

Kinetoplastid membrane protein-11 is present in promastigotes and amastigotes of *Leishmania amazonensis* and its surface expression increases during metacyclogenesis

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Kinetoplastid membrane protein-11 (KMP-11), a protein present in all kinetoplastid protozoa, is considered a potential candidate for a leishmaniasis vaccine. A suitable leishmaniasis vaccine candidate molecule must be expressed in amastigotes, the infective stage for mammals. However, the expression of KMP-11 in Leishmania amastigotes has been a subject of controversy. We evaluated the expression of this molecule in logarithmic and stationary growth phase promastigotes, as well as in amastigotes, of Leishmania amazonensis by immunoblotting, flow cytometry and immunocytochemistry, using a monoclonal antibody against KMP-11. We found that KMP-11 is present in promastigotes and amastigotes. In both stages, the protein was found in association with membrane structures (at the cell surface, flagellar pocket and intracellular vesicles). More importantly, its surface expression is higher in amastigotes than in promastigotes and increases during metacyclogenesis. The increased expression of KMP-11 in metacyclic promastigotes, and especially in amastigotes, indicates a role for this molecule in the parasite relationship with the mammalian host. The presence of this molecule in amastigotes is consistent with the previously demonstrated immunoprotective capacity of vaccine prototypes based on the KMP-11-coding gene and the presence of humoral and cellular immune responses to KMP-11 in Leishmania-infected humans and animals.

Key words: *Leishmania amazonensis* - KMP-11 - expression - amastigote - promastigote - metacyclogenesis

The leishmaniasis are a group of diseases caused by approximately 20 species of protozoan parasites belonging to the genus *Leishmania* (Herwaldt 1999, Desjeux 2004, Croft et al. 2006). They are present in 88 countries distributed in Africa, America, Asia and Europe, currently affecting around 12 million people, with two million new cases each year and these numbers are probably underestimated (Croft et al. 2006).

Because of their extremely diverse epidemiology (Desjeux 2004), an effective vaccine would be the most comprehensive means of prevention. Many candidate vaccines have been proposed, which can be broadly classified into first and second generations. First generation vaccine candidates consist of whole-cell extracts or fractions of killed *Leishmania* promastigotes, whereas second generation vaccine candidates constitute chemically defined preparations, such as recombinant proteins or DNA (Khamesipour et al. 2006). Various parasite molecules have been identified as potential

candidates for second-generation vaccines. One of these candidates is kinetoplastid membrane protein-11 or KMP-11 (Basu et al. 2005).

KMP-11 was discovered as a protein complex tightly associated with lipophosphoglycan, the major cell surface glycoconjugate of *Leishmania* promastigotes (King et al. 1987). After the demonstration that T cell responses to lipophosphoglycan preparations were in fact due to protein contaminants (Mendonça et al. 1991), these contaminants were identified and termed lipophosphoglycan-associated protein (Jardim et al. 1991). This protein has been shown to be highly antigenic for murine and human T cells (Jardim et al. 1991, Russo et al. 1992). Its present denomination, KMP-11, is due to the presence of this 11 kDa protein on the membrane of all kinetoplastid protozoa (Stebeck et al. 1995).

Leishmania parasites have a simple life cycle consisting of two stages: the non-motile amastigotes living in phagocytic cells of mammalian hosts and the motile flagellate promastigote forms that infect the alimentary tract of the sand fly vector (Pulvertaft & Hoyle 1960). Therefore, a suitable vaccine candidate antigen must be expressed in amastigotes, the infective stage for humans. KMP-11 has been shown to be expressed in promastigotes of all *Leishmania* species studied to date (Tolson et al. 1994, Stebeck et al. 1995) but, in amastigotes, the picture is less clear. Jardim et al. (1995a) found KMP-11 in *Leishmania donovani* amastigotes, but the amastigote levels were not compared

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with those found on promastigotes. However, Berberich et al. (1998) claimed that the expression of *Leishmania infantum* KMP-11 is promastigote stage-specific. Thus, in the present study, we evaluated KMP-11 expression in the two life-cycle stages of *Leishmania amazonensis*: promastigotes and amastigotes.

MATERIALS AND METHODS

Animals - Female BALB/c mice at 6-8 weeks of age were obtained from Oswaldo Cruz Institute Facilities. The protocols used in this study were approved by the Ethical Committee on Animal Use of Fundação Oswaldo Cruz (protocol L-0012/07).

Parasites and preparation of whole-cell extracts - IFLA/BR/67/PH8 is a *L. (L.) amazonensis* reference strain isolated from the sand fly *Lutzomyia flaviscutellata* in state of Pará, Brazil in 1967. This strain has been provided to us by the *Leishmania* Collection of Instituto Oswaldo Cruz. In our laboratory, it has been continuously maintained in mice in order to preserve its infectivity. Amastigotes from lesions were obtained as follows: BALB/c mice were inoculated subcutaneously with 10^6 stationary-phase *L. amazonensis* promastigotes (IFLA/BR/67/PH8) in 50 μ L of phosphate buffered saline (PBS) in the left hind footpad. Twelve weeks after infection, non-ulcerated lesions were aseptically excised in a laminar flow hood as a source of amastigotes and all subsequent manipulations were performed under sterile conditions. The tissues were minced and homogenised vigorously for several minutes with a glass tissue homogeniser in Schneider's medium supplemented with 10 mM L-glutamine, 200 IU penicillin, 200 μ g streptomycin/mL and 20% heat-inactivated bovine calf serum (BCS) (all from Sigma, St Louis, MO, USA) until completely disrupted. Large debris were discarded after 5 min of sedimentation and supernatant medium containing 10^7 - 10^8 amastigotes were harvested and centrifuged at 2.000 g for 5 min at 22°C. The amastigote pellet was resuspended in fresh growth medium for further use. Amastigotes from axenic cultures were obtained according to a previously described methodology (Cysne-Finkelstein et al. 1998).

L. amazonensis (IFLA/BR/67/PH8) promastigotes were grown in Schneider's medium supplemented with antibiotics (200 IU penicillin and 200 μ g streptomycin/mL) and 10% inactivated BCS (Sigma). We constructed a growth curve for cultures initiated with 10^4 organisms and defined the 4th and 8th day for harvesting logarithmic and stationary phase promastigotes, respectively. The proportion of metacyclics in logarithmic and stationary phase promastigote populations was determined using the complement lysis assay, as previously described (Cysne-Finkelstein et al. 1998). In order to obtain purified metacyclic promastigotes, we used a previously published Ficoll 400 (Sigma) density gradient centrifugation method (Spath & Beverley 2001).

Whole-cell extracts of *L. amazonensis* promastigotes and amastigotes were produced as follows: the parasites were washed three times in PBS and disrupted by 10 freeze and thaw cycles, followed by ultrasonication (Ultra-tip Labsonic System; Lab-Line, Melrose Park,

IL, USA) at 40 W for 15 min in an ice bath. All antigen preparations were adjusted to 20 mg of protein nitrogen/mL in PBS and all samples were stored at -70°C until use.

Because KMP-11 expression has been shown to decrease as a function of time and number of subcultures (Mukhopadhyay et al. 1998), the comparisons of KMP-11 expression were always performed between different life-cycle stages from the same isolate from infected BALB/c mice. Promastigotes and axenic amastigotes came from first-passage cultures.

Production of recombinant *L. amazonensis* KMP-11 - Two oligonucleotide primers were used to PCR amplify the complete KMP-11-coding region from *L. amazonensis* stationary phase promastigote cDNA. The forward primer was 5'-GATCCCCACGTACGAGGAG-3' and the reverse primer was 5'-AAGCTTGTGGTCTGATCTAA-3'. The first primer had a restriction target for BamHI and the second contained the TAA stop codon and a restriction target for HindIII. The PCR conditions were as follows: 30 cycles of 1 min at 95°C, 2 min at 41°C and 3 min at 72°C. The amplicon was purified with the Wizard Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Both the purified amplicon and the pQE-30 vector (Qiagen, Hilden, Germany), which contained the ATG initiation codon, were digested with BamHI and HindIII (Invitrogen Gibco, Grand Island, NY, USA) and ligated with T4 ligase (United States Biochemical Co, Cleveland, OH, USA) and the resulting construct was subsequently used to transform *Escherichia coli* strain DH5 α cells by electroporation. The positive clones were selected by PCR and double digestion with BamHI and HindIII to confirm the presence of the 273 bp insert. The *L. amazonensis* KMP-11 encoding sequence was confirmed by DNA sequencing (Sanger et al. 1977) with T3, T7 and internal primers, using the Sequenase kit (United States Biochemical Co). DNA sequence analysis was performed with the DNASTAR and GCG - Genetics Computer Group software packages (University of Wisconsin, USA). The *L. amazonensis* KMP-11 encoding sequence was deposited in the GenBank database (accession AF193432).

The expression host *E. coli* M15 was transformed with the recombinant plasmid by electroporation. Gene expression was induced by 1 mM IPTG and the bacteria were further grown for 4 h. Cells were harvested by centrifugation and the pellet, resuspended in lysis buffer (10 mM Tris, pH 8; 0.1% Triton X100; 0.5 mM phenylmethylsulphonyl fluoride; 1% lysozyme and 5 mM imidazole), was sonicated in an ice bath for four cycles of 15 s at 200 W, with intervals of 15 s and centrifuged. The recombinant protein was then purified in a Ni+2-NTA super flow column (Qiagen), according to the manufacturer's instructions. The protein was eluted in 8 M urea salt buffer with 60 mM imidazole and dialysed in pH 7.4 PBS.

Western blotting - *Leishmania* whole-cell extracts were lysed in lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.5% Triton X-100 and 1X protease inhibitor mixture set I, Sigma) and resolved

on SDS-PAGE. Cell lysates and KMP-11 recombinant protein were suspended in SDS-PAGE sample buffer and boiled for 5 min. Western blotting of lysates was performed using standard techniques (Sambrook et al. 1989). Thirty micrograms of the lysates and 10 µm of recombinant KMP-11 were subjected to SDS-PAGE on a 12% gel, followed by western blotting and immunochemical detection on a nitrocellulose membrane. Nonspecific interactions were blocked with 2% bovine serum albumin (BSA)/0.1% Tween-20 in PBS. KMP-11 was detected by using an anti-KMP-11 monoclonal antibody (Cedarlane, Hornby, Canada). The membranes were probed with primary antibodies at 4°C overnight and incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at RT. Proteins were visualised using the ECL kit (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions. Equal loading of *Leishmania* lysate proteins in each lane was confirmed by Ponceau S staining and by using an anti-β-actin monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) as a loading control (Xiao et al. 2006).

Flow cytometric analysis - Live promastigotes and amastigotes were subjected to cytofluorimetric analysis. Parasite suspensions (5.0×10^5 parasites/well) were washed in PBS/BSA/ NaN_3 (pH 7.2 PBS supplemented with 2% BCS and 0.1% NaN_3 , all purchased from Sigma) and stained for 30 min at 4°C with anti-KMP-11 monoclonal antibody (Cedarlane). Then, parasites were washed in PBS/BSA/ NaN_3 and co-stained with fluorescein isothiocyanate-conjugated anti-mouse IgG₁ antibody (Cedarlane) in the dilution recommended by the manufacturer. Stained cells were washed and fixed in PBS/BSA/ NaN_3 containing 1% paraformaldehyde (Sigma) for 15 min at 4°C. Parasites were also incubated with the secondary reagents and with or without an isotype control antibody (Cedarlane) as controls for nonspecific labelling. Fixed cells were then immediately applied to a FACSCalibur (BD Biosciences). The flow cytometric analysis was performed on Summit v4.3 software (DAKO) and both the percentage of positive cells and the density of activation marker expression (mean fluorescence intensity) were obtained. The results, obtained in five independent experiments, are represented by mean fluorescence intensity and normalised mean fluorescence intensity, defined as the percentage of positive lymphocytes multiplied by the corresponding mean fluorescence intensity values.

Ultrastructural immunocytochemistry - For analysis by transmission electron microscopy, the parasites were fixed in freshly prepared 4% paraformaldehyde/0.1% glutaraldehyde and 0.2% picric acid in a 0.1 M sodium cacodylate buffer, pH 7.2. Samples were dehydrated in methanol at progressively lower temperatures, embedded in Lowicryl K4M resin (Electron Microscopy Sciences, Hatfield, PA, US) and maintained at -20°C. Ultrathin sections were collected on 300 mesh nickel grids and incubated for 30 min at 25°C in 50 mM ammonium chloride in 0.1 M PBS, pH 8.0. Afterward, sections were

incubated for 10 min at RT in PBS containing 3% BSA and 0.2% Tween 20 (PBS-BSA-TW), pH 8.0 and then incubated overnight in the presence of anti-KMP-11 or isotype control antibodies (both from Cedarlane). Grids were rinsed in PBS-BSA and, finally, incubated for 60 min with a 1:20 dilution of goat anti-mouse antibody conjugated with 10 nm gold particles (Sigma) (Bendayan et al. 1987). The grids were subsequently washed with PBS and distilled water, stained with uranyl acetate and observed in a Zeiss EM 10C transmission electron microscope from Oswaldo Cruz Institute Platform.

RESULTS

Western blot analysis of KMP-11 expression in *L. amazonensis* promastigotes and amastigotes - In order to assess the expression of KMP-11 protein in different developmental stages, we performed immunoblot analysis of *L. amazonensis* promastigote and amastigote whole-cell extracts using anti-KMP-11 monoclonal antibody. The anti-KMP-11 antibody recognised bands of KMP-11 in whole-cell extracts from lesion amastigotes and from purified metacyclic promastigotes, as well as in the recombinant KMP-11 preparation. No clear labelling was observed in log phase promastigote extracts (Fig. 1).

Quantitative evaluation of KMP-11 expression on the surface of *L. amazonensis* promastigotes and amastigotes by flow cytometric analysis - In order to investigate whether KMP-11 is differentially expressed on the membrane of *L. amazonensis* promastigotes and amastigotes, we used a flow cytometric approach employing an anti-KMP-11 monoclonal antibody. Surface expression of KMP-11 was higher in amastigotes, both from lesion (Fig. 2C) and from axenic culture (Fig. 2D), compared to promastigotes (Fig. 2A, B). In addition, we observed that stationary growth phase promastigotes (Fig. 2B) expressed higher fluorescence intensity than logarithmic growth phase promastigotes (Fig. 2A). It is known that the proportion of metacyclics is increased when promastigote cultures reach the stationary growth phase (da Silva & Sacks 1987). In order to demonstrate that this was the case for the promastigote populations used in the present study, we determined the percentage of metacyclic organisms in the *L. amazonensis* log phase and

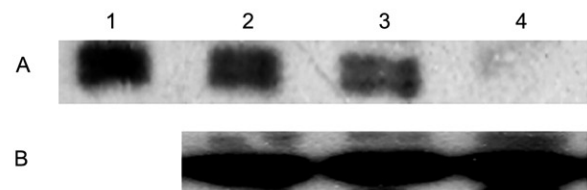


Fig. 1: western blot analysis of whole-cell lysates of *Leishmania amazonensis* amastigotes and promastigotes using anti-KMP-11 (A) and anti-β-actin monoclonal antibodies (B). Recombinant KMP-11 was loaded on Lane 1. The whole-cell lysates were distributed as follows: Lane 2: lysate from amastigotes isolated from cutaneous leishmaniasis lesions of BALB/c mice; 3: lysate from purified metacyclic promastigotes; 4: lysate from log-phase promastigotes.

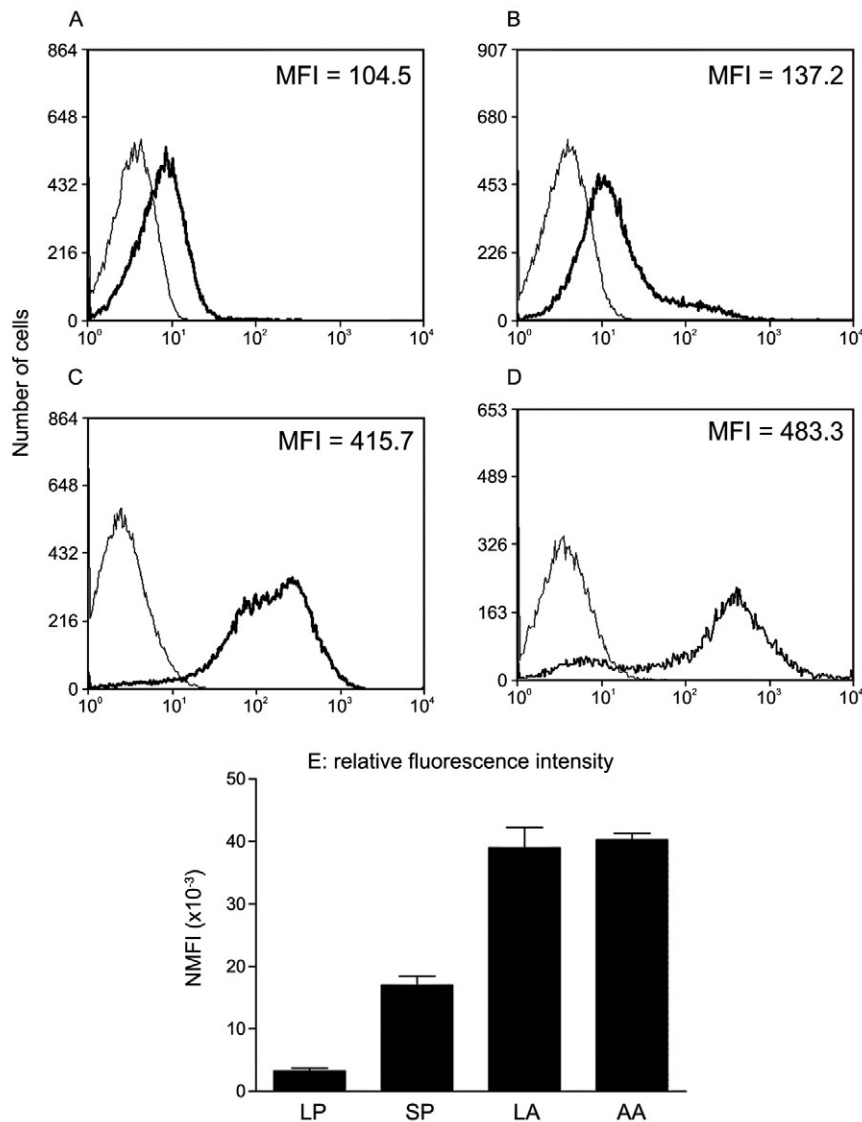


Fig. 2: flow cytometry analysis of KMP-11 surface expression in *Leishmania amazonensis* promastigotes and amastigotes. A: log phase promastigotes; B: stationary phase promastigotes; C: amastigotes from infected mouse tissue; D: amastigotes from axenic cultures. Histograms in bold represent staining with anti-KMP-11 mAb. Thin line histograms represent staining with the isotype control antibody; A-D: the results of a representative experiment from a series of five; E: expression of KMP-11, represented as normalized mean fluorescence intensity (NMFI), obtained by multiplying the percentage of positive cells by the mean fluorescence intensity (MFI) \pm standard error of five independent experiments; AA: axenic amastigotes; LA: lesion amastigotes; LP: logarithmic phase promastigotes; SP: stationary phase promastigotes.

stationary phase promastigote populations employed in the flow cytometry experiments. As expected, we found a higher proportion of metacyclic promastigotes in the stationary phase population (70%) compared to the log phase population (8.8%). The normalised mean fluorescence intensity values for logarithmic phase promastigotes, stationary phase promastigotes, lesion amastigotes and axenic culture amastigotes were, respectively, 289.0, 1721.9, 38953.8 and 40635.6 (Fig. 2E). Fig. 2A-D show the results of a representative experiment from a series of five. Data shown in Fig. 2E represent the means of those five independent experiments.

Immunolocalisation of KMP-11 in promastigotes and amastigotes - Ultrastructural analysis of the subcellular localisation of KMP-11 was performed in *L. amazonensis* promastigotes from log and stationary phase cultures and in amastigotes from axenic cultures or isolated from mouse cutaneous lesions. Amastigotes, both from axenic culture (Fig. 3A) and from lesions (Fig. 3C), presented labelling at the plasma membrane (arrows), at the flagellar pocket membrane (arrows), and in cytoplasmic vesicles (arrowheads). Both log (Fig. 3B) and stationary (Fig. 3D) phase promastigotes also showed labelling for this molecule at vesicular structures (arrowheads), at the surface

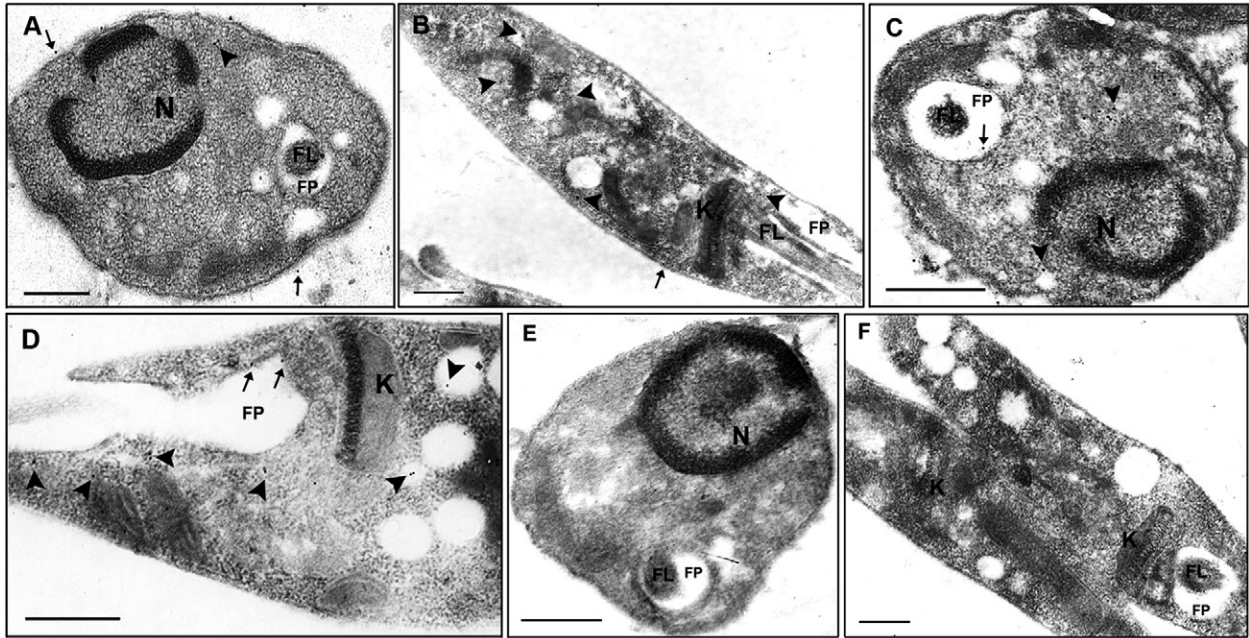


Fig. 3: electron micrographs showing subcellular localization of KMP-11 in *Leishmania (Leishmania) amazonensis* promastigotes and amastigotes using an anti-KMP-11 monoclonal antibody and a colloidal gold-conjugated secondary antibody. Amastigotes obtained from axenic cultures (A) and from mouse lesions (C) showed gold particles at the surface plasma membrane (arrows) at the flagellar pocket membrane (arrows) and in intracellular vesicles (arrowheads). In log (B) and stationary (D) phase promastigotes gold immunocomplexes were observed at the flagellar pocket (arrows) at the surface plasma membrane (arrows) and in intracellular compartments (arrowheads). Amastigotes (E) and promastigotes (F) incubated with an isotype control antibody showed no gold particle labeling. FL: flagellum; FP: flagellar pocket; K: kinetoplast; N: nucleus. Bar = 1 μ m.

plasma membrane (arrows), and at the plasma membrane of the flagellar pocket (arrows). Amastigote and promastigote forms incubated with the isotype control antibody showed no gold particle labelling (Fig. 3E, F).

DISCUSSION

There are several reports on the expression of KMP-11 through different life-cycle stages of kinetoplastid protozoa (Jardim et al. 1995a, Stebeck et al. 1995, Berberich et al. 1998, Thomas et al. 2000, Paba et al. 2004), but only a few of them have focused on parasites of the genus *Leishmania* (Jardim et al. 1995a, Berberich et al. 1998). A former study showed that KMP-11 is not only expressed in *L. donovani* promastigotes, but it is also present in amastigotes. However, it was not determined whether the amastigote levels were comparable with those found in promastigotes (Jardim et al. 1995a). In another study performed with *L. infantum*, it was claimed that the expression of KMP-11 is stage-specific and strongly down-regulated in the amastigote stage (Berberich et al. 1998). This statement is not easily reconciled with the immunoprotective capacity of vaccine prototypes based on the KMP-11-coding gene (Ramírez et al. 2001, Basu et al. 2005), with the T cell responses to KMP-11 found in leishmaniasis patients (Russo et al. 1992) or with the presence of anti-KMP-11 serum antibodies in *L. infantum*-infected dogs (Berberich et al. 1997), as well as in most leishmaniasis patients or asymptomatic

Leishmania-infected subjects (Ramírez et al. 1998). All of these observations are consistent with the presence of the protein in the amastigote form, the infective stage for the mammalian host. The contrast between the results obtained in the study from Berberich et al. (1998) and ours may be due to differences in the *Leishmania* species or the methodologies used. In the present study, we did not evaluate KMP-11 mRNA expression. Instead, we focused on protein expression using a quantitative approach. Moreover, it is interesting to note that expression of KMP-11 has been found in amastigotes from *L. donovani* (Jardim et al. 1995a, El Fadili et al. 2009), a species closely related to *L. infantum*, a member of the *L. donovani* complex (Lukes et al. 2007).

Here we provide evidence that, in *L. amazonensis*, KMP-11 is present in both promastigotes and amastigotes. The immunoblot analysis showed specific recognition of the molecule in whole-cell extracts from lesion amastigotes and also from metacyclic promastigotes, similar to that seen in the recombinant KMP-11 preparation (Fig. 1).

Flow cytometry has been extensively used for quantification of surface protein expression on mammalian cells (Ho & Springer 1983, Cole et al. 1999). This methodology has been used less frequently for parasite cells (Cuervo et al. 2008, Figueiredo et al. 2008). In order to compare KMP-11 expression levels on the surface of promastigotes and amastigotes, we followed a quantita-

tive approach using flow cytometry. Fig. 2 shows that not only KMP-11 is present in amastigotes but also its surface expression on this stage is markedly higher than on promastigotes. Among the promastigotes, we found that stationary phase promastigotes express more KMP-11 on their surfaces than do log phase promastigotes. Considering that the proportion of metacyclics is increased when promastigote cultures reach the stationary growth phase (da Silva & Sacks 1987), these results strongly indicate that KMP-11 expression is upregulated during metacyclogenesis. The high expression of KMP-11 in metacyclics is confirmed by the results from immunoblotting (Fig. 1). On the other hand, log phase promastigotes, with a low proportion of metacyclics (< 10%), showed no clear labelling on the immunoblot analysis.

Finally, we determined the localisation of this molecule in promastigotes and amastigotes. This was done by electron microscopy, using anti-KMP-11 monoclonal antibodies and colloidal gold-conjugated protein A. Fig. 3 shows that, in promastigotes, KMP-11 was localised at the flagellar pocket, at the surface plasma membrane, and associated with intracellular vesicular structures; whereas in amastigotes, it was found on the surface plasma membrane and on the plasma membranes of the flagellar pocket and intracellular vesicles. Our results confirm previous observations on the localisation of this molecule in promastigotes (Stebeck et al. 1995) and provide new information on its localisation in amastigotes. The localisation of KMP-11 in promastigotes and amastigotes is consistent with its marked affinity for lipid bilayers (Jardim et al. 1995b).

In conclusion, we demonstrated that KMP-11 is present in both life-cycle stages of *L. amazonensis* and that its expression is higher in amastigotes than in promastigotes. In both stages, the protein was found in association with membrane structures (on the cell surface, flagellar pocket and intracellular vesicles). Moreover, we also showed that, in *L. amazonensis*, KMP-11 expression increases during metacyclogenesis. The increased expression in metacyclic promastigotes and especially in amastigotes indicate a role for this molecule in parasite survival in the mammalian host. Interestingly, KMP-11 expression has been associated with virulence of *L. donovani* (Mukhopadhyay et al. 1998). Furthermore, peripheral blood mononuclear cells from American tegumentary leishmaniasis patients stimulated with KMP-11 produce high levels of interleukin-10 (de Carvalho et al. 2003, Carvalho et al. 2005), a cytokine responsible for pathogenesis and parasite persistence in leishmaniasis (Belkaid et al. 2001, Nylén & Sacks 2007).

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