Macrophage Damage by *Leishmania amazonensis* Cytolysis: Evidence of Pore Formation on Cell Membrane

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We have previously shown that both promastigotes and amastigotes of *Leishmania amazonensis* contain a lytic protein that damages erythrocytes and nucleated cells, including macrophages (F. S. M. Noronha, F. J. Ramalho-Pinto, and M. F. Horta, Infect. Immun. 64:3975–3982, 1996). Using the patch-clamp technique, we show here that cell damage by parasite extracts is mediated by the formation of nonselective pores on the target membrane. This demonstrates that *L. amazonensis* cytolsin is a pore-forming protein (PFP), here named leishporin. We show that the diameters of the pores formed by parasite extracts are heterogeneous, varying from ~1.6 to >6.1 nm according to cytolsin concentration or time. We also show that pore formation involves the binding of the PFP to the target cell membrane, a temperature-independent event that is necessary but not sufficient to lyse cells. This is followed by a temperature-dependent step that triggers lysis, probably the insertion and the polymerization of protein subunits in the lipid bilayer. We provide evidence that suggests that polymerization of single subunits must occur for pore formation. We show, in addition, that *L. amazonensis* expresses molecules antigenically homologous to other PFPS.

Leishmaniasis comprises a spectrum of diseases whose causative agents are protozoan, of the genus *Leishmania*. The vectors for these parasites are blood-sucking female sandflies, which harbor the flagellated promastigote forms, infective for vertebrates, including humans. Through the bite of the insect, promastigotes are inoculated into their new hosts and are internalized by cells of the mononuclear phagocytic system. In these cells, promastigotes transform into round nonmotile amastigotes, which can live and multiply within parasitophorous vacuoles, despite the continual fusion with lysosomes (24). Once taken up by macrophages, the parasite is easily disseminated throughout the mononuclear phagocytic system: host cells burst, releasing numerous amastigotes that are infective to bystander macrophages. This amplification culminates in the symptoms and pathology associated with the disease, which depend upon the species of the parasite and the immunological status of the host. These include self-healing skin ulcers, widespread thickening of the skin with lesions, mucocutaneous lesions, and visceral forms with fever, malaise, weight loss, coughing, and diarrhea accompanied by anemia, skin darkening, and hepatosplenomegaly. Visceral forms are often fatal if untreated (8, 18, 20).

The mechanisms of pathogenicity in leishmaniasis are poorly understood. One of the questions that has yet to be addressed is how infected macrophages are ruptured, releasing the amastigotes. Recently, we have reported that *Leishmania amazonensis* promastigotes and amastigotes have a cytolytic protein whose features are reminiscent of PFPS (21, 22). This cytolytic protein is heat labile with no phospholipase, proteolytic, or detergent-like activity and lyses both erythrocytes and nucleated cells, including the macrophage. Lysis is inhibited by macromolecules, indicating that it is caused by an osmotic imbalance that leads to water influx and cell rupture (colloid osmotic lysis), which is typical of a pore formation mechanism. The cytolytic activity is optimal at pH 5.0 to 5.5 and at 37°C, suggesting that it might be fully expressed in the acidic phagolysosome (22). We have therefore speculated that the parasite could use its membranolytic activity to leave the macrophages, by lysing their vacuolar and plasma membranes, to infect neighboring host cells (14, 21, 22).

In the present work, we show that the *L. amazonensis* cytolsin damages macrophages by forming discrete nonselective transmembrane pores, through a mechanism involving at least two distinct steps: binding to the membrane and, probably, insertion and polymerization of individual subunits into the lipid bilayer. We also show that *L. amazonensis* promastigotes contain proteins homologous to other PFPS. We discuss the possible role of this pore-forming activity in the pathogenesis of leishmaniasis.

**MATERIALS AND METHODS**

**Abbreviations.** PFP, pore-forming protein; cExt, promastigote crude extract; HuRBC, human red blood cells; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-phospholipase C; TC-TOX, *Trypanosoma cruzi* pore-forming protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEr, Stokes-Einstein radius; gp, glycoprotein; PEG, polyethylene glycol; BSA, bovine serum albumin.

**Parasites.** The PH8 (IFLA/BR/67/PHS) strain of *Leishmania (Leishmania) amazonensis*, used throughout this work, was provided by Maria Norma Melo (Depto. de Parasitologia, UFMG, Belo Horizonte, Brazil). Promastigotes were axenically cultured at 25°C in RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 20 mM HEPES (Sigma Chemical Co., St. Louis, Mo.) and 50 μg of gentamicin/ml and supplemented with 10% heat-inactivated fetal bovine serum (GIBCO). Promastigotes were harvested at the early stationary phase (day 4 to 5), at the peak of their cytolytic activity (22). Parasites were washed three times with PBS, and pellets, obtained by centrifugation at 1,000 × g, were kept at −80°C until required.

**Parasite extracts.** Promastigote extracts were prepared as previously described (22). Briefly, 2 × 10⁷ parasites/ml in 10 mM Tris-HCl, pH 7.5, were disrupted by five cycles of freezing and thawing and centrifuged at 1,000 × g for 10 min at 4°C. The supernatant, containing approximately 2 mg of protein/ml, was used as cExt. For some experiments cExt was further centrifuged at 100,000 × g for 1 h at 4°C, and the membrane-containing pellet was treated with 0.1 U of PI-PLC (American Radiolabeled Chemicals, St. Louis, Mo.)/ml in a solution containing 50 mM...
Tris-HCl (pH 7.5), 10 mM NaCl, and 2 mM CaCl2 for 2 h at 37°C with constant agitation. After centrifugation at 100,000 × g for 1 h at 4°C, pellet and supernatant were saved.

Hemolytic assays. Hemolysis was assessed as previously described (22). Briefly, 10 µl of serially diluted cExt was incubated in 96-round-bottomed-well microplates with 5 × 105 HuRBC in 200 µl of assay buffer (10 mM acetate buffer, 150 mM NaCl [pH 5.5]) at 37°C for 30 min. Hemolysis was determined by the hemoglobin release, quantitated by the absorbance of the supernatants at 414 nm. The percentage of lysis was calculated in relation to total lysis, obtained by incubation of the same number of HuRBC with 10 µl of 0.5% Triton X-100 in the same volume of assay buffer. Spontaneous lysis was determined for the supernatant from HuRBC incubated in assay buffer only.

For the osmotic protection experiments, molecules of various sizes were used in the hemolytic assays. Raffinose (molecular size, 504 g; SER, 0.55 nm), inulin (molecular size, 3,100 g; SER, 1.4 nm), or PEG of different sizes (molecular size, 1,500 g and SER, 1.2 nm; molecular size, 4,000 g and SER, 2.3 nm; molecular size, 6,000 g and SER, 3.05 nm) (25) was added to assay buffer at the final concentration of 30 mM. In these experiments, incubation periods of HuRBC with cExt varied from 10 to 90 min.

In some experiments, the hemolytic assay was carried out in two stages. During the first, HuRBC were incubated with cExt in Eppendorf tubes on ice for 30 min. The tubes were centrifuged at 500 × g for 10 min at 4°C, the supernatants were collected, and the absorbance at 414 nm was determined. In the second stage, the HuRBC pellet was then extensively washed with ice-cold assay buffer (500 × g, 10 min, 4°C) and resuspended to the original volume with the same buffer. In the second stage, this suspension was reincubated on ice or at 37°C for another 30 min and centrifuged, and the percentage of lysis was determined. Mock controls, in which HuRBC were added just before the last 30-min incubation, were carried out in parallel to ensure that lysis was not due to any particle-associated cytolsin sedimenting during HuRBC washes. To control for spontaneous lysis, all steps were performed with HuRBC alone in assay buffer. Total lysis was determined as described above.

Whole-cell patch clamp. The mouse macrophage-like cell line J774 was maintained in RPMI 1640 (pH 7.2), containing 50 µg of gentamicin/ml and 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Experiments using a conventional whole cell patch clamp technique were performed with soft glass pipettes of about 4 MΩ resistance. The pipettes were filled with a solution containing 120 mM K aspartate, 10 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, and 5 mM HEPES. The pH was adjusted to 7.2 with KOH. Disposable plastic petri dishes (Corning, Acton, Mass.) containing the cultured macrophage cell line J774 were mounted on an inverted microscope stage (IMT-2; Olympus, Tokyo, Japan), and the cells were washed with serum-free sterile RPMI 1640 medium. Currents were recorded with a patch clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, Calif.) at a holding potential of −60 mV and low-pass filtered at 2 KHz. The records were converted into digital arrays by an analog-to-digital converter (Digidata 1200; Axon Instruments). In some experiments, ramps of voltage from −160 to 40 mV (dV/dt = 4 V/s) were applied at 1-s intervals. The acquisition and analysis software was a suite of Axobasic programs (AXGOX 1.1; N. W. Davies, University of Leicester, Leicester, United Kingdom). At least 50 stable records were obtained before application of cExt.

Gel electrophoresis and immunoblotting. Aliquots of promastigote cExt, containing 20 µg of protein, were diluted in 10 µl of a solution containing 25% SDS, 20% glycerol, and 0.01% bromophenol blue in 125 mM Tris-HCl (pH 6.8) with 5% 2-mercaptoethanol and heated in boiling water for 5 min. SDS-PAGE was performed by the discontinuous buffer method (16), using a 10% resolving gel and a 4% stacking gel. Immunoblots were performed by electrotransferring proteins to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass.) at 100 V for 1 h at 4°C in 25 mM Tris–192 mM glycine (pH 8.3) with 20% vol/vol methanol. After blocking with 5% BSA in PBS for 1 h at room temperature, membranes were incubated with one of the following rabbit antisera: anti-mouse peroxidin, anti-human C8, anti-human C9 (dilution, 1:1,000) (kindly provided by Chau-Ching Liu, University of Pittsburgh, Pittsburgh, Pa.), anti-TC-TOX (dilution, 1:200), (a gift from Norma Andrews, Yale University, New Haven, Conn.), or anti-gp46 (L. amazonensis) (dilution, 1:2,000) (kindly provided by Diane McMahon-Pratt, Yale University) for 1 h at room temperature. Membranes were then washed 3 times in PBS with 0.05% Tween 20 and incubated with the second antibody (peroxidase-labeled anti-rabbit IgG, 125I-labeled anti-rabbit IgG, or 125I-labeled protein A, as specified in the legends to the figures) for 1 h at room temperature. Antibodies were diluted in PBS containing 1% BSA and 0.05% Tween 20. Proteins recognized by antibodies were developed with 4-chloro-1-naphthol and 3, 3′-diaminobenzidine in the presence of H2O2 for the peroxidase label or by autoradiography of dried membranes for the 125I label.

RESULTS

L. amazonensis extracts produce nonselective pores in the macrophage membrane. Previous osmotic protection experiments have indicated that L. amazonensis cytolytic protein damages cells by pore formation on the target membrane (22).

FIG. 1. Effect of L. amazonensis extract in the macrophage transmembrane current. cExt was added to J774 cells, and transmembrane current alterations were recorded with a patch clamp amplifier at a holding potential of −60 mV and low-pass filtered at 2 KHz, as described in Materials and Methods. At least 50 stable records were obtained before addition of cExt. These current alterations are representative of 30 measurements and were observed 4 min after addition of cExt. Arrows point to stepwise increases in current.

To verify whether the parasite cytolsin can form pores on cell membranes, we have used the whole cell patch clamp technique. Using the promastigolicell line J774 as the target and using promastigotes as a source of parasite cytolsin, we show that the addition of cExt to the external medium produced marked stepwise increases of membrane current, typical of channel formation (Fig. 1). The time required to observe these current alterations was variable and ranged from 2 to 20 min after addition of cExt. The steps of current had varying amplitudes: in 30 measurements, 20% of current steps had amplitudes in the narrow range of 11 to 15 pA (average, 12.3 ± 0.6 pA) and 27% had amplitudes in the range of 19 to 38 pA (average, 27.8 ± 2.6 pA), and values of current jumps as large as 888 pA were recorded. The larger events were less common, and 70% of all events had amplitudes lower than 138 pA. The smaller events occurred earlier than the larger ones. This progressive and stepwise increase of current was followed, 10 to 30 min later, by an enormous increase in current, probably as a result of the osmotic disruption of the membrane (not shown).

Control experiments with no cExt added had stable current records for up to 30 min.

Figure 2A shows the voltage dependence of the increase in transmembrane current. The control record shows the expected behavior of the resting macrophage membrane conductance. The main features are inward rectification, as shown by the higher conductance for negative (inward) currents, and a reversal potential (potential at 0 current) at −85.5 mV, which coincided with the calculated equilibrium potential for K+ (−85.4 mV), indicating a selective permeability of the membrane to this ion. Upon addition of cExt, a marked change in the voltage dependence of the current can be observed: there is a time-dependent increase in conductance for both inward and outward currents, and the reversal potential is shifted to less-negative values. Figure 2B represents the variation of current calculated by subtracting the currents at 2 and 4 min from the control current. It shows a quasi-linear voltage dependence for inward currents and a small outward rectification. The reversal potential for the variation of current is very near 0 mV, which strongly suggests that the increased current is not selective to any particular ion.
by cExt involves the formation of nonselective pores on the target cell membrane and that the cytolysin of *L. amazonensis* is a PFP, henceforth referred to as leishporin. The internal diameters of the pores are heterogeneous. The different amplitudes observed in the patch clamp experiments suggested that pores with different diameters are formed by the parasite cExt. To confirm this assumption, the internal diameters of the pores were estimated using osmotic protection experiments in which macromolecules of defined size were added to the extracellular compartment in hemolytic assays and assayed for their ability to prevent lysis. Inhibition of cell lysis implies the nontransferability of the macromolecule from the extra- to the intracellular compartment, and the molecule’s effective diameter is taken to exceed that of the functional pore (19). Raffinose, PEG 1500, inulin, PEG 4000, and PEG 6000, with Stokes-Einstein hydrodynamic diameters of 1.1 nm, 2.4 nm, 2.8 nm, 4.6 nm, and 6.1 nm, respectively, were used. We compared the patterns of protection afforded by these molecules over a wide range of cExt concentrations during a 30-min incubation at 37°C (Fig. 3A). Raffinose was unable to protect cells from lysis in all concentrations of cExt used. At the 1:320 dilution of cExt, where approximately 65% hemolysis is observed, molecules larger than 2.4 nm in diameter inhibited lysis by 80 to 100%. However, as the concentration of cExt increased, the degree of protection decreased, and it varied according to the molecule’s diameter. At the highest cExt concentration, only PEG 6000 was capable of giving almost full protection against lysis. These results show that in 30 min at 37°C and up to the highest concentration of cExt used, the diameters of the majority of the pores varied between >1.1 and <6.1 nm. It is apparent that there is a correlation between cytolysin concentration and the diameters of the pores formed.

We next examined the kinetics of pore formation in the presence of raffinose or PEG 6000, aiming to estimate the minimum functional pore diameter and the maximal pore diameter attainable. To determine the smallest pore diameter, we performed hemolytic assays in the presence of raffinose, with incubation periods varying from 5 to 30 min. To determine the maximum pore diameter, incubation periods of 30 to 90 min in the presence of PEG 6000 were used. After 5 min, in the absence of osmotic protection, the degree of hemolysis was only 15% above that of spontaneous lysis (not shown), which suggests the paucity of functional pores. Significant lysis was

![FIG. 2. Conductance alteration in the macrophage membrane mediated by *L. amazonensis* extract. The experiments were performed under the same conditions as those described in the legend to Fig. 1. After addition of cExt, ramps of voltage from −160 to 40 mV (dV/dt = 4 V/s) were applied at intervals of 1 s. (A) Membrane current of macrophages under control conditions and at 2 and 4 min after cExt addition. Arrows indicate reversal potentials at 0, 2, and 4 min after addition of cExt. (B) Variation of current, calculated by subtracting the current at 2 and 4 min from the control current, against membrane potential.](http://iai.asm.org/)

![FIG. 3. (A) Effects of different osmotic protectants in hemolysis mediated by *L. amazonensis* extract. HuRBC were incubated with serially diluted aliquots of cExt at pH 5.5 for 30 min at 37°C with or without 30 mM concentrations of the indicated compounds. (B) Effect of time on the diameters of pores formed by *L. amazonensis* extract. HuRBC were incubated with cExt (1:20 final dilution) at pH 5.5 for various periods of time at 37°C with or without 30 mM raffinose or PEG 6000. The percentages of inhibition were calculated by the equation: (\% lysis without osmotic protectant − \% lysis with osmotic protectant) / \% lysis without osmotic protectant × 100. The percentage of hemolysis was determined as described in Materials and Methods. Vertical lines represent the standard deviations of the means of duplicate samples.](http://iai.asm.org/)
Lysis was determined (second stage). Cells were extensively washed with ice-cold assay buffer, resuspended in the same suspension was centrifuged at 4°C, and lysis was determined (first stage). Cells were extensively washed with ice-cold assay buffer, resuspended in the same buffer to the initial volume, and incubated at 0 or 37°C for another 30 min, and lysis was determined (second stage).

**Leishporin binds to target membranes prior to lysing cells.**

To distinguish membrane binding from pore formation, we carried out a two-stage hemolytic assay. In the first stage, HuRBC were incubated with cExt for 30 min on ice, a temperature at which leishporin could probably bind to the target membrane without necessarily inflicting membrane damage. As we have previously shown (22), hemolysis mediated by cExt is barely detected at this temperature (Fig. 4). In the second stage, however, the treated HuRBC, extensively washed with ice-cold assay buffer to remove any residual cell-unbound material from the medium, were readily lysed upon incubation at 37°C for another 30 min. If, instead, these HuRBC were reincubated on ice for the same period, no lysis was observed. These results indicate that even at low temperatures, leishporin can bind firmly to HuRBC membranes. However, this binding is not sufficient to cause lysis; another event, which occurs only at higher temperatures, is necessary. The absence of hemolysis in mock controls, in which HuRBC were added just before the incubation at 37°C (not shown), certifies that lysis is not due to any particle-associated cytolysin that has sedimented during centrifugations.

**cExts contain molecules that cross-react with other PFPs.**

The presence of components antigenically homologous to other well-characterized PFPs in the cytolytic promastigotes extract was investigated. In Western blotting experiments, we used antibodies to mouse perforin (a PFP from killer cells), to human C8 or C9 (proteins of the membrane attack complex of the complement system) (29), and to TC-TOX (a C9-related PFP of *T. cruzi*) (2). Figure 5 shows that the hemolytic promastigotes cExt contains a protein of 76 kDa that strongly reacts to anti-mouse perforin (lanes 1) and, to a lesser extent, to anti-C8 (lane 2) and to anti-C9 (lane 3), but not with anti-TC-TOX (lane 4). Anti-perforin, but not anti-C8 or anti-C9, recognizes, in addition, a 46-kDa band (lane 1) which is also recognized by anti-TC-TOX (lane 4).

**Leishporin is not gp46.**

Because gp46 is a very abundant surface protein of *L. amazonensis* tethered to the membrane by a GPI anchor (15), the previous results prompted us to investigate whether this protein has cytolytic activity. The membrane fraction of cExt was treated with PI-PLC for 1 h at 37°C, to remove GPI-anchored molecules, and centrifuged at 100,000 × g. Both insoluble (pellet) and soluble (supernatant) fractions were assayed for hemolytic activity. Efficiency of solubilization of gp46 was monitored by Western blotting using anti-gp46. As shown in Fig. 6A, PI-PLC removed gp46 from the membrane fraction (lane 2) and transferred it to the soluble fraction (lane 3). Other GPI-anchored molecules, such as the protease gp63 (7), were also removed from the membrane fraction after PI-PLC treatment (not shown). Nevertheless, the gp46-free membrane fraction fully retained the hemolytic activity of cExt (Fig. 6B), whereas the soluble fraction, containing all the gp46, presented no hemolytic activity. This result shows that leishporin is not the same molecule as gp46 and probably is not a GPI-anchored protein.

**DISCUSSION**

PFPs are revealing themselves to be common molecules among pathogenic protozoan parasites and appear to act as virulence factors (3, 14, 23). Our recent report that *L. amazonensis* has a cytolytic protein that causes colloid-osmotic lysis strongly suggested the pore-forming nature of this cytolsin (22). In the present work, we demonstrate this hypothesis by direct measurements of the currents associated with the increase in membrane permeability. The striking feature of the records obtained with the whole-cell patch clamp technique is a stepwise increase of macrophage membrane current upon addition of cExt (Fig. 1). This is characteristic of pore formation, in which the assembling of each pore generates a pathway that causes a sudden increase of membrane current. The pores only detected after 10 min, when about 65% of the cells were lysed (not shown), yet raffinose was unable to inhibit lysis (Fig. 3B). This indicates that from the very beginning leishporin functional pores are larger than 1.1 nm in internal diameter. On the other hand, the osmotic protection of about 75%, conferred by PEG 6000 after 30 min, dropped gradually as the incubation period increased, reaching 30% in 90 min. This result shows that pores’ diameters increase with time, reaching values greater than 6.1 nm.

**FIG. 4.** Dissociation of the membrane binding and lytic activities of leishporin. HuRBC were incubated with cExt at pH 5.5 for 30 min on ice. The suspension was centrifuged at 4°C, and lysis was determined (first stage). Cells were extensively washed with ice-cold assay buffer, resuspended in the same buffer to the initial volume, and incubated at 0 or 37°C for another 30 min, and lysis was determined (second stage).

**FIG. 5.** Cross-reactivities between different PFPs and polypeptides of *L. amazonensis* extracts. cExt (20 μg) was fractionated in 10% SDS-PAGE under reducing conditions before Western blotting. Membrane strips were cut and incubated with antibodies to different PFPs: anti-mouse perforin (lane 1), anti-C8 (lane 2), anti-C9 (lane 3), and anti-TC-TOX (lane 4). Proteins recognized by the different anti-PFPs were detected by 125I-protein A (anti-perforin, anti-C8, and anti-C9) or by a peroxidase anti-rabbit IgG (anti-TC-TOX) and developed as described in Materials and Methods.
brane was incubated with antiserum anti-gp46, followed by 125I-anti-rabbit IgG, 10% SDS-PAGE under reducing conditions before Western blotting. The membrane fraction was treated with PI-PLC for 1 h at 37°C and centrifuged at 100,000 g. Supernatant (S), and untreated membrane fraction (U) were fractionated in inner diameter. We can estimate the diameters of the pores, if

\[ g_p = \Delta I_p / (E_m - E_{rev}) \]  

where \( g_p \) is the conductance step generated by the formation of the pore, \( \Delta I_p \) is the step of current, \( E_m \) is the holding membrane potential (−60 mV) and \( E_{rev} \) is the reversal potential (considered 0 mV). From the measurements described under Results, the smallest conductance jumps averaged 205 pS and comprised 20% of the data.

The conductance of a pore is markedly dependent on its inner diameter. We can estimate the diameters of the pores, if we assume that the ion mobility inside the pores is the same as in the bulk. The estimated resistance, and consequently the conductance, of the pore is given by:

\[ R_p = l/g_p = (l + \pi a^2) / \pi a^2 \]  

where \( g_p \) is the membrane conductance, \( a \) is the pore radius, \( l \) is the pore length, and \( \rho \) is the resistivity of the solution. As this equation is derived from macroscopic laws, its application to atomic dimensions must be handled carefully (13). In fact, it is logical that the narrower the pore, the more geometrically restricted will be the flow of ions, and more important will be effects such as ion-pore, ion-water, and pore-water interactions, thereby making ion mobility inside the pore slower than in the bulk solution. Equation 2 should therefore be regarded as setting the upper limit for ion conductance.

The maximum conductance of a pore with a radius of 3.05 (the SEar of PEG 6000) and a length of 8 nm (the width of the membrane) immersed in physiological solution (\( \rho = 100 \Omega \text{cm} \)) can be calculated as 2.3 nS, which under our conditions would give current steps of 138 pA (calculated from equation 1). This calculation estimates that the diameter of PEG 6000 would be greater than those of 70% of the pores recorded in our experiments in 30 min, showing that the electrical measurements are consistent with the osmotic protection data.

One important piece of information points to the heterogeneity of the pore diameters. We suggest that the pores are formed by the aggregation of subunits and that the number of subunits that form each pore will determine its diameter, thus generating the heterogeneity of the pore conductances. The current records show two features, unusual in preassembled ion-conducting pores, that support our proposal: (i) once formed, the pore remains open, and (ii) the steady level of each current step is usually preceded by a transient of current, which probably reflects transient conformation states during subunit aggregation. The relatively high frequency of low-conductance steps (205 pS) suggests that this is the “minimum” pore with a diameter of 1.6 nm, which can explain why raffinose (1.1 nm) failed to protect the HuRBC from lysis in all circumstances tested (Fig. 3). This hypothesis can explain the observation that the earliest pores formed are smaller than those found later in the experiments when, supposedly, more subunits have had time to incorporate into the membrane. Together with the results from osmotic protection showing that the diameters of pores increase with time and with leishporin concentration (Fig. 3), these results strongly suggest that functional pore formation is a result of aggregation or polymerization of single protein units. The maximal pore size was not determined, but the internal diameter can exceed 6.1 nm (Fig. 3B).

The early events of pore formation by leishporin seem to require the binding of the cytolsin to the cellular membrane, which occurs at temperatures as low as 0°C (Fig. 4). However, lysis itself is temperature dependent, occurring only at temperatures above 20°C (22) (Fig. 4). Therefore, binding appears to be followed by a second step that triggers cytolsis. These results indicate that pore formation requires at least two distinct stages: (i) the binding of the cytolsin to the membrane, a temperature-independent step that is necessary but not sufficient to cause lysis, and (ii) a temperature-dependent step that could be the insertion and the polymerization of protein subunits in the cellular membrane, which culminates in cytolsis.

Homology among different PFPs has already been demonstrated. For example, human C9 is homologous to perforin (28), to TC-TOX (2), and to mellitin from bee venom (17). We
show here that two parasite polypeptides of 46 and 76 kDa are antigenically related to mouse perfonin, human C8 and C9, or TC-TOX (Fig. 5). We have ruled out the possibility that gp46 is leishporin by showing that parasite membranes devoid of this protein fully retain the cytolytic activity of total extracts and that soluble gp46 is hemolytically inactive (Fig. 6). We can also rule out the possibility that other GPI-anchored proteins, such as gp63, removed from membrane extracts after treatment with PI-PLC (not shown), are involved with the parasite pore-forming activity. It is possible, though, that the cross-reactive 46-kDa polypeptide is different from gp46 and that the two cross-reactive bands are related to the cytolytic activity. In this case, three possibilities could be considered: (i) the cross-reacting bands are distinct polypeptides, (ii) the 76-kDa band corresponds to a dimer of the lower-molecular-weight molecule that could not be dissociated by SDS, or (iii) the 46-kDa band is a product of proteolytic cleavage of the higher-molecular-weight protein.

L. amazonensis cytolsin is optimally active at pH 5.0 to 5.5 and at 37°C (22), conditions that mimic the interior of the macrophage phagolysosomes that carry Leishmania amastigotes. This acid-active feature is shared by other PFPs, such as TC-TOX from T. cruzi (2, 4) and listeriolysin O from Listeria monocytogenes (10), which have been implicated in the escape of the parasites from the phagocytic vacuole into the cytosol (3, 11). Unlike TC-TOX (4) and listeriolysin O (10), leishporin is also active at a neutral pH (22), which would also favor its action inside the cytosol. Leishmania spp. do not escape the phagolysosome, but at later stages, macrophages disrupt and release amastigotes, which are infective for healthy adjacent cells. These facts led us to speculate that leishporin could play a role in rupturing the host cells (14, 22), acting first on the phagolysosomal membrane and shortly afterwards on the plasma membrane. This assumption leads to a shift in the current thinking that the burst of the macrophages is a direct result of excessive parasite burden and puts the cytolsin as a key molecule in the pathogenesis of leishmaniasis, acting not only as a tissue-damaging factor but also as an infection-spreading factor. It is also possible that L. amazonensis cytolsin can play a role at other stages of parasite development. Since leishporin is present in promastigotes (22), one could consider, for instance, that it may facilitate the penetration of the host cell by the parasite. Reports of promastigotes found inside nonphagocytic cells (6, 9, 26) and results showing that living L. amazonensis is better internalized by macrophages than killed parasites (5) suggest an active role of the parasite in the process of internalization by the host cell. In addition, the receptor-mediated phagocytosis, which is considered to be the main mechanism for Leishmania internalization (1), could be facilitated by an alteration of the permeability of the host cell membrane to the PFP, either by directly allowing calcium influx or by activating voltage-dependent calcium conductance (27). Since leishporin is also active at 23°C (22), it is also conceivable that inside the sandfly, it may be used to lyse the vertebrate host erythrocytes to obtain nutrients from hemoglobin. Although the actual role of leishporin waits to be demonstrated, the finding of a PFP in Leishmania makes the hypothesis of this cytolsin as a virulence factor quite attractive and opens a new field of investigation regarding the pathogenesis of leishmaniasis.

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