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Cellular and molecular mechanisms that underlie *Entamoeba histolytica* pathogenesis: prospects for intervention

Richard C. Laughlin and Lesly A. Temesvari

The protozoan parasite *Entamoeba histolytica* is the causative agent of amoebic dysentery. It is prevalent in developing countries that cannot prevent its fecal–oral spread and ranks second in worldwide causes of morbidity by parasitic infection. Improvements in sanitation would help curb disease spread. However, a lack of significant progress in this area has resulted in the need for a better understanding of the molecular and cellular biology of pathogenesis in order to design novel methods of disease treatment and prevention. Recent insight into the cellular mechanisms regulating virulence of *E. histolytica* has indicated that processes such as endocytosis, secretion, host cell adhesion and encystation play major roles in the infectious process. This review focuses on components of the molecular machinery that govern these cellular processes and their role in virulence, and discusses how an understanding of this might reveal opportunities to interfere with *E. histolytica* infection.

In his consolidating preliminary report on an invasive pathogenic amoeba, the zoologist Fritz Schaudinn assigned the name *Entamoeba histolytica* to the organism 'on account of its tissue-destroying ability' (Refs 1, 2). Few cells are known to possess a comparable assortment of cytolytic and biologically active molecules as *E. histolytica*. Originally documented by Fedor Lösch in 1875 from a fecal smear of a patient suffering from chronic and bloody diarrhoea, Lösch described, in great detail, the clinical course and symptoms of amoebic infection, as well as the ultrastructure of motile and quiescent amoebae (Refs 3, 4).

Since these early studies, an increasing knowledge of the basic cell biology of *E. histolytica* has aided the development of treatment strategies. Although studies have typically been performed in vitro, or using mammalian models of infection that may not fully represent the complexity of human infection, valuable insight has been gained

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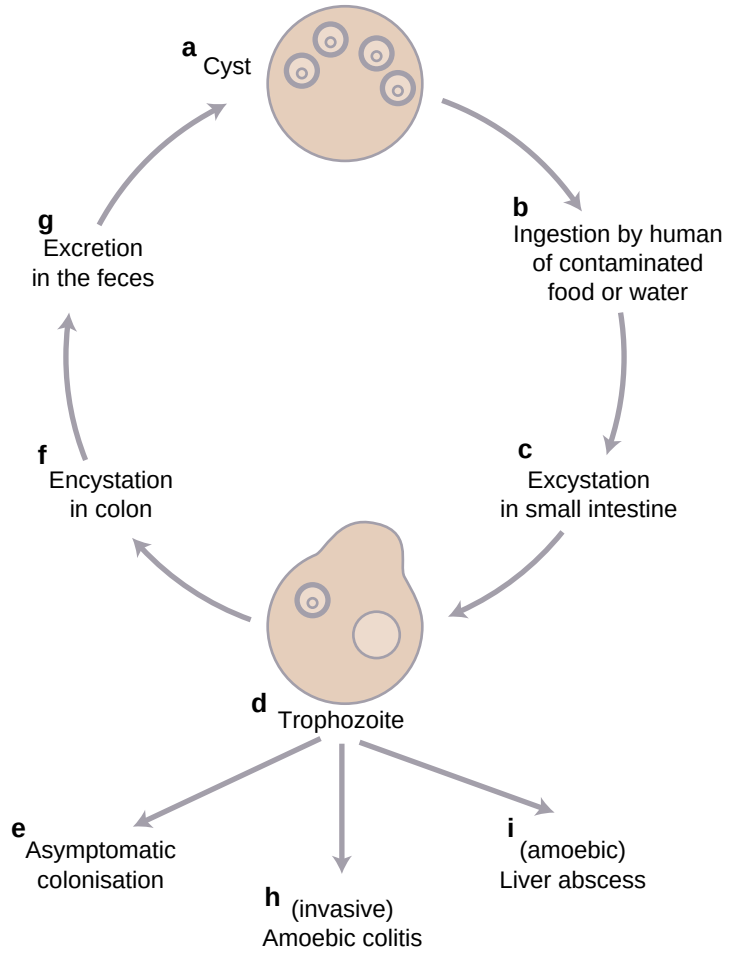
into the molecular mechanisms governing the basic cellular functions of *E. histolytica* and how these functions contribute to virulence and invasiveness of the parasite. For example, knowledge of the endo-lysosomal and secretory systems, the mechanisms and consequences of adhesion to host cells, and cues for encystation and excystation are important to appreciate fully this unique pathogen as well as to identify opportunities to interrupt the infectious and invasive cycles. After a brief introduction to the parasite, this review will focus on the molecular mechanisms underlying key cellular processes, before discussing putative chemotherapeutic and vaccine targets.

Introduction: transmission and diagnosis

The protozoan parasite infects humans and may infect some primates. It is passed through fecal–oral transmission and is most pervasive in areas marked by overcrowding, poverty and poor sanitation. The infectious form of the parasite, an environmentally stable cyst, is ingested by the human host through consumption of fecally contaminated food or water (Fig. 1) (Ref. 5). After



Life cycle of *Entamoeba histolytica* and the clinical manifestations of infection in humans

Figure 1. Life cycle of *Entamoeba histolytica* and the clinical manifestations of infection in humans. *(See next page for legend.)*

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Figure 1. Life cycle of *Entamoeba histolytica* and the clinical manifestations of infection in humans. *(Legend; see previous page for figure.) E. histolytica* is a protozoan parasite that causes amoebic colitis and liver abscess. Its life cycle consists of two stages: cysts and trophozoites. (a) Cysts measure 10–15 mm in diameter and typically contain four nuclei; (b) they are spread via the ingestion of fecally contaminated food or water. (c) During excystation within the lumen of the small intestine, nuclear division is followed by cytoplasmic division, giving rise to eight trophozoites. (d) Trophozoites, which measure 10–50 mm in diameter and contain a single nucleus with a central karyosome, reside in the lumen of the caecum and large intestine, where they adhere to the colonic mucus and epithelial layers. (e) Approximately 90% of individuals infected with *E. histolytica* are asymptomatically colonised; (f) re-encystation of the trophozoites occurs within the lumen of the colon, resulting in (g) the excretion of cysts in the feces and continuation of the life cycle. (h) Alternatively, the trophozoites can invade the colonic epithelium, causing amoebic colitis (in ~10% of infected people). Amoebic dysentery usually occurs gradually, with symptoms [such as abdominal pain and tenderness, and painful sudden bowel evacuation (tenesmus) and diarrhoea] developing over a period of one to several weeks, often followed by weight loss. *E. histolytica* can spread in the bloodstream (haematogenously) after it has penetrated the colonic epithelium (not shown) and can establish persistent extraintestinal infections, most commonly (i) amoebic liver abscess. Liver abscess is overwhelmingly the most common extraintestinal manifestation of amoebiasis. This complication is 10 times more common in adult men than in adult women. The typical patient with amoebic liver abscess is a 20–40-year-old male with a 1–2-week history of fever and diffuse or right, upper-quadrant abdominal pain. This figure, which was originally published by *Expert Reviews in Molecular Medicine* (Ref. 15), is reproduced with kind permission of Christopher D. Huston.

ingestion of the cysts, excystation occurs in the small intestine, during which nuclear and cytoplasmic division gives rise to eight trophozoites. The trophozoites colonise the colon, and either invade the colonic epithelium, causing amoebic colitis, or re-encyst within the lumen of the colon, resulting in the excretion of cysts in the feces and continuation of the life cycle. The endocytic, secretory and adhesion pathways of the parasite play crucial roles in nutrient uptake and host cell destruction. The endocytosis of gut-resident bacteria, erythrocytes and cell debris is the main nutrient source for the parasite (Ref. 6). Secretion of lytic molecules and pore-forming peptides act to destroy the surrounding intestinal lumen, which may lead to tissue invasion (Refs 7, 8). Interestingly, it has been reported that 90% of people infected with *E. histolytica* do not exhibit any symptoms and never develop amoebic dysentery (Ref. 5). However, in those with symptoms, amoebic infection may manifest itself differently depending on the severity and location of infection. Initial colonisation in the colon results in loose, bloody stools, whereas more-advanced infection results in amoebic colitis typified by weight loss, bloody diarrhoea and the formation of flask-shaped ulcers in the colon. Severe abdominal pain, fever and weight loss are symptomatic of amoebic liver abscess, which can occur independently of dysentery or colitis.

Industrialised nations have essentially eliminated the threat of outbreak through proven wastewater treatment regimens. However, developing countries often lack the infrastructure and resources to implement such treatment schemes. Thus, populations in underdeveloped or otherwise poverty-stricken countries have higher incidences of infection and mortality as compared with more-industrialised nations (Ref. 5). Although 40 000–100 000 people die of amoebic infection each year, it has recently become apparent that past estimations of infection are not reliable (Ref. 5). This is mainly a result of the difficulty in identifying the amoebae. Several factors have conspired to make diagnosis of *E. histolytica* infection difficult, including the occurrence of asymptomatic carriers and the existence of a morphologically identical, non-pathogenic amoeba, *Entamoeba dispar*, originally described in 1925 by Emile Brumpt (Ref. 9). The verification of this distinct, non-pathogenic species was not possible until the development of molecular techniques in the early 1970s, which demonstrated different lectin agglutination patterns between pathogenic and non-pathogenic species (Ref. 10). Subsequent isoenzyme patterning from cultured *Entamoeba* spp, gene sequence analyses and the development of monoclonal antibodies specific for *E. dispar* have supported the original distinction by Brumpt (Refs 11, 12, 13, 14).

The recognition of distinct pathogenic and non-pathogenic amoebae was important not only for taxonomy, but also for diagnosis. By accurately and reproducibly distinguishing *E. histolytica* from *E. dispar* in the laboratory, several new strategies

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have been designed to diagnose patients in the field. PCR-based detection and isoenzyme analysis from amoeba cultured from stool samples have proved very sensitive and reliable but may be technically prohibitive in the settings in which *E. histolytica* infection is normally found (Ref. 15). Enzyme-linked immunosorbent assays (ELISAs) that recognise an important cell-surface adhesin of *E. histolytica*, the galactose/*N*-acetyl-D-galactosamine (Gal/GalNAc)-inhibitible lectin, are currently the most convenient tests to be employed (Ref. 15). Several studies have recently reported prevention of amoebic liver abscess or intestinal amoebiasis by vaccination in rodent models of disease (see below; Refs 16, 17, 18). These reports mark some of the latest efforts to move from a treatment response to a proactive strategy to confer host immunity against the pathogen.

Endocytosis and secretion

Eukaryotic cells have evolved a sophisticated and highly regulated system for both the uptake of extracellular components (endocytosis) and the release of a variety of macromolecules (secretion). *E. histolytica* uses these basic cellular functions to initiate and propagate colonisation and infection. Cell debris, fluid-phase particles, bacteria, erythrocytes and other cells are endocytosed by *E. histolytica* through several distinct but related mechanisms. In addition, *E. histolytica* secretes several bioreactive molecules, such as pore-forming peptides, acid phosphatases and cysteine proteases, which degrade the extracellular matrix (ECM), destroy host cells and aid in immune system evasion. Because endocytosis and secretion are important to the pathogenicity of the parasite, a considerable effort has been made in understanding the molecular mechanisms governing these cellular processes.

Endocytosis

Trophozoites of *E. histolytica* are known to have robust endocytic capabilities, turning over approximately a third of their cellular volume every hour (Refs 19, 20). Endocytosis can be categorised into two general types: pinocytosis, which involves the uptake of fluid and small macromolecules; and phagocytosis, which is the regulated ingestion of large particles such as whole cells. As with other cell types, the pinocytic pathway of *E. histolytica* is a constitutive process that begins with the formation of early endosomes, whose lumens are typically the ambient pH of the local environment. Vesicles containing internalised material become increasingly acidic, representing late endosome or lysosome-like compartments (Ref. 21). Phagocytosis is a highly regulated receptor-mediated event that depends on a number of intracellular signalling components and the actin cytoskeleton, and may be mediated by one of several different adhesion molecules. Adhesion (which will be discussed in greater detail later in the article) is of vital importance to the pathogenicity of *E. histolytica* as much of the lytic capacity of the parasite is dependent on contact with the target host cell. Recently, a variety of proteins that regulate the endo-lysosomal system of *E. histolytica* have been identified and their contribution to virulence of the parasite has also been assessed. The molecular components of endocytosis in *E. histolytica* are summarised in Table 1.

The Ras-related superfamily of low-molecular-weight GTPases is extensively involved in a variety of cellular functions, including endocytosis, secretion, vesicle trafficking, membrane fusion events, intracellular signalling and actin cytoskeleton rearrangement in eukaryotic cells (Ref. 22). These 'molecular switches' cycle between active GTP-bound and inactive GDP-bound forms and typically associate with several different effector proteins for proper functioning. In *E. histolytica*, the identification and putative function of a variety of conserved and novel Ras-related superfamily proteins and effector molecules has been described. In many instances, the functions of these molecules in *E. histolytica* are consistent with the functions of related low-molecular-weight GTPases in other eukaryotic cells. Several Rab-like GTPases have been demonstrated to function in either pinocytosis or phagocytosis. EhRabB was shown to localise to intracellular vesicles and to translocate to phagocytic cups during erythrophagocytosis (Ref. 23). The role of EhRab5 in phagocytosis has been demonstrated through the characterisation of cell lines overexpressing wild-type or constitutively active EhRab5 (Ref. 24). In addition, the enrichment and localisation of EhRab7-like GTPases to early pinocytic vesicles and phagosomes has been shown (Refs 21, 24, 25, 26).

Low-molecular-weight GTPases typically involved in signal transduction or actin cytoskeleton organisation also function in

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Table 1. Factors proposed to regulate endocytic pathways in *Entamoeba histolytica*[a]

Factor	Pinocytosis[b]	Phagocytosis[c]
EhRabB	?	+
EhRab5	–	+
EhRab7 family	+	+
EhRacA	–	+
EhPAK	?	+
Actin	+	+
Myosin 1B	–	+
Phosphatidylinositol-3 kinase	+	+
Protein kinase C	?	+
Lipid rafts	+	?
Amoebapore A	?	+
Cysteine protease (CPs) 1, 2, 112	?	+
L220	?	+
Hgl, Lgl, Igl subunits of the Gal/GalNAc-inhibitible lectin	?	+

[a] *E. histolytica* acquires nutrients through endocytosis of cell debris, fluid phase particles, erythrocytes, bacteria and whole cells. The ability to endocytose has been correlated with the virulence of the organism. The endocytic process can be divided into two distinct pathways: pinocytosis and phagocytosis. Factors that influence these pathways either positively (+) or negatively (-) are shown. The precise effects of some factors are not fully known (?).
[b] Pinocytosis is a constitutive event that involves the uptake of small macromolecules or fluid-phase particles. Pinocytic vesicles become increasingly acidic as they mature.
[c] Phagocytosis is the regulated uptake of large particles and whole cells, such as erythrocytes.

endocytosis (Ref. 27). In other eukaryotes, Rac GTPases are known to be potent activators of actin polymerisation through interactions with members of the Wiscott-Aldrich Syndrome protein (WASp) family and with p21-activated serine/threonine kinase (PAK) (Ref. 27). In *E. histolytica*, EhRacA was shown to regulate actin polymerisation and, in doing so, influence phagocytosis (Ref. 28). EhPAK was shown to colocalise with the actin cytoskeleton and overexpression of its regulatory domain resulted in an increase in erythrophagocytosis (Ref. 29).

Signal transduction through reversible phosphorylation is a conserved mechanism for intracellular communication in both eukaryotic and prokaryotic cells. Large families of proteins that function to initiate and propagate phosphorylation-based signals are known and have been implicated in a variety of cellular events including endocytosis. Several studies have demonstrated the importance of molecules such as protein kinase C (PKC), tyrosine kinase and phosphatidylinositol-3 kinase (PI-3 kinase) in the pathogenicity of *E. histolytica* (Refs 28, 30, 31, 32). Endocytosis, especially erythrophagocytosis, has been found to be generally inhibited following the treatment of amoebae with inhibitors specific for each type of kinase (Refs 28, 30, 31). Studies identifying a role for PI-3 kinases in pathogenicity were also the first to implicate lipids as participants in *E. histolytica* virulence, since the substrate for PI-3 kinase is one of several inositol phospholipids. Inositol phospholipids that are phosphorylated by PI-3 kinase act as primary or secondary signalling molecules and can regulate many different cellular functions. Recently, lipid rafts (plasma membrane lipid microdomains) were shown to play a role in fluid phase pinocytosis in *E. histolytica* (Ref. 33). These microdomains, enriched in cholesterol, sphingolipids and saturated phospholipids, are more highly ordered, tightly packed and less fluid than other regions of the membrane (Ref. 34). In many other systems, lipid microdomains are known to function in the segregation of lipids, adhesion molecules and signalling proteins (Refs 34, 35). In *E. histolytica*, chemical disruption of the microdomains with methyl-β-cyclodextrin, a cholesterol-binding agent, inhibited the uptake of fluorescently labelled dextran (Ref. 33).

It has long been established that both actin and myosin are important structural components of eukaryotic cells and are vital to a variety of cellular

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functions, including vesicle trafficking and endocytosis (Refs 36, 37). The role of Rac-like GTPases, which are modulators of the actin cytoskeleton in endocytosis, is described above. Several additional studies have demonstrated an actin-dependent mechanism for the phagocytosis of erythrocytes and pinocytosis, as well as an increase in both actin polymerisation and actin transcript levels upon contact with host cells (Refs 38, 39, 40, 41). The actin-based motor protein, myosin IB, has also been shown to localise to the phagocytic cup of *E. histolytica* during erythrophagocytosis and around maturing phagosomes (Ref. 41). Additionally, upon overexpression of this unconventional myosin, erythrophagocytosis was decreased, whereas other myosin IB-related functions, such as pinocytosis and cell motility, were not altered (Ref. 41). Although Rabs are typically involved in vesicle trafficking events, the unique Rab GTPase of *E. histolytica*, EhRabA, is localised to the leading edge of motile trophozoites; overexpression of a dominant-negative EhRabA mutant results in altered cell shape and cytoskeleton arrangement, as well as mislocalisation of the Gal/GalNAc-inhibitable lectin (Refs 42, 43).

Secretion

Secretion, the process by which cells traffic and release a variety of proteins and molecules from intracellular organelles to the exterior of the cell, is utilised by *E. histolytica* to destroy the ECM, perforate host cell plasma membranes and evade detection by the immune system. Secreted proteins include pore-forming peptides, acid phosphatases and cysteine proteases. The release of these proteins can occur either constitutively or by a regulated mechanism in response to specific extracellular cues. In either case, the peptides and proteins secreted by *E. histolytica* are vital both for the virulence of the organism and its ability to invade. The molecular components of secretion in *E. histolytica* are summarised in Fig. 2.

One of the more unique molecules secreted from *E. histolytica* is the pore-forming peptide known as amoebapore. The 77-residue polypeptide is stored in lysosome-like granules and is trafficked to phagosomes or secreted in a regulated fashion into the extracellular space, where it can disrupt the membranes of bacteria or host cells (Refs 8, 44, 45). Sequence analysis of one of three isoforms of amoebapore, amoebapore A, identified the peptide as a saposin-like protein (SAPLIP); members of this family have a variety of biological functions and show a conserved ability to interact with lipids (Ref. 46). Given the relatedness among the amoebapore isoforms, it is likely that they are all members of the SAPLIP family. Further analysis of amoebapore A revealed a high degree of sequence similarity to other pore-forming peptides such as NK-lysin and granulysin (Refs 47, 48). Several structural analyses have revealed an alpha-helical secondary structure as well as the necessity for dimerisation in order to interact with membranes (Refs 46, 49). Additional multimeric assembly is necessary for the peptides to perforate the membrane (Refs 50, 51).

The expression of amoebapore A has been strongly linked to the virulence of *E. histolytica*. Trophozoites with reduced expression of this polypeptide through antisense inhibition were less virulent in hamster liver abscess models (Refs 52, 53). Additionally, transcriptionally silenced mutants lacking detectable expression of amoebapore A showed a decrease in the ability to lyse both eukaryotic and prokaryotic cells and to form liver abscesses in immunocompromised mice, but retained their ability to cause inflammation and tissue damage in human colonic xenografts (Refs 53, 54, 55).

A second class of molecules that are either secreted or otherwise trafficked to the plasma membrane in *E. histolytica* are the acid phosphatases. Acid phosphatases are known to be important to virulence and survival in other eukaryotic parasites, including *Leishmania*, *Trypanosoma* and *Giardia* (Refs 56, 57, 58). In *E. histolytica*, acid phosphatases have copurified with both early and late endosomes and have been shown to exist in either a membrane-bound (MAP) or secreted (SAP) form (Refs 21, 59, 60). Both MAP and SAP exhibit phosphotyrosine phosphatase activity and disrupt the actin cytoskeleton of cultured HeLa cells (Refs 61, 62). Interestingly, decreases in tyrosine phosphorylation have been observed in host cells after exposure to *E. histolytica* trophozoites (Ref. 63). Although a specific mechanism has not been described, it is postulated that acid phosphatases act on host surface phosphoproteins and disturb host adhesion molecule complexes through dephosphorylation of regulatory tyrosine residues. This, in turn, results in a loss of actin stress fibre organisation (Refs 61, 62).

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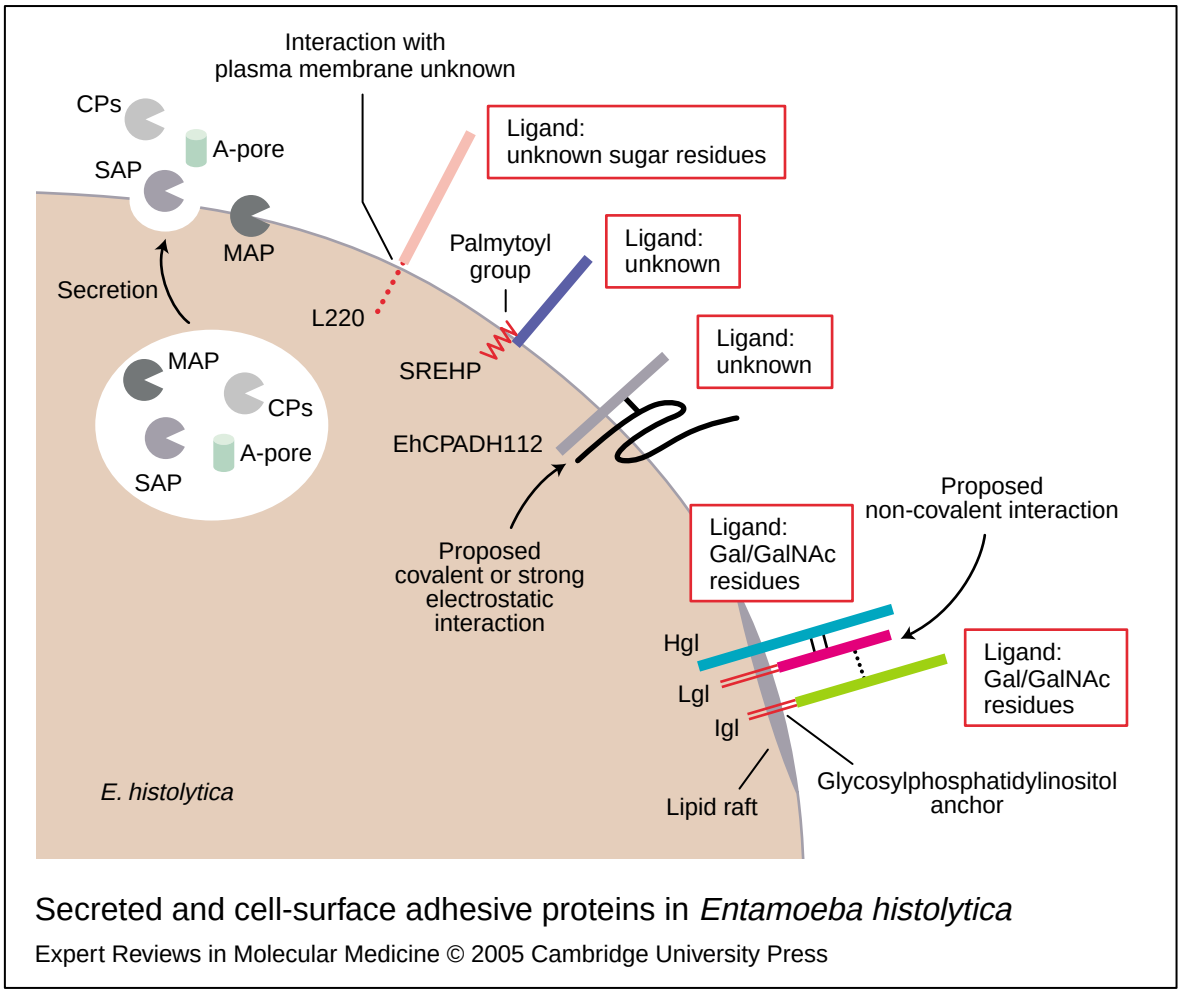

Secreted and cell-surface adhesive proteins in *Entamoeba histolytica*

Figure 2. Secreted and cell-surface adhesive proteins in *Entamoeba histolytica*. The ability of *E. histolytica* to traffic proteins to the cell surface or the extracellular space to participate in adhesion or degradation of host cell components, respectively, is critical to its virulence. Adhesion is mediated by several cell-surface proteins. One such adhesin is a multimeric protein complex with specific binding affinity for galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc). The complex comprises disulphide-linked heavy (Hgl) and light (Lgl) subunits and an intermediate (IgL) subunit postulated to interact non-covalently. Both the Hgl and Igl subunits are predicted to have Gal/GalNAc affinity. Hgl is a transmembrane protein that has been implicated in signal transduction and all three subunits reside in lipid rafts on the parasite plasma membrane. Additional adhesion molecules include a 220 kDa membrane protein (L220), a serine-rich *E. histolytica* protein (SREHP) and the multimeric adhesin/cysteine protease heterodimer EhCPADH112. The mechanism by which L220 interacts with the membrane is not known. SREHP exhibits a putative membrane-interacting region and palmytoylation, and EHCPADH112 has several predicted membrane-spanning sites. Although L220 has been shown to have lectin-like properties, the ligands of EhCPADH112 and SREHP have not been elucidated. All of these adhesion molecules have been proposed as vaccine targets. *E. histolytica* can secrete several factors into the extracellular milieu, including cysteine proteases (CPs), secreted acid phosphatase (SAP) and the pore-forming peptide amoebapore (A-pore). Interestingly, the amoebae can also secrete membrane-bound acid phosphatase (MAP). These factors are released constitutively (CPs, SAPs) or in a regulated fashion (A-pore) in response to host cell contact.

A final category of secreted proteins of *E. histolytica* that are known to have adverse effects on host cells, host ECM, and the host immune system is the cysteine proteases. These degradative enzymes are well known for their role in invasion in *Trypanosoma cruzi* and *Plasmodium* spp as well as in tumour metastasis (Refs 64, 65). To date, 20 genes are known to encode cysteine

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proteases in *E. histolytica*, with only a small subset of these expressed in axenically cultured *E. histolytica* (Ref. 66). Five proteins (EhCP1, 2, 3, 5 and 112) have been identified and localised to the plasma membrane, to endosomes, or secreted into the extracellular space. For EhCP1, 2 and 3, strong evidence supports their role in host monolayer destruction and phagocytosis through degradation of ECM proteins, as well as through their recruitment to phagocytic compartments (Refs 67, 68, 69, 70).

Membrane-bound EhCP5 is the best characterised of the cysteine proteases in *E. histolytica* and has been shown to perform a variety of functions contributing to invasion as well as to evasion of the host complement and immune system (Ref. 71). Antisense RNA silencing of EhCP5 resulted in cell lines that retained cytolytic and haemolytic capacities, but exhibited diminished phagocytosis and liver abscess formation. Furthermore, the host intestinal inflammatory response to this mutant was diminished as compared with wild-type *E. histolytica* (Refs 72, 73, 74). Interestingly, it was demonstrated that the EhCP5-deficient amoebae did not activate interleukin (IL)-1b or IL-8, two pro-inflammatory signalling molecules that stimulate the immune system and initiate a response (Ref. 74). Although EhCP5 can normally initiate a host immune response by cleavage of IL-1b or IL-8, it can also help to subvert the host immune system by proteolytic inactivation of IgA, IgG, IL-18 and complement system subunits C3 and C5 (Refs 75, 76, 77, 78).

A recently identified cysteine protease, EhCP112, is unique among *E. histolytica* cysteine proteases because it has been shown to associate with an adhesion molecule, EhAdh112, forming the EhCPADH112 complex (see below) (Ref. 79). Sequence analysis of EhCP112 has identified a putative transmembrane domain and an integrin attachment domain (RDG) (Ref. 79). A monoclonal antibody recognising the EhCPADH complex inhibited adhesion, phagocytosis and host monolayer destruction. Studies using cDNA or recombinant EhCP112 or EhAdh112 peptides suggest that this heterodimer has antigenicity and potential as a vaccine target (Refs 80, 81, 82).

Adhesion

An important step in the establishment of amoebic infection in the host is the adherence of the trophozoite to the intestinal mucosal layer. Adhesion is important not only for persistence and controlled migration of the pathogen, but also for the virulence of the parasite. Cell-contact-mediated lysis of host cells is a well-described feature of *E. histolytica* pathogenicity. Although human host cell targets have not been identified, it is postulated that the interaction is specific since *E. histolytica* interacts with varying affinity to cells, such as erythrocytes, derived from different animal species (Ref. 7). Thus far, four different adhesion molecules have been identified: the Gal/GalNAc-inhibitable lectin, the adhesin/cysteine protease heterodimer EhCPADH112, a 220 kDa membrane protein (L220) and a serine-rich *E. histolytica* protein (SREHP) (Refs 83, 84, 85, 86, 87, 88, 89). The molecular components of adhesion in *E. histolytica* are summarised in Fig. 2.

Gal/GalNAc-inhibitable lectin

The Gal/GalNAc-inhibitable lectin is one of the most extensively studied proteins of *E. histolytica* because of its role in adhesion, signal transduction and avoidance of the host complement system. It consists of a heavy subunit (Hgl; 170 kDa) that is disulphide linked to a glycosylphosphatidylinositol (GPI)-anchored light subunit (Lgl; 31/35 kDa) (Ref. 90). This heterodimer is noncovalently associated with a GPI-anchored intermediate subunit (Igl; 150 kDa) (Refs 83, 91). Although questions still remain regarding the exact mechanisms by which the adherence lectin modulates virulence and endocytosis, it is known that truncation of the N-terminus of Lgl results in a decreased ability of the amoebae to phagocytose erythrocytes (Ref. 92). Moreover, strains of *E. histolytica* with lower expression levels of Lgl displayed decreased virulence (Ref. 93). Proteomic analysis of isolated phagosomes have identified the association of Hgl, Lgl and Igl with this organelle (Ref. 26). Together, these observations further emphasise the connection between endocytosis, adhesion and virulence.

Functional analysis of Hgl has identified a carbohydrate-recognition domain (CRD) on the extracellular portion of the protein, as well as β2 and β7 integrin-like domains on the cytoplasmic C-terminus (Refs 90, 94). Treatment of trophozoites with free Gal or GalNAc reduces adherence to cultured cell monolayers by 90%, supporting the Gal/GalNAc binding specificity of the adhesion molecule. Additionally, changes

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in adhesion of *E. histolytica* to mutant Chinese hamster ovary (CHO) cells with altered glycosylation patterns further correlate this virulence function to the level of Gal or GalNAc modifications on the host cell proteins (Ref. 95). The inhibition of adhesion, in turn, significantly reduces the cytolytic capacity of the pathogen (Refs 86, 95, 96, 97). Cells overexpressing the cytoplasmic C-terminus of Hgl exhibit reduced host cell killing. This loss in cytolytic function is hypothesised to be due to an uninitiated signalling cascade normally mediated by an integrin-like homology motif found on the cytoplasmic C-terminal tail of this subunit (Ref. 94). Interestingly, integrins are known to be transmembrane allosteric signalling molecules with both cytoplasmic and extracellular binding domains and, in other eukaryotic systems, have been associated with the actin cytoskeleton as well as lipid microdomains (Ref. 98). Similarly, the *E. histolytica* Gal/GalNAc-inhibitable lectin has been localised within lipid microdomains and actin-rich regions of the membrane (Ref. 33).

Although lacking cytoplasmic domains, Igl and Lgl have been shown to be necessary factors in adhesion and virulence of *E. histolytica*. Adhesion to a cell monolayer was blocked by incubation of trophozoites with monoclonal antibodies to Igl. A Gal-binding affinity, independent from that of Hgl, suggests that the intermediate subunit may function as a co-receptor (Refs 83, 99). Antisense RNA inhibition of Lgl resulted in decreased host cell monolayer destruction and liver abscess formation (Ref. 93). Additionally, a decrease in virulence has been associated with prolonged co-culture with *E. coli* serotype O55, which is accompanied by a reduction in the expression of Lgl (Ref. 100). Although much is known about the Gal/GalNAc-inhibitable lectin and its role in virulence, several questions still remain regarding the identity of specific ligands, the regulation of ligand-binding events, the interactions between the three subunits, the influence of plasma membrane lipid components on lectin function and the role of protein intermediates responsible for signal transduction events that lead to the release of virulence factors.

EhCPADH112

Another protein complex known to participate in adhesion, EhAdh112, is found in the 112 kDa heterodimeric adhesin/cysteine protease complex, EhCPADH112. The EhCPADH112 complex has been localised to the plasma membrane and phagosomes, and has been shown to bind to host cells, although no specific extracellular or host-cell-associated ligand has been identified (Refs 79, 87). EhAdh112 has three putative transmembrane domains, and monoclonal antibodies to EhAdh112 diminished adhesion, phagocytosis and host cell monolayer destruction, strongly suggesting an important role in virulence for this adhesion molecule. Several studies have reported that hamsters are protected from forming liver abscesses after simultaneous innoculation with recombinant peptides or cDNAs encoding EhCP112 and EhAdh112 (Refs 80, 81).

220 kDa membrane protein (L220)

Originally isolated and purified from the *E. histolytica* HM1:IMSS strain, L220 has been localised to the plasma membrane of *E. histolytica* (Refs 88, 101). Although little is known about the function or extracellular ligand of the adhesin, it is known to have lectin-like properties and is highly immunogenic in mice, hamsters and rabbits (Refs 88, 101). The purified protein can bind to fixed host cells in vitro, block subsequent adhesion of trophozoites to the cell monolayer, and agglutinate human erythrocytes (Ref. 88). Antibodies to the adhesin have been shown partially to inhibit adhesion of trophozoites to erythrocytes and cultured host cells and to inhibit erythrophagocytosis (Ref. 101). Interestingly, whereas polyclonal antibodies to L220 recognised proteins of the same molecular weight in both pathogenic and non-pathogenic strains of *Entamoeba*, some monoclonal antibodies only recognised a protein from pathogenic HM1:IMSS or HM1:HM38 strains, suggesting that a specific isoform of the adhesin that is unique to pathogenic strains might exist (Ref. 101). More recently, a protocol to generate high antibody titres to a recombinant glutathione *S*-transferase (GST)-tagged L220 has been achieved (Ref. 102). Importantly, these antibodies, as well as antibodies generated to any other recombinant protein targets, may be useful as novel diagnoistic tools or to confer passive immunity to hosts.

Serine-rich *E. histolytica* protein (SREHP)

Stanley et al. initially identified SREHP by a differential cDNA screen comparing pathogenic *E. histolytica* HM1:IMSS and non-pathogenic *E. moshkovskii* Laredo (Ref. 89). This palmytoylated

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surface membrane protein has a high degree of post-translational modifications, a high number of serine residues, and tandomly repeated hydrophilic sequences (Refs 103, 104). Whereas antibodies to this membrane protein have been shown to block trophozoite adhesion to host cell monolayers, little is known about its actual physiological role or ligand (Ref. 89). Much of the research efforts have focused on exploring the potential of SREHP as a vaccine candidate, since several studies have identified an immune response to this protein (Refs 105, 106, 107). This work will be described in greater detail below.

Encystation/excystation

The infectious form of *E. histolytica* is an environmentally stable, quadranucleated cyst. Host infection begins with the ingestion of contaminated water or food containing this stage of the parasite. Excystation occurs in the small intestine with the release of a quadranucleated amoeba from the cyst wall, which rapidly divides to yield eight mature trophozoites. Encystation occurs in the large intestine as a result of unknown cues. Since no axenic, synchronous encystation protocols have been reported for *E. histolytica*, studies in this area have focused on a related reptilian parasite, *E. invadens*. The removal of glucose from the culture media causes *E. invadens* amoebae to round-up, complete at least one round of DNA replication, form multicellular aggregates, and then form chitin-coated cysts within these aggregates (Refs 108, 109, 110, 111, 112). Complete encystation of *E. histolytica* has not been observed in vitro; however, removal of serum and iron from the culture medium results in the formation of multinucleated cyst-like structures (Ref. 113).

Several components of the cyst wall have been described. Chitin, a polysaccharide composed of the aminosugar *N*-acetyl-glucosamine, and Jacob, a lectin with a series of chitin-binding domains, are known components of the cyst wall (Refs 112, 114). Although chitinases and chitin synthases have been identified in *E. histolytica, E. dispar* and *E. invadens*, little is known about the synthesis of chitin during *Entamoeba* encystation (Refs 115, 116). The Jacob protein, also present in all of these *Entamoeba* species, has been proposed to be responsible for the deposition and crosslinking of the polysaccharide on the surface of the cyst, contributing to the formation and environmental stability of the cyst wall (Ref. 114).

Several recent reports also provide some insight into the molecular mechanisms of cyst formation. EhRab11 was shown to translocate to the periphery of the cell during iron and serum starvation in *E. histolytica* cultures, suggesting a putative role for this small-molecular-weight GTPase in the encystation process (Ref. 113). In addition, ubiquitin and chitinase are among several genes that are upregulated in *E. invadens* cultures after 24 hours of Gal depletion (Ref. 117). This increased gene expression is reversible upon the addition of free Gal to the encystation media. This implies an important role for Gal-binding lectins in monitoring of the nutritional status of the local environment and in the subsequent signalling that governs the expression of the encystation gene regime. Interestingly, ubiquitin is a protein traditionally associated with the proteasome and it has been demonstrated that proteasome inhibitors can successfully inhibit encystation (Ref. 118). Given that inhibition of cyst formation would curtail spread of *Entamoeba*, great potential for the identification of novel chemotherapeutic targets lies in a better understanding of this cellular process.

Possible chemotherapeutic and vaccine targets: future directions

Elucidation of the molecular mechanisms that are central to the pathogenesis and virulence of *E. histolytica* has begun to pave the way for the development of vaccines and the identification of chemotherapeutic agents. Great strides have been made in the past several years to identify specific molecules whose function is critical to the survival or invasiveness of *E. histolytica*. The increased understanding of endocytosis, secretion, adhesion and encystation has yielded potential targets for prevention or treatment of infection.

Unfortunately, despite the importance of the Ras-related superfamily of GTPases to *E. histolytica* virulence, they may not serve as suitable targets for chemotherapeutic treatments because of the high degree of homology they share with related proteins in the host. Even novel members of the superfamily are not likely to be unique enough for specific chemical targeting. This is true not only for *E. histolytica*, but also for other parasitic protozoa such as *Trypanosoma brucei*, *Leishmania* spp, *Giardia lamblia* and *Toxoplasma gondii*. By contrast, the identification of unique GTPase-interacting proteins in these parasites has

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presented the opportunity to develop novel treatments. For example, prenyltransferases (farnesyltransferase and gerenyltransferase) are responsible for the addition of lipid anchors to the C-terminus of small GTPases. Most Ras-related GTP-binding proteins in eukaryotes undergo this post-translational modification, which facilitates membrane interaction. Chemical interruption of this modification is being investigated as an anti-cancer treatment and has been theorised as a strategy for anti-parasitic treatment, since parasites often grow and divide at a faster rate than host cells (Refs 119, 120). Unique farnesyltransferases have been identified in *T. brucei* and farnesyltransferase-targeted chemotherapeutic treatments have successfully inhibited the growth not only of *T. brucei*, but also of *G. lamblia*, *T. cruzi* and *Leishmania major* (Refs 121, 122, 123). Although prenyltransferases represent an anti-parasitic target not yet addressed for *E. histolytica*, a search of PubMed and *Entamoeba* Genome data has revealed predicted sequences for both farnesyltransferases and gerenyltransferases, suggesting this could be a promising future therapeutic target.

In general, cysteine proteases are initially expressed in an inactive form and are activated when the N-terminus of the protein, or pro-domain, is enzymatically cleaved and removed from this precursor. It is well documented that this pro-domain can also serve as a potent and specific inhibitor of the corresponding mature enzyme (Refs 124, 125, 126, 127). In other protozoan parasites such as *Leishmania mexicana*, *T. gondii* and *Trypanosoma* spp, natural cysteine protease inhibitors are known to exist, which probably protect the parasite from digestion by its own enzymes (Refs 128, 129, 130). Protease inhibitors have also been utilised to assess the importance of cysteine proteases in pathogenicity in *E. histolytica*. E-64 was shown to be a broad inhibitor of cysteine protease activity from *E. histolytica* and inhibited host monolayer destruction in vitro, as well as liver abscess formation in vivo (Refs 131, 132, 133, 134). Given the importance of cysteine proteases to the virulence functions of *E. histolytica*, it will be useful to identify protease inhibitors that might be used to treat amoebic infections.

The cell-surface location of Gal/GalNAc-inhibitable lectin make its potential as a vaccine target high. Antibody crossreactivity and sequence analysis have demonstrated homology between regions of Hgl and CD59, a human protein that inhibits the assembly of complement components C5a-9 (Ref. 135). This suggests that Hgl might participate in the inhibition of complement assembly. In contrast to this role in subverting the host immune system, Hgl is also proving to have antigenic potential. Acquired resistance in Bangladeshi children has been shown to be mediated by the development of an IgA-based immune response against the CRD of Hgl, supporting its potential as a vaccine target (Ref. 136). Interestingly, in this same group of children, inherited resistance to *E. histolytica* was associated with heterozygous expression of a particular HLA class II allele that did not correlate with the presence of stool IgA (Ref. 137). Recent vaccine trials in mice inoculated with either a recombinant fragment or native full-length Hgl were shown to develop anti-lectin IgA antibodies and resistance to post-inoculation challenge with active trophozoites (Ref. 18).

Other surface proteins, such as EhCPADH112 and SREHP, have also been explored for their antigenic capacity. Studies have shown the potential of the EhCPADH112 cysteine protease/adhesin complex as a vaccine target. Hamsters were protected from amoebic challenge after innoculation with either a mixture of recombinant proteins or a mixture of cDNA encoding the subunits for the complex (Ref. 81). In addition, both SCID mice and gerbils were protected from amoebic liver abscess formation by either treatment with anti-SREHP antibodies or vaccination with recombinant SREHP (Refs 106, 107). Interestingly, 80% of people with amoebic liver abscess were found to have antibodies to SREHP, further supporting the antigenic potential of this protein (Ref. 105).

New technological advances in the detection of *E. histolytica* will be critical for early and proper diagnosis of amoebic infection. A commercially available kit, *E. histolytica* II test (TechLab; http://www.techlabinc.com/product_details/t5017.htm), is an antigen detection test for the Gal/GalNAc-inhibitable lectin that is accurate and sensitive (Ref. 138). Importantly, this test is able to distinguish between *E. histolytica* and non-pathogenic *E. dispar*, overcoming a major difficulty in *E. histolytica* diagnosis. Detection of *E. histolytica* using real-time PCR analysis of stool samples was also recently shown to be highly accurate and discriminating (Refs 139, 140).

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Although this might be the most sensitive technique utilised in *E. histolytica* to date, it is limited to facilities with the appropriate resources.

A nitroimidazole antibiotic, metronidazole, is typically the first line of treatment in cases of infectious amoebiasis and may be co-administered with the luminal drug paramomycin (Refs 141, 142). However, incomplete resolution of *E. histolytica* infection is a common outcome of metronidazole treatment. Moreover, drug resistance in cultured *E. histolytica* has been correlated with the overexpression of P-glycoproteins 1 or 5 (EhPgp1 or EhPgp5), which are ATP-dependent pumps shown to decrease the intracellular concentration of chemotherapeutic agents (Refs 143, 144, 145, 146). Interestingly, EhPgp1 and EhPgp5 have partial sequence homology to the antigenic region of human proteinase 3, a specific autoimmune antigen for anti-neutrophil cytoplasmic autoantibody (ANCA) (Ref. 147). This might explain the autoimmune symptoms commonly seen in patients after treatment for *E. histolytica* and provides further insight into the mechanisms by which *E. histolytica* evades host defences. The potential for drug-resistant strains of *E. histolytica* to arise, as has been demonstrated by laboratory studies, underscores the necessity for vaccine development.

Concurrent with advances in the understanding of *E. histolytica* cell biology, important advances in bio-informatics, genomics / proteomics, high-throughput chemical library screening and RNA interference (RNAi) technologies will contribute to the design of better strategies for large-scale prevention and treatment. With the completion of the *E. histolytica* genome project (Ref. 148), researchers may now be able to identify potential therapeutic or vaccine targets rapidly and to elucidate systems critical to virulence. For example, Okada et al. recently undertook a proteomic study of isolated phagosomes (Ref. 26). Several phagosome-associated proteins were identified by mass spectroscopy including EhRacA, EhRacG, EhPAK, actin and several Rab7-related GTPases, further supporting their role in endocytosis (Ref. 26). With the advent of clinical RNAi and the recent knowledge that *E. histolytica* possesses the molecular machinery required to silence specific genes using a double-stranded RNA cue, great potential lies in designing deliverable RNA-based mechanisms that might silence genes linked to invasion or pathogenicity (Refs 149, 150). Still other technologies, such as automated high-throughput chemical screens, could be utilised to identify potential drug candidates that might interfere with any number of cell virulence functions, such as endocytosis, cysteine protease or acid phosphatase release, host adhesion, encystation, excystation, or critical signalling pathways.

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Further reading, resources and contacts

The *Entamoeba* Homepage, hosted by the London School of Hygiene and Tropical Medicine, provides information on the basics of *Entamoeba*, treatment of amoebic infection, links to relevant websites, a contact list of researchers and recent publications:
http://homepages.lshtm.ac.uk/entamoeba/

The Institute for Genomic Research (TIGR) *Entamoeba histolytica* Genome Project and the Sanger Institute *Entamoeba histolytica* Whole Genome Shotgun websites are excellent sources for sequence analysis and genome/proteome search options (e.g. BLAST, Gene Name, Contig, Locus and hidden Markov models):
http://www.tigr.org/tdb/e2k1/eha1/
http:www.sanger.ac.uk/Projects/E_histolytica/

World Health Organization (WHO):
http://www.who.org/

The American Society for Tropical Medicine and Hygiene (ASTMH):
http://www.astmh.org/

The Royal Society for Tropical Medicine and Hygiene:
http://www.rstmh.org/

Sterling, C.R. and Adam, R.D., eds (2004) The Pathogenic Enteric Protozoa: *Giardia*, *Entamoeba*, *Cryptosporidium* and *Cyclospora*. World Class Parasites (Vol. 8), Springer Publishers. This book provides an informative series of chapters on *E. histolytica*, including the history of amoebiasis, dissimilarity of *E. histolytica* and *E. dispar*, amoebic pathogenesis, and the implications of sequencing the genome to future research.

Features associated with this article

Figures

Figure 1. Life cycle of *Entamoeba histolytica* and the clinical manifestations of infection in humans.
Figure 2. Secreted and cell-surface adhesive proteins in *Entamoeba histolytica*.

Table

Table 1. Factors proposed to regulate endocytic pathways in *Entamoeba histolytica*.

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