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Human hololactoferrin: endocytosis and use as an iron source by the parasite *Entamoeba histolytica*

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Entamoeba histolytica is an enteric protozoan that exclusively infects human beings. This parasite requires iron for its metabolic functions. Lactoferrin is a mammalian glycoprotein that chelates extracellular iron on mucosal surfaces, including the surface of the large intestine, where *E. histolytica* initiates infection. This work examined the interaction *in vitro* of *E. histolytica* trophozoites with human hololactoferrin (iron-saturated lactoferrin). A minimum concentration of 50 μM Fe from hololactoferrin supported growth of the amoeba. Amoebic binding sites for hololactoferrin were different from those for human apolactoferrin, holotransferrin and haemoglobin. One amoebic hololactoferrrin-binding polypeptide of 90 kDa was found, which was not observed after treatment of trophozoites with trypsin. Hololactoferrin-binding-protein levels increased in amoebas starved of iron, or grown in hololactoferrin. Internalization of hololactoferrin was inhibited by filipin. Endocytosed hololactoferrin colocalized with an anti-chick embryo caveolin mAb in amoebic vesicles, and lactoferrin was further detected in acidic vesicles; amoebic caveolin of 22 kDa was detected by Western blotting using this antibody. Cysteine proteases from amoebic extracts were able to cleave hololactoferrin. Together, these data indicate that *E. histolytica* trophozoites bind to hololactoferrin through specific membrane lactoferrin-binding proteins. This ferric protein might be internalized via caveolae-like microdomains, then used as an iron source, and degraded.

INTRODUCTION

Iron is an essential nutrient for pathogens surviving in a mammalian host. Therefore, a host defence strategy against pathogens is to decrease the concentration of free iron in such a way that the extracellular environment contains a very low level of iron (Bullen, 1981; Weinberg & Weinberg, 1995; Testa, 2002). In humans, this defence strategy is accomplished through iron-withholding systems, such as transferrin (Tf) in serum, and lactoferrin (Lf) in exocrine and neutrophil secretions (Bullen, 1981; Finkelstein *et al.*, 1983; Masson *et al.*, 1966, 1969; Van Snick *et al.*, 1974). In order to survive, pathogens have developed mechanisms for scavenging iron from host supplies, such as iron-containing and iron-binding proteins; these mechanisms are considered to be virulence factors (Finkelstein *et al.*, 1983; Weinberg, 1999).

Abbreviations: Ab, antibody; AFC, ammonium ferric citrate; ApoLf, apolactoferrin; ApoTf, apotransferrin; EhLfbp, *E. histolytica* hololactoferrin-binding protein; Fl, fluorescence intensity; Hb, haemoglobin; HoloLf, hololactoferrin; HRP, horseradish peroxidase; Lf, lactoferrin; Lfbp, lactoferrin-binding protein; LY, Lucifer yellow; NEM, *N*-ethyl maleimide; pHMB, *p*-hydroxymercuribenzoate; Tf, transferrin.

In bacteria, iron-acquisition systems have been widely studied (Alderete *et al.*, 1988; Gray-Owen & Schryvers, 1996; Jarosik & Land, 2000). However, the mechanisms of iron acquisition in protozoa are less well understood (Loo & Lalonde, 1984; Murray *et al.*, 1991; Rodríguez & Jungery, 1986; Tachezy *et al.*, 1998). Some of these primitive eukaryotes acquire iron from Tf through receptor-mediated endocytosis (Coppens *et al.*, 1987; Reyes-López *et al.*, 2001). A receptor on *Trypanosoma brucei* binds and endocytoses Tf; inside vesicles, iron is released, apotransferrin (apoTf) is degraded, and the receptor is recycled (Steverding, 2000). Lf is also taken up by *Try. brucei* (Coppens *et al.*, 1987). Other parasites possess lactoferrin-binding proteins (Lfbp), and use Lf-iron for growth (Britigan *et al.*, 1998; Tachezy *et al.*, 1996; Weinberg, 1999).

Entamoeba histolytica is an extracellular parasitic protozoan that causes amoebiasis, a human intestinal and hepatic disease that is a significant source of morbidity and mortality in developing countries. *E. histolytica* cysts transform into trophozoites in the terminal ileum, and reproduce and invade the colonic mucosa, resulting in ulcerative lesions and dysentery. An inflammatory process develops in the

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large intestine, with abundant infiltration of neutrophils around amoebas. In further stages of the disease, amoebas can migrate to the liver and other organs (Espinosa-Cantellano & Martínez-Palomo, 2000).

E. histolytica trophozoites depend on exogenous iron sources for growth in axenic culture, and they can use both ferric and ferrous ions (Latour & Reeves, 1965; Serrano-Luna *et al.*, 1998a; Smith & Meerovitch, 1982). The capability of amoebas to use haemoglobin (Hb) as an iron source, and its cleavage by cysteine proteases, has been documented (Serrano-Luna *et al.*, 1998b). Also, the utilization of holotransferrin (holoTf) as an iron source, and iron acquisition through receptor-mediated endocytosis, have been described (Reyes-López *et al.*, 2001). Lf has been observed in *E. histolytica* tubular invaginations that did not show a typical clathrin coat (Batista *et al.*, 2000); however, it is not known whether human Lf supports growth of the amoeba.

The aim of this work was to determine how *E. histolytica* trophozoites take up and use human holoLf. We established that iron acquisition from this ferric protein can support growth of the amoeba. *E. histolytica* hololactoferrin-binding protein (EhLfbp) specifically recognized holoLf; this ferric protein was endocytosed by filipin-sensitive vesicles, which were recognized by an anti-caveolin mAb.

METHODS

Cultures. Trophozoites of *E. histolytica* strain HM-1 : IMSS were axenically grown in BI-S-33 medium (Diamond *et al.*, 1978) (Dibico) supplemented with 16 % (v/v) heat-inactivated bovine serum (BS) (Microlab, Mexico), at 37 °C. Unless otherwise specified, amoebas from 48 h cultures were chilled on ice, harvested by centrifugation at 500 ***g***, and washed twice in PBS (0·2 M NaH_2PO_4, 0·2 M Na_2HPO_4, 0·11 M NaCl, pH 7·4) before experiments. *Trichomonas vaginalis* was cultured in TYM (Dibico) supplemented with 10 % (v/v) heat-inactivated horse serum (Diamond, 1957) (Gibco). HEp-2 cells were cultured in humidified 5 % CO_2/95 % air at 37 °C in Eagle's medium modified by Dulbecco, and supplemented with 5 % (v/v) fetal BS (Hyclone), 1 % non-essential amino acids, 5 mM L-glutamine, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. Glass materials were treated with 6·0 M HCl for 24 h, and rinsed six times with double-distilled water before use. All chemicals were from Sigma. Bovine holoLf was kindly donated by Morinaga Milk (Tokyo, Japan).

Growth of *E. histolytica* in holoLf as an iron source

Media and iron concentrations used are shown in Table 1. The low-iron medium listed in Table 1 is BI-S-33 medium without ammonium ferric citrate that was treated with 5 g ml^{-1} of the chelating resin Chelex-100 in order to remove iron from the trace reagents in the medium. The resin was subsequently removed by filtration, and the medium was sterilized. This medium is henceforth referred to as 'low-iron'. In order to determine whether holoLf sustains trophozoite growth, the following methods were used.

Growth kinetics. Cells were kept for 2 h in BI-S-33 lacking BS, ammonium ferric citrate (AFC) and cysteine in order to synchronize the culture (Vohra *et al.*, 1998). Amoebas (10^4) were inoculated into 1 ml BI-S-33 or low-iron with or without holoLf incubated for 96 h, and samples were collected every 24 h to test cell viability by trypan blue exclusion, using light microscopy. Viability was also tested by double labelling: amoebas (10^4) were inoculated, as described above, in medium containing FITC-human holoLf (25 or 50 μM total iron), incubated for 48 h, centrifuged, and incubated for 10 min with 10 μg propidium iodide ml^{-1}. Amoebas were washed, and scanned by flow cytometry (FACScan; Beckton Dickinson).

Successive transfers. Amoebas (10^4) were inoculated into BI-S-33, low-iron and low-iron containing human holoLf (50 or 100 μM total iron). Amoebas were subcultured in each medium at least three times, and incubated for 48 h each time. Viability was measured by trypan blue exclusion.

Specific holoLf binding to the *E. histolytica* cell surface

The interaction of the amoebic cell surface and holoLf was investigated using the following methods.

Dot blot. Amoebas suspended in PBS (10^6 ml^{-1}) were fixed (4 % paraformaldehyde in PBS, pH 7·4, for 1 h at 37 °C). Cell suspensions (10 μl) were vacuum blotted onto a nitrocellulose membrane (Sigma) in a blotter apparatus (Bio-Rad). The membrane was blocked with PBS-T (5 % non-fat milk in PBS/0·05 % Tween 20, pH 7·4, for 1 h) at room temperature, washed, and incubated for 1 h with 3·0 μg HRP-holoLf ml^{-1} [holoLf was coupled to horseradish peroxidase (HRP) by using the method of Avrameas & Ternynck,

Table 1. Media used in this study

Medium	Iron concn (μM)*	Source of iron	Reference
BI-S-33	100	AFC, serum and iron traces from reagents	Diamond *et al.* (1978)
Low-iron	6·5	Serum	Serrano-Luna *et al.* (1998b)
Low-iron plus holoLf†	15, 25, 50 and 100	Lf-iron and serum	This work

*Iron concentration was measured using ferrozine reagent (Stookey, 1970) and an automatic analyser (Hitachi 747, Japan).

†Human or bovine holoLf was added to obtain the iron concentrations indicated.

1971]. The reaction was revealed with 3′,3-diaminobenzidine. BS and *Tri. vaginalis* were used as negative and positive controls, respectively.

Confocal microscopy. Trophozoites (2×10^5) were suspended for 30 min in BI-S-33, or low-iron lacking BS and AFC. The trophozoites were then washed, fixed, and incubated for 1 h with 100 μg ml^{-1} FITC-holoLf or FITC-apoLf [FITC-holoLf was prepared as described for holoTf (Reyes-López *et al.* 2001), and FITC-apoLf was prepared by depleting iron from FITC-holoLf, as described by Mazurier & Spik (1980)]. Samples were mounted in Vectashield on glass slides, and examined in a Leica TCS-SP2 confocal laser-scanning microscope, observing 10–20 optical sections from each cell.

Competition assays. To determine the specificity of the *E. histolytica* holoLf-binding sites, nitrocellulose membranes from the dot blot, and fixed amoebas from the confocal experiments, were preincubated for 1 h with 1 mg ml^{-1} of other iron-containing proteins (Tf and Hb), and with apoLf. Evidence of an EhLfbp was obtained by preincubating the membrane or the fixed cells with 1 mg holoLf ml^{-1} for 1 h, or by treatment of fixed amoebas with 24 μg trypsin ml^{-1} (1 h at 37 °C), before incubation with HRP-holoLf or FITC-holoLf, as indicated for dot blot or confocal microscopy, respectively.

Detection of EhLfbp in low-iron-containing medium, with and without holoLf. An amoebic total extract was obtained as reported by Serrano-Luna *et al.* (1998b) from 5×10^6 cells grown for 6 h in BI-S-33, low-iron, and low-iron plus holoLf (50 μM iron), or grown in BI-S-33, and then fixed and incubated with trypsin (see above). Proteins (30 μg per well) were separated (12 % SDS-PAGE) and stained with Coomassie blue. Protein concentration was determined by the method of Bradford (1976). For overlay assays, proteins were electrotransferred to a nitrocellulose membrane (1·5 h, 400 mA) (Towbin *et al.*, 1979), which was blocked (5 % non-fat milk in PBS-T, 3 h), incubated for 12 h with 3·0 μg holoLf ml^{-1}, washed, and incubated with rabbit anti-human Lf antibody (Ab) (Sigma, catalogue no. L 3262; 1 : 100). A secondary HRP-anti-rabbit IgG (1 : 1000) was added. The blotted dried membrane was then scanned and quantified by densitometry using SigmaGel software. Competition assays were performed in a similar way to the dot blots, but the membrane was incubated with HRP-holoLf for 12 h. Expression of holoLf-binding sites from *E. histolytica* grown in different iron sources was measured as follows: amoebas (1×10^6) were incubated for 30 min in BI-S-33 or low-iron, fixed (see above), washed, and incubated for 30 min with 100 μg FITC-holoLf ml^{-1}, and processed for flow cytometry. In other experiments, amoebas grown in low-iron or BI-S-33 were simultaneously exposed to FITC-holoLf and BI-S-33, fixed, washed, and processed. Fluorescence intensity (FI) was measured in 10^4 cells by flow cytometry, and the statistical significance of the difference between the two conditions was evaluated with the Kolmogorov–Smirnov test (Young, 1977).

The holoLf endocytic pathway in *E. histolytica*. To investigate whether *E. histolytica* is able to endocytose Lf, amoebas (2×10^5) were incubated in low-iron (without BS) containing FITC-apoLf or FITC-holoLf, for 5 or 30 min, fixed (see above), and processed for confocal microscopy. To determine the endocytosis pathway, amoebas (10^6) were incubated for 30 min in BI-S-33 without BS, but containing one of the following endocytosis-inhibitors: 100 μg filipin ml^{-1}, 8 μM chlorpromazine, 2 % (w/v) sucrose, 200 nM wortmannin or 100 mM chloroquine. Next, cells were incubated for 30 min with the inhibitor plus 100 μg ml^{-1} FITC-holoLf, and prepared for scanning by flow cytometry and confocal microscopy. To investigate whether caveolin or clathrin was participating in the holoLf endocytosis process, amoebas (2×10^5) were incubated for 30 min in BI-S-33 without BS, and then for 15 min in the presence of 200 μg FITC-holoLf ml^{-1}, fixed, permeabilized (0·2 % Triton X-100, 15 min), and finally incubated for 1 h with anti-chick embryo fibroblast caveolin-1 mAb (Zymed, catalogue no. 03-6000, clone Z034; 1 : 20) or goat anti-bovine brain clathrin Ab (Sigma, catalogue no. C 8034; 1 : 40). Some amoebas were also treated with 100 μg filipin ml^{-1} for 30 min before adding the Abs. Amoebas were then incubated for 1 h with secondary Abs: rabbit TRITC-anti-mouse IgG (for caveolin; Zymed; 1 : 50), and rabbit RITC-anti-goat IgG (for clathrin; Zymed; 1 : 50). The presence of caveolae-like vesicles in *E. histolytica* was determined by staining the following caveolae components: lipids (10 μg ml^{-1} Nile red for 30 min, as described by Kimura *et al.*, 2004; and Klinkner *et al.*, 1997) and caveolin [using anti-caveolin mAb as above, and, as a secondary Ab, goat anti-mouse IgG coupled to CY5 (Zymed; catalogue no. 62-6516; 1 : 100)]. Filipin was used to disrupt caveolae. In other assays, the traffic of endocytosed holoLf was visualized using Lucifer yellow (LY), which stains acidic vesicles. Cells were incubated for 35 or 45 min in BI-S-33 containing 1 mg LY ml^{-1} and 100 μg holoLf ml^{-1}. Amoebas were then fixed, permeabilized, and incubated for 1 h with rabbit anti-human Lf (see above), and then for 1 h with a secondary Ab coupled to CY5 (Zymed; mouse anti-rabbit IgG; 1 : 100). Samples were processed for confocal microscopy, and for fluorescence quantification by flow cytometry.

Immunodetection of clathrin and caveolin-like protein in *E. histolytica*. Lysates of HEp-2 cells or *E. histolytica* trophozoites (30 μg protein each) were separated by 13 % SDS-PAGE, and electrotransferred to a nitrocellulose membrane for 45 min at 4 °C. The blot was blocked with PBS-T at room temperature (see dot blot), and incubated for 2 h with anti-caveolin-1 or anti-clathrin Ab (1 : 300). HRP-conjugated goat anti-mouse IgG, or rabbit anti-goat IgG (Zymed) (each at 1 : 2000), was used as a secondary Ab. Blots were developed by chemiluminiscence (Amersham Pharmacia Biotech).

Substrate gel electrophoresis. To determine whether amoebic proteases cleave holoLf, the method of Heussen & Dowdle (1980) was followed. Cells were placed for 6 h in BI-S-33 or in low-iron (both without BS), then centrifuged, and total extracts were obtained from the pellet. Supernatants were precipitated with neat 2-propanol. In order to characterize the proteolytic activity, total-extract proteins from BI-S-33 cultures were incubated with an equal volume of protease inhibitor [*p*-hydroxymercuribenzoate (pHMB), *N*-ethylmaleimide (NEM), PMSF or EGTA], as reported by Serrano-Luna *et al.* (1998b). Samples (10 μg protein per well) were run in 12 % polyacrylamide gels copolymerized with 0·1 % holoLf (3 h, 100 V, 4 °C). Gels were washed, and incubated for 1 h with 2·5 % Triton X-100, rinsed, and incubated for 12 h with 10 mM $CaCl_2$ in either 1 M Tris-OH, pH 7·0, or 0·1 M sodium acetate-Tris/HCl, pH 4·0. Gels were rinsed one more time, and stained with Coomassie R-250.

RESULTS

Human holoLf is used as an iron source by *E. histolytica* trophozoites

To test whether holoLf supports the growth of *E. histolytica*, we used several holoLf concentrations (Table 1) in growth kinetics (Fig. 1a). Human holoLf (25–100 μM Fe) supported growth of the amoeba over a period of 96 h. Bovine holoLf was also used by *E. histolytica*, though less efficiently than human holoLf. In low-iron (6·5 μM Fe), the culture developed slowly until 48 h, and then the number of viable cells decreased. HoloLf sustained amoebic subcultures

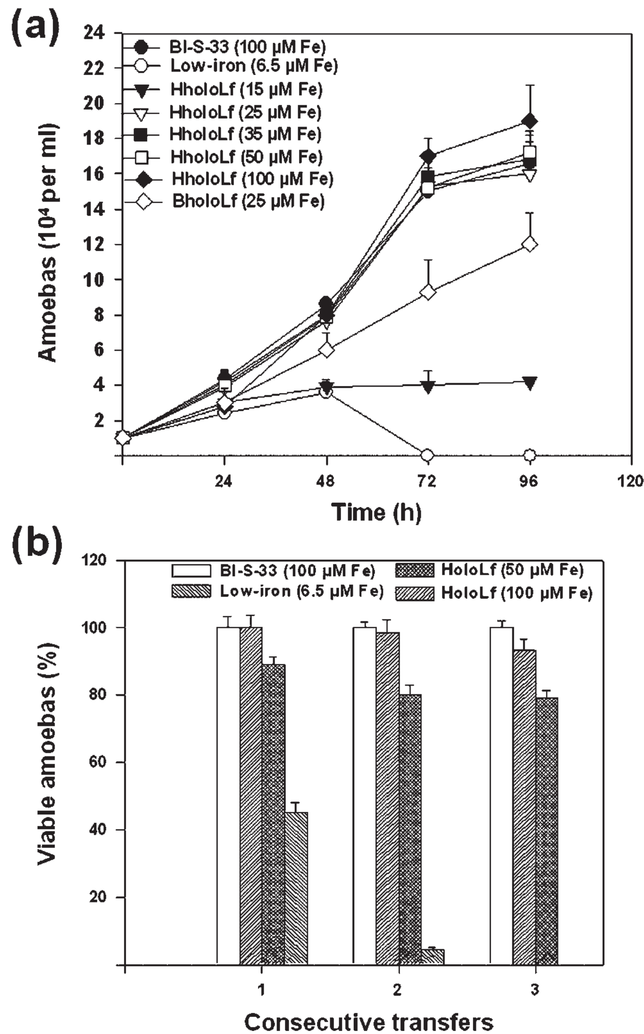

Fig. 1. *E. histolytica* growth in holoLf as an iron source. (a) Growth kinetics through 96 h in human holoLf (HholoLf) or bovine holoLf (BholoLf). The amoebic culture was synchronized (see Methods), 10^4 cells were inoculated into each medium, and viability was estimated every 24 h by trypan blue exclusion. (b) Amoebas were grown for 48 h, through three passages, in the media indicated. Viability was measured by trypan blue exclusion. Data are means of three independent experiments performed in triplicate. Error bars show SD.

(Fig. 1b), and the percentages of viable cells throughout three consecutive culture passages in low-iron plus 100 μM Lf-iron were 100, 98·5 and 93·2 %, respectively. When 50 μM Lf-iron was tested, the number of viable cells showed a slight reduction. In low-iron, trophozoite viability decreased considerably in the first passage, and decreased even more in the second passage. Next, we verified that human holoLf interacted with amoebas, and that cells remained viable (Table 2). In 25 μM Lf-iron, 83 % of amoebas were viable, and 65 % of these cells showed interaction with FITC-holoLf. In 50 μM Lf-iron, amoebas showed 98 % viability, and the percentage of labelled cells

Table 2. Viability of *E. histolytica*, and its interaction with FITC-holoLf

BF, basal fluorescence.

Medium	Iron concn (μM)*	Viability (%)†	FI (%)‡
BI-S-33	100	92 ± 6	BF
Low-iron	6·5	41 ± 4	BF
Low-iron plus FITC-holoLf	25	83 ± 2	65
Low-iron plus FITC-holoLf	50	98 ± 2	88

*Iron concentration was measured as described in Table 1.

†Viability was determined in 1×10^4 amoebas by exclusion of propidium iodide.

‡Percentages represent numbers of viable amoebas that interacted with FITC-holoLf. Values are means ± SD of three experiments in triplicate.

increased to 88 %. These data indicate that *E. histolytica* trophozoites use holoLf as an iron source by a mechanism in which the interaction between the parasite and holoLf is necessary.

E. histolytica cells bind holoLf specifically

A dot blot assay was used to demonstrate the interaction between holoLf and the trophozoite (Fig. 2a). HRP-holoLf was found to be strongly associated with cells (Fig. 2a, row 3, I). Specific binding to holoLf was determined in fixed cells incubated with an excess (tenfold) of unlabelled Hb, holoTf or apoLf, and then incubated with HRP-holoLf. Binding of HRP-holoLf was not inhibited by these proteins, and only unlabelled holoLf was able to compete (Fig. 2a, row 3, V). Since *Tri. vaginalis* trophozoites possess Lfbp (Peterson & Alderete, 1984), they were used as a positive control (Fig. 2a, row 2, I). BS was the negative control (Fig. 2a, row 1, I). To further analyse the binding of holoLf to the amoeba surface, confocal microscopy was carried out on fixed cells. FITC-holoLf showed a patch-like pattern on the cell surface (Fig. 2b, 2). Preincubation of amoebas with unlabelled holoLf prevented the binding of FITC-holoLf (Fig. 2b, 3). Neither holoTf nor Hb competed with FITC-holoLf for the surface of the amoeba (Fig. 2b, 4 and 5, respectively), suggesting that *E. histolytica* cells exhibit Lf binding sites distinct from those for Hb and Tf. On the other hand, treatment of amoebas with 24 μg trypsin ml^{-1} affected their sites for FITC-holoLf binding (Fig. 2b, 6), but cell integrity was preserved (not shown). To assess whether apoLf and holoLf bind to different amoeba proteins, competitive binding assays were carried out. Incubation of fixed iron-starved amoebas with apoLf prior to the interaction with FITC-holoLf (Fig. 2c, 2), or with holoLf prior to the interaction with FITC-apoLf (Fig. 2c, 3), did not affect

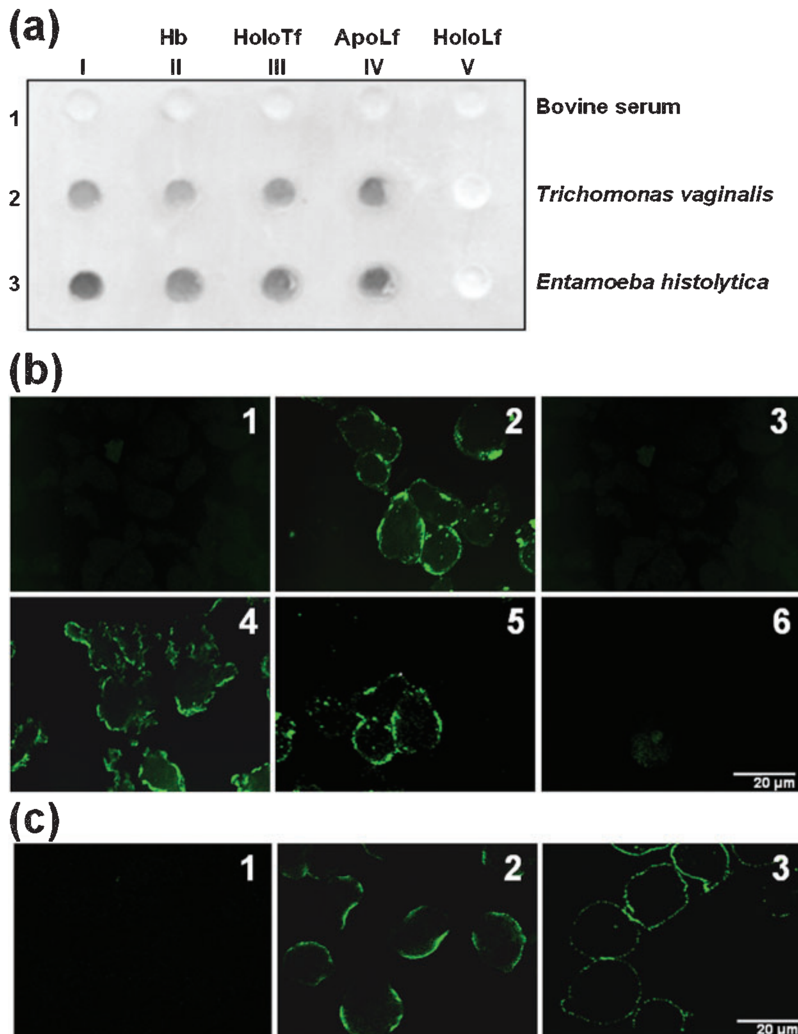

Fig. 2. Specific holoLf binding on the *E. histolytica* cell surface. (a) *E. histolytica* and *Tri. vaginalis* (positive control) (10^6 each) were fixed, adsorbed to filters, and incubated for 3 h with 3·0 μg HRP-holoLf ml^{-1} (I). BS was used as negative control. For competition assays, dots were incubated with 1 mg ml^{-1} unlabelled Hb (II), holoTf (III), apoLf (IV) or holoLf (V), and then with HRP-holoLf ml^{-1}. (b and c) Specific binding of FITC-holoLf on the cell surface. (b) Amoebas (2×10^5) in low-iron without serum were fixed and incubated for 30 min with 100 μg FITC-holoLf ml^{-1} (2–6), or without treatment (1). For competition assays, fixed cells were incubated with 1 mg ml^{-1} unlabelled holoLf (3), Hb (4) or holoTf (5), and then with FITC-holoLf. Cells treated with trypsin and then incubated with FITC-holoLf are shown in (6). (c) Amoebas in low-iron were fixed and incubated with unlabelled 1 mg ml^{-1} holoLf (2) or apoLf (3). After 1 h, samples were washed and incubated for 30 min with 100 μg ml^{-1} FITC-apoLf (2) or FITC-holoLf (3). Non-treated cells are shown in (1). Panels in (b) and (c) are representative of 100 cells. Fluorescence was observed in more than 80 % of amoebas. Bar, 20 μm.

the fluorescence on their surface. These results indicate that the *E. histolytica* trophozoite possesses specific proteins that bind holoLf, and that these proteins are localized on the cell surface.

Detection of EhLfbp

In conditions of insufficient iron availability, bacteria and protozoa express a high number of Lfbp (Lehker & Alderete, 1992; Gray-Owen & Schryvers, 1996). We decided to study the expression of Lfbp in amoebas grown in three different media: BI-S-33 (Fig. 3, lane 1), low-iron (Fig. 3, lane 2), and low-iron plus holoLf (Fig. 3, lane 3). Coomassie blue staining showed similar protein profiles in the three media. Overlay assays using holoLf and anti-Lf Ab were carried out to identify the EhLfbp. Results revealed a major band of 90 kDa, and a weak band of 45 kDa. The 90 kDa band was strongly recognized in iron-restricted amoebas (Fig. 3, lane 5), and in the presence of holoLf (Fig. 3, lane 6), compared with amoebas grown in BI-S-33 (Fig. 3, lane 4). This band was submitted to a densitometry analysis, and it showed a double intensity when amoebas were in low-iron or with holoLf. The lower band (80 kDa) observed in lane 6 corresponded to the holoLf added to the medium. The specificity of the 90 kDa polypeptide was demonstrated, given that unlabelled Hb, holoTf and apoLf did not prevent the binding of HRP-holoLf (Fig. 3, lanes 8–10, respectively). Only holoLf prevented the binding of HRP-holoLf to the 90 kDa band (Fig. 3, lane 7). Treatment of amoebas with trypsin inhibited the recognition of HRP-holoLf by the 90 kDa band (Fig. 3, lane 11). These results suggest that the 90 kDa polypeptide might correspond to a specific EhLfbp. In addition, the FITC-holoLf binding level was increased in iron-restricted amoebas (Table 3) compared with the basal binding measured in amoebas grown in BI-S-33. However, when iron-starved amoebas were exposed simultaneously to FITC-holoLf and BI-S-33, the FI decreased to a value similar to that in non-starved cells. These results suggest that there is an increase in amoebic holoLf-binding sites when this protein is the sole iron source, and cells are starved of iron. However, when iron-starved amoebas were exposed to holoLf plus ferric citrate, they returned to the basal levels of expression for holoLf-binding sites (Table 3).

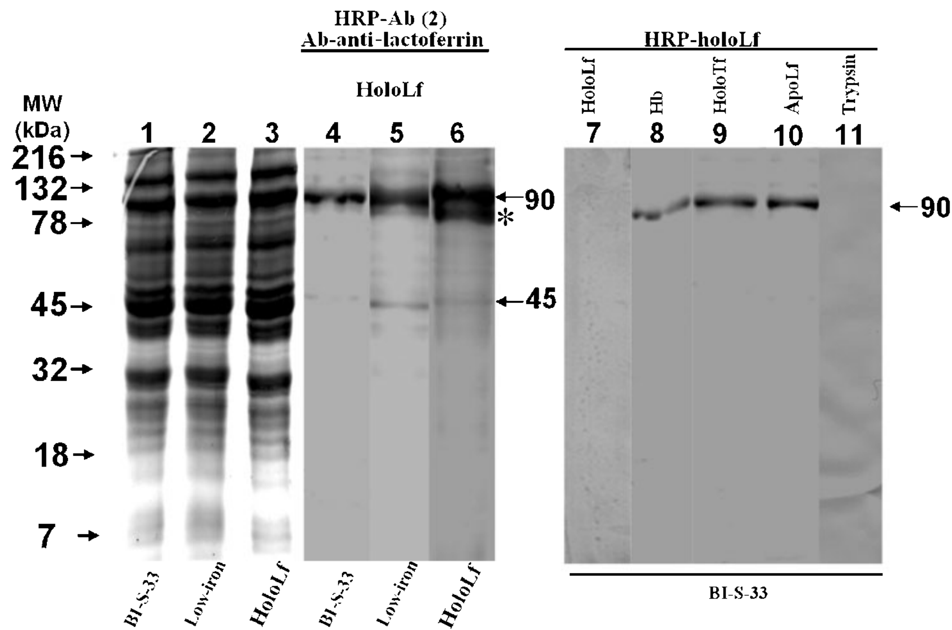

Fig. 3. Specific EhLfbp. Amoebas (5×10^6) were grown for 6 h in: BI-S-33 (lanes 1 and 4), low-iron (lanes 2 and 5), and low-iron plus holoLf (lanes 3 and 6). Total extracts (30 μg) were run in 12 % SDS-PAGE, stained with Coomassie blue (lanes 1–3), or transferred to a membrane and incubated with 3·0 μg holoLf ml^{-1}, which was immunodetected with anti-Lf (lanes 4–6; intensity in pixels, band in lane 4, 75 739 ± 28; lane 5, 146 234 ± 322; lane 6, 141 657 ± 132). The asterisk indicates lactoferrin. In competition assays, the membrane was incubated with 1 mg ml^{-1} holoLf (lane 7), Hb (lane 8), holoTf (lane 9) or apoLf (lane 10), and then with HRP-holoLf. Fixed cells were incubated with trypsin before obtaining the extract (lane 11). Data represent three experiments performed in triplicate.

HoloLf is mainly endocytosed via caveola-like vesicles by the *E. histolytica* trophozoites

In *E. histolytica*, holoLf has been observed in tubular invaginations, and in vesicles that do not show a typical clathrin coat (Batista *et al.*, 2000). However, it is not known if apoLf is internalized by the parasite. Confocal microscopy was used to observe the internalization of holoLf and aopoLf. Amoebas incubated for 5 min with FITC-holoLf showed a clearly labelled surface (Fig. 4, 2), and at 30 min, the protein was localized in vesicle-like structures (Fig. 4, 3). However, cells incubated for 5 min with FITC-apoLf showed the protein on the surface (Fig. 4, 4), but at 30 min few cells remained attached to the slide, and no label was found (Fig. 4, 5). These results show that holoLf is bound and internalized by amoebas, whereas apoLf is bound, but not internalized. Also, amoebas incubated for 30 min with FITC-apoLf plus BI-S-33 showed the label internally (Fig. 4, 6), suggesting that apoLf immediately sequesters iron from ferric citrate, and then internalizes it as holoLf. On the other hand, we used specific inhibitors to characterize the endocytic holoLf pathway in *E. histolytica* (Fig. 5a). If endocytosis by untreated amoebas is taken as 100 %, filipin (which disrupts caveolae structure and function) inhibited FITC-holoLf entrance by 70 %. Inhibitors of clathrin-mediated endocytosis (Wu *et al.*, 2003), such as sucrose, chloroquine, and the cationic amphiphilic drug chlorpromazine (not shown), did not affect the internalization of FITC-holoLf (< 10 % inhibition). Wortmannin, which inhibits fluid-phase endocytosis (Clague *et al.*, 1995), did not have an effect either. Similar results were obtained using these drugs in confocal microscopy assays (not shown). Further, entry of FITC-holoLf was inhibited by prior incubation of *E. histolytica* trophozoites with the anti-caveolin mAb, whereas anti-clathrin Ab did not impede holoLf entry (data not shown). As holoLf entry into amoebas was filipin-sensitive,

Table 3. *E. histolytica* trophozoites bind more holoLf when they are stressed for iron

Medium	FI*	
	FITC-holoLf†	FITC-holoLf plus BI-S-33‡
BI-S-33	124 ± 6	130 ± 6
Low-iron	204 ± 8	138 ± 4

*Determined in 1×10^4 cells by flow cytometry.
†Amoebas were incubated for 30 min in BI-S-33 or low-iron, incubated with FITC-holoLf for a further 30 min, fixed, and then FI was measured. Values are means ± SD of three experiments in triplicate.
‡Amoebas were incubated for 30 min in BI-S-33 or low-iron, and then incubated for a further 30 min with FITC-holoLf plus BI-S-33, fixed, and their FI was measured. Values are means ± SD of three experiments in triplicate.

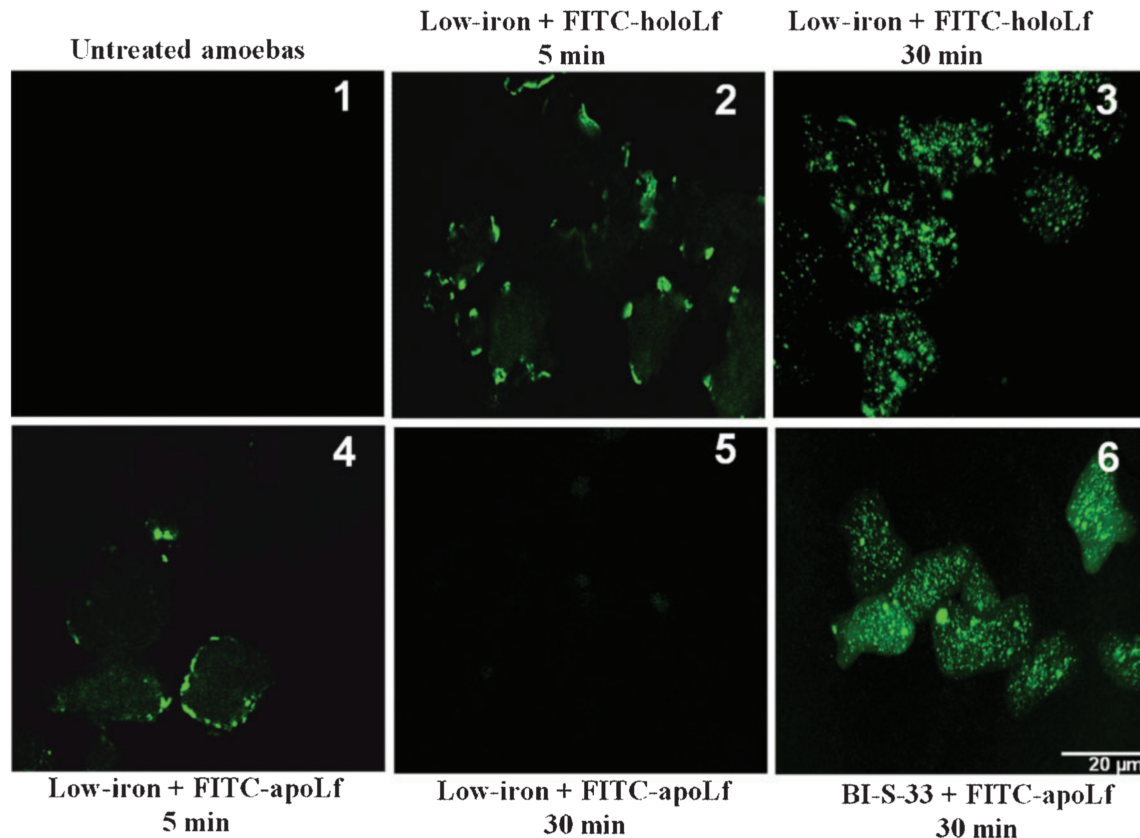

Fig. 4. Binding and endocytosis of human apo- and holoLf by *E. histolytica*. Control cells without treatment, cultured in BI-S-33 (1); cells maintained in low-iron, and incubated for 5 min (2) or 30 min (3) with 100 μg FITC-holoLf ml^{-1}; or incubated for 5 min (4) or 30 min (5) with 100 μg FITC-apoLf ml^{-1}; cells incubated for 30 min in BI-S-33 plus FITC-apoLf (6). Bar, 20 μm.

this process could be mainly via caveolae-like structures; thus we designed an experiment to detect caveolae markers (lipids and caveolin) in *E. histolytica*. Lipids (stained with Nile red; Fig. 5b, 2, red) and caveolin (immunolocalized using mAb; Fig. 5b, 3, blue), co-localized in amoebas (Fig. 5b, 4, violet). Filipin was used to corroborate the sensitivity of caveolae-like structures in amoebas. This drug disrupted lipids (Fig. 5b, 6), and diminished the recognition by anti-caveolin mAb (Fig. 5b, 7), as well as the co-localization between lipids and caveolin (Fig. 5b, 8); the FI was measured by flow cytometry, and is shown in the figure panels. Next, the participation of amoebic caveolae-like structures in the holoLf endocytosis was determined, since, at 15 min of incubation, FITC-holoLf (green) co-localized with caveolin-like coated vesicles (detected with anti-caveolin mAb and a secondary Ab in red) as a yellow fluorescent mark (Fig. 5c, 1). It seems that clathrin-coated vesicles do not participate in holoLf endocytosis, since the anti-clathrin Ab did not co-localize with holoLf (Fig. 5c, 2). In order to confirm the presence of caveolin-like proteins and clathrin in *E. histolytica*, Western blot assays were performed. Anti-caveolin mAb recognized amoebic proteins of 22 and 24 kDa, which might correspond to caveolin-1 like isoforms (Fig. 5d). Anti-clathrin Ab recognized a 180 kDa protein that is possibly the heavy chain of clathrin (Fig. 5d); this Ab has been used to localize amoebic clathrin (Tovar *et al.*, 2000). Our attempt to identify the amoebic caveolin gene was not successful.

Taken together, these results show that the mechanism responsible for internalizing holoLf in *E. histolytica* trophozoites is not clathrin dependent; however, caveolin-like containing vesicles might be involved in this process.

Traffic of holoLf in acidic vesicles, and proteases that cleave holoLf

Since the *E. histolytica* trophozoite is a multivesicular cell in which non-acidified and acidified vesicles have been found (Aley *et al.*, 1984; Swanson, 1989; Batista *et al.*, 2000), we investigated whether holoLf reaches acidic vesicles, an environment in which Lf-iron is probably released. LY (green and red) (Fig. 6a, 1 and 2) and holoLf (blue) (Fig. 6a, 3) co-localized in low amounts in acidic vesicles (purple and light blue) after 35 min incubation (Fig. 6a, 4); at 45 min, co-localization was clearly observed (Fig. 6a, 8, light blue). This co-localization was quantified by flow cytometry (not shown). These results suggest that holoLf may follow the vesicular traffic from endosomes, and reach the acidic vesicles in amoebas. In parasites, the Lf-iron release inside

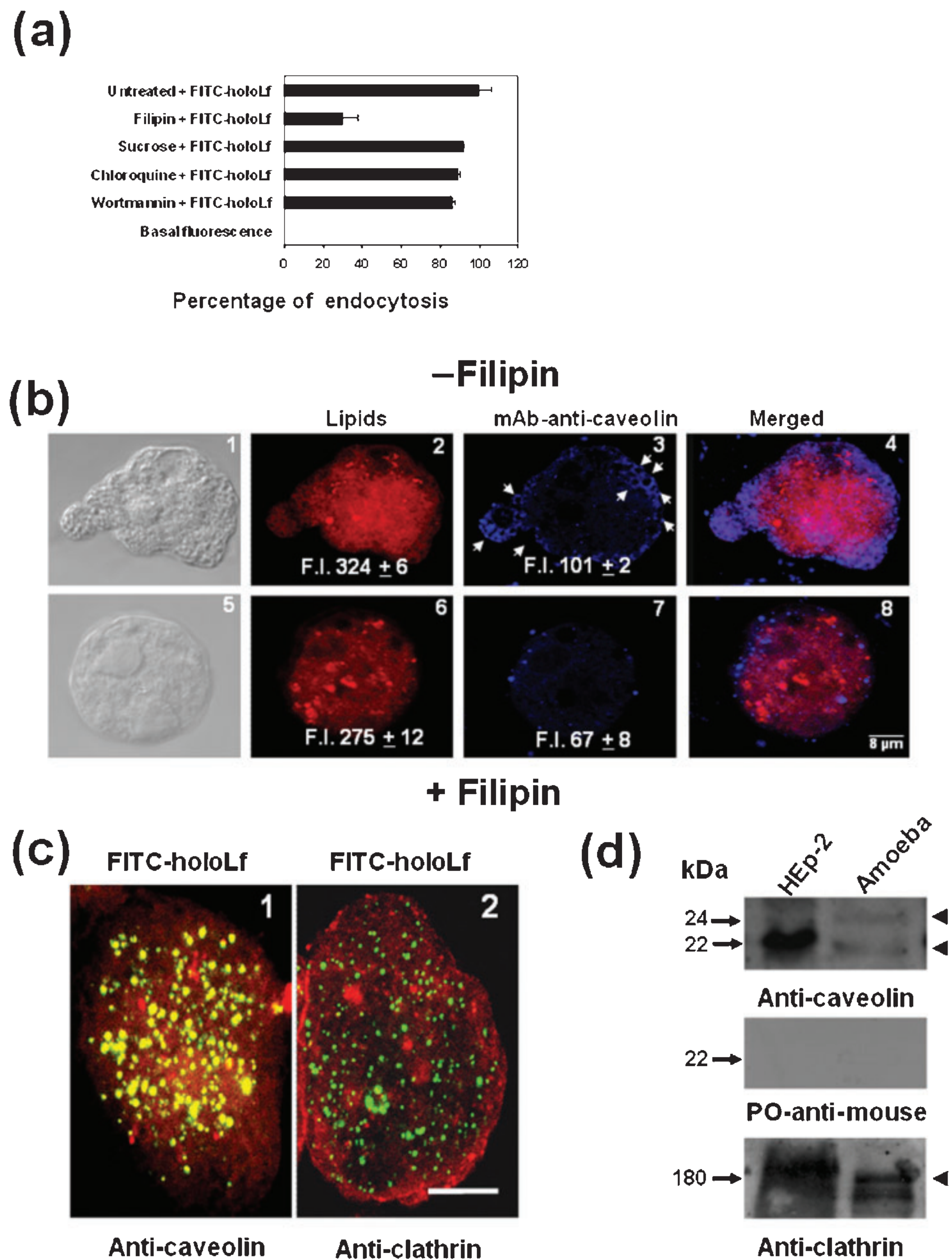

Fig. 5. Participation of lipids and caveolin-like proteins in *E. histolytica* holoLf endocytosis. (a) Amoebas were incubated for 30 min in BI-S-33 and the indicated drug. They were then incubated in BI-S-33 containing 100 µg FITC-holoLf ml^{-1} plus the indicated drug for a further 30 min, and processed for flow cytometry. Error bars show SD. (b) Amoebas cultured in BI-S-33 were either treated with filipin (+Filipin) or not (−Filipin), stained with Nile red for 30 min, fixed, and then incubated with an anti-caveolin-1 mAb for 1 h, followed by a secondary Ab coupled to CY5. FI in 10^4 cells was measured by flow cytometry. (c) Amoebas were incubated with 100 µg FITC-holoLf ml^{-1} for 15 min, fixed, permeabilized, and incubated with anti-caveolin or anti-clathrin Abs for 1 h, followed by TRITC-conjugated secondary Abs. Panels in (b) and (c) are representative of 100 cells. Fluorescence was observed in more than 80 % of cells. Bar, 8 µm. (d) Western blot of HEp-2 and amoebic extracts. Membranes were incubated with anti-caveolin or anti-clathrin Abs for 24 h at 4 °C, and then with an HRP-conjugated secondary Ab. The negative control for the secondary Ab was HRP-anti-mouse IgG.

acidic vesicles is only partial, therefore alternative mechanisms, such as proteases, could be involved in the holoLf cleavage; thus, to determine whether proteases cleave holoLf, amoebic extracts and culture supernatants were run in substrate gels with holoLf or gelatin (positive control). Proteases of 250, 100, 40 and 22 kDa from amoebic extracts cleaved holoLf at pH 7 (Fig. 6b, lane 2); however, the activity increased considerably at pH 4 (Fig. 6b, lane 9).

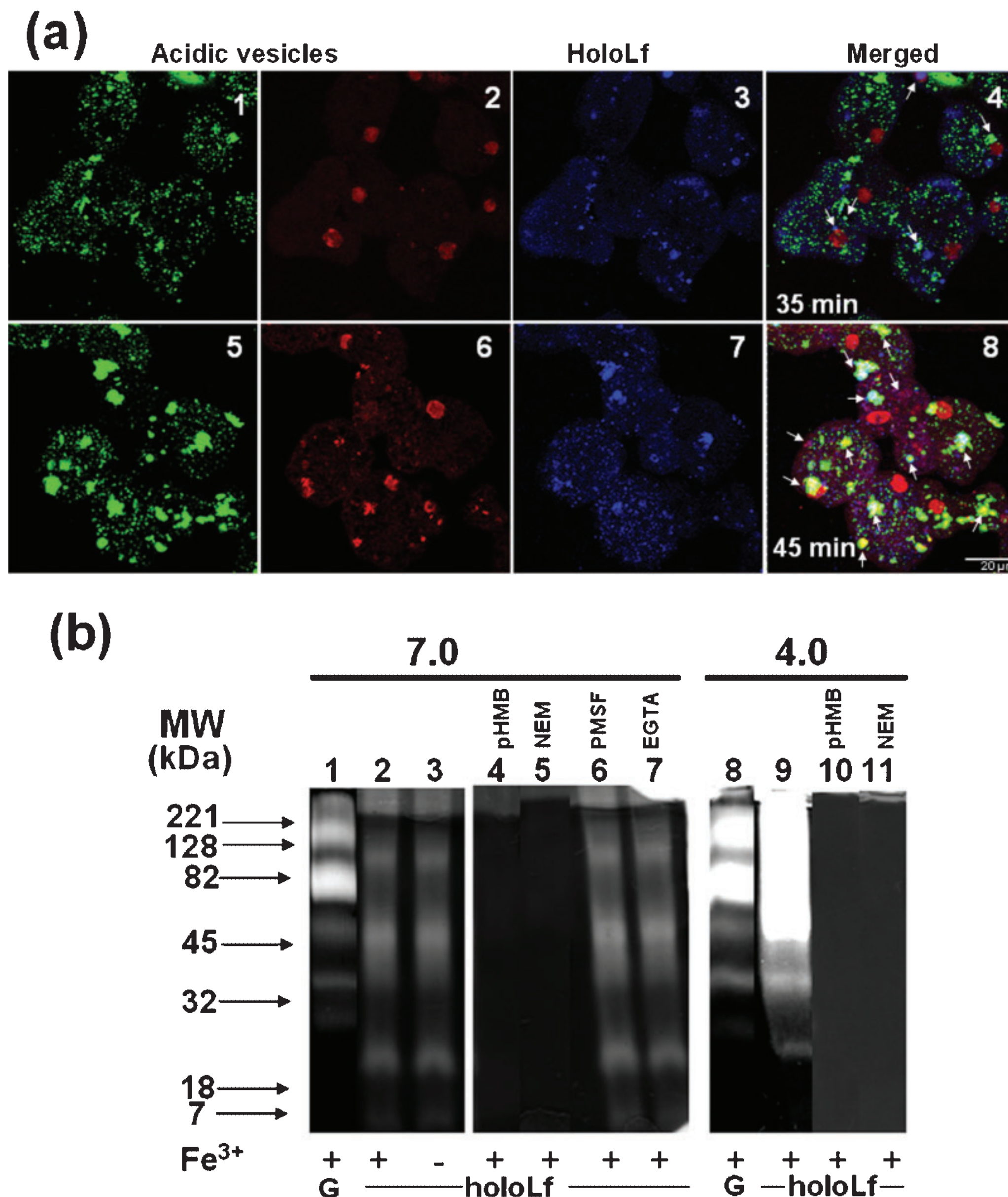

Fig. 6. Traffic of holoLf in *E. histolytica* acidic vesicles, and protease zymograms. (a) Amoebas (10^5) were incubated for 20 min in BI-S-33 (without serum) containing 1 mg LY ml^{-1}, and then with 100 µg holoLf ml^{-1} for 35 or 45 min. After fixation and permeabilization, Lf was immunodetected using a rabbit anti-Lf Ab; the secondary Ab was coupled to Cy5. Blue corresponds to holoLf, and red and green correspond to acidic vesicles. Co-localization of acidic vesicles and holoLf is observed in pink and light blue. (b) A protease zymogram from total extracts of amoebas grown in holoLf as the substrate at pH 4 and 7. Lanes 1 and 8, gelatin was used as the substrate. Extracts treated with protease inhibitors are indicated (lanes 4–7, 10 and 11). Amoebas were obtained from 48 h cultures in BI-S-33 with (+) or without (–) AFC in the medium.

Similar results were found in extracts of iron-starved amoebas (Fig. 6b, lane 3). No activity was found in supernatants (not shown). All activities were inhibited by the cysteine protease inhibitors pHMB and NEM (Fig. 6b, lanes 4 and 10, and 5 and 11, respectively). There was no inhibition by the serine (PMSF) and metallo- (EGTA) protease inhibitors (Fig. 6b, lanes 6 and 7). The data suggest that cysteine proteases could cleave holoLf in acidic vesicles, where the pH allows the release of Lf-iron, which is then utilized by *E. histolytica* for growth.

DISCUSSION

The success of parasitism depends on the ability of pathogens to take up nutrients, such as iron, from host

sources. On mucosal surfaces, iron is mainly sequestered by Lf, and its concentration has been used as a parameter to identify intestinal inflammatory disorders, such as ulcerative colitis (Kane *et al.*, 2003; Masson *et al.*, 1966; Uchida *et al.*, 1994). This study was undertaken to investigate whether *E. histolytica* is able to use Lf as a source of iron for growth. A possible mechanism of the Lf-iron acquisition by the parasite was also assessed.

The iron content of BI-S-33 medium supplemented with BS ranged between 90 and 105 μM depending on the reagent batch; however, the minimum iron requirement for amoebas is >50 μM. This requirement surpasses that of the majority of eukaryotic and prokaryotic cells, and is due to the presence of amoebic iron-containing proteins (Weinbach *et al.*, 1980). Human Lf-iron (>25 μM) supported *E. histolytica* growth, and 50–100 μM Lf-iron sustained growth through several passages. Lf-iron was the growth support, since a low-iron environment and absence of this ferric protein promote amoebic death. Thus, Lf-iron seems to be more efficient than ferric citrate for amoebic growth. In addition, pathogens grown *in vitro* require higher iron concentrations from ferric citrate, ferric nitrilotriacetate (Fe-NTA), $FeSO_4$ and other sources than from host proteins, such as Hb, Lf and Tf (Wilson *et al.*, 1994; Tachezy *et al.*, 1996; Jarosik *et al.*, 1998; Serrano-Luna *et al.*, 1998b; Reyes-López *et al.*, 2001). The results also show that amoebas have a constitutive level of expression of binding sites for holoLf. However, in conditions of iron deficiency, the number of binding sites for holoLf is increased. Interestingly, in the presence of holoLf plus ferric citrate, amoebas expressed the basal level of binding sites, which could be due to Lf-iron acquisition requiring receptor expression and synthesis, endocytosis and iron release at acidic pH, whereas iron ferric citrate acquisition may consist of a more straightforward iron release and entry process.

Suchan *et al.* (2003) reported that human Lf-iron (up to 100 μM) was unable to support *E. histolytica* HM-1 : IMSS growth in TYI-S-33 containing the iron chelator 2,2-dipyridyl (100 μM). A possible explanation could be the characteristics of Lf in its N-terminal arginine-rich part, which is important for binding to some receptors (van Veen *et al.*, 2002). We used the Chelex-100 resin to eliminate ferrous and ferric iron from traces of reagents in the media, since the resin can be subsequently removed from media, and then the culture is not affected. Chelex-100 also sequesters calcium, magnesium and zinc (Giles & Czuprynski, 2004), but they are restored by the serum added after chelation. Our data also show that bovine holoLf can sustain amoebic growth, but to a lower extent than human holoLf. It has been reported that Lf glycans are specific for different animal species (Spik *et al.*, 1988), however, since we tested human and bovine Lf only, it remains to be determined whether or not the use of Lf-iron by *E. histolytica* is species specific. It is important to state that the holoLf concentrations used by amoebas in this work can be found in physiological conditions. HoloLf present in the large intestine could be providing iron to amoebic cells, which is important for amoebic colonization and invasion.

We found specific holoLf-binding sites distributed in a patch-like pattern on the amoebic surface, which is in agreement with the observations of Batista *et al.* (2000) by electron microscopy. Other iron-containing proteins, such as Tf and Hb, and apoLf, were unable to prevent holoLf recognition by the *E. histolytica* sites located on the cell surface. HoloLf recognition was affected by unlabelled holoLf only, or by degradation of amoebic membrane proteins with trypsin, a serine protease. Therefore, different receptors for all these proteins may be present in *E. histolytica*. Protozoa such as *Tritrichomonas foetus*, *Leishmania donovani* and *Tri. vaginalis* bind ferric Lf in a specific receptor-mediated fashion to use it as iron source (Peterson & Alderete, 1984; Lehker & Alderete, 1992; Tachezy *et al.*, 1996). However, *Leishmania chagasi* binds Tf and Lf through the same protein, indicating that a specific receptor is not present (Wilson *et al.*, 2002). All these results corroborate that *E. histolytica* possesses specific holoLf-binding proteins on the surface. *E. histolytica* Lfbp might be recognizing amino acid residues or structural motifs unique to the holoLf molecule. In mammalian cells, Lf receptors bind equally to apoLf and holoLf (Testa, 2002). As a result of its conformation, ApoLf (iron-free Lf) displays different properties compared with the ferric-saturated form of Lf (Mazurier & Spik, 1980; Testa, 2002). Since human apoLf did not compete with holoLf for the binding sites in amoebas, this suggests that amoebas could have different receptors for the two Lfs. In addition, apoLf was able to bind to amoebas, but the cells became round and died; we presume that apoLf had an amoebicidal effect that damaged the cellular membrane, and we are currently studying this possibility.

Amoebic proteins of 70 and 140 kDa that bind Tf have been reported (Reyes-López *et al.*, 2001). Here, we report an *E. histolytica* surface protein of 90 kDa that is specifically recognized by holoLf. This recognition was not affected by Tf, Hb or apoLf; it was only inhibited with an excess of holoLf, or by treatment of amoebas with trypsin. On the other hand, since the 90 kDa band was detected in amoebas grown with ferric citrate, in low-iron, and with holoLf, this protein could be constitutively synthesized; however, its expression could be stimulated by holoLf or iron stress. Whether the EhLfbp is involved in the virulence of the parasite remains to be determined. With these results, it is tempting to speculate that amoebas could bind and use several iron-containing proteins at the time of invasion, e.g. Lf from mucosal surfaces, Tf from serum, and Hb from destroyed erythrocytes. Use of multiple iron sources by *E. histolytica* explains its successful survival in different organs, and it should be highly advantageous at sites with different iron environments.

The ability of amoebas to bind and endocytose holoLf, and the presence of a specific *E. histolytica* 90 kDa protein on the cell surface, provide some indication of the mechanism of

Lf-iron acquisition. Protein endocytosis may occur through several different pathways, such as clathrin-coated pits, caveolar structures, and macropinocytosis (Mellman, 1996; Pelkmans & Helenius, 2002). By using specific inhibitors of vesicular traffic and endocytosis, we found that only filipin inhibited the holoLf entry in amoebas. Filipin disrupts cholesterol, a major component of membrane glycolipid microdomains, and thus affects the structure and organization of caveolar components (Schnitzer *et al.*, 1994; Orlandi & Fishman, 1998). Filipin has been recently used in *E. histolytica* to detect the presence of raft-like vesicles, and investigate their participation in virulence events (Laughlin *et al.*, 2004). We localized caveolae-like structures in *E. histolytica* by using an anti-caveolin mAb and lipid staining (Fig. 5b); this mAb recognizes both α and β isoforms of caveolin-1 in fibroblasts from humans, mice and rats (Okamoto *et al.*, 1998), and, in amoebas, it recognized two proteins of 22 and 24 kDa that could correspond to caveolin-1-like isoforms. Further evidence of caveolae-like structures in *E. histolytica* included the inhibition of co-localization between lipids and caveolin when amoebas were treated with filipin. This inhibition could be due to loss of integrity of caveolae-like structures, as a result of cholesterol disruption and caveolin dissolution. The participation of *E. histolytica* caveolae-like vesicles in holoLf internalization was further supported by endocytosis experiments, which showed co-localization of holoLf and the anti-caveolin signal in vesicles after 15 min of holoLf endocytosis. Clathrin-coated pits did not participate in the holoLf endocytosis.

After endocytosis of holoLf via amoebic caveolae-like structures, we found that this protein reached acidic vesicles. In the environment of these vesicles, holoLf probably releases iron. The vesicular traffic of caveolae has been the subject of some controversy; however, recent studies have shown that caveolar structures can interact with both the caveosome and the lysosome pathway, and that caveolin-enriched regions can incorporate cargo in a regulated manner (Escriche *et al.*, 2003; Peters *et al.*, 2003; Parton, 2004; Schnitzer *et al.*, 1995). Furthermore, in amoebic extracts, we found proteases that cleave holoLf. It has been reported that *E. histolytica* endocytic vesicles are enriched in cysteine proteases which contain acid phosphatase (Temesvari *et al.*, 1999); the presence of holoLf in acid-phosphatase-containing vacuoles has been described in *Trt. foetus* and *E. histolytica* (Affonso *et al.*, 1994; Batista *et al.*, 2000). *E. histolytica* acidic vesicles show a pH below 4, (Aley *et al.*, 1984), a pH at which Lf-iron is partially released. These results suggest that holoLf could be releasing iron in the amoeba cysteine-protease-enriched acidic vesicles.

Together, the results indicate that human holoLf can be used by the parasite as an iron source for growth. The amoeba might acquire Lf-iron in a specific receptor-mediated endocytosis mechanism via caveolae-like filipin-sensitive vesicles. The acidic environment of amoebic vesicles, and the presence of cysteine proteases, may be factors that contribute to Lf-iron release.

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