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# Membrane binding requirements for the cytolytic activity of *Leishmania amazonensis* leishporin

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ABSTRACT

**To lyse cells, some pore-forming proteins need to bind to receptors on their targets. Studying the binding requirements of *Leishmania amazonensis* leishporin, we have shown that protease-treated erythrocytes are as sensitive to leishporin-mediated lysis as untreated cells, indicating that protein receptors are dispensable. Similarly, carbohydrate receptors do not seem to be needed, since several sugars do not inhibit leishporin-mediated hemolysis. Conversely, dipalmitoylphosphatidylcholine (DPPC), but not cholesterol, completely inhibits leishporin-mediated lysis. DPPC liposomes, with or without cholesterol, are lysed by leishporin and remove its lytic activity. Our results demonstrate that leishporin is a cholesterol-independent cytolysin that binds directly to phospholipids.**


## 1. Introduction

Protozoa of the genus *Leishmania* comprise many species that cause leishmaniasis, an endemic disease in several continents. The parasite is spread to mammalian hosts through the bite of a *Phlebotomus* or *Lutzomia* sandfly containing the infective metacyclic promastigotes, which end up infecting macrophages. Inside phagolysosomes, they change into amastigotes, proliferate and, ultimately, exit the host cell, to infect healthy ones. The parasite's life cycle is completed when, during a blood meal, the sandfly ingests amastigotes, which evolve again to metacyclic promastigotes.

A crucial but poorly understood step in the pathogenesis of intracellular microorganisms is their exit from host cells. Its consequences are patent both in the amplification of the infection, thus exacerbating the disease and the rate of transmission, and in the outcome of the immune response. Over the last years, we have described and studied a cytolytic activity in *Leishmania amazonensis* that functions optimally at 37 °C and pH 5.5 (conditions found inside phagolysosome), but also at lower temperatures and/or neutral pH [1,2]. This cytolytic activity is due to pore-formation on target membranes by the action or on the dependence of a protein (or proteins) [2,3] and results from either proteolysis [4] or dissociation of an oligopeptide inhibitor (unpublished result). We have been referring to the component(s) responsible for the pore-formation as leishporin [3–5], although the putative protein(s) remain(s) unidentified. The above features are compatible with a function of lysing membranes from both inside the phagolysosome and, later on, the cytosol. We have then postulated that the exit of *Leishmania* from macrophages is not simply a consequence of pathogen burden or cell stress, as usually assumed, but could be caused by the action of leishporin [1–6]. In fact, the active egress of

*Abbreviations:* CHAPS, 3[(3-cholamidopropyl)dimethyl-ammonium]-2-hydroxy-propanesulfonate; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; $H_{50}$, 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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intracellular pathogens from within the host cell can be orchestrated by the pathogens themselves through a variety of strategies, including pore-formation [7,8].

Pore-forming proteins (PFPs) require binding to specific molecules on their target membranes, which can be lipids, carbohydrates or proteins [9–11]. Here, we have studied the requirements of leishporin to bind to and lyse cells. Using erythrocytes and liposomes as targets, we showed that, to lyse membranes, leishporin bind directly to phospholipids not requiring cholesterol, proteins or carbohydrates.

## 2. Materials and methods

### 2.1. Parasites

The PH8 (IFLA:PA:67:PH8) strain of *Leishmania* (*Leishmania*) *amazonensis* was used. Promastigotes were grown in Schneider medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) (CULTILAB, Campinas, Brazil) and 50 μg/ml gentamycin (Sigma). Four-day cultured parasites were washed with phosphate-buffered saline (PBS) at 1000×*g*, and pellets were kept at −80 °C until required.

### 2.2. Parasite membrane extract

Promastigotes were resuspended in 50 mM borate buffer, pH 7.0, to a density of $2 \times 10^9$ cells/ml and subjected to five cycles of freeze-and-thaw [2]. The extract was centrifuged at 1000×*g* for 10 min to sediment intact cells and nuclei and the supernatant centrifuged for 1 h at 16 000×*g*. The membrane-rich pellet was resuspended in the same buffer containing 0.4% (sub-lytical) CHAPS (3[(3-cholamidopropyl) dimethyl-ammonium]-2-hydroxy-propanesulfonate) and kept on ice for 1 h with occasional agitation. The suspension was centrifuged at 100 000×*g* for 1 h, and the supernatant, corresponding to the solubilized membrane molecules, was referred to as promastigotes membrane detergent-soluble extract (mExt).

### 2.3. Liposomes

Liposomes were made of DPPC (Lα-dipalmitoylphosphatidylcholine) or DOPC (dioleylphosphatidylcholine), with or without cholesterol (10, 15, 20 or 30% w/w). To prepare large unilamellar vesicles, a lipid film was hydrated with PBS containing 30 mM phospholipids. The suspension was subjected to 10 cycles of freeze-and-thaw and extruded through 200-nm pore size polycarbonate membranes. To obtain calcein-containing small unilamellar liposomes, the lipid film was hydrated with a 75 mM calcein solution, pH 7.4, and the suspension was ultrasonicated (Sonics Vibra-Cell, USA). Calcein-loaded liposomes were separated from free calcein by Sephadex G50.

### 2.4. Hemolytic assay

Hemolysis was assessed in human erythrocytes (HuE) [2]. Briefly, $5 \times 10^6$ cells in 200 μl 20 mM acetate buffer, pH 5.5, containing 150 mM NaCl, were incubated in 96 round-bottomed well microplates with 10 μl of 1:2 serially diluted mExt treated or not as described below. After 30 min at 37 °C, microplates were centrifuged for 10 min at 500 × *g* and hemolysis was quantified through the absorbance of the supernatant at 414 nm. The percentage of lysis was in relation to total lysis, obtained by addition of 10 μl of 0.25% Triton X-100 to the same number of HuE. Hemolytic activity was reported as percentage of lysis *versus* dilution factor or as $H_{50}$, the inverse of the dilution that caused 50% of hemolysis.

Alternatively, HuE were previously incubated for 1 h at 37 °C with trypsin, Pronase® or proteinase K (12.5, 25, 50 or 100 μg/ml) in 10 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 10 mM $CaCl_2$ and washed three times with PBS. For the competition experiments, mExt was previously incubated for 30 min at 37 °C with fructose, galactose, glucose, lactose, maltose or mannose (12.5, 25, 50 or 100 μM), DPPC or cholesterol (12.5, 25, 50 or 100 mg/μl).

### 2.5. Removal of the lytic activity from mExt

Ten microliters of mExt in a final volume of 200 l were incubated on ice with HuE, *Lactobacillus acidophilus* or liposomes as follows: (1) HuE: $0.2 \times 10^7$, $1 \times 10^7$ or $2 \times 10^7$, acetate buffer, pH 5.5, 30 min; (2) bacteria: $1 \times 10^{10}$/ml (heated at 100 °C, 10 min or treated with 1 mg/ml lysozyme at 37 °C, 30 min), Tris–HCl buffer, pH 8.0, 15 min; (3) liposomes: 5, 15 or 20 μl of suspension, acetate buffer, pH 5.5, 30 s. Cells and liposomes were removed by centrifugation and mExt was assayed for hemolytic activity. All centrifugations in this work were carried out at 4 °C.

### 2.6. Liposome lysis assay

Ten microliters of calcein-loaded liposomes were diluted in 90 μl of acetate buffer, pH 5.5, and incubated at 0 °C or at 37 °C with (1) mExt heated or not at 100 °C or previously incubated with calcein-free liposomes or (2) liposomes previously incubated with mExt. Twenty-microliters aliquots were collected at 5- or 10-min interval and diluted in 2 ml acetate buffer in a quartz cuvette before reading the fluorescence (wavelengths: exciting – 490 nm; emission: 515 nm) (Varian Cary Eclipse Fluorimeter). Lysis was reported as the percentage of total lysis, obtained after addition of 5 μl of 4% CHAPS. All experiments in this work were repeated at least three times and all data correspond to a typical result.

## 3. Results

### 3.1. Binding of the leishporin to cell membranes

At least two steps are required for pore-formation by mExt on erythrocytes: (1) binding of the cytolysin to the target membrane, which occurs even at 0 °C with no hemolysis and (2) the pore-formation itself that probably consists of oligomerization and insertion of subunits, occurring only at higher temperatures [2,3]. To verify whether HuE remove hemolytic activity from mExt, we incubated both components on ice. As expected, HuE were sedimented without hemolysis and the hemolytic activity of the supernatant was determined. We verified that HuE removes the hemolytic activity of mExt in a dose-dependent manner (Fig. 1A). Similar results were observed when erythrocytes were replaced by prokaryotic cells. Fig. 1B shows that the Gram-positive *L. acidophilus* partially or completely removes hemolytic activity of mExt if previously heated at 100 °C or treated with lysozyme, respectively. The Gram-negative bacterium *Escherichia coli* also partially removes hemolytic activity if heated at 100 °C (not shown). This indicates that the elimination or disruption of bacterial cell wall exposes cytolysin-binding sites on the cell, showing that leishporin is able to bind to membranes other than eukaryotic ones. We next sought whether the cytolysin had a particular receptor, investigating the participation of carbohydrates, proteins and lipids.

### 3.2. Involvement of carbohydrates, proteins and lipids in the binding of leishporin to target membranes

To investigate whether hemolysis was dependent on the binding of the cytolysin to proteins, we treated HuE with trypsin, Pronase®