Trypanosoma cruzi heparin-binding proteins present a flagellar membrane localization and serine proteinase activity

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SUMMARY

Heparin-binding proteins (HBPs) play a key role in *Trypanosoma cruzi*-host cell interactions. HBPs recognize heparan sulfate (HS) at the host cell surface and are able to induce the cytoadherence and invasion of this parasite. Herein, we analysed the biochemical properties of the HBPs and also evaluated the expression and subcellular localization of HBPs in *T. cruzi* trypomastigotes. A flow cytometry analysis revealed that HBPs are highly expressed at the surface of trypomastigotes, and their peculiar localization mainly at the flagellar membrane, which is known as an important signalling domain, may enhance their binding to HS and elicit the parasite invasion. The plasmon surface resonance results demonstrated the stability of HBPs and their affinity to HS and heparin. Additionally, gelatinolytic activities of 70 kDa, 65·8 kDa and 59 kDa HBPs over a broad pH range (5·5–8·0) were revealed using a zymography assay. These proteolytic activities were sensitive to serine proteinase inhibitors, such as aprotinin and phenylmethylsulfonyl fluoride, suggesting that HBPs have the properties of trypsin-like proteinases.

Key words: *Trypanosoma cruzi*, heparin-binding protein, plasmon surface resonance, serine proteinase.

INTRODUCTION

Chronic chagasic cardiomyopathy, which is caused by *Trypanosoma cruzi*, is an important clinical manifestation of Chagas' disease and causes morbidity and mortality in Latin America (Tanowitz *et al*. 2009). In endemic areas, *T. cruzi* is transmitted to humans primarily via triatomine feces during blood meals, but food-borne transmission has also emerged as an important mechanism of *T. cruzi* infection (Kribs-Zaleta, 2010; Toso *et al*. 2011; Yoshida *et al*. 2011). For a successful infection in mammalian hosts, infective forms of *T. cruzi* must recognize molecules on the surfaces of the host cells that trigger the invasion process of the parasite (reviewed by Caradonna and Burleigh, 2011). Many glycoproteins on the surface of the parasite have been demonstrated to participate in the recognition and invasion process (reviewed by De Souza *et al*. 2010). However, the expression of these molecules depends on the strain and developmental stage of *T. cruzi*. For example, variations in the expression of glycoproteins such as gp82, gp90 and gp35/50 in metacyclic trypomastigotes can define the invasiveness of the parasite in the host cell (Ruiz *et al*. 1998). The binding of these molecules to their receptors triggers signalling cascades involved in Ca^{2+} mobilization from different cellular compartments (Yoshida and Cortes, 2008), which leads to a mechanism of invasion dependent on or independent of the actin cytoskeleton (Ferreira *et al*. 2006). Members of the trans-sialidase (TS) superfamily also play a key role in the attachment and invasion of the parasite (Eugenia Giorgi and de Lederkremer, 2011). Gp85/trans-sialidase, for instance, interacts with multiple ligands at the cell surface of the host. The highly conserved peptide sequence of this protein (FLY peptide) mediates binding to cytokeratin 18 on the surface of epithelial cells (Magdesian *et al*. 2007) and may also selectively promote parasite tissue tropism (Tonelli *et al*. 2010). Additionally, *T. cruzi* peptidases, such as members of the propyl oligopeptidase family of serine proteinase (oligopeptidase B and Tc-80), are also involved in the invasion of host cells (reviewed by Cazzulo, 2002). It has been shown that *T. cruzi* oligopeptidase B is engaged in the generation of the Ca^{2+} signalling agonist required for the lysosome-dependent mechanism of invasion in mammalian cells (Burleigh *et al*. 1997; Caler *et al*. 1998), whereas POP Tc-80 may degrade extracellular matrix (ECM) components and activate molecules on the parasite and/or host cell ECM that are essential for *T. cruzi* invasion (Grellier *et al*. 2001). The invasion process also involves proteins that are able to bind to heparin (Ortega-Barria and Pereira, 1991;

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Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008), but little is known about these proteins identified in *T. cruzi*.

Heparin-binding protein (HBP) was first reported in trypomastigote forms of *T. cruzi* by Ortega-Barria and Pereira (1991). This 60 kDa protein named penetrin has the ability to bind to heparin and ECM components (heparan sulfate and collagen), promoting parasite entry into mammalian cells (Ortega-Barria and Pereira 1992; Calvet *et al*. 2003) in a mechanism distinct from the TS-sialic acid route (Herrera *et al*. 1994). After purification using Triton X-114 and heparin-affinity chromatography, 2 proteins (59 kDa and 65·8 kDa) have been identified in the trypomastigote and amastigote forms of *T. cruzi* that bind sulfated GAGs (Oliveira-Jr *et al*. 2008). Additionally, we have demonstrated the involvement of the heparan sulfate proteoglycans (HSPG) in the attachment to and invasion of cardiomyocytes by trypomastigotes (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008) and amastigotes (Bambino-Medeiros *et al*. 2011). Our previous data also suggested that the N-acetylated/N-sulfated domain of the HS chain is involved in the selective binding of HS to *T. cruzi* ligands to trigger parasite entry, whereas chondroitin sulfate (CS) had no effect on the invasion of mammalian cells (Oliveira-Jr *et al*. 2008). Recently, the presence of HBPs has been demonstrated in epimastigotes, suggesting that these HBPs play a role in vector-*T. cruzi* interaction (Oliveira *et al*. 2012).

Therefore, because HBPs play a key role in the host cell-parasite recognition process and are observed in different stages of the life cycle of *T. cruzi*, a better characterization of these proteins is essential for a more complete knowledge of the physiological function of HBPs and the potential of these proteins as drug target for the treatment of Chagas' disease. In the present study, attention was focused on the spatial distribution and enzyme properties of HBPs. We provide evidence that the HBPs are located primarily at the flagellar membrane and bind to sulfated GAGs such as heparin and HS. Additionally, we provide evidence that the HBPs have characteristics of serine proteinases.

MATERIALS AND METHODS

Reagents

The detergents [Triton X-100 (TX-100), Triton X-114 (TX-114) and sodium dodecyl sulfate (SDS)], proteinase inhibitors [transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), aprotinin (Apo), 1,10-phenanthroline (o-phe), pepstatin A (Pep A) and phenylmethylsulfonyl fluoride (PMSF)], reducing reagents [dithiothreitol (DTT) and β-mercaptoethanol (β-ME)], gelatin, bovine serum albumin (BSA), penicillin and the aprotinin-agarose column (Sigma-Aldrich; 1·5 × 2·5 cm) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). The heparin-Sepharose column (HiTrap-Heparin; 1·5 × 2·5 cm) was purchased from GE Healthcare (Piscataway, NJ, USA). Fetal bovine serum (FBS) was purchased from Cultilab S/A (Brazil). Heparin (Hep) from bovine lungs was purchased from INORP Laboratories (Buenos Aires, Argentina). Heparan sulfate (HS) from the bovine pancreas was a kind gift from Dr P. Bianchini (Opocrin Research Laboratories, Modena, Italy). All biotinylated GAGs (Hep and HS) were prepared as previously described (Bouças *et al*. 2008).

Parasites

Vero cells were cultivated in Dulbecco's modified Eagle medium (DMEM; Sigma) and maintained at 37 °C in an atmosphere of 5% CO_2. The cells grown in 150 cm^2 tissue culture flasks were infected for 24 h with 10^7 *T. cruzi* trypomastigotes, clone Dm28c. Free trypomastigotes were harvested from the supernatant of the *T. cruzi*-infected cultures after 4 days of infection.

Triton X-114 extraction and chromatographic procedures

The trypomastigotes were washed 3 times with phosphate-buffered saline (PBS), pH 7·2, and the detergent-soluble proteins were collected using the TX-114 phase separation technique. Briefly, 5×10^{10} trypomastigotes were extracted for 40 min on ice with 2% TX-114 in TBS (150 mM NaCl, 10 mM Tris, pH 7·4), and the soluble proteins were obtained after condensation at 37 °C followed by centrifugation at 12 000 ***g*** for 15 min.

The hydrophobic phase was washed 3 times with PBS and subjected to a sequential affinity-chromatography procedure on 2 columns. First, the hydrophobic proteins were applied to a heparin affinity column, as previously described (Oliveira-Jr *et al*. 2008). After washing, the retained proteins were eluted with 0·5 M NaCl in PBS. The proteins were dialysed in a second equilibrium buffer (10 mM Tris-HCl, pH 7·5), concentrated using a Centriprep YM-10 and passed through an aprotinin-agarose column that was previously equilibrated with the second equilibrium buffer. The column was washed with the second equilibrium buffer, and the retained proteins were eluted using the same buffer with an increased concentration of NaCl (1·5 M).

All of the chromatography washes and elution steps were accompanied by spectrophotometric measurements at 280 nm (Ultrospec 1100 pro; Amersham Biosciences, UK) and were performed in a refrigerated room. The eluted proteins were concentrated by ultra-filtration in Centriprep 10 filters (Millipore Corporation, Bedford, USA), and the protein concentration was determined colourimetrically

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using a BCA-Protein assay kit (PIERCE) with BSA as a standard.

Electrophoresis assay

SDS-PAGE was performed at room temperature using 12% polyacrylamide gels in Laemmli´s buffer (Laemmli, 1970). The samples (10 μg) were dissolved in SDS-PAGE sample buffer (80 mM Tris-HCl, pH 6·8, 2% SDS, 12% glycerol and 0·015% bromophenol blue) that was supplemented with 5% β-mercaptoethanol and then boiled for 5 min. After electrophoresis, the protein bands were revealed using silver staining (Gonçalves *et al*. 1990).

Zymographic assays with gelatin

The proteinase activity was determined using SDS-PAGE with gelatin co-polymerized in the gel (substrate-SDS-PAGE), as previously described (Heussen and Dowdle, 1980; Alves *et al*. 1993). Briefly, the soluble proteins were subjected to electrophoresis under reducing conditions (in sample buffer) using 12% acrylamide gels that were co-polymerized with 0·1% gelatin. Following electrophoresis, the gel was washed (1 h, 25 °C) with 2·5% Triton X-100 and then incubated (16 h, 37 °C) with activation buffers: 10 mM sodium acetate, pH 3·5, for aspartic proteinases; 10 mM Tris–HCl, pH 5·5, containing 1·0 mM of DTT for cysteine proteinases; 10 mM Tris–HCl, pH 7·5, for serine proteinases; and 10 mM Tris–HCl, pH 8·0, for metalloproteinases. The inhibition assays were performed by adding the inhibitor of each proteinase into the activation buffer. The hydrolysis of the gelatin was detected by staining the gels with 0·1% (w/v) amide black, which was prepared in a methanol: acetic acid:water (3:1:6, v/v/v) solution.

Fluorescence microscopy and flow cytometry assays

The culture-derived trypomastigotes (3×10^6 cells) were incubated for 1 h on ice with 20 μg/ml sulfated glycosaminoglycans (GAGs) conjugated with biotin, including heparin (Hep) and heparan sulfate (HS) in DMEM supplemented with 0·5% BSA; Sigma). The incubation step was followed by fixation with 4% paraformaldehyde (PFA) in PBS, pH 7·2. After washing with PBS, the parasites were incubated for 1 h at room temperature with FITC-conjugated streptavidin (1:200) in PBS followed by DNA detection with 10 μg/ml 4,6-diamidino-2-phenylindole dye (DAPI; Sigma). Then, the parasites were settled onto poly-*L*-lysine coated cover slips and mounted with 2·5% 1,4-diazabicyclo-(2,2,2)-octane (DABCO; Sigma) in PBS, pH 7·2, containing 50% glycerol. Also, adhered trypomastigotes were permeabilized with 0·5% Triton X-110 and incubated with anti-4D9 antibody (1:200) for 60 min at 37 °C. After washing, the samples were incubated with TRITC-conjugated anti-mouse IgG (1:200) and the DNA stained with DAPI. The controls were prepared by omitting the biotinylated GAGs and primary antibody. As an additional control, parasites were treated for 5 min at 37 ºC with 500 μg/ml of trypsin prior to biotinylated-GAGs incubation. Images of the samples were acquired using a Zeiss Axio Imager M1 epifluorescence microscope (Zeiss) equipped with an AxioCam HRm (AxioVision Digital Image Processing Software).

Additionally, the samples were excited at 488 nm and quantified using a flow cytometer (FACSCalibur, BD Bioscience, USA) equipped with a 15 mW argon laser emitting at 488 nm. Each experimental population was then mapped using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population ($n = 10\,000$) was analysed for the log of green fluorescence using a single parameter histogram.

Binding assays using surface plasmon resonance (SPR)

The SPR assays were performed using a sensor chip with a carboxyl surface coated with neutravidin (Biocap; Nomadics, USA), as previously described (Oliveira *et al*. 2012; Côrtes *et al*. 2012). Briefly, the chip surface was covered with biotinylated heparin (0·5 μg) and used in the interaction with BSA (0·1–0·001 μg) or whole trypomastigotes (3×10^6 cells). To perform the inhibition assays, the trypomastigotes were pre-incubated for 1 h on ice with different concentrations of sulfated GAGs (0·1 μg–0·001 μg). Prior to the interaction with the sensor chip surface, the trypomastigotes were fixed for 1 h at 4 °C with 1% PFA and washed 3 times by centrifugation (800 ***g***, 10 min, 4 °C) in PBS. The SPR assays were performed at 25 °C with 100 μl of material injected at a flow rate of 10 μl/min. The binding assays were performed in PBS and registered in real time using a sensorgram. The resonance signals of the samples were analysed after subtraction of the resonance unit (RU) values from the reference channel to avoid methodological artifacts. The SPR experiments were conducted in an optical biosensor SensiQ Pioneer instrument (Icx Nomadics, USA), and the data were analysed using Qdat software (Icx Nomadics, USA). The dissociation RU values presented here are representative of the average response between 1,250 and 1,550 sec in all assays.

Statistical analysis

To compare the results, Student's *t*-test was applied, assuming an equal variance between samples. The data matrices were considered statistically distinct when the *P*-value was lower than 0·05.

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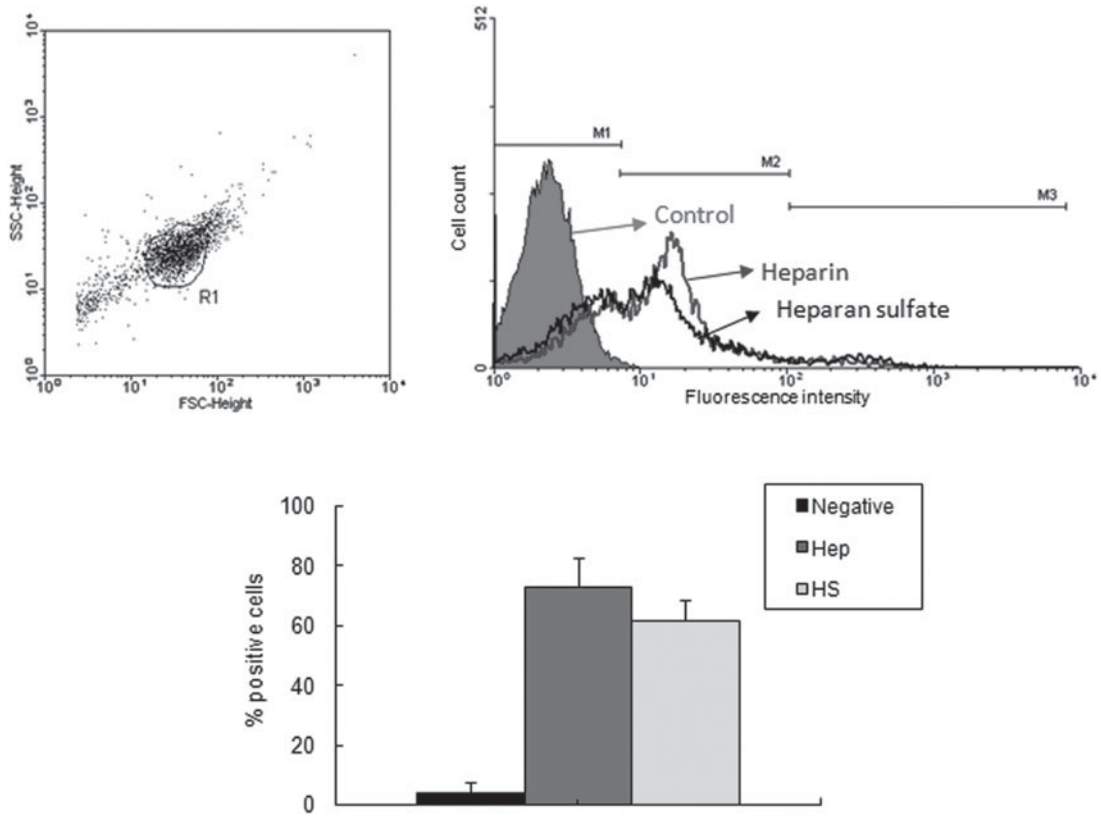

Fig. 1. Flow cytometric analysis showing the expression of heparin-binding proteins in the culture-derived trypomastigotes of *Trypanosoma cruzi* clone Dm28c. Live parasites were incubated with 20 μg/ml biotinylated-heparin and biotinylated-heparan sulfate on ice prior to fixation, and this incubation was followed by detection with streptavidin-FITC. The negative controls (M1) were prepared in the absence of sulfated glycosaminoglycans (GAGs). M2 and M3 represent different levels of fluorescence intensity of *T. cruzi* population. The results are expressed as the mean ± S.D. ($n = 3$).

RESULTS

Following the previous work in which we identified 2 proteins with heparin-binding properties in *T. cruzi* and discussed the ability of these proteins to recognize sulfated glycosaminoglycans (Oliveira-Jr *et al*. 2008), we now extend the study to investigate the expression, spatial distribution and other biochemical features of these proteins in this parasite. Fluorescence assays were performed to evaluate the expression and subcellular localization of these proteins in trypomastigotes. The availability of the HBPs at the surface of cultured-derived trypomastigotes was investigated by pre-incubation of living parasites with 20 μg/ml sulfated glycosaminoglycans (GAGs) conjugated with biotin, including heparin and heparan sulfate (HS), on ice prior to the fixation step, and this incubation was followed by detection using streptavidin-FITC. Flow cytometric analysis revealed a homogeneous profile in the expression of the HBPs on the surface of trypomastigotes that recognize heparin and HS (Fig. 1). The histogram of the fluorescence intensity indicates that 78% and 62% of the parasite population expresses proteins that are able to bind heparin and heparan sulfate, respectively. A small fraction (~5%) of this population displays high levels of expression of HBPs, as detected by higher fluorescence intensity.

Subsequently, the binding specificity of HBPs on the surface of trypomastigotes to GAGs was determined using an SPR analyses with a Biocap sensor chip. Thus, trypomastigotes were incubated or not with different concentrations of heparin and HS and then, passed through a biotinylated heparin-coated sensor chip to determine the parasite attachment onto the chip surface. Detection of association and dissociation events leads to increase and decrease, respectively, of resonance unit (RU value) in the sensograms. Therefore, the HBPs-GAG affinity and stable binding is determined by the dissociation values. In the SPR assay, in which parasites were not submitted to GAG treatment, the dissociation value was 83·3 ± 2·0 RU. Pre-incubation of trypomastigotes with sulfated GAGs led to an inhibition of parasite binding to immobilized heparin, and lower dissociation RU values obtained compared with those of the control assay. The significant residual binding RU was dose independent and reached 15·7 ± 2·2 ($P = 0{\cdot}00058$) for HS and 19·5 ± 2·5

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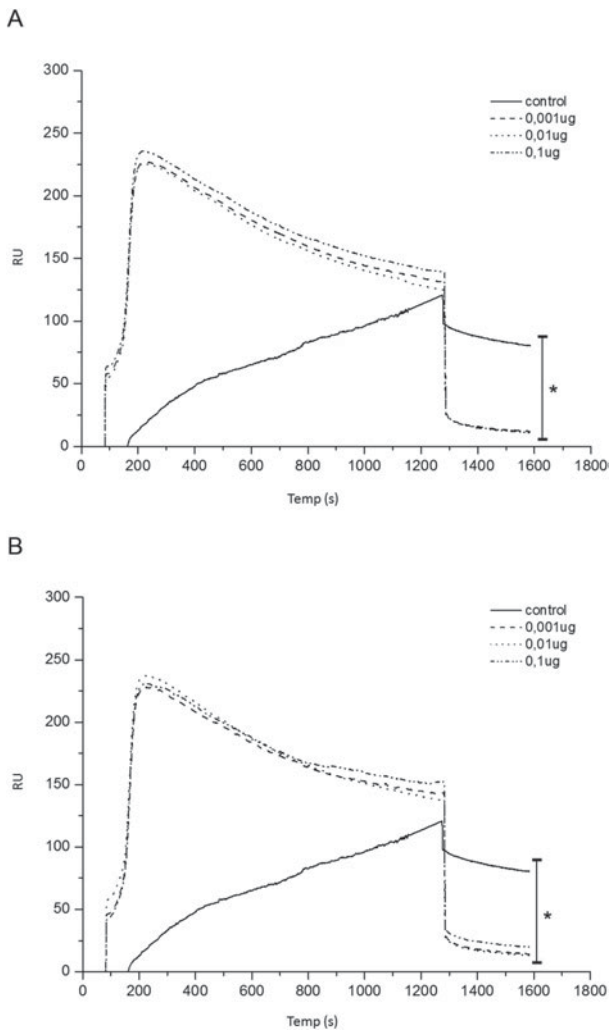

Fig. 2. Analysis of the interaction between trypomastigotes and heparin using surface plasmon resonance. These assays were performed with 10^6 parasites in a final volume of 100 μl at a flow rate of 10 μl/s. The biocap sensor chips were covered with biotinylated heparin, and the parasites were passed over their surface. The inhibition assays were performed following the incubation of the parasites with glycosaminoglycans (GAGs), such as heparin (A) and heparan sulfate (B). The parasites were assayed either without pre-incubation or after pre-incubation with 0·001 μg/ml, 0·01 μg/ml or 0·1 μg/ml GAGs. The interaction assays were performed in PBS, and a significant inhibition was achieved: (*), $P<0·05$. The resonance signals are represented by sensorgrams, which were analysed after subtraction of a reference line using the Qdat software. The data are presented in arbitrary resonance units (RU) and are representative of 4 independent experiments.

($P=0·0013$) for heparin, corresponding to an inhibition of 81·1% and 76·6%, respectively (Fig. 2). Additionally, control assays using 3 different concentrations of BSA were processed in parallel using the same SPR assays, as previously described (Oliveira *et al*. 2012). The dissociation values were consistent with the lack of relevant binding between BSA and heparin (data not shown), demonstrating the specificity of the binding assays performed with trypomastigotes.

Additionally, because the HBPs of trypomastigotes play a key role in the recognition and invasion process in mammalian cells (Ortega-Barria and Pereira, 1991: Calvet *et al*. 2003), we wondered whether these proteins localize to a particular membrane domain in trypomastigotes. Therefore, HBP-biotinylated-GAG binding at the parasite surface was investigated using streptavidin-FITC, and the samples were analysed using fluorescence microscopy. Interestingly, the fluorescence images revealed an intense labelling mainly localized in the flagellar membrane after pre-treatment of the parasites with heparin or HS, whereas no signal could be detected when the GAG treatment was omitted (Fig. 3) or parasites were pre-treated with trypsin (data not shown). To confirm the localization of HBPs at the flagellar domain, we detected a high molecular weight protein located on the cell body side of the flagellar-cell body attachment zone (Mortara *et al*. 2001; Rocha *et al*. 2006). Labelling with monoclonal antibody 4D9, showed staining emerging from the flagellar pocket and continuously along the flagellar attachment zone (FAZ), and revealed a similar pattern of distribution of parasites labelled with biotinylated-GAGs (Fig. 3), supporting the flagellar membrane location of HBPs.

Because many proteins that are involved in the invasion of *T. cruzi* exhibit enzymatic properties, we addressed the question of whether the HBPs have proteinase activity. The strategy of 2 chromatography steps allowed the elution of protein fractions with hydrophobic properties that had previously been concentrated in the detergent Triton X-114. The first affinity chromatography step using a heparin-Sepharose column yielded approximately 0·26 ± 0·04 mg of HBPs, corresponding to 10% of the total hydrophobic protein applied. As a result of this chromatography procedure, we observed a protein profile ranging from 250 kDa to 30 kDa, showing an intense protein band with molecular mass of 65·8 kDa, that is mainly revealed after subsequent elution of proteins bound to the aprotinin column (Fig. 4).

The proteolytic activities of the HBPs against gelatin were determined using SDS-PAGE zymography. This analysis showed a gelatinolytic activity of 70 kDa, 65·8 kDa, 59 kDa and 30·0 kDa over a pH range from 5·5 to 8·0 (Fig. 5), but no activity was detected at pH 3·0 (data not shown). The activity of these bands was sensitive to inhibition by aprotinin at pH 7·5, and no inhibition was evident in the gels incubated with other proteinase inhibitors, suggesting a serine proteinase-like activity (Fig. 5).

In light of the fact that a predominant serine proteinase activity was observed in the zymography assays, a second affinity chromatography step was performed using an aprotinin-agarose column. This chromatography step yielded approximately

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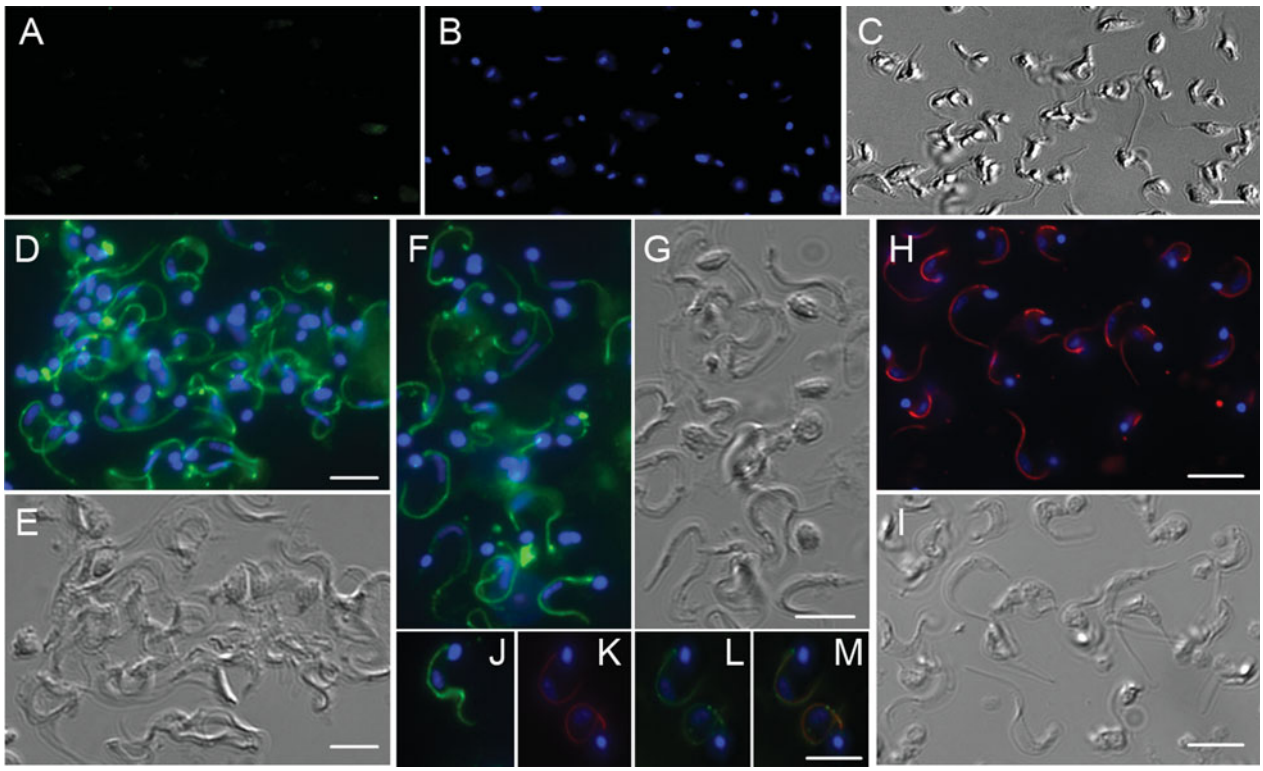

Fig. 3. Spatial distribution of heparin-binding proteins in *Trypanosoma cruzi* trypomastigotes. (A–C) The negative controls were prepared in the absence of biotinylated-GAGs. The HBPs were detected by incubating living trypomastigotes with 20 μg/ml biotinylated-GAGs, such as heparin (D and L) and heparan sulfate (F and J), on ice followed by streptavidin-FITC (green) and DAPI staining (blue). The FAZ region protein was detected with the 4D9 antibody (H and K; red). Note the localization of the HPBs predominantly at the flagellar membrane of the trypomastigotes, which co-localize with the FAZ region protein (K–M; merge M). Differential interference contrast image (DIC; C, E, G and I). Scale Bars = 10 μm.

0·03 ± 0·001 mg of protein, corresponding to 12% of the HBP applied to the column. In general, a major band of 65·8 kDa was identified by SDS-PAGE after silver staining, regardless of the 3 protein bands of 70 kDa, 65·8 kDa and 59 kDa that have always been detected using the zymography assay (Fig. 6). All of the gelatinolytic bands were inhibited by Apo and PMSF inhibitor, but they were not inhibited by other proteinase inhibitors such as Pep A, E-64 and o-phe (Fig. 6).

DISCUSSION

The establishment and persistence of intracellular pathogens in mammalian hosts relies on the recognition and invasion of the target cells. Multiple molecules at the surface of the parasites have been described to mediate cytoadhesion (reviewed by De Souza *et al*. 2010; Sahar *et al*. 2010). Heparin-binding proteins have been identified as potential parasite ligands implicated in the recognition of the host cell surface glycosaminoglycans (Tossavainen *et al*. 2006; Bosetto and Giorgio, 2007; Wu and Wang, 2012). In *Trypanosoma cruzi*, HBPs modulate the adhesion to both insect and mammalian cells and constitute an important protein in the parasite life cycle (Calvet *et al*. 2003; Bambino-Medeiros *et al*. 2011; Oliveira *et al*. 2012). However, the HBPs-GAG interaction is not completely understood. In this article, we have described the expression, subcellular localization and proteolytic activity of trypomastigote HBPs.

Our data demonstrated a high level of HPBs at the surface of trypomastigotes with the ability to bind heparin and HS which corroborate our previous findings on the role of these proteins on parasite invasion (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008). The differential expression of surface proteins implicated in *T. cruzi* invasion appears to be essential to induce signalling pathways that trigger parasite entry. A heterogeneous expression of cruzipain, a surface protein involved in parasite invasion, was observed between *T. cruzi* populations (TCI and TCII), suggesting that the level of cruzipain expression may interfere with the parasite virulence (Fampa *et al*. 2010). Additionally, the balance between cruzipain and chagasin appears to influence the ability of the parasite to invade human smooth muscle cells (Scharfstein and Lima, 2008). Similarly, the differential expression of glycoproteins involved in Ca^{2+} signalling, such as gp82, gp35/50 and gp90, appears to be directly responsible for the ability of

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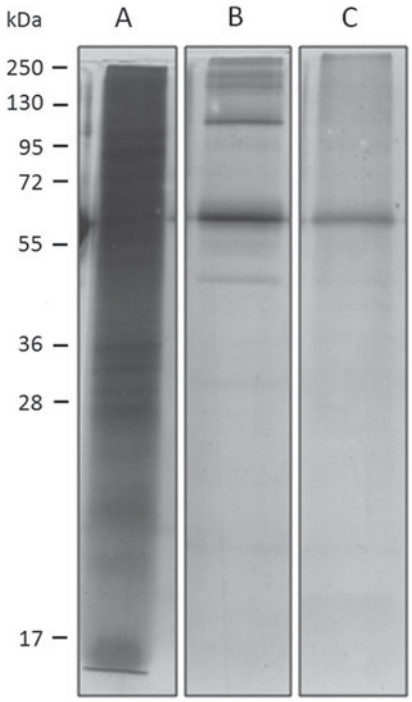

Fig. 4. Denaturing electrophoresis assays of trypomastigote proteins. The hydrophobic protein samples (10 μg) were collected prior to (A) or after separation on heparin-Sepharose (B) and aprotinin-agarose (C) columns, which was a second step after heparin chromatography. The proteins were subsequently submitted to SDS-PAGE and then visualized using silver staining. These results are representative of 4 independent experiments. The molecular mass markers are indicated (kDa).

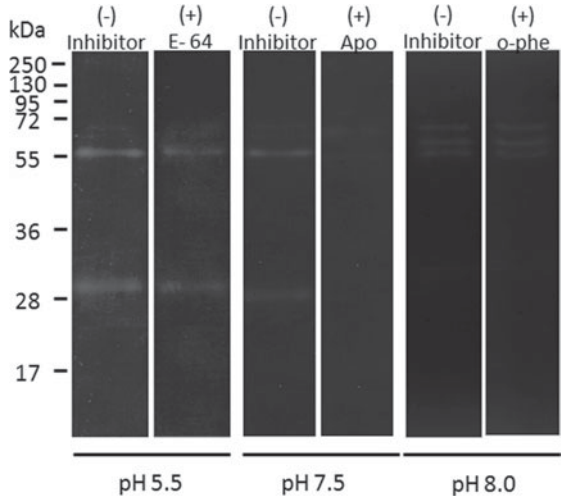

Fig. 5. Proteinase activity in the trypomastigote proteins eluted from a heparin-affinity chromatography column. The proteinase activity was determined by hydrolysis of gelatin that was co-polymerized with polyacrylamide. After electrophoresis of the samples eluted from a heparin-Sepharose column, the gels were incubated with different buffers (pH 5·5, pH 7·5 and pH 8·0) in the absence (−) or presence (+) of specific inhibitors for different classes of proteinases: E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. These results are representative of 4 independent experiments. The molecular mass markers are indicated (kDa).

T. cruzi strains to invade host cells (Ruiz *et al*. 1998), leading to a lysosome-dependent (reviewed by Yoshida, 2006; Yoshida and Cortez, 2008) or actin cytoskeleton-dependent (Ferreira *et al*. 2006) mechanism of invasion. Although the role of HBPs in the parasite invasion of mammalian cells is well known (Calvet *et al*. 2003; Oliveira-Jr *et al*. 2008; Bambino-Medeiros *et al*. 2011), the signalling pathway triggered by this receptor-ligand recognition is still unclear.

One striking feature is the peculiar localization of HBPs mainly at the flagellar membrane. Although most of the studies to date have demonstrated that surface proteins involved in the parasite invasion, including trans-sialidase, gp82, gp35/50 and gp90, are distributed throughout the parasite body (Cordero *et al*. 2008; Penã *et al*. 2009; Buschiazzo *et al*. 2012), this specific subcellular localization of HBPs may facilitate the interaction of the parasite ligand to its host cell surface receptor and other key cellular components to promote parasite invasion. Interestingly, the flagellar membrane domain is enriched with lipid raft microdomains that are involved in protein sorting and signalling (Tyler *et al*. 2009). Therefore, it is possible that the localization of HBPs in the flagellar membrane, an important signalling site, may potentiate the activation of signalling pathways involved in parasite invasion. This field is an interesting area of investigation and will be the focus of future research.

The analysis of HBP-heparin binding using SPR demonstrated that the *T. cruzi* HBPs strongly bind to GAG, thus reinforcing the putative function of these trypomastigote proteins in the life cycle of this parasite. The physicochemical assay effectively confirmed the strength and specificity of the binding of trypomastigote HBPs to heparin immobilized onto the sensor chip surface, as has also been demonstrated for the HBPs of epimastigotes (Oliveira *et al*. 2012). The biosensing surface procedures have been used to elucidate the adhesion between intracellular pathogens and host cells, with a focus on the parasite surface proteins and their interaction with glycosaminoglycans. In this context, the interaction induced by heparin has been assessed in the *Plasmodium falciparum* circumsporozoite protein during the invasion of liver cells (Rathore *et al*. 2001) and in the interaction between the measles virus in SLAM-negative cell lines (Terao-Muto *et al*. 2008). In addition, the localization of HBPs on the surface of *Leishmania (Viannia) braziliensis* promastigotes was identified using a biosensing assay in both flagellar and membrane protein fractions. However, the HBPs from the flagellar membrane have a higher affinity to bind to heparin (Côrtes *et al*. 2012), supporting

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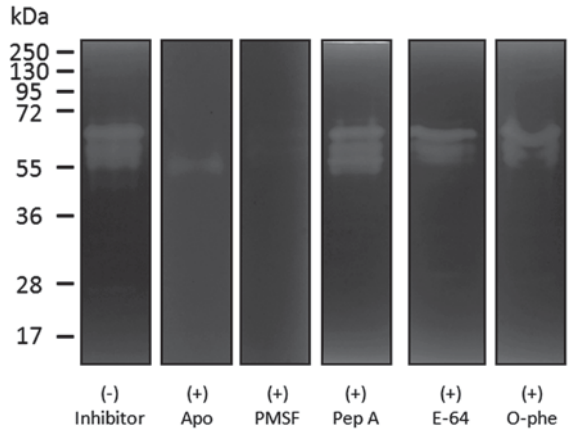

Fig. 6. Proteinase activity in the trypomastigote proteins eluted from an aprotinin-affinity chromatography column. The proteins eluted from the heparin-Sepharose column were submitted to a second chromatography step on an aprotinin-agarose column, and the proteinase activity was measured by hydrolysis of gelatin that was co-polymerized with polyacrylamide. After electrophoresis, the gels were incubated with 10 mM Tris-HCl, pH 7·5, in the absence (−) or presence (+) of specific inhibitors for different classes of proteinases: Pep A, E-64, PMSF and o-phe. The gelatinolytic bands were detected by negative staining with a Coomassie blue solution. These results are representative of 4 independent experiments. The molecular mass markers are indicated (kDa).

the involvement of the parasite flagellum in the adhesion to the host cells (Bates and Rogers, 2004; Bates, 2008).

An important property of HBPs described in this work was its proteinase activity. Our data show that trypomastigote HBPs exhibit serine proteinase activity associated with the 70 kDa, 65·8 kDa and 59 kDa protein bands that were responsible for the hydrolysis profile observed in the zymography assays. Serine proteinases catalyse the cleavage of peptide bonds using a nucleophilic serine residue within the enzyme active site (Hedstrom, 2002). Serine proteases are classified into 2 fundamental families: chymotrypsin-like (trypsin-like) and subtilisin-like (Madala *et al*. 2010). These enzymes have broad substrate specificity including proteins involved in digestion, immune response, blood coagulation and reproduction (Hedstrom, 2002; Antalis *et al*. 2011). Because the proteinase activity of HPBs was sensitive to incubation with inhibitors such as aprotinin and PMSF, it is possible that these HBPs possess the properties of trypsin-like proteinases.

Different classes of proteases such as cysteine proteinases (Rangel *et al*. 1981; Ashall, 1990; McKerrow *et al*. 2006), serine proteinases (Burleigh *et al*. 1997; Bastos *et al*. 2005), metalloproteinases (Lowndes *et al*. 1996; Cuevas *et al*. 2003; Kulkarni *et al*. 2009; Nogueria-Melo *et al*. 2010) and aspartyl proteinases (Pinho *et al*. 2009) have already been described in *T. cruzi*, and most of them have been reported to be implicated in the parasite invasion process. The serine proteinase, the second most studied proteinase in *T. cruzi*, was first described as a 200 kDa protein with esterase and transamidase activities (Bongertz and Hungerer, 1978). Only 20 years after its description, a cytosolic serine endopeptidase of 80 kDa was identified as a requirement for the generation of Ca^{2+} signalling in mammalian cells during parasite entry (Burleigh and Andrews, 1998). Subsequently, the extended substrate binding site of the recombinant 80 kDa oligopeptidase B enzymes (OPBTc) was characterized in *T. cruzi* and *Trypanosoma brucei*, and the specificity of their S3, S2, S1′, S2′ and S3′ subsites was evaluated (Hemerly *et al*. 2003). Recently, it was proposed that the OPBTc enzyme has a dimeric structure and is fully active at temperatures up to 42 °C. OPBTc has a highly stable secondary structure over a broad range of pH values; it undergoes tertiary structural changes at low pH and is less stable under moderate ionic strength conditions (Motta *et al*. 2012). Some molecular, functional and structural properties of the 80 kDa prolyl oligopeptidase (Tc80) were proposed as requirements for parasite entry into mammalian cells (Bastos *et al*. 2005). Additionally, a 75 kDa protein was identified as an excretory product in epimastigotes and may be involved in metacyclogenesis (Silva-Lopez *et al*. 2008). In fact, serine proteinases have been described in many species of parasites, and these proteins are related to interesting biological aspects of the host-parasite interaction, including the invasion properties and degradation of the extracellular matrix (McKerrow *et al*. 2006; Ghosh and Jacobs-Lorena 2011; Meyer-Hoffert and Schröder 2011). The hydrolysis of large substrates, such as fibronectin and native collagen, was also proposed for the 80 kDa *T. brucei* serine proteinase (Bastos *et al*. 2010), allowing the parasite to migrate through tissue barriers. These enzymes, which have been extensively studied in *Leishmania* spp., another protozoan parasite of the family Trypanosomatidae, have a broad molecular mass range of 115 kDa to 45 kDa (Silva-Lopez *et al*. 2004; Morgado-Diaz *et al*. 2005; Silva-Lopez *et al*. 2005; Guedes *et al*. 2007; Silva-Lopez *et al*. 2010) and may play a key role in the parasite life cycle.

Herein, we propose that *T. cruzi* trypomastigotes express 70 kDa, 65·8 kDa and 59·0 kDa serine proteinases on their surfaces, and these enzymes have the ability to bind to GAGs such as heparin and heparan sulfate. Our hypothesis is that the HBPs may act as protagonists of protein cleavages that trigger signalling pathways involved in the penetration of the parasite. The signal transduction pathway involved in this mechanism of the *T. cruzi*-mammalian cell invasion will be the focus of a future investigation.

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