Acetaldehyde/alcohol dehydrogenase-2 (*EhADH2*) and clathrin are involved in internalization of human transferrin by *Entamoeba histolytica*

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Transferrin (Tf) is a host glycoprotein capable of binding two ferric-iron ions to become holotransferrin (holoTf), which transports iron in to all cells. *Entamoeba histolytica* is a parasitic protozoan able to use holoTf as a sole iron source *in vitro*. The mechanism by which this parasite scavenges iron from holoTf is unknown. An *E. histolytica* holoTf-binding protein (*EhTfbp*) was purified by using an anti-human transferrin receptor (TfR) monoclonal antibody. *EhTfbp* was identified by MS/MS analysis and database searches as *E. histolytica* acetaldehyde/alcohol dehydrogenase-2 (*EhADH2*), an iron-dependent enzyme. Both *EhTfbp* and *EhADH2* bound holoTf and were recognized by the anti-human TfR antibody, indicating that they correspond to the same protein. It was found that the amoebae internalized holoTf through clathrin-coated pits, suggesting that holoTf endocytosis could be important for the parasite during colonization and invasion of the intestinal mucosa and liver.

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INTRODUCTION

Iron is a vital element for nearly all living organisms but it is bound to proteins to prevent tissue damage by oxygen free-radical formation. In mammals, iron is an enzyme cofactor and forms part of haemoproteins. In addition, iron is sequestered by proteins such as the iron-transporter transferrin (Tf) in serum and the iron-withholding defence lactoferrin (Lf) in mucosae. Thus, the free ionic-iron concentration ($\sim 10^{-18}$ M) is far too low to sustain the growth of pathogens (Bullen *et al.*, 1978).

Bacterial pathogens have developed several mechanisms for obtaining host iron; one of these is the use of receptors that can bind iron-containing proteins, such as holoTf, holoLf, and haemoglobin, with very high host specificity (Weinberg, 2009). In Gram-negative bacteria, receptors for holoTf

generally consist of two iron-regulated outer-membrane proteins, termed TbpA and TbpB (Genco & Desai, 1996), and iron is removed from its receptor in an energy-dependent process. A transferrin-binding protein A (StbA, also known as IsdA), has been reported in *Staphylococcus aureus* strain RN6390 (Maresso & Schneewind, 2006; Mazmanian *et al.*, 2003; Taylor & Heinrichs, 2002). Homologues of the *isd* gene are also found in Gram-positive bacilli (Bierne *et al.*, 2004; Gat *et al.*, 2008; Maresso & Schneewind, 2006). These pathogens have several elaborate mechanisms for iron sequestration.

Furthermore, it has been reported that *S. aureus* strain BB and *Staphylococcus epidermidis* strain 138 utilize the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Gap or Tpn) to bind holoTf (Modun & Williams, 1999). Interestingly, the parasite *Trypanosoma brucei* also makes use of GAPDH for holoLf binding rather than for holoTf binding (Tanaka *et al.*, 2004). Enzymes that bind holoTf have not previously been described in parasitic protozoa.

Iron uptake in mammalian cells is initiated by the binding of holoTf to the TfR on the cell surface. Then, via clathrin-coated pits, the Tf–TfR complex becomes trapped within

Abbreviations: Ab, antibody; *EhADH2*, *Entamoeba histolytica* acetaldehyde/alcohol dehydrogenase-2; *EhTfbp*, *Entamoeba histolytica* holoTf-binding protein; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; holoTf, holotransferrin; Lf, lactoferrin; Tf, transferrin; TfR, transferrin receptor; TRITC, tetramethylrhodamine isothiocyanate.

endocytic vesicles, which are rapidly acidified, and the iron is removed from holoTf and transported across the endosomal membrane into the cytosol. Apotransferrin (apoTf, Tf without iron) is thus formed and, still bound to the TfR, is sorted into exocytic vesicles that fuse with the plasma membrane. The complex is then exposed to the extracellular pH, where apoTf has a very low affinity for the TfR and is thus dissociated from it, ready for another cycle of iron capture and endocytosis (Dautry-Varsat *et al.*, 1983; Thorstensen & Romslo, 1990).

The presence of clathrin has been demonstrated in several protozoa (Corrêa *et al.*, 2007; Hernandez *et al.*, 2007; Morgan *et al.*, 2001; Robibaro *et al.*, 2001) and also in *Entamoeba histolytica* (León-Sicaireo *et al.*, 2005; López-Soto *et al.*, 2009a). The gene encoding the clathrin protein has been identified in the *E. histolytica* genome (Loftus *et al.*, 2005). Clathrin vesicles in protozoa seem to play a crucial role in the acquisition of nutrients. HoloTf endocytosis has been reported as a clathrin-independent but cholesterol-dependent process in *Trypanosoma cruzi* (Corrêa *et al.* 2008). However, in its relatives *Trypanosoma brucei* and *Trypanosoma congolense* holoTf enters by receptor-mediated endocytosis in clathrin-coated vesicles (Grab *et al.*, 1992; Liu *et al.*, 2000; Merschjohann & Steverding, 2006; Natesan *et al.*, 2007). The transferrin binding-protein (Tfbp) is encoded by two expression-site-associated genes, *ESAG 6* and *ESAG 7*. The Tf-Tfbp complex is internalized and transported to lysosomes where Tf is proteolytically degraded (Maier & Steverding, 1996).

E. histolytica causes amoebiasis, a disease characterized by dysentery and abscesses in the large intestine and liver. Amoebiasis affects the human population worldwide, mainly in developing countries. This parasite requires about 100 µM iron for growth; thus it has developed mechanisms to scavenge iron, for example through the removal of iron from Hb, holoTf, holoLf and ferritin (León-Sicaireo *et al.*, 2005; López-Soto *et al.*, 2009a, b; Reyes-López *et al.*, 2001; Serrano-Luna *et al.*, 1998). Previously, we reported the detection of *E. histolytica* holoTf-binding proteins (*EhTfbp*) in extracts from *E. histolytica* trophozoites cultured *in vitro*; they were detected using the anti-human transferrin receptor B3/25 mAb. This mAb co-localized with FITC-holoTf on the amoeba membrane and also in the cytoplasm, as observed by confocal laser-scanning microscopy (Reyes-López *et al.*, 2001). In this work we suggest that *E. histolytica* possesses at least two ways to internalize holoTf: one via a specific binding protein, *EhTfbp*, reported here, and another via a receptor-independent process (Welter *et al.*, 2006). It is suggested that, due to its absolute requirement for the iron, the parasite must obtain iron through multiple mechanisms. Our data demonstrate the involvement of the *E. histolytica* acetaldehyde/alcohol dehydrogenase-2 (*EhADH2*) enzyme and clathrin-coated vesicles in the binding and internalization of human holoTf. To our knowledge ADH2 has not been previously found to be involved in holoTf binding in either bacteria or protozoa.

METHODS

Cultures. Trophozoites of *E. histolytica* strain HM-1:IMSS were grown axenically in BI-S-33 medium (Dibico) (Diamond *et al.*, 1978a) supplemented with 16% (v/v) heat-inactivated bovine serum (Microlab). Cultures were grown in glass screw-cap tubes at 37 °C for 48 h and incubated for 6 h in medium without serum before starting the experiments. Tubes were placed in an ice bath for 10 min and the amoebae were harvested by centrifugation at 500 g for 5 min and washed once with PBS. Iron-saturated human transferrin was purchased from Sigma-Aldrich. Cell viability was determined by the exclusion of trypan-blue dye by live cells when observed under a light microscope in a Neubauer chamber.

Purification of *EhTfbp* by immunoprecipitation with an anti-human TfR mAb. Amoebae were suspended in 50 mM HEPES (pH 7) containing an inhibitor cocktail (10 mM *p*-hydroxymercurybenzoate, 3 mM *N*-ethylmaleimide, 1 mM PMSF, 30 mM EDTA) and disrupted by five cycles of freezing and thawing before total protein concentration quantification (Bradford, 1976). Next, 5 µg anti-human TfR mAb H68.4 (Zymed, catalogue no. 13-6800) was added to 500 µg amoebic protein. The mixture was incubated overnight at 4 °C, after which 15 µl Sepharose-coupled protein A was added, followed by further incubation. The precipitate was washed with a buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate (SD), and the inhibitor cocktail, centrifuged for 20 s at 12 000 g and washed again with the above buffer. It was then centrifuged and washed again with the same buffer but without NaCl, centrifuged, boiled for 5 min with 5% β-mercaptoethanol and separated by 10% SDS-PAGE.

To test whether the precipitated *EhTfbp* was still able to bind holoTf, it was electro-transferred (1.5 h, 400 mA) to a nitrocellulose membrane (Towbin *et al.*, 1979), which was blocked for 1 h with 5% non-fat milk in PBS with 0.05% Tween 20 (pH 7.4), incubated overnight at 4 °C with 115 nM HRP-holoTf and washed twice with PBS. The reaction was detected either with 3,3'-diaminobenzidine and H₂O₂ or by chemiluminescence.

Mass spectrometry. To identify the amoebic protein that was able to bind holoTf and could be recognized by the anti-human TfR, the band obtained was excised from the gel and digested *in situ* with trypsin. The digestion products were extracted from the gel fragments and analysed by MS/MS in Qtrap 3200 equipment.

Cross-reaction between *EhTfbp* and *EhADH2*. *EhADH2* was purified and a rabbit antiserum was prepared against the 95 kDa protein band excised from the gel (Avila *et al.*, 2002). To determine whether the purified *EhADH2* and the immunoprecipitated *EhTfbp* were the same protein, they were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane blocked with PBS-T-milk for 1 h at room temperature (RT). *EhTfbp* and *EhADH2* proteins were incubated overnight at 4 °C with anti-*EhADH2* Ab (1:1000) and anti-human TfR H68.4 mAb (1:100). Total amoebic protein, molecular mass markers (Bio-Rad) and a purified non-related protein of *Giardia lamblia* were used as controls. Secondary HRP-anti-rabbit and HRP-anti-mouse Abs were then added and incubated for 2 h at RT. The reaction was detected by chemiluminescence.

The cross-reaction between *EhADH2* and *EhTfbp* was also studied by 2D gel electrophoresis. *EhADH2* was first resolved by IEF in a pH 6–9 strip, using the IPG-Phor System (Invitrogen). The 2D electrophoresis was performed using 10% SDS-PAGE. The gel was transferred to a nitrocellulose membrane and incubated with the anti-human TfR mAb (1:1000) overnight at 4 °C. The membrane was washed and incubated for 2 h at RT with a secondary anti-mouse Ab, and the reaction was revealed by chemiluminescence.

Participation of *EhADH2* in the holoTf binding/internalization

Confocal microscopy. Amoebae (10^4) were incubated for 30 min at 37 °C with anti-*EhADH2* Ab (1:100), washed and then incubated with FITC-holoTf (115 nM) for 1, 2, 5, 10, 15, 20 or 30 min. Next, they were fixed for 30 min with 2 % paraformaldehyde and incubated with tetramethylrhodamine isothiocyanate (TRITC)-anti-rabbit Ab for 1 h. The same experiment was repeated but incubating first with FITC-holoTf and then with anti-*EhADH2* Ab. Samples were mounted in Vectashield medium on glass slides and examined with a Leica TCS-SP2 confocal laser-scanning microscope, observing between 10 and 20 optical sections from each cell.

Flow cytometry. Amoebae (2×10^5) were incubated at 37 °C (allowing holoTf binding and endocytosis to take place) or 4 °C (avoiding endocytosis) with 115 nM FITC-holoTf for 0.5, 1, 5, 15, 30, 45 or 60 min. After incubation, each sample was washed twice, fixed, washed again and suspended in 500 µl PBS for scanning in a FACSsort flow cytometer (Becton Dickinson).

To study the effect of holoTf concentration on its internalization, amoebae were incubated with different concentrations of FITC-holoTf (1.15 fM–115 µM) for 30 min at 37 °C or at 4 °C. They were then treated for scanning in the flow cytometer.

To determine whether *EhADH2* participates in the binding of holoTf to the cell, trophozoite cultures were adjusted to 10^6 cells ml⁻¹ in PBS containing different dilutions of the anti-*EhADH2* Ab (1:1, 1:10, 1:100, and 1:1000) and incubated for 15 min at 37 °C. FITC-holoTf (115 nM) was added to each tube and incubated for additional 15 min. The amoebae were washed, fixed and then suspended in 500 µl PBS for scanning in the flow cytometer. The fluorescence data obtained were compared with those of tests using FITC-apoTf (iron-lacking Tf) and with FITC-holoLf.

Localization of clathrin, *EhADH2* and holoTf in *E. histolytica*. Trophozoite cultures (2×10^5 cells) were incubated for 0, 2, 5, 10, 15 or 30 min with FITC-holoTf (1.15 µM) in medium without serum. Samples were fixed, permeabilized (0.5 % Triton X-100, 10 min), and incubated for 1 h at 37 °C with an anti-bovine brain clathrin Ab (Sigma, cat. no. C8034, 1:50). Then, TRITC-anti-rabbit IgG was added and incubated for 2 h. Samples were washed and examined by confocal microscopy. A negative control with only the secondary Ab was included to test the specificity of the reaction.

A similar experiment was performed using anti-*EhADH2* Ab to observe *EhADH2* alongside clathrin during holoTf internalization. In addition, the anti-human TfR Ab was used in cells incubated with holoTf and stained for clathrin.

Effect of endocytosis inhibitors on holoTf internalization. First, the maximal concentration of inhibitor that would not affect cell viability was determined. Amoebae (10^6) were incubated for 30 min at 37 °C with the following inhibitors: 1–10 % sucrose; 0.5–100 mM chloroquine diphosphate; 0.1–10 µM filipin; 1–2 µM colchicine; 10 µM cytochalasin D; and 50–300 µM wortmannin. Fresh medium containing 1.15 µM FITC-holoTf along with the inhibitor to be tested was added, and the amoebae were incubated for another 30 min. The samples were fixed, washed and prepared for scanning by flow cytometry.

For confocal microscopy the same treatment was employed but with the amoebae being placed onto glass slides and allowed to adhere at 37 °C for 15 min before treatment with inhibitors and FITC-holoTf. The samples were then fixed, washed and prepared with Vectashield. Between ten and twenty optical sections were observed from each cell.

RESULTS

Purification of *EhTfbp* and its identification by mass spectrometry

A polypeptide of 95 kDa was the main protein precipitated from amoeba extracts with the anti-TfR H68.4 mAb (Fig. 1a, lane 1); this polypeptide was still able to bind HRP-holoTf (Fig. 1a, lane 2). When the polypeptide was analysed by MS/MS, and after searching in public databases with ProteinPilot software, two peptides, TRNPVFSFHPSALK and KIFIVSDRM, were identified with 99 % confidence in two independent experiments. The two peptides belong to *EhADH2* (ADH2_ENTHI with accession number Q24803) previously described by Yang *et al.*, (1994), Bruchhaus & Tannich, (1994) and Avila *et al.*, (2002).

Cross-reaction between *EhTfbp* and *EhADH2*

Since the MS analyses of *EhTfbp* revealed a high identity with *EhADH2*, the latter protein was purified and an anti-*EhADH2* Ab was used to find out whether *EhTfbp* and *EhADH2* are related. Both proteins showed the same molecular mass by SDS-PAGE (Fig. 1a, lanes 1 and 4, respectively) and were recognized by the anti-*EhADH2* Ab (Fig. 1a, lanes 3 and 5, respectively). *EhADH2* was also recognized by the anti-human TfR mAb (Fig. 1a, lane 6). To determine whether other non-related proteins are detected by these antibodies, amoebic total protein extracts (Fig. 1a, lane 7), were analysed. A main band of 95 kDa was recognized by the anti-*EhADH2* Ab and the anti-human TfR mAb, and it also bound biotinylated holoTf (Fig. 1a, lanes 8, 9 and 10, respectively), which was observed by using the purified proteins. A protein from *Giardia lamblia* that is not involved in Tf internalization (Fig. 1a, lanes 11 and 12) and non-related molecular mass marker proteins (Fig. 1a, lanes 13–15) were used as controls; none of them were recognized by the anti-human TfR mAb (Fig. 1a, lanes 12–15). These results suggest that *EhTfbp* and *EhADH2* are either similar or strongly related. When trophozoites were incubated without iron or in the presence of holoTf, the 95 kDa protein was still observed and in a similar quantity, suggesting constitutive expression of *EhTfbp* (data not shown).

To rule out any non-specific signal during 1D gel electrophoresis, an IEF experiment was performed with the purified *EhADH2*. Three different isoforms with pIs of 6.6, 6.52 and 6.32 were obtained (Fig. 1b, I), which were recognized by the anti-TfR mAb (Fig. 1b, II). The third isoform displayed a higher affinity to the anti-TfR mAb than the others. This result is consistent with the results of the previous cross-reactions, suggesting that the proteins are either the same or at least share high similarity.

EhADH2 is involved in the binding/internalization of human holoTf by *E. histolytica*

Confocal microscopy. The *EhADH2* protein was localized by using the anti-*EhADH2* Ab. In permeabilized amoebae,

Fig. 1. (a) Cross-reaction between *EhTfbp* and *EhADH2*. Lanes 1–3: *EhTfbp*, 95 kDa polypeptide immunoprecipitated by using an anti-human TfR mAb. 1, Coomassie blue staining; 2, overlay with HRP-holoTf; 3, Western blot with anti-*EhADH2* Ab. Lanes 4–6: purified *EhADH2*. 4, Coomassie blue staining; 5, Western blot with anti-*EhADH2* Ab; 6, Western blot with anti-human TfR mAb. Lanes 7–10: *E. histolytica* total protein. 7, Coomassie blue staining; 8, Western blot with anti-*EhADH2* Ab; 9, Western blot with anti-human TfR mAb; 10, overlay with biotinylated holoTf. Lanes 11 and 12: a non-related protein purified from *Giardia lamblia*. 11, Coomassie blue staining; 12, Western blot with anti-human TfR mAb. Lanes 13–15: molecular mass markers (MMM). 13, Coomassie blue staining; 14, Western blot with anti-human TfR mAb; 15, Western blot with anti-*EhADH2* Ab. Lanes 16 and 17: Western blot of *E. histolytica* total protein only with the secondary Ab. 16, HRP-anti-mouse IgG; 17, HRP-anti-rabbit IgG. (b) 2D gel electrophoresis of *EhADH2*. (I) *EhADH2* isoforms stained with Coomassie blue after being separated in the first dimension by IEF (pH 6–9) and in the second dimension by 10% SDS-PAGE. (II) Proteins were transferred to a nitrocellulose membrane and incubated with anti-human TfR mAb. They were then incubated with a secondary HRP-anti-mouse Ab and revealed by chemiluminescence.

EhADH2 was shown to be distributed in the cell membrane and in the cytoplasm (Fig. 2a, red), as was reported by Flores *et al.*, (1996) and Avila *et al.*, (2002). In non-permeabilized amoebae, *EhADH2* was observed to be bound only on the cell membrane (Fig. 2b). To determine whether *EhADH2* participates in the binding/internalization of holoTf, amoebae were incubated with or without anti-*EhADH2* Ab in the presence of FITC-holoTf, and observed over time. Fig. 2(c) shows holoTf in vesicles (green). When amoebae were first incubated with anti-*EhADH2* Ab (Fig. 2d), the holoTf internalization and Ab recognition was diminished, demonstrated by few green vesicles and a very low red signal, respectively. This colocalization was probably due to a small fraction of holoTf still being bound to *EhADH2* (Fig. 2d). This result suggests that the anti-*EhADH2* Ab blocked the binding/internalization of holoTf. Interestingly, under these conditions, a small amount of holoTf seemed to diffuse into the cytoplasm, suggesting that it was internalized independent of a binding protein, perhaps by fluid-phase endocytosis. A similar blocking of internalization was observed when amoebae were incubated with FITC-holoTf and then with anti-*EhADH2* Ab (not shown). However, when amoebae were incubated with anti-human TfR mAb and then with holoTf, the blocking effect was not observed (not shown).

Importantly, FITC-apoTf was neither bound nor internalized by amoebae without (Fig. 2e) or with (Fig. 2f) anti-*EhADH2* Ab, suggesting that the presence of iron is necessary for this interaction.

Flow cytometry. HoloTf binding/internalization in *E. histolytica* was evaluated by incubation with FITC-holoTf at 37 and 4 °C (Fig. 3a). At 37 °C, amoebae showed rapid and continued endocytosis of holoTf, and by ~15 min of incubation saturation levels were reached. At 4 °C, however, very low fluorescence was detected at all stages of incubation due to the lack of internalization. This implies that entry of holoTf is regulated and, at temperatures allowing internalization, eventually limited by the presence of a Tfbp on the cell surface, rather than being internalized passively or via an alternative mechanism.

Effect of holoTf concentration on its binding/internalization

Amoebae were incubated with different concentrations of FITC-holoTf to evaluate the effect on holoTf binding/internalization. At 37 °C, holoTf was regularly internalized up to 11.5 nM, at which point the amoebae achieved saturation; however, at 4 °C the holoTf internalization was

Fig. 2. Immunolocalization of *EhADH2* by confocal microscopy. Amoebae were incubated with anti-*EhADH2* Ab. (a) Permeabilized and (b) non-permeabilized amoebae. (c) Amoebae incubated with FITC-holoTf. (d) Amoebae incubated with anti-*EhADH2* Ab and then with FITC-holoTf. (e) Amoebae incubated with FITC-apoTf. (f) Amoebae incubated with anti-*EhADH2* Ab and then with FITC-apoTf. Trophozoites were fixed and incubated with TRITC-anti-rabbit IgG.

reduced and saturation of the cell-membrane binding sites was reached at very low holoTf concentration (Fig. 3b). Furthermore, at higher holoTf concentrations, internalization seems to be independent of a Tfbp because no

saturation effect was observed at either temperature (not shown).

To determine the presence of a Tfbp, holoTf binding/internalization was quantified in trophozoites incubated with different dilutions of anti-*EhADH2* Ab and then with FITC-holoTf (Fig. 4). The fluorescence obtained was compared with that of FITC-apoTf and with FITC-holoLf (a protein that *E. histolytica* binds and internalizes by a filipin-inhibitable mechanism) (León-Sicaireo *et al.*, 2005). The anti-*EhADH2* Ab blocked the holoTf binding/internalization, confirming that this enzyme participates specifically in holoTf recognition. In contrast, holoLf internalization was not inhibited by the anti-*EhADH2* Ab, even at high concentration (1 : 1 dilution). In addition, apoTf showed almost no binding to the amoebae, suggesting that iron is a requisite for the binding process; this verifies the results obtained by confocal microscopy.

Clathrin participates in the internalization of holoTf by *E. histolytica*

To find out whether the protein clathrin is involved in holoTf internalization in *E. histolytica*, we used an anti-bovine brain clathrin Ab. This Ab recognized clathrin vesicles mainly on the cell-surface membrane (Fig. 5b, red). At the beginning of the holoTf entry process, FITC-holoTf (Fig. 5c, green) co-localized with clathrin-coated vesicles (Fig. 5d, yellow). After longer periods of time (15–45 min), holoTf and clathrin were found to be separately localized (not shown). To test the specificity of the reaction, a negative control with only the secondary Ab was included (not shown). This result suggests that holoTf is endocytosed via clathrin-coated pits.

To investigate whether *EhADH2* interacts with clathrin vesicles, the amoebae were incubated with non-labelled holoTf for 2 min at 37 °C, fixed and treated with anti-*EhADH2* Ab (Fig. 5e, red) and after that with the

Fig. 3. Binding and internalization of holoTf reaches saturation, and is temperature, time, and concentration-dependent in *E. histolytica*. Amoebae (2×10^5 cells) were incubated with FITC-holoTf (115 nM) for the indicated times (a) and with the indicated concentration of FITC-holoTf (b). They were then fixed, washed, and analysed by flow cytometry.

Fig. 4. Effect of anti-*EhADH2* Ab on the binding/internalization of holoTf. Amoebae were incubated with anti-*EhADH2* Ab for 15 min and then with FITC-holoTf (hTf), FITC-apoTf (aTf) or FITC-holoLf (hLf) for 15 min. Fluorescence was then quantified by flow cytometry. * $P \leq 0.001$; ** $P \leq 0.05$, compared with holoTf.

anti-clathrin Ab (Fig. 5f, green). We did not observe co-localization of clathrin and the enzyme; while *EhADH2* was observed on the cytoplasmic membrane, clathrin was distributed throughout the cytoplasm.

Inhibitors of clathrin-coated vesicle endocytosis block the internalization of holoTf

The inhibitor wortmannin affected holoTf endocytosis considerably (91 % inhibition) (Fig. 6a, V). Specific inhibitors of clathrin-dependent endocytosis, chloroquine and sucrose (Fig. 6a, III and IV, respectively), blocked the entry of holoTf into the amoebae by 80–85 %. In contrast, filipin (Fig. 6a, VI) did not affect Tf internalization.

Cytochalasin D, which affects the integrity of the actin cytoskeleton, inhibited the holoTf endocytosis by 85 % (Fig. 6b). However, colchicine had a minor effect (30 % inhibition). These results indicate that one route of access for holoTf is clathrin- and actin-dependent. Therefore, this parasite increases its ability to endocytose holoTf by constitutively expressing clathrin in coated pits.

DISCUSSION

E. histolytica trophozoites depend on host iron for their growth. Given the diverse habitats it occupies in the human body during the course of infection, it would be

Fig. 5. Participation of clathrin in holoTf endocytosis in *E. histolytica*. Amoebae were incubated with 1.15 μ M FITC-holoTf for different times. Samples were incubated with a rabbit anti-bovine brain clathrin antibody and then TRITC-anti-rabbit IgG was added. (a) Differential interference contrast microscopy (Nomarski) of amoebae. (b) Amoebae with anti-clathrin Ab. (c) Amoebae with FITC-holoTf. (d) Co-localization of both anti-clathrin Ab and FITC-holoTf (yellow) at 1 min of incubation. (e) Amoebae with anti-*EhADH2* Ab. (f) Amoebae with anti-*EhADH2* Ab and anti-clathrin Ab.

Fig. 6. Effect of endocytosis inhibitors on holoTf internalization. Amoebae (10^6 cells) were incubated with inhibitors and then with $1.15 \mu\text{M}$ FITC-holoTf plus inhibitors; they were then prepared for both confocal microscopy and flow cytometry. (a) Confocal microscopy. I and II, HoloTf without inhibitors; III, 50 mM chloroquine; IV, 5% sucrose; V, $50 \mu\text{M}$ wortmannin; VI, $10 \mu\text{M}$ filipin. The same concentration of inhibitors was used for both confocal microscopy and flow cytometry analysis (b).

advantageous for the parasite to possess several mechanisms that can acquire iron from multiple sources, including holoTf. The physiological role of Tf-iron in amoebiasis has not yet been elucidated. Patients suffering hepatic abscesses show lower iron levels and a lower percentage of Tf saturation in serum than healthy controls. Thus, the host reacts by inducing a hypoferrhaemic state, a protective response similar to that of bacterial infections (Diamond *et al.*, 1978b). For amoebae, holoTf could be important during the invasive process because this parasite crosses the natural barriers of the intestine and spreads into other organs via the blood vessels.

We previously reported an amoebic protein recognized by the anti-human TfR mAb B3/25, which was prepared against the TfR extracellular region (Reyes-López *et al.*, 2001). This mAb was discontinued by the supplier, so the H68.4 mAb, which recognizes TfR cytoplasmic residues, was used instead. This mAb detected a 96 kDa protein in the amoebae that binds holoTf. The EhTfbp was purified and identified by mass spectrometry as EhADH2. Given that two EhTfbp peptides were 99% identical to EhADH2 (results were obtained in two independent experiments), and that there is no significant similarity between the EhADH2 amino acid sequence and that predicted for the human TfR site, recognition by the anti-TfR mAb could only be explained by certain structural similarities.

The growth and survival of *E. histolytica* trophozoites depend absolutely on EhADH2; it is used by amoebae to obtain energy through glucose fermentation and converts acetyl-CoA into ethanol. EhADH2 has been purified and studied by several groups (Avila *et al.*, 2002; Bruchhaus & Tannich, 1994; Chen *et al.*, 2004; Espinosa *et al.*, 2001, 2004, 2009; Flores *et al.*, 1996; Yang *et al.*, 1994) and has the ability to bind to extracellular matrix (ECM) proteins. Interestingly, it is a typical cytoplasmic enzyme, although it is also found in the cell membrane. Due to its requirement for iron, it has been proposed that iron chelators could be used as anti-amoebics (Espinosa *et al.*, 2009).

EhADH2 shares a striking sequence similarity with several acetaldehyde dehydrogenase (ADH) enzymes of prokaryotic origin that possess both ADH and alcohol dehydrogenase (ALDH) activities, such as the *Clostridium acetobutylicum* alcohol/aldehyde dehydrogenase (AAD) and the *Escherichia coli* and *Lactococcus lactis* ADH enzymes. This similarity suggested an analogous functional organization in the amoebic enzyme (Bruchhaus & Tannich, 1994) and its essential character was demonstrated by lack of growth in its absence as well as by an ALDH activity dependent on an intact ADH activity (Espinosa *et al.* 2001). The fact that the predicted amino acid sequences of EhADH2 and *E. coli* ADHE are 48% identical (Yang *et al.*, 1994), and that the EhADH2 gene

displays a 90 % AT bias in its third codon position – a feature characteristic of *Entamoeba* spp. – indicates that this protein may have been derived from a common prokaryotic ancestor and acquired by horizontal gene transfer (Andersson *et al.*, 2006) coupled with vertical evolution (mutational rate tied with natural selection) within *Entamoeba* spp. (Paz-y-Miño & Espinosa, 2010). The identification of the *EhADH2* protein as a member of the ADHE-like family provides evidence for similar enzymic mechanisms in the fermentation pathway in the gut inhabited by anaerobic protozoa and bacteria, as was reported for other enzymes of the glycolytic pathway (Reeves *et al.*, 1977).

In this work, we found that the *EhTfbp* shares epitopes with *EhADH2*. In addition, *EhADH2*-bound holoTf and its three isoforms (which were also purified and identified by mass spectrometry, not shown), were recognized by the anti-human TfR mAb. The binding of *EhADH2* to holoTf could be explained by the iron content since the predicted sequence of *EhADH2* does not have a binding site for holoTf. ApoTf was not bound by the *E. histolytica* trophozoites; this demonstrates that amoebae can discriminate between Tfs containing and not containing iron, perhaps through *EhADH2*, which needs iron for its activity. In a similar way, it was suggested that the staphylococcal GAPDH participates in holoTf binding by removing iron (Modun & Williams, 1999). In *Trichomonas vaginalis* GAPDH is upregulated in iron-depleted conditions (Lama *et al.*, 2009), suggesting that it could utilize the enzyme with the purpose of sequestering iron. In *E. histolytica*, *EhADH2* would be acting as a receptor for obtaining iron and as a binding protein for ECM components. Other glycolytic enzymes have also been shown to have several functions, such as enolase, which regulates the activity of cytosine 5-methyltransferase 2 (Dnmt2), an enzyme that catalyses DNA and tRNA methylation in *E. histolytica* (Tovy *et al.*, 2010). This multi-functionality could confer additional advantages for tissue invasion.

At 37 °C, holoTf binding and endocytosis was initially extremely fast and remained continuous until 15 min, at which point saturation was reached; at this temperature, amoebic cytoplasmic-membrane molecules are in constant turnover, and the new Tfbps are actively binding and endocytosing the ligand. This effect, observed by confocal microscopy (Reyes-López *et al.*, 2001), suggests that the Tfbp and an endocytosis mechanism could be involved in regulating the uptake and utilization of holoTf by trophozoites. Since the holoTf binding/internalization was continuous and also saturable, dependent on time, temperature and holoTf concentration, our results support the hypothesis that the process of endocytosis is Tfbp- or receptor-dependent. However, when the endocytic process was followed using higher holoTf concentrations, it was also Tfbp-independent. This result is in agreement with Welter *et al.* (2006), who did not obtain saturation when using concentrations of holoTf in the micromolar range.

This result, together with the the protein's apparent high affinity for holoTf (1.81×10^{-9} M), suggests that the process involves a Tfbp in the parasite. In addition, amoebae showed two affinity constants for holoTf (1.1 and 5.7 nM), perhaps due to the presence of two Tfbps or a single protein with two affinities. In bacteria, the affinity for holoTf was reported to be lower (0.7×10^{-7} or 4×10^{-7} M) (Pintor *et al.*, 1993), and in the protozoan *T. cruzi* the affinity was even smaller (about 2.8×10^{-6} M) (Testa, 2002). The comparatively high affinity of amoebae for holoTf suggests that the parasite has a greater ability to bind human holoTf to obtain iron.

Since at 37 °C both the binding and endocytosis mechanisms are active, we also performed incubations at 4 °C in order to only detect the binding. At this low temperature there is no membrane turnover, even in *E. histolytica*, which normally has an extremely active endocytic process (Bailey *et al.*, 1985; Gitler & Mirelman, 1986; Meza & Clarke, 2004). Trophozoites showed very low fluorescence, probably due to the saturation of a specific *EhTfbp*, and because they did not internalize holoTf at 4 °C.

When amoebae were incubated with anti-*EhADH2* Ab, the binding/internalization of holoTf was inhibited. This effect can be explained by the anti-*EhADH2* Ab and holoTf sharing a binding site on *EhTfbp*; alternatively, steric hindrance is also possible. This result suggests that *EhADH2* participates in the binding of holoTf to the cell. HoloLf (a protein that shares 60 % identity with holoTf) does not share the *EhTfbp*, because the anti-*EhADH2* Ab blocked only the holoTf binding, showing that this enzyme binds holoTf specifically. Consistent with this, in a previous study we reported that holoLf binding was not blocked by holoTf in amoebae (León-Sicairens *et al.*, 2005). The anti-human TfR mAb also did not block holoTf binding to *EhTfbp*. This suggests that the TfR mAb requires a different holoTf binding site from that of the *EhTfbp*.

Endocytosis is a broadly conserved mechanism in cells of higher organisms and protozoa (Hernandez *et al.*, 2007; Morgan *et al.*, 2001; Robibaro *et al.*, 2001). Endocytosis of ferritin through clathrin-coated vesicles has been described in *E. histolytica* (López-Soto *et al.*, 2009a). Caveolin-like protein is present in amoebae and is apparently involved in holoLf endocytosis (León-Sicairens *et al.*, 2005). Other proteins implicated in endocytosis, such as Rab GTPases, have been found in amoebae (Juárez *et al.*, 2001; Romero-Díaz *et al.*, 2007; Temesvari *et al.*, 1999; Welter *et al.*, 2002). As we found that the uptake of holoTf was likely to be dependent on a receptor, we studied the holoTf endocytosis mechanism by using a number of inhibitors. Wortmannin, which inhibits phosphatidylinositol 3-kinase (PI3K) activity, inhibited holoTf internalization by about 90 %. Since PI3K contributes to various endocytosis mechanisms in *E. histolytica*, such as phagocytosis (Ghosh & Samuelson, 1997; Marion *et al.*, 2005; Nakada-Tsukui *et al.*, 2009), pinocytosis (Batista & de Souza, 2004) and

macropinocytosis (Meza & Clarke, 2004), the high level of inhibition observed in holoTf internalization suggests that holoTf could be internalized through several pathways.

To test whether holoTf is endocytosed via clathrin-coated vesicles, chloroquine, a specific inhibitor of this kind of endocytosis, which prevents the separation of nascent clathrin-coated pits from the plasma membrane, was used; it gave ~80 % inhibition. Sucrose, which specifically prevents receptor-mediated uptake in polymorphonuclear leukocytes (Daukas & Zigmond, 1985) and inhibits clathrin-coated pit formation (Heuser & Anderson, 1989), diminished holoTf internalization by 85 % in *E. histolytica*. This result together with that of the wortmannin experiment suggests that holoTf mainly enters via the clathrin route and relies to a lesser extent on other mechanisms. Recently, it was reported that holoTf could be internalized by fluid-phase endocytosis in *E. histolytica* (Welter *et al.*, 2006). Similarly, in rat hepatocytes, it was found that holoTf is endocytosed by two pathways (Qian & Tang, 1995; Richardson & Ponka, 1997).

HoloTf endocytosis decreased by about 87 % in amoebae treated with cytochalasin D, suggesting that microfilaments are crucially important for the endocytosis of this ligand. The connection between receptor-mediated endocytosis and the actin cytoskeleton during the formation and detachment of newly formed coated pits from the plasma membrane has been observed in other cells (Neuhaus *et al.*, 2002; Qualmann *et al.*, 2000; Slepnev & De Camilli, 2000). In *E. histolytica*, actin has been implicated in phagocytosis (Marion *et al.*, 2005), fluid-phase endocytosis (Sahoo *et al.*, 2004), vesicle exocytosis (Ravdin *et al.*, 1988), erythrophagocytosis (de la Garza *et al.*, 1989; Marion *et al.*, 2005) and macropinocytosis (Meza & Clarke, 2004). However, microtubules do not seem to be involved in holoTf endocytosis as a high colchicine concentration had no effect on this process. Nevertheless, microtubules participate in holoTf endocytosis in liver sinusoidal endothelial cells, in which clathrin-dependent vesicles are organized by microtubules (Falkowska-Hansen *et al.*, 2007).

To obtain further evidence for the involvement of clathrin in holoTf endocytosis, a heterologous anti-clathrin Ab was used. Both holoTf and clathrin co-localized at 2–3 min after internalization was started. Hence, holoTf is internalized inside structures containing clathrin, which can then travel through the endocytic route. To see if *EhTfbp* is internalized with holoTf as a complex inside the clathrin-structures, amoebae supplied with holoTf were incubated with anti-*EhADH2* and anti-clathrin Abs. The results revealed that *EhTfbp* did not co-localize with clathrin in amoebae incubated with holoTf at any time tested. Perhaps holoTf interacts rapidly with *EhTfbp* because even at shorter times the blocking effect could be observed. It is also possible that holoTf might bind to *EhTfbp* and then to another protein or receptor before being internalized in clathrin-coated vesicles.

In conclusion, our findings, together with those reported for holoTf use in *E. histolytica*, show the following. (1) Iron

is important for amoebic growth and enzymic activities in this parasite. (2) Trophozoites bind, internalize and use holoTf as an iron source but do not bind or internalize apoTf, perhaps due to its lack of iron. (3) The *EhTfbp* is recognized by two different anti-human TfR mAbs, the B3/25 mAb, which forms a complex with holoTf that is then internalized, and the H.68.4 mAb, which was observed co-localizing with holoTf only at the amoebic surface. (4) The protein recognized by the H.68.4 mAb corresponds to the *EhADH2* enzyme, which is essential for the survival of amoebae and uses iron as a cofactor, indispensable for structural and functional stability. (5) HoloTf is bound with a high affinity by amoebae and this binding is not blocked by apoTf or holoLf, suggesting the presence of a specific *EhTfbp*. (6) Amoebae can also bind holoTf with low affinity. (7) HoloTf internalization is time-, temperature- and concentration-dependent. Finally, (8) although fluid phase endocytosis may also participate in this process, holoTf internalization is carried out mainly through the endocytosis of clathrin-coated vesicles. We propose that *E. histolytica* obtains iron from holoTf through a specific and elaborate pathway that could involve several proteins, including the *EhADH2* enzyme, which discriminates between ferric and non-ferric Tf. Additionally, another protein could be necessary to internalize holoTf in clathrin-coated vesicles.

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REFERENCES

- Andersson, J. O., Hirt, R. P., Foster, P. G. & Roger, A. J. (2006). Evolution of four gene families with patchy phylogenetic distributions: influx of genes into protist genomes. *BMC Evol Biol* **6**, 27.
- Avila, E. E., Martínez-Alcaráz, E., Barbosa-Sabanero, G., Rivera-Baron, E., Arias-Negrete, S. & Zazueta-Sandoval, R. (2002). Subcellular localization of the NAD⁺-dependent alcohol dehydrogenase in *Entamoeba histolytica* trophozoites. *J Parasitol* **88**, 217–222.
- Bailey, G. B., Day, D. B. & Gasque, J. W. (1985). Rapid polymerization of *Entamoeba histolytica* actin induced by interaction with target cells. *J Exp Med* **162**, 546–558.
- Batista, E. J. & de Souza, W. (2004). Involvement of protein kinases on the process of erythrophagocytosis by *Entamoeba histolytica*. *Cell Biol Int* **28**, 243–248.
- Bierne, H., Garandeau, C., Pucciarelli, M. G., Sabet, C., Newton, S., Garcia-del Portillo, F., Cossart, P. & Charbit, A. (2004). Sortase B, a new class of sortase in *Listeria monocytogenes*. *J Bacteriol* **186**, 1972–1982.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Bruchhaus, I. & Tannich, E. (1994). Purification and molecular characterization of the NAD⁺-dependent acetaldehyde/alcohol dehydrogenase from *Entamoeba histolytica*. *Biochem J* **303**, 743–748.

- Bullen, J. J., Rogers, H. J. & Griffiths, E. (1978). Role of iron in bacterial infection. *Curr Top Microbiol Immunol* **80**, 1–35.
- Chen, M., Li, E. & Stanley, S., Jr (2004). Structural analysis of the acetaldehyde dehydrogenase activity of *Entamoeba histolytica* alcohol dehydrogenase 2 (EhADH2), a member of the ADHE enzyme family. *Mol Biochem Parasitol* **137**, 201–205.
- Corrêa, J. R., Atella, G. C., Menna-Barreto, R. S. & Soares, M. J. (2007). Clathrin in *Trypanosoma cruzi*: in silico gene identification, isolation, and localization of protein expression sites. *J Eukaryot Microbiol* **54**, 297–302.
- Corrêa, J. R., Atella, G. C., Batista, M. M. & Soares, M. J. (2008). Transferrin uptake in *Trypanosoma cruzi* is impaired by interference on cytosome-associated cytoskeleton elements and stability of membrane cholesterol but not by the obstruction of clathrin-dependent endocytosis. *Exp Parasitol* **119**, 58–66.
- Daukas, G. & Zigmond, S. (1985). Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. *J Cell Biol* **101**, 1673–1679.
- Dautry-Varsat, A., Ciechanover, A. & Lodish, H. (1983). pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc Natl Acad Sci U S A* **80**, 2258–2262.
- de la Garza, M., Gallegos, B. & Meza, I. (1989). Characterization of a cytochalasin D-resistant mutant of *Entamoeba histolytica*. *J Protozool* **36**, 556–560.
- Diamond, L. S., Harlow, D. R. & Cunnick, C. C. (1978a). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg* **72**, 431–432.
- Diamond, L. S., Harlow, D. R., Phillips, B. P. & Keister, D. B. (1978b). *Entamoeba histolytica*: iron and nutritional immunity. *Arch Invest Med (Mex)* **9**, 329–338.
- Espinosa, A., Yan, L., Zhang, Z., Foster, L., Clark, D., Li, E. & Stanley, S. L., Jr (2001). The bifunctional *Entamoeba histolytica* alcohol dehydrogenase 2 (EhADH2) protein is necessary for amoebic growth and survival and requires an intact C-terminal domain for both alcohol dehydrogenase and acetaldehyde dehydrogenase activity. *J Biol Chem* **276**, 20136–20143.
- Espinosa, A., Clark, D. & Stanley, S., Jr (2004). *Entamoeba histolytica* alcohol dehydrogenase 2 (EhADH2) as a target for anti-amoebic agents. *J Antimicrob Chemother* **54**, 56–59.
- Espinosa, A., Pedrizet, G., Paz-Y-Miño, C. G., Lanfranchi, R. & Phay, M. (2009). Effects of iron depletion on *Entamoeba histolytica* alcohol dehydrogenase 2 (EhADH2) and trophozoite growth: implications for antiamoebic therapy. *J Antimicrob Chemother* **63**, 675–678.
- Falkowska-Hansen, B., Falkowski, M., Metharom, P., Kronic, D. & Goerdts, S. (2007). Clathrin-coated vesicles form a unique net-like structure in liver sinusoidal endothelial cells by assembling along undisturbed microtubules. *Exp Cell Res* **313**, 1745–1757.
- Flores, B. M., Stanley, S., Jr, Yong, T., Ali, M., Yang, W., Diedrich, D. & Torian, B. (1996). Surface localization, regulation and biologic properties of the 96 kDa alcohol/aldehyde dehydrogenase (EhADH2) of pathogenic *Entamoeba histolytica*. *J Infect Dis* **173**, 226–231.
- Gat, O., Zaide, G., Inbar, I., Grosfeld, H., Chitlaru, T., Levy, H. & Shafferman, A. (2008). Characterization of *Bacillus anthracis* iron-regulated surface determinant (Isd) proteins containing NEAT domains. *Mol Microbiol* **70**, 983–999.
- Genco, C. A. & Desai, P. J. (1996). Iron acquisition in the pathogenic *Neisseria*. *Trends Microbiol* **4**, 179–184.
- Ghosh, S. K. & Samuelson, J. (1997). Involvement of p21^{racA}, phosphoinositide 3-kinase, and vacuolar ATPase in phagocytosis of bacteria and erythrocytes by *Entamoeba histolytica*: suggestive evidence for coincidental evolution of amoebic invasiveness. *Infect Immun* **65**, 4243–4249.
- Gitler, C. & Mirelman, D. (1986). Factors contributing to the pathogenic behaviour of *Entamoeba histolytica*. *Annu Rev Microbiol* **40**, 237–261.
- Grab, D. J., Wells, C. W., Shaw, M. K., Webster, P. & Russo, D. C. (1992). Endocytosed transferrin in African trypanosomes is delivered to lysosomes and may not be recycled. *Eur J Cell Biol* **59**, 398–404.
- Hernandez, Y., Castillo, C., Roychowdhury, S., Hehd, A., Aley, S. B. & Dasc, S. (2007). Clathrin-dependent pathways and the cytoskeleton network are involved in ceramide endocytosis by a parasitic protozoan, *Giardia lamblia*. *Int J Parasitol* **37**, 21–32.
- Heuser, J. E. & Anderson, R. G. (1989). Hypertonic media inhibit receptor mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108**, 389–400.
- Juárez, P., Sánchez-López, R., Stock, R. P., Olvera, A., Ramos, M. A. & Alagón, A. (2001). Characterization of the *Ehrab8* gene, a marker of the late stages of the secretory pathway of *Entamoeba histolytica*. *Mol Biochem Parasitol* **116**, 223–228.
- Lama, A., Kucknoor, A., Mundodi, V. & Alderete, J. (2009). Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of *Trichomonas vaginalis*. *Infect Immun* **77**, 2703–2711.
- León-Sicaire, N., Reyes-López, M., Canizalez-Roman, A., Bermúdez-Cruz, R. M., Serrano-Luna, J., Arroyo, R. & de la Garza, M. (2005). Human holo-lactoferrin: endocytosis and use as an iron source by the parasite *Entamoeba histolytica*. *Microbiology* **151**, 3859–3871.
- Liu, J., Qiao, X., Du, D. & Lee, M. G. (2000). Receptor-mediated endocytosis in the procyclic form of *Trypanosoma brucei*. *J Biol Chem* **275**, 12032–12040.
- Loftus, B., Anderson, I., Davies, R., Alsmark, U. C., Samuelson, J., Amedeo, P., Roncaglia, P., Berriman, M., Hirt, R. P. & other authors (2005). The genome of the protist parasite *Entamoeba histolytica*. *Nature* **433**, 865–868.
- López-Soto, F., González-Robles, A., Salazar-Villatoro, L., León-Sicaire, N., Piña-Vázquez, C., Pérez, E. & de la Garza, M. (2009a). *Entamoeba histolytica* uses ferritin as an iron source and internalises this protein by means of clathrin-coated vesicles. *Int J Parasitol* **39**, 417–426.
- López-Soto, F., León-Sicaire, N., Reyes-López, M., Serrano-Luna, J., Ordaz-Pichardo, C., Piña-Vázquez, C., Ortiz-Estrada, G. & de la Garza, M. (2009b). Use and endocytosis of iron-containing proteins by *Entamoeba histolytica* trophozoites. *Infect Genet Evol* **9**, 1038–1050.
- Maier, A. & Steverding, D. (1996). Low affinity of *Trypanosoma brucei* transferrin receptor to apo-transferrin at pH 5 explains the fate of the ligand during endocytosis. *FEBS Lett* **396**, 87–89.
- Maresso, A. W. & Schneewind, O. (2006). Iron acquisition and transport in *Staphylococcus aureus*. *Biomaterials* **19**, 193–203.
- Marion, S., Laurent, C. & Guillen, N. (2005). Signalization and cytoskeleton activity through myosin IB during the early steps of phagocytosis in *Entamoeba histolytica*: a proteomic approach. *Cell Microbiol* **7**, 1504–1518.
- Mazmanian, S. K., Skaar, E. P., Gaspar, A. H., Humayun, M., Gorincki, P., Jalenska, J., Joachmiak, A., Missiakas, D. M. & Schneewind, O. (2003). Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* **299**, 906–909.
- Merschjohann, K. & Steverding, D. (2006). *In vitro* growth inhibition of bloodstream forms of *Trypanosoma brucei* and *Trypanosoma congolense* by iron chelators. *Kinetoplastid Biol Dis* **5**, 3.
- Meza, I. & Clarke, M. (2004). Dynamics of endocytic traffic of *Entamoeba histolytica* revealed by confocal microscopy and flow cytometry. *Cell Motil Cytoskeleton* **59**, 215–226.

- Modun, B. & Williams, P. (1999). The staphylococcal transferrin-binding protein is a cell wall glyceraldehyde-3-phosphate dehydrogenase. *Infect Immun* **67**, 1086–1092.
- Morgan, G. W., Allen, C. L., Jefries, T. R., Hollinshead, M. & Field, M. C. (2001). Developmental and morphological regulation of clathrin-mediated endocytosis in *Trypanosoma brucei*. *J Cell Sci* **114**, 2605–2615.
- Nakada-Tsukui, K., Okada, H., Mitra, B. N. & Nozaki, T. (2009). Phosphatidylinositol-phosphates mediate cytoskeleton reorganization during phagocytosis via a unique modular protein consisting of RhoGEF/DH and FYVE domains in the parasitic protozoan *Entamoeba histolytica*. *Cell Microbiol* **11**, 1471–1491.
- Natesan, S. K., Peacock, L., Matthews, K., Gibson, W. & Field, M. C. (2007). Activation of endocytosis as an adaptation to the mammalian host by trypanosomes. *Eukaryot Cell* **6**, 2029–2037.
- Neuhaus, E. M., Almers, W. & Soldati, T. (2002). Morphology and dynamics of the endocytic pathway in *Dictyostelium discoideum*. *Mol Biol Cell* **13**, 1390–1407.
- Paz-y-Miño, C. G. & Espinosa, A. (2010). Integrating horizontal gene transfer and common descent to depict evolution and contrast it with “Common Design”. *J Eukaryot Microbiol* **57**, 11–18.
- Pintor, M., Ferreira, C. M. & Criado, M. T. (1993). Characterization of the transferrin-iron uptake system in *Neisseria meningitidis*. *FEMS Microbiol Lett* **112**, 159–165.
- Qian, Z. M. & Tang, P. L. (1995). Mechanisms of iron uptake by mammalian cells. *Biochim Biophys Acta* **1269**, 205–214.
- Qualmann, B., Kessels, M. M. & Kelly, R. B. (2000). Molecular links between endocytosis and the actin cytoskeleton. *J Cell Biol* **150**, F111–F116.
- Ravdin, J. I., Murphy, C. F. & Schlesinger, P. H. (1988). The cellular regulation of vesicle exocytosis by *Entamoeba histolytica*. *J Protozool* **35**, 159–163.
- Reeves, R. E., Warren, L. G., Susskind, B. & Lo, H. (1977). An energy conserving pyruvate to acetate pathway in *Entamoeba histolytica*. *J Biol Chem* **252**, 726–731.
- Reyes-López, M., Serrano-Luna, J. J., Negrete-Abascal, E., León-Sicaire, N., Guerrero-Barrera, A. L. & de la Garza, M. (2001). *Entamoeba histolytica*: transferrin binding proteins. *Exp Parasitol* **99**, 132–140.
- Richardson, D. R. & Ponka, P. (1997). The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim Biophys Acta* **1331**, 1–40.
- Robibaro, B., Hope, H. C., Yang, M., Coppens, I., Ngô, H. M., Stedman, T. T., Pprotka, K. & Joiner, K. A. (2001). Endocytosis in different lifestyles of protozoan parasitism: role in nutrient uptake with special reference to *Toxoplasma gondii*. *Int J Parasitol* **31**, 1343–1353.
- Romero-Díaz, M., Gómez, C., López-Reyes, I., Martínez, M., Orozco, E. & Rodríguez, M. (2007). Structural and functional analysis of the *Entamoeba histolytica* *EhrabB* gene promoter. *BMC Mol Biol* **8**, 82.
- Sahoo, N., Labruyère, E., Bhattacharya, S., Sen, P., Guillén, N. & Bhattacharya, A. (2004). Calcium binding protein 1 of the protozoan parasite *Entamoeba histolytica* interacts with actin and is involved in cytoskeleton dynamics. *J Cell Sci* **117**, 3625–3634.
- Serrano-Luna, J. J., Negrete, E., Reyes-López, M. & de la Garza, M. (1998). *Entamoeba histolytica* HM1:IMSS: haemoglobin-degrading neutral cysteine proteases. *Exp Parasitol* **89**, 71–77.
- Slepnev, V. I. & De Camilli, P. (2000). Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat Rev Neurosci* **1**, 161–172.
- Tanaka, T., Abe, Y., Inoue, N., Kim, W., Kumura, H., Nagasawa, H., Igarashi, I. & Shimazaki, K. (2004). The detection of bovine lactoferrin binding protein on *Trypanosoma brucei*. *J Vet Med Sci* **66**, 619–625.
- Taylor, J. M. & Heinrichs, D. E. (2002). Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Mol Microbiol* **43**, 1603–1614.
- Temesvari, L. A., Harris, D. N., Stanley, S. L. & Cardelli, J. (1999). Early and late endosomal compartments of *Entamoeba histolytica* are enriched in cysteine proteases, acid phosphatase and several Ras-related Rab-GTPases. *Mol Biochem Parasitol* **103**, 225–241.
- Testa, U. (2002). Transferrin. In *Proteins of Iron Metabolism*, pp. 143–247. Edited by U. Testa. Boca Raton, FL: CRC Press.
- Thorstensen, K. & Romslo, I. (1990). The role of transferrin in the mechanism of cellular iron uptake. *Biochem J* **271**, 1–9.
- Tov, A., Tov, R. S., Gaentzsch, R., Helm, M. & Ankri, S. (2010). A new nuclear function of the *Entamoeba histolytica* glycolytic enzyme enolase: the metabolic regulation of cytosine-5 methyltransferase 2 (Dnmt2) activity. *PLoS Pathog* **6**, e1000775.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.
- Weinberg, E. D. (2009). Iron availability and infection. *Biochim Biophys Acta* **1790**, 600–605.
- Welter, B. H., Laughlin, R. C. & Temesvari, L. A. (2002). Characterization of a Rab7-like GTPase, EhRab7: a marker for the early stages of endocytosis in *Entamoeba histolytica*. *Mol Biochem Parasitol* **121**, 254–264.
- Welter, B. H., Powell, R. R., Laughlin, R. C., McGugan, G. C., Bonner, M., King, A. & Temesvari, L. A. (2006). *Entamoeba histolytica*: comparison of the role of receptors and filamentous actin among various endocytic processes. *Exp Parasitol* **113**, 91–99.
- Yang, W., Li, E., Kairong, T. & Stanley, S. L., Jr (1994). *Entamoeba histolytica* has an alcohol dehydrogenase homologous to the multi-functional *adhE* gene product of *Escherichia coli*. *Mol Biochem Parasitol* **64**, 253–260.

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