Subcellular localization of an intracellular serine protease of 68 kDa in *Leishmania (Leishmania) amazonensis* promastigotes

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Here we report the subcellular localization of an intracellular serine protease of 68 kDa in axenic promastigotes of Leishmania (Leishmania) amazonensis*, using subcellular fractionation, enzymatic assays, immunoblotting, and immunocytochemistry. All fractions were evaluated by transmission electron microscopy and the serine protease activity was measured during the cell fractionation procedure using α-N-r-tosyl-L-arginine methyl ester (L-TAME) as substrate, phenylmethylsulphone fluoride (PMSF) and L-1-tosylamino-2-phenylethylchloromethylketone (TPCK) as specific inhibitors. The enzymatic activity was detected mainly in a membranous vesicular fraction (6.5-fold enrichment relative to the whole homogenate), but also in a crude plasma membrane fraction (2.0-fold). Analysis by SDS-PAGE gelatin under reducing conditions demonstrated that the major proteolytic activity was found in a 68 kDa protein in all fractions studied. A protein with identical molecular weight was also recognized in immunoblots by a polyclonal antibody against serine protease (anti-SP), with higher immunoreactivity in the vesicular fraction. Electron microscopic immunolocalization using the same polyclonal antibody showed the enzyme present at the cell surface, as well as in cytoplasmic membranous compartments of the parasite. Our findings indicate that the internal location of this serine protease in* L. amazonensis *is mainly restricted to the membranes of intracellular compartments resembling endocytic/exocytic elements.*

Key words: *Leishmania* - serine protease - subcellular localization - trypanosomatid

Study of parasitic proteases has received considerable attention, since their physiological role elucidation may help to develop strategies for exploiting these enzymes as novel chemotherapic targets (Cazzulo 2002, Sajid & McKerrow 2002). In eukaryotic organisms, serine protease are the most studied proteases, while cysteine and metallo-type enzymes have been the most investigated in trypanosomatid protozoa of the *Leishmania* genus (Coombs & Mottram 1997, Mottram et al. 1998, Alves et al. 2000, Jaffe & Dwyer 2003). Serine peptidases are among the most extensively studied enzymes. They are found in all organisms studied and participate in blood clotting and complement activation cascade reactions, phage maturation and fertilization, as well as any number of other fields of biological phenomena (Rawling & Barret 1994). Protozoan serine proteases play crucial roles in host-parasite interaction (Rosenthal 1999). The most notorious serine proteases are found in species of *Plasmodium*. These malarial enzymes digest proteins of the cytoplasmatic membrane of the red blood cells thereby affording invasion and infection by the parasite performing several functions such as mediation of the merozoite entry into host erythrocytes (Braun-Breton et al. 1992, Braun-Breton & Pereira da Silva 1993) and formation of the parasitophorous vacuole (Roggwille et al. 1996), affording invasion and infection of erythrocytes by the parasites.

Trypanosomatid protozoa serine peptidases have been identified and characterized, and play crucial roles in host infection (Burleigh & Woolsey 2002, Silva-Lopez & Giovanni De Simone 2004). *Trypanosoma cruzi* oligopeptidase B mediates intracellular $[Ca^{2+}]_i$-transients in mammalian cells promoting actin rearrangements and lysosome-plasma membrane fusion, which is required for efficient parasite invasion (Burleigh & Woolsey 2002). In this same protozoan a prolyl oligopeptidase, representative of a new serine peptidase family (EC 3.4.21.26) was characterized. This enzyme hydrolyzes components of extracellular matrix, allowing the parasites to migrate through the host tissue (Grellier et al. 2001). Activity of both *T. cruzi* peptidases suggests that they might be attractive targets for studies on drug design. In *Leishmania amazonensis* promastigotes, previous studies have reported the biochemical characterization of a serine oligopeptidase, without proteolytic activity (Ribeiro de Andrade et al. 1998) and a 68 kDa serine protease displaying important hydrolytic activity on proteins (Silva-Lopez & Giovanni De Simone 2004). It is important to point out that both enzymes were purified from water-soluble intracellular extracts and have demonstrated different properties including molecular mass, pI, inhibition profile, and substrate specificity. Although the biochemical proper-

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ties of 68 kDa *Leishmania* serine protease are known, its function in the parasite physiology and in the host-parasite interaction is still unclear. Studies on subcellular localization will help to understand the function of this enzyme. Recently, our group isolated a 56 kDa serine protease from the culture supernatant of promastigotes of the same parasite, produced an antiserum against this enzyme and its intracellular location was analyzed by immunocytochemistry (Silva-Lopez et al. 2004).

So, in order to improve the knowledge on the parasite physiology and create a possibility to develop a rational design of compounds that may be useful in the treatment of the leishmaniasis, here we report the subcellular localization of *L. amazonensis* promastigotes intracellular 68 kDa serine protease, on the basis of some biochemical properties.

MATERIALS AND METHODS

Parasite - Promastigote forms of *L. (L.) amazonensis* (IOC 575; IFLA/BR/67/PH8) were maintained in brain heart infusion medium (Difco, Detroit, US) supplemented with 10% (v/v) heat-inactivated fetal-calf serum. The cultures were kept at room temperature (25°C) in Roller bottles using a Cel-Gro Rotator (Lab-Line Model, Thomas Scientific, New Jersey, US).

Parasite lysis and subcellular fractionation - Cells were harvested by centrifugation (Beckman centrifuge Model J2-21, Palo Alto, California, US) at 3800 g for 10 min and washed three times in P buffer (10 mM Tris-HCl, pH 7.0, and containing 0.25 M sucrose). Thereafter, the cellular pellet was ressuspended for 30 min in hypotonic solution (P buffer without sucrose) and the cells were disrupted in a Dounce type homogenizer (~ 80 strokes). The homogenization process was stopped while some unbroken cells were still present. A concentrated sucrose solution (0.5 M), prepared in 10 mM Tris-HCl, pH 7.0, was immediately added to the lysate to make a final concentration of 0.25 M, in order to minimize the osmotic damage caused by the hypotonic solution. Cell swelling and rupture was monitored by phase contrast microscopy. Lysed cells were submitted to a first centrifugation for 10 min at 4300 g, and the resulting pellet containing unbroken parasites, nuclei, mitochondria, and some kinetoplast-mitochondrial complexes was discarded. The post-nuclear supernatant was harvested and then centrifuged for 15 min at 12,000 g. The pellet was collected and the second supernatant was spun again for 45 min at 35,000 g. Aliquots of the last supernatant and the pellets (called P-12,000 g and P-35,000 g, respectively) were harvested to subsequent analysis. All centrifugation steps and other operations were performed at 4ºC or on ice.

Assay of protease activity and effect of inhibitors - Enzyme activity was measured as previously described (Silva-Lopez & Giovanni De Simone 2004), with slight modifications. Briefly, the reaction mixtures, which comprised 300 µl of 100 mM Tris-HCl buffer, pH 8.0, containing 100 µl of sample preparations, were incubated with 100 µl of 0.1 mM (final concentration) α-N-r-tosyl-L-arginine methyl ester (L-TAME). Following incubation at room temperature (25ºC) for 30 min, the solutions were centrifuged to remove insoluble material and the absorbance increase at 247 nm was measured in the supernatant (Hitachi U-2000 spectrophotometer, Schaumburg, Illinois, US). One unit of enzymatic activity was defined as the amount of enzyme required to cause an increase of 0.1 in the absorbance under standard conditions.

In order to investigate the serine protease presence in parasite subcellular fractions, inhibition assays were performed. Protease inhibitors were incubated with each fraction (50 mg protein/µl) for 30 min in Tris-HCl 0.1M pH 8.0 at room temperature. The following protease inhibitors were used: 10 mM phenymethanesulphonyl fluoride (PMSF), 1 mM L-1-tosylamino-2-phenylethylchloromethylketone (TPCK), 1 mM trans-epoxysuccinyl-leucylamide (4-guanidino) butane (E-64), 2 mM ethylenediaminetetraacetic acid (EDTA), 1mM o-phenanthroline (OP) and 25 µM Pepstatin-A (PA). The reaction started upon addition of the substrate (0.25 mM L-TAME) in Tris-buffer at 28°C for 30 min. The tests were performed in triplicate in standardized conditions (Silva-Lopez & Giovanni De Simone 2004). Appropriate controls were carried out in parallel using the same enzyme solutions without inhibitors. Inhibition was expressed as the percentage of the appropriate control activity. All inhibitors and the substrate were purchased from Sigma Chemical Co. (St. Louis, Missouri, US).

Gelatin substrate gel electrophoresis - Protease activity in the different parasite fractions was also determined using gelatin substrate gel electrophoresis. Samples were applied to 12% SDS-PAGE with 0.1% gelatin and electrophoresis conducted at 4ºC in a Mini Protean II Apparatus (BioRad, Richmond) without prior reduction or heating, according to previously described conditions (Silva-Lopez & De Simone 2004). After electrophoresis, gels were washed under agitation for 60 min at room temperature with 2.5% Triton X-100, and incubated overnight at 37ºC in 0.1 M Tris-HCl buffer, pH 8.0. The gel was then stained with 0.1% amide black and distained using methanol/acetic acid/distilled water (3:1:6).

Antiserum production - In order to produce the antibody against the 68 kDa serine protease, the enzyme was purified from a whole extract of *L. amazonensis* promastigotes by freezing-thawing. The purification was a combination of affinity chromatography on aprotinin-agarose and gel filtration high performance liquid chromatography, as previously described (Silva-Lopez & Giovanni De Simone 2004). The anti-*L. amazonensis* serine protease antiserum (anti-SP) was raised in rabbit by injecting 20 µg of the homogeneous heat inactivated (2 min at 100°C) 68 kDa enzyme emulsified in complete (first booster) and incomplete (subsequent boosters) Freud's adjuvant. Rabbits were bled after the fourth injection and the antibody reactivity was checked by immunoblotting.

Electrophoretic analysis and immunoblotting - Discontinuous SDS-PAGE (12%) was performed using the conditions described by Laemmli (1970). The proteins from SDS-PAGE were electroblotted onto nitrocellulose sheets using a semidry blotting apparatus (BioRad, Richmond, UK) at 10 V for 60 min, as described (Towbin et al. 1979).

For immune staining, the nitrocellulose sheets were incubated for 60 min with the rabbit anti-SP serum (1:400 dilution). The sheets were then soaked for 60 min in peroxidase-conjugated goat anti-rabbit IgG and the blots developed with 3,3'-diaminobenzidine/H_2O_2.

Electron microscopy and immunocytochemistry - Routine transmission electron microscopy analysis was carried out to control the subcellular fractionation process. Samples were fixed by the addition of an equal volume of 5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, and containing 0.25 M sucrose. Post-fixation was carried out with 1% osmium tetroxide in cacodylate buffer containing 0.8% potassium ferricyanide and 5 mM $CaCl_2$, followed by dehydration in acetone and embedding in PolyBed 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a Zeiss EM10C transmission electron microscope.

The polyclonal antiserum (anti-SP) was used to determine the subcellular localization of the serine protease in *L. amazonensis* promastigotes, by using previously described methods for immunogold labeling (Bendayan et al. 1987). Briefly, the parasites were fixed in freshly prepared 4% paraformaldehyde/1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Samples were dehydrated in methanol and embedded at progressively lowered temperature in Lowicryl K4M resin. Thin sections were collected on 400 mesh uncoated nickel grids, incubated for 30 min at room temperature in phosphate buffered saline (PBS) containing 1.5% bovine serum albumin (BSA) and 0.01% Tween 20 (PBS-BSA-Tw), pH 8.0, and then for 60 min in the presence of the anti-SP antibody diluted 1:50 in PBS-BSA-Tw, pH 8.0. Grids were rinsed in PBS-BSA-Tw and finally incubated for 60 min with a 1:200 dilution of goat anti-rabbit antibody labeled with 10-nm gold particles (Bendayan et al. 1987). The grids were subsequently washed with PBS and distilled water, stained with uranyl acetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope. Under these conditions, controls incubated only with the secondary gold-labeled antibody showed little, if any, background reaction.

Protein determination - The protein content in the different fractions was estimated with the BioRad (Richmond, UK) protein assay kit using bovine serum albumin (BSA) as standard.

RESULTS

Isolation of subcellular fractions - Using the homogenization procedure described here, 90% of cell disruption was obtained as ascertained by phase contrast microscopy. The fractionation methodology was also controlled by transmission electron microscopy. Nuclei and mitochondria in the resulting homogenate presented no apparent morphological membrane lesion (data not shown). The general aspect of the P-12,000 g and P-35,000 g fractions obtained by differential centrifugation is shown in Figs 1 and 2, respectively. The major elements found in the P-12,000 g fraction were plasma membrane profiles, as recognized by the attached subpellicular microtubules, although some flagella could also be seen.

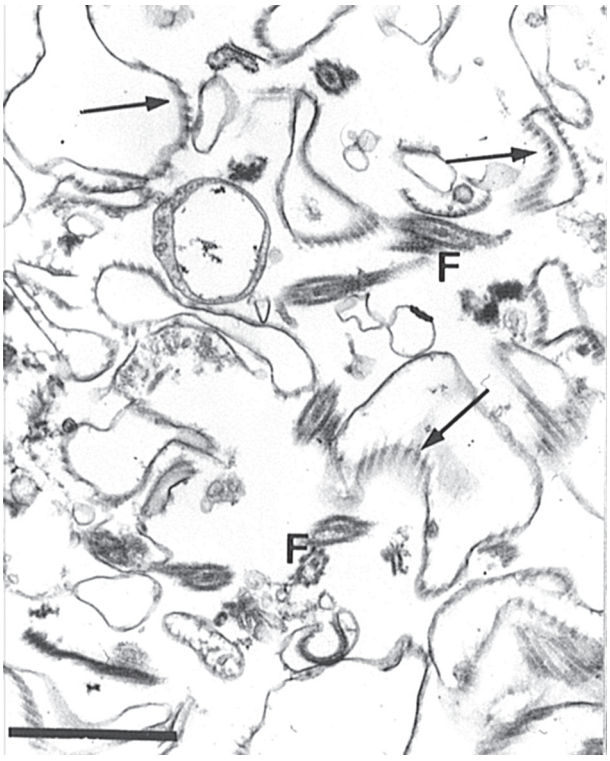

Fig. 1: electron micrograph of the 12,000 g pellet obtained by subcellular fractionation. Most isolated membranes are characterized to be from surface, associated with microtubules (arrows). F: flagella. Bar = 1 µm.

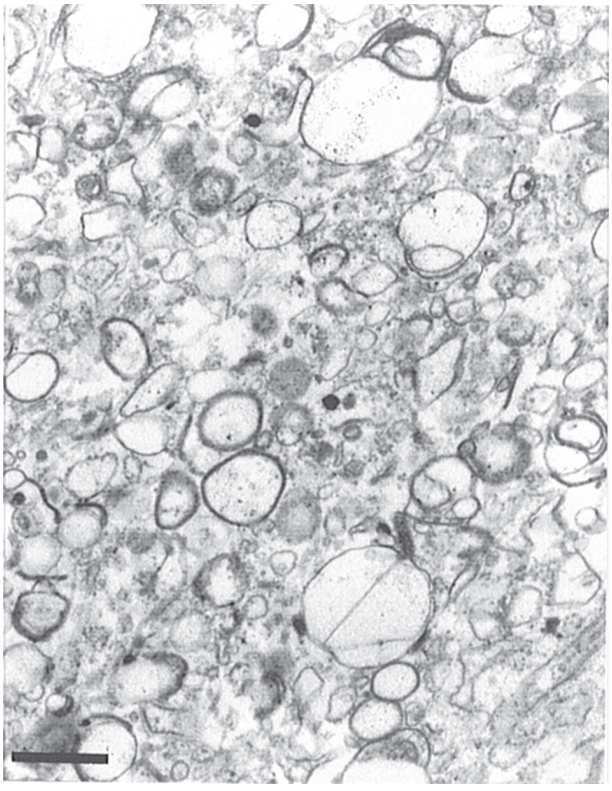

Fig. 2: transmission electron microscopy showing the general aspect of the 35,000 g pellet. It is possible to observe vesicles of different sizes as the main component. Bar = 0.5 µm.

Ultrathin sections of the P-35,000 g fraction showed that its main component was a population of spherical membrane bound vesicles of different sizes, which possibly represent elements of the endocytic/exocytic compartments.

Immunoreactivity analysis - Specificity of the polyclonal serum to serine protease was evaluated by western blot analysis employing the four fractions obtained in the subcellular fractionation. The antibody to serine protease recognized a major band of 68 kDa in all fractions, with the highest immunoreactivity in the vesicular fraction (Fig. 3). Interestingly, the antiserum revealed two additional bands of 50 and 45 kDa in the whole homogenate, the membrane fraction and the vesicular fraction.

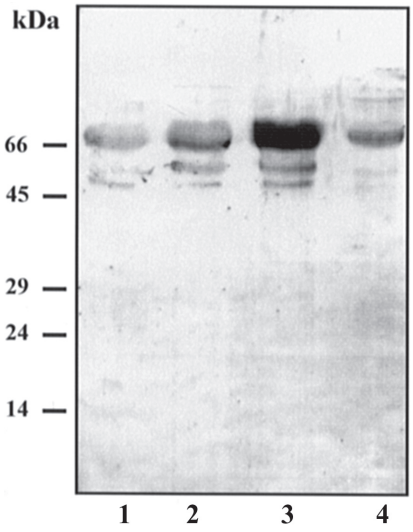

Fig. 3: western blotting analysis of *Leishmania amazonensis* fractions using anti-SP antibody. Lanes - 1: whole homogenate; 2: P-12,000 g fraction; 3: P-35,000 g fraction; 4: final supernatant. Fifteen µg of protein were loaded in each lane. Values of the molecular weight markers are indicated on the left side of gel.

In order to localize the serine protease in the parasites, electron microscopy immunolocalization by labeling the cells with the anti-SP was performed. The antibody reacted against the parasite surface and internal structures in most (about 95%) analyzed cells (Fig. 5A). Cytoplasmic gold particles were localized predominantly in vesicular structures close to the flagellar pocket region, morphologically similar to that of the endocytic/exocytic pathways (Fig. 5B). The gold markers were seen bound to the inner membrane leaflet of the cytoplasmic vesicles (Fig. 5C).

Enzymatic activity detection in subcellular fractions - Previous experiments showed that extracts of *L. amazonensis* were able to hydrolyze L-TAME at pH 8.0. Thus, we have used this chromogenic substrate to evaluate protease activity in the different fractions obtained by the fractionation methodology. Table I shows the subcellular distribution profile of protease activity in the whole

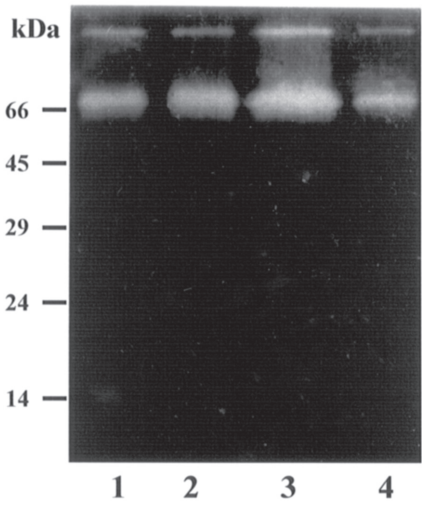

Fig. 4: proteolytic activity of *Leishmania amazonensis* fractions. The fractions were submitted to SDS-PAGE gelatin, incubated overnight at pH 8.0 and 37ºC and stained with amido black. Lanes - 1: whole homogenate; 2: P-12,000 g; 3: P-35,000 g; 4: final supernatant. Fifteen µg of protein were loaded in each lane. Values of the molecular weight markers are indicated on the left side of gel.

homogenate, in the P-12,000 g and P-35,000 g fractions, and in the final supernatant. Both the P-12,000 g fraction and the final supernatant showed some enrichment in activity (2- and 1.5-fold, respectively) in relation to the whole homogenate, but the highest activity was found in the P-35,000 g fraction. This later fraction corresponded to the membranous vesicles and displayed an enrichment of 6.5-fold in the enzymatic activity, with 42% recovery (Table I). Interestingly, the enzyme displayed no latency when the enzymatic activity was checked in samples of freshly isolated fractions incubated in the presence of Triton X-100 (data not shown).

In order to identify the *L. amazonensis* protease activity in the subcellular fractions, inhibition experiments were carried out using L-TAME as substrate. The activity was not inhibited by *o*-phenanthroline, EDTA or Pepstatin A, was weakly sensitive to E-64 (an inhibitor of cysteine proteinase), but the activity in the four fractions was PMSF- and TPCK-sensitive, both serine protease inhibitors. The enzymatic activity was reduced 60-88% in the presence of PMSF and 50-80% in the presence of TPCK (Table II), supporting the hypothesis that the activity was due to a serine-type peptidase. Similar results were observed for the purified serine enzyme (Silva-Lopez & Giovanni De Simone 2004).

Proteolytic activity of the fractions was also screened using SDS-PAGE gelatin at pH 8.0. As shown in Fig. 4, a single majority protein was associated with the four fractions analyzed, although noticeable differences in activity could be seen among them. For instance, the enzymatic activity was higher in the membranous vesicular fraction (lane 3) than in the other fractions. In all cases the protein migrated with the same electrophoretic mobility (68 kDa). Additionally, a minor protein about ≥ 100 kDa was also detected in the fractions.

TABLE I

Distribution and recovery of the serine type protease activity in subcellular fractions of *Leishmania amazonensis* promastigotes prepared by differential centrifugation. Values are means of three independent experiments

Fractions	Total protein[a] (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Enrichment (-fold-)
Whole homogenate	90.00	7.38	0.082	100	1.0
P-12,000 g	9.40	1.55	0.16	21	2.0
P-35,000 g	5.80	3.10	0.53	42	6.5
Final supernatant	15.00	1.90	0.12	25	1.5

a: from 2×10^{10} cells

TABLE II

Effects of inhibitors on the L-TAME activity of subcellular fractions of *Leishmania amazonensis* promastigotes. The fractions (50 µg protein/ml) were incubated previously for 30 min with the respective inhibitors and the residual activity was measured at 25ºC at pH 8.0 as described in Materials and Methods. The percentage of inhibition was calculated taking the control values (without inhibitor) as 100%. The assays represent the means of at three independent determinations. Standard deviations were always lower than 5%

	Residual activity (%)					
Fractions	PMSF	TPCK	E-64	EDTA	OP	PA
Whole homogenate	22	15	85	100	100	100
P-12,000 g	30	44	78	100	100	100
P-35,000 g	40	50	85	100	100	100
Final supernatant	12	19	88	100	100	100

PMSF: phenymethanesulphonyl fluoride; TPCK: L-1-tosylamino-2-phenylethylchloromethylketone; E-64: trans-epoxysuccinyl-leucylamide (4-guanidino) butane; EDTA: ethylene-diaminetetraacetic acid; OP: O-phenanthroline; PA: Pepstatin-A

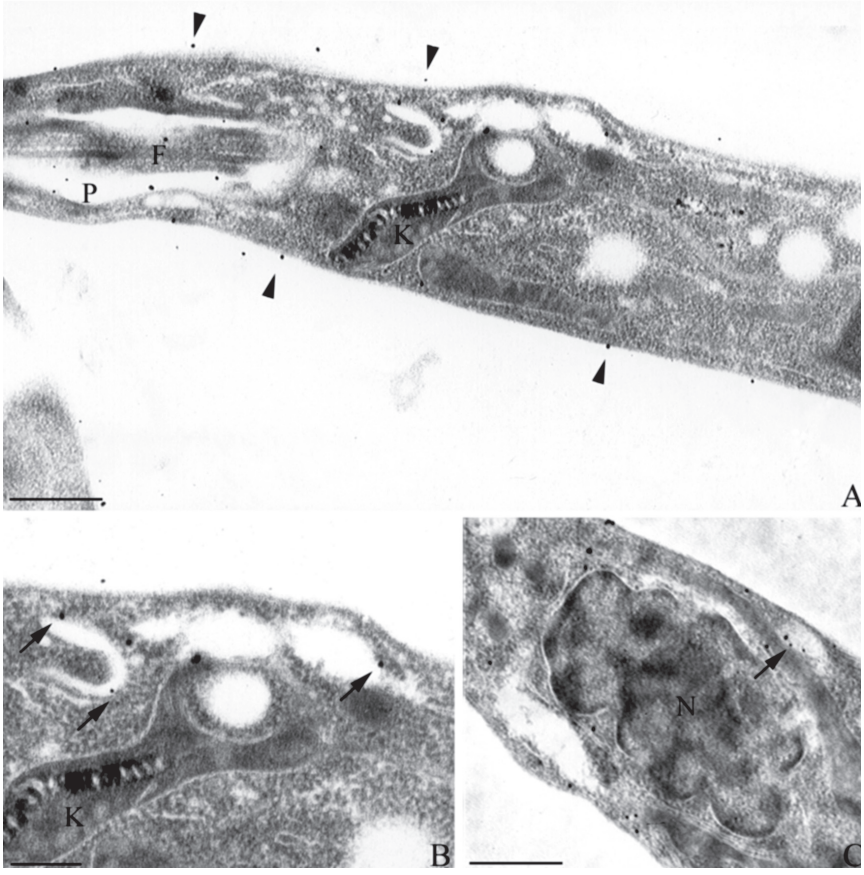

Fig. 5: subcellular localization of serine protease in *Leishmania amazonensis* promastigotes using the polyclonal anti-SP antibody. A: gold particles are seen bound to the external surface (arrowheads) and to the flagellar pocket membrane; B: high magnification showing labeling in cytoplasmic vesicles and tubulovesicular structures close to the flagellar pocket (arrows); C: high magnification showing labeling (arrow) in cytoplasmic vesicles. P: flagellar pocket; F: flagellum; k: Kinetoplast; N: nucleus. Bar = 0.25 µm.

DISCUSSION

Parasite proteases have been demonstrated to play important roles in the pathogenesis of a variety of diseases (McKerrow et al. 1993), but their identification and subcellular localization are important prerequisites for understanding their function in the infection process. Immunolocalization studies associated with cell fractionation techniques are essential to achieve these goals, but in trypanosomatid protozoa, particularly in *Leishmania* species, serious difficulties have been associated with cell rupture, due mainly to their own morphological characteristics (Coombs et al. 1982, Mottram & Coombs 1985). The cell rupture method used here has proved to be ideal in obtaining a homogenate in which the subcellular structures were well preserved, as confirmed by transmission electron microscopy.

Subcellular distribution of serine protease in *L. amazonensis* promastigotes using the chromogenic substrate (L-TAME) indicated that the enzymatic activity was mainly associated to the membranous vesicular fraction. Protease activity was also recovered in the crude plasma membrane fraction, but in minor amounts, indicating that the enzyme may, in some way, be associated with membranes of intracellular compartments, instead of being enclosed in a soluble form in the lumen of these compartments. The finding that the enzymatic activity displayed no latency when vesicle fraction was incubated with Triton X-100 (data not shown) together with the immunocytochemical data further suggests that the enzyme is membrane-bound. The small amount of activity in the final supernatant probably was derived from proteases released from vesicle membranes, as suggested by the identical molecular weight of the enzyme, although the possibility that it represents a different cytosolic form of the enzyme cannot be excluded. Previous studies in *T. cruzi* have demonstrated that an alkaline serine peptidase involved in the generation of a novel Ca^{2+}-signaling factor for mammalian cells was found in soluble form after disruption of parasites by freezing and sonication, but some activity was also associated with a crude membrane fraction (Burleigh & Andrews 1995, Burleigh & Woolsey 2002). Furthermore, since the activity could be removed by repeated washing, it was concluded that the enzyme was not tightly associated to the membranes (Tardieux et al. 1994). Other efforts to obtain the same enzyme have also been carried out using different cell breakage methodologies. For example, in *T. cruzi* and *Crithidia fasciculata* both detergent Nonidet P-40 (Ashall et al. 1992, Healy et al. 1992) and freezing-thawing (Grellier et al. 2001) methods were used. Freezing and thawing, followed by Triton X-100 treatment, was also used to rupture *T. brucei brucei* (Troeberg et al. 1996). It is important to note that in these latter studies, as well as to *L. amazonensis* oligopeptidase (Ribeiro de Andrade et al. 1998), the enzyme was always recovered in a soluble form, but subcellular fractionation or immunocytochemistry investigations were not carried out.

Using gel enzymography the proteolytic activity of a *L. amazonensis* serine protease corresponding to 68 kDa was observed as the main component in all four fractions. Apparently, this protease activity corresponds to the mature protease, while the proteolytic activity detected at higher molecular mass (≥ 100 kDa) is likely due to associations of the protease or precursor molecules, or both, considering the relatively mild conditions used for protein separation. On the other hand, western blotting analysis using anti-SP revealed two proteins with 50 and 45 kDa. These proteins represent auto-proteolysis products yielded from the highly active 68 kDa serine protease during the purification procedures, since they do not display hydrolytic activity and are present after the chromatography steps and in stored samples (Silva-Lopez & Giovanni De Simone 2004).

By immunoelectron microscopy cytoplasmic labeled structures of *L. amazonensis* demonstrated to have striking morphological similarities with compartments of the endocytic/exocytic pathways, as previously reported in cryosections of *Leishmania* species (Yahiaoui et al. 1993). Detailed examination of electron micrographs showed that the label was associated with membranes of cytoplasmic vesicles and tubules. Such endocytic/exocytic-like elements have also been described in other trypanosomatids (Soares et al. 1992, Porto-Carreiro et al. 2000, McConville et al. 2002). It is possible that in *L. amazonensis* promastigotes most of these structures co-sediment with the vesicular fraction obtained in the subcellular fractionation, as suggested by the higher enzymatic activity and immunoreactivity to serine protease found in this fraction. Further studies using suitable biochemical markers for endocytic/exocytic components in these parasites (reviewed in Overath et al. 1997, McConville et al. 2002) are necessary in order to characterize the cell compartments (and/or compartment domains) present in our subcellular fractions. Other interesting observation is that the structures described in the present study were located between the nucleus and the flagellar pocket, which also resembles the localization of endocytic/exocytic structures of other trypanosomatids (Webster & Fish 1989, McConville et al. 2002). On the contrary of the 68 kDa intracellular serine protease here reported, to be mainly located in membranes of intracellular compartments and plasma membrane, the 56 kDa extracellular serine protease, previously described, reacted poorly with the parasite surface and moderately with internal structures. However, it was predominantly located in the flagellar pocket and structures that are morphologically similar to the compartments that found in mammalian endocytic/exocytic pathways (Silva-Lopez et al. 2004), which justified the released into the extracellular environment. If the intracellular location of this enzyme correspond a correlated enzymatic activity or intracellular trafficking, remains to be demonstrated.

In conclusion, we reported the subcellular localization of the 68 kDa intracellular serine protease in *L. amazonensis* promastigote forms, providing useful data in future investigations on cellular trafficking route in order to elucidate the role of this enzyme in the parasite.

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