Membrane-bound acid phosphatase (MAP) from *Entamoeba histolytica* has phosphotyrosine phosphatase activity and disrupts the actin cytoskeleton of host cells

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SUMMARY

Protein tyrosine phosphatases (PTPases) have been described as virulence factors in different pathogenic microorganisms. The pathogenic process by *Entamoeba histolytica* is a multifactorial phenomenon that occurs in 3 steps: adhesion, cytolytic and cytotoxic effect, and phagocytosis. Lytic enzymes may participate during the second part of this process. In this work, we determined that purified membrane-bound acid phosphatase (MAP) from *E. histolytica* trophozoites has PTPase activity. The enzyme specifically dephosphorylated *O*-phospho-L-tyrosine at optimum pH of 5·0, with little activity towards *O*-phospho-L-serine, *O*-phospho-L-threonine, and ATP. It was inhibited by ammonium molybdate and sodium tungstate, and trifluoperazine did not show any effect. A monoclonal antibody against the catalytic domain of the human placental PTPase 1B, cross-reacted with a 55 kDa molecule present in the solubilized fraction. The interaction of the amoebic PTPase with HeLa cells resulted in the alteration of the cell actin cytoskeleton by disruption of the actin stress fibres.

Key words: acid phosphatase, actin stress fibres, *Entamoeba histolytica*, phosphotyrosine phosphatase, signal transduction.

INTRODUCTION

Phosphorylation of proteins is one of the most common mechanisms for regulation of intracellular processes. This intracellular phosphorylation is highly controlled by protein kinases and protein phosphatases (Hunter, 1995). Phosphorylation can occur on serine/threonine (Ser/Thr) or tyrosine (Tyr) residues of intracellular proteins, and is involved in processes such as cell growth, proliferation and differentiation. The enzymes responsible for phosphorylation of Tyr residues are protein tyrosine kinases (PTKs) (Stewart, 1993), and the enzymes that dephosphorylate Tyr residues are protein tyrosine phosphatases (PTPases) (Fauman & Saper, 1996).

PTPase activities have been reported as pathogenic factors in various infectious microorganisms, such as viruses (Ninfa & Dixon, 1994), bacteria (Guan & Dixon, 1990), and parasites (Wimmer *et al*. 1998). The PTPase of *Yersinia*, YopH, inhibits bacterial phagocytosis (Rosqvist, Bolin & Wolf-Watz, 1988) through the disruption of the actin stress fibres by dephosphorylation of specific proteins such as p130^{cas} and FAK (Bliska *et al*. 1991). This enzyme also inhibits the oxidative burst (Bliska & Black, 1995; Green *et al*. 1995). Moreover, the PTPase from *Salmonella typhimurium* (SptP) is translocated into epithelial cells, and microinjection of purified GST-SptP results in disruption of the actin cytoskeleton and the disappearance of stress fibres (Fu & Galán, 1998).

Entamoeba histolytica is the causal agent of amoebiasis. The mechanisms of pathogenesis of this protozoan parasite are still under investigation. Although many enzymatic activities have been reported in *E. histolytica*, the presence of kinases and phosphatases has been poorly studied. Previously, our group reported the existence, in different strains of amoebas, of an acid phosphatase bound to the membrane (MAP) (Anaya-Ruiz, Rosales-Encina & Talamás-Rohana, 1997) as well as one secreted to the extracellular medium (SAP) (Aguirre-García, Rosales-Encina & Talamás-Rohana, 1997). An interesting finding was that although the content of MAP in all strains was similar, SAP was found only in *E. histolytica* strains and not in *Entamoeba dispar* (Talamás-Rohana *et al*. 1999). Later on, MAP was further purified and characterized. As part of this characterization, specific inhibitors were tested and the results showed that MAP activity present in the solubilized fraction was inhibited by micromolar concentrations of ammonium molybdate and sodium orthovanadate (Aguirre-García, Cerbón & Talamás-Rohana, 2000), suggesting the possibility that *E. histolytica* MAP could also have PTPase activity.

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In the present study we tested the activity of the purified enzyme against *O*-phospho-L-tyrosine (P-Tyr), a substrate for PTPases (Ansai *et al*. 1998); we also analysed the behaviour of purified MAP or PTPase activities towards PTPase inhibitors and Ser/Thr phosphatase inhibitors. Additionally, we studied if there was cross-reactivity of a monoclonal antibody, against the human placental PTPase 1B, with the amoebic enzyme, and analysed the effect of purified amoebic PTPase on the actin cytoskeleton of HeLa cells.

MATERIALS AND METHODS

Cell culture

Entamoeba histolytica HM-1 : IMSS strain was cultured under axenic conditions as described (Diamond, Harlow & Cunnick, 1978). Trophozoites were harvested during logarithmic growth by centrifugation at 200 ***g***. Cell pellets were washed several times with cold PBS, pH 7·4.

Human epithelial (HeLa) cells were routinely cultured in Dulbecco's modified Eagles medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated foetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 in air humidified incubator at 37 °C.

Purification of the membrane-bound acid phosphatase

Acid phosphatase (AP) from *E. histolytica* was purified as described (Aguirre-García *et al*. 2000). Briefly, the solubilized fraction was applied to a Concanavalin A (Con A)–Sepharose column and the enzyme was eluted with PBS, Ca^{2+}, Mg^{2+} (0·68 mM $CaCl_2$, 0·49 mM $MgCl_2.6H_2O$) containing 0·1 M methyl-α-D-mannopyranoside (α-MM). Further enrichment of the enzyme was achieved on an anion-exchange column (DEAE–Cellulose) equilibrated with 10 mM Tris–HCl containing 50 mM NaCl, 10 μM α-MM, pH 7. The enzyme was eluted with a NaCl gradient (50–400 mM) in 10 mM Tris, containing 10 μM α-MM, pH 7.

Acid phosphatase activity

Purified MAP was used to analyse the effect of specific inhibitors. Ammonium molybdate, sodium tungstate, and sodium orthovanadate (100 μM), known inhibitors of PTPases, and trifluoperazine (100 μM), and sodium fluoride (5 mM) inhibitors of phosphatases, were added to tubes containing the enzyme in the reaction mixture (1 ml), and were pre-incubated for 10 min at room temperature before adding the substrate *p*-nitrophenyl phosphate (*p*-NPP). The incubation was continued for another 30 min at 37 °C, and the enzyme activity was determined as described (Aguirre-García *et al*. 2000).

Phosphotyrosine phosphatase (PTPase) activity

PTPase activity was assayed using *O*-phospho-L-tyrosine as substrate. Samples were incubated at 37 °C for 30 min in a total volume of 500 μl of assay buffer containing 100 mM sodium acetate, pH 5·0, 1 mM EDTA, 0·1% Triton X-100. The reaction was initiated by the addition of 7 mM phosphotyrosine substrate, and it was stopped with 100 μl of ice-cold 30% trichloroacetic acid (Novak *et al*. 1994). The amount of released phosphate in a portion of the supernatant (200 μl) was determined by the method of Chen, Toribara & Warner (1956), using K_2HPO_4 as a standard. ATP, *O*-phospho-L-threonine, and *O*-phospho-L-serine at a final concentration of 7 mM were also tested. The effect of different compounds on phosphotyrosine phosphatase activity was tested using *O*-phospho-L-tyrosine as substrate.

Preparation of polyclonal antibodies against MAP

Purified enzyme (6 μg/100 μl) was acetone precipitated, resuspended in 20 μl of sample buffer, and loaded in a non-denaturing gel. The band of activity was cut and electroeluted in a total volume of 220 μl. BALB/c mice were immunized subcutaneously 3 times, each one with the 20 μl of electroeluted enzyme mixed with Titer Max adjuvant the first time and with Melox the following times.

Western blot

Acetone precipitated samples (1/10, v/v) from DEAE-eluted material, or solubilized fractions were run in 10% SDS–PAGE and transferred to nitrocellulose paper (NCP). Blots were incubated with the polyclonal anti-amoebic acid phosphatase antibody 1 : 1000 dilution, or with a commercial monoclonal antibody (Oncogene, DH01) generated against the catalytic domain of a recombinant human placental PTPase 1B (1 μg/ml), followed by incubation with a 1 : 1000 dilution of the secondary antibodies conjugated with horseradish peroxidase. Blots were developed by chemiluminiscence (Pierce).

Fluorescence microscopy

Trophozoites were fixed with 4% *p*-formaldehyde for 1 h at 37 °C and either permeabilized or not with PBS-T (0·2% Triton X-100) for 20 min at room temperature. Cells were incubated with the anti-AP antibody (1 : 200), then incubated with a FITC-conjugated secondary goat anti-mouse antibody (1 : 50), and visualized by confocal microscopy. HeLa cells were plated on cover-slips and incubated in culture medium for 24 h to obtain a semiconfluent monolayer. Cells were rinsed with PBS and incubated in the presence of purified PTPase activity, and at the indicated times (0 and 30 min, 1, 2, 3, and

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8 h) they were fixed with 4% paraformaldehyde for 1 h at 37 °C. Cells were permeabilized with PBS-T (0·2% Triton X-100) for 20 min at room temperature. Actin was stained with rhodamine phalloidin (Molecular Probes, 1 : 80) for 30 min. Cover-slips were rinsed and mounted with Vecta-Shield (Vector); cells were viewed using a Nikon Eclipse E600 microscope.

RESULTS

Substrate specificity of MAP

Functional distinctions such as substrate dephosphorylation and sensitivity to inhibitors has helped to differentiate phosphatases specific for Tyr from those specific for Ser and Thr (Brautigan, 1992). In order to demonstrate that purified MAP also had PTPase activity, dephosphorylation assays were done using *O*-phospho-L-tyrosine. We found that purified MAP was able to dephosphorylate the specific substrate in a dose-dependent manner, starting at 3 mM, and reaching the plateau at 10 mM (Fig. 1A). Next we tested the optimum pH for the PTPase activity, finding that the optimum pH was 5·0 (Fig. 1B). To confirm the specificity of the PTPase activity, different substrates were tested at optimum pH 5·0. As shown in Fig. 1C, if we take as 100% the dephosphorylation of *O*-phospho-L-tyrosine, the enzyme was able to dephosphorylate 45% of *O*-phospho-L-serine, 13% of ATP, and 10% of *O*-phospho-L-threonine.

Effect of various compounds on purified MAP and PTPase activities

The effect of different compounds was tested using *p*-NPP as the substrate for MAP activity and *O*-phospho-L-tyrosine as the substrate for PTPase activity. We found that purified MAP activity was inhibited by 100 μM of ammonium molybdate, sodium orthovanadate, and sodium tungstate, all of them described as PTPases inhibitors. Regarding the PTPase activity, we were able to confirm that this activity was inhibited by PTPase inhibitors, except for sodium orthovanadate which did not have any effect at 100 μM. MAP activity was not inhibited but instead activated by 100 μM of trifluoperazine, a ser/thr phosphatases inhibitor, in contrast with PTPase activity which was not affected by this compound. Both activities (MAP and PTPase were 100% inhibited by sodium fluoride (Table 1).

Identity of the amoebic PTPase with the human placental PTPase 1B

Additional evidence for the presence of a PTPase in *E. histolytica* was obtained comparing the recognition pattern between a commercial monoclonal anti-human placental PTPase 1B antibody, directed against the catalytic site of this enzyme, and a polyclonal antibody prepared against the amoebic enzyme. Purified enzyme obtained from the DEAE-column was analysed by 10% SDS–PAGE and silver staining (Fig. 2A, lane 1) and was also run in substrate native gels (Aguirre-García *et al.* 2000) (Fig. 2A, lane 2); the activity band from the substrate native gel was cut, electroeluted, and analysed again by 10% SDS–PAGE and silver staining, showing the same 55 kDa band (Fig. 2A, lane 3), present in the DEAE-fraction. This material was used to immunize BALB/c mice and the antibodies induced recognized a 55 kDa band

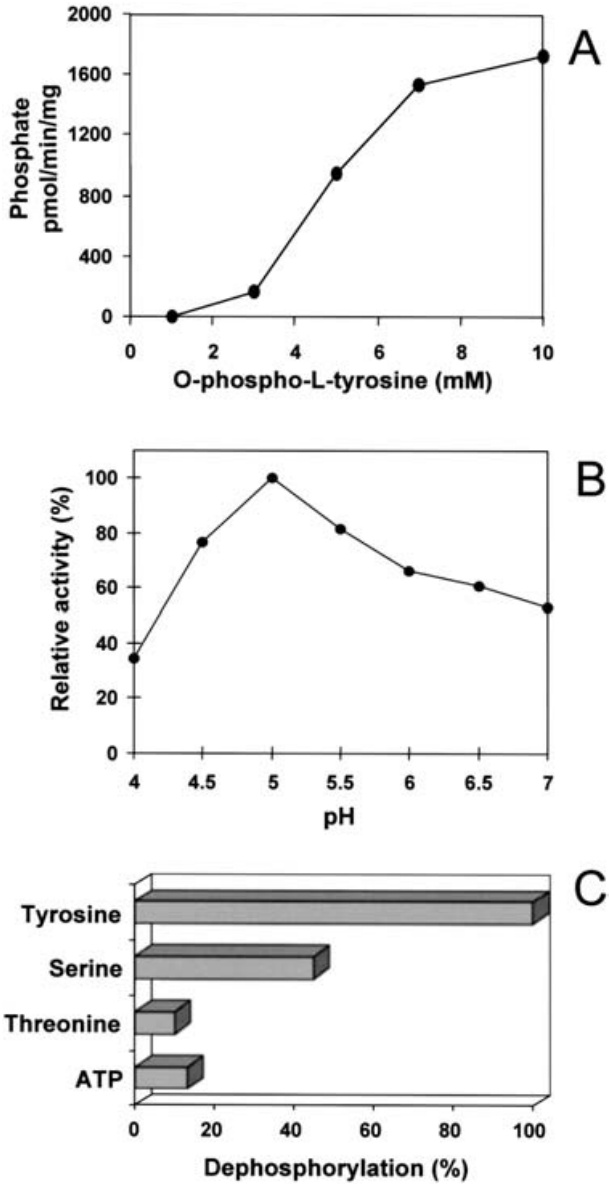

Fig. 1. Kinetics saturation, optimum pH, and substrate specificity of *Entamoeba histolytica* PTPase activity. (A) Substrate saturation curve. (B) PTPase activity was determined at various pH using *O*-phospho-L-tyrosine as substrate. (C) Specificity of PTPase activity was determined comparing the dephosphorylation percentage of different phospho-aminoacids and ATP. Data are representative of 2 independent experiments.

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Table 1. Effect of various compounds on purified membrane-bound acid phosphatase (MAP) and protein tyrosine phosphatase (PTPase) activities

Modulator		(% Original activity) MAP*	PTPase†
None		100	100
PTPase inhibitors			
Ammonium molybdate	(100 μM)	1·37	1·14
Sodium tungstate	(100 μM)	9·24	0·0
Sodium orthovanadate	(100 μM)	10·00	98·54
Ser/Thr phosphatase inhibitor			
Trifluoperazine	(100 μM)	238·46	110·62
Phosphatase inhibitor			
Sodium fluoride	(5 mM)	0·0	0·0

* *p*-NPP.
† *O*-phospho-L-tyrosine.

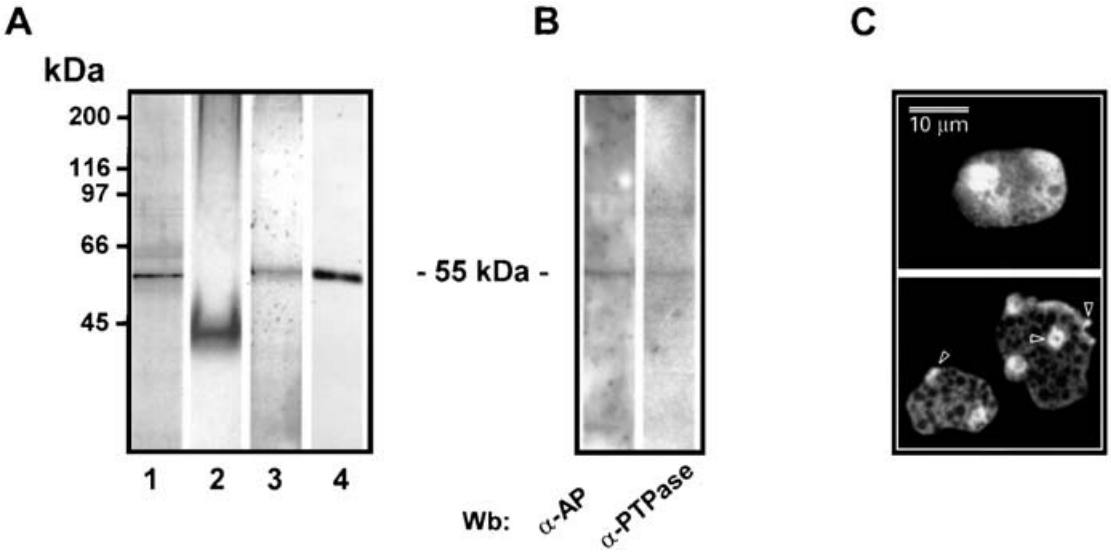

Fig. 2. Western blot reactivity of amoebic acid phosphatase with anti-amoebic phosphatase and anti-human placental PTPase 1B antibodies. (A) Purified MAP (lane 1) was run in native gels (lane 2); the activity band was cut and electroeluted and the material analysed by silver staining after SDS–PAGE (lane 3). Polyclonal antibodies produced were reacted against purified MAP (lane 4). (B) Polyclonal anti-amoebic phosphatase (α-AP), and anti-human placental PTPase 1B (α-PTPase) recognized a 55 kDa molecule present in the solubilized fraction. The purification procedure has been repeated more than 20 times. Results in (B) are representative of 2 independent experiments. (C) Indirect immunofluorescence staining of non-permeabilized (upper panel) and permeabilized (lower panel) trophozoites with the anti-AP antibody.

present in the DEAE-eluted fraction (Fig. 2A, lane 4). Western blot assays were done using the polyclonal anti-amoebic antibody (Fig. 2B, α-AP) as well as the monoclonal anti-PTPase 1B antibody (Fig. 2B, α-PTPase). Both antibodies recognized the 55 kDa molecule present in solubilized fractions. In order to learn more about the subcellular localization of the enzyme in the trophozoite, indirect immunofluorescence assays were done with the α-AP antibody. In non-permeabilized trophozoites, the staining pattern showed a surface localization of the antigen in a patch-like manner (Fig. 2C, upper panel). Permeabilized cells, showed the staining in well-defined zones (Fig. 2C, lower panel), which resemble phagocytic invaginations, typical amoebic structures formed by polymerized actin.

Amoebic PTPase activity disrupts the actin cytoskeleton of HeLa cells

As phosphorylation in a key mechanism in cytoskeleton regulation, cytoskeleton structures constitute target organelles for signalling modifying toxins. We therefore explored the possibility that the PTPase

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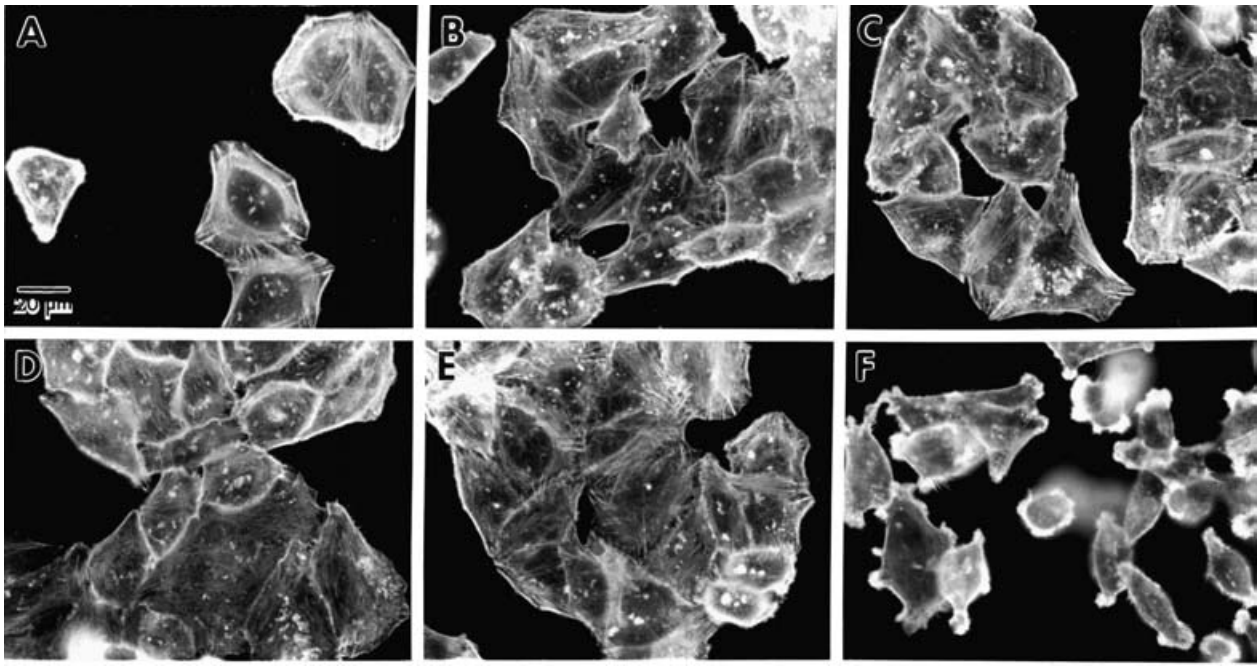

Fig. 3. The membrane-bound PTPase from *Entamoeba histolytica* affects the integrity of actin cytoskeleton in HeLa cells. HeLa cells, either not incubated (A), or incubated for the indicated periods of time with the PTPase activity; 30 min, (B); 1 h, (C); 2 h, (D); 3 h, (E); and 8 h (F), were fixed and permeabilized. Cellular F-actin was stained with rhodamine–phalloidin, and the actin cytoskeleton was visualized by fluorescence using a Nikon Eclipse E600 microscope. Results are representative of 5 independent experiments.

from *E. histolytica* could have an effect on the integrity of the actin cytoskeleton in target cells. The interaction of PTPase with HeLa cells at short times (Fig. 3B–E) had no effects on actin cytoskeleton and showed the same type of staining pattern as in control cells (Fig. 3A). In these cells, selective staining of F-actin with phalloidin revealed a peripheral ring of cortical actin and cytoplasmic fibres. However, the F-actin stress fibres were progressively disrupted with increasing time of interaction, and after 8 h (Fig. 3F), these structures were completely absent. The cortical actin, however, could still be seen as actin patches lining the margin of the cytotoxically affected cells.

To confirm the effect of the PTPase at 8 h of interaction (Fig. 4A), HeLa cells were incubated with a fraction eluted from the DEAE-Cellulose column but without PTPase activity; this fraction had no destructive effects on the actin stress fibres (data not shown). These cells showed the same staining pattern as in control cells (Fig. 4B). In contrast with the results found with the PTPase at 8 h (Fig. 4A), cells incubated with previously heat-inactivated enzyme (95 °C, 15 min), were not affected and the actin cytoskeleton remained intact (Fig. 4C). Cells were also incubated with dialysed purified enzyme; the results showed that even after dialysis, there was a loss of actin stress fibres in these cells (Fig. 4D). The involvement of PTPase activity of the amoebic enzyme was confirmed when the enzyme was added to the cells in the presence of ammonium molybdate. In this condition, the actin stress fibres were not disrupted after 8 h of interaction (Fig. 4E). The addition of this inhibitor alone to HeLa cells, did not affect the actin cytoskeleton (Fig. 4F).

DISCUSSION

PTPases are a family of enzymes that function in concert with protein tyrosine kinases to modulate the level of tyrosine phosphorylation involved in signal transduction processes. However, PTPases from pathogenic micro-organisms appear to be involved in the breakdown of signal transduction pathways in many cell types, including those of the immune system, enabling bacteria to avoid phagocytosis (Virji, 1996; DeVinney, 2000).

It has been reported previously, that some AP have PTPase activity (Lau, Farley & Baylink, 1989). In order to test whether the amoebic MAP had PTPase activity, dephosphorylation experiments using *O*-phospho-L-tyrosine were done, showing that this enzyme was able to dephosphorylate this substrate in a dose-dependent manner, its optimum pH with this substrate was slightly more acidic than the optimum pH of 5·5 for the MAP activity reported previously (Lau *et al.* 1989; Aguirre-García *et al.* 2000); the substrate specificity was also confirmed at the optimum pH of 5·0 comparing its ability to dephosphorylate different phospho-aminoacids.

The use of specific inhibitors or activators has been widely used for the preliminary characterization of acid phosphatases as tyrosine phosphatases

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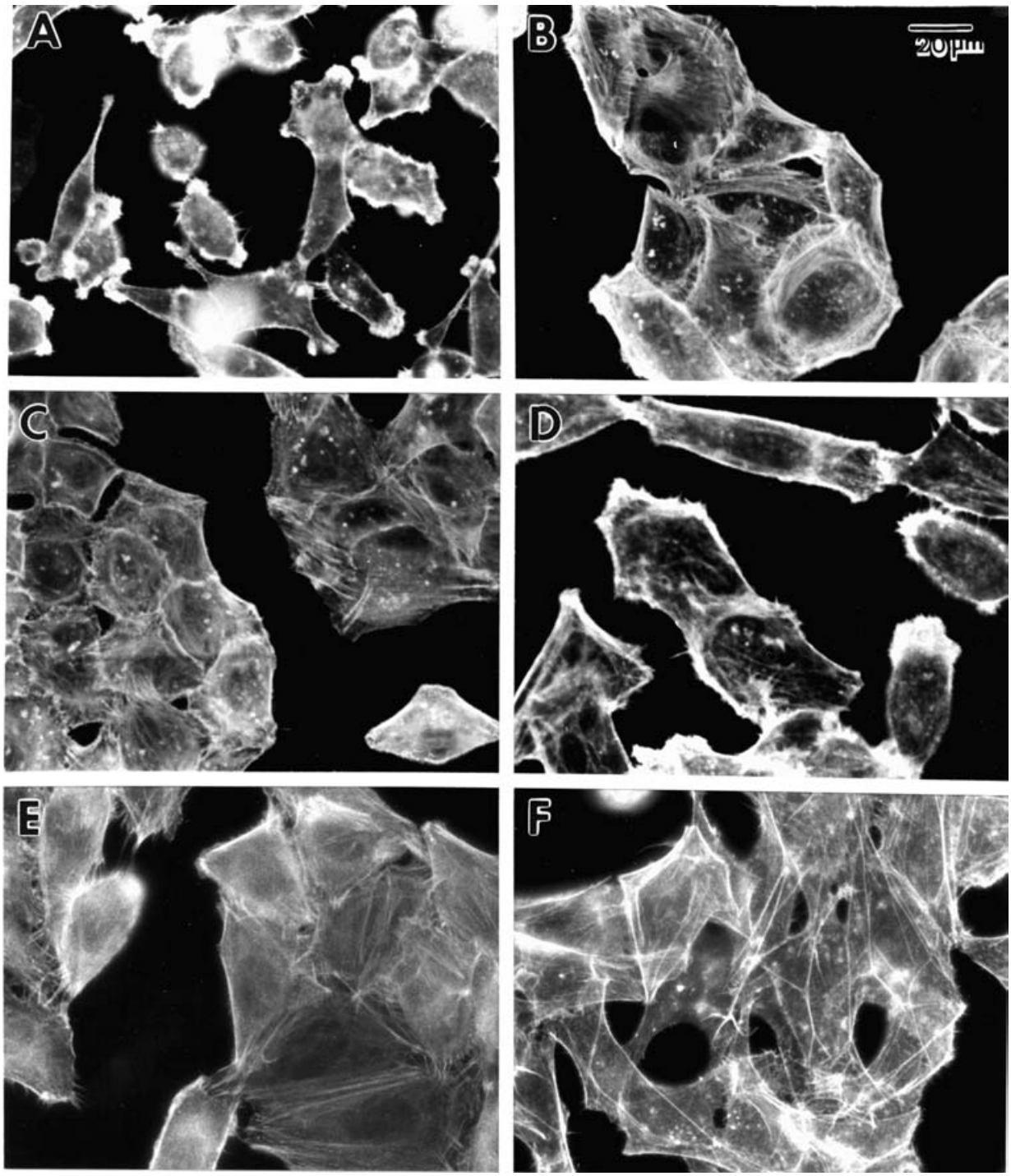

Fig. 4. The PTPase activity from *Entamoeba histolytica* elicits disruption of the actin microfilament structure in HeLa cells. HeLa cells were incubated for 8 h, at 37 °C with the following: purified PTPase (A); DMEM medium (B); inactive PTPase (95 °C) (C); dialysed PTPase (D); purified PTPase plus ammonium molybdate (E); ammonium molybdate (F). Cells were fixed, and the microfilaments were stained with rhodamine–phalloidin and visualized by fluorescence microscopy. Results are representative of 3 independent experiments.

(Fukami & Lipman, 1982; Zhang & Van Etten, 1990; Hardie, 1993; Zhang & Dixon, 1994; Chen *et al.* 1999); therefore, the effect of several compounds was tested. The inhibition of MAP by micromolar concentrations of competitive inhibitors such as tungstate, orthovanadate, and molybdate, strongly suggested the presence of PTPase activity. These inhibitors were also tested using *O*-phospho-L-tyrosine as substrate; under this condition we confirmed the results obtained with MAP activity, except that the purified enzyme was not inhibited by sodium orthovanadate, although higher concentrations of this inhibitor were not tested. In contrast with MAP activity, PTPase activity was not activated by

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trifluoperazine, a Ser/Thr phosphatase inhibitor. At this moment, we do not know whether the two activities belong to the same enzyme or if they are independent activities. The differences in sensitivity and activation between MAP and PTPase activities in the presence of orthovanadate and trifluoperazine respectively, may depend on the catalytic mechanism of every activity, their substrate specificity or their association with other regulatory molecules (Lau *et al*. 1989). It has also been reported that vanadate is probably not a specific inhibitor of all PTPases, and that these enzymes are heterogeneous with respect to sensitivity to inhibition by P_i analogues.

PTPases from different sources are highly conserved at the catalytic site (Fauman & Saper, 1996). PTP-1B is known to regulate cell proliferation negatively; it is also known that PTP-1B and the *Yersinia* PTPase, bind to and dephosphorylate $p130^{cas}$, a protein involved in connecting integrins to the actin cytoskeleton (Liu, Sells & Chernoff, 1988; DeVinney, 2000). The recognition of the amoebic enzyme by a specific monoclonal antibody against the conserved catalytic domain of the human placenta PTP-1B, strongly suggest that this site may be present in the amoebic PTPase. A cross-reactivity of this antibody with a 55 kDa molecule in *T. brucei* (Bakalara *et al*. 1995) and in *A. suum* (Schmid *et al*. 1996) has been reported, and the molecular weight (Mr) of the amoebic enzyme is similar to that of other PTPases from bacteria (Ansai *et al*. 1998) and parasites (Bakalara *et al*. 1995; Schmid *et al*. 1996).

As macrophages are one of the first line of defence during hepatic amoebiasis (Tsutsumi *et al*. 1984), a murine macrophage cell line (J774) was used to test the effect of the amoebic PTPase activity on the actin cytoskeleton. However, these cells do not form typical stress fibres and the only effect observed, was a rounding up and later on a detachment of the cells (data not shown). Thus, in this study, we examined the effect of the amoebic PTPase activity on the cytoskeleton of HeLa cells. Interestingly, the addition of active PTPase resulted in the disappearance of stress fibres and the formation of patches of F-actin fragments; therefore, it is likely that this enzyme mediated the change in the morphology of the HeLa cells, as a consequence of a direct effect of the amoebic PTPase on the integrity of actin cytoskeleton. The inhibition of cell alterations by the use of ammonium molybdate, demonstrates that the amoebic PTPase damages the host cells during the interaction, and that such damage requires the activity of PTPase. Most of the intermediate steps in signal transduction cascades include activation of enzymes through phosphorylation (Hunter, 1995). Quantitatively, the cytoskeletal proteins and their associated proteins seem to constitute a significant group of substrate proteins for the homeostasis-related phosphorylation, as well as important targets for toxins affecting cell signalling (Toivola & Eriksson, 1999). These results strongly suggest that PTPases participate in signal transduction processes that are involved in the cytoskeleton rearrangement.

Together, these findings support the hypothesis that the amoebic phosphatase could be an important element in the pathogenic mechanisms of the parasite. The presence of a PTPase activity could play a role altering the phosphorylation level of different proteins involved in signal transduction. To date we do not know how the amoebic enzyme reaches the host cell cytoplasm. The ability of the enzyme to bind to Con A, strongly suggests that this enzyme is glycosylated; therefore, in the *in vitro* assays, the route of entry could be through sugar receptors. In the case of the whole parasite, the enzyme may penetrate with the help of other pathogenic factors such as proteases and the amoebapore (Stanley, 2001). Another possibility is that the amoebic PTPase activity is on the surface of the trophozoites, as is the case of an ecto-protein tyrosine phosphatase in *T. cruzi* (Furuya *et al*. 1998), affecting primarily surface molecules in the host cells that initiate the signalling involved in stress fibre formation. More experiments need to be done in order to confirm any of these alternatives.

Additionally, to understand the function of the amoebic PTPase as a virulence factor, it will be necessary to identify the target substrates. The known targets for Yop H in HeLa cells are paxillin, $p125^{FAK}$, and $p130^{CAS}$, all of them participating importantly in the formation of actin stress fibres; therefore, it is tempting to speculate that these proteins could be a possible target for the *E. histolytica* enzyme, which we are currently investigating.

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