In Vivo and In Vitro Phosphorylation and Subcellular Localization of Trypanosomatid Cytoskeletal Giant Proteins

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Promastigote forms of Phytomonas serpens, Leptomonas samueli, and Leishmania tarentolae express cytoskeletal giant proteins with apparent molecular masses of 3,500 kDa (Ps 3500), 2,500 kDa (Ls 2500), and 1,200 kDa (Lt 1200), respectively. Polyclonal antibodies to Lt 1200 and to Ps 3500 specifically recognize similar polypeptides of the same genera of parasite. In addition to reacting with giant polypeptides of the Leptomonas species, anti-Ls 2500 also cross reacts with Ps 3500, and with a 500-kDa polypeptide of Leishmania. Confocal immunofluorescence and immunogold electron microscopy showed major differences in topological distribution of these three proteins, though they partially share a common localization at the anterior end of the cell body skeleton. Furthermore, Ps 3500, Ls 2500, and Lt 1200 are in vivo phosphorylated at serine and threonine residues, whereas, in vitro phosphorylation of cytoskeletal fractions reveal that only Ps 3500 and Ls 2500 are phosphorylated. Heat treatment (100°C) of high salt cytoskeletal extracts demonstrates that Ps 3500 and Ls 2500 remain stable in solution, whereas Lt 1200 is denatured. Kinase assays with immunocomplexes of heat-treated giant proteins show that only Ps 3500 and Ls 2500 are phosphorylated. These results demonstrate the existence of a novel class of megadalton phosphoproteins in promastigote forms of trypanosomatids that appear to be genera specific with distinct cytoskeletal functions. In addition, there is also evidence that Ps 3500 and Ls 2500, in contrast to Lt 1200, seem to be autophosphorylating serine and threonine protein kinases, suggesting that they might play regulatory roles in the cytoskeletal organization. Cell Motil. Cytoskeleton 47: 25–37, 2000. © 2000 Wiley-Liss, Inc.

Key words: promastigote; cytoskeleton; microtubules; flagellum; kinase

INTRODUCTION

The family Trypanosomatidae belongs to the order of Kinetoplastida protozoa, which is one of the most primitive organisms in eukaryotic evolution to have mitochondria and peroxisomes [Vickerman and Preston, 1976]. These parasites have different life cycles that involve one (monoxenous) or two hosts (heteroxenous). The latter involves an invertebrate that acts as a vector between other vertebrates or plants. Their general morphology and the kinetoplast position, which varies according to the genus and life cycle stage, can distinguish them. During differentiation in the insect gut and in culture, they appear as promastigotes (Phytomonas, Leptomonas, and Leishmania), trypomastigotes, epimastigotes (Trypanosoma), amastigotes (Trypanosoma and Leishmania), and opisthotmotigote in Herpetomonas [Vickerman and Preston, 1976].

Amongst eukaryotic cells, the trypanosomatids represent one of the simplest models for biochemical, molecular, and cellular studies.

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lecular biology, and functional studies of the cytoskeletal elements, but most genera have not been extensively analysed. All trypanosomatids have a kinetoplast and a cytoskeletal organization, which is essentially a cortical array of microtubules [Vickerman and Preston, 1976]. However, investigations on the ultrastructure and biochemistry of this filamentous structure have been centered on two genera, the heteroxenous Trypanosoma brucei, and the monoxenous Crithidia fasciculata [Kohl and Gull, 1998].

Recently, novel cytoskeletal giant proteins with apparent molecular masses of 700–2,000 kDa were identified in epimastigote and trypomastigote forms of Trypanosoma cruzi [Moreno et al., 1995] and of 3,500 kDa in promastigote forms of Phytomonas serpens [Baqui et al., 1996]. These two proteins do not share common biochemical properties or antigenic determinants, but both are located at the region where the flagellum and the cell body are in contact. The differences in biochemical and immunochemical properties of these two giant proteins could be due to the distinct morphology and to the large evolutionary distance, as inferred from sequences studies of the small RNA genes of these two parasites [Maslov et al., 1996; Lukes et al., 1997].

In the present report, we extend our studies on the properties of the P. serpens giant protein and demonstrate that the promastigote of Leishmania tarentolae and of Leptomonas samueli also express cytoskeletal mega-dalton proteins. Although, these proteins are all phosphorylated in vivo at serine and threonine residues, they have different molecular masses, immunochemical properties, and subcellular localizations. Moreover, in vitro phosphorylation of cytoskeletal fractions of these parasites reveal that the giant proteins of Phytomonas and Leptomonas are phosphorylated at the same residues as those determined in vivo, whereas, the Leishmania giant protein is not phosphorylated. In addition, the giant proteins of Phytomonas and Leptomonas appear to be auto-phosphorylating serine and threonine protein kinases.

**MATERIALS AND METHODS**

**Organisms**

The following organisms came from the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo, São Paulo, Brazil (TCC/USP): Phytomonas serpens (060), P. spp: Cbe (233), P. ssp: Jma (066), Leptomonas samueli (003), L. seymouri (011), L. collosoma (013), and Leishmania tarentolae (017). They were grown at 25–28°C in liver infusion-tryptose (LIT medium) supplemented with 10% fetal calf serum [Camargo, 1964]. Promastigote forms of Leishmania (Leishmania) amazonensis LV 79 (MPRO/BR/72/M1841) and amastigote-like forms of Leishmania (Viannia) braziliensis (MHOM/BR/75/M2903) were kindly provided by Dr. Silvia C. Alfieri, of the Department of Parasitology, University of São Paulo, São Paulo, Brazil.

**Cytoskeletons**

Cells (10^6/ml) were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM K_2HPO_4, pH 7.2) and were incubated in lysis buffer (50 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), pH 6.9, 5 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 2 mM MgCl_2) containing 1.0% Nonidet P-40 (NP-40), and protease inhibitors (0.5 mM phenylmethylsulfonylfluoride (PMSF); 2 mM benzamidine-hydrochloride and 5 μM of each: leupeptin, antipain, pepstatin A, and chymostatin) and left on ice for 10 min. After centrifugation at 14,000 g for 10 min at 4°C, the insoluble fraction (cytoskeleton) was washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM K_2HPO_4, pH 7.4). The cytoskeletal preparations were either used immediately or suspended in PBS/glycerol (v/v) and kept at −80°C until further use [Baqui et al., 1996].

**Metabolic Labeling**

Metabolic labeling experiments were carried out by incubating 10^8 cells/ml with 100 μCi/ml of [35S]methionine (1 mCi/mmol) (Amersham, Amersham Pharmacia Biotech, Bucks, UK) in methionine-free RPMI 1640 medium (GIBCO, Gaithersburg MD/USA) for 2 h at 28°C. The reaction was terminated by centrifugation at 5,000 g for 5 min. The cell pellet was washed twice with RPMI medium and the radiolabeled cells were subjected to non-ionic detergent extraction as described above.

**In Vivo Phosphorylation**

Cells (10^6/ml) were harvested by centrifugation and washed in phosphate free-RPMI 1640 medium (GIBCO, Gaithersburg, MD), then suspended in 1 ml of phosphate free-RPMI 1640 medium containing 100 μCi/ml of ^32Porthophosphoric acid (10 mCi/mm) (NEN, Boston, MA) and incubated for 2 h at room temperature. Labeled parasites were washed twice in PBS and submitted to non-ionic detergent extraction as described above in the presence of phosphatase inhibitors (1 mM Na_3VO_4 and 10 mM NaF).

**High Ionic Strength Extraction**

Cytoskeleton recovered from 5 × 10^8 cells were suspended in 1 ml of 1M NaCl in 50 mM Tris[hydroxy-methyl]ammonomethane (Tris) pH 7.2 plus protease inhibitors [Hemphill et al., 1992]. After vortexing vigorously,
the suspension was left on ice for 30 min, and subsequently centrifuged at 14,000g for 20 min at 4°C or at 100,000g for 60 min at 4°C. The supernatant contains solubilized cell body skeletons and the pellet consists of the flagellar skeleton (axoneme and paraflagellar rod).

**Heat Treatment of the High Ionic Strength Extracts**

The supernatant fractions of the 1M NaCl treatment of *L. samueli, P. serpens*, and *L. tarentolae* were heated for 5 min at 100°C, quenched on ice for 10 min and centrifuged at 100,000g for 30 min at 4°C [Hemphill et al., 1992]. The insoluble fraction contains thermostabilized proteins and the supernatant the heat-resistant proteins.

**Gel Electrophoresis (SDS-PAGE) and Immunoblotting**

Fractions were solubilized in SDS-sample buffer (100 mM Tris, pH 8.0, 4% Sodium Dodecyl Sulfate [SDS], 20% glycerol, 10% β-ME, 0.002% Bromophenol blue), heated at 100°C for 5 min and were analyzed by SDS-PAGE using a 2.5–12.5% polyacrylamide linear gradient gel without a stacking gel [Wang, 1982; Pudles et al., 1990]. As molecular mass standards, we used rabbit skeletal muscle myofibrils [Wang, 1982]: titin (3,000–4,000 kDa), nebulin (800 kDa), myosin heavy chain (205 kDa), α-actinin (95 kDa), actin (43 kDa), tropomyosin (32 kDa), and troponin (23 kDa). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Burlingame, CA).

For immunoblot analysis, cytoskeletal proteins fractionated by SDS-PAGE were electrophoretically transferred to nitrocellulose filters as described by Palade and Gershoni [1983]. Then the filters were blocked for 1 h at room temperature by TBS solution (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% defatted milk powder. Strips were incubated for 1 h at room temperature or overnight at 4°C with the primary antibody in TBS blocking solution. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgG (dilution 1:1,000) for 1 h at room temperature and blots were visualized by phosphatase substrates (Bio-Rad Laboratories).

**Production and Affinity Purification of Antibodies**

The polyclonal mouse antiserum against the giant proteins was prepared as previously described by Baqui et al. [1996]. For affinity purification of the antibodies [Hall et al., 1984], cytoskeletal proteins were transferred to nitrocellulose (Bio-Rad) and strips containing the giant protein were excised and incubated with the giant protein antiserum, at 1:50 dilution. The elution of bound antibodies was carried out using 200 mM Glycine-HCl, pH 2.8, 150 mM NaCl, 10 μg/ml BSA (Bovine Serum Albumin), 0.05% Tween 20, for 2 min at room temperature. The eluted material was removed and neutralized by addition of 1 M Tris, pH 12.

**Immunoprecipitations**

Immunoprecipitations were performed under the following two conditions.

**Condition 1.** Cytoskeletons fractions labeled with [35S]methionine or with [32P]orthophosphoric acid were solubilized in 2% SDS/PBS, then diluted with addition of 10% Triton X-100, T-NET (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA), to give a final concentration of 0.2% SDS and 0.7% Triton X-100 plus protease inhibitors. To the fractions, preimmune mouse serum (dilution 1:50) was added, and incubated for 2 h with intermittent agitation at 4°C. A suspension of protein A-Sepharose Cl-4B (Pharmacia, Biotech, Bucks/UK) in T-NET (25% final concentration) was added and incubated for a further 30 min at 4°C with agitation, followed by centrifugation at 12,000g. The resulting supernatant was incubated with the affinity purified antibody raised against the specific giant protein (dilution 1:50) or against polyclonal anti-tubulin (Sigma Chemical Co., St. Louis, MO) (dilution 1:50) and further steps were carried out as described above for the preimmunoprecipitation.

**Condition 2.** The supernatants of the 1M NaCl treatment (solubilized cell body skeletons) and of the heat-resistant proteins were desalted by dialysis at 4°C against 500 mM NaCl in 50 mM Tris, and then against 50 mM Tris, 150 mM NaCl (both solutions containing protease inhibitors, 1 mM EGTA, and 1 mM adenosine 5’-triphosphate [ATP]). After centrifugation at 100,000g for 30 min at 4°C, 10% Triton X-100, protease inhibitors were added to the supernatants and submitted to immunoprecipitation as described above except that SDS was omitted.

Pellets recovered after protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) treatment of both conditions were analysed by SDS-PAGE or submitted to in vitro kinase assay (described below). The gel was dried, exposed to a Kodak film (X-Omat AR-5) at ~80°C or to phosphor screen (Molecular Dynamics) and scanned in a STORM 840 (Molecular Dynamics).

**In Vitro Phosphorylation of Cytoskeletal Proteins**

Parasites (10⁶) were washed twice in PBS and submitted to non-ionic detergent extraction as described above in the presence of phosphatase inhibitors (1 mM Na₃VO₄ and 10 mM NaF). Cytoskeletal preparation (50 μg) were labeled endogenously by addition of 40 μl of kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 5 mM MgCl₂, 5 mM MnCl₂, or 0.2 mM CaCl₂ and 5 μg/ml CaM (calmodulin was
kindly provided by Prof. R. Larson and by Prof. S. Gomes, University of São Paulo, São Paulo, Brazil) containing 15 μM ATP, 5 μCi [γ-32P]ATP. After 10 min at room temperature, the cytoskeletons were pelleted and the reaction was stopped by addition of SDS-sample buffer and boiled for 2 min. The samples were run on SDS-PAGE and autoradiographed or exposed to phosphor screen and scanned in a STORM 840.

In Vitro Kinase Assay

Immunoprecipitates of condition 2 were washed three times with kinase buffer then incubated for 20 min at room temperature in 40 μl of kinase buffer containing 25 μM ATP, 10 μCi [γ-32P]ATP (10 mCi/ml, (Amersham, Amersham Pharmacia Biotech)) as performed by Okano et al. [1995]. After SDS-PAGE, the gel was dried, exposed to a Kodak film (X-Omat AR-5) at ~80°C or to phosphor screen and scanned in a STORM 840.

Phosphoamino Acids Analysis

Immunoprecipitated giant proteins of *P. serpens*, *L. samueli*, and *L. tarentolae* labeled in vivo or in vitro were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Millipore, MA) [Palade and Gershoni, 1983]. The giant protein bands were excised from the blot and submitted to partial acid hydrolysis with 250 μl of 6 N HCl for 2 h at 110°C as described by Kamps and Sefton [1991]. Phosphoamino acids were separated by one-dimensional High Performance Thin Layer Chromatography (HPTLC, Merck, Darmstadt, Germany) silica gel 60 plates [Munoz and Marshall, 1990]. After migration and drying, phosphoamino acids were detected by autoradiography and compared with the ninhydrin-stained phosphoamino acid standards.

Confocal Immunofluorescence Assays and Immunogold Electron Microscopy

Parasites were washed twice in PBS, and settled onto glass slides containing poly-lysine 0.1%; then were extracted for 10 min at 4°C with 0.5% NP-40 in PBS plus protease inhibitors. After three washes with PBS, they were directly fixed in 2% paraformaldehyde in PBS for 10 min and rehydrated in PBS for 10 min. Cytoskeletons were treated for 10 min in blocking solution (2% BSA in PBS), followed by incubation for 30 min, at 37°C, with affinity purified polyclonal antibody against the specific giant protein (dilution 1:50) together with polyclonal anti-tubulin (dilution 1:50) in blocking solution. Unbound antibody was removed by 3 dip-washes in blocking solution and subsequently incubated for 30 min at 37°C with FITC-conjugated goat anti-mouse IgG and RITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) at a final dilution 1:50 and 4', 6-diamidino-2-phenylindole (DAPI). The glass slides were mounted in Mowiol containing 1 mg ml⁻¹ of paraphenylenediamine and examined under laser scanning fluorescence confocal microscopy (Bio Rad MRC 1024/UV system, BIO-RAD, Hercules, CA). Images were collected through a 40× 1.2/water immersion or a 100×/1.4 oil immersion PlanApochromatic objective (Zeiss), Kalman averaging at least 15 frames using a 2-mm iris (pinhole).

For immunoelectron microscopy, cells were washed twice in PBS and applied for 30 min to carbon-formvar-polysine coated nickel grids. Extraction was performed by floating grids on top of a drop of 1% NP-40 in PEME buffer (100 mM PIPES (piperazine-N, N’-bis [2-ethanesulfonic acid]), pH 6.9, 1 mM MgSO₄, 0.1 mM EDTA, 2 mM EGTA) plus protease inhibitors. Samples were then fixed with 3.7% paraformaldehyde plus 0.01% glutaraldehyde in PEME for 10 min, washed in 20 mM glycine in PBS, followed by 1% BSA in PBS. Grids were then incubated with the affinity purified polyclonal antibody against the specific giant protein (diluted 1:50) at room temperature, during 1 h. After washing in 1% BSA in PBS, samples were incubated with the second antibody goat anti-mouse, 15-nm gold conjugates (Amersham, Amersham Pharmacia Biotech) diluted 1:25 in 1% goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) containing 1 mg ml⁻¹ of para-phenylenediamine and examined under laser scanning fluorescence confocal microscopy (Bio Rad MRC 1024/UV system, BIO-RAD, Hercules, CA). Images were collected through a 40× 1.2/water immersion or a 100×/1.4 oil immersion PlanApochromatic objective (Zeiss), Kalman averaging at least 15 frames using a 2-mm iris (pinhole).

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RESULTS

Identification of Megadalton Proteins in the Cytoskeleton and Immunoblot Analysis

Detergent extraction of *L. samueli* and of *L. tarentolae* reveals very stable cytoskeletons similar to those described for *P. serpens* [Baqui et al., 1996], *T. brucei* [Hemphill et al., 1992], and *T. cruzi* [Moreno et al., 1995]. Electrophoresis in a gradient of SDS-PAGE (Fig. 1A) shows that like *P. serpens* (Fig. 1A, lane b), *L. samueli* (Fig. 1A, lane c) and *L. tarentolae* (Fig. 1A, lane d) also express cytoskeletal megadalton polypeptides. It is interesting to remark that few similarities are observed in the polypeptide patterns on the gel for the three species.

Earlier work from this laboratory [Baqui et al., 1996] demonstrated that the *Phytomonas* giant polypeptide has about the same electrophoretic mobility as titin, that was considered by Wang [1982], to have an apparent molecular mass of ~2,500 kDa. Based on this value, we named the *Phytomonas* protein as Ps 2500. However, recent data obtained by sequence determination indicates
that titin has \(\sim 3,000 - 4,000\) kDa [Labeit and Kolmerer, 1995]. Because of this data, Ps 2500 is now called Ps 3500. The Leptomonas (Fig. 1A, lane c) and the Leishmania (Fig. 1A, lane d) giant polypeptides have apparent molecular mass of \(\sim 2,500\) kDa (Ls 2500) and \(\sim 1,200\) kDa (Lt 1200) respectively. Similar to Ps 3500 [Baqui et al., 1996] and to T. cruzi giant protein [Moreno et al., 1995], the molecular masses of Ls 2500 and Lt 1200 are not affected by harsher denaturation conditions of the cytoskeletal proteins or by carboxymethylation (not shown).

Immunoblot analysis of the parasite cytoskeletal preparations, described in Figure 1A, using affinity purified polyclonal antibodies raised against each of the giant proteins, are shown in Figure 1B. Anti-Ls 2500 recognizes the giant protein in L. samueli (Fig. 1B, lane c) and in L. seymouri and L. collosoma (not shown). Furthermore, it cross-reacts with Ps 3500 (Fig. 1B, lane b) and with a polypeptide of apparent molecular mass of 500 kDa in Leishmania, but does not recognizes Lt 1200 (Fig. 1B, lane d). In contrast, anti-Ps 3500 does not cross react with Ls 2500 (Fig. 1B, lane c) but only recognizes Ps 3500 (Fig. 1B, lane b) or polypeptides of similar molecular masses from different Phytomonas species [Baqui et al., 1996]. In addition, anti-Lt 1200 only detected Lt 1200 in L. tarentolae (Fig. 1B, lane d) and in promastigote forms of L. amazonensis and amastigote-like forms of L. braziliensis (not shown). Moreover, anti-Ls 2500 and anti-Lt 1200, similarly to anti-Ps 3500 [Baqui et al., 1996] do not react with titin in rabbit muscle myofibril preparations and or with T. cruzi giant protein (not shown).

**Confocal Microscopy**

The distribution of these giant components under laser scanning fluorescence confocal microscopy was performed on cytoskeletons using double labeling and DAPI staining (Fig. 2). Ps 3500 is exclusively located at the anterior end of the cell body skeleton (Fig. 2b) as described in Baqui et al. [1996]. In L. samueli cytoskeleton, Ls 2500 is distributed along the cell body skeleton (Fig. 2f) and colocalizes with the subpellicular array of microtubules at the cell body level (evidenced by the yellow/orange color in Fig. 2h). In L. tarentolae cy-
Fig. 2. Distribution of giant proteins and tubulin as determined by double labeling using the anti-giant proteins and anti-tubulin antibodies in trypanosomatid cytoskeletons under laser confocal microscopy. a–d: Phytomonas serpens; d: colocalization of Ps 3500 and tubulin at the anterior end of the cell body skeleton. e–h: Leptomonas samueli; h: colocalization of Ls 2500 and tubulin throughout the subpellicular microtubules. i–m: Leishmania tarentolae; m: colocalization of Lt 1200 and tubulin at the anterior end of the cell body skeleton and along the flagellum. a,e,i: DAPI images (blue); b,f,j: Anti-giant proteins labeling (green): anti-Ps 3500; anti-Ls 2500, anti-Lt 1200, respectively. c,g,l: anti-tubulin labeling (red); d,h,m: superimposition (yellow/orange). Arrowheads indicate, in all parasites, a strong fluorescence at the level of the flagellar pocket area of the cell body skeleton. Bar = 10 μM.
Fig. 3. Immunogold labeling of negatively stained whole-mount cytoskeletons. 

a: *Phytomonas serpens* probed with anti-Ps 3500. Note that gold lies exclusively at the anterior end of the cell body skeleton.

b: *Leptomonas samueli* probed with anti-Ls 2500. Gold particles are distributed throughout the subpellicular microtubules.

c: *Leishmania tarentolae* probed with anti-Lt 1200. Gold label extends along the flagellum.

Kinetoplast (k), nucleus (n), flagellum (f); flagellar pocket (fp). Bar = 0.5 μM.
toskeleton, the giant protein distributes along the flagellum and displays a strong punctate fluorescence at the anterior region of the cell body skeleton (Fig. 2j). It is interesting to note that the three proteins partially share a common colocalization with the microtubule at the anterior end of the cell body (Fig. 2d, h, m arrowhead). No fluorescence is detected either in live or in fixed parasites (not shown), indicating that the antigens are not exposed to the outside. They are only accessible to the antibodies when the cell membrane is removed by detergent treatment, as was previously observed for \textit{T. cruzi} [Moreno et al., 1995] and \textit{P. serpens} [Baqui et al., 1996].

**Immunogold Electron Microscopy**

Immunogold labeling of Ps 3500 in \textit{P. serpens} cytoskeletons revealed that the gold beads are exclusively located at the flagellar pocket area at the anterior end of the cell body skeleton (Fig. 3a), confirming our previous results [Baqui et al., 1996]. In \textit{L. samueli} cytoskeletons, Ls 2500 is distributed throughout the cytoskeletal array of microtubules, as well as at the pocket area at the anterior end of the cell body skeleton (Fig. 3b). In cytoskeletal preparations of \textit{L. tarentolae}, the labeling of Lt 1200 extends along the flagellum (Fig. 3c). Furthermore, similarly to Ls 2500 and to Ps 3500, Lt 1200 is partially located in the anterior end of the cell body. These results further confirm the confocal microscopy analysis.

**Giant Proteins Are In Vivo Phosphorylated at Serine and Threonine Residues**

To determine whether the giant proteins are submitted to post-translational modification by phosphorylation, the parasites are labeled in vivo with $^{32}$Porthophosphoric acid. The cytoskeletal fractions recovered from \textit{P. serpens}, \textit{L. samueli}, and \textit{L. tarentolae} show, after SDS-PAGE and autoradiography, the presence of numerous phosphorylated polypeptides bands including the giant proteins (Fig. 4A, lanes b). In Figure 4A, lane c

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**Fig. 4.** In vivo phosphorylation of cytoskeletal giant protein and phosphoamino acids analysis. **A:** Identification of in vivo phosphorylated cytoskeletal proteins. Lane a: cytoskeletal fractions metabolically labeled with $^{35}$Smethionine (control); lane b: phosphorylated cytoskeletal proteins; lane c: immunoprecipitation of phosphorylated giant proteins (see Materials and Methods, condition 1). Arrowheads show the phosphorylated giant proteins; arrow indicates the top of the gel. At the left, molecular mass standard (rabbit muscle myofibrils).

**B:** Phosphoamino acid analysis of in vivo labeled of (lane a) Ps 3500, (lane b) Ls 2500, and (lane c) Lt 1200. The giant proteins were resolved by SDS-PAGE, excised, and analysed by one-dimensional HPTLC. The relative positions of \(s\) phosphoamino acid standards: phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y).
reveals that Ps 3500, Ls 2500, and Lt 1200 are immuno-
precipitated under phosphorylated form.

Phosphoamino acid analysis, after acid hydrolysis, is performed to determine the relative distribution of phosphoamino acids in immunoprecipitated fraction of Ps 3500, Ls 2500, and Lt 1200. As shown in Figure 4B, all these proteins are phosphorylated at both serine and threonine residues and the latter is the major phosphoamino acid. In Lt 1200, phosphoserine is present at a much lower level (Fig. 4B, lane c).

In Vitro Phosphorylation of the Cytoskeletal Fractions

In vitro phosphorylation assay is performed on whole cytoskeletal fractions of P. serpens, L. samueli, and L. tarentolae that are labeled endogenously by addition of \([\gamma^3P]\)ATP and analysed by SDS-PAGE and autoradiography. Arrowheads show giant proteins, arrow indicates the top of gels. At the left, molecular mass standard (rabbit muscle myofibrils).

is possible that the protein kinase responsible for the in vivo phosphorylation of Lt 1200 has been removed by the non-ionic detergent extraction.

In order to detect protein kinase activity in the cytoskeletal fractions, kinase renaturation assays were performed after SDS-PAGE according to Ferrel and Martin [1991] and also as described by Hutchcroft et al. [1991]. However, though several polypeptides with lower molecular masses, distinct for each parasite, showed autophosphorylating activities, no detectable signal was observed at the level of Ps 3500 and of Ls 2500 and as expected for Lt 1200 (not shown).

Kinase Assay With Immunocomplexes

Since the kinase renaturation assays were negative, it was important to use an alternative method to verify if Ps 3500 and Ls 2500 possess kinase activity. Several reports show that protein kinases can be determined with immunocomplexes in non-denaturing conditions [Okano et al., 1995; Maroto et al., 1992]. However, since in non-denaturing conditions, contaminating proteins may also coimmunoprecipitate, it was necessary to verify the purity of immunocomplexes prior to determine the eventual kinase activities of Ps 3500, Ls 2500, and Lt 1200. For this reason, control experiments were carried out with high ionic strength cytoskeletal extracts metabolically labeled with \([^{35}S]\)methionine (Fig. 6). These experiments show that the three giant proteins coimmunoprecipitate with a polypeptide band of about 55 kDa, which appears to be tubulin (Fig. 6d). This was further confirmed with anti-tubulin that also induces coimmunoprecipitation of the giant proteins with tubulin (not shown).

It has been reported that some high molecular mass proteins like MAP 2 of brain [Vallee, 1985] and MARPs of T. brucei [Hemphill et al., 1992] are not affected when high ionic strength cytoskeletal fractions are submitted to heat treatment, whereas tubulin is thermodenatured.

Heat treatment of the 1 M NaCl cytoskeletal extracts of Phytonomas, Leptomonas, and Leishmania shows that Ps 3500 and Ls 2500 remain stable in solution, whereas the majority of Lt 1200 is thermodenatured (Fig. 6e). It is worth noting in the Leishmania heat-treated cytoskeletal extract, the presence of an intense band of a heat stable tubulin fraction. This fraction is not found in the heat-treated cytoskeletal extracts of Phytonomas and of Leptomonas, thus indicating than an important tubulin fraction of L. tarentolae cell body skeleton presents distinct physicochemical properties. Furthermore, after heat treatment, Ps 3500 and Ls 2500 immunoprecipitate without being contaminated by other cytoskeletal components and Lt 1200 is not found immunoprecipitated (Fig. 6f).
Kinase assays (Fig. 7A) performed with non-radiolabeled immunocomplexes obtained under the same conditions as described above show that phosphorylation of Ps 3500 and Ls 2500 are dependent on Mg\(^{2+}\)/Mn\(^{2+}\) (Fig. 7A, lanes a) while Ca\(^{2+}\)/CaM (Fig. 7A, lanes b) has no significant stimulatory effect. Moreover, the presence of phosphorylated bands of about 55 kDa can also be observed. The latter seem to be the same as those coimmunoprecipitated with giant proteins that were metabolically labeled with \([35 \text{S}]\)methionine (see Fig. 6d). Control kinase assays of Lt 1200 immunocomplexes failed to show any trace of phosphorylation (Fig. 7A, lanes a,b).

Kinase assays performed under similar conditions with immunocomplexes obtained after heat treatment, using Lt 1200 as a negative control, show that Ps 3500 and Ls 2500 are the only phosphorylated protein bands (Fig. 7A, lanes c).

Phosphoamino acid analysis was similar to those observed in vivo, both Ps 3500 and Ls 2500 are phosphorylated at serine and threonine residues (Fig. 7B). However, in contrast to the in vivo experiment, the proportion of phosphorylated serine is higher than threonine (see Fig. 4B).

Although we cannot entirely rule out the eventual existence, in catalytic amounts, of thermostable protein kinases, nonetheless, the experimental evidence strongly indicates that Ps 3500 and Ls 2500 appear to be auto-phosphorylating serine/threonine protein kinases.

**DISCUSSION**

In the present study, we have demonstrated that promastigotes of the trypanosomatids *L. tarentolae* and *L. samueli* express cytoskeletal associated megadalton proteins that migrate in SDS-PAGE as polypeptides with apparent molecular masses of 1,000–1,200 kDa and 2,100–2,500 kDa respectively. We use the abbreviation Lt 1200 for *Leishmania* and Ls 2500 for *Leptomonas*.

Western blot and immunoprecipitation revealed that the immunoaffinity purified polyclonal antibodies against Lt 1200 specifically recognize cytoskeletal giant proteins of *Leishmania* species (*L. (L.) amazonensis* and *L. (V.) braziliensis*). Antibodies raised against Ls 2500 cross reacted with polypeptides of similar masses of *L. seymouri* and *L. collosoma*, with *P. serpens* giant polypeptide Ps 3500 and with a yet uncharacterized 500 kDa polypeptide from the *Leishmania* cytoskeleton. However, by immunofluorescence microscopy no signal was observed when *Leishmania* cytoskeleton was treated with anti-Ls 2500 antibody (not shown). Furthermore, polyclonal antibodies anti-Ps 3500 did not recognize Ls 2500 [Baqui et al., 1996]. The anti-Ps 3500, the anti-Ls 2500, and the anti-Lt 1200 did not react with the cytoskeletal giant protein of *T. cruzi*.

Confocal immunofluorescence and immunogold electron microscopy demonstrated that Ls 2500 is distributed along the subpellicular microtubules, whereas Ps 3500 is exclusively located at the anterior end of the cell body.
skeleton. Contrasting with above proteins, Lt 1200 is essentially located along the Leishmania flagellum. Despite these major differences, all antigens appear to partially share a common colocalization with the microtubules at the level of the flagellar pocket area of the cell body skeleton.

Notwithstanding, these major immunocytochemical differences, the three giant proteins are all phosphorylated in vivo at both serine and threonine residues, while the latter residue is the major phosphoamino acid. In vitro phosphorylation of Phytomonas, Leptomonas, and Leishmania cytoskeletal fractions reveal that Ps 3500 and Ls 2500 are phosphorylated, but Lt 1200 is not. This in vitro phosphorylation of Ps 3500 and Ls 2500 occurs at the same amino acids types as those described in vivo (not shown) and are dependent on Mg\(^{2+}\)/Mn\(^{2+}\) or Ca\(^{2+}\)/CaM, though this latter cofactor is much less efficient. It is interesting that the phosphorylation of two former proteins in cytoskeletal fractions may now be included with the other examples of specific cytoskeletal associated protein kinases [Dash et al., 1995; Weernink and Rijksen, 1995; Walker et al., 1997].

The majority of cytoskeletal giant proteins that have been characterized belong to titin/connectin families [Wang et al., 1979; Maruyama et al., 1981; Trinick et al., 1984] that encompass vertebrate muscle titins (3,000–4,000 kDa) and invertebrate muscle mini titins called projectin (700 kDa) found in both honeybee and Drosophila [Saide, 1981; Ayme-Southgate et al., 1991]; and twitchin (750 kDa) in Caenorhabditis elegans [Waterson et al., 1980; Moerman et al., 1988; Benian et al., 1989]. All these titins, though they appear to have distinct functions [Ayme-Southgate et al., 1995], are associated with myosin II, and are autophosphorylating serine and threonine protein kinases [Maroto et al., 1992; Takano-Ohmuro et al., 1992; Heierhorst et al., 1994; Lei et al., 1994; Mayans et al., 1998].

Using Lt 1200 as the negative control, we investigated whether Ps 3500 and Ls 2500 have kinase activities. Kinase renaturation experiments were performed after SDS-PAGE of cytoskeletal fractions. However, no phosphorylation was observed at the level of Ps 3500, Ls 2500, and Lt 1200. These results indicated that after denaturation either the eventual kinase domains of Ps 3500 and Ls 2500 do not recover their native conformation, or protein kinases associated with these proteins are removed during migration in the gel electrophoresis. In contrast, when the kinase assays are performed with the immunocomplexes of Ps 3500, Ls 2500, and Lt 1200, prepared from high salt cytoskeletal extracts, after removal of excess salt, the two former proteins are phosphorylated as well as a polypeptide of about 55 kDa. A similar coimmunoprecipitation was observed when con-

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**Fig. 7.** Kinase assay of giant protein immunocomplexes. A: In the presence of (lanes a, c) Mg\(^{2+}\)/Mn\(^{2+}\) or (lanes b, d) Ca\(^{2+}\)/CaM. Lanes a, b: immunoprecipitated giant proteins of the 1M NaCl soluble fractions; lanes c, d: immunoprecipitated giant proteins of the heat stable 1M NaCl soluble fractions. Arrowheads indicate the phosphorylated giant proteins; asterisks indicate the phosphorylated 55 kDa protein; arrow indicates the top of the gel. At the left, molecular mass standard (rabbit muscle myofibrils). B: Phosphoamino acid analysis of giant proteins from the heat stable fractions that are autophosphorylated in the presence of Mg\(^{2+}\)/Mn\(^{2+}\). Lane a: Ps 3500; lane b: Ls 2500 and (s) phosphoamino acid standards: phosphoserine a(S), phosphothreonine (T), and phosphotyrosine (Y).
control experiments were performed with proteins metabolically labeled with [35S]methionine. At present, we do not have any evidence indicating whether the 55-kDa phosphorylated band is related to tubulin.

Surprisingly, when high salt cytoskeletal extracts are submitted to heat treatment (100°C), Ps 3500 and Ls 2500 remain in solution and, after immunoprecipitation, no radiolabeled contaminants can be detected, whereas, the majority of the Lt 1200 fraction is thermodenatured.

It is noteworthy that determination of the kinase activities of immunocomplexes prepared from non-radiolabeled, heat stable Ps 3500 and Ls 2500, show that they are the only phosphorylated polypeptide bands detected in the autoradiograms. In addition, like in vivo as well as in vitro phosphorylation of these proteins in cytoskeletal fractions, the same amino acids types are phosphorylated and their phosphorylations are essentially Mg2+/Mn2+ dependent.

In conclusion, these results describe the existence of novel giant phosphoproteins associated with the cytoskeleton of trypanosomatid promastigotes. Furthermore, evidence is presented that strongly supports the idea that Ps 3500 and Ls 2500 are autophosphorylating serine and threonine protein kinase, implying that like the titin/connectin family of proteins, they could perform regulatory functions. On the other hand, since these proteins have distinct molecular masses, immunochemical regulatory functions. On the other hand, since these proteins have distinct molecular masses, immunochemical properties, and subcellular localizations, it suggests that each might have a specific functional role. We are then tempted to postulate that these phenotype differences may be related to the parasite’s way of life, and represent adaptations of their cytoskeleton to the environmental constraints that these organisms are submitted to in the different physiological medium of their specific hosts.

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REFERENCES


