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Evidence of a Laminin Binding Protein on the Surface of *Leishmania donovani*

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Both the promastigote and amastigote forms of the intracellular parasite, *Leishmania donovani* bind the basement membrane glycoprotein laminin with high affinity ($K_d = 3.56 \times 10^{-9}$ M and 3.98×10^{-9} M respectively) with ~9000 and ~800 sites per cell. Bound laminin was identified by direct autoradiography and the binding protein through analysis of the parasite extract by SDS-PAGE and immunoblotting. A major component of 67 kDa was detected. The same protein was obtained when parasite outer membrane proteins were adsorbed to laminin-sepharose affinity matrix and subsequently eluted with SDS. The binding affinity of the isolated receptor was similar to that of the whole cells. Such a receptor isolated in *Leishmania* for the first time, may function as one of the bridging molecules for extracellular matrix recognition.

The infective *Leishmania donovani* promastigote, the causative agent of visceral leishmaniasis, is introduced into the human blood stream when its vector, the sandfly takes a blood meal. The blood borne promastigotes take refuge almost exclusively in the liver and the spleen macrophages. Although much light has been shed on parasite-macrophage interactions, very little is known about the molecular recognitions that enable the promastigotes to cross the epithelial barrier of the basement membrane and the extracellular matrix (ECM) of liver and spleen. In all likelihood, *L. donovani* promastigotes express on their plasma membrane, molecules capable of recognizing the ECM macromolecules which help them to glide through the interstitial tissue during their transit from blood to target cells. In the absence of such recognition systems, the promastigotes might get entrapped within the meshwork of ECM. Several such recognition proteins/molecules have been reported on the surface of *T. cruzi* (1-4), *C. albicans* (5), *P. brasiliensis* (6) and trichomonads (7). In this report, we show for the first time the existence for laminin, a glycoprotein uniquely localized in the ECM and basement membrane, a specific binding protein on *L.donovani* membrane, that could help the parasite to cross the ECM and the basement membrane.

MATERIALS AND METHODS

Parasites. Leishmania donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis (8). Parasites were maintained in BALB/c mice by intravenous passage every 6 weeks and cultured in medium 199 as described earlier (9).

Laminin binding assay. Binding assay was carried out according to Love et al., (10). In brief, *L. donovani* parasites after several washings were suspended in PBS containing 0.1% bovine serum albumin (BSA) to reduce non-specific binding and then incubated with graded concentrations of laminin at 4° C for 30 min. Radiolabeled laminin was separated from bound [^{125}I] laminin by layering the parasites on an oil gradient consisting of one part bisphthalate and one and a half parts dibutyl phthalate (Eastman Kodak). Laminin (100 μg) was iodinated with 1 mCi of Na[^{125}I] (Carrier free, Amersham) by the chloramine T method (11) to a specific activity of $3\text{-}5 \times 10^6$ cpm/μg. Non-specific binding was determined in the presence of 100-fold excess of unlabeled ligand and was <15% of the total binding.

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Electrophoresis and autoradiography. *L. donovani* promastigotes were allowed to bind iodinated laminin as described in the binding assay. The iodinated laminin bound parasites (2×10^6) were treated at 4° C with 500 μl of PBS containing 1% Triton-X-100, 10 μM leupeptin, 1 μM phenylmethylsulfonyl fluoride (PMSF) and 50 μg/ml soybean trypsin inhibitor for 1 h. After centrifugation at 30,000g for 20 min, samples were dissolved in a buffer containing 50 mM Tris-HCl, pH 7.0, 2% (w/v) SDS, 10% (w/v) glycerol and 5% (v/v) 2-mercaptoethanol, boiled for 2 min and subjected to 3.5% polyacrylamide gel electrophoresis under reducing conditions as described by Laemmli (12). Gels were dried and autoradiographs were prepared by exposing the dried gel to X-Omat RP film (Eastman Kodak) at −70° C. For comparison, iodinated laminin was run in a separate lane after reduction with 2-mercaptoethanol.

Immunoblotting. For immunoblots, parasites (2×10^8) were lysed in 1.0 ml of electrophoresis sample buffer containing 2% SDS, 5% 2-mercaptoethanol and 1% Triton-X-100 for 1 h at 4° C, boiled for 3 min, centrifuged at 30,000 g for 20 min and aliquots (50 μg protein) of the clear supernatant was electrophoresed on a reducing 12.5% SDS-polyacrylamide gel with 3% stacking gel. The separated proteins were transferred to nitrocellulose paper (Schleicher & Schuell, 0.45 μm) in a Bio-Rad transfer apparatus in a transfer buffer containing 20 mM Tris, 150 mM glycine and 20% (v/v) methanol as described (13). Residual binding sites on the filters were blocked by incubation with 5% nonfat dry milk, 1% ovalbumin, 5% FCS and 7.5% glycine for 30 min at room temperature with gentle shaking. The membranes were washed 3 times for 5 min each in TBS (20 mM Tris, pH 7.4, 0.9% NaCl) containing 0.1% NP-40 (TBSN) and incubated for 1 h at 37°C with laminin (50 μg/ml) in TBSN supplemented with 1% BSA (TBSN-BSA). After washing again for 3 times with TBSN, the membranes were incubated with rabbit antilaminin antibody (Sigma) at a dilution of 1:100 in TBSN-BSA at 37° C for 30 min. Blots were washed as described earlier and incubated for 30 min at 37° C with secondary antibody (goat anti-rabbit IgG $F(ab')_2$ conjugated to alkaline phosphatase; Sigma) diluted 1:500 in TBSN-BSA. After washing, the protein bands were developed with nitro blue tetrazolium (NBT) (330 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (165 μg/ml) in 50 mM Tris, pH 9.5, 150 mM NaCl and 5 mM $MgCl_2$ as substrate (14).

Biotinylation of intact L. donovani outer membrane proteins. Parasite membrane proteins were biotinylated according to Hurley et al., (15). 100μg biotin (Sulfo-NHS-Biotin, Pierce Chemical Co., Rockford, IL) was added to 2×10^8 parasites and incubated for 10 min at 22°C. After washing off excess unbound biotin, cells were lysed by sonication in 1.0 ml of 50 mM Tris-HCl, pH 8 containing 1% Triton-X-100 and an antiprotease cocktail (10 mM pepstatin, 10 mM leupeptin, 10 mM iodoacetamide, 10 μg/ml soybean trypsin inhibitor and 2 mM PMSF) (lysis buffer). The insoluble material was removed by centrifugation at 100,000 g for 1 h at 4°C and the supernatant incubated with 500 μl streptavidin-agarose (Pierce Chemical Co., Rockford, IL) for 2 h at 4°C with shaking. The unbound proteins were washed off from the agarose beads by the lysis buffer. The membrane proteins were eluted with 25 mM Tris-HCl, pH 7.5 containing 5 mM $MgCl_2$ and 30 mM β-octylglycoside and dialysed against the lysis buffer.

Affinity purification. The dialysed biotinylated membrane proteins from *L. donovani* promastigotes were mixed with an equal volume of laminin-Sepharose. Affinity matrix was prepared by coupling laminin (25 μg) with 100 μl of cyanogen bromide activated Sepharose CL 4B (Pharmacia Biotech, Uppsala, Sweden) and incubated for 16 h at 4° C with shaking. Resins were washed twice with 10 mM Tris-HCl, pH 8 containing 0.1% Triton-X-100 and 150 mM NaCl and once with 50 mM Tris-HCl, pH 7.2. Bound material was eluted with 2% SDS, dialyzed and concentrated. Eluted proteins were run on 12.5% SDS-PAGE under reducing conditions and silver stained. Affinity purified proteins were identified on a 12.5% SDS-PAGE by transfer to nitrocellulose paper and immunoblotting as detailed earlier.

RESULTS

Binding of parasites to laminin. Preliminary experiments showed that *L. donovani* promastigotes adhere to laminin immobilized on plastic surfaces, but not to IgG- or BSA-coated surfaces. These suggested the presence of laminin-binding activity on promastigotes. To detect this activity, binding studies were carried out with promastigotes using [^{125}I] laminin as ligand. Incubation of promastigotes (10^6 cells) with [^{125}I] laminin (20 ng) corresponding to 0.01 × saturating concentration resulted in a time-dependent binding of the protein (data not shown). The binding was rapid and a saturation curve was obtained within 60 min. When the promastigotes were incubated with increasing concentrations of [^{125}I] labeled laminin for 60 min, the amount of ligand bound increased to a saturation level of 9.7 ng of laminin bound per 10^6 cells at a laminin concentration of 20 μg/ml (Fig. 1A). Scatchard analysis of the specific binding data revealed a straight line (Fig. 1A; inset) indicating one class of laminin receptor sites with high affinity and yeilded a K_d value of 3.56×10^{-9} M and approximately ~ 9000 receptor sites per cell. The binding of radiolabeled laminin was almost completely inhibited by excess nonradioactive laminin, but binding was not inhibited by excess heparin or chondroitin sulphate or hyaluronic acid or vitronectin (Table 1). Binding studies were extended to *L.*

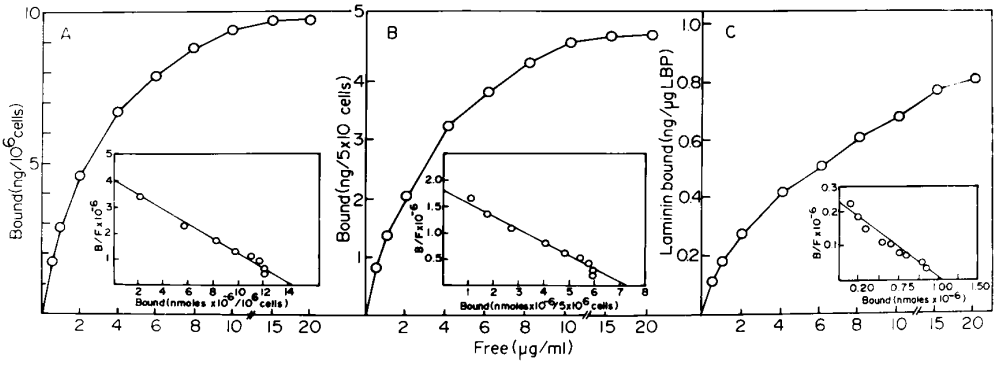

FIG. 1. Radiolabeled laminin binding to *L. donovani* parasites. Increasing amounts of radiolabeled laminin (1.2×10^6 cpm/μg) were added to a constant number (1×10^6) of promastigotes (Fig. 1A) and (5×10^6) of amastigotes (Fig. 1B). Non-specific binding (i.e. binding in the presence of 100-fold molar excess of unlabeled laminin) was subtracted from the total. A Scatchard plot (inset) was constructed from the specific binding data, yeilding a binding constant (K_d) of 3.56×10^{-9} M for promastigotes (Fig. 1A, inset) and 3.98×10^{-9} M for amastigotes (Fig. 1B, inset) and a stoichiometry of $\sim$ 9000 and $\sim$ 800 receptors per cell for promastigotes and amastigotes respectively. Radiolabeled laminin binding to isolated laminin receptor (Fig. 1C) also display linear kinetics consistent with a single class of receptors yeilding a K_d value of 5.65×10^{-9} M. Data represent duplicate determinations of three separate experiments.

donovani amastigotes and a similar type of dose-dependent, specific and saturable binding of radiolabeled laminin to *Leishmania* amastigotes was observed ($K_d = 3.98 \times 10^{-9}$ M with approximately $\sim$ 800 sites per cell, Fig. 1B).

Identification of laminin binding protein from the surface of L. donovani. Direct demonstration of laminin binding to parasite surface was revealed by SDS-polyacrylamide gel electrophoresis autoradiography of promastigote extract after binding with iodinated laminin (Fig. 2). The autoradiograph showed two bands corresponding to the two subunits of laminin run as standard in a separate lane. To obtain information on the surface proteins that bind laminin, promastigote proteins were extracted by SDS and Triton-X-100 and run on a 12.5% reducing SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose paper and treated sequentially with laminin, rabbit antilaminin antibody and goat antirabbit antibody alkaline phosphatase conjugate. On treatment of the paper with NBT-BCIP, a major band at 67 kDa became

TABLE 1
Inhibition of Radiolabeled Laminin Binding to *L. donovani* Promastigotes by Soluble Glycoasaminoglycans

Competitor	Cpm bound[a]	Laminin bound[b] (ng)
None	22,465 ± 3,050	7.5 ± 1.0
Laminin	3,724 ± 1,621	1.2 ± 0.5
Heparin	20,372 ± 2,758	6.8 ± 0.9
Chondroitin sulfate	18,464 ± 2,467	6.2 ± 0.8
Hyaluronic acid	17,695 ± 2,122	5.9 ± 0.7
Vitronectin	19,620 ± 2,654	6.5 ± 0.9

[a] Unlabeled competitors were used at a final concentration of 1 mg/ml.

[b] The amount of [^{125}I]-labeled laminin per 10^6 promastigotes. Data represent mean ± SD of triplicate determinations.

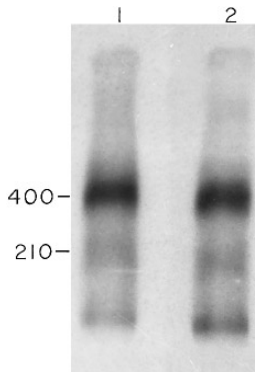

FIG. 2. Autoradiograph of SDS-PAGE of Triton-X-100 extracted *L. donovani* promastigotes after their incubation with [^{125}I] labeled laminin (lane 1). Reduced [^{125}I] labeled laminin (10 ng) was run as control in lane 2. Lane 1 was exposed to radiographic film for 2 days whereas lane 2 was exposed for 6 h.

visible (Fig. 3A, lane 2). Two faint minor bands were also visible raising the possibility that other minor proteins may also bind laminin. Membrane proteins of promastigotes isolated by biotinylation and streptavidin-agarose extraction and immunoblotted under the same conditions, also showed 67 kDa protein as the major bound protein (Fig. 3B, lane 5). No bands were visible in the control experiments suggesting the specificity of the reaction. The binding of laminin to a 67 kDa protein could also be seen on the surface of *L. donovani* amastigotes. Biotinylated membrane protein extracts of amastigotes were subjected to Western blot, incubated or not with laminin, followed by probing with rabbit anti-laminin antibodies. A clear reaction could be seen only when the membrane extract was incubated with laminin (Fig. 3C, lane 7). The binding of laminin to a 67 kDa surface protein was confirmed by affinity

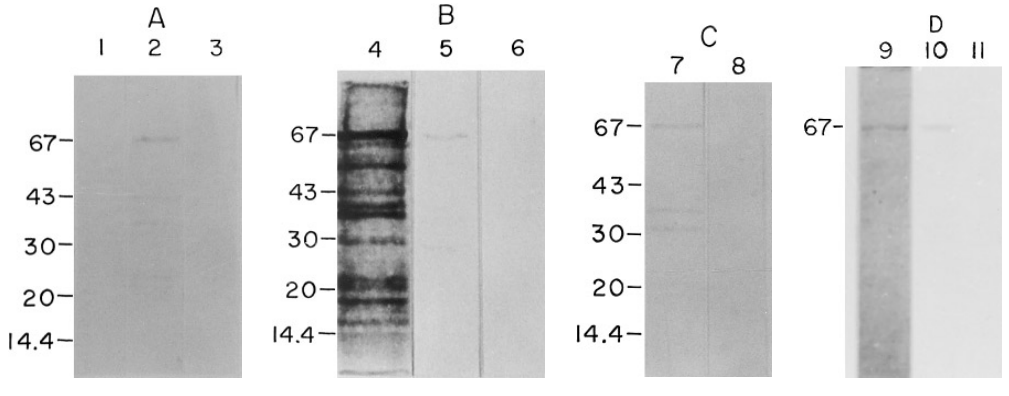

FIG. 3. Immunoblot analysis of leishmanial laminin binding protein. *L. donovani* promastigote extract or membrane proteins isolated by biotinylation and streptavidin-agarose were applied to 12.5% polyacrylamide slab gel (50 μg protein per lane) and transferred to nitrocellulose. Blots were sequentially treated with laminin, rabbit anti-laminin antiserum and alkaline phosphatase conjugated goat anti-rabbit IgG. Finally, the reaction was developed by NBT-BCIP system. (A) Western blot of promastigote extract. A major band of 67 kDa was revealed with some minor bands (lane 2). No bands were detected when laminin was omitted (lane 1) or when an irrevelant immune serum was used (lane 3). (B) Western blot of promastigote membrane proteins in presence (lane 5) or absence (lane 6) of laminin. Silver stained promastigote membrane proteins are in lane 4. (C) Western blot of amastigote membrane proteins in presence (lane 7) and absence (lane 8) of laminin. (D) Western blot of the affinity purified binding protein revealed a band at 67 kDa (lane 10). No bands were detected when an irrelevant immune serum was used (lane 11). Silver stained affinity purified protein in lane 9.

chromatographic isolation. Promastigote membrane proteins were incubated with laminin-Sepharose beads, washed thoroughly and the matrix-bound proteins analyzed by SDS-PAGE under reducing conditions. Silver staining of the resolved protein showed a single band at 67 kDa (Fig. 3D, lane 9). Western blot analysis of the affinity purified protein showed the presence of this same band at 67 kDa (Fig. 3D, lane 10). Under the same experimental conditions, no 67 kDa protein was visible when membrane extracts were incubated with BSA -Sepharose, gelatin-Sepharose or Sepharose 4B beads (data not shown).

Assay of laminin binding to purified proteins. The protein eluted from the laminin affinity matrix was tested for its ability to bind exogenous laminin using the nitrocellulose filter disc assay according to Malinoff and Wicha (17). A dose-dependent, specific and saturable binding was observed (Fig. 1C) with an affinity ($K_d = 5.65 \times 10^{-9}$ M) which is comparable to the binding of laminin to intact *L. donovani* promastigotes indicating thereby that the eluted protein is the laminin binding protein.

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 (16). The infective *L. donovani* promastigotes 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 the liver and the spleen. Our results show the presence of a *L. donovani* membrane protein that binds strongly with laminin, a major adhesive glycoprotein in the ECM and basement membrane. Using radioligand binding assay we have shown that both the amastigotes and the promastigotes have one class of laminin receptors which bind laminin with dissociation constant of 3.98×10^{-9} M and 3.56×10^{-9} M respectively and the number of receptors are ~9000 for promastigotes and ~ 800 for amastigotes. This receptor number is much lower than the number of available receptors on the surface of eukaryotic cells where the number of laminin receptors is 10^4 to 10^5 (17,18). Taking into consideration the larger size of eukaryotic cells, the density of receptors appears to be as high or higher on parasite surfaces as on eukaryotic cell surfaces. The binding was specific since excess amounts of unlabelled laminin almost completely inhibited the binding of iodinated laminin whereas the other ECM proteins tested had no or low inhibitory effect.

Laminin binding to promastigote was also shown by autoradiography. However, the conclusive evidence for the presence of the binding protein came from immunoblot experiments. Following gel electrophoresis and electroblotting of promastigote and amastigote total proteins, soluble laminin bound preferrentially to a protein of molecular weight 67 kDa. Furthermore only a protein of this molecular weight was isolated by affinity chromatography of promastigote membrane proteins on laminin-sepharose. This protein may be similar to the previously described laminin receptor, which is present on many cells (17, 18, 19) and is involved in cell adhesion to laminin through the Tyr-Ile-Gly-Ser-Arg site on the B1 chain of laminin (20). Interaction with ECM proteins has been correlated with the invasive ability of different cells (21-24). It has been shown that recognition of laminin may influence the pathogenesis of several microorganisms and receptors have been identified in various species of bacteria (25-28) and parasites (5, 6). Our aim would be to check if the soluble laminin binding protein is an effective agent in neutralizing infection of host cells, as they are in other *in vitro* systems (4). They could then be used effectively in designing of ligand and immunotherapy of leishmaniasis.

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