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Membrane orientation of laminin binding protein
An extracellular matrix bridging molecule of *Leishmania donovani*

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Earlier we presented several lines of evidence that a 67-kDa laminin binding protein (LBP) in *Leishmania donovani*, that is different from the putative mammalian 67-kDa laminin receptor, may play an important role in the onset of leishmaniasis, as these parasites invade macrophages in various organs after migrating through the extracellular matrix. Here we describe the membrane orientation of this *Leishmania* laminin receptor. Flow cytometric analysis using anti-LBP Ig revealed its surface localization, which was further confirmed by enzymatic radiolabeling of *Leishmania* surface proteins, autoradiography and Western blotting. Efficient incorporation of LBP into artificial lipid bilayer, as well as its presence in the detergent phase after Triton X-114 membrane extraction, suggests that it may be an integral membrane protein. Limited trypsinization of intact parasite and subsequent immunoblotting of trypsin released material using laminin as primary probe revealed that a major part of this protein harbouring the laminin binding site is oriented extracellularly. Carboxypeptidase Y treatment of the whole cell, as well as the membrane preparation, revealed that a small part of the C-terminal is located in the cytosol. A 34-kDa transmembrane part of LBP could be identified using the photoactive probe, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID). Partial sequence comparison of the intact protein to that with the trypsin-released fragment indicated that N-terminal may be located extracellularly. Together, these results suggest that LBP may be an integral membrane protein, having significant portion of N-terminal end as well as the laminin binding site oriented extracellularly, a membrane spanning domain and a C-terminal cytosolic end.

Keywords: *Leishmania donovani*; extracellular matrix; laminin binding protein; topological distribution; integral membrane protein.

One of the primary events in the initiation of a disease is thought to be the attachment of the causative pathogen to the host epithelial cells and subsequently, penetration into these cells and inner tissue lining lead to disease progression. Besides specialized cells, the tissue and organ contain macromolecules like collagen, laminin, fibronectin, elastin, vitronectin, etc., that constitute the extracellular matrix (ECM) and basement membrane (BM). In the case of leishmaniasis, the causative parasite, *Leishmania donovani*, invades mammalian cells, primarily the resident macrophages of liver and spleen, where in successive steps they adhere, penetrate, transform into amastigotes and replicate. During this process, the host macrophage is lysed, parasites move in search of fresh target cells and infection is spread to the neighbouring cells [1]. In order to migrate from blood vessels, where they are introduced by the carrier sand fly bite, to the interior of the cell lysosome, where they differentiate, these parasites have to surpass the formidable barrier of the ECM and BM. The ability to adhere to ECM components may represent a mechanism by which the pathogen may avoid entrapment within the ECM, thus playing an important role in pathogenesis. Interaction with ECM proteins has been correlated with the invasive ability of different pathogens [2]. We earlier reported the presence of a 67-kDa glycoprotein on the surface of *L. donovani* that binds to laminin, a major protein of ECM [3]. This was found to be different from the putative mammalian 67-kDa laminin receptor based on computational analysis of internal sequences and Western blot analysis. Detailed characterization revealed that it might act as an adhesin that may constitute the basis for the homing of the parasites to its 'physiological address' [4–6]. Understanding of the mechanisms mediating the adherence of *L. donovani* to the ECM or host cells could lead to the development of antiparasitic agents whose mechanism of action would involve competition with the endogenous ligands for binding to pathogen receptors or adhesins. For this, the knowledge of membrane organization is crucial for the deduction of the functional mechanism of a surface binding protein. In the present paper, we have undertaken a detailed topological study of the 67-kDa laminin binding protein (LBP) on the surface of *L. donovani* promastigotes. We provide evidence that LBP is an integral membrane protein,

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Abbreviations: ECM, extracellular matrix; BM, basement membrane; LBP, laminin binding protein; TID, 3-(triflouromethyl)-3-(*m*-iodophenyl)diazirine; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-indolyl-3-phosphate.

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having a significant N-terminal end, a laminin binding site oriented extracellularly, a membrane spanning domain and a C-terminal cytosolic end.

Materials and methods

Parasites

L. donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis (undertaken with the understanding and written consent of the subject) [7] and subsequently maintained in BALB/c mice by intravenous passage every 6 weeks. Promastigotes, obtained by *in vitro* transformation of liver and spleen-derived amastigotes, were cultured at 22 °C in medium 199 (Invitrogen, Carlsbad, CA, USA) with Hanks salt containing Hepes (12 mM), L-glutamine (20 mM), 10% heat-inactivated fetal bovine serum, 50 $U \cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ streptomycin. *L. donovani* promastigotes (2.5×10^7 in 800 μL $NaCl/P_i$, pH 7.2) were surface-labeled with ^{125}I using lactoperoxidase-glucose oxidase as described earlier [8] and labeled metabolically with [^{35}S]methionine according to Kahl and McMohan [9].

Anti-LBP Ig

Polyclonal antibody to the LBP was raised by intraperitoneal injection of 20 μg LBP emulsified in complete Freund's adjuvant into male New Zealand rabbit. Three booster doses were administered at an interval of 2 weeks by injecting LBP emulsified in incomplete Freund's adjuvant. After 10 days from the fourth injection, blood was collected from rabbit ears and the anti-LBP Ig separated according to Hall *et al.* [10].

Flow cytometric analysis

After thorough washing with phosphate buffered saline ($NaCl/P_i$), *Leishmania* promastigotes were first treated with blocking solution ($NaCl/P_i$ containing 2% goat serum). After 1 h at room temperature, cells were treated with 100 μL rabbit anti-LBP Ig [1 : 50] in blocking solution for 1 h at room temperature. Cells were washed twice in $NaCl/P_i$, incubated for 30 min at room temperature in 100 μL fluorescein isothiocyanate (FITC) conjugated goat anti-(rabbit IgG) (Sigma Chemical Co) at a 1 : 50 dilution in blocking solution. Following another two washes with $NaCl/P_i$, cells were suspended in $NaCl/P_i$ containing 1% paraformaldehyde, and then analysed with a FACS Calibur cytofluometer using the CELLQUEST software (BD Biosciences, San Jose, CA, USA). The area of positivity was determined using preimmunized serum.

Incorporation of laminin binding protein into the liposome

Multilamellar liposomes were prepared with egg lecithin and cholesterol (Sigma) in a molar ratio of 1 : 1 according to the method described previously [11]. Radioiodinated LBP (2×10^6 c.p.m.$\cdot \mu g^{-1}$), labeled by the chloramine-T method [12] or the unlabeled protein was added to the liposome suspension at a protein to lipid ratio of 1 : 100, incubated at room temperature for 2 h and unbound material separated by size exclusion chromatography with Sepharose-CL-4B. Binding studies were carried out as described earlier [3] with liposome associated binding protein using [^{125}I]laminin as the ligand.

Separation of integral membrane proteins, electrophoresis and immunoblotting

The promastogote integral membrane proteins were separated according to the method of Bouvier *et al.* [13]. Briefly, 10^8 promastigotes from the stationary phase of growth were suspended in 10 mL of 10 mM Tris/HCl, pH 7.4 containing 150 mM NaCl and 1% Triton X-114, incubated at 0 °C for 10 min and centrifuged at 15 000 ***g*** for 15 min at 4 °C. The clear supernatant was overlaid on to a sucrose cushion [6% (w/v) sucrose in 10 mM Tris/HCl, pH 7.4 containing 150 mM NaCl and 0.06% Triton X-114], incubated for 3 min at 30 °C and then centrifuged at 1000 ***g*** for 10 min. The oily droplets that settled at the bottom of the centrifuge tubes were collected. These were subjected to re-phase separation twice more to yield an enriched integral-membrane protein preparation. These proteins were dissolved in SDS sample buffer, electrophoresed and immunoblotted as described previously [5]. Briefly, proteins were transferred to nitrocellulose membranes (0.45, Scheleicher and Schuell, Keene, NH, USA). The residual binding sites were blocked by incubation with 5% (w/v) nonfat dry milk, 1% (w/v) ovalbumin, 5% (v/v) fetal bovine serum and 7.5% (w/v) glycine for 30 min at room temperature with gentle shaking. The membranes were washed for 5 min each with 20 mM Tris/HCl (pH 7.4)/50 mM NaCl (TBS) containing 0.1% (v/v) Nonidet P40 (TBSN) and incubated for 1 h at 37 °C with laminin ($50 \mu g \cdot mL^{-1}$) in TBS supplemented with 1% (w/v) BSA (TBS/BSA). After washing with TBSN, membranes were treated with anti-laminin Ig in TBS/BSA at 37 °C for 30 min followed by another round of washing and incubation with alkaline phosphatase-conjugated goat anti-(rabbit IgG) $F(ab')_2$ (Sigma Chemical Co) at 1 : 500 dilution in TBS/BSA. The protein bands were developed with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) in 50 mM Tris/HCl, pH 9.5 containing 150 mM NaCl and 5 mM $MgCl_2$ [14]. In some cases, the nitrocellulose membranes were incubated with anti-LBP Ig and the second antibody instead of laminin and anti-laminin Ig to visualize the LBP.

Limited trypsin digestion of *L. donovani* promastigotes

L. donovani promastigotes (2×10^6) were incubated with 1 mL 0.1% trypsin in serum free medium. After incubation for 30 min at 37 °C, 1.5 mL of ice cold 0.15 M NaCl containing 0.1% (w/v) egg white trypsin inhibitor and 0.5% (w/v) BSA were added to stop the reaction. The trypsin treated cells were centrifuged (2100 ***g***, 10 min) to obtain the cell pellet and supernatant containing the trypsin released material. The cell pellet was then lysed under cold conditions (4 °C) in lysis buffer (5 mM Tris/HCl, pH 7.5, 0.5% Triton X-100, 25 mM KCl, 5 mM $MgCl_2$) in the presence of protease inhibitors [0.5 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ aprotinin, 50 $\mu g \cdot mL^{-1}$ soyabean trypsin inhibitor and 10 $\mu g \cdot mL^{-1}$ phenylmethanesulfonyl fluoride (PMSF)]. This

cell lysate and the supernatant containing the trypsin released material were subjected separately to immunoblotting using either anti-LBP IgG or anti-laminin IgG.

Proteolytic digestion of *L. donovani* membrane

L. donovani membrane preparations were made according to the method described previously [15]. Membrane preparations (100 μL) were incubated in the absence or presence of 0.1% (v/v) Triton X-100 with 0.025% (w/v) carboxypeptidase Y in a final volume of 150 μL. Incubation with carboxypeptidase was performed at pH 5.4 (adjusted with 1 M sodium acetate, pH 4.0). After incubation for 45 min at 37 °C, reaction was terminated by chilling on ice and adding 4 μL of 0.25 M PMSF and 60 μL of 0.5% (w/v) egg white trypsin inhibitor in 0.15 M NaCl. From the protease treated and untreated membrane preparations, LBP was isolated by immunoprecipitation with anti-LBP Ig.

[^{125}I]TID photolabeling of *L. donovani* promastigote integral membrane proteins

Trypsin treated or untreated *L. donovani* promastigotes (2.5×10^7 in 3 mL NaCl/P_i, pH 7.2) were equilibrated with 500 μCi of hydrophobic photoactivable probe, [^{125}I]TID (10 Ci·mM^{-1}, Amersham) for 15 min at 0 °C. In control experiments, 5 mM glutathione was added. To perform photolysis, the reaction mixture was kept under 400 W medium pressure mercury lamp for 10 min. Promastigotes were washed four times in NaCl/P_i, pH 7.2 containing 1.0% (w/v) BSA and then twice in NaCl/P_i, pH 7.2 without BSA. LBP was then immunoprecipitated with anti-LBP Ig from [^{125}I]TID-labeled *Leishmania*.

Amino acid sequence

Electrophoresis of the purified LBP was performed using 12% SDS/PAGE in a slab gel apparatus, the protein was subsequently transblotted onto a nitrocellulose membrane. The protein band was visualized by staining briefly with 0.1% Ponceau S in 1% acetic acid (v/v) and was destained quickly with (deionized) water. The stained band was excised and hydrolysed *in situ* with endopeptidase LysC in an Eppendorf tube at 37 °C overnight in 10 mM Tris HCl, pH 9.0 according to the process described elsewhere [16]. After digestion, the whole reaction mixture was loaded onto a C18 HPLC column (Hitachi HPLC, type, 7000) equilibrated with 0.1% (v/v) trifluoroacetic acid to separate the proteolytic peptides. The peptides were eluted with a shallow gradient of acetonitrile in 0.1% (v/v) acetic acid. The fractions were collected and stored at −20 °C. The amino acid sequence anlayses of the peptides were carried by a protein sequencer (Hewlett Packard N-sequence, Y1005A) and the amino acid residues were identified as phenylthiohydantoin derivatives.

Results

Surface localization of the laminin binding protein

As a part of molecular mechanism underlying the invasion of the extracellular matrix of solid organs like liver and spleen, a laminin binding component was detected previously from this laboratory in the protozoan parasite *L. donovani* [3]. Detail biochemical characterization revealed that it is a 67-kDa glycoprotein [4] and may act as an adhesin [5]. In order to determine the cellular localization of this laminin binding protein, flow cytometric analysis were performed using monospecific antibodies directed against affinity purified LBP followed by FITC-conjugated secondary antibody. With anti-LBP Ig, a strong fluorescence was obtained when compared to controls, where a preimmune serum was used as primary antibody (Fig. 1). It was further established by repeating the same experiment in presence of saponin, a detergent that makes cells permeable to antibody molecules. Similar mean fluorescence intensities (MFI) in the presence or absence of saponin indicated the surface localization of LBP (Fig. 1, inset). To further ascertain the membrane localization of LBP, *L. donovani* promastigotes were surface-labeled with ^{125}I by lactoperoxidase-glucose oxidase. Membrane proteins were then isolated by biotinylation and streptavidin/agarose extraction, analysed by SDS/PAGE and autoradiographed. The 67-kDa LBP, together with other membrane proteins were found to be intensely labeled (Fig. 2, lane 1). When the membrane proteins were transferred to nitrocellulose membrane and subjected to Western blot analysis using anti-LBP Ig, a single band at 67 kDa region was obtained (Fig. 2, lane 2), suggesting LBP to be one of the membrane proteins of *Leishmania* which were surface iodinated.

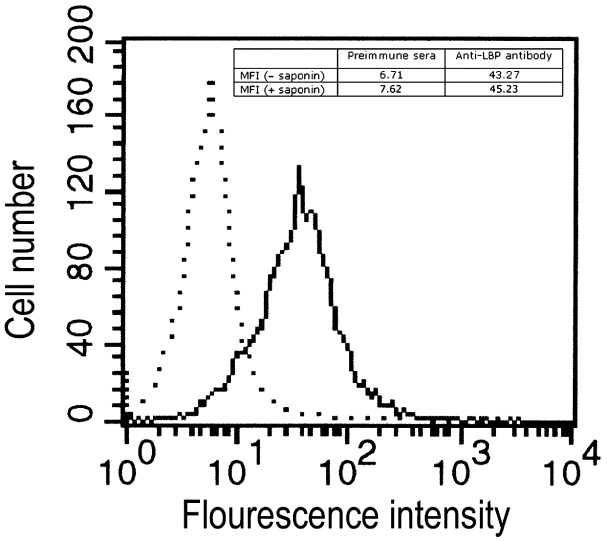

Fig. 1. Surface localization of LBP on *Leishmania* promastigotes by flow cytometry. Promastigotes were treated with preimmune serum (dotted line) or anti-LBP Ig (solid line) followed by goat anti-(rabbit IgG) coupled to FITC and then analyzed by flow cytometry. Mean flourescence intensity (MFI, inset) was compared for saponin-treated cells to that with untreated cells.

LBP is an integral membrane protein

The hydrophobic nature of LBP was confirmed by reconstituting the purified protein into a liposome. Almost 70% of the protein was found to be associated with the liposome

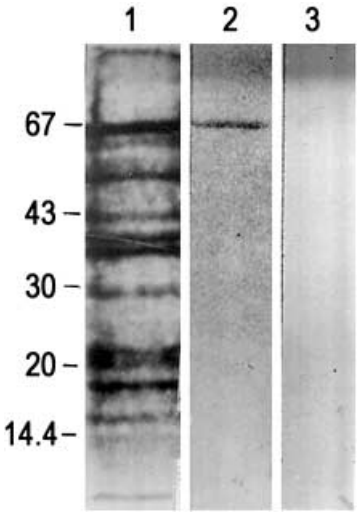

Fig. 2. Identification of LBP from radioiodinated Leishmania membrane proteins. Membrane proteins, isolated from surface iodinated *L. donovani* promastigotes, were resolved under denaturing conditions in 12.5% SDS/PAGE. Lane 1, isolated membrane proteins were subjected to autoradiography. Lane 2, membrane proteins were transferred onto nitrocellulose membrane and subjected to indirect immunoblot analysis using laminin as primary probe followed by rabbit anti-(laminin IgG), goat anti-(rabbit IgG), BCIP and NBT. Lane 3, transferred proteins were incubated with BSA instead of laminin.

fraction when separated in a Sepharose-4B column and liposome-incorporated LBP specifically bound [^{125}I]laminin with the same high affinity ($K_d = 5.64 \times 10^{-9}$ M) (Fig. 3) as did intact *L. donovani* promastigotes [5]. In contrast, purified LBP showed approximately 100-fold lower affinity ($K_d = 3.52 \times 10^{-7}$ M) compared with promastigotes that may be attributed to the presence of detergents. In order to ascertain whether LBP is an integral membrane protein, an extract of *L. donovani* promastigotes was made in Triton X-114, a detergent known to selectively accumulate integral membrane proteins in the detergent phase. SDS/PAGE analysis of the proteins of both the aqueous and detergent phases was performed and the separated proteins were transformed to nitrocellulose membrane for Western blot analysis. LBP was found to be present only in the detergent phase and not in the aqueous phase (Fig. 4A). Partitioning of LBP in Triton X-114 phase together with its efficient incorporation into liposomes suggests that it may be an integral membrane protein.

External orientation of the laminin-binding moiety

Leishmania promastigotes were subjected to mild trypsinization and centrifuged. This resulted in two fractions, a cell free supernatant containing trypsin released material and the cell pellet. Both the trypsin released material and the cell pellet lysate were then subjected to direct immunoblotting using anti-LBP Ig (Fig. 4B) as well as indirect immunoblotting using laminin as primary probe (Fig. 4C) followed by rabbit anti-laminin IgG and alkaline phosphatase conjugated goat anti-(rabbit IgG). Significantly, cross-reactive material could be detected in both supernatant and pellet in case of direct immunoblot, suggesting that anti-LBP Igs could detect reactive epitopes in both the trypsin released supernatant and the cell pellet. In other words, both the 27-kDa fragment of LBP released by trypsin as well as the 34-kDa fragment retained in the cell membrane could be detected by anti-LBP Ig (Fig. 4B, lanes 1 and 2). In contrast, in the case of indirect immunoblotting, signals could be detected only in the supernatant containing the 27-kDa part, implying thereby the presence of laminin binding region in the portion of LBP released by trypsin digestion (Fig. 4C, lanes 1 and 2).

Succeptibility of LBP to carboxypeptidase Y

L. donovani promastigotes were treated with carboxypeptidase Y for 30 min at 37 °C and pH 5.4. There was no change in the apparent molecular mass of LBP isolated from the enzyme treated parasites (Fig. 5, lane 1). However, when a membrane fraction isolated from *L. donovani* was digested with carbodypeptidase Y, there was a decrease in the apparent molecular mass of LBP by about 6 kDa (Fig. 5, lane 2). Upon addition of Triton X-100, the decrease in molecular mass caused by digestion with carboxypeptidase Y was about 9 kDa compared with 6 kDa in the absence of detergent (Fig. 5, lane 3). The relative resistance of intact cells to carboxypeptidase Y

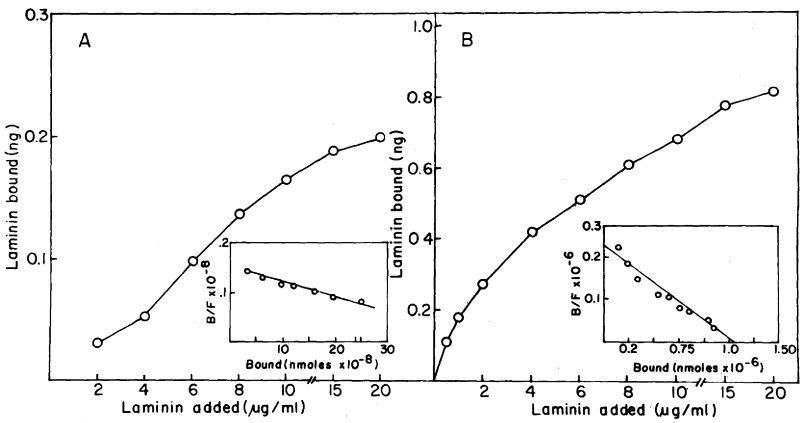

Fig. 3. Radiolabeled laminin binding to (A) isolated laminin receptor and (B) liposome incorporated receptor. Nitrocellulose discs were spotted with 1 μg of affinity purified LBP and then increasing amounts of [^{125}I]laminin were added to the discs in the presence or absence of 100-fold excess of unlabeled laminin. Binding assays in (A) and (B) were performed as described in the text. Insets show Scatchard analysis of specific binding data. B and F (see insets) represent concentrations of bound and free iodinated laminin, respectively.

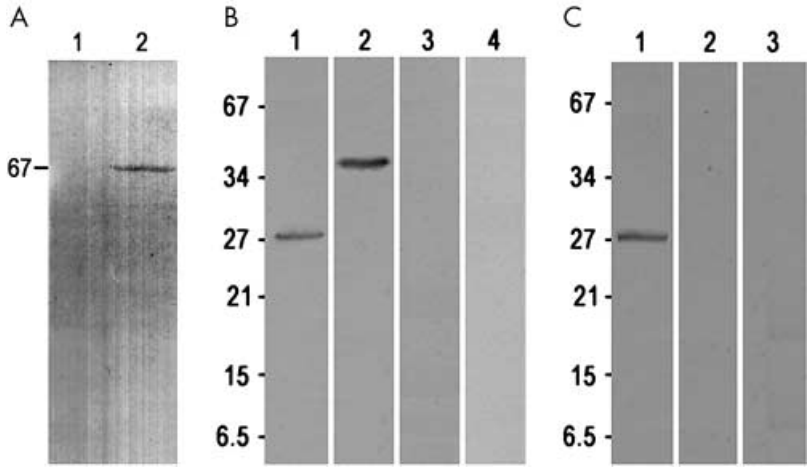

Fig. 4. Phase separation of LBP by Triton X-114 and external orientation of laminin binding domain of LBP. (A) *L. donovani* promastigote membrane proteins were extracted by Triton X−114 and subjected to phase seperation. The proteins partitioned in the aqueous, as well as in the detergent phases, were resolved on a 12.5% SDS/PAGE, transferred to nitrocellulose membrane and subjected to direct immunoblot analysis using rabbit anti-LBP Ig as the primary probe followed by alkaline phosphatase conjugated goat anti-(rabbit IgG), BCIP and NBT. Lanes 1 and 2 represent proteins extracted in the aqueous and detergent phases, respectively. (B) *L. donovani* promastigotes were trypsinized and centrifuged to obtain pellet and supernatant. These two parts were separately resolved on 12.5% SDS/PAGE (1 μg per lane), transferred onto nitrocellulose membrane and subjected to direct immunoblot analysis using rabbit anti-(LBP Ig) as the primary probe. Lane 1, cell supernatant; lane 2, cell pellet; lanes 3 and 4, supernatant and pellet, respectively, treated with pre-immune serum. (C) Transferred proteins were subjected to indirect immunoblot analysis using laminin as primary probe followed by anti-laminin IgG and alkaline phosphatase conjugated secondary antibody. Lane 1, cell supernatant; lane 2, cell pellet and lane 3, cell supernatant where the blot was incubated with BSA instead of laminin.

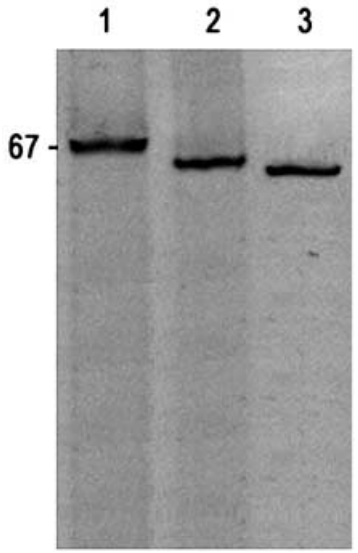

Fig. 5. Carboxypeptidase Y treatment of LBP. ^{35}S-metabolically labeled *L. donovani* promastigotes (1×10^4 cells) and membrane preparations (100 μg) were incubated at pH 5.4 for 30 min at 37 °C with carboxypeptidase Y as described in Materials and methods. LBP was immunoprecipitated, subjected to 15% SDS/PAGE and autoradiography. Lane 1, LBP immunoprecipitated from carboxypeptidase Y treated *L. donovani* promastigotes; lanes 2 and 3, LBP immunoprecipitated from carboxy peptidase Y treated membrane preparation in presence and absence of Triton X-100.

treatment together with the susceptibility of the isolated membrane to the enzyme suggest that C-terminal of LBP may be oriented intracellularly.

Intramembranous domain of LBP

To identify the intramembranous domain of LBP, a series of radiolabeling reactions involving the photoactivable hydrophobic probe, [^{125}I]TID was performed. Following photolabeling of *Leishmania* promastigotes with [^{125}I]TID, LBP was one of the most prominently radiolabelled proteins (data not shown). Leishmanial LBP can be cleaved by treating parasite with trypsin at one site generating a 27-kDa and a 34-kDa peptide, both of which can be immunoprecipitated from solubilized promastigotes using anti-LBP Igs. After tryptic digestion of photolabeled parasites, the 34-kDa peptide was found to be labeled by [^{125}I]TID (Fig. 6, lane 2). It was also recognized by anti-LBP Ig in the direct Western blot analysis (Fig. 6, lane 4). However, this 34 kDa peptide did not give any signal in the indirect Western blot analysis where laminin was used as primary probe (Fig. 6, lane 5). Control experiments with photolabeled but trypsin undigested promastigotes only highlighted a protein band in the 67 kDa region after immunoprecipitation and autoradiography (Fig. 6, lane 1), showing specificity of the TID incorporation. To determine if the labeling of the tryptic peptide was due to the presence of [^{125}I]TID in the aqueous phase, photolabeling was performed in the presence of 5 mM reduced glutathione that scavenges [^{125}I]TID present only in the aqueous phase [17]. Reduced glutathione did not affect the photolabeling of tryptic peptide of LBP (Fig. 6, lane 3), indicating that the photolabeling was not due to the presence of [^{125}I]TID in the aqueous phase. All these data indicate that the 34 kDa tryptic peptide identified by TID involves the intramembraneous region and the 27 kDa, unable to incorporate TID, involves the laminin binding region.

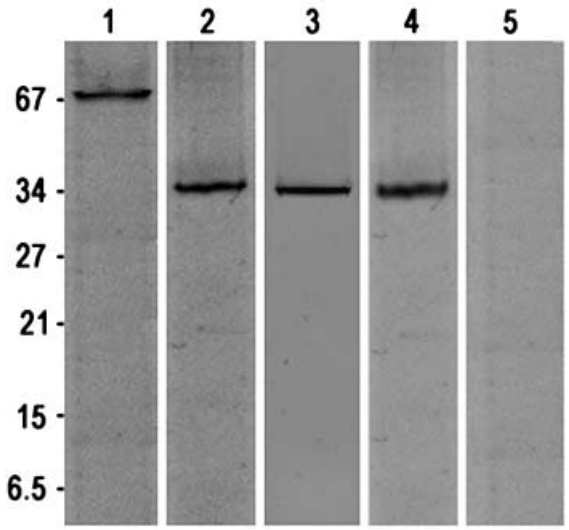

Fig. 6. Analysis of [^{125}I]TID-labeled LBP. LBP, isolated from trypsin treated and untreated [^{125}I]TID-labeled *L. donovani* promastigotes were subjected to autoradiography as well as direct and indirect Western blotting. Lane 1, LBP isolated from TID labeled promastigotes was run on a 12.5% SDS/PAGE and autoradiographed. Lanes 2 and 3, LBP isolated from trypsin-digested TID-labeled cells, in the presence and absence of 5 mM glutathione, respectively, were run on 12.5% SDS/PAGE and autoradiographed. Lanes 4 and 5, LBP, isolated from trypsin-digested TID-labeled cells were resolved on 12.5% SDS/PAGE, transferred onto nitrocellulose paper and monitored by both direct and indirect Western blotting as described in the legend of Fig. 4.

Extracellular orientation of the N-terminus

Preliminary attempts to determine the N-terminal sequence of LBP were not successful. In this case, no predominant phenylthiohydantoin-derivative was estimated through 1–10 cycles of Edman degradation. It was assumed therefore, that the N-terminal amino acid residue might be modified. Endopeptidase Lys C and CNBr digests of highly purified LBP were separated and purified by serial HPLC on an Ultrasphere C_8 reversed phase HPLC followed by re-chromatography on an C_{18} reverse phase column. The N-terminal amino acid sequences of three such oligopeptides were determined using a protein sequencer (LNILHRPGFIEXQR, IQWRNGDQQVLFDDL and IVGMYTRGAN). These sequences were checked for homology with other proteins in protein database using BLASTP server (NCBI, Bethesda, MD, USA) [18]. The search against all known protein sequences failed to reveal significant similarity (more than 60% match) to the peptides derived from LBP.

In order to ascertain the orientation of the N-terminal end of LBP, attempts were made to sequence the ectodomain released by mild trypsin digestion of the promastigote. If the N terminus of LBP is intracellular or associated with the cell membrane, trypsin treatment, which cleaves the cell surface LBP between its membrane-associated lipophilic domain and its extracellular domain, should generate either a new free N terminus or new multiple free N termini if there are multiple trypsin cleavage sites. Alternatively, if the N terminus is extracellular, no new free N termini will be formed as a result of trypsin treatment. Attempts to sequence the trypsin-released ectodomain revealed a high background that diminished rapidly during successive cycles of sequencing, indicating that either the N terminus is blocked or the concentration was not adequate to obtain a sequence. To distinguish these possibilities, the same filter was treated with CNBr to free the putatively blocked N terminus. An enhanced signal was obtained, yielding an identifiable signal sequence for eight cycles (XMYTRGXN), which matched with one of the LBP sequences (IVGMYTRGAN). Thus, the amount of material on the filter was sufficient for sequencing, indicating that the cell surface LBP has a blocked N terminus. As trypsin cleavage of the core protein would not produce a blocked amino acid residue, the N terminus of the cell surface LBP is likely to be located extracellularly.

Discussion

The general agreement among scientists about the most critical step in the establishment of a disease like leishmaniasis, by the obligate intracellular parasite *L. donovani*, involves the adherence of the parasite to the host cell plasma membrane [19]. ECM binding proteins on *Leishmania* surface are thought to play a crucial role in the onset of leishmaniasis as the ineffective parasites introduced into the blood when the sandfly bites, must come in contact with the ECM during their transit in the interstitial tissue on their way to liver and spleen. Towards this end, we have already identified and isolated an *L. donovani* surface protein that binds strongly to laminin, a major adhesive glycoprotein of ECM and basement membrane [3,5]. Preliminary evidence indicates that this protein may behave as an adhesin [4] and is involved in cell adhesion to laminin through the Tyr-Ile-Gly-Ser-Arg site on the B1 chain of laminin [6]. This protein may be similar to the previously described laminin receptor, which is present on many cells [20,21]. It has been shown that the recognition of laminin may influence the pathogenesis of several microorganisms, and receptors have been identified in various species of bacteria [22,23], parasites [24,25] and fungi [26,27]. The present study represents an initial attempt to map the organization of this protein in the parasite membrane in terms of its structural domain. This 67 kDa protein was purified from the promastigote membrane fraction by a three step procedure involving DEAE-cellulose, Con A-Sepharose and laminin-Sepharose affinity chromatography. Cell surface localization of the protein was demonstrated by (a) extracellular flowcytometry with anti-LBP Ig and (b) the fact that the protein can be labeled readily by surface radioiodination of intact cells. Efficient incorporation of LBP into liposomes may suggest its hydrophobic nature. Both the insolubility of LBP in aqueous buffer without detergent and its ability to incorporate into liposome support the notion that it may be an integral membrane protein. This was further confirmed by direct immunoblot experiments with Triton X-114 partitioning of the promastigote lysate.

In this study, we have used limited proteolysis and C-terminal exopeptidase together with direct and indirect immunoblotting to identify the orientation of three readily cleaved domains, the extracellular amino terminal region

that contains bound carbohydrate, the large intramembranous domain and the carboxy terminal intracellular region. The formation of a distinct peptide of 27 kDa after trypsin treatment of cells indicates the existence of one large extension protruding at the external side of the plasma membrane. From the size of the fragment it may be inferred that a major part of LBP is exposed at the external site of the plasma membrane and correspondingly, at the luminal side of intracellular membrane-delimited organelles. The extension protruding at the external site contains the binding site(s) for laminin as revealed by indirect immunoblotting experiments using laminin as primary probe. Furthermore, as the effect of treatment with tunicamycin and endoglycosidase F demonstrates that LBP contains N-linked carbohydrate [4] and as N-glycosylation normally takes place only on the luminal side of the endoplasmic reticulum, this region is likely to be extracellular [28]. Amino acid sequencing of intact and CNBr fragments of both the LBP and the trypsin-released ectodomain establishes that both of them share a blocked N terminus and an identical partial amino acid sequence. These results suggest that LBP is oriented at the cell surface with its N terminus located extracellularly. This orientation would put the cell surface LBP among type I cell surface receptors [29], e.g. glycophorin [30], lymphocyte histocompatibility antigens [31] and vesicular stomatitis virus G-protein [32].

[^{125}I]TID has been a useful tool for identifying transmembrane domains of proteins [17,33,34]. [^{125}I]TID partitions efficiently into membrane lipid bilayers and photolabeling of proteins with [^{125}I]TID occur predominantly in domains that are in direct contact with membranes [17]. Hydrophobic photolabeling data demonstrated the intramembranous nature of LBP. Control experiments involving labeling in the presence of glutathionine followed by immunoblot analysis confirmed the hydrophobic specificity of the reagent. Quantitatively, incorporation of [^{125}I]TID into LBP was consistent with similar labeling with another intramembranous protein [9]. Carboxypeptidase Y was found to have no effect on intact cells. However, treatment of isolated membranes with the enzyme led to an apparently homogeneous product that is smaller in size by 6 kDa. This indicates that only a small part is exposed at the cytosolic side of the membrane and forms the C terminus of LBP.

Taken together, this leads to a topographic model for LBP in which the intramembranous domain is associated with the lipid bilayer, and is flanked by an extracellular N-terminal domain containing N-linked carbohydrate chains and the laminin binding domain and a transmembrane span with the extreme C-terminal residues exposed at the intracellular surface. This topological arrangement is consistent with the sensitivity of the protein to externally added proteases as well as the fact that the protein was accessible to laminin binding in intact cells and was readily radioiodinated. Apart from its importance as a possible mediator for homing of the parasites to their physiological address, the fact remains that the plasma membrane of these cells represents an important biological interface between host and parasite and, as such, probably occupies a pivotal position in multiple signaling pathways. Naturally, these topics are among the most important areas of research in molecular parasitology and a crucial step toward improving our understanding of these processes must be the complete characterization of the plasma membrane proteins that mediate them.

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