Isolation of a laminin-binding protein from the protozoan parasite *Leishmania donovani* that may mediate cell adhesion

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Extracellular matrix (ECM)-binding proteins on the surface of *Leishmania* are thought to play a crucial role in the onset of leishmaniasis, as these parasites invade mononuclear phagocytes in various organs after migrating through the ECM. In a previous report, we presented several lines of evidence suggesting that *Leishmania* has a specific receptor for laminin, a major ECM protein, with a K_d in the nanomolar range. Here we describe the identification, purification and biochemical characterization of the *Leishmania* laminin receptor. When the outer membrane proteins of *L. donovani* were blotted on to nitrocellulose paper and probed with laminin, a prominent laminin-binding protein of 67 kDa was identified. The purified protein was isolated by a three-step process involving DEAE-cellulose, Con A (concanavalin A)-Sephacrose and laminin-Sepharose affinity chromatography and was used to raise a monospecific antibody. The same protein was obtained when parasite membrane extracts

were adsorbed to antibody affinity matrix and eluted with glycine. The affinity-purified protein bound to laminin in a detergent-solubilized form as well as after integration into artificial bilayers, and was subsequently characterized as an integral membrane protein. Metaperiodate oxidation and metabolic inhibition of glycosylation studies indicate the binding protein to be glycoprotein in nature and that N-linked oligosaccharides play a part in the interaction of laminin with the binding protein. Surface-labelled parasites attached to microtitre wells coated with laminin and the 67 kDa protein blocked the adhesion to laminin substrate. We propose that the 67 kDa protein is an adhesin involved in the attachment of *Leishmania* to host tissues.

Key words: binding protein, extracellular matrix, integral membrane protein, liposomes, visceral leishmaniasis.

INTRODUCTION

Visceral leishmaniasis, a parasitic disease arising from *Leishmania donovani* infection, is one of the major killer diseases afflicting the Third World, especially those people living below the poverty level. The parasite is introduced by its vector, the infected sandfly, when it regurgitates the promastigote form of the parasite at the time of taking a blood meal from the human body. The blood-borne promastigotes take shelter predominantly inside the liver and spleen macrophages. These macrophages serve as congenial thriving grounds for the parasites, which transform into the amastigote form inside the phagolysosomes and multiply in number. The macrophage eventually bursts open to release the amastigotes, which are phagocytosed by the adjacent macrophages. In this process a chain reaction gets initiated leading to the colonization of an organ like the liver and can prove fatal if chemotherapy is not started in the early stages of the disease.

Organs like the liver and spleen are not just conglomerations of cells endowed with specialized functions. They also contain components, like the extracellular matrix (ECM) and the basement membrane, which play important roles in tumour metastasis, tissue invasion, neurite spreading and differentiation. The ECM and the basement membrane are comprised of structural glycoproteins, like collagen and elastin, and adhesive glycoproteins, like laminin and fibronectin. Although extensive literature is available regarding host-parasite interactions in leishmaniasis [1], very little is known about the molecular recognitions that enable the parasite to cross the epithelial barrier of the basement membrane and the ECM of the liver and the spleen. In the absence of such recognition systems, the

parasites might get entrapped within the meshwork of the ECM. ECM proteins are implicated in the attachment of a variety of extracellular and intracellular pathogens to host tissues or cells [2–4]. Laminin is the major adhesive glycoprotein of the ECM and the basement membrane. It is a large (850 kDa) multimeric, ubiquitously distributed, glycoprotein which plays a critical role not only in normal cell adhesion but also during tissue invasion and metastases by tumour cells and certain pathogens. These functions of laminin are attributed to the presence of laminin-specific receptors on the surface of both normal [5,6] and tumour [7,8] cells and it is postulated that its recognition may influence the pathogenicity of several micro-organisms, including bacteria [9–11], protozoa [2,12,13] and fungi [3,14,15]. In this report we discuss the purification and characterization of a laminin-binding protein which we detected earlier on the surface of *L. donovani* [16].

MATERIALS AND METHODS

Parasites

L. donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis [17] 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 (Gibco, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal calf serum and 50 units/ml penicillin and streptomycin. *L. donovani* promastigotes were surface-labelled with ¹²⁵I by using lactoperoxidase-glucose oxidase as described

Abbreviations used: ECM, extracellular matrix; TBS, Tris-buffered saline; TBSN, Tris-buffered saline containing Nonidet P40; BCIP, 5-bromo-4-chloro-indol-3-yl phosphate; LBP, laminin-binding protein; TCM, Tris calcium magnesium buffer; Con A, concanavalin A.

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earlier [18] and metabolically labelled with [^{35}S]methionine according to [19].

Laminin-binding assay

The binding of ^{125}I -labelled laminin to *L. donovani* was quantified as described previously [16]. In brief, *L. donovani* promastigotes were incubated with graded concentrations of laminin at 4 °C for 30 min in PBS containing 1 % BSA. After thorough washing with PBS, cells were assayed for bound radioactivity. Laminin (100 μg) was iodinated with 1 mCi of ^{125}I (carrier-free; Amersham, Arlington Heights, IL, U.S.A.) by the chloramine-T method [20] to a specific activity of $(3\text{--}5) \times 10^6$ c.p.m./ μg . Non-specific binding was determined in the presence of 100-fold excess of unlabelled ligand and was not more than 15 % of the total binding.

Isolation of promastigote plasma-membrane proteins

L. donovani promastigotes (2×10^8) were incubated at 22 °C for 10 min with 100 μg of sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL, U.S.A.). After extensive washing with PBS to remove excess unbound biotin, parasites were lysed in 1 ml 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 $\mu\text{g}/\text{ml}$ leupeptin/1 $\mu\text{g}/\text{ml}$ aprotinin/50 $\mu\text{g}/\text{ml}$ soya bean trypsin inhibitor/10 $\mu\text{g}/\text{ml}$ PMSF), centrifuged at 12000 *g* for 30 min at 4 °C and the supernatant adsorbed on to a strept-avidin-agarose column (1 ml, Pierce Chemical Co.). Membrane proteins were eluted with 25 mM Tris/HCl (pH 7.5), supplemented with 5 mM MgCl_2 /30 mM β -octylglycoside and dialysed against the lysis buffer before storage at -70 °C.

Purification of laminin-binding protein (LBP)

The promastigote membrane proteins were loaded on to a DEAE-cellulose column (1 cm $\times$ 10 cm) previously equilibrated with 50 mM Tris/HCl (pH 7.4) containing 1 mM EDTA/0.5 mM PMSF/25 units/ml aprotinin (buffer I). The column was washed with 50 ml of buffer I and the bound material eluted with 100 ml of a linear gradient of 0–400 mM NaCl in buffer I. The eluate was dialysed against 10 mM Tris/HCl (pH 7.4) containing 0.2 M NaCl/0.1 % Nonidet P40 (buffer II), and was passed through a concanavalin A (Con A)-Sepharose column (1 cm $\times$ 41.5 cm) previously equilibrated with buffer II. The bound proteins were eluted with 10 mM Tris/HCl (pH 7.4)/1 mM NaCl/0.1 % Nonidet P40/1 M α -methyl-D-mannopyranoside and dialysed against 10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1 % Nonidet P40. The dialysate was mixed with an equal volume of laminin-Sepharose, which was prepared by coupling laminin (25 μg) with 100 μl of cyanogen bromide-activated Sepharose CL-4B, and incubated for 16 h at 4 °C with shaking. The resins were washed twice with 10 mM Tris/HCl (pH 8.0)/0.1 % Triton X-100/150 mM NaCl and once with 50 mM Tris/HCl (pH 7.2). The bound substances were eluted with 2 % SDS, dialysed against 10 mM Tris/HCl (pH 7.4), concentrated to approx. 1 mg/ml and stored at -70 °C.

Electrophoresis and immunoblotting

The promastigote membrane proteins and the eluates from the DEAE-cellulose, and Con A- and laminin-Sepharose columns were resolved by 7.5 % SDS/PAGE under reducing conditions and transferred to nitrocellulose membranes (0.45 μm ; Schleicher and Schuell, Keene, NH, U.S.A.) using a Bio-Rad semi-dry transfer apparatus (Bio-Rad, Hercules, CA, U.S.A.). The residual binding sites were blocked by incubation with 5 % non-fat dry

milk/1 % ovalbumin/5 % fetal calf serum/7.5 % 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)/150 mM NaCl (TBS) containing 0.1 % Nonidet P40 (TBSN) and incubated for 1 h at 37 °C with laminin (50 $\mu\text{g}/\text{ml}$) in TBS supplemented with 1 % BSA (TBS-BSA). After washing with TBSN, membranes were treated with rabbit anti-laminin antibody (1:100 dilution, Sigma) 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) at 1:500 dilution in TBS-BSA. The protein bands were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-indol-3-yl phosphate (BCIP) in 50 mM Tris/HCl (pH 9.5)/150 mM NaCl/5 mM MgCl_2 [21]. In some experiments the nitrocellulose membranes were incubated with anti-LBP antibody and the second antibody instead of laminin and anti-laminin antibody to visualize the LBP.

Preparation of antibodies to the LBP

Polyclonal antibody to the LBP was raised by intraperitoneal injection of 20 μg of LBP emulsified in complete Freund's adjuvant into male New Zealand rabbits. Three booster doses were administered at intervals of 2 weeks by injecting LBP emulsified in incomplete Freund's adjuvant. Blood was collected 10 days after the fourth injection from rabbit ear and the anti-LBP antibodies separated according to [22].

Immunoprecipitation

Metabolically labelled (with [^{35}S]methionine) *L. donovani* promastigotes (2×10^6) were lysed in 50 mM Tris/HCl (pH 7.5)/1 % Nonidet P40/0.25 % sodium deoxycholate/150 mM NaCl/1 mM NaF/1 mM orthovanadate/1 mM PMSF/10 $\mu\text{g}/\text{ml}$ aprotinin/10 $\mu\text{g}/\text{ml}$ leupeptin/2 $\mu\text{g}/\text{ml}$ pepstatin A (lysis buffer) under ice-cold conditions as outlined elsewhere [23]. The lysate was centrifuged, anti-LBP IgG (1:250 dilution) was added to the clear supernatant and kept at 4 °C for 18 h. Protein A-agarose was added and the mixture incubated with shaking for another 2 h at 4 °C. After washing with lysis buffer, agarose beads were boiled in SDS sample buffer for 2 min to release the immune complexes. The released proteins were then electrophoresed under reducing conditions in 12.5 % SDS/PAGE. In control experiments, instead of anti-LBP antibody, preimmune serum was used. Electrophoresis gels were then dried and exposed to X-Omat RP film (Eastman Kodak Co., Rochester, NY, U.S.A.) at -70 °C for autoradiography.

Binding assay of affinity-purified protein

Binding assay of the purified protein was done according to Malinoff and Wicha [24]. Nitrocellulose discs (6 mm diameter) were spotted with 1 μg of protein each in a total volume of 10 μl and blocked by 5 % BSA in PBS at 37 °C for 1 h. Increasing amounts of [^{125}I]laminin were added to the discs with or without a 100-fold excess of unlabelled laminin in a final volume of 50 μl and incubated for 30 min at 20 °C. The discs were then washed thrice with 5 % BSA solution and counted for radioactivity retained in them.

Reconstitution of binding proteins into liposomes

Multilamellar liposomes were prepared with egg lecithin and cholesterol (Sigma) in a molar ratio of 1:1 according to the method described earlier [25]. Radio-iodinated LBP (2×10^6 c.p.m./ μg), labelled by the chloramine-T method [20], or

the unlabelled 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 detailed earlier with liposome-associated binding protein using [125 I]laminin as ligand.

Tunicamycin and metaperiodate treatment

Tunicamycin and metaperiodate treatments were carried out according to Chammas et al. [26]. *L. donovani* promastigotes were treated for 24 h with tunicamycin (solubilized in DMSO; Sigma) at a final concentration of 5 μ g/ml. LBP or LBP transferred to nitrocellulose strips were treated with various concentrations of metaperiodate (1–20 mM) in 50 mM sodium acetate buffer (pH 5.5) for 15 min in the dark at 4 °C. Control and experimental strips were then rinsed with 50 mM sodium acetate buffer, pH 5.5, and exposed to 50 mM sodium borohydride in PBS for 30 min at room temperature.

Separation of integral membrane proteins

The promastigote integral membrane proteins were separated according to the method of Bouvier et al. [27]. Briefly, 10^8 promastigotes from the stationary phase of growth were suspended in 10 ml of 10 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 % Triton X-114, incubated at 0 °C for 10 min and centrifuged at 15000 *g* for 15 min at 4 °C. The clear supernatant was overlaid on to a sucrose cushion [6 % (w/v) sucrose/10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.06 % Triton X-114], incubated for 3 min at 30 °C and then centrifuged at 1000 *g* for 10 min. The oily droplets 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 and electrophoresed in a 12.5 % SDS/PAGE and immunoblotted as described earlier.

Lectin staining of the LBP

The affinity-purified LBP was electrophoresed under reducing conditions in a 12.5 % SDS/PAGE and transferred to nitrocellulose membrane as mentioned earlier. After rinsing the membrane with 25 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM CaCl_2 /0.5 mM MgCl_2 (TCM), residual binding sites were blocked by incubating in 2 % BSA in TCM for 1 h at room temperature. The membrane was then incubated in a solution containing 10 μ g/ml Con A and 2 % BSA in TCM for 30 min at room temperature, washed three times, each for 10 min in TCM, and allowed to incubate with 10 μ l/ml horseradish peroxidase/2 % BSA in TCM for 30 min at room temperature [28]. After three more washes with TCM, the blot was developed with 0.5 mg/ml 4-chloro-1-naphthol in PBS containing 0.01 % hydrogen peroxide. Con A incubated in the presence of 100 mM methylmannoside was used as control.

Solid-phase binding assay

In adhesion assays, microtitre wells (Costar, Cambridge, MA, U.S.A.) were coated with 50 μ l of laminin (100 μ g/ml) and blocked with BSA as reported earlier. To the wells, 125 I-labelled parasites (5×10^5 parasites/ml) were added and allowed to incubate for 60 min at 22 °C. The wells were then washed extensively with PBS containing 0.1 % Tween 20 and the radioactivity measured. All readings were corrected for background values which represented radioactivity recovered in wells coated with BSA alone.

RESULTS

Characterization of laminin binding to *L. donovani*

Preliminary experiments showed that *L. donovani* promastigotes adhere to laminin immobilized on plastic surfaces, but not to IgG- or BSA-coated surfaces. This suggested the presence of laminin-binding activity on promastigotes. The binding of [125 I]-laminin to *L. donovani* promastigotes was very rapid and essentially complete within 60 min. Prolonged incubation did not result in additional binding of [125 I]laminin. The specificity of laminin binding by *L. donovani* was examined by incubating parasites with [125 I]laminin in the presence of excess amounts of a variety of unlabelled proteins. Addition of unlabelled laminin effectively blocked the binding of labelled ligand. Other molecules tested included heparin, chondroitin sulphate, hyaluronic acid, collagen, fibronectin and vitronectin, none of which affected the ligand binding. When increasing concentrations of native and denatured laminin were tried as inhibitors of [125 I]laminin binding to parasites we found that the native laminin was a more potent

Figure 1 Radiolabelled laminin binding to *L. donovani* parasites

(A) Parasites (1×10^6) were incubated for 1 h with [125 I]laminin (1.2×10^6 c.p.m./ μ g) in the presence of increasing concentrations of native (○) or denatured (Δ) laminin, or BSA (●). The amount of bound radiolabelled laminin was determined. Inhibition is expressed as a percentage of [125 I]laminin bound to parasites in the absence of any potential inhibitors. (B) Increasing amounts of radiolabelled laminin (1.2×10^6 c.p.m./ μ g) were added to a constant number (1×10^6) of promastigotes. Parasites were incubated for 30 min at 4 °C. The amount of [125 I]laminin bound to the parasites was determined. Non-specific binding (i.e. binding in the presence of 100-fold molar excess of unlabelled laminin) was subtracted from the total. A Scatchard plot (inset) was constructed from the specific binding data. B and F (inset) represent concentrations of bound and free iodinated laminin respectively. Data represent duplicate determinations in three separate experiments.

Figure 2 Purification profile of LBP from *L. donovani* promastigotes by SDS/PAGE and immunoblot analysis

(A) *L. donovani* promastigote membrane proteins isolated by biotinylation and streptavidin–agarose extraction were resolved under denaturing conditions in a 7.5% SDS/PAGE (lane 1) and transferred to nitrocellulose membrane. The transferred proteins were subjected to indirect immunoblot analysis using laminin as the primary probe followed by rabbit anti-laminin IgG, goat anti-rabbit IgG, BCIP and Nitro Blue Tetrazolium (lane 2). Lane 3 was incubated with BSA instead of laminin. (B) The isolated promastigote membrane proteins were passed through various chromatographic columns. The eluates from DEAE–cellulose (lane 1), Con A–Sepharose (lane 2), laminin–Sepharose (lane 3) and anti-LBP–Sepharose (lane 4) were analysed by 7.5% SDS/PAGE under reducing conditions and silver-stained. (C) The eluates from the various chromatographic columns after being resolved by gel electrophoresis (as described in B) were transferred to nitrocellulose membrane and subjected to indirect immunoblot analysis as described in (A). Lane 2, DEAE–cellulose; lane 3, Con A–Sepharose; lane 4, laminin–Sepharose; lanes 1 and 5, anti-LBP–Sepharose. Lane 1 was treated with BSA instead of laminin. (D) The eluates from the various chromatographic columns were subjected to direct immunoblot analysis using rabbit anti-LBP antiserum as primary probe. Lane 1, biotinylated membrane proteins; lane 2, DEAE–cellulose; lane 3, Con A–Sepharose; lane 4, laminin–Sepharose; lanes 5 and 6, anti-LBP–Sepharose; lane 6 was treated with preimmune serum. The molecular masses are indicated to the left of each panel.

inhibitor compared with the denatured forms (Figure 1A), indicating that native laminin displayed a higher affinity than denatured laminin for the leishmanial laminin-binding component. Incubation of *L. donovani* with increasing concentrations of [125 I]laminin showed that the cells could be saturated with labelled ligand (Figure 1B). The amount of bound ligand increased to a saturation level of 9.7 ng of laminin bound per 10^6 cells at a laminin concentration of 20 μ g/ml. Scatchard analysis of the specific binding data revealed a straight line (Figure 1B, inset), indicating one class of laminin receptor sites with high affinity, and yielded a K_d value of 3.56×10^{-9} M and approx. 9000 sites per cell.

Purification of LBP from *L. donovani*

Mild digestion of *L. donovani* promastigotes with trypsin and Pronase E markedly reduced binding of [125 I]laminin, suggesting that the leishmanial laminin receptor contained a protein component. To assess whether the interaction of laminin with parasites is mediated by specific protein components, membrane proteins of *L. donovani* promastigotes were obtained by biotinylation and streptavidin–agarose extraction and subjected to Western-blot analysis with laminin. Membrane proteins were first resolved in a 7.5% reducing SDS/PAGE and transferred to a nitrocellulose membrane. After blocking the residual unbound sites, the membrane was sequentially treated with laminin, rabbit anti-laminin antibody and alkaline phosphatase-conjugated goat anti-rabbit antibody. The reaction of the Nitro Blue Tetrazolium–BCIP complex with alkaline phosphatase revealed a major 67 kDa protein in the experimental strip (Figure 2A, lane 2). The control nitrocellulose strip, which was devoid of laminin treatment, failed to reveal any band, thereby suggesting the specificity of the reaction (Figure 2A, lane 3).

To isolate the laminin-binding component, *L. donovani* membrane proteins were subjected to a three-step purification procedure involving DEAE–cellulose, Con A–Sepharose and an affinity chromatography of laminin–Sepharose. Analysis by PAGE showed that the material bound to the final step of laminin–Sepharose and subsequently eluted with 2% SDS contained a single band of molecular mass 67 kDa (Figure 2B, lane

4). When the Con A–Sepharose eluate was passed over a collagen–Sepharose column and the column subsequently eluted with SDS, the 67 kDa protein was not seen in the eluate. Moreover, when the flow-through from the collagen–Sepharose column was applied to the laminin–Sepharose column, the 67 kDa protein was bound to the column (results not shown). The presence of a laminin-binding component of similar molecular mass was demonstrated in experiments in which samples from different steps of purification were separated by electrophoresis, electroblotted on to nitrocellulose membrane and probed with laminin, anti-laminin antibody and alkaline phosphatase-conjugated second antibody (Figure 2C). In the membrane fraction as well as in various chromatographic steps most of the laminin that bound nitrocellulose was associated with a 67 kDa component. However, lower-molecular-mass species visible in some of the earlier fractions indicated the possibility that other minor proteins may also bind laminin. This could also have been the result of non-specific interactions and/or degradation of the parent protein. An antiserum generated by immunizing a rabbit with purified 67 kDa protein was found to react with a single band both in the membrane fraction and in various purification fractions with mobility identical to the antigen (Figure 2D). Preimmune IgG did not give a detectable signal with the material blotted, as reported in Figure 2(D), lane 6. The affinity-purified protein, which was incubated with other ECM proteins like fibronectin, collagen or vitronectin on the nitrocellulose membrane did not show any band in the Western-blot analysis.

Detection of LBP by immunoprecipitation

In order to demonstrate the authenticity of the raised antiserum, we used anti-LBP antiserum and protein A–Sepharose beads to selectively precipitate out and catch the LBP from metabolically labelled *L. donovani* promastigotes. When these immune complexes were dissociated, run on SDS/PAGE and autoradiographed, we observed a single band at 67 kDa position in the lane containing anti-LBP-antibody-treated parasite extract (Figure 3). No band was present in the lane which contained parasite lysate treated with preimmune serum.

Figure 3 Immunoprecipitation of the LBP

Promastigotes were labelled metabolically with [35 S]methionine, lysed and the LBP was immunoprecipitated by anti-LBP antibody and autoradiographed. Lanes 1 and 2 represent promastigote lysate treated with preimmune sera and anti-LBP antibody, respectively.

Assay of laminin binding to affinity-purified protein

To ascertain if the 67 kDa purified protein represented a soluble form of the laminin receptor, the protein was tested for its ability to specifically bind exogenous laminin using the nitrocellulose-filter disc assay. A dose-dependent specific and saturable binding was observed (Figure 4A) with approx. 100-fold lower affinity ($K_d = 3.52 \times 10^{-7}$ M) compared with promastigotes, which may be attributed to the presence of detergents. Attempts were made to study the binding in the absence of detergents by inserting LBP into liposomes. Almost 70% of LBP was found to be associated with the liposome fraction when separated in a Sepharose-4B column. In contrast to detergent-solubilized protein, liposome-incorporated LBP specifically bound [125 I]laminin with the same high affinity (Figure 4B, $K_d = 5.65 \times 10^{-9}$ M) as did intact *L. donovani* promastigotes.

Figure 4 Radiolabelled laminin binding to (A) isolated laminin receptor and (B) liposome-incorporated receptor

Nitrocellulose discs were spotted with 1 μ g of affinity-purified LBP in 10 μ l of PBS and then increasing amounts of [125 I]laminin were added to the discs in the presence or absence of 100-fold excess of unlabelled laminin. Binding assays in (A) and (B) were performed as described in the text. Insets show Scatchard analyses of specific binding data. B and F (see the insets) represent concentrations of bound and free iodinated laminin respectively.

Figure 5 Immunoblot analysis of *L. donovani* proteins solubilized by Triton X-114

The promastigote membrane proteins were extracted by Triton X-114 and subjected to phase separation. The proteins partitioned in the aqueous as well as in the detergent phases were resolved on a 12.5% denaturing gel, transferred to nitrocellulose membrane and subjected to Western-blot analysis. Lanes 1 and 2 represent proteins extracted in the aqueous and detergent phases, respectively.

Biochemical characterization of LBP

LBP is an integral membrane protein

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. An SDS/PAGE analysis of the proteins of both the aqueous and detergent phases was performed and the separated proteins were transferred to nitrocellulose membrane for Western-blot analysis. LBP was found to be present only in the detergent phase and not in the aqueous phase (Figure 5). Although Triton X-114 partitions integral membrane proteins along with glycosyl-phosphatidyl-inositol-anchored proteins and a number of other proteins in the

Figure 6 Lectin staining of the laminin-Sepharose affinity-purified protein from *L. donovani* promastigotes

The affinity-purified LBP was electrophoresed under reducing conditions on 12.5% SDS/PAGE and transferred to the nitrocellulose membrane. Lectin staining was performed by incubating the membrane with Con A-peroxidase in the presence (lane 1) and absence (lane 2) of methylmannoside.

Table 1 Binding of radiolabelled laminin to metaperiodate-oxidized LBP

Binding of native laminin to native LBP was considered as 100%. Data represent means $\pm$ S.D. of determinations made in triplicate.

Ligand	Binding (%)	
	Native LBP	Oxidized LBP
Native laminin	100 $\pm$ 4	74 $\pm$ 8
Oxidized laminin	44 $\pm$ 5	25 $\pm$ 2

detergent phase [27], the efficient incorporation of LBP into liposomes suggests that it may be an integral membrane protein.

LBP is a glycoprotein

The glycoprotein nature of LBP was first demonstrated by lectin staining when purified LBP was probed with Con A-peroxidase on nitrocellulose membrane and developed with hydrogen peroxide and 3,3'-diaminobenzidine for the colour reaction (Figure 6). No such characteristic staining was observed in the nitrocellulose membrane when LBP was incubated with Con A-peroxidase in the presence of methylmannoside. In order to further determine the glycoconjugate nature of LBP, graded concentrations of either native or metaperiodate-oxidized [125 I]-laminin were incubated with a fixed concentration of either native or metaperiodate-oxidized LBP under optimal binding conditions, as described earlier. Significant decreases in binding activities were observed irrespective of whether oxidized laminin was used as ligand or oxidized LBP was used as receptor (Table 1). These results suggest that sugar residues of both the interactants play a part in this binding and that both laminin and LBP may be considered as lectin-like molecules. To further ascertain the role of LBP oligosaccharide moieties in its interaction

Figure 7 Laminin binding to metaperiodate-oxidized LBP and tunicamycin-treated *L. donovani* promastigotes

(A) Affinity-purified LBP was separated on 12.5% SDS/PAGE and transferred to nitrocellulose filters. Strips were then treated with increasing concentrations of metaperiodate and subjected to indirect immunoblot analysis as described in the legend of Figure 2. Lanes 1 and 2 were treated with laminin and BSA respectively. Lanes 3–5 represent strips treated with 1, 5 and 20 mM metaperiodate respectively. Lane 6 is same as lane 5 but immunoblotted directly with anti-LBP antibody. (B) *L. donovani* promastigotes cultured in the presence or absence of tunicamycin were extracted, electrophoresed under reducing conditions and electroblotted on to nitrocellulose membrane. Lanes 1 and 2 represent control cells; lanes 3 and 4, tunicamycin-treated cells. Lanes 2 and 4 were incubated with laminin whereas lanes 1 and 3 were incubated with BSA.

with laminin, metaperiodate oxidation and metabolic deglycosylation studies were performed and LBP binding was evaluated by SDS/PAGE and immunoblotting as described earlier. Figure 7(A) shows the effect of metaperiodate oxidation on affinity-purified LBP transferred to nitrocellulose membrane. Strips were treated with various concentrations of metaperiodate (1, 5 and 20 mM) and binding activities were determined. A significant decrease was observed with concentrations greater than 5 mM, suggesting the involvement of sugar residues other than sialic acid, which gets oxidized at low metaperiodate concentrations. Metaperiodate treatment did not cause any damage to the polypeptide backbone as evidenced by immunoblot analysis with anti-LBP antibody (Figure 7A, lane 6). It may be mentioned that endoglycosidase H treatment of the purified protein also significantly abolished its binding to laminin (results not shown). *L. donovani* promastigotes were pretreated with tunicamycin, an inhibitor of N-glycosylation [29], under conditions that did not alter protein synthesis or cell viability. Membrane proteins from tunicamycin-treated cells were analysed with regard to laminin-binding ac-

Figure 8 Kinetics of attachment of *L. donovani* promastigotes to laminin-coated microtitre wells (A) and the influence of various proteins on their adherence to laminin-coated wells (B)

(A) Laminin-coated surfaces ($2 \mu\text{g}/\text{well}$) were overlaid with 5×10^5 cells of a suspension of ^{125}I -labelled parasites and incubated for the indicated periods of time. After extensive washing of the unbound parasites with PBS, the adherence of parasites was determined by counting the wells in a gamma counter. (B) Parasites (1×10^6) were surface-labelled with ^{125}I and incubated for 1 h at 22°C with laminin-coated microtitre wells in the presence of $2 \mu\text{g}$ of various proteins. Data are means from incubations performed in triplicate. The amount of attached cells is given as a percentage of the number of cells that were attached to the wells in the absence of proteins.

tivity. Figure 7(B) shows complete destruction of this activity after tunicamycin treatment, thereby suggesting that LBP may have N-linked oligosaccharides in its composition.

LBP may act as an adhesin

The ability of *L. donovani* promastigotes to bind laminin with high affinity prompted us to analyse the role of this interaction in parasite attachment to laminin-containing substrates. Radio-labelled parasite cells were incubated in microtitre wells coated with laminin or BSA. Adhesion of *L. donovani* to laminin-containing substrata was time-dependent and reached a maximum after 3 h of incubation, whereas a negligible amount of parasites had attached to BSA after 3 h of incubation (Figure 8A). Various proteins were then tested for potential competitive inhibitions of leishmanial adherence to laminin-coated substrate. Among the proteins analysed, only laminin and the solubilized 67 kDa protein showed inhibitory activity (Figure 8B). These results therefore, suggest that laminin may serve as a substrate of leishmanial adherence and that the 67 kDa protein can act as an adhesin.

DISCUSSION

One of the primary events in the initiation of any disease is thought to be the attachment of the causative pathogen to the host epithelial cells and, subsequently, penetration of these cells and inner-tissue linings lead to disease progression. A disease like leishmaniasis usually starts with the bite of the sandfly, which in the process delivers the motile promastigotes into the bloodstream. These injected promastigotes then migrate through the bloodstream into various definite organs, like the liver and the spleen, where they, in successive steps, adhere, penetrate, transform into amastigotes and replicate, primarily in the

resident macrophages like the Kupffer cells. In this process the host macrophage is lysed and when released in the interstitial tissue, the parasites move in search of fresh target cells, where they seek refuge only to multiply in number, lyse the host cell and thus the cycle is repeated. Evidently, during transit in the interstitial tissue, these intracellular parasites must be in contact with the ECM. In this report, we have identified, isolated and characterized an *L. donovani* surface protein that binds strongly to laminin, a major adhesive glycoprotein of ECM and basement membrane.

We first demonstrated that the binding of laminin to *L. donovani* promastigotes exhibits the properties of a typical receptor–ligand interaction. Binding of laminin was specific and saturable, and cells bound exogenous laminin with high affinity. Incubation of promastigotes with proteolytic enzymes resulted in a rapid loss of laminin binding (results not shown), suggesting the protein nature and surface location of the receptor. Moreover, since only outer membrane proteins get biotinylated, detection of the laminin-binding component in the membrane fraction prepared by biotinylation and streptavidin–agarose extraction also corroborated the surface location of the receptor. On the basis of these preliminary results we then isolated a 67 kDa LBP from the promastigote membrane fraction by a three-step procedure involving DEAE–cellulose, Con A–Sephacrose and laminin–Sephacrose affinity chromatography. The LBP behaved as one would expect of a laminin receptor. Binding of laminin to *L. donovani* promastigotes was inhibited in a concentration-dependent manner by the purified 67 kDa protein. Although the isolated receptor protein bound laminin with almost 100-fold lower affinity, it bound laminin with the same high affinity as intact cells when incorporated into artificial bilayers. Moreover, the 67 kDa protein could be positively detected in the biotinylated membrane fraction by an anti-67-kDa-protein antiserum, suggesting a surface location of the isolated laminin receptor. Taken

together, these results strongly suggest that the isolated 67 kDa protein is a cell-surface receptor and is responsible for the binding of laminin to *Leishmania* cells.

Efficient incorporation of LBP into liposomes may suggest its hydrophobic nature. Both the insolubility of the LBP in aqueous buffer without detergents and its ability to incorporate into liposomes 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. The glycoprotein nature of LBP was revealed by both the diminished activity of the metaperiodate-oxidized LBP as well as the complete loss of binding activity after tunicamycin treatment. Metaperiodate oxidation of either native laminin or LBP caused significant reduction in the binding activity, suggesting the involvement of sugar residues of both laminin and LBP. These studies further indicate that the LBP synthesized in the presence of tunicamycin, which blocks the initial step in the formation of N-linked oligosaccharide, cannot bind laminin or are not transported normally through intracellular organelles to the cell surface.

The first step in the process of tissue invasion is probably an association of the parasite with the host epithelial cell surface via a receptor–adhesion-like interaction. Recent reports have indicated that ECM components provide a mechanism of adherence for several human pathogens. *Candida albicans*, *Paracoccidioides brasiliensis* and trichomonads express laminin-receptor-like molecules which mediate cellular attachment to eukaryotic host cells [13,30,31]. Herpes simplex virus [32] and cytomegalovirus [33] bind to heparan sulphate proteoglycan on mammalian cells and this interaction represents the first step in viral infection. Several pathogenic bacteria bind to fibronectin and collagen and this binding is implicated in the bacterial adherence and colonization of a tissue [34–38]. A receptor for the malaria circumsporozoite protein has been identified as heparan sulphate proteoglycan [39]. A heparin-binding protein has also been identified in *Trypanosoma cruzi* [40]. This molecule is believed to aid in migration of the organism through ECM by attaching to ECM components. Love et al. [41] identified a high-affinity heparin-binding activity on *L. donovani* amastigote surface and proposed that this activity mediates attachment of amastigotes to mammalian cells by binding to cellular proteoglycan containing heparan sulphate. The preliminary results of the present study indicate that the 67 kDa LBP may behave as an adhesin. In fact, incubation of parasites with the laminin-coated microtitre wells in the presence of the soluble receptor protein resulted in a specific and significant inhibition of promastigote attachment. Our future objective is to see whether this *in vitro* interaction promoted by the 67 kDa protein facilitates the establishment of leishmanial infections and thus contributes to the pathogenicity of this parasite.

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