually a single pseudopodium (lobopodium) with hyaline sub-pseudopodia (acanthopodia)

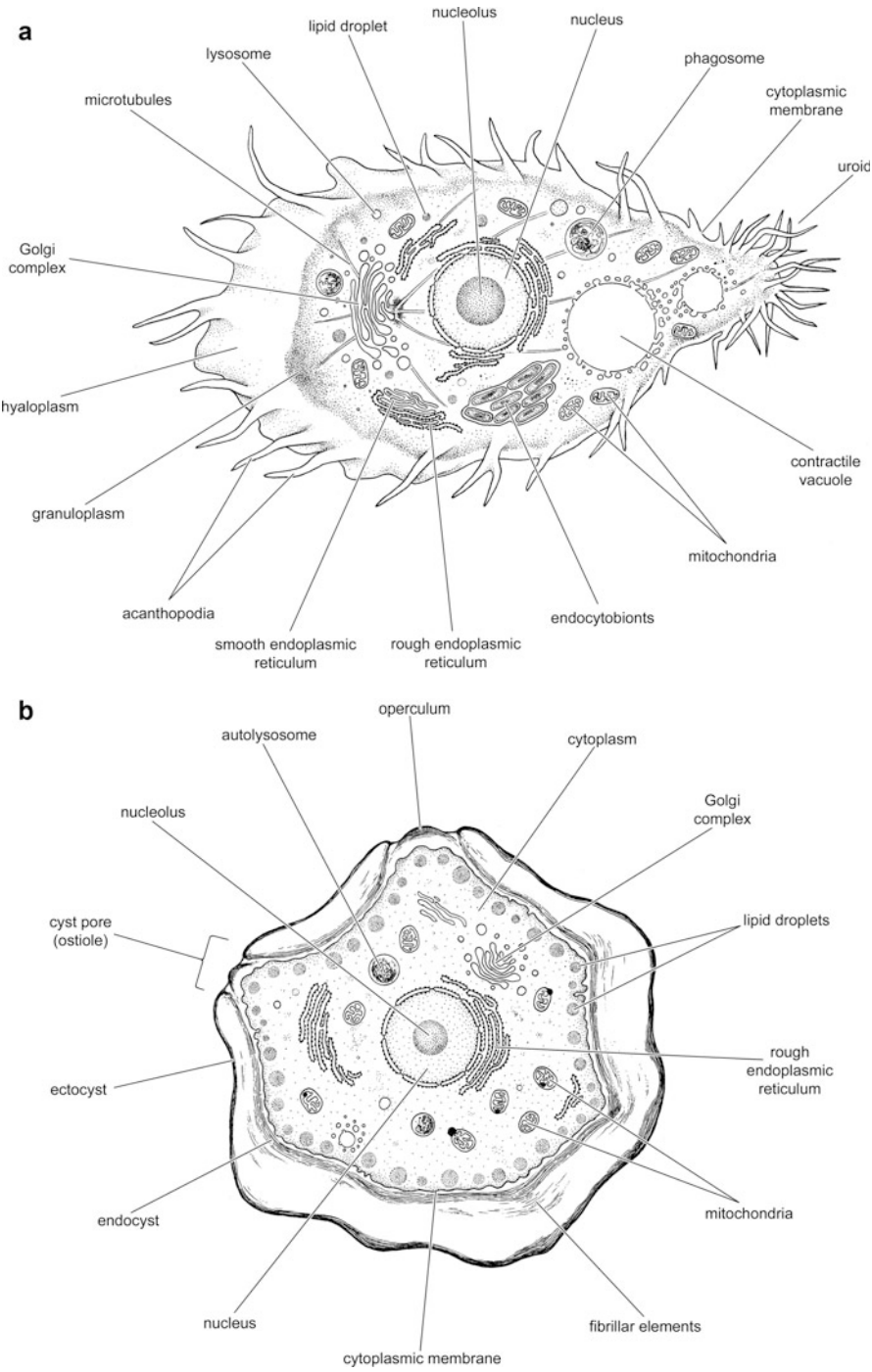

Fig. 10.2 Sketch of the ultrastructure of *Acanthamoeba* trophozoite (**a**) and cyst (**b**). Simplified and not entirely true to scale. Orig

on the entire surface of the cell. Acanthamoebae move with approximately 0.8 µm per second, accomplished by actin polymerisation anteriorly and accordingly a breakdown posteriorly in the advancing cell (Preston and King 1984). Actin, which constitutes about 20 % of the total protein, and myosin, together with more than 20 cytoskeletal proteins, are responsible for movement, intracellular transport and cell division (Gordon et al. 1976). The most important actin-binding proteins in *Acanthamoeba* are two classes of myosin, myosin-I and myosin-II (Pollard and Korn 1973a, b), two actin monomer-binding profilins (Reichstein and Korn 1979), actophorin (Cooper et al. 1986), an actin filament capping protein (Isenberg et al. 1980) and actin filament cross-linking proteins, the so-called gelation proteins (Pollard 1984). A typical feature of the genus are the characteristic spiny surface projections, the so-called acanthopodia, which are built up by actin fibres and networks (González-Robles et al. 2008) and which were shown to be particularly important in the infection process, since they allow the trophozoite to interact with the cell surfaces of the host (Omaña-Molina et al. 2004). Actin fibres and networks are also found in diverse endocytic structures that sometimes extend far away from the cell body (González-Robles et al. 2008).

Ingestion is linked very tightly to locomotion and represents a typical phagocytosis after cell-cell contact and consecutive invagination of the cell membrane. The trophozoite will phagocytose any particle of adequate size that it encounters, including nonorganic particles (Weisman and Korn 1967). Phagocytosed particles reach the phagosome, which subsequently fuses with a lysosome. Excretion is accomplished by the contractile vacuole, which empties and reappears in an actively growing culture within 1–2 min.

Trophozoites multiply rapidly in a favourable environment, growth rate depending on species. Under optimal conditions, they multiply every 6–8 h. Most species prefer temperatures of around 30 °C; however, many isolates can grow also at elevated temperatures, up to even 45 °C. Acanthamoebae divide by conventional mitosis, in which the nucleolus and the nuclear membrane disappear during cell division. For exponentially growing cells, cell division is largely occupied with the G2 phase (up to 90 %) and negligible with the G1 phase, 2–3 % M phase (mitosis) and 2–3 % S phase (synthesis) (Band and Mohrlok 1973; Byers et al. 1990, 1991). Mitosis is characterised by an early breakdown of the nuclear envelope as well as disintegration of the nucleolus and differs from the typical eukaryotic pattern in that the centrioles seem to be anomalous, although spindle fibres do terminate in centriolar equivalents (Bowers and Korn 1968).

10.2.3.2 Cysts

Under adverse environmental conditions, the amoebae form highly resistant cysts, whose morphologies were used for classification before molecular genotyping became standard.

Acanthamoeba cysts (Fig. 10.2b) are 12–32 µm in size. They have a double-layered cyst wall, an outer ectocyst consisting of proteins and polysaccharides (Neff and Neff 1969) and an inner endocyst, mostly composed of cellulose, which is only found in the cysts, not in the trophozoites (Neff and Benton 1962). Interestingly, besides glucose, galactose was found to be a major constituent of *Acanthamoeba* cyst walls (Dudley et al. 2009). The ectocyst and endocyst are separated by an

intercystic space and join only at the ostioles, pores covered by opercula, from which the amoebae emerge in the course of excystation (Chávez-Munguía et al. 2005). The ectocyst usually has an irregular surface, while the endocyst is rather smooth. Moreover, the ectocyst is more than twice as thick as the endocyst (650 nm versus 290 nm) and has numerous vesicles (67–167 nm) particularly towards the outer surface. The space between the two cyst walls has an average thickness of 301 nm and appears to be filled with 11-nm-thick filaments connecting the two walls (Lemgruber et al. 2010). The endocyst is primarily composed of cellulose, and it is assumed that cellulose is secreted through vesicles in the periphery region of the encysted amoeba (Lemgruber et al. 2010).

Acanthamoeba cysts maintain viability in the natural environment for at least 25 years (Mazur et al. 1995).

10.2.4 Life Cycle

10.2.4.1 Encystment

The encystment process of *Acanthamoeba* (Fig. 10.3) has been of specific interest since the pathogenic potential of these organisms became more apparent, not only because cyst formation is a typical reaction to treatment, with cysts being highly resistant against a broad variety of agents, but also due to the fact that pathogenic bacteria can persist in the cysts, well-protected from adverse environmental conditions. Encystment is accompanied by morphological changes, termination of cell growth and biochemical modifications. Initially, the amoebae become rounded, followed by a phase, in which the two cyst walls are synthesised. The first wall that is formed gives rise to the ectocyst. Subsequently, after the appearance of a well-defined layer, the endocyst is synthesised (Weisman 1976). Cyst wall synthesis is usually accompanied by a decrease of intracellular macromolecules such as proteins, glycogen and RNA and, particularly, a decrease in cytoplasmic mass by approximately 80 % through gradual dehydration, thereby causing retraction of the protoplast from the cyst wall (Bowers and Korn 1969).

In order to initiate the complex encystment ‘machinery’, acanthamoebae have to sense and react to adverse environmental conditions. In general, natural triggers for encystment are starvation and osmotic stress, while under laboratory conditions, catecholamines (epinephrine, norepinephrine), magnesium and taurine can efficiently induce encystment (Köhsler et al. 2008; Verma et al. 1974). Since high cell densities correlate with higher encystment rates, also quorum-sensing molecules have been suggested to be involved (Fouque et al. 2012). Similar to quorum-sensing molecules, an encystment-enhancing activity (EEA) secreted by amoebae and able to stimulate encystment in a density-dependent manner was reported by Akins and Byers (1980). The genome of *Acanthamoeba* encodes 35 G protein-coupled receptors (GPCRs), which act as sensors for extracellular stimuli and hence constitute potential candidates for the first step in signal transduction at the beginning of encystment (Clarke et al. 2013). This is consistent with the fact that biogenic amines as ligands for GPCRs sufficiently induce encystment and an involvement of GPCRs, in particular β-adrenergic receptors for catecholamines, in the encystment of *Acanthamoeba* has been demonstrated (Krishna Murti and

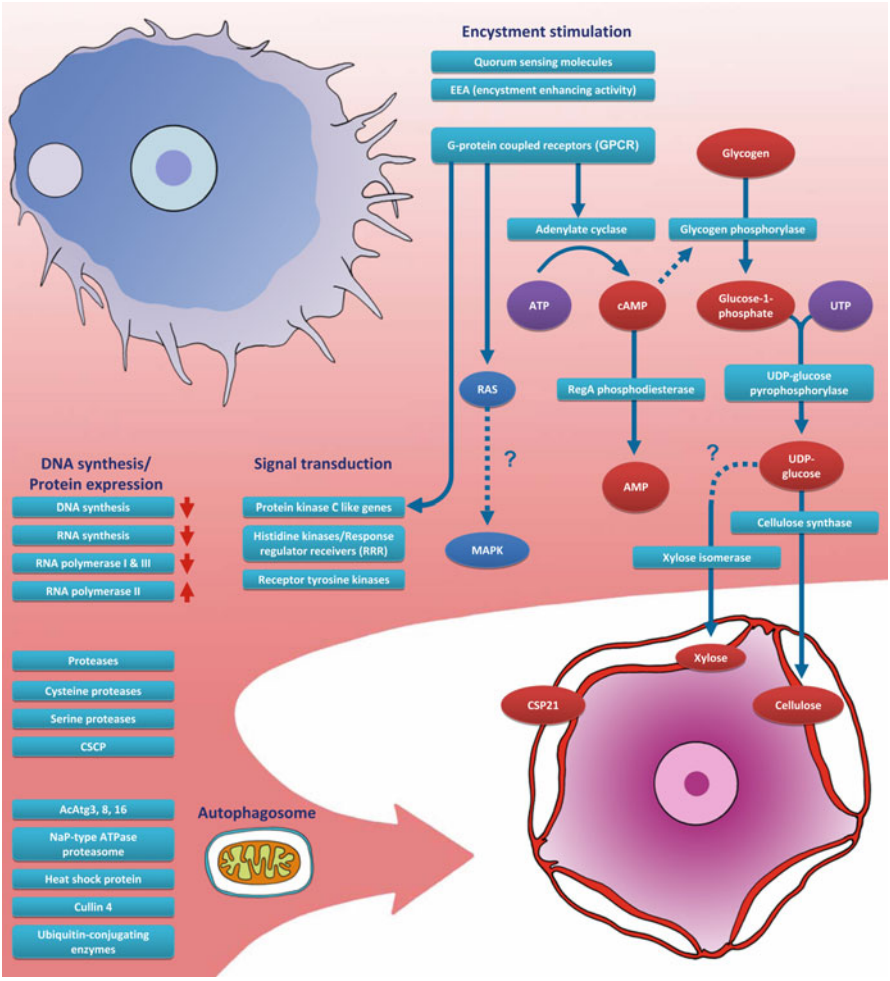

Fig. 10.3 Encystment pathway. Quorum-sensing molecules, EEA, GPCRs, histidine kinases/RRR and receptor tyrosine kinases are suggested to be involved in stimulation and signal transduction of encystment. GPCRs are supposed to activate protein kinase C-like genes, Ras and adenylate cyclase. Adenylate cyclase catalyses the rise of cAMP, leading to activation of glycogen phosphorylase breaking down glycogen for the synthesis of cellulose and xylose. Changes on the DNA/RNA level are indicated by upwards/downwards arrows. Highly expressed proteins involved in the encystment process are shown. *AcAtg Acanthamoeba* autophagy-related protein, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *CSCP* cyst-specific cysteine protease, *CSP21* cyst-specific protein 21, *RRR* response regulator receivers, *UDP* uridine diphosphate, *UTP* uridine triphosphate. Orig

Shukla 1984), and inhibition of β-adrenergic receptors reduces encystment in *Acanthamoeba* (Aqeel et al. 2015). GPCRs are involved in the activation of Ras, a small GTPase, which also has been shown to be involved in encystment, since an inhibitor of farnesylation of Ras leads to decreased encystment. In general, Ras activates the mitogen-activated protein kinase (MAPK) pathway, which, however could not yet be demonstrated during encystment (Dudley et al. 2009). Also activation of adenylate cyclase depends on GPCR-mediated activation of G proteins.

Adenylate cyclase catalyses the conversion from adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Adenylate cyclase activity of *Acanthamoeba* during exponential growth phase is generally low but rises two- to fourfold during the stationary phase to a peak, roughly at the time when cysts are detectable in the cultures (Achar and Weisman 1980). Correspondingly, cAMP levels rise two- to threefold within the first 8 h of encystment (Achar and Weisman 1980). The role of cAMP in encystation is thought to convert glycogen phosphorylase to the active form for glycogen degradation in order to synthesise cellulose for cyst wall formation (Siddiqui et al. 2012). cAMP degradation into AMP on the other hand is catalysed by the enzyme phosphodiesterase, which has been shown to play a crucial role during encystment as well, since inhibition of the phosphodiesterase RegA strongly stimulates encystation and causes a tenfold increase in cellular cAMP levels (Du et al. 2014).

The exact mechanisms for the regulation of signal transduction are far from being fully understood; however, the detection and response to a number of stress conditions are likely to be accomplished with a large set of sensor histidine kinases, with potential receptor function (Clarke et al. 2013). Additionally, response regulator receiver domains usually corresponding to membrane-bound histidine kinases have been detected (Anderson et al. 2005). Also tyrosine kinase-mediated signalling was suggested to be involved in encystment (Dudley et al. 2009) which is corroborated by the detection of potential receptor tyrosine kinases in the genome (Clarke et al. 2013).

Other signalling molecules, which have been suggested to participate in signal transduction pathways during encystation, are protein kinase C-like genes (PKC), which are activated through GPCRs and known to participate in signal transduction pathways that regulate cellular proliferation and differentiation (Hug and Sarre 1993). A great number of PKCs are highly expressed during the early stage of encystment with some of them localised in the cell membrane or nuclear membrane (Moon et al. 2012). The PKCs in *Acanthamoeba* have been identified as atypical PKCs, lacking a Ca^{2+} binding domain and a diacylglycerol (DAG) binding domain, indicating a more primitive composition. Moreover, signalling molecules interacting with PKCs have been detected in expressed sequence tags (ESTs) of *Acanthamoeba* cysts, such as G protein alpha subunit, G protein beta subunit, DAG kinase, tyrosine kinase, tyrosine phosphatase receptor, MAP kinase and calcium binding mitochondrial carrier, corroborating the involvement of PKCs in signal transduction during encystment (Moon et al. 2011).

Glycogen is a storage polysaccharide and is the most rapidly degraded molecule during encystment, since it is used as a precursor of the glucosyl residues of cellulose for cell wall synthesis (Bowers and Korn 1969). Cellular levels of glycogen decrease to between one-half and one-third within the first 4 h of encystment (Weisman et al. 1970). The glycogen breakdown during early encystment has been shown to be at least partly accomplished by the action of glycogen phosphorylase, which catalyses the cleavage of $\alpha(1 \rightarrow 4)$ glycosidic linkages of glycogen, releasing glucose-1-phosphate. The *Acanthamoeba* genome also includes a gene for a predicted glycogen debranching enzyme helping in glycogen breakdown. Interestingly, silencing of glycogen phosphorylase results in incomplete cyst formation, in particular, assembly of the cellulose-containing inner cyst wall could not be completed (Lorenzo-Morales et al. 2008). The expression of glycogen phosphorylase is

restricted to early encystment and appears to be repressed in growing cells. UDP (uridine diphosphate) glucose is synthesised from glucose-1-phosphate and uridine triphosphate catalysed by UDP-glucose pyrophosphorylase. UDP glucose levels increase fivefold within the first 4 h after induction of encystment. Subsequently, UDP glucose is incorporated into the cyst wall as $\beta(1\rightarrow4)$-glycans through the action of cellulose synthase (Aqeel et al. 2013; Moon et al. 2014; Weisman 1976).

Additionally, xylose isomerase appears to be crucial for wall formation (Aqeel et al. 2013). Analysis of cyst walls of *A. castellanii* has revealed that in addition to $\beta(1\rightarrow4)$-glucan-containing cellulose, an important constituent is xylose (Dudley et al. 2009). Obviously, both, cellulose synthase and xylose isomerase, contribute significantly to cyst wall formation (Aqeel et al. 2013; Moon et al. 2014).

Naturally, changes also occur on the DNA and RNA level. Nuclear activity is clearly required during encystment (Roti and Stevens 1975), but normal DNA synthesis is continued only at a significantly reduced rate (Byers et al. 1991). RNA levels decrease by 50 % during encystation and rRNA synthesis is completely shut down after the first 7 h of encystment (Weisman 1976). This is due to a covalent modification of RNA polymerase I, which prevents contact with the DNA-bound transcription factor TF-IB, and a loss of transcription factor TF-IIIA (Matthews et al. 1995). Activities of RNA polymerases I and III decrease during early encystment, while transcription by RNA polymerase II increases significantly (Orfeo and Bateman 1998). It has been shown that the transcription rates of many protein-coding genes are not substantially reduced during the first 16 h of encystment and mRNA pools persist even into the mature cyst stage. However, the increase of polymerase II indicates that additional genes, which might be actively repressed in trophozoites, are transcribed during encystment. Chromatin remodelling was suggested as a mechanism for repression and has been demonstrated to be involved in encystment to a certain extent (Köhsler et al. 2009). Hirukawa et al. (1998) reported a protein expressed exclusively in cysts, the cyst-specific protein CSP21, which is produced during early stages of encystment and associated with the cyst walls. This protein has been shown to be actively repressed in trophozoites; however, since chromatin remodelling was ruled out as repressing mechanism for CSP21, the exact mode of action remains to be established (Chen et al. 2004).

Cytoskeleton rearrangements are crucial for encystment. Rho kinase, which is involved in regulation of actin polymerisation, and the gelation factor, involved in actin cross-linking, have both been demonstrated to play an important role in the course of differentiation (Bouyer et al. 2009; Dudley et al. 2009).

Likewise, several proteases play an important role during the encystment process. A subtilisin-like serine protease, demonstrated in encysting cells, was suggested to be involved in promoting autolysis, since it associates with autophagosomes (Moon et al. 2008). Leitsch et al. (2010) demonstrated the involvement of cysteine proteases and suggested a promoting role for serine proteases, which could serve as mediators for the release of cysteine proteases by promoting the maturation of autophagosomes. Additionally, a specific role for cyst-specific cysteine proteases (CSCPs) in mitochondrial autolysis has been demonstrated. Since cysts require significantly fewer mitochondria, large numbers are degraded in the course of encystment mediated by CSCPs, probably providing macromolecules for building up the cyst walls (Moon et al. 2012).

Other factors related to differentiation of *Acanthamoeba* are P-type ATPases, proteasome and heat shock proteins, cullin 4, and ubiquitin-conjugating enzymes (Moon et al. 2008). Additionally, specific *Acanthamoeba* autophagy-related proteins (AcAtg) which are essential components of the autophagic machinery during encystment have been detected. AcAtg8 is involved in the formation of the autophagosomal membrane, while AcAtg3 plays an important role in AcAtg8 lipidation (Moon et al. 2009). AcAtg16 was found to be associated with small or large vesicular structures that partially co-localise with autophagolysosomes (Song et al. 2012).

Although encystment has been a focus of research in *Acanthamoeba* and many factors contributing to this complex process have been established lately, the interplay of all molecules involved is still far from being fully understood and clearly an important subject for future studies since the encystment process constitutes a promising target for specific therapeutic measures in *Acanthamoeba* infections.

10.2.4.2 Excystment

For the excystment process of *Acanthamoeba*, only very limited information is available. Excystment occurs under favourable conditions through the ostioles after removal of the operculum, leaving behind the outer shell. While the actual hatching from the cysts only lasts a couple of minutes, the entire excystment process, excystment takes around 12 h (Mattar and Byers 1971). The evidence of genes in the *Acanthamoeba* genome encoding cellulase and cellobiosidase corroborates the hypothesis that the amoebae break down cellulose from their own cyst walls and utilise it as a food source in the course of excystment (Anderson et al. 2005). This may, however, depend on general food supply, as under lab conditions with an oversupply of nutrients, intact empty cyst walls can be observed frequently.

10.2.5 Metabolism

Acanthamoeba has a heterotrophic metabolism and generally requires organic substrates for its growth and development. However, the metabolic and biosynthetic capabilities of acanthamoebae are very broad and they can indeed thrive on almost anything.

Generally, acanthamoebae produce energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation in the mitochondria with a functional tricarboxylic acid (TCA) cycle and electron transport chain (ETC) similar to those found in other aerobic eukaryotes (Fig. 10.4). In short, under aerobic conditions, pyruvate is decarboxylated via the pyruvate dehydrogenase (PDH) multi-enzyme complex and passed as acetyl-CoA into the TCA cycle, where reducing equivalents in the form of NADH (nicotinamide adenine dinucleotide) are generated and subsequently oxidised by the electron transport chain, resulting in an electrochemical gradient, which is utilised by the ATP synthase for the synthesis of ATP. In *A. castellanii*, the presence of all components of PDH, all subunits associated with the enzymatic activities of the TCA cycle and an ETC comprising all five complexes for oxidative phosphorylation has been confirmed (Gawryluk et al. 2012, 2014).

In addition, also a functional glyoxylate cycle, an anabolic variant of the TCA cycle for the synthesis of carbohydrates from acetyl-CoA by β-oxidation of fatty

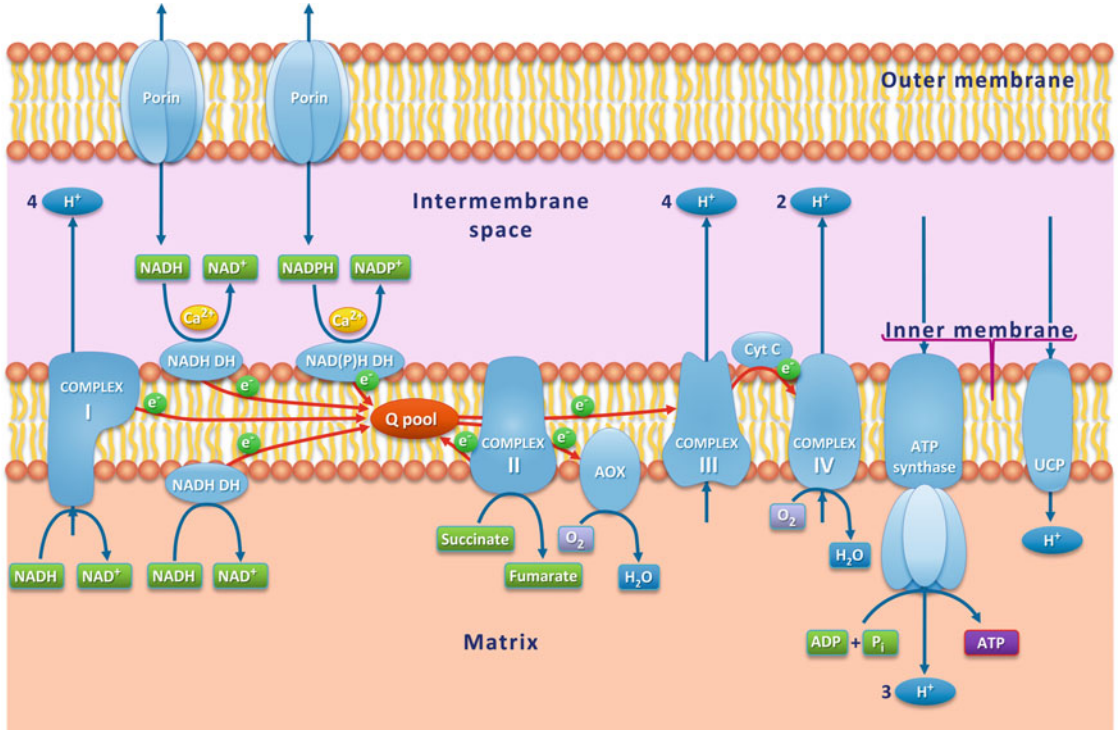

Fig. 10.4 ETC and ATP synthesis of *Acanthamoeba*. *Acanthamoeba* possess a plant-type respiratory chain. In addition to the four standard protein complexes (I–IV), the ETC contains additional enzymes for electron transport – an internal and external NADH DH and an external NAD(P)H DH. Alternative NADH DHs and NAD(P)H DH and complex I and II reduce ubiquinone (Q). AOX can bypass complexes III and IV and act as terminal oxidase, while UCP can bypass ATP synthase, both resulting in a lower ATP yield. *AOX* alternative oxidase, *DH* dehydrogenase, *NADH* nicotinamide adenine dinucleotide, *NADPH* nicotinamide adenine dinucleotide phosphate, *Q* ubiquinone (coenzyme Q), *UCP* uncoupling protein. Orig

acids has been demonstrated. The two key enzymes, isocitrate lyase (ICL) and malate synthase (MalS), have been detected and suggested to be fused in the mature protein (Gawryluk et al. 2012; Tomlinson 1967). ICL and MalS have also been suggested to be involved in the encystment process by providing glucose from lipid sources (Mehdi and Garg 1987). Interestingly, while in other organisms the glyoxylate cycle takes place in specialised peroxisomes, the glyoxysomes, in *Acanthamoeba* the site of the glyoxylate cycle appears to be the mitochondria. The presence of MalS and ICL as well as other glyoxylate cycle enzymes, such as citrate synthase, aconitate hydratase and malate dehydrogenase, has been demonstrated in the mitochondria (Gawryluk et al. 2012, 2014).

The mitochondria of *A. castellanii* possess a plant-type respiratory chain, which is highly branched and has additional electron transport complexes, bypassing the conventional complexes of oxidative phosphorylation, thereby dissipating redox energy instead of building up an electrochemical proton gradient. Complex I, the rotenone-sensitive NADH:ubiquinone oxidoreductase, situated in the inner mitochondrial membrane, can be bypassed by alternative NADH and NAD(P)H dehydrogenases (DH), namely, a rotenone-insensitive internal NADH DH (Hryniewiecka 1986), a rotenone-insensitive external NADH DH, located on the outer surface of the inner mitochondrial membrane (Hryniewiecka et al. 1980; Lloyd and Griffiths 1968), and a rotenone-insensitive, calcium-dependent external NAD(P)H DH (Antos-Krzeminska and Jarmuszkiewicz 2014). These alternative NADH and NAD(P)H DHs transport electrons but do not contribute to proton pumping. By oxidising cytosolic and matrix NADH and NAD(P)H, alternative NADH and NAD(P)H DHs together with complex I and II reduce a common ubiquinone pool. The exact physiological role of these alternative enzymes in *Acanthamoeba* has not been established yet; however, it has been suggested that these enzymes might enable the cell to adapt to changes in the surrounding environment more rapidly and make the mitochondrial respiratory chain more flexible by regulating the redox state of cytoplasmic and mitochondrial matrix NAD(P)H pools (Antos-Krzeminska and Jarmuszkiewicz 2014; Rasmusson et al. 2004). Possible functions were also assumed to be thermogenesis, oxidation of excess carbohydrates, oxidation of excess reductant for continuation of metabolic pathways, cold resistance and avoidance of reactive oxygen species (ROS) formation (Rasmusson et al. 2004).

In addition to alternative DHs, a cyanide-insensitive alternative oxidase (AOX) constitutes a bypass for complexes III and IV (Edwards and Lloyd 1978), and an alternative proton transporter, the uncoupling protein (UCP), allows proton flow from the intermembrane space to the matrix without ATP synthesis, thereby bypassing ATP synthase (Jarmuszkiewicz et al. 1999). Both systems lead to a decrease in ATP synthesis yield and are supposed to control the cellular energy balance as well as prevent the cell against the production of ROS.

An alternative respiratory pathway in *Acanthamoeba* spp. was first reported in 1978 (Edwards and Lloyd 1978), and AOX was first described in 1997 (Jarmuszkiewicz et al. 1997). In general, the AOX pathway branches from the main respiratory chain after ubiquinone, where AOX can potentially act as terminal oxidase. However, in response to low temperatures, it has been shown that AOX is upregulated without disturbing the conventional cytochrome pathway of complexes

III and IV (Jarmuszkiewicz et al. 2001). AOX was shown to be stimulated by purine nucleoside 5′-monophosphates, with guanosine monophosphate (GMP) being the most efficient. In addition to the binding of GMP, its activity is determined by the redox state of ubiquinone (coenzyme Q) as well as the matrix pH (Jarmuszkiewicz et al. 2002a, b). A possible function of AOX in *Acanthamoeba* is thought to be an alternative route for respiration during periods of oxidative stress (Czarna and Jarmuszkiewicz 2005; Jarmuszkiewicz et al. 2001).

Also, the *Acanthamoeba* UCPs are well studied and were first demonstrated in 1999 (Jarmuszkiewicz et al. 1999). UCPs are members of the mitochondrial anion carrier protein (MACP) family and are present in the mitochondrial inner membrane, where they mediate the free fatty acid (FFA)-activated, PN (purine nucleotide)-inhibited H+ leak that can divert energy from oxidative phosphorylation, thereby modulating the coupling of mitochondrial respiration and ATP synthesis (Jarmuszkiewicz et al. 2005). Additionally, UCP is activated by 4-hydroxy-2-nonenal (HNE), a reactive unsaturated aldehyde, which was shown to induce PN-sensitive uncoupling in *A. castellanii* (Woyda-Ploszczyca and Jarmuszkiewicz 2012). HNE is the most abundant end product of membrane lipid peroxidation, for which increased ROS production is a prerequisite. It has been shown that the redox state of membranous coenzyme Q is particularly important for the inhibition by PNs of FFA- and HNE-activated UCP in phosphorylating and non-phosphorylating mitochondria of *A. castellanii*, with a high coenzyme Q reduction level being essential for UCPs' activity, while the inhibitory effect of PNs can only be observed when coenzyme Q is sufficiently oxidised (Jarmuszkiewicz et al. 2005; Swida et al. 2008; Woyda-Ploszczyca and Jarmuszkiewicz 2013).

Through its redox state, coenzyme Q appears to be an ideal regulator for both AOX and UCP activity since it is able to sense the ATP demand of the cell. At a high ATP demand and low substrate availability, a low reduction level of coenzyme Q leads to inactivation of UCP and AOX and efficient ATP synthesis, while at low ATP demand and high substrate availability through a high reduction state of coenzyme Q, UCP and AOX are activated and might work as a safety valve to avoid overload in reducing power and ROS formation (Jarmuszkiewicz et al. 2010). In contrast to other organisms, where UCP and AOX do not occur simultaneously or are co-regulated by FFAs with an inhibitory effect on AOX (plants, fungi), in *Acanthamoeba* UCP and AOX appear to work in concert with a cumulative effect on the efficiency of oxidative phosphorylation. In particular, the protection against mitochondrial ROS production appears to be the physiological role of UCP and AOX, which might act as antioxidant systems to prevent damage to the cell at the level of energy production, but at the expense of oxidative phosphorylation (Czarna and Jarmuszkiewicz 2005; Czarna et al. 2007).

In addition to conventional aerobic respiration the presence of a complete hydrogenosome-like ATP generation pathway with a complete set of enzymes has been detected, probably functioning under anaerobic conditions (Leger et al. 2013). This anaerobic ATP generation pathway is similar to that found in *T. vaginalis* hydrogenosomes and includes the following enzymes: pyruvate ferredoxin

oxidoreductase (PFO); FeFe-hydrogenase; maturases HydE, HydG and HydF; and succinate CoA transferase. Interestingly, this hydrogenase exists in the presence of aerobic mitochondria and might thus, in contrast to the ones found, e.g. in *Trichomonas* or *Entamoeba*, constitute an oxygen-resistant hydrogenase, meaning that it rather stably could produce hydrogen even in the presence of air.

10.2.6 Pathogenesis

Different *Acanthamoeba* strains do not seem to be equally pathogenic, but it has not yet been really clarified, whether pathogenicity is a distinct character of specific strains or whether all strains are potentially pathogenic. Pathogenicity of *Acanthamoeba* isolates is usually assessed by animal inoculation, either into the eye (pig or hamster) or intranasally into the brain (mouse), or by evaluating cytopathic effects on various cell lines. Also, virulence usually correlates to high growth rate and temperature tolerance (Griffin 1972; Walochnik et al. 2000), but all these characters have shown to be rather epigenetically regulated than genetically defined (Köhsler et al. 2009; Pumidonming et al. 2010).

Generally, pathogenesis in *Acanthamoeba* is characterised by adhesion to the host cells, contact-mediated cytolysis and immunoreaction of the host. A scheme of the current understanding of this process is given in Fig. 10.5. Most studies on *Acanthamoeba* pathogenesis have focused on *Acanthamoeba* keratitis; however, the general steps can be assumed to be comparable in *Acanthamoeba* GAE. In AK, the amoebae particularly accumulate around the corneal nerves, which leads to radial neuritis and is the reason for the violent pain.

10.2.6.1 Adhesion

The acanthopodia allow the trophozoite to interact with other cells, the adhesins in the membrane anchoring to the cytoskeleton network (González-Robles et al. 2008). *Acanthamoeba* has a complex repertoire of surface proteins and glycoproteins that can potentially act as receptor adhesins to the host cells.

In *Acanthamoeba* keratitis, the first critical step in the pathogenesis is the adhesion of the amoebae to the surface of the cornea, which has been shown to be primarily mediated by a lectin, the mannose-binding protein (MBP), recognising mannosylated glycoproteins in the membrane of other cells and adhering to them (Garate et al. 2004, 2005; Yang et al. 1997). This *Acanthamoeba* lectin binds methyl-α-D-mannopyranoside and α – 1-3-D-mannobiose with highest affinity, and this adhesion is necessary for amoeba-induced cytolysis of target cells (Cao et al. 1998). The mannosylated glycoproteins on the cornea have been shown to be increasingly expressed in the course of corneal abrasion, a leading risk factor for the development of AK (Jaison et al. 1998). MPB is a 400 kDa protein that is composed of multiple 130 kDa subunits. It consists of a large N-terminal extracellular domain, a transmembrane domain and a short C-terminal cytoplasmic domain and has characteristics of a typical cell surface receptor (Garate et al. 2004). The cytoplasmic domain contains a number of phosphorylation sites and

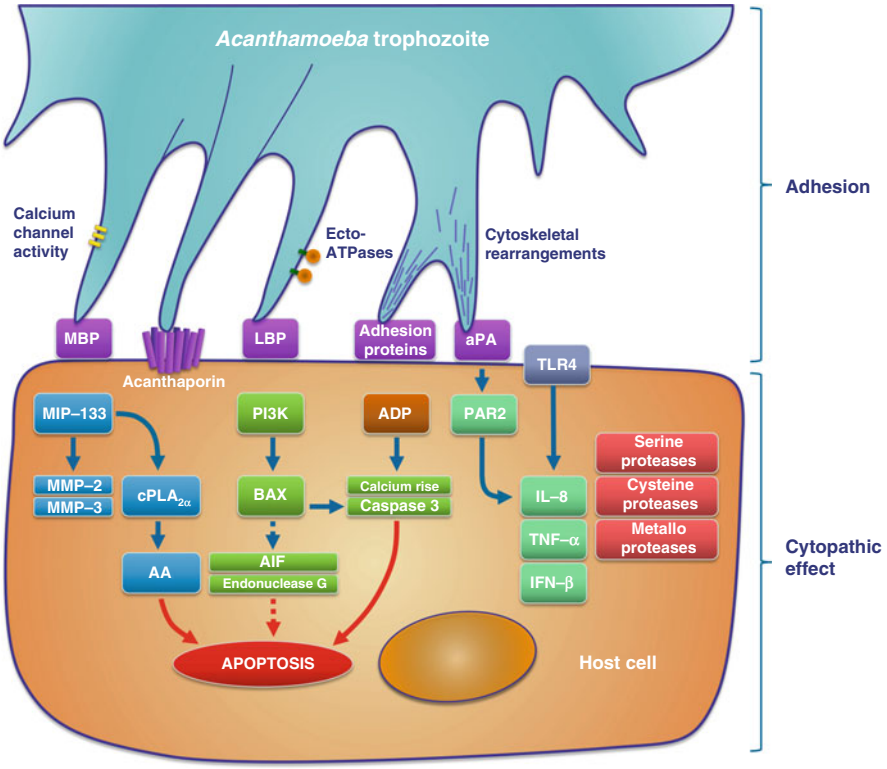

Fig. 10.5 Pathogenesis of *Acanthamoeba*. Adhesion to host cells is accompanied by calcium channel activity and cytoskeletal rearrangements and accomplished by MBP, LBP, ecto-ATPases and other adhesion proteins. Additionally, host cells can be permeabilised by the pore-forming protein acanthaporin. The secretion of MIP-133 leads to activation of MMPs and cPLA2α, the latter leading to the release of AA stimulating apoptosis. Alternatively, apoptosis might be induced by PI3K signalling pathways and ADP release. Host cell receptors involved are PAR2, activated by aPA resulting in IL-8 production and TLR4, inducing the secretion of IL-8 and TNF-α or IFN-β. Additionally, several proteases contribute to the cytopathic effect of *Acanthamoeba*. *AA* arachidonic acid, *ADP* adenosine diphosphate, *AIF* apoptosis-inducing factor, *aPA Acanthamoeba* plasminogen activator, *cPLA* cytosolic phospholipase A, *IFN* interferon, *IL* interleukin, *LBP* laminin-binding protein, *MBP* mannose-binding protein, *MMP* matrix metalloprotease, *PAR* protease-activated receptor, *PI3K* phosphatidylinositol 3-kinase, *TLR* toll-like receptor, *TNF* tumour necrosis factor. Orig

an NPLF motif, known for its ability to participate in cell signalling events leading to cell spreading and shape change. Size, number and location of the carbohydrate recognition domains (CRDs) of the MBP of *Acanthamoeba* remain to be determined; however, it appears that MBP contains at least one novel CRD, which lacks sequence similarity to well-characterised lectin CRDs (Panjwani 2010). For initiation of AK, amoebae adhere to the surface of the cornea via the CRD of the MBP, which leads to signal transduction events via the cytoplasmic domain, eventually resulting in the expression of components inducing the cytopathic effect (CPE).

However, since the adherence to host cells is a complex process, numerous other molecules have been suggested to be involved as well. For example, a 207 kDa adhesion molecule that does not bind to mannose has been detected by monoclonal antibodies blocking the adhesion of trophozoites to human corneal epithelial cells (HCEs) (Kennett et al. 1999). Furthermore, 16 potentially involved surface proteins, eight mannose glycoproteins and eight glycoproteins with N-acetyl glucosamine residues, were detected in *Acanthamoeba* (Soto-Arredondo et al. 2014). Since HCEs express proteins with Glc*N*Ac and Man residues on their surface (Panjwani et al. 1995), these proteins might represent potential receptor adhesins, corroborated by the fact that most of these proteins have been demonstrated to interact with neuronal and epithelial cells (Soto-Arredondo et al. 2014). Interestingly, *Acanthamoeba* seems to possess all ER glycosyltransferases involved in N-glycosylation as well as unusual fucosyl- and pentosyltransferases in the Golgi (Schiller et al. 2012).

Additionally, the interplay of amoebae and components of the host extracellular matrix (ECM) may be essential for adhesion and subsequent invasion. It has been reported that *A. polyphaga* binds to three different elements of the ECM: the basement membrane components laminin and collagen IV and the adhesive glycoprotein fibronectin (Gordon et al. 1993). In that context, a 28.2 kDa laminin-binding protein (LBP), which shows a definite binding specificity to mammalian laminin and higher expression levels in pathogenic strains (Hong et al. 2004), and a 54 kDa LBP protein, in that study only expressed in a pathogenic *A. culbertsoni* strain, have been described (Rocha-Azevedo et al. 2010). However, the actual involvement of other proteins than the MBP in the course of infection remains to be determined.

In addition to binding molecules, an important prerequisite for the establishment of a CPE are calcium channel activity and cytoskeletal rearrangements involving Rho-associated pathways (Taylor et al. 1995). Cytoskeletal participation is not only obvious due to formation of membrane structures like acanthopodia but also based on the fact that adhesins are strongly anchored to the cytoskeleton and disintegration of cytoskeletal elements leads to physiological changes for adhesins (Soto-Arredondo et al. 2014).

10.2.6.2 Cytopathic Effect

The next step in an AK is the release of a 133-kDa serine protease termed mannose-induced protein (MIP) 133, which is produced upon exposure to mannose and interacts with mannose receptors on the cell membrane of *Acanthamoeba* (Leher et al. 1998a). The expression of MIP-133 and the ability to cause disease have been shown to be directly related, since MIP-133 is expressed in clinical but not in soil isolates (Hurt et al. 2003a). The generation of MIP-133 seems to be a crucial component of the pathogenic cascade of *Acanthamoeba* keratitis, and several mechanisms have been described how the interaction of MIP-133 and host cells leads to cytolysis and desquamation. MIP-133 activates matrix metalloproteases (MMP), which have been shown to be expressed by corneal cells in response to pathogenic microorganisms (Fini et al. 1992). They are secreted as inactive enzymes and activated extracellularly via pathway-dependent proteases and participate in tissue degradation and remodelling under physiological and pathological conditions. In

HCEs the interaction with MIP-133 has been shown to lead to a two- to fourfold increase of the expression of MMP-2 and MMP-3, potentially facilitating the invasion of trophozoites (Alizadeh et al. 2008). Furthermore, MIP-133 can degrade human collagen types I and IV and is able to induce apoptosis via a caspase-3-dependent pathway (Hurt et al. 2003a). The mechanism to induce apoptosis through MIP-133 is supposed to be an interaction with phospholipids on the plasma membrane of corneal cells in order to activate cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) (Tripathi et al. 2012). cPLAs are involved in cell signalling processes, such as inflammatory responses, and lead to the release of arachidonic acid (AA), which stimulates apoptosis through activation of the mitochondrial pathway and is associated with loss of cell viability, caspase activation and DNA fragmentation (Taketo and Sonoshita 2002). $cPLA_{2\alpha}$ is regulated via phosphorylation by MAPKs at amino acid residue serine-505 or by Ca^{2+} to become its soluble form (p-$cPLA_{2\alpha}$), which translocates to the plasma membrane (Leslie 1997). In *Acanthamoeba* it still has to be determined, whether phosphorylation by MAPKs or interaction of MIP-133 with phosphatidylserine, a phospholipid exposed on the outer cell membrane of HCEs during apoptosis, induce $cPLA_{2\alpha}$. Furthermore, $cPLA_{2\alpha}$ appears to be involved in the production of chemokine (C-X-C motif) ligand 2 (CXCL2), which has a chemotactic effect on neutrophils (Tripathi et al. 2013). Collectively, activation of $cPLA_{2\alpha}$ induced by MIP-133 was shown to initiate a rapid immune response in corneal epithelial cells by inducing cytolysis and stimulating the accumulation of polymorphonuclear leukocytes (PMNs), accompanied by the production of IL-8, IL-6, IFN-γ and IL-1β (Tripathi et al. 2012). However, in human brain microvascular endothelial cells (HBMEC) activation of phosphatidylinositol 3-kinase (PI3K) was shown to be a prerequisite for apoptosis, since direct inhibition of PI3K significantly reduced *Acanthamoeba*-mediated HBMEC death (Sissons et al. 2005). In myeloma cells, it was demonstrated that downstream effectors of PI3K-mediated apoptosis involve activation of the pro-apoptotic proteins Bak and Bax (Thyrell et al. 2004).

Generally, activation of Bax (and other pro-apoptotic proteins) results in increased permeability of the outer mitochondrial membrane, which gives rise to the release of cytochrome c and other pro-apoptotic factors, leading to activation of caspases. In fact, in neuroblastoma cells, evidence for an involvement of the protein Bax in apoptosis via the mitochondrial pathway induced by *Acanthamoeba* has been demonstrated (Chusattayanond et al. 2010). In addition to the caspase-dependent pathway, also a caspase-independent pathway via upregulation of Bax was proposed, possibly involving apoptosis-inducing factor (AIF) and endonuclease G, which are released upon membrane permeabilisation induced by Bax activity and lead to apoptosis in a caspase-independent manner. They translocate to the nucleus and induce chromatin condensation and/or large-scale DNA fragmentation (Bröker et al. 2005).

An alternative way on how acanthamoebae accomplish to induce host cell death, which must be distinguished from protein-induced apoptosis and may reflect an additional strategy to overcome the host defence, is by release of adenosine diphosphate (ADP), which in vitro affects human epithelial cells by a

process that begins with a rise of cytosolic free-calcium concentrations and culminates in apoptosis (Mattana et al. 2001). In human monocytes, the effect of ADP is mediated by specific $P2y_2$ purinergic receptors (purinoceptors) expressed on the monocyte cell membrane and leads to a substantial rise of calcium and caspase-3 activation. Furthermore, ADP together with other excreted not yet identified soluble compounds has been shown not only to induce apoptosis but also to stimulate the secretion of pro-inflammatory cytokines (TNF-α and IL-1β) and IL-6 in human monocytes (Mattana et al. 2001, 2002). In this context, a potential role of ecto-ATPases in *Acanthamoeba* pathogenesis was suggested as well, since pathogenic and non-pathogenic *Acanthamoeba* strains exhibit distinct ecto-ATPases. In pathogenic strains, inhibition of these leads to decreased host cell cytotoxicity. Ecto-ATPases are glycoproteins in the plasma membrane with their active sites facing outward and hydrolysing extracellular ATP to ADP and phosphate. Since ecto-ATPases are supposed to be involved in cellular adhesion, also an association of ecto-ATPases and the MBP has been suggested, since mannose increases ecto-ATPase activities in pathogenic strains (Sissons et al. 2004).

Another important enzyme involved in the pathogenesis of AK is a 40 kDa *Acanthamoeba* plasminogen activator (aPA), a serine protease expressed from pathogenic, but not from non-pathogenic strains, activating plasminogen from several mammalian species including humans (Mitra et al. 1995). It has been demonstrated that the pathogenic potential of *Acanthamoeba* species closely correlates with aPA secretion, which facilitates the penetration of trophozoites through the basement membrane (Alizadeh et al. 2007). Four protease-activated receptors (PARs) have been identified so far (PAR1-4). Among these, functional PAR1 and PAR2 were shown to be expressed by human corneal epithelial cells (Lang et al. 2003). PARs are cleaved by proteases at an activation site, which eventually leads to activation and signalling. For *Acanthamoeba*, a specific activation of the PAR2 pathway by aPA has been demonstrated, resulting in the expression and production of IL-8 (Tripathi et al. 2014).

Also suggested to participate in the pathogenic cascade of *Acanthamoeba* infections is a newly identified pore-forming protein of pathogenic acanthamoebae, the so-called acanthaporin (Michalek et al. 2013). Pore-forming proteins are well-known virulence factors and are considered essential for host tissue destruction. In other pathogenic 'amoebae', including *Entamoeba histolytica* (amoebapore A) and *Naegleria fowleri* (naegleriapores), the existence and function of amoebapores have been described years ago (Leippe et al. 1994; Young and Lowrey 1989). Structurally, these proteins are members of the saposin-like protein (SAPLIP) family (Kolter et al. 2005). Interestingly, acanthaporin does not resemble previously described SAPLIP proteins, but its structure represents an entirely new protein fold, with which membranes can be permeabilised. Acanthaporins seem to be amphipathic; thus, a switch from the soluble form to a membrane-inserted state might be facilitated. Additionally, a very distinct activation mechanism for pore formation has been proposed. While in amoebapore A, at low pH, a protonated single histidine residue triggers the formation of an active dimer from inactive monomers to form a

hexameric pore (Leippe et al. 2005), acanthaporin becomes activated after protonation of histidine residues within the C-terminal helix by the transition from an inactive dimer to the active monomer resulting in the formation of oligomeric pores. Acanthaporin has been shown to be cytotoxic for human neuronal cells and a variety of bacterial strains by permeabilising their membranes, and its proposed primary function could be the combat against the growth of engulfed bacteria inside phagosomes. However, based on the contact-dependent cytotoxic reactions observed upon interaction of acanthaporin and HCEs, acanthaporin might as well take an effect extracellularly and contribute to the pathogenic cascade ultimately resulting in host cell death.

Several proteases have been described in *Acanthamoeba* that might play a role in phagocytosis, but might as well contribute to the penetration and dissolution of host tissue since proteolytic activities have been demonstrated for most of them. Some of these proteases seem to be specifically excreted by clinical isolates, indicating their potential as virulence factors. Upon contact to host cells, a 97 kDa serine protease is markedly upregulated as well as a cytotoxic 80 kDa metalloprotease, which, however, depends on mannose-mediated adhesion (Cao et al. 1998). A 65 kDa cysteine protease only expressed in pathogenic strains has also been suggested to be involved in pathogenicity, as well as a 150 kDa metalloprotease from an *Acanthamoeba* strain isolated from a GAE patient (Alsam et al. 2005; Hadas and Mazur 1993).

10.2.6.3 Immunobiology

Disseminating *Acanthamoeba* infections are clearly linked to immunodeficiency. And despite the ubiquitous nature of the acanthamoebae and many million contact lens wearers worldwide, also AK is very rare, suggesting that besides an impaired corneal surface, also factors of the host's immune system might influence the incidence and severity of this infection (Niederkorn 2002). Acanthamoebae generally are a source of constant antigenic stimulation. It has been shown in numerous studies that the vast majority of humans, including AK patients, have specific antibodies against these amoebae.

The most abundant immunoglobulin in mammalian tears is IgA, and IgA provides an immunological barrier for microorganisms, blocking their adherence to epithelial cells. AK patients seem to have lowered IgA levels, and this might constitute a supplementary risk factor for generating AK. If trophozoites achieve to bind to epithelial cells, other IgA-dependent elements of the immune system may be activated, including the complement system or inflammatory cells. The innate immune system, particularly macrophages, neutrophils and the complement system, is assumed to play an important role in the resistance against infections with *Acanthamoeba*. The presence of IgA together with the activation of the alternative pathway might serve as a first-line defence (Cursons et al. 1980; Niederkorn et al. 2002; Walochnik et al. 2001).

Macrophages may provide protection against AK, particularly in the very early stage of the infection. Macrophages are chemotactically attracted to *Acanthamoeba* trophozoites and have been shown to kill them in vitro, which is

even enhanced in the presence of specific antibodies (Marciano-Cabral and Toney 1998). Inflammatory infiltrates in *Acanthamoeba* infections are mainly composed of neutrophils, indicating that also neutrophils play an important role in the immunity to acanthamoebae. Also, neutrophils are more efficient than macrophages in killing *Acanthamoeba* cysts (Hurt et al. 2003b). Further, although the main localisations of *Acanthamoeba* in the human body, the eye and the brain, are immune-privileged sites, also complement components are continuously active at low levels at these sites (Bora et al. 2008; Woodruff et al. 2010). Thus, strains causing infections have to circumvent the action of activated complement proteins to invade and multiply. In vitro, acanthamoebae are killed by a complement-dependent process activated via the alternative pathway (Pumidonming et al. 2011). A concerted action of C5, C6, C7, C8 and C9 leads to membrane damage and in the following to lysis of the amoeba cell (Ferrante and Rowan-Kelly 1983). This is consistent with findings that membranes of acanthamoebae lack sialic acid (Ulsamer et al. 1971). Sialic acid on plasma membranes prevents the activation of the alternative pathway; thus, organisms with sialic acid are spared with this natural host defence mechanism. The uncontrolled activation of complement is also at least partially responsible for oedema and damage to blood vessel walls that are characteristic in *Acanthamoeba* infections.

Specific antibodies increase the amoebolytic capability of the immune system, inhibit host cell adherence and phagocytosis and seem to block the action of amoebal enzymes, thus neutralising their cytopathogenic effects (Ferrante and Abell 1986; Marciano-Cabral and Toney 1998; Stewart et al. 1994). They are most likely even transferred placentally as anti-*Acanthamoeba* antibodies can also be found in human cord blood (Cursons et al. 1980). However, immunological memory is not established in AK. In GAE, generally, immunoglobulin levels below the normal range are typical (Im and Kim 1998).

Host cell receptors that have been shown to be involved in *Acanthamoeba* recognition are Toll-like receptors (TLR), in particular TLR4 (Ren et al. 2010). TLRs recognise specific pathogen-associated molecular patterns leading to the activation of an inflammatory signalling cascade producing pro-inflammatory cytokines and chemokines. TLR4 is a unique member of the TLR family in that it signals through two distinct signalling pathways, the MyD88-dependent and TRIF-dependent pathway (the TIR domain-containing adapter inducing IFN-β). On ligand binding, most TLRs recruit the adapter molecule myeloid differentiation protein 88 (MyD88), which eventually results in induction of cytokines such as IL-6, IL-8 and TNF-α and chemokines (MyD88-dependent pathway). The TRIF-dependent pathway (or MyD88-independent pathway) uses TRIF to induce the activation of IFN-β and interferon-induced genes (Medzhitov et al. 1998; Yamamoto et al. 2002). Upon interaction of HCEs with *Acanthamoeba* trophozoites, the TLR4–MyD88 pathway is activated early and induces the secretion of IL-8 and TNF-α, while the TLR4–ERK1/2 pathway is activated later and induces the production of IFN-β (Ren et al. 2010; Ren and Wu 2011). Additionally, it was shown that clinical but not soil isolates induce an upregulation of TLR4 with production of IL-8 and CXCL2 gene

expression, which is of particular interest since IL-8 and CXCL2 are chemoattractants known to attract PMN to a site of infection, which is critical to disease severity in AK (Alizadeh et al. 2014).

Immunisation of mice with sonicated *Acanthamoeba* antigen induces a significant and moreover highly specific protection against a lethal challenge with this amoeba (Rowan-Kelly and Ferrante 1984). Successful immunisation against *Acanthamoeba* keratitis has also been demonstrated in a pig model (Alizadeh et al. 1995) and in Chinese hamsters (Leher et al. 1998b). However, recrudescence in AK suggests that corneal infection does not induce protective immunity. Moreover, it has been demonstrated that pathogenic acanthamoebae can evade the antibody-dependent amoebicidal activity of macrophages, are more resistant to the lytic activity of complement and can degrade human IgG and IgA antibodies (Kong et al. 2000; Marciano-Cabral and Toney 1998; Toney and Marciano-Cabral 1998).

In summary, one can assume that amoebae invading the body via skin lesions or lung tissue are opsonised by antibodies and complement. Antibodies may control the invasiveness of the amoebae by preventing amoebic adherence to tissue cells, neutralising cytolytic molecules and inhibiting amoebic phagocytosis. Subsequently phagocytic cells such as neutrophils are attracted to the infection site. A decreased antibody production, lowered complement levels, inhibited leukocyte function or an impaired lymphokine production result in a reduced resistance. A persistent non-ocular *Acanthamoeba* infection is the consequence of an immunodeficiency. In AK, immunobiology certainly also plays role; however, the details remain to be fully established.

10.3 Perspectives and Open Questions

Acanthamoeba is a particularly easy-to-culture eukaryote with very low requirements but a complex and highly evolved cellular repertoire. It thus represents an excellent model organism for cell biological studies in general, and with its similarity to phagocytotic cells of the mammalian immune system, it is also an interesting model for investigating infection processes at the cellular level. The completion of the first genome project and further genome projects expected to be finished soon gave this field of research a significant push forward, and it will certainly further expand in the future.

Also the surface structures of *Acanthamoeba* spp. are highly interesting and partially even unique. As there is evidence for differences in the glycomes between clinical and non-clinical isolates and as cell-cell attachment is the crucial step in the infection process, the elucidation of these structures will significantly advance our understanding of *Acanthamoeba* pathogenicity. Fact is, they are capable of synthesising a large number of highly uncommon N-glycans, including several entirely novel structures still waiting to be explored. Apart from their importance for attachment, these molecules may also represent interesting targets for specific treatment, which is still lacking.

Further, the role of *Acanthamoeba* spp. as permanent and transient host cells and vehicles for microorganisms, including not only bacteria of various clades but also fungi and viruses, has received increasing attention in the recent past. The

dimension and the importance of microbial interactions have long been seriously underestimated and are just now beginning to be understood. This field will certainly bring novel and fascinating findings in the next years.

And finally, the Amoebozoa, being phylogenetically relatively close to the animals and an extremely diverse group, are also highly interesting with respect to evolution, particularly to the emergence of multicellularity and sex. The complex cell communication machinery of *Acanthamoeba* possibly representing a precursor of multicellularity and the availability and significance of meiosis in amoebozoan taxa remain to be elucidated.

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