Leishmanolysin (gp63 metallopeptidase)-like activity extracellularly released by *Herpetomonas samuelpessoai*

C. G. R. ELIAS†, F. M. PEREIRA†, B. A. SILVA, C. S. ALVIANO, R. M. A. SOARES *and* A. L. S. SANTOS*

Departamento de Microbiologia Geral, Instituto de Microbiologia Prof. Paulo de Góes (IMPPG), Centro de Ciências da Saúde (CCS), Universidade Federal do Rio de Janeiro (UFRJ), Ilha do Fundão, Rio de Janeiro, RJ 21941-590, Brazil

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SUMMARY

In previous studies, we showed that *Herpetomonas samuelpessoai* produced a large amount of a surface-located metallopeptidase that presented similar biochemical properties to that of gp63 from *Leishmania* spp., which is a well-known virulence factor expressed by these digenetic parasites. The present study aims to identify the proteolytic activity released by living *H. samuelpessoai* cells. In this context, the parasites were incubated in phosphate buffer up to 4 h, and the supernatants were obtained by centrifugation and filtration steps and were then applied on SDS–PAGE to determine the secretory protein profile and on gelatin-SDS–PAGE to identify the proteolytic activity. The results demonstrated that *H. samuelpessoai* secreted at least 12 polypeptides and an extracellular peptidase of 66 kDa. This enzyme had its activity diminished by 1,10-phenanthroline, EDTA and EGTA. This metallopeptidase was active in a broad spectrum of pH, showing maximum activity at pH 6·0 at 37 °C. Casein was also cleaved by this secretory proteolytic enzyme, while bovine serum albumin and haemoglobin were not degraded under these conditions. Fluorescence microscopy and flow cytometry using anti-gp63 antibody against leishmanolysin of *L. amazonensis* demonstrated the presence of similar molecules on the cell-surface of *H. samuelpessoai*. Moreover, immunoblot analysis showed the presence of a reactive polypeptide in the cellular extract and in the supernatant fluid of *H. samuelpessoai*, which suggests immunological similarities between these two distinct trypanosomatids. The zinc-metallopeptidase inhibitor 1,10-phenanthroline was able to inhibit the secretion of the 66 kDa metallopeptidase in a dose-dependent manner, while the phospholipase C inhibitor (*p*-CMPS) did not alter the secretion pattern. Additionally, anti-cross-reacting determinant (CRD) antibody failed to recognize any secreted polypeptide from *H. samuelpessoai*. Collectively, these results suggest that the gp63-like molecule was released from the *H. samuelpessoai* surface by proteolysis instead of phospholipolysis, in a similar mechanism to that observed in *Leishmania*.

Key words: *Herpetomonas samuelpessoai*, leishmanolysin, metallopeptidases, secretion, trypanosomatids.

INTRODUCTION

Kinetoplastid protozoa, including the genera *Trypanosoma* and *Leishmania*, are responsible for many parasitic diseases of humans and animals worldwide (McGhee and Cosgrove, 1980). The peptidases present in different protozoa appear to be relevant for several aspects of host-parasite interactions, quite apart from their obvious participation in the nutrition of the parasite at the expense of the host (McKerrow *et al*. 1993; Yao, Donelson and Wilson, 2003). Metallopeptidases have been described in a number of trypanosomatids, but only those present in *Leishmania* spp. have been thoroughly characterized (reviewed by Yao *et al*. 2003). Parasites that belong to the *Leishmania* genus are digenetic trypanosomatids that cycle between the gut of the sand fly vector (promastigote forms) and the phagolysosome of a mammalian host macrophage (amastigote forms) (McGhee and Cosgrove, 1980). Several *Leishmania* surface molecules facilitate survival in these diverse host environments. For instance, *Leishmania* spp. express a major glycosylphosphatidylinositol (GPI)-anchored glycoprotein of 63 kDa named gp63 or leishmanolysin (Etges, Bouvier and Bordier, 1986; Medina-Acosta, Beverley and Russell, 1993). Leishmanolysin is a Zn^{+2}-dependent HE*XX*H metallopeptidase with a broad range of substrate specificity and optimum pH activity (Yao *et al*. 2003). Structural and biochemical similarities exist between gp63 and members of the metzincin class of matrix metallopeptidases (Stöcker and Bode, 1995). The latter are important for enhancing the migration of some tumor cells through the extracellular matrix and basement membrane, aiding in their metastasis (Stöcker and Bode, 1995). In this context, *Leishmania* species engineered to express high levels of the surface metallopeptidase gp63 had enhanced capacity of migration through extracellular matrix, since type IV collagen and fibronectin were extensively degraded *in vitro* (McGwire, Chang and Engman, 2003).

* Corresponding author: Tel: +55 21 2562 6740. Fax: +55 21 2560 8344. E-mail: andre@micro.ufrj.br
† These authors contributed equally to this work.

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The amount of gp63 protein expressed by *Leishmania* spp. is modulated in different parasite growth-phase and life-cycle stages (Yao *et al*. 2003). In this sense, gp63 is abundantly expressed on the surface of the promastigote forms, up-regulated in infectious metacyclic promastigotes, and has a low but detectable expression level in the intracellular amastigote stage (Medina-Acosta *et al*. 1989). Leishmanolysin plays different roles in the host-parasite interactions and has been postulated as a virulence factor (Joshi *et al*. 2002; Yao *et al*. 2003). The finding of gp63 gene homologues in the insect trypanosomatids *Crithidia fasciculata* and *Herpetomonas samuelpessoai* (Inverso *et al*. 1993; Yao *et al*. 2003) suggests that leishmanolysin may be important for the interaction with the invertebrate host. However, gp63 is apparently not essential for insect stages of the life-cycle, since *L. major* promastigote-specific genes can be knocked out with no deleterious effects on the growth and development of the parasite in 3 Old World *Phlebotomus* species (Joshi *et al*. 2002). In contrast, Hajmová and co-workers (2004) reported that the down-regulation of gp63 in a *L. amazonensis* clone adversely affects its early development in the neotropical *Lutzomyia longipalpis* sand fly. The possibility exists that gp63 may function differently for these two distinct *Leishmania* species in their interactions with different invertebrate vector species.

The identification, characterization and cloning of parasite cellular antigens have been the focus of extensive investigations. Previous studies of our group and others, showed the production of a similar cell-surface metallopeptidase activity of 60–66 kDa in some monoxenous trypanosomatids, including *C. fasciculata* (Etges, 1992), *C. deanei*, *C. desouzai* and *C. oncopelti* (d'Avila-Levy *et al*. 2001), *C. guilhermei* (Melo *et al*. 2002), *C. luciliae* (Jaffe and Dwyer, 2003), *H. samuelpessoai* (Etges, 1992; Santos *et al*. 2003), *H. roitmani* and *H. anglusteri* (Santos *et al*. 1999), *Leptomonas seymouri* (Jaffe and Dwyer, 2003) and *Blastocrithidia culicis* (Santos *et al*. 2001*a*; d'Avila-Levy *et al*. 2005). On the other hand, less is known about the identity and functions of molecules secreted or released during the growth of these parasites into their environment. In the present study, we showed that *H. samuelpessoai* cells extracellularly released a metallopeptidase of 66 kDa, which shared biochemical and immunological similarities with the leishmanolysin of the human pathogen *L. amazonensis*.

MATERIALS AND METHODS

Chemicals

Media constituents, reagents used in electrophoresis, buffer components, nitrocellulose membrane and reagents for chemiluminescence detection were purchased from Amersham Life Science (Little Chalfont, England). The proteolytic inhibitors (phenylmethylsulfonyl fluoride [PMSF], *trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane [E-64], ethylene glycol-bis(β-aminoethyl ether) [EGTA], ethylenediaminetretraacetic acid [EDTA] and 1,10-phenanthroline), the protein substrates (gelatin, bovine serum albumin [BSA], haemoglobin and casein), *p*-chloromercuriphenylsulfonic acid (*p*-CMPS), *Bacillus thuringiensis* phospholipase C (*Bt*PLC), nitro-blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and the secondary antibodies were products of Sigma Chemical Co. (St Louis, MO, USA). Sulfosuccinimidyl-6-(biotinamide) hexanoate (Sulfo-NHS-LC-biotin) and avidin-alkaline phosphatase conjugated (AAPC) were products of Pierce (Rockford, IL, USA).

Parasites and growth conditions

Herpetomonas samuelpessoai (CT–IOC–067) was kindly provided by Dr Maria Auxiliadora de Sousa (Coleção de Tripanosomatídeos, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil). The trypanosomatid was maintained by weekly transfers in a complex medium (brain heart infusion – BHI) at 26 °C. *Leishmania amazonensis* Josefa strain (MHOM/BR/75 Josefa) promastigote forms were maintained at 26 °C for 4 days in BHI medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Soares *et al*. 2003).

Secretion experiment

For this experiment, *H. samuelpessoai* was grown at 26 °C in 1000 ml flasks containing 500 ml of BHI medium. Parasite cells were harvested at the log growth phase (48 h) by centrifugation at 2500 ***g***, for 15 min at 4 °C, and washed 3 times with cold PBS (150 mM NaCl; 20 mM phosphate buffer, pH 7·2). Cellular growth was estimated by counting the parasites in a Neubauer chamber. The intact cells ($1{\cdot}0 \times 10^{10}$) were resuspended in 10 ml of sterile isotonic PBS. Aliquots of 2·5 ml (containing $2{\cdot}5 \times 10^{9}$ parasites) were separated in 4 tubes, which were incubated for different periods of time (from 1 to 4 h) at 26 °C. After intervals of 1 h, the cells were removed by centrifugation (2500 ***g***/20 min/4 °C) and the supernatants were passed over a 0·22-μm membrane (Millipore), to obtain the PBS-conditioned supernatants (d'Avila-Levy *et al*. 2003).

Modulation of the proteolytic secretion by metallopeptidase and phospholipase C inhibitors

Living *H. samuelpessoai* ($2{\cdot}5 \times 10^{8}$ cells) were pretreated or not with 1 and 10 mM 1,10-phenanthroline (a zinc-metallopeptidase inhibitor) for 30 min at room temperature. Then, the cells were washed 5 times with PBS and the secretion experiment was

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repeated as described above. Alternatively, the same number of parasites was incubated in the absence and in the presence of 0·1 and 1 μM *p*-CMPS (a PLC inhibitor) for 1 h, and the reaction mixtures were obtained after centrifugation and filtration steps (Santos *et al*. 2002*a*). All supernatants were analysed by proteolytic activity as described below.

Cellular viability

The survival of the parasites along the incubation period in the isotonic phosphate buffer as well as after the treatment with 1,10-phenanthroline and *p*-CMPS was assessed by mobility, trypan blue cell dye exclusion and by measurement of lactate dehydrogenase activity, a cytoplasmic enzyme, in the secretion supernatant fluids (Santos *et al*. 2002*c*, 2005).

Cellular parasite extract

The parasites ($1{\cdot}0 \times 10^8$ cells) were harvested by centrifugation for 5 min at 1500 ***g*** at 4 °C, and washed 3 times with cold PBS. The cells were resuspended in 100 μl of PBS and lysed by the addition of 1% (w/v) sodium dodecyl sulfate (SDS). The cells were broken in a vortex by alternating 1 min shaking and 2 min cooling intervals, followed by a centrifugation at 5000 ***g*** for 15 min at 4 °C. The supernatant obtained after centrifugation corresponded to the whole parasite cellular extract (Santos *et al*. 2001*b*).

Secretory protein profile

Protein concentration was determined by the method described by Lowry and co-workers (1951), using BSA as standard. Samples containing 5 μg of released proteins were added to 10 μl of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (125 mM Tris, pH 6·8; 4% SDS; 20% glycerol; 0·002% bromophenol blue) supplemented with 5% (v/v) β-mercaptoethanol, followed by heating at 100 °C, for 5 min. Proteins were analysed in 12% SDS–PAGE using the method described by Laemmli (1970). Electrophoresis was carried out at 4 °C, at 120 V, and the gels were silver stained (Santos *et al*. 2001*b*). The molecular mass of sample polypeptides was calculated from mobility of GIBCO BRL (Grand Island, NY, USA) molecular mass standards.

Quantitative proteolytic activity assay

The extracellular peptidase activity was measured spectrophotometrically using the substrate gelatin, according to the method described by Jones and co-workers (1998). Briefly, 50 μl of each *H. samuelpessoai* PBS-conditioned supernatant and 350 μl of 50 mM sodium phosphate buffer, pH 6·0, were added to 600 μl of the substrate solution (1% (w/v) gelatin in distilled water) and the mixtures were incubated at 37 °C for 2 h. A control, where the substrate was added just after the reactions were stopped, was used as blank. A 300 μl sample was removed from each reaction mixture and added to 400 μl of cold isopropanol. After centrifugation at 16 000 ***g*** for 10 min, the supernatants were removed and the absorbance was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0·01 in absorbance unit, under standard assay conditions.

Gelatin-SDS–PAGE analysis

The secretory proteolytic activity was assayed and characterized by 10% SDS–PAGE with 0·1% (w/v) gelatin incorporated into the gel as substrate (Heussen and Dowdle, 1980). The gels were loaded with 25 μl (equivalent to 5 μg of protein) of each PBS-conditioned supernatant per slot. After electrophoresis, at a constant current of 120 V at 4 °C, SDS was removed by incubation with 10 volumes of 1% Triton X-100 for 1 h at room temperature under constant agitation. Then, the gels were incubated in 50 mM sodium phosphate buffer, pH 6·0, for 20 h, to promote the proteolysis. The gels were stained for 2 h with 0·2% (w/v) Coomassie brilliant blue R-250 in methanol-acetic acid-water (50 : 10 : 40) and destained overnight in a solution containing methanol-acetic acid-water (5 : 10 : 85), to intensify the digestion halos (Santos *et al*. 2005). Molecular mass of the peptidase was calculated by the comparison of the mobility of GIBCO BRL SDS–PAGE standards (Grand Island, NY, USA). The gels were dried, scanned and the densitometric analysis was performed with the use of the Kodak Digital Science EDAS 120 software (Soares *et al*. 2003).

Effect of pH, temperature and proteolytic inhibitors on the extracellular proteolytic activity

In this set of experiments proteolytic activity was evaluated using the standard assay conditions described above. The effect of pH was determined incubating the gel strips at 37 °C for 20 h in the following buffers: 10 mM sodium citrate (pH 2·0–4·0), 50 mM sodium phosphate (pH 5·0–8·0) and 20 mM glycine-NaOH (pH 9·0–10·0). Extracellular proteolytic enzyme was also analysed on gelatin-SDS–PAGE at different temperatures (4, 26, 37, 50 and 65 °C) for 20 h in 50 mM sodium phosphate buffer, pH 6·0. Then, the gel strips containing the released peptidases were incubated for 20 h at 37 °C in 50 mM sodium phosphate buffer, pH 6·0, in the absence and in the presence of the following peptidase inhibitors: 10 mM PMSF, 10 mM 1,10-phenanthroline, 10 mM EDTA, 10 mM EGTA and 10 μM E-64 (Santos *et al*. 2002*a*).

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Effect of different protein substrates on the extracellular proteolytic activity

The ability of the extracellular peptidase to degrade different proteinaceous substrates was also evaluated by 10% SDS–PAGE containing 0·1% (w/v) gelatin, casein, haemoglobin and BSA (Melo *et al*. 2002). Electrophoresis was performed as described before and gel strips were incubated in 50 mM sodium phosphate buffer, pH 6·0, for 20 h at 37 °C.

Flow cytometry and fluorescence microscopy for cell-surface gp63

Parasites ($5{\cdot}0 \times 10^6$ cells) used for these experiments were fixed at 4 °C with 4% paraformaldehyde in PBS (pH 7·2) for 5 min, followed by extensive washing in the same buffer. These fixed cells maintained their morphological integrity, as verified by microscopical observation. They were incubated for 1 h with a 1 : 250 dilution of rabbit anti-gp63 antibody raised against *L. amazonensis* (kindly provided by Dr Kwang-Poo Chang, University of Health Sciences, Chicago Medical School, USA), and then incubated for an additional hour with a 1 : 250 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit immunoglobulin G (IgG). These cells were washed 3 times in PBS and observed in a Zeiss epifluorescence microscope (Axioplan). For flow cytometry analysis, these cells were examined in an EPICS ELITE flow cytometer (Coulter Electronics, Hialeah, Fla.) equipped with a 15 mW argon laser emitting at 488 nm. Untreated cells and those treated only with the secondary antibody were used as controls. Each experimental population was then mapped by using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population ($n = 10\,000$) was then analysed for log green fluorescence by using a single-parameter histogram (Brittingham *et al*. 1995).

PLC treatment

Parasites ($1{\cdot}0 \times 10^8$ cells) were fixed for 15 min in 0·4% (v/v) paraformaldehyde, washed exhaustively in PBS (pH 8·0) and then reacted with 0·2 mg/ml Sulfo-NHS-LC-biotin for 30 min at 4 °C. The cells were washed 3 times with PBS to remove non-reacted biotin (Santos *et al*. 2001*b*) and then incubated with 0·5 U/μl of *Bt*PLC in a final reaction mixture (400 μl) containing 100 mM Tris-acetate, pH 7·4, supplemented with 0·1% Triton X-100 for 1 h at 37 °C. Control cells were subjected to the same experimental conditions, except for the presence of *Bt*PLC. The supernatants from the reaction mixtures were collected by centrifugation (1500 ***g***, 10 min), filtered in a 0·22-μm membrane (Millipore) and mixed with SDS–PAGE sample buffer for the further analysis (Santos *et al*. 2002*a*). Alternatively, PBS-conditioned supernatant was also incubated in the absence or in the presence of 0·5 U/μl *Bt*PLC for 1 h at 37 °C.

Western blotting

Protein extracts were separated in 12% SDS–PAGE and the polypeptides electrophoretically transferred at 4 °C at 100 V/300 mA for 2 h to a nitrocellulose membrane. The membrane was blocked in 5% (w/v) low-fat dried milk in TBS (150 mM NaCl; 10 mM Tris, pH 7·4) containing 0·5% Tween 20 (TBS/Tween) for 1 h at room temperature. Then, membranes were washed 3 times (10 min each) with the blocking solution and incubated separately with the anti-gp63 antibody at 1 : 500 dilution and with the anti-cross-reacting determinant (CRD) (kindly provided by Dr Michael A. J. Ferguson and Dr Maria Lucia S. Guther, University of Dundee, UK) at 1 : 400 dilution for 2 h. The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG at 1 : 2500. Immunoblots were exposed to X-ray film after reaction with ECL reagents for chemiluminescence (Brittingham *et al*. 1995). Alternatively, cell-surface biotinylated proteins were also transferred to nitrocellulose membranes, blocked for 1 h, and washed 5 times (5 min per wash) in blocking solution. Membranes were then incubated for 1 h in a 1 : 500 dilution of AAPC in TBS/Tween, and then washed as described above. Bands were visualized by reaction with 30 mg/ml NBT plus 15 mg/ml BCIP in detection reagent (100 mM Tris, 500 mM $MgCl_2$, pH 9·5). The blots were washed with several changes of distilled water and air-dried (Santos *et al*. 2001*a*).

Statistical analysis

All experiments were performed in triplicate. The mean and standard error of at least 3 distinct experiments were determined. Statistical analysis was calculated by using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software by using Student's *t*-test.

RESULTS

In previous studies (Santos *et al*. 2002*a*, 2003), we showed that *Herpetomonas samuelpessoai* produced a large amount of a surface-located metallopeptidase (60–70 kDa) that presented similar biochemical properties with the gp63 of *Leishmania* spp., which is a well-known virulence factor expressed by these digenetic human parasites (Yao *et al*. 2003). In order to confirm that the cell-surface and the released peptidase produced by *H. samuelpessoai* is a member of the gp63 family, cellular and secretory extracts were probed with anti-gp63 polyclonal antibody by Western blotting (Fig. 1). This antibody strongly

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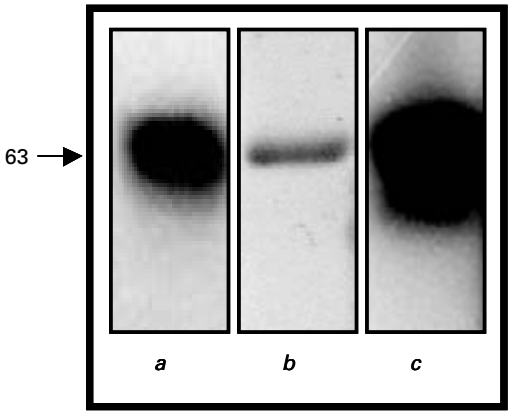

Fig. 1. Western blotting showing the leishmanolysin-like polypeptide detected in the whole cellular extract (*a*) and in the released polypeptides (*b*) from *Herpetomonas samuelpessoai* cells. The total cellular extract of promastigotes from *Leishmania amazonensis* was used as a positive control (*c*). The number on the left indicates the apparent molecular mass of the reactive polypeptide, expressed in kilodaltons.

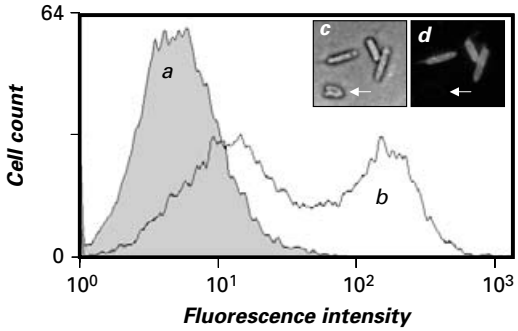

Fig. 2. Flow cytometry analysis showing the anti-leishmanolysin antibody binding to the cell-surface of *Herpetomonas samuelpessoai*. The cells were incubated in the absence (*a*) and in the presence (*b*) of anti-gp63 antibody as described in the Material and Methods section, and then analysed by flow cytometry. Phase contrast (*c*) and fluorescence microscopy images of parasites sequentially incubated with anti-gp63 and FITC-secondary antibodies (*d*) are shown in the inset. Note that in the FACS analysis 2 distinct populations were clearly observed, 1 of them was inaccessible to the anti-gp63 antibody. Corroborating this result, fluorescence microscopy showed that some cells were not labelled with this antibody (arrow in *c* and *d*).

recognized a single 63 kDa polypeptide band in *L. amazonensis* lysate (Fig. 1, lane c), as well as a similar molecular mass component in the cellular extract (Fig. 1, lane a) and in the released polypeptides (Fig. 1, lane b) from *H. samuelpessoai*. In addition, flow cytometry analysis provided measurements for the relative levels of *H. samuelpessoai* surface gp63-like molecules (Fig. 2). Fluorescence microscopy corroborated with the fact that the anti-gp63 antibody recognized a similar molecule on the cell-surface of *H. samuelpessoai* (Fig. 2, inset). Nevertheless, some cells were not fluorescently labelled with the anti-gp63 antibody, corroborating the flow cytometry analysis in which 2 distinct populations were clearly observed (Fig. 2).

Figure 3 shows the time-course of protein and peptidase released by *H. samuelpessoai* cells during the 4 h incubation in PBS. The results demonstrated that *H. samuelpessoai* secreted several polypeptides and an extracellular peptidase of 66 kDa. The total protein content increased linearly from 2 to 4 h and the maximal peptidase liberation was detected in the third hour of incubation with PBS (Fig. 3A). The cellular viability was assessed throughout the incubation period in PBS by monitoring the mobility of cells and trypan blue cell dye exclusion. Non-motile or dead cells were not detected during the 4 h incubation in PBS (data not shown). In addition, lactate dehydrogenase, an intracellular enzyme, was not found in the supernatant fluids, indicating that the extracellular peptidase detected in this study was not released by autolysis (data not shown). SDS–PAGE analysis showed that at least 12 polypeptides, ranging from 40 to 100 kDa, were released by the parasite cells (Fig. 3B). The slight augmentation in the 66 kDa peptidase was also apparent when the supernatant fluids (from 1 to 3 h) were examined by gelatin-SDS–PAGE (Fig. 3D) or by immunoblotting using the anti-leishmanolysin antibody (Fig. 3C).

The pH dependence of the 66 kDa extracellular peptidase was determined by densitometric measurements of the digestion halo after gelatin-SDS–PAGE (Fig. 4A). On the gelatin substrate, the enzyme exhibited the maximal activity at pH 6·0. However, the enzyme activity decreased markedly at pH values below 4·0 and above 8·0 (Fig. 4A). The optimum temperature of 66 kDa peptidase was 37 °C, and a decrease by approximately 80% in its maximum activity was detected at 65 °C (Fig. 4B). This extracellular peptidase had its activity inhibited ($\approx$90%) by 10 mM 1,10-phenanthroline, a zinc-metallopeptidase inhibitor (Fig. 5). However, a total inhibition on the 66 kDa peptidase activity was observed when the gel strip was incubated in the presence of 20 mM 1,10-phenanthroline (data not shown). The metallopeptidase inhibitors EDTA and EGTA also promoted a significant decrease on the 66 kDa activity. E-64, a cysteine peptidase inhibitor, weakly inhibited the enzyme (Fig. 5), this could indicate the presence of critical thiol group(s) near the active site as previously suggested (Melo *et al.* 2002; Santos *et al.* 2002*a*). PMSF did not interfere significantly with the enzyme behaviour (Fig. 5). The 66 kDa metallopeptidase exhibited selective substrate utilization on SDS–PAGE, being active with gelatin and casein, while haemoglobin and BSA were not hydrolysed (Fig. 6).

Previously, we showed that *H. samuelpessoai* cells express at least 4 major surface polypeptides and that

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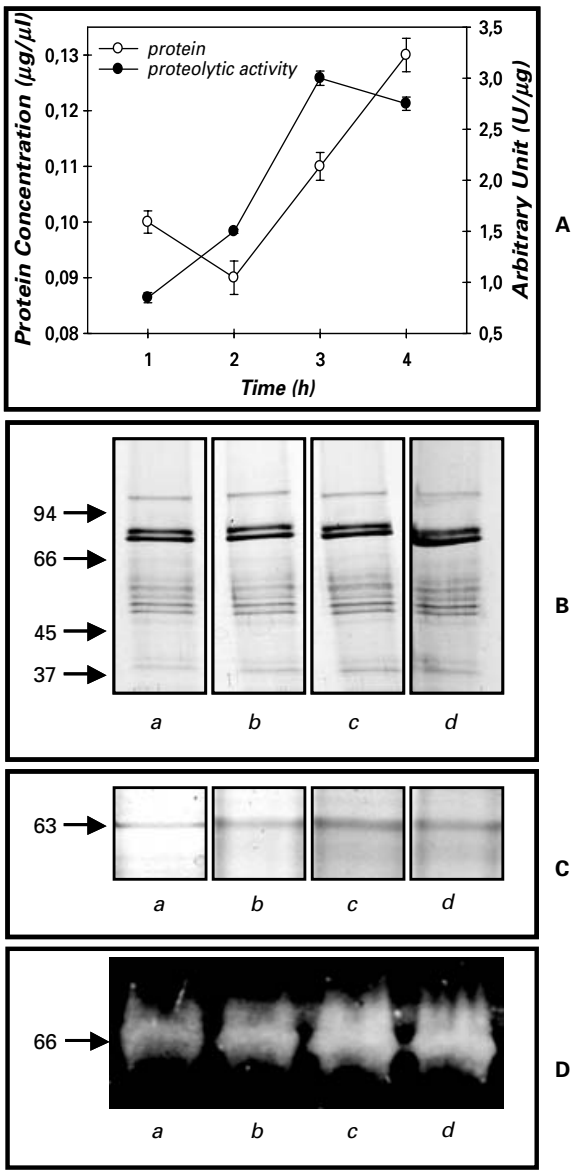

Fig. 3. Quantitative measurements of proteolytic activity, using gelatin as soluble substrate, and protein released to the extracellular medium (A). SDS–PAGE showing the secretory protein profile revealed by silver staining (B), Western blotting showing the leishmanolysin-like polypeptide (C) and gelatin-SDS–PAGE evidencing the 66 kDa extracellular peptidase (D) released from *Herpetomonas samuelpessoai* during 1 (*a*), 2 (*b*), 3 (*c*) and 4 h (*d*) of incubation in isotonic PBS. The numbers on the left indicate the apparent molecular masses, expressed in kilodaltons.

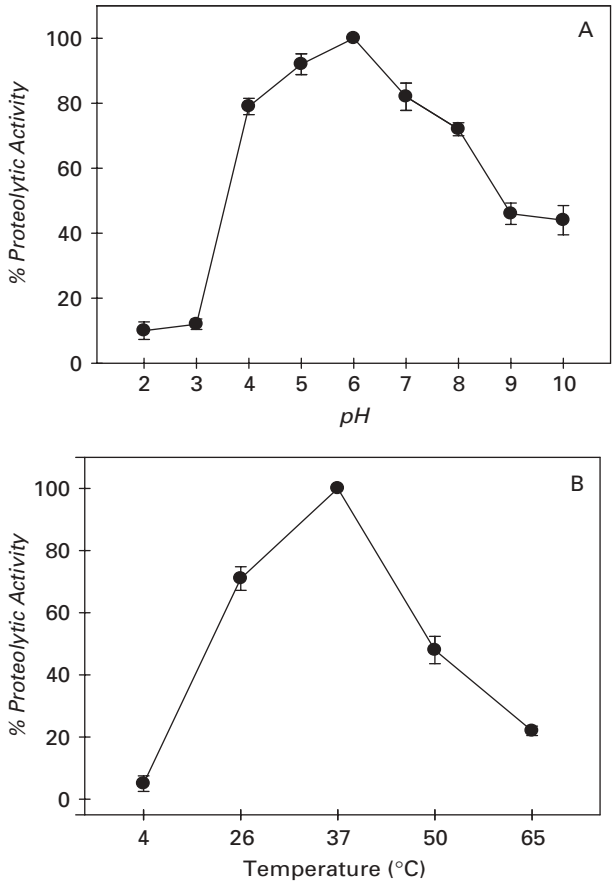

Fig. 4. Effect of pH (A) and temperature (B) on the 66 kDa peptidase secreted by *Herpetomonas samuelpessoai* after 3 h of PBS incubation. The gel strips were incubated in different buffers (pH 2·0–10·0), as described in the Materials and Methods section, for 20 h at 37 °C. Alternatively, the gel strips were incubated in phosphate buffer (pH 6·0) for 20 h at different temperatures (ranging from 4 to 65 °C). Proteolytic activity of each control system is shown as 100%. The percentage values represent means ± standard error of 3 independent densitometric measurements of the digestion halos after gelatin-SDS–PAGE analysis.

living parasites normally released these 4 polypeptides to the extracellular milieu, including the 66 kDa surface metallopeptidase activity (Santos *et al*. 2001*b*, 2002*a*). Conversely, we showed that paraformaldehyde-fixed parasites were incapable of releasing proteins (data not shown) and surface polypeptides to the extracellular environment (Fig. 7A, lane b). However, when the fixed parasites were treated with *Bt*PLC, we observed in the supernatant reaction mixture at least 4 bands (Fig. 7A, lane d), corresponding to the major cell-surface polypeptides, which were recognized by anti-CRD antibody, including a 63 kDa polypeptide component (Fig. 7B, lane d_1) that also reacted with the anti-gp63 antibody (Fig. 7B, lane d_2), showing that these polypeptides were anchored to the parasite surface via GPI anchor. Control fixed cells, which were not treated with *Bt*PLC, did not produce any positive reaction when probed with the 2 tested antibodies (Fig. 7B, lanes b_1 and b_2).

Here, we also looked for a probable reminiscent GPI-anchor in the released polypeptides, especially in the gp63-like component of *H. samuelpessoai*. The anti-CRD antibody, which recognizes the inositol cyclic phosphate on the reminiscent glycosyl moiety of the protein, failed in recognizing any secreted polypeptide from living *H. samuelpessoai* (Fig. 8, lane b). A comparable result was obtained when the

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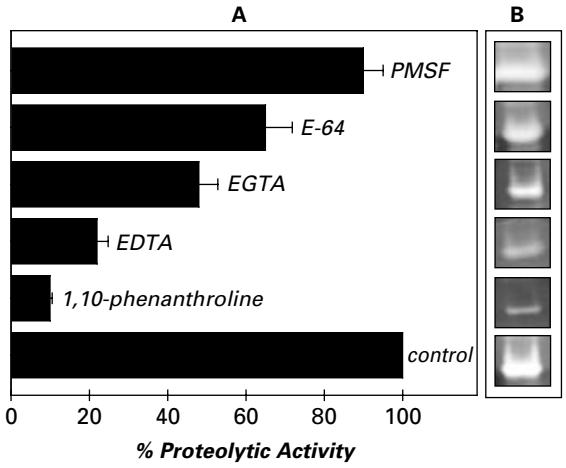

Fig. 5. Gelatin-SDS–PAGE showing the modulation of the 66 kDa extracellular proteolytic activity from *Herpetomonas samuelpessoai*, when the gel strips were incubated in the absence (control) and in the presence of different proteolytic inhibitors: 10 mM 1,10-phenanthroline, 10 mM EDTA, 10 mM EGTA, 10 mM PMSF and 10 μM E-64 (B). The graphic represents the densitometric measurements of the digestion halos (A). Proteolytic activity of control is shown as 100%. The percentage values represent means $\pm$ standard error of 3 independent measurements.

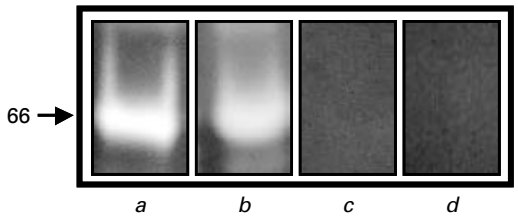

Fig. 6. Effect of different protein substrates incorporated into 10% SDS–PAGE on the 66 kDa extracellular peptidase activity: gelatin (*a*), casein (*b*), BSA (*c*) and haemoglobin (*d*). The gel strips were incubated in phosphate buffer (pH 6·0) for 20 h at 37 °C. The number on the left indicates the apparent molecular mass, expressed in kilodaltons, of the peptidase activity.

PBS-derived supernatant was treated with *Bt*PLC (Fig. 8, lane c). Moreover, as similarly demonstrated in *Leishmania* species (McGwire *et al*. 2002), 1,10-phenanthroline-treated *H. samuelpessoai* cells showed a considerably reduction in release of 66 kDa peptidase to the extracellular medium (Fig. 9A). Inhibition of the proteolytic secretion was dose dependent, increasing from 56 to 87% as 1,10-phenanthroline concentration rose from 1 to 10 mM (Fig. 9A). On the other hand, *p*-CMPS, a PLC inhibitor, at 0·1 and 1 μM did not interfere with the 66 kDa peptidase secretion (Fig. 9B). Additionally, these 2 inhibitors did not affect the parasite viability when tested under the conditions employed in this work. Collectively, these results corroborated with the fact that the metallopeptidase of 66 kDa detected in the extracellular environment could be released on

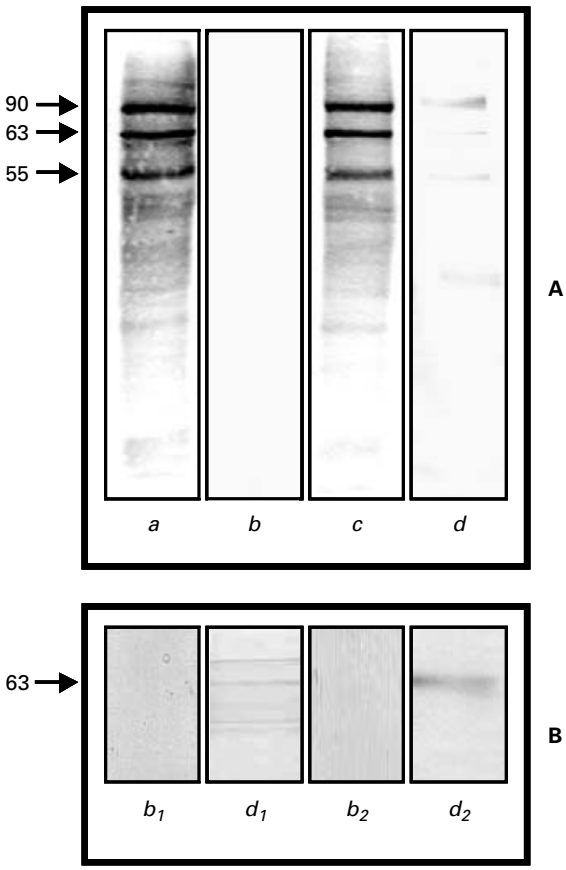

Fig. 7. Detection of polypeptides GPI-anchored to the cell-surface of *Herpetomonas samuelpessoai*. Formaldehyde-fixed *Herpetomonas samuelpessoai* promastigotes were biotin-labelled with Sulfo-NHS-LC-biotin for 30 min. Then parasite cells were non-treated (*a*) or treated (*c*) for 1 h with *Bt*PLC. After this period, the supernatants (reaction mixtures) were obtained (*b*, *d*) after centrifugation. Cellular extracts and supernatants were electrophoretically transferred to nitrocellulose membranes and then revealed with AACP to show the cell-surface composition (*a*, *c*) as well as the released polypeptides (*b*, *d*) (A). The reaction mixture supernatants of both non-treated (*b*) and *Bt*PLC-treated cells (*d*) were probed with anti-CRD (*1*) and anti-gp63 (*2*) antibodies (B). The number on the left indicates the apparent molecular mass, expressed in kilodaltons.

the cell-surface from *H. samuelpessoai* through proteolysis instead of phospholipolysis.

DISCUSSION

A variety of microorganisms possess membrane-bound and/or secreted peptidases that aid in their interaction with host tissues. In parasites of the genus *Leishmania*, leishmanolysin molecules on the surface of promastigotes probably form a significant part of the interface between the invading parasite cell and the mammalian host at the time of infection (Yao *et al*. 2003). Approximately 75% of gp63 from *Leishmania* species are located on the cell-surface according to surface biotinylation, fluorescence microscopy and

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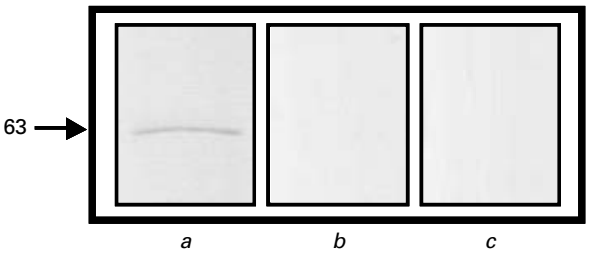

Fig. 8. Detection of reminiscent GPI-anchor in the extracellular leishmanolysin-like protein of *Herpetomonas samuelpessoai*. PBS-conditioned supernatant was incubated in the absence (*a*, *b*) and in the presence of *Bt*PLC (*c*) for 1 h at 37 °C. These mixtures were submitted to SDS–PAGE and then the polypeptides were transferred to nitrocellulose membranes. The membranes were incubated separately with anti-gp63 (*a*) and anti-CRD (*b*, *c*) antibodies. The number on the left indicates the apparent molecular mass, expressed in kilodaltons.

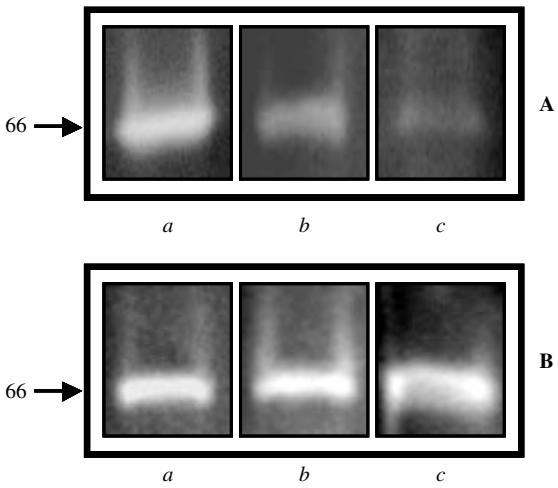

Fig. 9. Effect of metallopeptidase and PLC inhibitors on the 66 kDa secretion by living *Herpetomonas samuelpessoai* cell. Parasites were treated for 30 min in the absence (*a*) and in the presence of 1 (*b*) and 10 mM 1,10-phenanthroline (*c*), then cells were exhaustively washed in PBS, centrifuged and then allowed to secrete for an additional hour (A). Otherwise, cells were incubated for 1 h in the absence (*a*) and in the presence of 0·1 (*b*) and 1 μM *p*-CMPS (*c*), and the supernatants were obtained after centrifugation and filtration steps (B). All these supernatants were analysed on 10% gelatin-SDS–PAGE, to evidence the proteolytic activity. The number on the left indicates the apparent molecular mass, expressed in kilodaltons, of the peptidase activity.

immunoelectron microscopy (Weise *et al*. 2000). Previous studies (Etges, 1992; Santos *et al*. 2003) showed a cell-surface located gp63-like molecule in *H. samuelpessoai* by biochemical enzymatic characterization. Here, we corroborated those early studies demonstrating an immunological cross-reactivity between *H. samuelpessoai* and anti-leishmanolysin antibody using Western blotting, flow cytometry and fluorescence microscopy analyses. Intriguingly, 2 distinct populations with different affinities for the anti-gp63 antibody were clearly identified in *H. samuelpessoai*, indicating that gp63-like molecules are not equally expressed on the surface of parasite cells. A first explanation for this observation would be that the lack of equal expression is correlated to the *H. samuelpessoai* growth phase, since flagellate cultures were not synchronized. Furthermore, the occurrence of distinct subpopulations could alternatively denote a different expression of surface gp63-like molecules in the promastigote, paramastigote and opisthomastigote developmental stages (Santos *et al*. 2003) or even a diminished accessibility to external ligands in cell subsets, as previously reported for other cell-surface molecules expressed in *H. samuelpessoai* (Santos *et al*. 2002*b*).

In addition, several groups recently reported that a significant proportion of leishmanolysin is released by *Leishmania* promastigotes (Weise *et al*. 2000; McGwire *et al*. 2002; Yao *et al*. 2002; Jaffe and Dwyer, 2003). In this context, we also showed the expression of a leishmanolysin-like activity secreted by living *H. samuelpessoai* cells. As previously proposed, the existence of a leishmanolysin homologue in monoxenous trypanosomatids, which are insect parasites with no mammalian host, suggests a primary role in the midgut of the insect vector. Interestingly, the prevailing pH range of the insect gut (pH 6–7), the normal habitat for *Herpetomonas* (McGhee and Cosgrove, 1980), coincides with the pH range in which higher values for proteolytic activity were observed in this paper. However, there is disagreement about the pH optimum of *Leishmania* gp63. Using native proteins as substrates, Chaudhuri and Chang (1988) observed a pH optimum of 3·0–4·0 for *L. mexicana amazonensis* promastigote gp63, Tzinia and Soteriadou (1991) reported 2 peaks of activity at pH 5·8 and pH 7·2, for the cleavage of insulin in 7 different *Leishmania* strains, while Etges *et al*. (1986) found that *L. major* promastigote gp63 was most active against azocasein above pH 7·0. Some of the differences may be explained by the use of native, globular substrates versus denatured, linear substrates (Yao *et al*. 2003). Additionally, in a previous study, we reported that *H. samuelpessoai* produced a cell-surface metallopeptidase of 60–70 kDa that presented a broad spectrum of activity ranging from pH 5·0 to 10·0 (Santos *et al*. 2003).

In *H. samuelpessoai*, a differential expression of cell-surface metallopeptidase at 26 and 37 °C was recently determined (Santos *et al*. 2003). Here, we also compared the activity of the 66 kDa extracellular metallopeptidase at different temperatures. Similarly, at 26 °C (insect vector temperature), this proteolytic activity was lower than in the vertebrate temperature (37 °C). The different optimal temperatures for these enzymes might reflect adaptations of the parasite to the distinct environments it might confront during its life-cycle. Accordingly, Jansen,

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Carreira and Deane (1988) demonstrated that the scent glands of experimentally infected opossum *Didelphis marsupialis* support growth of *H. samuelpessoai* for many months. In addition, *H. samuelpessoai* grows well at 37 °C in complex and chemically defined media (Roitman, Roitman and Azevedo, 1972). Consequently, the evolutionary proximity of *H. samuelpessoai* to important human pathogens, including *Leishmania* species (Hughes and Piontkivska, 2003), is possibly reflected in the similarity of some aspects of the basic cellular machinery. Therefore, biochemical and immunological similarities between the *Leishmania* gp63 enzyme and that of *H. samuelpessoai* suggests that the metallopeptidase was represented in a common ancestor that predated invasion of the vertebrate host. This is evidence that the peptidase activity is important for the survival of the trypanosomatid inside its invertebrate vector, but it does not necessarily preclude it from fulfilling its role in the vertebrate host (Yao *et al.* 2003).

The effect of inhibitors on the proteolytic activity provides the reliable information concerning the catalytic type of a peptidase. In this sense, extracellular leishmanolysin-like enzyme from *H. samuelpessoai* demonstrated close similarities to the cell-surface gp63 from *Leishmania*, including sensitivity towards the metal-chelating 1,10-phenanthroline and modulation by divalent cations (data not shown). Other lower trypanosomatids also release peptidases that can be inhibited by 1,10-phenanthroline (Santos *et al.* 1999, 2001*a*, 2005; D'Avila-Levy *et al.* 2001; Melo *et al.* 2002; Almeida *et al.* 2003; Jaffe and Dwyer, 2003; Vermelho *et al.* 2003). E-64, a cysteine peptidase inhibitor diminished the 66 kDa metallopeptidase activity secreted by *H. samuelpessoai*. Likewise, the cysteine residue detected in the *L. major* gp63 protein demonstrated involvement in a cysteine switch mechanism of peptidase activity, binding the zinc atom at the active site and inhibiting enzyme activity (MacDonald *et al.* 1995). Moreover, leishmanolysin is an enzyme capable of degrading many protein substrates including gelatin, casein, azocasein, albumin, haemoglobin and fibrinogen (Bouvier *et al.* 1990). Indeed, its ability to hydrolyse such a range of substrates has made it a convenient protein to interact with molecules encountered in diverse host or vector environments. In contrast, the extracellular leishmanolysin-like molecule released from *H. samuelpessoai* hydrolysed gelatin and casein, but not BSA and haemoglobin, showing a more evident strict substrate specificity under the conditions employed in our experiments.

The extracellular gp63-like enzyme identified herein showed similar biochemical properties with the major metallopeptidase associated to the surface of *H. samuelpessoai* (Santos *et al.* 2003). The surface and released molecules synthesized by *H. samuelpessoai* could form the interface between the parasites and the microenvironments provided by its vector. In addition, this fact suggests that the metallopeptidase may be released into the culture medium by a mechanism that could be similar to that observed for the *Leishmania* gp63, since *H. samuelpessoai* surface metallopeptidase is also GPI-anchored to the plasma membrane (Etges, 1992; Schneider and Glaser, 1993; Santos *et al.* 2002*a*). Although the GPI anchor can be cleaved by *Bt*PLC revealing the CRD epitope, evidence using anti-CRD antibody indicates that the GPI anchor is absent or not enzymatically cleaved during the release of surface gp63-like molecule from *H. samuelpessoai* cells. McGwire and co-workers (2002) demonstrated that release of GPI-anchored gp63 into the extracellular medium was dramatically reduced by 1,10-phenanthroline or in the case of a leishmanolysin mutation at the zinc-binding motif. This result suggests that gp63 release is dependent on autoproteolysis, consistent with early reports that gp63 is able to cleave a synthetic peptide substrate corresponding to the pro-peptide cleavage site (Bouvier *et al.* 1990) and with a later study of *L. major* leishmanolysin active site mutants (MacDonald *et al.* 1995). In this sense, we also showed that 1,10-phenanthroline significantly inhibited the release of the 66 kDa metallopeptidase, which suggests a resembling mechanism of secretion in these 2 distinct trypanosomatids. Corroborating these findings, the PLC inhibitor *p*-CMPS did not alter the 66 kDa metallo-enzyme secretion pattern.

Recently, gp63-like activity has been described in other human pathogenic trypanosomatids, including *T. brucei* and *T. cruzi*. In the former, it was suggested that a metallopeptidase surface activity is responsible for the shedding of variant surface glycoprotein (VSG) during cellular differentiation (Bangs *et al.* 2001). In fact, one of the *T. brucei* gp63 families is involved in the release of transgenic VSG from procyclic cells (Lacount *et al.* 2003). *T. cruzi* possesses a family of gp63 genes composed of multiple groups (Cuevas, Cazzulo and Sanchez, 2003). Two of these groups, *Tcgp63-I* and *–II*, are present as high-copy-number genes and antibodies against *Tcgp63-I* partially blocked the infection of Vero cells by trypomastigotes, which suggests a possible role for this metallopeptidase during the infection process *in vitro* (Cuevas *et al.* 2003).

A myriad of functions can be hypothesized for extracellularly released metallopeptidases. For instance, secreted gp63 could allow the parasites to evade a variety of anti-microbial factors in the extracellular environment, including degradation of serine peptidases that are mediators in many aspects of invertebrate immunity such as haemolymph coagulation, activation of anti-microbial peptide synthesis and melanotic encapsulation (Gorman and Paskewitz, 2001). Furthermore, within the sand fly vector, released gp63 could favour the nutrient utilization present in the bloodmeal during the early

stages of development, as well as to protect the parasite against the sand fly digestive enzymes (Schlein *et al*. 1990). We are currently testing the potential role(s) of the extracellular gp63-like molecule from *H. samuelpessoai* and *L. amazonensis* in these processes.

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