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A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron

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Summary

***Trichomonas vaginalis*, a human sexually transmitted protozoan, relies on adherence to the vaginal epithelium for colonization and maintenance of infection in the host. Thus, adherence molecules play a fundamental role in the trichomonal infection. Here, we show the identification and characterization of a 120 kDa surface glycoprotein (AP120) induced by iron, which participates in cytoadherence. AP120 is synthesized by the parasite when grown in 250 μM iron medium. Antibodies to AP120 and the electro-eluted AP120 inhibited parasite adherence in a concentration-dependent manner, demonstrating its participation in cytoadherence. In addition, a protein of 130 kDa was detected on the surface of HeLa cells as the putative receptor for AP120. By peptide matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), the AP120 adhesin showed homology with a hydrogenosomal enzyme, the pyruvate:ferredoxin oxidoreductase (PFO) encoded by the *pfoa* gene. This homology was confirmed by immunoblot and indirect immunofluorescence assays with an antibody to the carboxy-terminus region of the *Entamoeba histolytica* PFO. Reverse transcription polymerase chain reaction (RT-PCR) assays showed that a *pfoa*-like gene was better transcribed in trichomonads grown in iron-rich medium. In conclusion, the homology of AP120 to PFO suggests that this novel adhesin induced by iron could be an example of moonlighting protein in *T. vaginalis*.**

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Introduction

Trichomonas vaginalis, a flagellated protozoan, is the aetiological agent of trichomonosis, one of the most common non-viral sexually transmitted diseases in humans. Parasite adherence to the vaginal epithelium is essential for initiating and maintaining the infection and fundamental for the parasites to survive in the human host. Trichomonal cytoadherence is mediated by ligand-receptor-type interactions (Alderete and Garza, 1985; Alderete *et al*., 1988), in which at least four surface proteins (AP65, AP51, AP33 and AP23) participate (Alderete and Garza, 1988; Arroyo *et al*., 1992) fulfilling criteria of adhesins (Beachey *et al*., 1988). A relationship between levels of cytoadherence, amount of adhesins (Arroyo *et al*., 1992) and surface delivery of synthesized adhesins (García *et al*., 2003) has been demonstrated. Likewise, levels of adherence and adhesin synthesis are regulated by iron concentrations (Lehker *et al*., 1991) and cellular contact (Arroyo *et al*., 1993; García *et al*., 2003). Moreover, three of the four adhesins (AP65, AP51 and AP33) encoded by multigene families have homology to metabolic enzymes and are upregulated by iron at the transcriptional level (Alderete *et al*., 1995a; 2001; Engbring *et al*., 1996; Engbring and Alderete, 1998), except one of the AP51 genes (Alderete *et al*., 1998).

Recent work by Alderete's group (García *et al*., 2003) demonstrated that trichomonads are part of a growing list of microbial pathogens that possess surface-associated enzymes with alternative, non-enzymatic functions (Alderete *et al*., 2001). This type of proteins have been referred as moonlighting proteins that can switch between functions because of changes in cellular localization, expression in a novel cell type, oligomeric state, and cellular concentration of a ligand, substrate, cofactor or product. Switching between the distinct functions of a protein without involving gene fusions, splice variants or multiple proteolytic fragments adds another dimension to cellular complexity (Jeffery, 1999; 2003). Here we identified and characterized a novel trichomonad adhesin of 120 kDa (AP120) expressed on the surface of parasites grown in high iron concentrations with homology to the parasite hydrogenosomal enzyme pyruvate:ferredoxin oxidoreductase (PFO). Our data suggest that this sexually transmit-

ted protozoan has several moonlighting proteins working as adhesins such as AP120 described in here, AP65, AP51 and AP33 (Arroyo *et al.*, 1992). All are surface proteins that have high identity to hydrogenosome metabolic enzymes.

Results

Presence of a T. vaginalis *120 kDa protein with affinity to HeLa cell surfaces in parasites grown in high iron*

First, we determined whether the clinical *T. vaginalis* isolate CNCD 188 is an iron-responsive isolate. Parasites grown in 20 or 250 μM iron medium and labelled with [^{3}H]-thymidine were incubated with HeLa cell monolayers. Trichomonads grown in 250 μM iron showed 95% of cytoadherence levels to HeLa cell monolayers; whereas only 45% was observed on parasites grown in 20 μM iron medium (Fig. 1A), suggesting that this is indeed an iron-responsive isolate.

Then, cell-binding assays were performed incubating fixed HeLa cells with protein extracts from the same cell equivalents of parasites grown in 20 and 250 μM iron medium. Figure 1B shows the electrophoretic patterns of the cell-binding proteins. Two (AP65 and AP51) adhesin bands were observed by SDS-PAGE (7%) in greater amounts in parasites grown in 250 μM iron (lane 4) than those grown in 20 μM iron medium (lane 3). No protein bands were observed in the negative control (lane 5) in which fixed HeLa were incubated only with the interaction buffer. Interestingly, a 120 kDa (AP120) protein band in total-protein extracts with affinity to the surface of HeLa cells was observed only in parasites grown in 250 μM iron concentrations (Ex, lanes 2; Adh, lane 4). In addition, AP120 was also observed in five clinical trichomonad isolates grown in 250 μM iron medium after a cell-binding assay (data not shown). Therefore, our working hypothesis was that the AP120 protein could be involved in the host–parasite interaction probably as an iron-induced adhesin.

The protein of 120 kDa is synthesized by T. vaginalis

To investigate whether AP120 is synthesized by the parasite, trichomonads were metabolically labelled with ^{35}S-Met-Cys before cell-binding assays (Fig. 1C). Autoradiography of the cell-binding proteins showed a 120 kDa ^{35}S-labelled protein band in parasites grown in 250 μM iron medium (lane 4) that was absent in trichomonads grown in 20 μM iron medium (lane 3). Even though the same cell equivalents were used, the AP65 adhesin band was also more intense in parasites grown in 250 μM than in 20 μM iron concentrations (lanes 3 and 4); whereas, an unknown protein of about 110 kDa appeared with similar intensity in both iron conditions (lanes 3 and 4). Similar patterns for the 120 kDa labelled-protein band were also observed in the corresponding total-protein extracts (lanes 1 and 2). These data demonstrate that AP120 is a parasite protein, which is induced by iron.

Anti-AP120 antibodies recognize the 120 kDa protein in parasites grown with 250 μM iron concentrations

The AP120 was electro-eluted and inoculated in rabbits to obtain antibodies, which were titrated and used

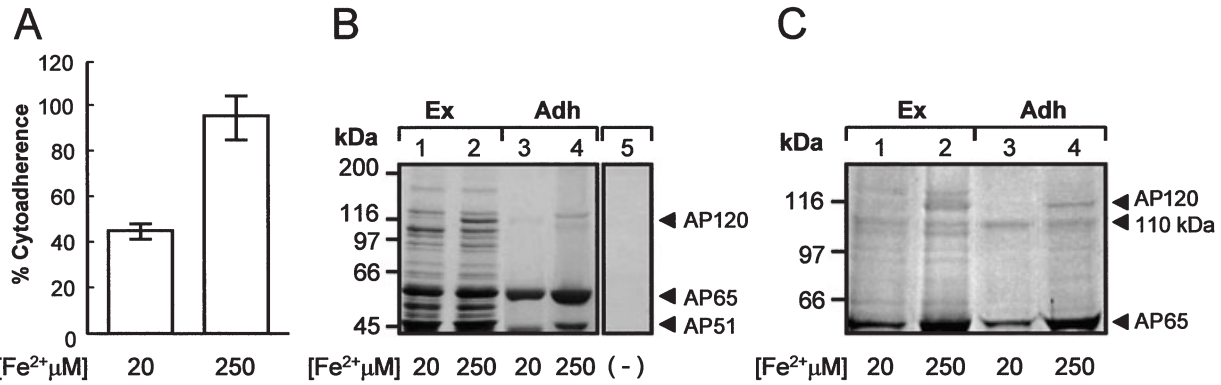

Fig. 1. A 120 kDa protein (AP120) with affinity to the surface of HeLa cells is induced by 250 μM iron concentration.
A. Percentage of cytoadherence of *T. vaginalis* isolate CNCD 188 grown in 20 or 250 μM iron medium over HeLa cell monolayers. Error standard bars are shown.
B. Coomassie brillant blue-stained SDS-PAGE (7.5%) of total-parasite extracts (Ex, lanes 1 and 2) or proteins obtained after a cell-binding assay (Adh, lanes 3 and 4) from parasites grown in 20 (lanes 1 and 3) or in 250 μM iron (lanes 2 and 4) medium. Lane 5 (–), a mock experiment with fixed HeLa cells incubated only with interaction buffer as a negative control.
C. The same experiment as in (B), but using metabolically labelled (^{35}S-Met-Cys) parasites grown in 20 (lanes 1 and 3) or 250 μM iron (lanes 2 and 4) medium. Proteins were separated by SDS-PAGE (5%) and analysed by autoradiography on a Kodak X Omat XR film. kDa, molecular size markers. Arrowheads show position of the adhesin bands.

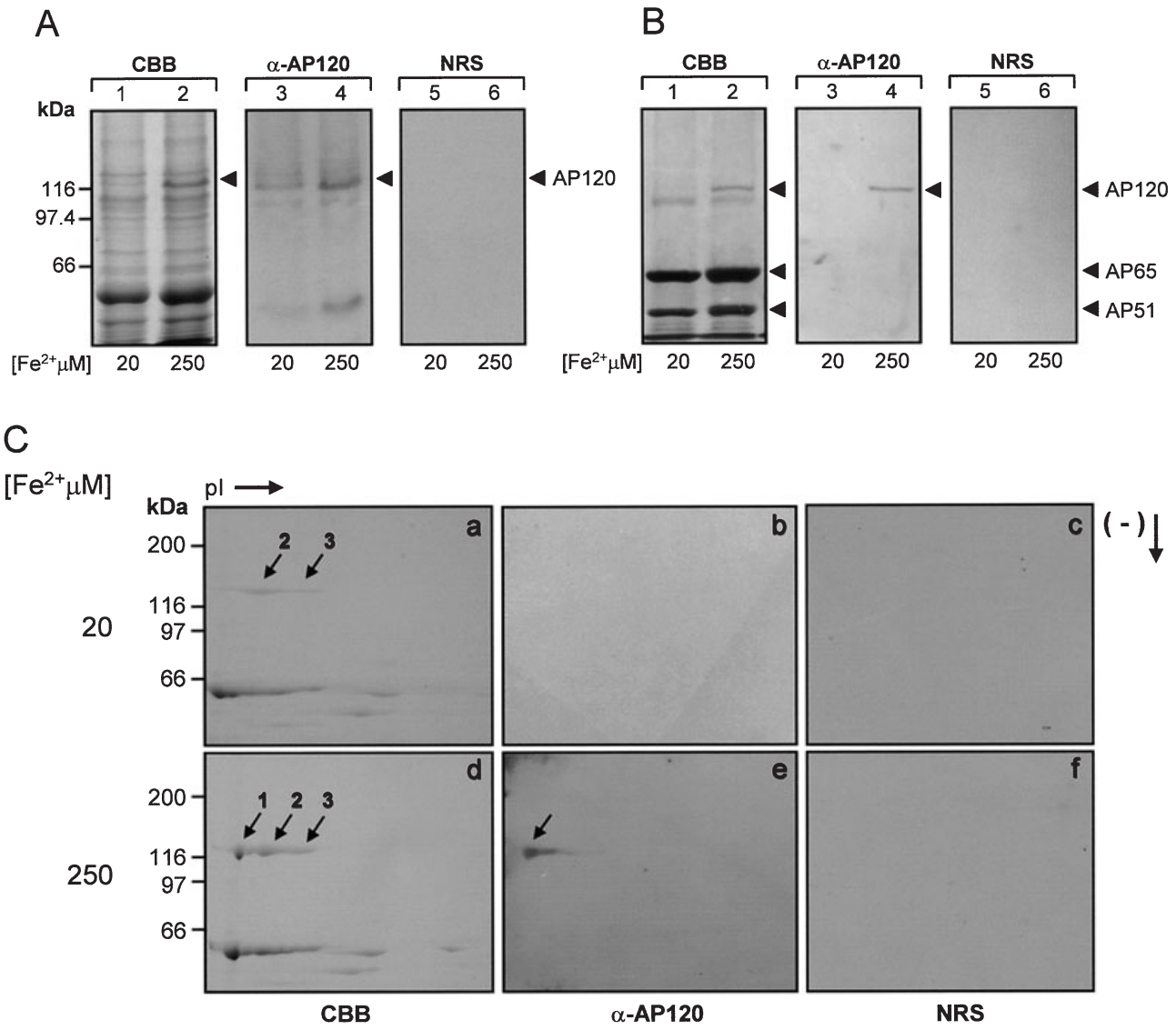

Fig. 2. Specificity of the anti-AP120 antibody.
A. Coomassie brillant blue-stained SDS-PAGE (7%) of total-protein extracts from parasites grown in 20 or 250 μM iron medium (CBB, lanes 1 and 2). Duplicate Western blot using the anti-AP120 (α-AP120, lanes 3 and 4) or pre-immune serum (NRS, lanes 5 and 6) (1:10 000) developed by chemiluminescence. Arrowheads show position of AP120.
B. Coomassie brillant blue-stained SDS-PAGE (7%) of cell-binding proteins obtained after a cell-binding assay with extracts from parasites grown in 20 or 250 μM iron medium (CBB, lanes 1 and 2). Duplicated Western blot assay using the same treatments as in (A) (lanes 3–6). Arrowheads show position of the adhesin bands.
C. Coomassie brilliant blue 2-D SDS-PAGE (7%) of total proteins of *T. vaginalis* grown in 20 or 250 μM iron medium separated using conditions for very basic proteins (CBB, a and d). Duplicate Western blots with the anti-AP120 (α-AP120, b and e) or pre-immune (NRS, c and f) rabbit serum were performed as in (A). Arrows show position and number of spots corresponding to proteins contained in the 120 kDa protein band. kDa, molecular size markers; pI, direction of isoelectrofocusing from basic to acidic conditions; (–)↓, direction of the SDS-PAGE by size.

for different experiments. In one-dimensional (1-D) Western blot assays, using extracts of parasites grown in 20 and 250 μM iron medium (Fig. 2A), the anti-AP120 antibodies reacted with a 120 kDa band, which was more intense in proteins from parasites grown in iron-rich medium (Fig. 2A, lanes 3 and 4). In these experiments, we observed other low-molecular-weight bands that could be degradation products from AP120. After cell-binding assays (Fig. 2B), the anti-AP120 antibody also reacted with the AP120 protein from parasites grown in 250 μM iron medium (Fig. 2B, lane 4), whereas no reaction was observed on similar proteins from parasites grown in 20 μM iron medium (Fig. 2B, lane 3). The control pre-immune serum did not react with any of the proteins tested (Fig. 2A and B, lanes 5 and 6).

The AP120 protein band analysed by basic two-dimensional (2-D) gel electrophoresis corresponds to the spot induced in 250 μM iron concentrations

The anti-AP120 antibody was also tested by two-dimensional (2-D) Western blot of total-protein extracts from parasites grown in both iron conditions (Fig. 2C). A differential basic protein pattern from parasites grown in 20 and 250 μM iron medium was observed on 2-D Coomassie brillant blue-stained gels (CBB). The 120 kDa region exhibited two spots (2 and 3) in extracts from parasites grown in 20 μM iron and three spots (1, 2 and 3) in those grown in 250 μM iron medium (Fig. 2C, a and d). It is noteworthy that spot 1 was only observed in proteins from parasites grown in 250 μM iron medium (Fig. 2C, d). In 2-D Western blot assays, the anti-AP120 antibody mainly recognized spot 1 only on proteins from parasites grown in 250 μM iron (Fig. 2C, e, arrow), although the same cell equivalents were used. As expected, no reaction was observed with the control pre-immune serum (Fig. 2C, c and f).

The AP120 is localized on the surface of parasites grown in 250 μM iron medium

We performed a cellular fractionation of parasites grown in 20 and 250 μM iron concentrations to determine the localization of the AP120 protein in *T. vaginalis.* Each fraction was analysed by SDS-PAGE. Figure 3A shows that protein patterns from the membrane fraction of parasites grown in 250 μM iron medium have a 120 kDa protein band (lane 4), which was observed as a light band in the corresponding cytoplasm fraction (lane 5) as compared with the total-protein patterns (lane 6). This protein was not detected under our experimental conditions in cellular fractions from parasites grown in 20 μM iron medium (lanes 1 and 2). However, a light band of 120 kDa was observed in total-protein extracts from the same parasites (lane 3).

Biotin labelling of live parasites before cell-binding assays confirmed these results. Figure 3B shows that the AP120 protein band was only present in the adhesin preparation from biotin-labelled parasites grown in 250 μM iron medium (lane 4), confirming that AP120 is in the plasma membrane. In addition to the absence of the AP120 band in parasites grown in 20 μM iron (lane 2), the other three biotinylated adhesin bands, AP65, AP51 and AP33, were in lower amounts in spite of using the same cell equivalents.

Moreover, comparative immunocytochemistry with the anti-AP120 antibody was performed with cryosections obtained from parasites grown in 20 and 250 μM iron medium to further confirm surface localization of AP120. Figure 3C shows a representative experiment of semi-thin cryosections incubated with the anti-AP120 antibody showing fluorescence reactivity on the surface of 95% of parasites grown in 250 μM iron medium (b). Some parasites showed also fluorescence signal in the cytoplasm. In contrast, weak fluorescence was observed in 18% of the parasites grown in 20 μM iron medium (d). No fluorescence was detected with the secondary antibody used as a negative control (f). Figure 3C (a, c and e) corresponds to the phase contrast microscopy of the same samples analysed by indirect immunoflurescence. In addition, duplicate immunogold labelling experiments showed approximately threefold more gold particles (34 particles) detecting AP120 on the trichomonad surface, cytoplasm, and vacuoles of parasites grown in 250 μM iron medium (Fig. 3D, a, arrows) than the samples from parasites grown in 20 μM iron medium (11 particles) (Fig. 3D, b, arrows). Control cryosections incubated with the secondary antibody showed no recognition (Fig. 3D, c), thus confirming that the AP120 protein is located on the surface of *T. vaginalis* when grown in iron-rich medium.

The AP120 protein participates in trichomonal adherence to HeLa cells

To demonstrate the involvement of AP120 in parasite attachment to the host cells, different concentrations of the anti-AP120 IgG fraction or different amounts of the electro-eluted AP120 were tested on trichomonal cytoadherence over HeLa cell monolayers (Fig. 4). *T. vaginalis* adherence was inhibited in a concentration-dependent manner when parasites were incubated with 100–300 μg ml^{-1} of the anti-AP120 IgG fraction before interaction with the HeLa cell monolayers. Although 100 μg ml^{-1} antibody did not affect parasite attachment, raising the antibody concentration twofold (200 μg ml^{-1}) and threefold (300 μg ml^{-1}) reduced trichomonal cytoadherence 25% and 46% respectively. The IgGs from control serum had no effect (Fig. 4A). The antibody concentration (300 μg ml^{-1}) that gave the greatest cytoadherence inhibition did not cause agglutination or affect parasites mobility (data not shown).

The effect of AP120 on trichomonal cytoadherence was also evaluated incubating fixed HeLa cell monolayers with different amounts (0.5–10.0 μg) of electro-eluted AP120 before the addition of ^{3}H-labelled parasites. The AP120 protein also reduced *T. vaginalis* cytoadherence to HeLa cell monolayers in a concentration-dependent manner, with a maximum inhibition of 64%, using 10 μg of AP120. Interestingly, only 1 μg of AP120 inhibited 35% of trichomonal cytoadherence, 2.5 μg inhibited 45% and 5.0 μg 60%. Bovine serum albumin (BSA) used under identical conditions did not affect parasite adherence (Fig. 4B). The integrity of AP120 used in these experiments was verified by electrophoresis in Coomassie brillant blue-stained gels (Fig. 4C).

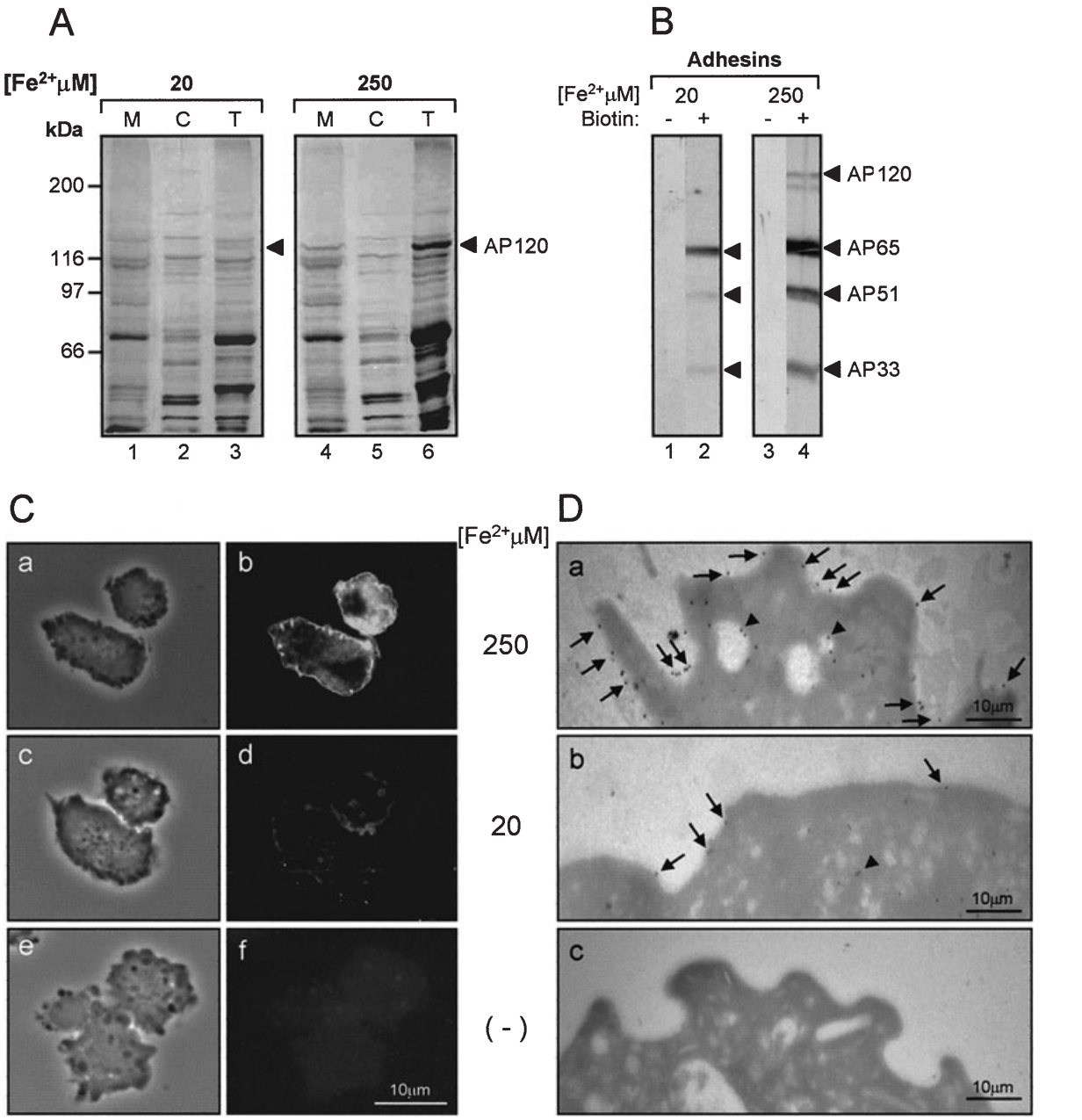

Fig. 3. Localization of AP120 on the *T. vaginalis* cell surface.
A. Coomassie brilliant blue-stained SDS-PAGE (7.0%) of proteins from different cellular fractions of *T. vaginalis* grown in 20 (lanes 1–3) or 250 μM iron medium (lanes 4–6). M, membrane proteins; C, cytoplasmic proteins; T, total-protein extract; kDa, molecular size markers. Arrowhead shows position of AP120.
B. Biotin surface labelling of parasites grown in 20 or 250 μM iron medium (lanes 1 and 2 or lanes 3 and 4 respectively). Then, a cell-binding assay was performed and proteins (Adhesins) were separated by SDS-PAGE (10%), transferred to NC membranes and developed with streptavidin-HRPO and 4-Cl-naphtol. Unlabelled [(–), lanes 1 and 3] or biotin-labelled [(+), lanes 2 and 4] adhesins. Arrowheads show position of AP120, AP65, AP51 and AP33 protein bands.
C and D. Semi-thin (C) and thin (D) cryosections from parasites grown in 20 or 250 μM iron medium incubated for 1 h at 25°C with the anti-AP120 antibody (1:50), washed and incubated for 30 min or 1 h at 25°C with the goat anti-rabbit secondary antibody coupled to FITC (1:100) or colloidal gold (1:50) respectively. Samples were analysed by epifluorescence or transmission electron microscopy (C or D). As a negative (–) control cryosections were treated with the corresponding secondary antibody only. Arrows show plasma membrane localization of the colloidal gold grains. Arrowheads show vacuolar localization of the colloidal gold grains.

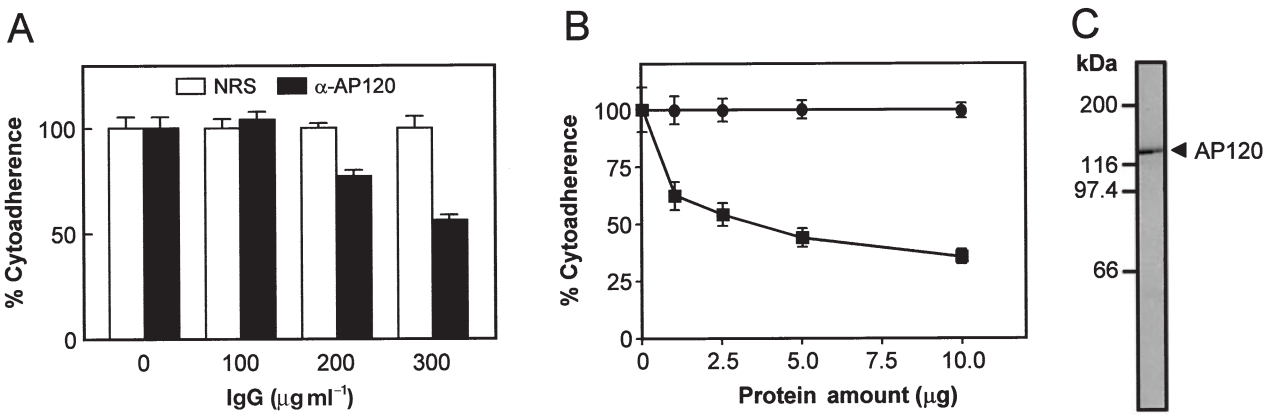

Fig. 4. Effect of the anti-AP120 antibody and the AP120 protein in *T. vaginalis* cytoadherence.
A. Effect of the anti-AP120 antibody on trichomonal adherence. Percentage of parasite adherence over HeLa cell monolayers after incubation of tritiated parasites with different concentrations (0, 100, 200 and 300 μg ml^{-1}) of anti-AP120 (■) or NRS (□) IgG fraction. Each bar represents the mean of the percentage of cytoadherence of two experiments with triplicate samples, and error bars represent the standard deviations.
B. Effect of the AP120 protein on trichomonal cytoadherence. The parasite adherence over fixed HeLa cell monolayers incubated with 0, 1.0, 2.5, 5.0 and 10.0 μg of electro-eluted AP120 or bovine serum albumin (BSA) before adding tritiated parasites. Each point represents the mean of the percentage of cytoadherence in the presence of AP120 (■) or BSA (●) used as a negative control on duplicate experiments with triplicate samples. Bars represent standard deviations.
C. Coomassie brillant blue-stained SDS-PAGE (7.5%) of the electro-eluted AP120 protein used in the inhibition experiments. kDa as in Fig. 1; arrowhead shows position of AP120.

AP120 recognizes a 130 kDa membrane protein of HeLa cells

To determine the presence of putative receptors for the AP120 on the surface of HeLa cells, we incubated fixed HeLa cells with biotin-labelled AP120 (AP120-B), streptavidin-FITC and the cells were analysed by confocal microscopy. Fluorescence was present on the surface of HeLa cells that interacted with AP120-B with a ring and patch distribution (Fig. 5A, arrows). No signal was detected on HeLa cells incubated with biotin-labelled-BSA or only with streptavidin-FITC (data not shown).

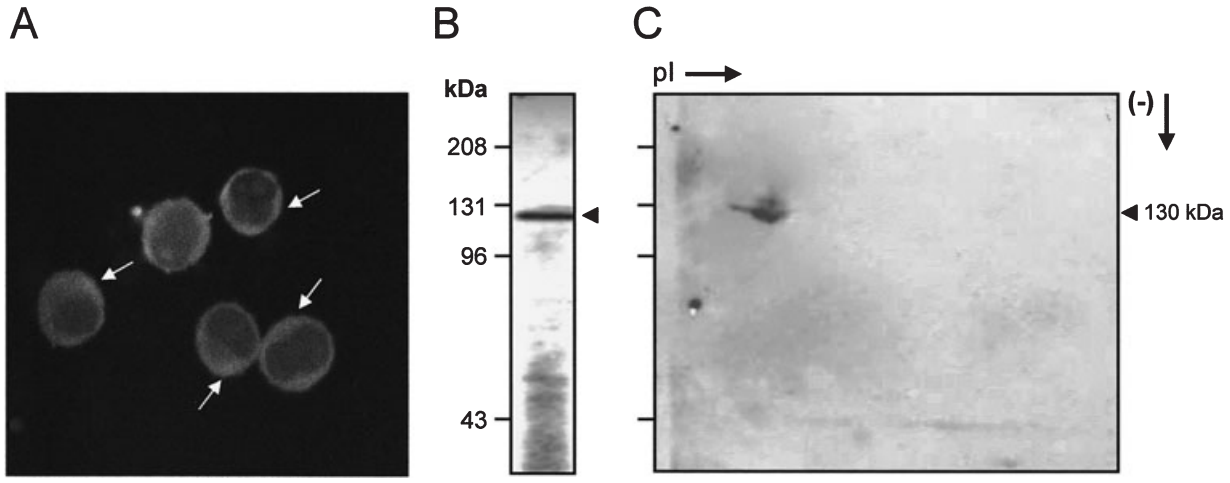

Fig. 5. A putative receptor of 130 kDa on the surface of HeLa cells.
A. The electro-eluted AP120 labelled with biotin (AP120-B) was used in a binding assay to fixed HeLa cells and analysed by confocal microscopy at 40×. Fixed HeLa cells were incubated with AP120-B (2.5 μg per 10^6 cells) for 2 h at 37°C, washed with PBS and incubated with streptavidin-FITC (1:500). Arrows show patches of fluorescence.
B and C. 1-D (B) and 2-D (C) SDS-PAGE (9%) of HeLa cell proteins were transferred to NC membranes for overlay assays using the proteins from parasites grown in high iron medium and interacting them with the NC membranes for 18 h at 4°C. Then, NC membranes were incubated with the anti-AP120 antibody (1:1000), with a secondary goat anti-rabbit horseradish peroxidase (HRP) antibody (1:3000) and developed with 4-Cl-naphtol. Arrows show the putative receptor for trichomonad AP120, a band or spot of 130 kDa.

Next, to investigate whether AP120 recognized a putative receptor in the HeLa cell membrane proteins, we carried out overlay assays. Membrane HeLa proteins were separated by 1-D and 2-D SDS-PAGE and transferred to nitrocellulose (NC) filters (Fig. 5B and C). Then, filters were incubated with parasite membrane fractions from parasites grown in iron-rich medium and the receptor–AP120 complex was displayed with the anti-AP120 antibody. After incubation of filters containing HeLa proteins with parasite proteins, the anti-AP120 antibody recognized a 130 kDa membrane protein band (Fig. 5B) that corresponded to a single spot with pI 6.0 (Fig. 5C). When we omitted the incubation of NC filters containing HeLa proteins with trichomonad proteins, the anti-AP120 antibody did not react (data not shown). Incubation of NC filters containing HeLa proteins directly with biotin-labelled AP120 also recognized the same 130 kDa protein band and spot by 1-D and 2-D overlay assays respectively (data not shown). In addition, this 130 kDa protein band and spot appeared biotin-labelled after live HeLa cells were surface-labelled with biotin (data not shown).

The AP120 adhesin is a glycoprotein

We investigated also whether the AP120 adhesin is a glycoprotein by 1-D and 2-D Western blot assays using biotin-labelled concanavalin A lectin (ConA-B). As expected, many parasite proteins separated in 1-D and 2-D gels reacted with ConA-B including the two or three spots forming the 120 kDa band present in the protein extracts from parasites grown in 20 and 250 μM iron medium (data not shown). No label was observed on the BSA band used as a negative control (data not shown). These data indicate that the AP120 adhesin (Fig. 2C, d, arrow 1) is a glycoprotein present only in parasites grown in iron-rich medium.

AP120 adhesin shares homology with the hydrogenosomal enzyme PFO-A

To identify the novel AP120 adhesin induced by high iron concentrations, a tryptic mapping of spot 1 detected in 2-D gels (Fig. 2C, d, arrow 1) was performed and analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The masses of 22 peptides obtained from the AP120 protein spot 1 corresponded to peptides masses from the hydrogenosomal PFO of *T. vaginalis* encoded by the *pfoa* gene (Hrdy and Muller, 1995). Some of these peptides overlapped such as 17 with 22, 24 with 29, and 26 with 27 (Table 1). After these results, we also analysed by MALDI-TOF-MS spots 2 and 3 from the 120 kDa region of parasites grown in 250 μM iron medium (Fig. 2C, d, arrows 2 and 3). Interestingly, these two spots were also identified as PFO-A-related proteins with 24 and 21 identical mass peptides respectively (Table 1). It is noteworthy that tryptic digestion of the three spots showed common and distinct mass peptides among them (Table 1), which could partially explain the differences observed in the pI. These differences may reflect distinct unknown post-translational modifications or unidentified PFO genes in this parasite.

*The anti-*E. histolytica *PFO (anti-EhPFO) antibody recognizes the surface of* T. vaginalis *and cross-reacts with the AP120 adhesin*

Based on the 45% sequence identity between the PFO of *Entamoeba histolytica* and *T. vaginalis* (Rodríguez *et al*. 1996), the homology of AP120 with PFO was first analysed by immunolocalization assays with an anti-*E. histolytica* PFO (anti-EhPFO) antibody. This antibody was prepared against a recombinant fragment of 648 amino acid residues (from nucleotide 1260 to 3498) of the carboxy-terminus of the *E. histolytica* PFO (EhPFO) (Rodríguez *et al*., 1996). Eleven of the 22 peptides identified in AP120 were found in the same *T. vaginalis* PFO (TvPFO) region (Table 1; Fig. 6A, clear boxes I–XI). It is noteworthy that three of them, peptides V, VII and VIII, showed high identity (85%) at the amino acid level with the EhPFO (data not shown) and peptides V and VII were present in the three PFO-like spots (Table 1); whereas, peptides I and X were only identified in AP120 (Table 1).

Immunofluorescence assays with cryosections from the same preparation used above (Fig. 3C) showed that the anti-EhPFO antibody recognized the surface of *T. vaginalis* parasites grown in a 250 μM iron medium with greater intensity than in a 20 μM iron medium (Fig. 6B, b and d). This antibody also shows cytoplasm recognition on hydrogenosome-like membranes.

The cross-reactivity of the anti-EhPFO antibody with the AP120 protein was also analysed over the *T. vaginalis* and *E. histolytica* proteins by Western blot with both anti-*T. vaginalis* AP120 (anti-TvAP120) and anti-EhPFO antibodies. Figure 6C shows that the anti-EhPFO antibody reacted with the 120 kDa protein band from total extract (lane 2, α-EhPFO) and with the adhesin preparation (lane 4, α-EhPFO) of *T. vaginalis* cultivated in 250 μM iron, in addition to the homologue *E. histolytica* protein (lane 5, α-EhPFO). Likewise, the anti-TvAP120 antibody recognized EhPFO (lane 5, α-TvAP120), in addition to its homologues *T. vaginalis* proteins (lanes 2 and 4, α-TvAP120) from parasites grown in iron-rich medium. No recognition was observed with the secondary antibody used as a negative control [lanes 1–5 (–)]. Figure 6C also shows the Coomassie brillant blue patterns from each set of protein preparations used in this experiment (lanes 1–5, CBB). These results show that there is a cross-recognition between the antibodies to these two proteins, which

Table 1. Peptides identified after tryptic digestion and mass spectrometry (MALDI-TOF-MS) analysis of the three P120 protein spots obtained from *Trichomonas vaginalis* parasites grown in high iron conditions.

Peptide number[a]	m/z (av)[b]	Position[c]	Peptides identified by MALDI-MS[d]				Amino acid sequence[g]
			PFO-A[e]	Spot 1 (AP120)[f]	Spot 2[f]	Spot 3[f]	
1	917.0157	1145–1151	XI	+	+	+	(K)TRYEGYK(K)
2	984.1504	1084–1091	IX	+	+	+	(K)TGYLNLFR(Y)
3	1006.1569	1117–1125		–	+	+	(K)GENRFAALK(D)
4	1015.1211	457–466		–	+	+	(K)SGGVTTSHLR(F)
5	1104.3057	776–785		–	–	+	(R)GHLLPPTNVR(N)
6	1166.2436	210–219		+	+	+	(R)SLNPEHPSER(G)
7	1302.4793	1106–1116	X	+	–	–	(K)QPDYNLDPLVK(G)
8	1325.5605	368–379		+	+	+	(R)DFAPVHVEAIVK(N)
9	1387.6293	237–248		–	+	+	(R)KPFOALPTEVEK(M)
10	1406.5940	260–270		+	+	+	(R)YYKPYQYAGPR(D)
11	1507.7436	616–628	III	+	+	–	(K)AEWLNAPVEPRPK(H)
12	1527.7533	597–609	II	+	+	+	(K)NWDMVDHALQGLK(E)
13	1547.7213	444–456		+	+	+	(K)LYGQAYFAYDAHK(S)
14	1569.8754	325–338		–	+	+	(K)ICVLDKVVDPTGPR(E)
15	1578.8580	536–549		–	+	+	(K)LYTIDATQIAIDLK(L)
16	1620.7239	905–918	VI	+	+	+	(K)VWDQDKESGDLAEK(I)
17	1630.8500	339–353		+	+	+	(R)EPLFEDVAAALIGER(N)
18	1656.9784	235–248		+	–	–	(K)LRKPFQALPTEVEK(M)
19	1702.0635	103–117		–	+	–	(K)IIGEMCPAVFHISAR(C)
20	2361.8325	303–323		+	+	–	(K)VHLFRPFSVEMENAAIPASVK(K)
21	2408.7367	504–526	I	+	–	–	(K)GGVFVINFPGSADLNKDLPGSFR(K)
22	2452.7875	331–353		+	–	–	(K)VVDPTGPREPLFEDVAAALIGER(N)
23	2546.7978	847–869	V	+	+	+	(R)GHGPAWANSLFEDNAEFGYGMFK(A)
24	2790.0318	179–201		+	+	+	(R)TSHEINTYEELDNETLWPLIDQK(A)
25	2822.2544	390–415		+	+	+	(R)FTVGVVNPETQLPLGKPFDHLPEGTK(Q)
26	3177.5226	947–976	VIII	+	+	–	(K)QVWIVGGDGWAYDIGYGGLDHVLASGENVK(I)
27	3305.6978	946–976	VII	+	+	+	(K)KQVWIVGGDGWAYDIGYGGLDHVLASGENVK(I)
28	3370.8439	634–664	IV	+	+	+	(R)HIIDMSILQEGESVSVDEMVEIGLVPNDTAK(Y)
29	3419.7966	179–207		+	+	+	(R)TSHEINTYEELDNETLWPLIDQKALAAFR (A)

a. Consecutive number assigned to the identified peptides.
b. Peptide mass average [m/z (av)] identified by MALDI-TOF-MS after tryptic digestion of the three P120 protein spots obtained from a duplicate 2-D SDS-PAGE of protein extracts from *T. vaginalis* grown in high iron conditions (Fig. 2C, d).
c. Position in amino acids residues of the identified peptides (start–end) in the deduced amino acid sequence of the *T. vaginalis pfoa* gene reported by Hrdy and Muller (1995).
d. Presence (+) or absence (–) of the peptides identified by MALDI-TOF-MS on each of the three P120 protein spots.
e. Arbitrary nomenclature used to describe 11 of the 22 identified peptides in AP120 spot 1 (AP120) (Fig. 6A). These peptides were localized, after theoretical tryptic digestion, in the carboxy-terminus region of the *T. vaginalis* PFO-A-deduced amino acid sequence.
f. Protein spots obtained by 2-D SDS-PAGE of protein extracts from *T. vaginalis* grown in high iron medium (Fig. 2C, d, arrows 1, 2 and 3) and analysed by MALDI-TOF-MS.
g. Amino acid sequence of the peptides obtained from a theoretical tryptic digestion of *T. vaginalis* PFO-A-deduced amino acid sequence.

agrees with the peptide mapping results. These data suggest that the AP120 surface protein identified in this study could be a moonlighting protein related with the hydrogenosomal PFO of *T. vaginalis* that works as an adhesin when located on the surface of *T. vaginalis* in the presence of high iron concentrations.

Iron upregulates a pfoa*-like transcript*

Our data show that the expression of AP120, a *pfoa*-like gene, is upregulated by iron at the protein level. Thus, to investigate whether the expression is also affected at the transcriptional level by iron, we performed comparative reverse transcription polymerase chain reaction (RT-PCR) assays with RNA from trichomonads grown in 20 and 250 μM iron medium. The primers used were localized inside the 648-amino-acid carboxy-terminus region of the TvPFO-A to amplify a 1.1 kb fragment, which encodes seven of the 22 identified peptides in AP120 (Fig. 6A, arrows). Figure 6D shows the expected 1100 bp product obtained only from parasites grown in 250 μM iron medium [*pfoa* (30), lane 2]. However, adding five more cycles to the PCR, a light band product appeared now in the RNA from parasites grown in 20 μM iron medium [*pfoa* (35), lane 1]. These data indicate that a *pfoa*-like gene is better transcribed in the presence of 250 μM iron concentration (*pfoa*, lane 2). As a control, we amplified the β-tubulin gene from the same samples used above (León-Sicairos *et al*., 2004). An identical band of 112 bp from the β-tubulin gene was amplified using RNA from both iron growth conditions (β-Tub, lanes 1 and 2). Densitometry analysis of the RT-PCR products confirmed these results

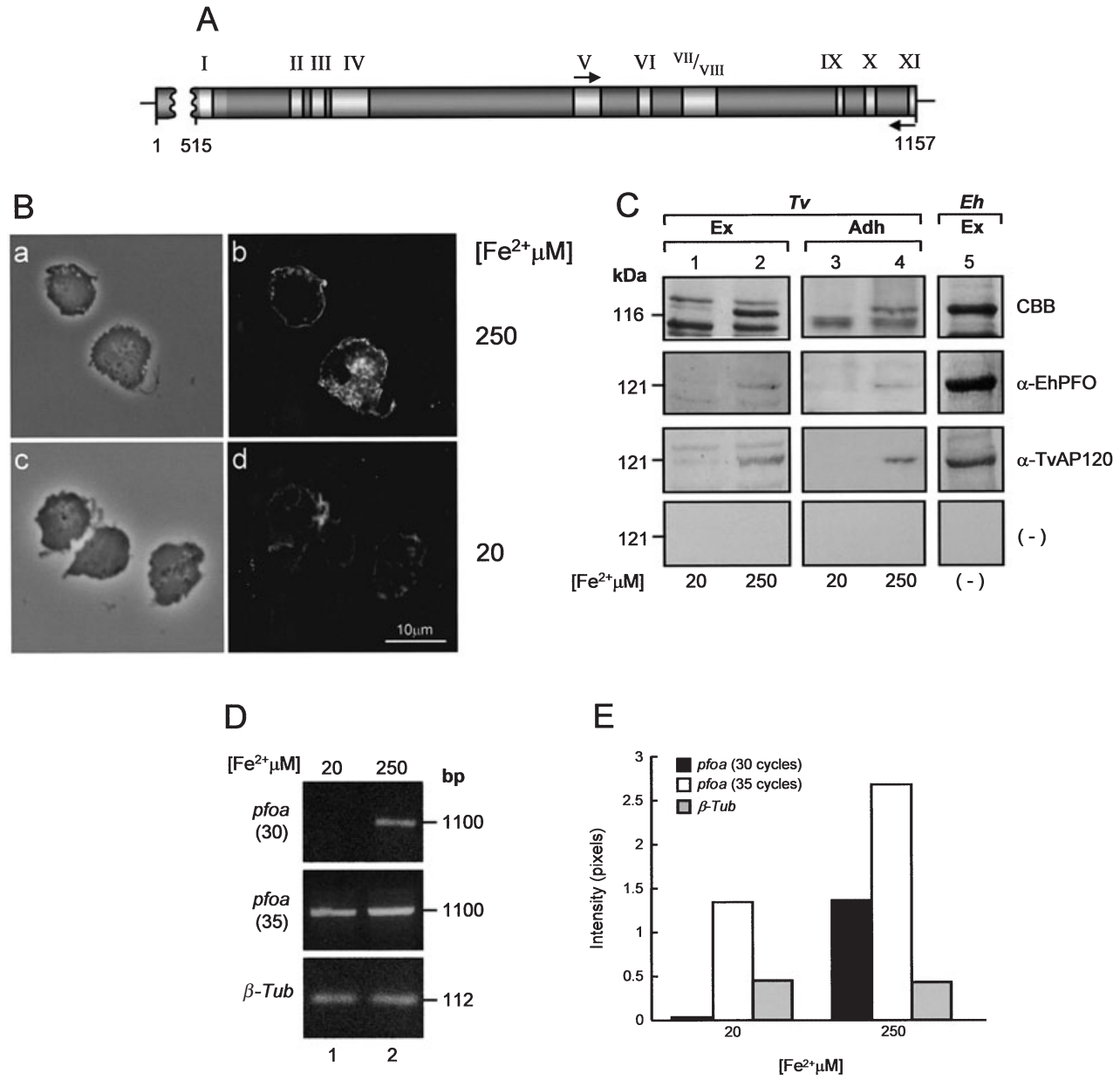

Fig. 6. Cross-recognition of the anti-PFO and anti-AP120 antibodies.
A. Schematic representation of the *T. vaginalis pfoa* gene product (TvPFO-A) indicating the position of 11 of the 22 peptides identified by MALDI-TOF-MS in AP120 localized in the homologue carboxy-terminus fragment (648 amino acid residues) of a recombinant protein of the *E. histolytica* PFO. A polyclonal antibody (anti-EhPFO) produced against this *E. histolytica* recombinant protein was used for immunolocalization and Western blot assays described below. Arrows show the position of the sense and anti-sense primers used to amply a 1.1 kb fragment of the carboxy-terminus region of the trichomonad *pfoa* gene by RT-PCR assays.
B. Semi-thin cryosections of *T. vaginalis* grown in 250 μM (b) or 20 μM (d) iron medium incubated with the anti-EhPFO antibody (1:250) and analysed by epifluorescence microscopy. Phase contrast microscopy (a and c) of the same samples analysed by fluorescence (b and d).
C. Coomassie brilliant blue-stained (CBB) 1-D SDS-PAGE (7%) of protein extracts (Ex, lanes 1 and 2) or adhesins (Adh, lanes 3 and 4) of *T. vaginalis* and protein extracts of *E. histolytica* (Ex, lane 5). Duplicate gels were transferred to NC membranes and incubated with anti-EhPFO (1:1000) (α-EhPFO), anti-AP120 (1:100) (α-TvAP120) antibodies, and normal mouse serum (1:100) (–). In this figure only the gel fragments of the 120 kDa region are shown. kDa as in Fig. 1.
D. RT-PCR with specific primers for *pfoa* gene (*pfoa*, 1100 bp) using 30 (30) or 35 (35) cycles for PCR or β-tubulin gene (*β-Tub*, 112 bp), used as a control, and cDNA from parasites grown under 20 μM and 250 μM iron medium.
E. Densitometrical analysis of the RT-PCR products from (D) using Quantity One software.

(Fig. 6E). These data show that a *pfoa*-like transcript is induced in iron-rich medium, which could correspond to the AP120 protein spot induced in 250 μM iron concentrations (Fig. 2C, d, arrow 1).

The 1100 bp fragment was sequenced and then compared with the reported *pfoa* gene (Hrdy and Muller, 1995), showing a size of 1178 bp with 99% identity at the nucleotide level with the reported *pfoa* gene. At the amino acid level only three amino acid residues in positions 1014, 1024 and 1127 changed from K to E, from R to K and from I to T respectively (data not shown). These sequence data have been submitted to the GenBank database under Accession No. AY652962.

Immunogenicity of the AP120 adhesin

Finally, the *in vivo* relevance of AP120 during trichomonal infection was investigated in sera from 14 patients attending the Centro Nacional de Clínicas de Displasias (CNCD) at the Hospital General de México (HGM). To determine the presence of antibodies to the AP120 putative adhesin in human sera, a Western blot assay was performed on NC membranes containing only the electro-eluted AP120 protein (Fig. 4C). Of the 14 sera tested from patients with clinically diagnosed trichomonosis, only 10 were confirmed by *in vitro* culture (Hernández-Gutiérrez *et al*., 2004). Four sera from healthy women were used as controls. Sera from all patients with culture-positive trichomonosis (10/10) recognized the AP120 protein; whereas, sera from patients negative by *in vitro* culture and sera from healthy people did not recognize the AP120 protein (data not shown). Anti-trichomonad and preimmune rabbit sera were used as positive and negative controls respectively (data not shown). These data show that the AP120 adhesin is immunogenic during trichomonal infection probably due to the homology with the PFO hydrogenosomal enzyme, which is absent in the human host.

Discussion

Trichomonal cytoadherence is a multifactorial event mediated by ligand–receptor interactions (Alderete *et al*., 1995b) that also requires proteinase activity (Arroyo and Alderete, 1989; Mendoza-López *et al*., 2000), involves transmembranal signalling processes (Arroyo *et al*., 1993) and is regulated by cell contact and iron concentrations (Lehker *et al*., 1991; Arroyo *et al*., 1993; García *et al*., 2003). Trichomonads have evolved to successfully parasitize the constantly changing and nutrient-limiting female urogenital region, partially through functional diversity of proteins (García *et al*., 2003).

In this article, we present the identification and characterization of a novel 120 kDa protein with homology to a hydrogenosomal enzyme as an adhesin, AP120, of *T. vaginalis* that participates in host-cell attachment when parasites are exposed to high iron concentrations and binds to the surface of HeLa cells through a putative cell receptor. This novel adhesin has been observed as a light band during the studies of the effect of iron on parasite cytoadherence and amount of adhesins (Lehker *et al*., 1991). Even though the 120 kDa protein exhibited affinity to the surface of HeLa cells, it was not studied further thinking that this protein probably corresponded to the laminin receptor of 118 kDa reported by Silva-Filho *et al*. (1988). It was not surprising that we observed this band again studying six new clinical *T. vaginalis* isolates grown in high iron medium. Therefore, we decided to test the hypothesis that the 120 kDa protein observed in high iron conditions is another *T. vaginalis* adhesin.

Despite that four-trichomonad surface adhesins: AP65, AP51, AP33 and AP23, have been identified and characterized previously (Alderete and Garza, 1988; Arroyo *et al*., 1992), no putative receptors on the human urogenital cells have been identified for any of them yet. Nevertheless, in this work, we showed the identification of a putative receptor to the AP120 adhesin in HeLa cells, a 130 kDa surface protein. Interestingly, a surface protein in HeLa cells of this size has been identified as the receptor for adenovirus serotype 3 (Di Guilmi *et al*., 1995). We do not know at this time whether these two molecules of 130 kDa are related. Work is in progress to clarify it, to characterize this putative receptor and to map the position of the cell-binding domain in the AP120 adhesin.

Interestingly, a tryptic mapping of AP120 showed that this adhesin shares homology with PFO, a trichomonad metabolic enzyme responsible for the oxidative decarboxylation of pyruvate to acetyl-Co-A, which is localized on the membrane of hydrogenosomes (Hrdy and Muller, 1995). It is noteworthy that the homology between AP120 and PFO was supported by the cross-reactivity shown by the anti-EhPFO and the anti-AP120 antibodies to the surface of *T. vaginalis* and with the AP120 protein, and to EhPFO respectively (Fig. 6). The behaviour of AP120 is similar to the AP65, AP51 and AP33 adhesins (Arroyo *et al*., 1992). These adhesins have also high identity to hydrogenosomal enzymes (Engbring *et al*., 1996; Alderete *et al*., 2001), are located on the parasite surface and are upregulated by cell contact and high iron concentrations (Lehker *et al*., 1991; Arroyo *et al*., 1993; García *et al*., 2003). These data suggest that four (AP120, AP65, AP51 and AP33) of the five trichomonad adhesins could be examples of moonlighting proteins (Jeffery, 1999). These proteins work as metabolic enzymes and adhesins when located on the hydrogenosomes and on the surface of trichomonads respectively (Arroyo *et al*., 1992; Hrdy and Muller, 1995; Engbring *et al*., 1996; Alderete *et al*., 2001; García *et al*., 2003). Thus, in *T. vaginalis* iron could

be one of the cofactors that trigger the switching mechanism between the distinct functions of these proteins, modulating their compartmentalization outside the hydrogenosomes (García *et al*., 2003).

Likewise, a similar phenomenon might be occurring with EhPFO, which is localized both on the kinetoplast-like organelle (EhkO) (Rodriguez *et al*., 1996; Luna-Arias *et al*., 2003) and on the plasma membrane of different species of *Entamoeba*-including *E. histolytica* (Rodriguez *et al*., 1998). Thus, the EhPFO may also be working as an adhesin when located on the surface of this parasite. This is not surprising, as numerous microbial pathogens have surface-associated metabolic enzymes that show alternative functions (Alderete *et al*., 2001). An example of functional diversity of metabolic enzymes is the mammalian enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Pancholi and Fischetti, 1993; Alderete *et al*., 2001; Maeda *et al*., 2004), which is classified as one of the typical moonlighting proteins in nature (Jeffery, 1999).

Finally, one of the differences between the AP120 and the other four adhesins (AP65, AP51, AP33 and AP23) (Arroyo *et al*., 1992) is the AP120 immunogenicity. This could result from the homology of AP120 to PFO, an enzyme that is absent in the human host, whereas three of the four adhesins (AP65, AP51 and AP33) have homology to metabolic enzymes common in host and trichomonads (Engbring *et al*., 1996). This apparent disadvantage of AP65, AP51 and AP33 is, however, part of the molecular mimicry strategies used for immune evasion by *T. vaginalis* (Alderete *et al*., 2001). This could explain the expression of AP120 on the surface of trichomonads only in high iron concentrations, as a way to avoid host recognition by the immune system. Thus, the immunogenicity of AP120 could potentially make this protein useful as a diagnostic tool or even as a possible vaccine target; however, the role of the anti-AP120 antibody response in host protection is still unclear.

In conclusion, this work confirms the existence of another surface protein with homology to a hydrogenosomal enzyme PFO as a novel adhesin participating in parasite attachment to the surface of host cells, thus contributing to understand the multifactorial and complex mechanism of cytoadherence, and supports the functional diversity of trichomonad proteins.

Experimental procedures

Parasite and HeLa cell cultures

Trichomonas vaginalis parasites from a fresh clinical isolate CNCD 188 (Alvarez-Sánchez *et al*., 2000) were cultured in trypticase–yeast extract–maltose (TYM) medium (Diamond, 1957) supplemented with 10% heat-inactivated horse serum (HIHS) and incubated at 37°C for 24 h. The TYM–HIHS medium was considered to have 20 μM iron concentration as described by Gorrell (1985). For parasites grown in high iron concentrations, the culture medium was supplemented with 250 μM ferrous ammonium sulphate solution (Lehker *et al*., 1991). For metabolic labelling, parasites were incubated for 24 h at 37°C with PRO-MIX: L-[^{35}S]-methionine and [^{35}S]-cysteine (14.3 mCi ml^{-1}; total activity 7.15 mCi, AMERSHAM). HeLa cells were grown in Dulbeco's Minimal Essential Medium (DMEM) (Gibco Laboratories) supplemented with 10% HIHS at 37°C for 48 h in a 5% CO_2 atmosphere to obtain confluent cell monolayer (Arroyo *et al*., 1992).

Cytoadherence assay

Cytoadherence assay was performed over confluent HeLa cell monolayers on 96-well microtitre plates, as previously described (Arroyo *et al*., 1992; Mendoza-López *et al*., 2000). For inhibition assays [^{3}H]-thymidine-labelled parasites were incubated for 30 min at 4°C with 100, 200 and 300 μg ml^{-1} IgG fractions from the anti-AP120 or pre-immune serum before interaction with HeLa cell monolayers. In addition, fixed HeLa cell monolayers were incubated for 30 min at 4°C with 1–10 μg of electro-eluted AP120 before the addition of tritium-labelled parasites.

Cell-binding assay

A cell-binding assay or ligand assay for isolation of adhesin proteins was performed as before (Arroyo *et al*., 1992). Briefly, 1×10^6 glutaraldehyde-fixed HeLa cells were incubated, with a detergent extract derived from 2×10^7 solubilized parasites and, after incubation, cells were washed well to remove loosely associated trichomonad proteins. Boiling cells for 3 min in Laemmli buffer (Laemmli, 1970) eluted the epithelial cell-binding proteins. These proteins were then analysed by SDS-PAGE, stained with Coomassie brilliant blue, or blotted onto NC membranes for immunodetection with antibody (Arroyo *et al*., 1992). In addition, gels from ^{35}S-labelled parasite proteins were analysed by autoradiography.

Cell fractioning

Washed parasites (2×10^8 ml^{-1}) suspended in ice-cold buffer R (20 mM Tris, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM β-mercaptoethanol) were lysed in the presence of 1 mM TLCK and 0.2 mM leupeptin (Sigma) by 10–20 strokes in a Dounce homogenizer. Nuclei and cellular debris were removed by centrifugation at 2500 *g* for 10 min at 4°C. Membrane proteins were recovered by centrifugation at 25 000 *g* for 1.5 h at 4°C and suspended in buffer R without β-mercaptoethanol (Campbell *et al*., 1995). Protein concentration was determined by the Bradford method (Bio-Rad).

Biotin labelling of live cells surface or isolated proteins

For surface labelling, parasites or HeLa cells were suspended in PBS, pH 8.0 (4×10^7 parasites ml^{-1} or 1.2×10^7 cells ml^{-1}, respectively), incubated for 30 min with 0.5 mg ml^{-1} sulpha-NSH-

LC-biotin (Pierce) diluted in PBS and washed three times with cold PBS to eliminate free biotin. The cell viability was assessed by the trypan blue exclusion method before and after biotin treatment. Next, parasites were lysed for the cell-binding assay, as described above; whereas HeLa cell proteins were obtained by trichloroacetic acid (TCA) precipitation as for 2-D gel analysis. Unlabelled parasites or HeLa cells were used as controls. After SDS-PAGE, proteins were transferred to NC membranes, processed as for Western blot and developed with streptavidin-peroxidase (Pierce). To label AP120 with biotin (AP120-B), electro-eluted AP120 (10 μg) was incubated with 100 μg of biotin in 0.1 M bicarbonate buffer, pH 8.2 with 0.8% NaCl, for 1 h at room temperature and dialysed against PBS, pH 7 for 24 h, to remove unbound biotin.

Production of a polyclonal antibody against AP120

Rabbits were inoculated subcutaneously eight times at 3 week intervals with acrylamide gel fragments containing ≈100 μg of AP120 protein in the presence of Freund's adjuvant (Gibco). The immune serum (anti-AP120) was obtained 7 days after the last inoculation (Harlow and Lane, 1988). For cytoadherence inhibition assays, the IgG fractions from serum was obtained by the caprylic acid method (Harlow and Lane, 1988).

Western blot assays

Proteins in gels were transferred to NC membranes (Bio-Rad) (Towbin *et al*., 1979) and incubated with the anti-AP120 serum (1:10 000), human patient sera (1:100) or biotin-labelled Concanavalin-A lectin (ConA-B) (1:25 000) for 18 h at 4°C. Then, membranes were incubated with peroxidase-conjugated secondary antibodies (Bio-Rad; 1:3000) or with streptavidin-peroxidase (1:5000) for 2 h at 25°C, respectively, and developed with 4-chloro1-naphtol or luminol (Bio-Rad).

Two-dimensional gel electrophoresis

Parasite or HeLa cell total proteins were precipitated with 10% TCA and solubilized with 2-D sample buffer (9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 2% bio-Lyte 3/10 ampholytes and 0.05% bromophenol blue) followed by high-speed centrifugation. Isoelectrofocusing gels and total-protein samples were prepared using standard protocols (O'Farrell, 1975; Hochstrasser *et al*., 1988). HeLa cell protein samples were loaded and electro-focused as recommended by the Mini-Protean II 2-D Cell Instruction Manual (Bio-Rad). For parasite proteins, special conditions for very basic proteins using non-equilibrium pH gradient electrophoresis (NEPHGE) were used (Ausubel *et al*., 1999). Focused gels were used immediately for SDS-PAGE on 7% or 9% acrylamide gels and stained with Coomassie brilliant blue. Duplicate gels were transferred to NC for Western blot assays.

Immunolocalization assays

Parasites were fixed with 4% paraformaldehyde–0.5% glutaraldehyde for 1 h at 25°C and embedded in 2.3 M sucrose in 2.5% polyvinylpirrolydone (dissolved in 10 mM glycine) for 1 h. The mixture was changed once and parasites were incubated for another 18 h at 25°C in it. Then, samples were frozen in liquid nitrogen and semi-thin (1 μM) and thin (60 nM) cryosections were obtained with a cryoultramicrotome (Ultracut E, Reichert-Jung). For indirect immunofluorescence (IFI) assays, the semi-thin cryosections were mounted in glass slides. For the transmission electron microscopy (TEM) immunogold localization, thin cryosections were placed on nickel grids. Both type of samples were incubated with the anti-AP120 (1:50) for 1 h at 25°C. Next, samples for IFI were incubated with the FITC-conjugated secondary antibody (Pierce, 1:100) for 30 min at 25°C, mounted with Vectashield resin (Vector Laboratories), and analysed by epifluorescence microscopy (Axiophot, Zeiss). For immunogold localization, samples were incubated with the 15 nM gold-conjugated secondary antibody (ZYMED, 1:50) for 1 h at 25°C. Then, the grids were contrasted with 2% methylcellulose containing 0.3% uranyl acetate for 2 min on ice, and analysed by TEM (Morgagni 268 D, Philips).

Overlay assays

The NC membranes containing membrane proteins from HeLa cells separated in 1-D and 2-D gels were incubated for 18 h at 4°C with total-protein extracts or with membrane proteins obtained from 2×10^7 parasites grown in high iron conditions. Then, NC membranes were washed three times with PBS–0.1% Tween 20, incubated with the anti-AP120 antibody diluted 1:10 000 as for Western blot and developed by chemoluminescence.

Protein identification

The protein spots of interest from a 2-D gel were cut out of the gel. A tryptic mapping was performed by the MALDI-MS peptide mass mapping method and analysed on a ToFSPec SE MALDI-TOF mass spectrometer (Protein Unit of the Columbia University in California). To search genomic databases, a program developed by UC, which uses fragment ion masses to search the NCBI databases for matches to peptides from known proteins, was used.

RT-PCR assays

RT-PCR assays were performed using the Superscript RNAse H^- Reverse Transcriptase kit (Stratagene) as recommended by the manufacturer. Total RNA from parasites grown in both iron conditions was reverse-transcribed using AMV reverse transcriptase and the oligo (dT) primer. Then, a fragment of 1100 bp of the *pfoa* gene was amplified by PCR using a sense primer PFOR5′ 5′-GAAGAGGGCAACTGGGA-3′ at position 2279–2299 nt and PFOA3′ primer 5′-ATCTTCTTGTAGCCCTCGTAA-3′ at position 3436–3457 nt (Fig. 6A). A fragment of 112 bp of the *T. vaginalis* β-tubulin gene was amplified by PCR using BTUB9 primer: 5′-CATTGATAACGAAGCTCTTTACGAT-3′ and BTUB2 primer: 5′-GCATGTTGTGCCGGACATAACCAT-3′, as an internal control (León-Sicairos *et al*., 2004).

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