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ORIGINAL PAPER

Activation of *Leishmania* spp. leishporin: evidence that dissociation of an inhibitor not only improves its lipid-binding efficiency but also endows it with the ability to form pores

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Abstract We have previously shown that various species of *Leishmania* produce a lytic activity, which, in *Leishmania amazonensis*, is mediated by a pore-forming cytolysin, called leishporin. It is toxic for macrophages in vitro and optimally active at pH 5.0 to 5.5 and at 37 °C, suggesting that it might be active inside phagolysosomes. Leishporin from both *L. amazonensis* (a-leishporin) and *Leishmania guyanensis* (g-leishporin) can be activated by proteases, suggesting either a limited proteolysis of an inactive precursor or a proteolytic degradation of a non-covalently linked inhibitor. Here, we show that both a- and g-leishporin are also activated in dissociating conditions, indicating the second hypothesis as the correct one. In fact, we further demonstrated that inactive leishporin is non-covalently associated with an inhibitor, possibly more than one oligopeptide that, when removed, renders leishporin hemolytically active. This activation was shown to be the result of both the improvement of leishporin's ability to bind to phospholipids and the emergence of its pore-forming ability. In vitro results demonstrate that leishporin can be released by the parasites, as they evolve in axenic cultures, in an inactive form that can be activated. These results are compatible with our hypothesis that leishporin can be activated in the protease-rich, low pH, and dissociating environment of parasitophorous vacuoles, leading to disruption of both vacuoles and plasma membranes with the release of amastigotes.

Abbreviations

CHAPSO	3[(3-Cholamidopropyl) dimethyl-ammonio]-2-hydroxy-propanesulfonate
DPPC	Dipalmitoylphosphatidyl choline
(g-HCl)	Guanidine-HCl
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_{50}	Inverse of the dilution that caused 50 % of hemolysis
HuE	Human erythrocytes
mExt	Promastigotes membrane detergent-soluble extract
PFP	Pore-forming protein(s)
PBS	Phosphate-buffered saline

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Introduction

The protozoan *Leishmania* spp. is the causative agent of a group of diseases known as leishmaniasis. In humans, infection with *Leishmania* produces a variety of outcomes. Upon infection through the bite of phlebotomine sandflies, *Leishmania* promastigotes invade macrophages where they transform into amastigotes. The amastigotes live and multiply inside parasitophorous vacuoles of macrophages and, eventually, escape the host cells, invading neighboring healthy ones (Handman 1999; Wilson et al. 2005).

Escaping the host cell is a crucial step for causing disease, since the invasion of healthy adjacent cells results in the amplification of the infection. The mechanisms whereby *Leishmania* amastigotes are released from macrophages are not truly understood. It is usually assumed that the burden on the host cell by unrestricted replication of amastigotes forces the cell to burst (Handman 1999; Wilson et al. 2005). Based on previous findings, we have hypothesized that leishporin, a pore-forming cytolysin that we have described in more detail in *Leishmania amazonensis*, but also in other species, is involved in the release of amastigotes from macrophages (Noronha et al. 1994, 1996, 2000; Almeida-Campos et al. 2002). In fact, it is becoming apparent that a variety of exit strategies is governed by the intracellular pathogens, and release of the microorganisms is not simply a consequence of the physical or metabolic burden imposed on the host cell (reviewed by Hybiske and Stephens 2008).

Leishporin lyses target cells at acid and neutral pHs and at temperature ranging from 20–37 °C, but its cytolytic activity is optimal at pH 5.0–5.5 and at 37 °C (Noronha et al. 2000), conditions found inside the parasitophorous vacuole. While characterizing *L. amazonensis* and *Leishmania guyanensis* a- and g-leishporin, respectively, we had verified that these cytolysins can be activated by proteases. This led us to propose that activation may occur either by limited proteolysis of an inactive precursor or by proteolytic degradation of an inhibitor non-covalently bound to the active molecule (Almeida-Campos and Horta 2000). Here, we present evidence that supports the second hypothesis by showing that the activation of both types of leishporin also occurs in dissociating conditions and that, at least for a-leishporin, it is due to the removal of a non-covalently linked inhibitor molecules, possibly a mixture of oligopeptides. We also present evidence that leishporin can be released by the parasites in an inactive form that can be activated, hypothesizing that the same may occur in the acidic, protease-rich, and dissociating environment of the parasitophorous vacuole.

Materials and methods

Parasites

L. amazonensis [PH8 (IFLA/PA/67/PH8)] and *L.* (*Viannia*) *guyanensis* [M4147 (MHOM/BR/75/M4147)] were provided by Dr. M.N. Melo (Departamento de Parasitologia, UFMG, Belo Horizonte, Brazil). Promastigotes were kept in Roswell Park Memorial Institute (RPMI) - 1640 with 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 50-μg/ml gentamycin (Sigma Chemical Co., St. Louis, MO), and 10 % heat-inactivated FBS (GIBCO, Grand Island, NY) (23 °C). Four/five-day cultured parasites were washed with phosphate-buffered saline (PBS), pelleted (1,000×*g*), and kept at −80 °C until required.

Parasite extracts

Promastigotes extracts were prepared as described (Castro-Gomes et al. 2009). Briefly, parasites were resuspended in 50 mM borate buffer for pH 7.0 (2×10^9 cells/ml) and subjected to 5 cycles of freeze-and-thaw. The extracts were centrifuged (1,000×*g*, 10 min, 4 °C), and supernatants were centrifuged (47,000×*g*, 1 h, 4 °C). The membrane-enriched pellet was resuspended to the original volume with borate buffer pH 7.0 containing a non-lytic concentration (0.4 %) of CHAPSO (3[(3-cholamidopropyl) dimethyl-ammonio]-2-hydroxy-propanesulfonate) (Sigma) and kept for 1 h on ice, with occasional agitation. This fraction was centrifuged (100,000×*g*, 1 h, 4 °C), and the supernatant, corresponding to the solubilized membrane molecules, was referred to as detergent-soluble membrane extract (mExt).

Cytolytic activity assay

Cytolytic activity was assessed by a hemolytic assay using human erythrocytes (HuE) (Noronha et al. 2000). Briefly, in 96 round-bottomed well microplates, 5×10^6 HuE in 200 μl of assay buffer (20 mM acetate, 150 mM NaCl, and pH 5.5), were incubated (30 min, 37 °C) with 10 μl serially diluted mExt either before or after different treatments and/or incubations. Microplates were centrifuged (500×*g*, 10 min), and hemolysis was quantified by determining supernatant A_{414}. The percentage of lysis was determined in relation to lysis obtained by incubation of the same number of HuE with 10 μl of 0.25 % Triton X-100. Hemolytic activity was reported either as percentage of lysis or as H_{50} (inverse dilution causing 50 % hemolysis), as calculated by linear regression of percentage of lysis versus dilution curves.

Hemolytic activity in dissociating conditions was investigated using a two-step hemolytic assay (Almeida-Campos and Horta 2000) in which (1) mExt was incubated with HuE in either assay or universal buffer [7 mM (Tris, citric acid, boric acid, and KH2PO4)] (30 min, 4 °C-binding, no lysis) and (2) the cells were washed with PBS and re-incubated in assay buffer (30 min, 37 °C-binding, lysis). The dissociating conditions (125, 250, or 500 mM KCl, guanidine-HCl (g-HCl) or urea), non-neutral pHs, or thermal stress were used at usually the first, but also the second step, or both. Hemolysis was determined as described above. Alternatively, mExt was dialyzed in a 12-kDa mesh membrane in universal buffer pH 7.0 with 250 mM g-HCl (32 h, 4 °C), and their hemolytic activity was determined at different time intervals.

Assay of leishporin release

Parasites from different days of in vitro culture were washed in PBS and re-suspended to 1×10^9 parasites/mL in RPMI-1640 pH 7.2, without pH indicator, with 20 mM HEPES. The samples were incubated for different periods of time at 25 °C. Five microliters of each sample was diluted in 1 mL cold PBS and stained with 100 μL of 0.4 % erythrosine B in PBS to verify viability of the parasites. The rest of the sample was centrifuged for 10 min at 1,000×*g* at 4 °C. The supernatants were collected and 200 μL were assayed as to their hemolytic activity. The pH of the samples was adjusted to pH 5.5 immediately before hemolytic assay and 5×10^6 HuE were added to each sample. The rest was carried out as described above for the cytolytic assay. For some experiments, the parasite pellets were used for mExt preparation as described above. In certain experiments, the supernatants were further centrifuged at 150,000×*g* (Sorvall Ultra Pro 80, DuPont Instruments, Delaware, USA) before cytolytic activity assay. In other experiments, 62.5, 125, 250, or 375 mM g-HCl was added to the hemolytic assay.

Fractionation of mExt and inhibition of hemolytic activity

mExt was treated with 500 mM g-HCl and filtered through a 10-kDa mesh membrane (Amicon®, Millipore, Billerica, MA, USA). The fraction containing >10 kDa molecules was recovered, and the fraction containing <10 kDa molecules was concentrated approximately 15 times in a 3-kDa mesh filter (Centricon®, Millipore). Hemolytic activity of the fractions containing >10 kDa (Ac), 3–10 kDa (I), or <3 kDa molecules was assayed as described above. The ability of fraction I to inhibit the hemolytic activity of fraction A was tested by mixing both at different proportions before the hemolytic assay. Alternatively, the test was made in the two-step hemolytic assay with the mixing made at the Ac/I ratio of 1:10 during either the binding or the pore-formation, or both steps. Data represent the mean of hemolytic activity (H_{50}) of duplicates ± standard deviation. Fraction I was further submitted either to a reverse phase (C18) or to a gel filtration (Superdex TM 30, Prep grade, HiLoad TM 16/60) chromatography using HPLC. Fractions obtained were tested for their leishporin inhibitory activity.

Gel electrophoresis and elution of bands

Molecules were separated by either 10 % SDS-PAGE or tricine 16 % SDS-PAGE. All samples were diluted into sample buffer containing 5 % β-mercaptoethanol and boiled for 3 min. Tricine-SDS gels were sliced at every 2 mm and molecules from each slice were eluted in borate buffer pH 7.0 (overnight, 25 °C). SDS was removed by dialysis and samples were concentrated. Their ability to inhibit hemolytic activity of the >10 kDa fraction was tested by hemolytic assay.

Liposomes and binding assays

Multilamellar vesicles of L-α-dipalmitoyl-phosphatidylcholine (DPPC) (Sigma) and cholesterol (Sigma) were prepared at 1:0.8 molar ratio in PBS to a final concentration of 45 mM, according to the lipid film hydration method (Bangham et al. 1965).

mExt was incubated with different volumes of a liposome suspension for different periods of time (4 °C). Hemolytic activity of supernatant, after centrifugation (10 min, 15.000×*g*) of liposomes suspension, was tested. This supernatant was treated either with proteinase K (10 mg/mL) or g-HCl (250 mM), tested for hemolytic activity, re-incubated with liposomes, and tested again for hemolytic activity.

In order to determine whether active and inactive forms of leishporin binds to lipids with different strengths, mExt was incubated with a pre-standardized amount of liposomes, with no excess whatsoever, just enough to remove active, but not inactive, a-leishporin. After sedimentation of liposomes, 500 mM g-HCl were added to the hemolytically inactive mExt to activate leishporin before incubation with HuE for 1 to 10 min at 4 °C. After these periods of time, HuE were further incubated for 30 min at 37 °C. Alternatively, the hemolytically inactive mExt was incubated with HuE for the same periods of time at 4 °C. Cells were extensively washed to remove unbound mExt molecules and then incubated for

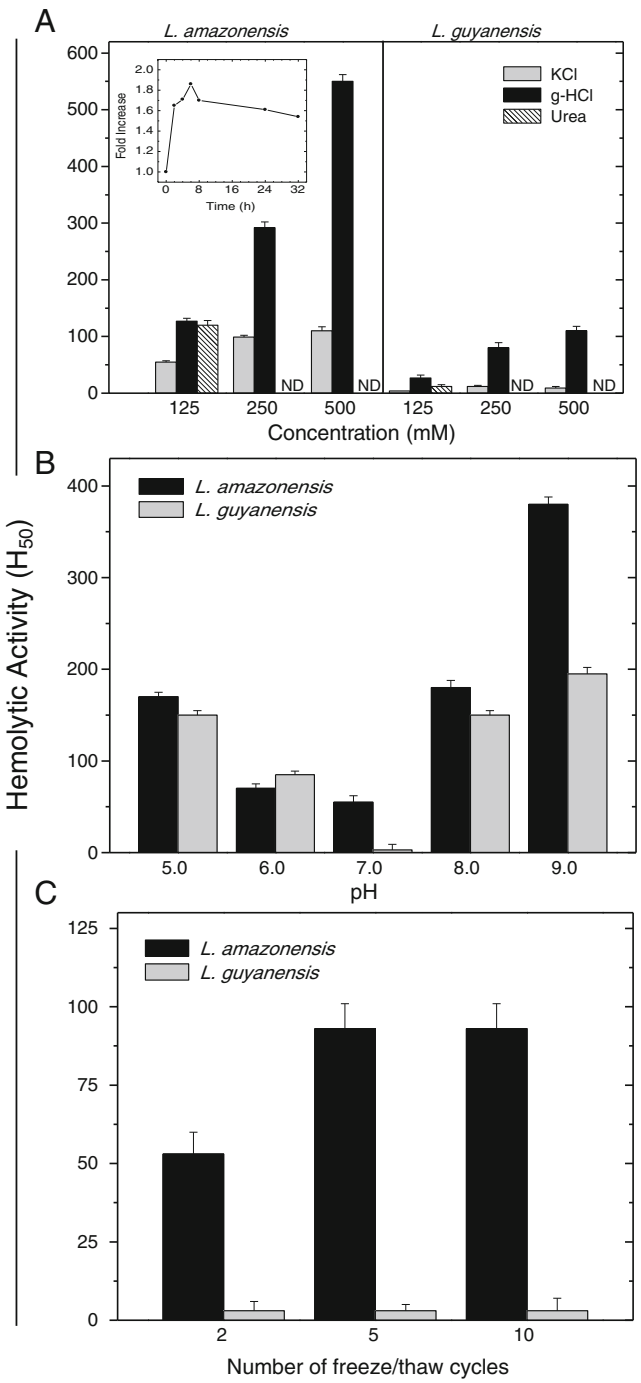

Fig. 1 Generation of hemolytic activity in mExt by dissociating conditions. *L. amazonensis* or *L. guyanensis* mExt were incubated with HuE (30 min, 4 °C) in assay buffer in the presence of KCl, g-HCl, or urea at the indicated concentrations (**a**) or in universal buffer at the indicated pHs (**b**). After extensive washing, HuE were incubated in assay buffer (30 min, 37 °C), centrifuged, and the hemolytic activity of the supernatants determined. HuE are not lysed in the absence of mExt, in any of the dissociating conditions regardless of pH or temperature. Hemolytic activity of freshly prepared *L. amazonensis* and *L. guyanensis* mExt was 55 and 0, respectively. *ND* not done. Alternatively, mExt were submitted to increasing number of freeze-and-thaw cycles and assayed for hemolytic activity (**c**). Data represent the mean of hemolytic activity (H_{50}) of duplicates ± standard deviation. Inset *Figure*—generation of hemolytic activity in mExt during dialysis in g-HCl *L. amazonensis* mExt were dialyzed in a 12-kDa mesh membrane in universal buffer pH 7.0 containing 250 mM g-HCl for 32 h. The hemolytic activity mExt was determined at the indicated times. Data represent the fold increase of hemolytic activity

30 min at 37 °C in the presence of 500 mM g-HCl. Hemolysis was determined for both types of assays. Hemolysis was also determined at periods of time earlier than 30 min at 37 °C after the incubation period of 8 min at 4 °C for both types of assay.

All experiments in this study were carried out in duplicate, and the results shown are representative of at least three independent experiments.

Results

Leishporin is activated in dissociating conditions

Because a-leishporin is better activated by non-specific than by specific proteases (Almeida-Campos and Horta, 2000), we have postulated that it is activated by proteolysis of a non-covalently linked peptide inhibitor, and not by limited proteolysis. If so, activation could also be achieved by non-proteolytic dissociation of the putative inhibitor. To test this hypothesis, we initially investigated whether dissociating conditions (dissociating agents, non-neutral pHs, and thermal stress) could generate hemolytic activity in mExt, to where leishporin is localized.

L. amazonensis or *L. guyanensis* mExt were incubated with HuE (4 °C) in the presence of KCl, g-HCl, or urea at various concentrations (Fig. 1a) or at pHs 5.0–9.0 (Fig. 1b). After removing unbound mExt molecules, cells were incubated in the absence of the dissociating agents (pH 5.0, 37 °C) and hemolysis was determined. In the presence of dissociating agents (Fig. 1a) or non-neutral pHs (Fig. 1b), both *L. amazonensis* and *L. guyanensis* mExt have their hemolytic activity increased, in a concentration- or pH-dependent manner, as compared to untreated controls (see legend) or to pH 7.0, respectively. Final hemolytic activities were always much higher for a-leishporin than for g-leishporin. g-HCl was the most potent in generating hemolytic activity in mExt, increasing its original hemolytic activity in up to tenfold for *L. amazonensis*. Urea, at concentrations >125 mM, caused hemolysis by itself and was not used. When *L. amazonensis* mExt is dialyzed in a 12-kDa mesh membrane in the presence of g-HCl and its hemolytic activity is periodically determined after removal of g-HCl, its activity increases and reaches a peak in 6 h, which is maintained during the period observed (Fig. 1a insert). Because further results (Fig. 2a) refute that the dissociation is irreversible, this suggests that the putative inhibitor is <12 kDa. Non-neutral pHs also caused increases

in *L. amazonensis* and *L. guyanensis* mExt activities, reaching up to sevenfold for *L. amazonensis* (pH 9.0). At pHs 5.0, 6.0, and 8.0, activation of both types of leishporin are comparable (Fig. 1b). It is unlikely that the treatments interfere in erythrocytes susceptibility to lysis, since the incubation of HuE in all conditions used in the experiments, previously to incubation with untreated mExt, did not show any increase in hemolysis (results not shown). Thermal stress induced by

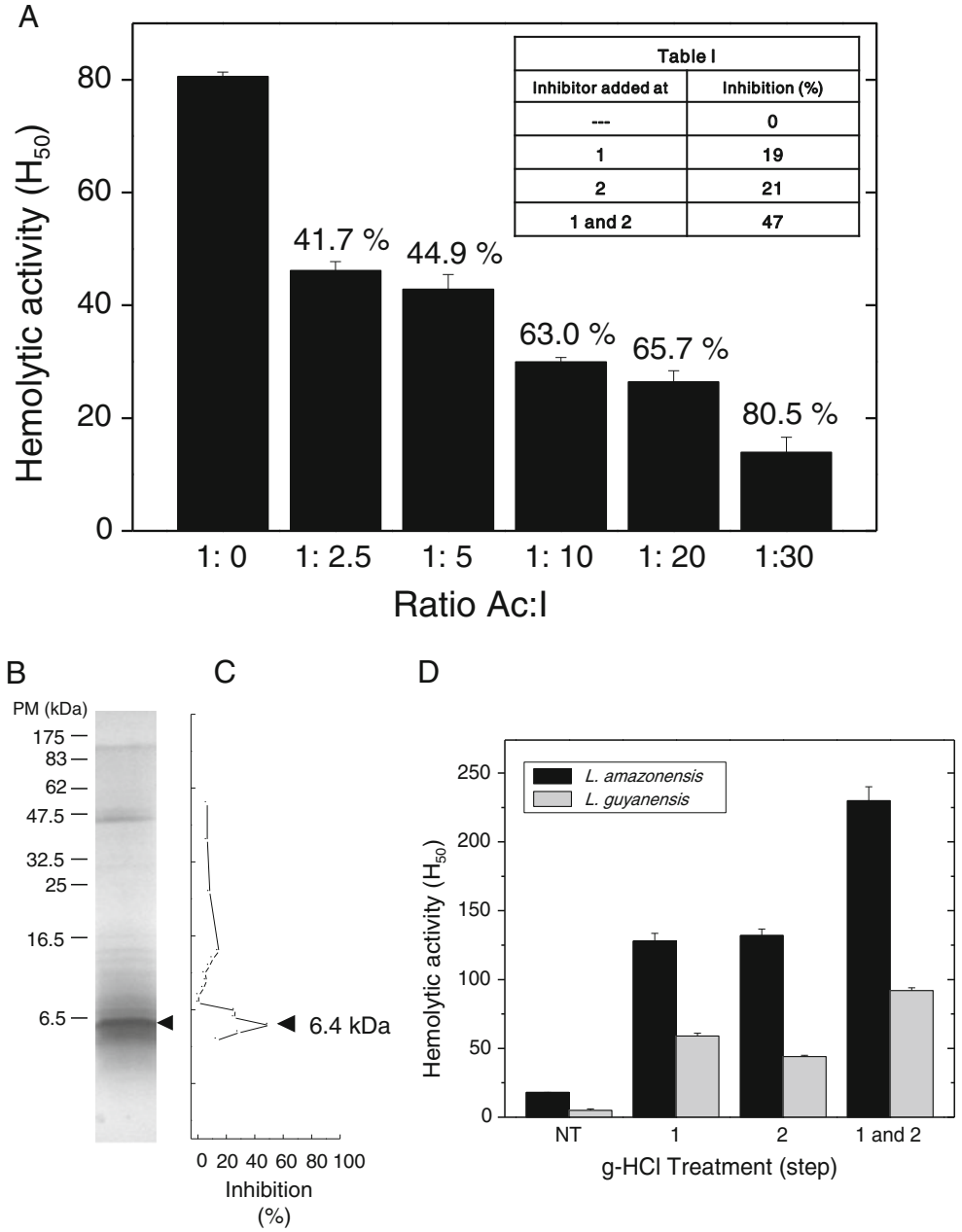

Fig. 2 Separation of *L. amazonensis* mExt into a hemolytically active fraction and an inhibitory fraction. *L.amazonensis* mExt was treated with 500 mM g-HCl and filtered through a 10-kDa mesh membrane. The hemolytically active fraction containing >10 kDa molecules (*Ac*), was reconstituted with the indicated proportions of the hemolytically inactive fraction containing the <10 kDa molecules (*I*), previously concentrated in a 3-kDa mesh filter. Data represent the mean of hemolytic activity (H_{50}) of duplicates ± standard deviation (**a**). Inset *Table I*—inhibition at different steps of pore formation—the hemolytically active fraction (*Ac*) was reconstituted with the inhibitory fraction (*I*) at the ratio Ac/I=1:10 at the step of binding (30 min, 4 °C) (*1*) and at the step of pore-formation (30 min, 37 °C) (*2*) or at both steps. Addition of *I* at step 1 was followed by washings of erythrocytes with PBS before step 2. *L. amazonensis* mExt fraction *Ac* was separated by Tricine SDS-PAGE (**b**). A duplicate gel was sliced at every 2 mm and the molecules in each slice were eluted. The ability of each eluate to inhibit hemolytic activity of the fraction *Ac* was tested by hemolytic assay (**c**). In another experiment, *L. amazonensis* or *L. guyanensis* mExt were incubated with HuE in assay buffer in the presence of 250 mM g-HCl at the step of binding (30 min, 4 °C) (*1*), at the step of pore-formation (30 min, 37 °C) (*2*), or at both steps (**d**). Data on hemolytic activity represent the mean of hemolytic activity (H_{50}) of duplicates ± standard deviation. *NT* not treated

several cycles of freeze-and-thaw also causes an increase in the hemolytic activity of *L. amazonensis*, but not of *L. guyanensis* mExt (Fig. 1c).

Together, these results support our hypothesis that leishporin can be activated by the dissociation of a non-covalently bound inhibitory molecule.

L. amazonensis mExt can be fractionated into hemolytically active and inhibitory fractions

If a-leishporin is activated by the dissociation of a non-covalently bound <12 kDa molecule (Fig. 1a, insert), then it would be possible to separate the two molecules through molecular sieving. To verify this possibility, g-HCl-treated *L. amazonensis* mExt was filtered through a 10-kDa mesh membrane and the flow-through fraction was further filtered through a 3-kDa mesh membrane. The generated hemolytic activity was recovered in the >10 kDa fraction (Ac) (Fig. 2a), and no hemolytic activity was detected in the 3–10 kDa or in the <3 kDa flow-through fraction (data not shown). When the 3–10 kDa fraction (I) (Fig. 2a) is added back to the active fraction (Ac), the hemolytic activity is inhibited in a concentration-dependent fashion. This demonstrates that the 3–10 kDa fraction holds an inhibitor of a-leishporin activity.

At least two distinct events are required for pore formation by a-leishporin: (1) binding to cell phospholipids, temperature-independent, occuring even at 4 °C and (2) pore-formation itself, temperature-dependent (probably insertion/oligomerization of subunits into lipid bilayer), occurring only at higher temperatures (optimal, 37 °C) (Noronha et al. 2000; Almeida-Campos and Horta 2000; Castro-Gomes et al. 2009). To investigate at which step a-leishporin inhibitor acts, we carried out the two-step hemolytic assay, adding the inhibitory fraction at either one of each step or both. We observed that the inhibitor interferes with both pore-formation steps, inhibiting about 20 % of the hemolytic activity if added during one of the steps and almost 50 % if added at both (Fig. 2a, inset Table I). This shows that fraction I inhibits the binding and the subsequent step(s) of pore formation. The additive effect suggests that not all molecules were inhibited at each step. These results also show that the dissociation of the molecules is reversible.

a-Leishporin is activated by the removal of a <6.5 kDa molecule

In an attempt to identify a-leishporin inhibitor, we separated the components of the fraction I using tricine SDS-PAGE. Several fainter bands and a major band of 6.4 kDa are seen (Fig. 2b). When eluates of each 2 mm slice of duplicate gels are tested for inhibitory ability over the >10 kDa fraction, all inhibitory activity of fraction I is associated with the 6.4 kDa band (Fig. 2c). These results suggest that a-leishporin precursor is a molecule non-covalently associated with a <6.5 kDa inhibitor. Several attempts were made to purify a-leishporin inhibitor. We have submitted fraction I to a reverse phase (C18) or to a gel filtration (Superdex TM 30, Prep grade, HiLoad TM 16/60) chromatography using HPLC. A number of fractions were obtained by both methods but the inhibitory activity could not be recovered in any of the chromatographic fractions, even using high concentrations of parasite extracts (results not shown). One possibility is that more than one molecule could be acting together as inhibitor, but not when separated.

Both active and inactive leishporin bind to target cell membrane lipids

To investigate whether the dissociation of the inhibitor is necessary for leishporin binding or for pore-formation, we carried out the two-step hemolytic assay, adding g-HCl at either one of each steps or both. We observed that the hemolytic activities of *L. amazonensis* or *L. guyanensis* mExt generated are similar regardless of the step at which g-HCl is added (Fig. 2d). The generated activity may achieve two- to tenfold the original activity, depending on the experiment and on the *Leishmania* species. These results suggest that both a- and g-leishporin can bind to cell membrane irrespective to their form, but that lysis only occurs after dissociation from the inhibitor. For both species of

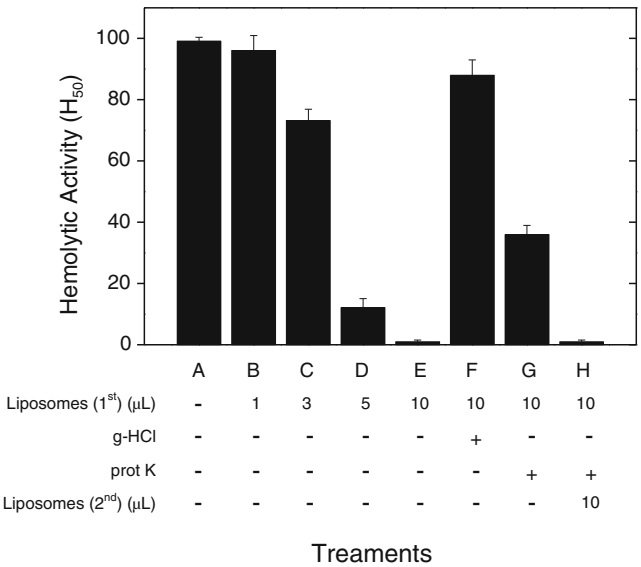

Fig. 3 Binding of leishporin to membrane lipids. *L. amazonensis* mExt was first incubated (*B* to *E*) or not (*A*) with different volumes of a liposome suspension. After centrifugation, the hemolytic activity of supernatant was tested. The hemolytically inactive liposome-treated mExt (*E*) were further treated with g-HCl (250 mM) (*F*) or proteinase K (10 µg/mL) (*G*), and the latter were incubated a second time with 10 µL liposomes suspension (*H*). Data represent the mean of hemolytic activity (H_{50}) of duplicates ± standard deviation

Leishmania, the hemolytic activity generated is usually twice as high when g-HCl is added at both steps than when added at one of them. This additive effect suggests that activation of all molecules may not be completed in 30 min of each step.

The interaction with cell membrane is faster for active than for inactive a-leishporin

a-leishporin is a cholesterol-independent cytolysin that binds directly to target membrane phospholipids (Castro-Gomes et al. 2009). To compare the phospholipid-binding abilities of active and inactive a-leishporin, we have incubated *L. amazonensis* mExt with increasing amounts of liposomes (4 °C). Liposomes were able to remove all mExt hemolytic activity in a concentration-dependent manner, showing that a-leishporin is able to bind to the lipid (Fig. 3). However, if the liposome-treated hemolytically inactive mExt is further treated with proteinase K or g-HCl, it regains activity, demonstrating that the liposomes did not remove all inactive a-leishporin. The newly generated hemolytic activity can also be removed with liposomes in a concentration-dependent manner (shown only for 10 μL) (Fig. 3). This suggests that either active a-leishporin binds to phospholipids with higher strength than inactive form, or the latter prevails over the former in freshly prepared mExt.

To verify these possibilities, we used mExt previously depleted of only active a-leishporin. This inactive mExt was incubated with HuE for varying times, either before or after treatment with g-HCl (4 °C). The resulting hemolysis (30 min, 37 °C) shows that active a-leishporin (dissociated from its inhibitor before incubation with HuE) binds to the cells faster than its inactive form (rendered active after cell binding). This is most clearly observed from 2–8 min of HuE contact (Fig. 4a). The high hemolysis obtained by 8 min incubation with active a-leishporin (Fig. 4a) is observed as early as 5 min (37 °C), whereas a-leishporin activated after HuE binding only attains the same level of activity at 60 min, corroborating that, during the same time, much less inactive than active a-leishporin had bound to HuE (Fig. 4b).

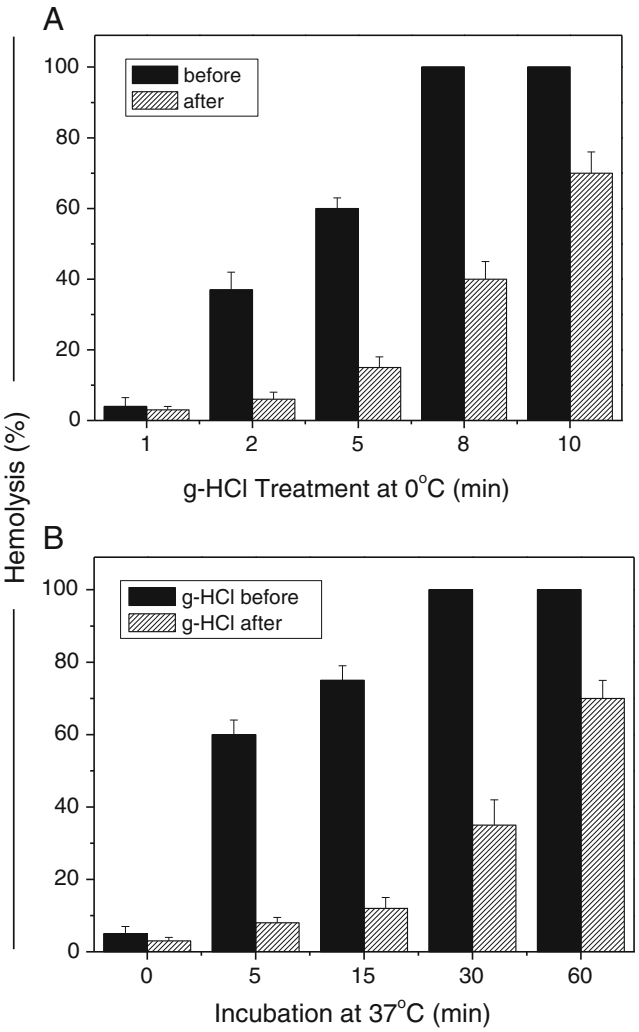

Fig. 4 Active leishporin binds to membrane lipids faster than the inactive molecule. *L. amazonensis* mExt was incubated with liposomes in a concentration known to remove only active a-leishporin. The hemolytically inactive mExt obtained was then treated with 500 mM g-HCl (4 °C) to activate leishporin *before* incubation with HuE for the indicated periods of time. After these periods of time, HuE were further incubated for 30 min at 37 °C. Alternatively, the hemolytically inactive mExt was incubated with HuE for the same periods of time at 4 °C. Cells were extensively washed to remove unbound mExt molecules and then incubated at 37 °C in the presence of 500 mM g-HCl to activate leishporin *after* binding to HuE. After 30 min, hemolysis was determined for both types of assay (**a**). Hemolysis was also determined at the indicated periods of time, earlier than 30 min, at 37 °C, after the 8-min incubation at 4 °C in both situations described above (**b**)

Release of soluble leishporin by promastigotes

To investigate the possibility that inside the macrophage parasitophorous vacuole leishporin could be released by the parasite, we examined whether leishporin can be released by the parasite in vitro. Promastigotes were incubated in culture medium without FBS or pH indicators, and both parasite pellets and supernatants were assayed for hemolytic activity. Figure 5a shows that in a 30-min incubation, promastigotes' supernatants already presented some hemolytic activity that peaked in 1.5 h but vanished by 2 h. The hemolytic activity remained in the supernatants even after further centrifugation at 150,000×*g* for 1 h (not shown), demonstrating that leishporin was released by promastigotes in a soluble form.

In vitro, axenic promastigotes cultures show an exponential growth from 2 to 4 days, followed by a stationary phase of growth (Fig. 5b). We then carried out the above assay using promastigotes of different days of in vitro culture, this time investigating the hemolytic activity of both supernatants and parasite pellets. We observed that during the

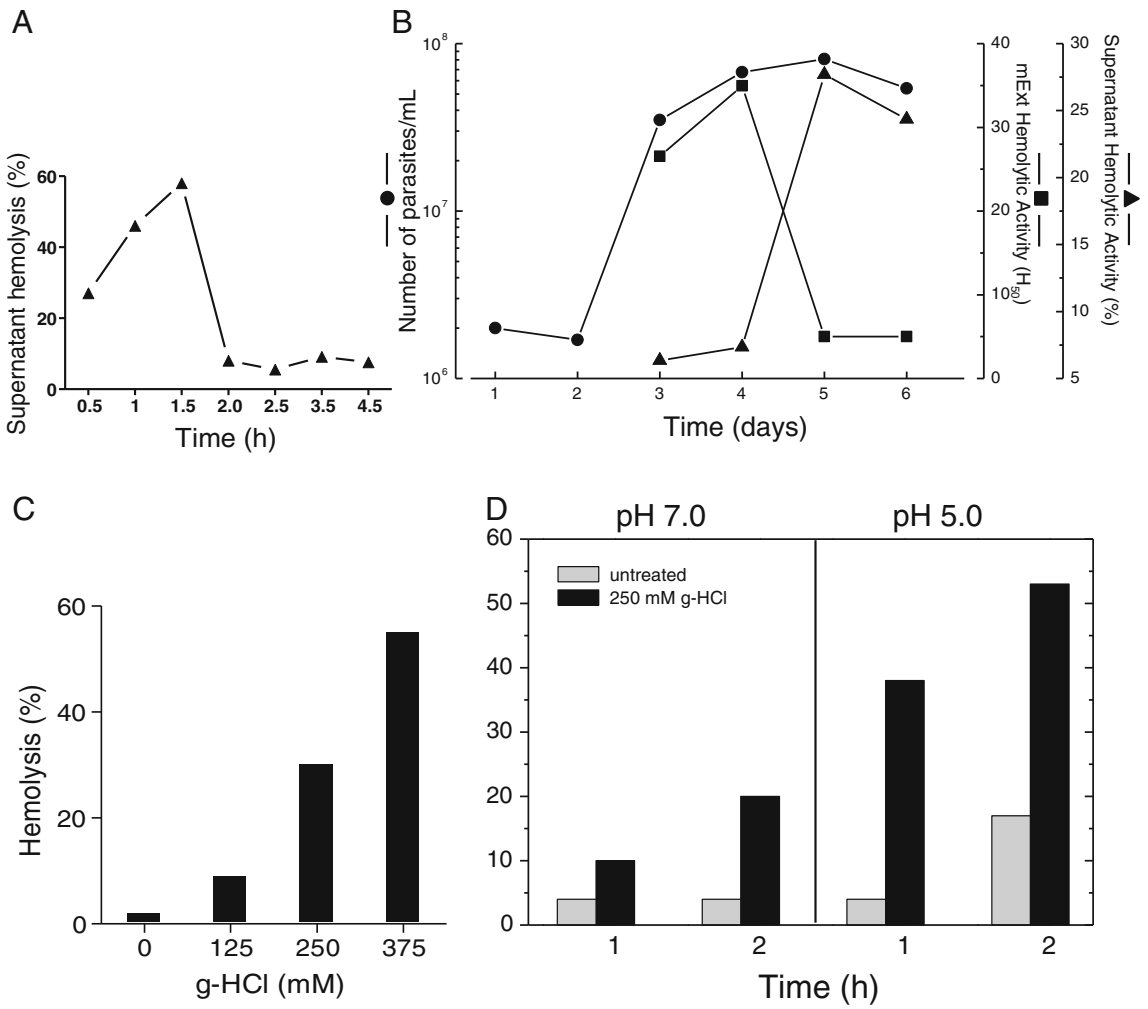

Fig. 5 Release of leishporin by promastigotes and its activation with g-HCl. Promastigotes were washed in PBS and re-suspended to the 1×10^9 parasites/mL in RPMI-1640 pH 7.2, without pH indicator. The samples were incubated at 25 °C for the indicated periods of time (**a**, **d**) or for 2 h (**b**, **c**) and centrifuged for 10 min at 1,000×*g* at 4 °C. Supernatants had their pH adjusted to 5.5 before assayed for their hemolytic activity. For some experiments, pellets were used to prepare mExt before assaying their hemolytic activity (**b**). In some experiments, g-HCl was added to the supernatants at the indicated concentrations before being assayed for hemolytic activity (**c**, **d**)

exponential phase to the beginning of the stationary phase, there is no release of leishporin by the promastigotes. Release of leishporin occurs during the stationary phase (fifth and sixth days) (Fig. 5b). Conversely, the hemolytic activity of the parasites' mExt gradually increases during the log phase and disappears in the stationary phase, just as leishporin is released.

In order to investigate whether leishporin was also being released in its inactive form, the supernatants of promastigotes incubated for 2 h in pH 7.0 were treated with increasing concentrations of g-HCl. Figure 5c shows that whereas untreated supernatants are not hemolytically active, they become hemolytic when treated with g-HCl, in a concentration-dependent manner (Fig. 5c). Release of inactive leishporin is more efficient in pH 5.0 than in pH 7.0. At pH 7.0, only inactive leishporin is detected in the supernatants by 1 or 2 h, which can be activated by g-HCl (Fig. 5d). However, g-HCl generates much more hemolytic activity in hemolytically inactive supernatants obtained by 1 h incubation of parasites at pH 5.0. After 2 h, the release of inactive leishporin may not be higher than in 1 h. It is possible that the higher activity achieved in 2 h with g-HCl may be due to the previous activation of leishporin, which can be seen in untreated mExt, by the acidic pH itself (as shown in Fig. 1b) after its release as inactive form.

Discussion

Pore-forming proteins (PFP) or peptides are molecules that can destroy cells or affect their function. PFPs from protozoan pathogens have been reported to play an important role

in pathogenesis (Almeida-Campos et al. 2002; Andrews and Portnoy 1994; Horta 1997; Roiko and Carruthers 2009). A recurrent mechanism to impede pores to be formed on the pathogen itself is the regulation of the PFP lytic activity by the expression of inactive molecules that can be activated when needed (Abrami et al. 1998; Leippe et al. 2005).

We have previously shown that a- and g-leishporin need to be activated to lyse cells (Almeida-Campos and Horta 2000). Because the cytolysins could be proteolitically activated, two hypotheses were drawn from our data: (1) limited proteolysis of an inactive precursor and (2) proteolytic degradation of an inhibitor. The fact that proteinase K and Pronase®, two broad-spectrum proteases, were the most effective activators that inclined us to neglect the first and consider the second hypothesis as the probable mechanism activation. In fact, the first evidence that inactive forms of leishporin are non-covalently bound to an inhibitory molecule was the increase in the hemolytic activity of *L. amazonensis* and *L. guyanensis* mExt submitted to dissociating conditions (Fig. 1). Contrasting with a-leishporin, g-leishporin is inactive in freshly prepared mExt, is only activated in the presence of proteases (Almeida-Campos and Horta 2000) or dissociating conditions, and is less efficiently activated (Fig. 1). Interestingly, *L. amazonensis*, but not *L. guyanensis*, soluble extracts contain serine-proteases that activate both a- and g-leishporin (Almeida-Campos and Horta 2000). It is probable that the active forms of a-leishporin are generated only during the in vitro preparation of the extract and, like g-leishporin, might not be found in live parasites. Our results show that dissociating conditions activate leishporin. The differences between the two species of *Leishmania* are in agreement with our previous findings (Almeida-Campos and Horta 2000).

The above results suggested that the removal of a non-covalently linked molecule could be the mechanism of leishporin activation. This was confirmed by the demonstration that two discrete fractions, one (>10 kDa) with a-leishporin activity and one (3–10 kDa) with a-leishporin inhibitory ability (Fig. 2a), were separated by molecular sieving after g-HCl treatment and that a 6.4-kDa band held the a-leishporin inhibitory ability (Fig. 2b, c). Therefore, and because a-leishporin is activated by serine-proteases (Almeida-Campos and Horta 2000), we speculate that a-leishporin is primarily expressed as a precursor molecule non-covalently associated with a <6.5-kDa oligopeptide inhibitor. This bond is reversible, since the inhibitor can be added back to mExt, abolishing leishporin activity (Fig. 2a). If this is also true for g-leishporin, our present (Figs. 1 and 2d) and past results (Almeida-Campos and Horta 2000) suggest that its inhibitor might be more tightly bound and resistant to proteases than that of a-leishporin.

There are at least two distinct steps in the lysis mediated by a-leishporin: (1) binding to the target membrane, which occurs even at 4 °C and, by itself, does not result in lysis and (2) lysis itself, which only occurs when temperature is raised (possibly insertion and/or polymerization) (Almeida-Campos et al. 2002). Leishporin inhibitor seems to prevent lysis by hindering either the cytolysin binding or the pore formation (inset Table I of Fig. 2). Although both inactive and active a- and g-leishporin can bind to cell membrane, causing the same degree of hemolysis (Fig. 2d), active a-leishporin binds faster than its inactive form (Fig. 4).

a-Leishporin binds directly to membrane phospholipids and DPPC liposomes. DPPC liposomes are readily lysed by promastigotes mExt, while removing its cytolytic activity (Castro-Gomes et al. 2009). Interestingly, mExt rendered inactive by a short treatment with liposomes can be further activated, demonstrating that inactive forms of leishporin were left. This is supported by the removal of the newly generated hemolytic activity by fresh liposomes (Fig. 3). However, if mExt is incubated for longer periods or with higher amount of liposomes, activation is no longer possible (results not shown), indicating that liposomes can bind to the inactive forms. This is corroborated by the activation of inactive forms after their binding to the erythrocytes (Fig. 2d). Two non-exclusive possibilities can be considered: (1) abundance of inactive over active a-leishporin in freshly prepared mExt (higher amount of liposomes or longer period to be removed) and (2) higher lipid-binding strength of active over inactive a-leishporin (selectively removed). The second hypothesis is supported by the striking decrease of the time required for the cytolysin to bind to the cells, and consequently for hemolysis, if dissociation is carried out previously to, as compared to subsequently to, incubation with erythrocytes (Fig. 4). Although inactive leishporin can bind to cell membrane, lysis only occurs in the presence of g-HCl (Fig. 2d), showing that the dissociation of the two molecules also turns inactive bound a-leishporin into a pore-former.

Together, our results indicate that (1) leishporin is expressed in promastigotes as inactive non-covalently linked heteromers, containing a moiety that acts as inhibitor of its pore-forming activity, which, in the case of a-leishporin, seems to be molecules <6.5 kDa and (2) the removal of the inhibitor from a-leishporin leads to changes that not only make it bind faster to its phospholipid receptor but also renders it able to form pores.

We showed here that promastigotes can release leishporin during incubation in vitro in culture medium (Fig. 5a). During in vitro growth of promastigotes, the release of leishporin is highest in stationary phase parasites, in an inverse correlation with the hemolytic activity retained in the parasite cells, suggesting that, as parasites evolve, they release leishporin (Fig. 5b). Moreover, we showed that leishporin is released in an inactive form that can be further activated (Fig. 5c) and that this release is more effective in pH 5.0 (Fig. 5d).

The present results are compatible with the view that, in vivo, inside macrophages, amastigotes could release inactive

leishporin which, in the protease-rich and low pH/dissociating environment of the parasitophorous vacuole, would dissociate its inhibitor, gaining its pore-forming activity. Our hypothesis is that leishporin-mediated rupture of the phagolysosome and, subsequently, of the plasma membrane, would release infective amastigotes, amplifying the infection.

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