Eur. J. Biochem. **269**, 1622–1629 (2002) © FEBS 2002

High affinity binding between laminin and laminin binding protein of *Leishmania* is stimulated by zinc and may involve laminin zinc-finger like sequences

Keya Bandyopadhyay, Sudipan Karmakar, Abhijit Ghosh and Pijush K. Das

Molecular Cell Biology Laboratory, Indian Institute of Chemical Biology, Jadavpur, Calcutta, India

In the course of trying to understand the pathogenesis of leishmaniasis in relation to extracellular matrix (ECM) elements, laminin, a major ECM protein, has been found to bind saturably and with high affinity to a 67-kDa cell surface protein of *Leishmania donovani*. This interaction involves a single class of binding sites, which are ionic in nature, conformation-dependent and possibly involves sulfhydryls. Binding activity was significantly enhanced by Zn^{2+}, an effect possibly mediated through Cys-rich zinc finger-like sequences on laminin. Inhibition studies with monoclonals against polypeptide chains and specific peptides with adhesive properties revealed that the binding site was localized in one of the nested zinc finger consensus sequences of B1 chain containing the specific pentapeptide sequence, YIGSR. Furthermore, incubation of *L. donovani* promastigotes with $C(YIGSR)_3$-NH_2 peptide amide or antibody directed against the 67-kDa laminin-binding protein (LBP) induced tyrosine phosphorylation of proteins with a molecular mass ranging from 115 to 130 kDa. These studies suggest a role for LBP in the interaction of parasites with ECM elements, which may mediate one or more downstream signalling events necessary for establishment of infection.

Keywords: *Leishmania donovani*; laminin; laminin-binding protein; zinc finger sequence; cell adhesion.

Protozoan parasites of the genus *Leishmania* cause a diverse group of diseases collectively called leishmaniases, which range in severity from spontaneously healing cutaneous ulcers to potentially fatal visceral disease. These parasites have a digenetic life cycle, passing from the infected sand fly vector to the mammalian host as the vector takes a blood meal. The flagellated promastigote invades mammalian cells, primarily the resident macrophages, where in successive steps they adhere, penetrate, transform into amastigotes and replicate. In this process the host macrophage is lysed, parasites move in search of fresh target cells and thus infection is spread to the neighbouring cells. In order to migrate from blood vessels, where they circulate, to the interior of the cell lysosome, where they differentiate, these parasites have to surpass the formidable barrier of the extracellular matrix (ECM) and basement membrane (BM). The ability to adhere to ECM components may represent a mechanism by which pathogens avoid entrapment within the ECM, thus playing an important role in pathogenesis. Pathogens like trichomonads, *Paracoccidioides brasiliensis and Candida albicans* possess cell surface molecules capable of interacting with ECM [1–3]. Trypomastigotes of *Trypanosoma cruzi* express a set of surface glycoproteins known collectively as Tc-85, at least one member of which has adhesive property to laminin [4]. We have recently reported the presence of a 67-kDa transmembrane glycoprotein on the surface of *Leishmania donovani* that binds to laminin, the major glycoprotein of ECM and BM [5]. Detailed characterization has revealed that it may act as an adhesin [6]. However, neither the mode of binding nor the possible factors cooperating in binding protein are understood in any detail. Laminin is a glycoprotein consisting of three chains (A, B1 and B2), which are joined by disulfide bonds into a cruciform structure with three N-terminal short arms and one C-terminal long arm. Many of the functional sites exist on individual chains of laminin, while others seem to be formed by folding of all three chains. It is also possible that some sites are cryptic in native trimeric protein and become exposed under certain conditions [7]. Although various functional sites of laminin have been identified using proteolytic fragments and synthetic peptides, little is known about the physical nature of these binding sites or the regulatory factors that govern these interactions.

A recent study focussing on BM assembly showed the involvement of zinc and implicated laminin zinc finger-like sequences [8]. The assembly of BM is believed to involve the independent polymerization of collagen type IV and laminin, as well as high affinity interactions between laminin, enactin/nidogen, perlecan and collagen type IV. Zn^{2+} was found to be most effective in enhancing laminin–enactin and laminin–collagen type IV binding. Previously, the enactin binding site was mapped to one of the zinc-finger containing repeats on the laminin A chain [9]. More recently, high affinity binding between laminin and Alzheimer's amyloid precursor protein, serum amyloid A, was

Correspondence to P. K. Das, Molecular Cell Biology Laboratory, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Jadavpur, Calcutta 700 032, India.
Fax: + 91 33 473 5197, Tel.: + 91 33 473 6793,
E-mail: pijush@cal2.vsnl.net.in

Abbreviations: ECM, extracellular matrix; BM, basement membrane; LBP, laminin binding protein.

(Received 26 October 2001, revised 10 January 2002, accepted 17 January 2002)

attributed to be mediated through Cys-rich zinc finger-like sequences on laminin [10].

Attempts have been made in the present study to reveal the physicochemical nature of the binding between laminin and laminin-binding protein (LBP) of *Leishmania,* believed to be important for the homing of the parasites. We investigated the influence of pH and various essential ions on laminin–LBP interactions. Of all the essential ions tested, zinc was the most effective at enhancing laminin–LBP interactions. The zinc effect was saturable and the binding site was localized in one of the nested zinc finger consensus sequences of B1 chain containing the specific pentapeptide sequence, YIGSR. It is now beginning to be believed that cell–matrix interactions do not merely provide structural anchors, but, at least in some cases, transmit signals that trigger downstream biochemical events [11,12]. We here provide evidence that YIGSR, the binding motif of laminin, as well as polyclonal anti-LBP Ig induce protein tyrosine phosphorylation.

MATERIALS AND METHODS

Parasites

L. donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with visceral leishmaniasis [13]. Parasites were maintained in BALB/c mice by intravenous passage every 6 weeks. For experiments involving promastigotes, parasites were used at or near the stationary phase of growth from passages 2–5 after *in vitro* transformation from liver and spleen-derived amastigotes. Promastigotes were cultured at 22 °C in medium 199 with Hanks salts (Gibco laboratories, Grand Island, NY, USA) containing Hepes (12 mM), L-glutamine (20 mM), 10% fetal bovine serum, 50 $U{\cdot}mL^{-1}$ penicillin and 50 $\mu g{\cdot}mL^{-1}$ streptomycin. *L. donovani* promastigotes were surface-labelled with ^{125}I by using lactoperoxidase-glucose oxidase as described previously [14] and metabolically labelled with [^{35}S]methionine according to [15].

Purification of LBP

Membrane proteins were isolated by biotinylation and streptavidin–agarose extraction. *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, USA). Cells were then washed and lysed in 1 mL lysis buffer [5 mM Tris/HCl (pH 7.5), 0.5% Triton X-100, 25 mM KCl, 5 mM $MgCl_2$, 0.5 $\mu g{\cdot}mL^{-1}$ leupeptin, 1 $\mu g{\cdot}mL^{-1}$ aprotinin, 50 $\mu g{\cdot}mL^{-1}$ soybean trypsin inhibitor, 10 $\mu g{\cdot}mL^{-1}$ phenylmethanesulfonyl fluoride. Cells were then centrifuged at 12 000 ***g*** for 30 min at 4 °C, supernatant absorbed on to a streptavidin–agarose column (1 mL, Pierce Chemical Co.) and membrane proteins eluted with 25 mM Tris/HCl (pH 7.5) containing 5 mM $MgCl_2$/30 mM β-octylglucoside.

Membrane proteins were first passed through a DEAE-cellulose column (1 × 10 cm) previously equilibrated with buffer I [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 25 $U{\cdot}mL^{-1}$ aprotinin]. Bound proteins were eluted with 100 mL of a linear gradient of 0–400 mM NaCl in buffer I. The eluate was then passed through a Con A–Sepharose column previously equilibrated with buffer II [10 mM Tris/HCl (pH 7.4), 0.2 M NaCl, 0.1% Nonidet P40) and eluted with buffer II containing 1 M α-methyl-D-mannopyranoside. The purified LBP was obtained by mixing the eluate with an equal volume of laminin–Sepharose [prepared by coupling Engelbreth-Holm-Swarm laminin (25 μg, Sigma Chemical Co., St Louis, MO, USA) with 100 μL of cyanogen bromide-activated Sepharose CL-4B] and incubated for 16 h at 4 °C. The bound protein was eluted with 2 M glycine, dialyzed against 10 mM Tris/HCl (pH 7.4) and stored at −70 °C. Authenticity of the purified protein was checked by autoradiography of immunoprecipitated protein from metabolically ([^{35}S]methionine) labelled parasites as well as direct and indirect immunoblotting as described previously [6]. Direct immunoblotting denotes treatment of nitrocellulose paper containing proteins with anti-LBP Ig followed by alkaline phosphatase conjugated secondary antibody whereas indirect immunoblotting denotes sequential treatment with laminin, anti-laminin Ig and secondary antibody.

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 intervals of 2 weeks by injecting LBP emulsified in incomplete Freund's adjuvant. After 10 days from the fourth injection blood was collected from rabbit ear and the anti-LBP Ig separated according to Hall *et al*. [16].

Peptides and antibodies

The synthetic peptides RNIAEIIKDI, GPRPPERHQS, SIKVAV, LRYESK, YIGSR, HEIPA, RGD, LGTIPG, RYVVLPR, $C(YIGSR)_3NH_2$ and CYKNVRSKIGSTE NIKHQPGGGKV were synthesized on a 430-A peptide synthesizer (Applied Biosystems) and further purified by HPLC. Before use, the peptides were dissolved in 10 mM HCl and immediately added to indicated buffer. Anti-laminin and anti-(P-Tyr) Ig were from Sigma Chemical Co. Monoclonal antibodies against human laminin A, B1 and B2 chains were from Life Technologies Inc.

Zinc analysis

Laminin zinc content was assayed by atomic absorption spectroscopy using elemental zinc standards (0–2 p.p.m.). Laminin was assayed either directly or after loading with $ZnCl_2$, which involved sequential dialysis first against NaCl/Tris [20 mM Tris/HCl (pH 7.4), 150 mM NaCl] containing 50 μM $ZnCl_2$, then against NaCl/Tris containing 0.1 mM EDTA and finally against NaCl/Tris to remove unbound Zn^{2+}. Samples at 0.5 $mg{\cdot}mL^{-1}$ protein were dissolved in 2% nitric acid prior to analysis.

Assay of laminin binding to LBP

Laminin binding to pure LBP was assayed according to Malinoff & Wicha [17]. Nitrocellulose discs (6 mm diameter) were spotted with 200 ng of protein each in a total volume of 10 μL and blocked by 5% BSA in NaCl/P_i at 37 °C for 1 h. The discs were incubated in presence of

^{125}I-labelled 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 and measured for radioactivity retained in them. Laminin was iodinated with 1 mCi of ^{125}I (carrier-free, Amersham, Arlington Heights, IL, USA) by the chloramine-T method [18] to a specific activity of (3–5) × 10^6 c.p.m.μg^{-1}. The binding of ^{125}I-labelled laminin to *L. donovani* was quantified as described previously [5].

Solid phase adhesion assay

Microtiter wells were coated with 50 μL of laminin (100 μg·mL^{-1}) and blocked with BSA. To the wells, ^{125}I-labelled parasites (5×10^5 parasites·mL^{-1}) were added and allowed to incubate for 60 min at 22 °C. The wells were then washed extensively with NaCl/P_i 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.

Tyrosine phosphorylation

L. donovani promastigotes (2×10^8) at log phase culture were first washed twice with medium M199 devoid of fetal bovine serum and then suspended in 1 mL of the same medium. Then, 100 μg·mL^{-1} of either C(YIGSR)$_3$-NH_2 or an unrelated peptide as negative control was added. The cells were incubated at 22 °C for various time periods, washed twice with ice cold NaCl/P_i and immediately frozen in liquid nitrogen. Cells were lysed in 100 μL of SDS/PAGE sample buffer by boiling for 5 min, proteins were resolved by means of 7.5% SDS/PAGE and analysed by immunoblotting with monoclonal anti-(P-Tyr) antibody followed by alkaline phosphatase conjugated goat anti-(rabbit IgG) Ig as secondary antibody. Protein bands were developed with Nitro Blue tetrazolium and 5-bromo-4-chloro-indolyl-3-phosphate in 50 mM Tris/HCl (pH 9.5), 150 mM NaCl, 5 mM $MgCl_2$ [19]. For selective adhesion to coated polystyrene latex beads, these (0.05 mL) were first suspended in 0.45 mL NaCl/P_i containing 100 μg of C(YIGSR)$_3$-NH_2 peptide amide or 100 μg of anti-LBP Ig followed by incubation for 30 min at room temperature, centrifugation at 2000 ***g*** for 10 min and resuspending in 0.5 mL NaCl/P_i. Serum-starved *L. donovani* promastigotes (0.2 mL, 5×10^7 cells) were mixed with 0.1 mL (2.1×10^8) latex beads coated with C(YIGSR)$_3$-NH_2 peptide amide or anti-LBP Ig, incubated at room temperature for 30 min and harvested by centrifugation for 10 min at 2000 ***g***. Cells were solubilized by boiling in SDS sample buffer for 5 min and the extracted proteins were resolved by means of 7.5% SDS/PAGE followed by immunoblotting with anti-(P-Tyr) Ig.

RESULTS

Isolation of LBP

To isolate the laminin-binding component, *L. donovani* promastigote membrane proteins obtained by biotinylation and streptavidin–agarose extraction were subjected to a three-step purification procedure involving DEAE-cellulose, Con A–Sepharose and a laminin–Sepharose affinity chromatography. Silver staining of the purified protein showed a single band of molecular mass of 67 kDa (Fig. 1, lane 1).

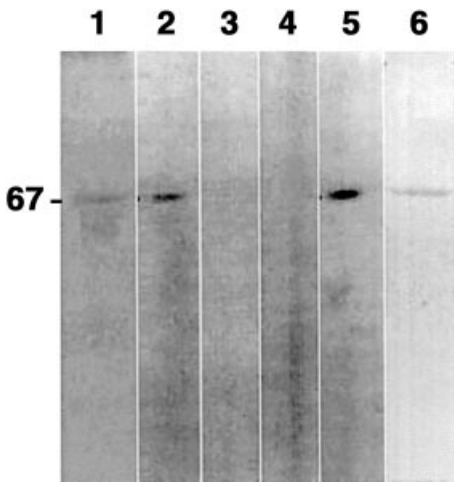

Fig. 1. Isolation and identification of LBP. *L. donovani* membrane proteins isolated by biotinylation and streptavidin–agarose extraction and passed through DEAE-cellulose, Con A–Sepharose and laminin–Sepharose were analysed by 7.5% SDS/PAGE under reducing conditions. The gel was silver stained (lane 1). The molecular masses are indicated to the left of the panel. Affinity purified protein from laminin–Sepharose was transferred to nitrocellulose membrane and subjected to indirect immunoblot analysis using laminin as the primary probe followed by rabbit anti-laminin IgG, goat anti-(rabbit IgG) Ig, Nitro Blue tetrazolium and 5-bromo-4-chloro-indolyl-3-phosphate; (lane 2). Lane 3 was incubated with BSA instead of laminin. Lane 4 represents immunoblot analysis using avidin as the primary probe and anti-(rabbit avidin) IgG as the secondary antibody. Affinity purified protein was subjected to direct immunoblot analysis using rabbit anti-LBP antiserum as primary probe (lane 5). Promastigotes were metabolically labelled with [^{35}S]methionine, lysed and the LBP was immunoprecipitated by anti-LBP Ig and autoradiographed (lane 6).

Indirect immunoblotting revealed a 67-kDa protein band using laminin as the primary probe followed by treatment with anti-laminin Ig and alkaline phosphatase-conjugated secondary Ig (lane 2). The control nitrocellulose strip (lane 3), which was devoid of laminin treatment, failed to reveal any band thereby suggesting the specificity of the reaction. Blotting with avidin probes also did not reveal any band (lane 4). Direct immunoblotting using anti-LBP Ig and secondary antibody also resulted in a 67-kDa band (lane 5) confirming the authenticity of the protein. Finally, the parasitic origin of the protein was demonstrated by immunoprecipitating LBP from metabolically labelled *L. donovani* using anti-LBP Ig and protein A–Sepharose beads. When these immune complexes were dissociated and run on SDS/PAGE and autoradiographed, we observed a single band at 67 kDa (lane 6).

Requirements for optimal laminin-LBP binding

Denaturation by heat had similar effects on both laminin and LBP (Fig. 2A). The binding activities of both laminin or LBP were completely destroyed by heat denaturation (100 °C, 5 min) indicating that the conformation of both the receptor and ligand are essential for binding. Changes in pH of the binding buffer also had marked effect on binding constant with a change of as little as 0.5 pH units from pH 7.5 being enough to lower specific binding activity

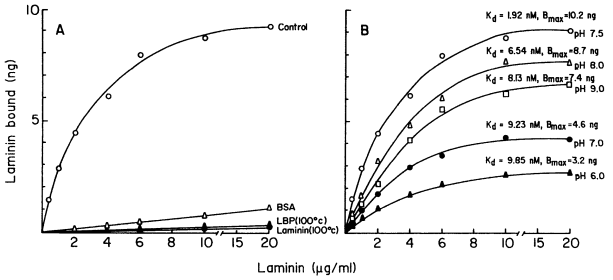

Fig. 2. Laminin binding activity for LBP (A) after heat denaturation and (B) at different pH. (A) Binding experiments were carried out after heating laminin in 20 mM Tris/HCl (pH 7.4), 150 mM NaCl and LBP in 20 mM Na_2CO_3, $NaHCO_3$ (pH 9.6), 4 M urea at 100 °C for 5 min. Binding of untreated laminin to BSA is also included. (B) Laminin-LBP binding was carried out at different pH levels: pH 6.5 and 7.0 (20 mM phosphate), pH 7.5 and 8.0 (20 mM Tris/HCl) and pH 9.0 (20 mM glycine/NaOH) with usual amount of NaCl (150 mM). Dissociation constants and binding maxima (where applicable) are shown for each curve on graph. All binding was carried in presence of 15 μM $ZnCl_2$ and are represented as mean of three separate experiments.

(Fig. 2B). Both affinity and binding maxima were optimum at pH 7.5. Nonspecific binding to BSA was not changed over the pH range (data not shown). Involvement of surface charge in the binding may be one of the reasons for pH dependence. A number of compounds were also found to affect laminin–LBP interaction (Fig. 3A). The protein denaturant urea at 2 M prevented binding, indicating again that the interaction is conformation-dependent. Increasing the NaCl concentration to 0.3 M also significantly reduced binding suggesting the ionic nature of the binding sites. Free sulfhydryl groups were also implicated as alkylation of laminin with *N*-ethylmaleimide without reduction of disulfide bonds also reduced the binding significantly. No such reduction in binding was observed when LBP was treated with *N*-ethylmaleimide (data not shown). The inhibition of laminin binding activity with EDTA suggested the involvement of divalent metal ions and a series of common trace elements were tested at their respective plasma concentrations (Fig. 3B). Zn^{+2} was found to be the most effective of all metal ions tested at enhancing the laminin-binding activity ($K_d = 1.92 \pm 0.42$ nM and $B_{max} = 10.20 \pm 0.90$ ng). Mn^{2+} and Cu^{2+} are the other two metals, which promoted binding to a small extent whereas Ca^{2+} and Mg^{2+} showed inhibitory effect compared with EDTA. The zinc effect on laminin binding was saturable with optimal binding occurring at physiological Zn^{2+} concentration (15 μM), above which the amount of nonspecific binding increased. Preincubation of LBP with either Zn^{2+} or EDTA (Fig. 3C) did not alter the binding activity suggesting thereby that the cofactor requirement of Zn^{2+} is for laminin only. Treatment of laminin with diethyl pyrocarbonate, a histidine modifying agent, did not change the binding parameters (Fig. 3A) suggesting thereby that Zn^{2+} binding did not occur via the His-Xaa-His sites, which are known to bind certain metals with high affinity [20]. Significant reduction in binding after alkylation with *N*-ethylmaleimide on the other hand may suggest the involvement of cysteine sulfhydryl groups in Zn^{2+} binding. Laminin (0.5 mg·mL^{-1}) dialyzed against an excess of $ZnCl_2$ (50 μM), followed by extensive dialysis against NaCl/Tris to

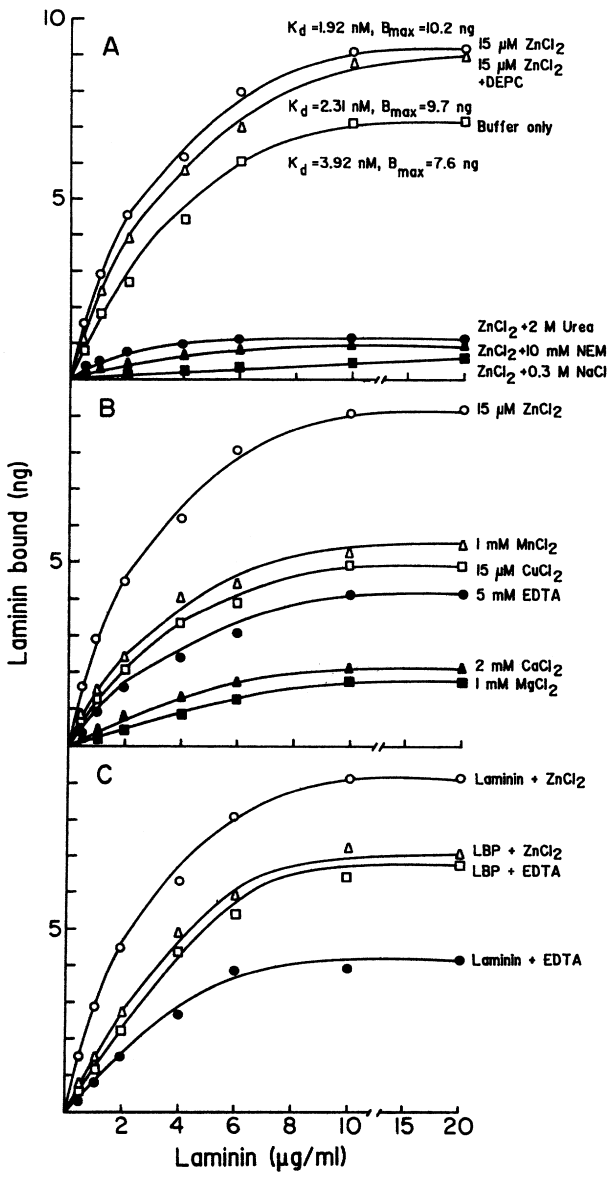

Fig. 3. Effect of various agents on laminin-LBP binding. (A) LBP was coated onto nitrocellulose discs and incubated with increasing concentrations of laminin under different conditions (shown on the right of the graph). (B) The influence of different divalent metal ions on binding was evaluated at their respective plasma concentrations (2 mM $CaCl_2$, 15 μM $CuCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 15 μM $ZnCl_2$). (C) Binding was carried out after pretreating either laminin or LBP with Zn^{2+} and EDTA. Data represent mean of three separate experiments.

remove free metal, was found to contain 9.84 ± 1.51 nmol of Zn^{2+} per mol of laminin. A small amount of Zn^{2+}, 1.21 ± 0.32 nmol·mol^{-1} of laminin was also detected in control laminin preparation not dialyzed against $ZnCl_2$. Incidentally, laminin has 42 Cys-rich repeats found on the amino terminal ends of its three subunits (A, B1 and B2), of which 12 contained nested zinc-finger consensus sequences known to be involved in several protein–protein interactions [21].

Table 1. Inhibition of radiolabelled laminin binding to *L. donovani* promastigotes. Data represent mean ± SD of triplicate determinations. Values include the significance (*$P < 0.001$) of the difference between inhibition in the presence and absence of inhibitors as determined by analysis of variance.

	Bound c.p.m.	Bound laminin (ng)[b]
(A) By soluble glycosaminoglycans		
Competitor[a]		
None	20 987 ± 2868	7.20 ± 0.98
Laminin	2846 ± 845	0.98 ± 0.29*
Heparin	18 467 ± 2255	6.34 ± 0.77
Chondroitin sulfate	16 870 ± 2032	5.79 ± 0.70
Hyaluronic acid	17 121 ± 1983	5.87 ± 0.68
(B) By purified LBP		
LBP ($\mu g \cdot mL^{-1}$)		
0.25	13 897 ± 1835	4.77 ± 0.63
0.50	8658 ± 1246	2.97 ± 0.43*
0.75	5396 ± 887	1.85 ± 0.30*
1.00	2124 ± 636	0.73 ± 0.22*

[a] Unlabelled competitors were used at a final concentration of 1 $mg \cdot mL^{-1}$. [b] The amount of ^{125}I-labelled laminin per 10^7 promastigotes.

Localization of the binding region of laminin

The binding of radiolabelled laminin was almost completely inhibited by excess nonradioactive laminin, but not by excess heparin or chondroitin sulfate or hyaluronic acid or vitronectin (Table 1). Binding of radiolabelled laminin was also inhibited by purified LBP in a concentration-dependent manner (Table 1). Consistent with this finding is the observation that polyclonal anti-laminin serum resulted in abolishing the parasite adherence to laminin-coated wells (Fig. 4A). In order to determine which polypeptide chain of laminin harbour the LBP binding site, monoclonal antibodies against various laminin chains were tested for their potential of competitive inhibitions of leishmanial adherence to laminin-coated substrata (Fig. 4A). Of the various monoclonals tested, only that against B1 chain could abrogate parasite adherence to laminin-coated wells. To further localize the domain of laminin responsible for LBP binding, we took advantage of the fact that a number of peptides responsible for the attachment activity for a variety of cell types have been derived from laminin. The first peptide, YIGSR, a component of the B1 chain of laminin, is included in the major cell binding and cell migration site of laminin [22,23]. The second one, RNIAEIIKDI, a component of B2 chain of laminin, is associated with the promotion of neurite outgrowth and cell binding [24]. The hexapeptide, SIKVAV, a component of the A chain of laminin has been described as an angiogenic factor *in vivo* [25]. Control peptides of the same length, but with different structures were also included for all the sequences. Of all these peptides tested in adherence inhibition studies only YIGSR and $C(YIGSR)_3$-NH_2 were found to inhibit laminin binding significantly (59% and 65%, respectively) (Fig. 4B). In order to ascertain whether YIGSR in a protein environment would be more active, YIGSR fused to protein A was also tested. The inhibitory effect was similar to that of

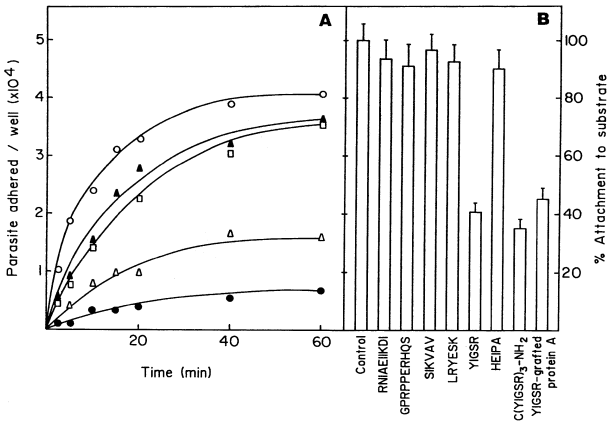

Fig. 4. Inhibition of attachment of *L. donovani* promastigotes to laminin-coated micro titer wells by (A) various antibodies and (B) synthetic peptides. (A) Laminin-coated surfaces (5 μg per well) were overlaid with 5×10^5 cells of a suspension of ^{125}I-labelled parasites and incubated for the indicated periods of time in presence of (○) none (●) anti-laminin Ig (△) anti-B1 chain Ig (□) anti-B2 chain Ig and (▲) anti-A chain Ig. All antibodies were at 1 : 10 dilution. After extensive washing of the unbound parasites with $NaCl/P_i$, 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 micro titer wells in the presence of 0.1 $mg \cdot mL^{-1}$ of various synthetic peptides. Data are mean ± SD from incubations performed in triplicate. The amount of attached cells is given as a percent of the number of cells that were attached to the wells in the absence of peptides. For the decapeptide RNIAEIIKDI related to the cell binding site from the B2 chain of laminin, the decapeptide GPRPPERHQS was used as control. For the hexapeptide SIKVAV related to the A chain, LRYESK was used as control whereas for the pentapeptide YIGSR related to the B1 chain, HEIPA was used as control.

the pentapeptide (Fig. 4B). Other signature sequences of B1 chain with adhesion property such as RYVVLPR (21), LGTIPG [26] and RGD [27] did not show any inhibitory activity (data not shown). All these molecules with adherence inhibitory activity could effectively block laminin binding to LBP (Table 2).

Tyrosine phosphorylation through LBP

Results suggest that the zinc finger motif of B1 chain of laminin containing YIGSR sequence may provide the

Table 2. The effect of various agents on laminin-LBP binding. Means of three determinations ± SD. Values include the significance (* $P < 0.001$) of the difference between inhibition in the presence and absence of inhibitors as determined by analysis of variance.

Agents applied	% Inhibition
None	0 ± 3
Laminin B1	81 ± 6*
YIGSR	66 ± 5*
HEIPA	8 ± 2
$C(YIGSR)_3$-NH_2	76 ± 6*
YIGSR grafted protein A	53 ± 5*

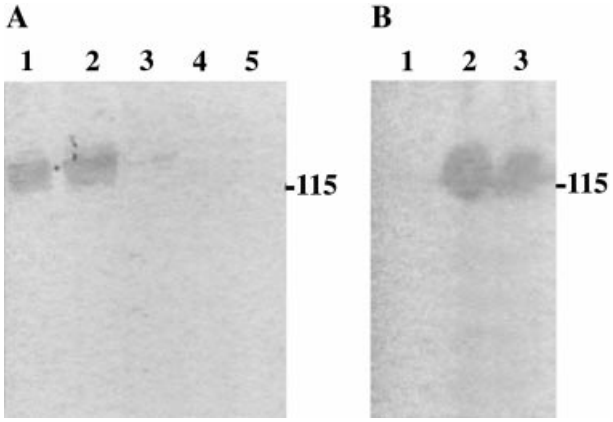

Fig. 5. Tyrosine phosphorylation via LBP. (A) *L. donovani* promastigotes (2×10^8 cells) were washed twice with medium M199 and incubated with 100 $\mu g \cdot mL^{-1}$ of either $C(YIGSR)_3$-NH_2 for 1 min (lane 1), 5 min (lane 2) or 15 min (lane 3) or with 100 $\mu g \cdot mL^{-1}$ of unrelated peptide for 1 min (lane 4) and 5 min (lane 5). Cells were washed with ice-cold NaCl/P_i, lysed, subjected to 7.5% SDS/PAGE and transferred to nitrocellulose membrane. The blotted membranes were incubated with anti-(P-Tyr) monoclonal antibodies followed by alkaline phosphatase conjugated secondary antibody and developed by Nitro Blue tetrazolium and 5-bromo-4-chloro-indolyl-3-phosphate. (B) Serum-starved promastigotes (5×10^7 cells) were incubated with uncoated latex beads (lane 1), latex beads coated with $C(YIGSR)_3$-NH_2 (lane 2) or with antibodies directed against the 67 kDa LBP (lane 3). Following incubation, cells were collected, lysed, subjected to SDS/PAGE and blotted with anti-(P-Tyr) monoclonal antibodies.

physiological scaffolding required for LBP binding. It is likely that binding of laminin to cell surface LBP through YIGSR sequence may involve specific downstream signalling events, one of which may be phosphorylation of tyrosine residues of some intracellular proteins. We therefore analysed the response of *L. donovani* promastigotes to the presence of $C(YIGSR)_3$-NH_2 as compared to an unrelated peptide. Exposure of 2×10^8 promastigotes to 100 $\mu g \cdot mL^{-1}$ of $C(YIGSR)_3$-NH_2 peptide induced tyrosine phosphorylation of several proteins with a molecular mass of 115–130 kDa (Fig. 5A). The induction of tyrosine phosphorylation was rapid and transient, reaching a maximum level within 1 min. In contrast, when cells were exposed to an unrelated polypeptide (CYKNVRSKIGSTENIKHQPGGGKV) of similar length, and the same molar concentration, tyrosine phosphorylation of these proteins was hardly detected (Fig. 5A, lanes 4 and 5). It seems therefore that at least some high molecular mass proteins of 115–130 kDa underwent phosphorylation on tyrosine residues following binding of YIGSR repeat to the cell surface 67-kDa LBP. In order to further ascertain that the induction of tyrosine phosphorylation is not due to any growth factors, serum-starved parasites were allowed to adhere in suspension to polystyrene latex beads coated with $C(YIGSR)_3$-NH_2 for 1 min at 22 °C. As shown in Fig. 5B (lane 2), the same high molecular mass proteins of 115–130 kDa underwent phosphorylation on tyrosine residues. Phosphorylation was not detected in the presence of uncoated beads (lane 1). In order to know whether clustering of LBP by anti-LBP Ig also could induce tyrosine phosphorylation, serum-starved cells were allowed to adhere in suspension to polystyrene latex beads coated with anti-LBP Ig and incubated for 1 min at 22 °C. Figure 5B (lane 3) shows that clustering of LBP by the corresponding antibody resulted in phosphorylating the same group of proteins that were phosphorylated in response to $C(YIGSR)_3$-NH_2 coated beads.

DISCUSSION

Adhesion of pathogen to host tissue is a prerequisite for many types of infections. Diseases such as leishmaniases are is generally initiated when sand fly, the vector, regurgitates promastigote form of the parasite at the time of taking a blood meal from human body. This developmental form migrates through the blood stream into various definite organs like liver and spleen and ultimately takes refuge within the resident macrophages where it transforms into the amastigote form and multiplies in number. Eventually parasites are released into the interstitial tissue by macrophage lysis, invade fresh cells and the cycle is repeated. This way the entire reticuloendothelial system becomes progressively infected. Evidently during transit in the interstitial tissue, these intracellular parasites must be in contact with the extracellular matrix and the basement membrane. We have identified and characterized a laminin binding protein (LBP) from the surface of *L. donovani* that may mediate cell adhesion by helping the parasite to home in their physiological address [5,6]. Laminin is a multidomain molecule [24], and it is known that there are several specific binding domains on laminin for each of the laminin binding proteins. Studies with proteolytic fragments, domain-specific antibodies, and synthetic peptides have identified different regions of laminin with biological activity [21]. This paper is mainly concerned with the identification of a specific domain of laminin mediating the binding of leishmanial LBP.

The purified 67-kDa LBP isolated from the membrane fraction behaved as one would expect of a laminin receptor and laminin binding to LBP was found to be dose-dependent, specific and saturable. Laminin–LBP interaction also involved a single class of binding sites, which appeared to be conformation-dependent, ionic in nature, and significantly enhanced by Zn^{2+}. Detailed binding studies at various pH indicated the presence of His and Cys at the binding site. However, the unaltered binding parameters after diethyl pyrocarbonate treatment preclude the possibility of the presence of His at the binding site. It may be mentioned that the ionization state of amino-acid residues is influenced by their unique microenvironment; therefore, predicting the impact of the residues based solely on theoretical pK_a of their individual side chains is speculative.

The positive effect of zinc on laminin binding activity suggests that it could be a potential metal cofactor for *L. donovani* interaction with ECM and BM. Both Zn^{2+} and free sulfhydryls may be required for LBP binding site on laminin as evidenced by the stimulatory and inhibitory effects of $ZnCl_2$ and *N*-ethylmaleimide, respectively. Preincubating LBP with $ZnCl_2$ did not enhance laminin-binding activity, indicating that zinc was affecting laminin only. Moreover, treating LBP with EDTA had little effect on its binding with laminin, consistent with the indication of the role of zinc as laminin-specific cofactor. Laminin is known

to contain 42 Cys-rich repeats of which 12 represent the consensus sequence for Cys-rich Zn^{2+} fingers. Taken together, the data therefore suggest that Zn^{2+} finger like sequence may represent the actual LBP binding site or at least contribute to it significantly. Laminin bound zinc detected by flame atomic absorption spectroscopy was about 10 mol·mol^{-1}. The amount is consistent with the predicted number of zinc finger sequences. It is now well known that metal-binding domains, particularly Zn^{2+} finger motifs, play central roles in mediating interactions between proteins and many different macromolecules [28]. This may be due to the formation of bumps and ridges that extend from the surfaces of proteins that are well suited for interactions with other macromolecules. Laminin zinc fingers are known to participate in binding to Alzheimer's amyloid precursor protein and collagen IV [8,29]. The enactin binding site was recently mapped to Cys-rich repeats on the laminin B2 chain which happens to contain Zn^{2+} finger like sequence [9]. Although the present study was carried out with mouse laminin, the putative zinc-finger motifs are known to be highly conserved between human [30–32], mouse [33,34] and *Drosophila* [35–37]. Inhibition studies with Fab fragments of monoclonal antibodies against various chains of laminin are indicative of the presence of LBP binding site on the B1 chain of laminin. Moreover, a number of small peptide recognition sequences have been reported to date in laminin, which are attributed to various biological activities of laminin [38]. YIGSR, a short sequence of the B1 chain of laminin, was reported to be a potential binding site for specific laminin binding proteins, particularly 67-kDa laminin receptor present on normal and cancer cell surface [39, 40]. This sequence is not present in the A and B2 chains. Competitive inhibition of laminin-LBP binding by YIGSR indicates that interaction of LBP with this peptide is specific. However, YIGSR grafted in protein A could not enhance the inhibitory effect over that of the peptide alone. All these studies suggest that zinc finger motif of B1 chains containing YIGSR sequence, may provide the physiological scaffolding required for LBP binding.

Cell–matrix interactions have recently been shown to trigger many signalling processes [11,12]. For example, tyrosine phosphorylation is involved in collagen signalling in amoebas, which might play a role in the invasiveness capability of this parasite [41]. In the present studies one class of proteins was found to be phosphorylated in response to the interaction of $C(YIGSR)_3$-NH_2 with the 67-kDa LBP. These proteins had a molecular mass of 115–130 kDa, but their identity remains to be determined. It is possible that the above proteins may undergo autophosphorylation on a tyrosine residue, which generally implies that it encodes a phosphotyrosine kinase, as a result of activation by cell adhesion to YIGSR sequence. Alternatively, the proteins may be phosphorylated by another unknown phosphotyrosine kinase. As an antibody directed against the 67-kDa LBP can induce tyrosine phosphorylation of these proteins, it is likely that dimerization or oligomerization of LBP is required for activating an associated tyrosine kinase.

The ability of *L. donovani* LBP to bind a major ECM protein like laminin probably plays a role in pathogenesis of the disease process this species exhibits in mammalian host. The ECM protein binding ability of the leishmanial LBP could allow the parasite to persist within the host and thus contribute to virulence. For example, binding of ECM protein to the surface of the parasite via LBP could block or reduce host's immune response to the parasite by sterically masking immunogenic epitope. The ability to bind ECM proteins might also facilitate adhesion of the pathogen to host cells such as macrophages via laminin receptors present on the cell surface. The elucidation of the binding region of laminin may therefore help in better understanding the pathogenesis as well as developing effective therapeutic strategies.

ACKNOWLEDGEMENTS

We are indebted to the Council for Scientific and Industrial Research and the Department of Biotechnology, Government of India for financial help.

REFERENCES

1. Silva Filho, F.C., de Souza, W. & Lopes, L.D. (1988) Presence of laminin-binding proteins in *trichomonads and* their role in adhesion. *Proc. Natl Acad. Sci. USA* **85**, 8042–8046.
2. Vicentini, A.P., Gesztesi, J., Franco, M.F., deSouza, W., deMoraes, J.Z., Travassos, L.R. & Lopes, J.D. (1994) Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp 43 leads to enhancement of fungal pathogenesis. *Infect. Immun.* **62**, 1464–1469.
3. Lopez-Ribot, J.L., Casanova, M., Monteagudo, C., Sepulveda, P. & Martinez, J.P. (1994) Evidence for the presence of a high affinity laminin receptor-like molecule on the surface of *Candida albicans* yeast cells. *Infect. Immun.* **62**, 742–746.
4. Giordano, R., Chammas, R., Veiga, S.S., Colli, W. & Alves, M.J.M. (1994) An acidic component of the heterogeneous Tc-85 protein family from the surface of *Trypanosoma cruzi* is a laminin binding glycoprotein. *Mol. Biochem. Parasitol.* **65**, 85–94.
5. Ghosh, A., Kole, L., Bandyopadhyay, K., Sarkar, K. & Das, P.K. (1996) Evidence of a laminin binding protein on the surface of *Leishmania donovani*. *Biochem. Biophys. Res. Commun.* **226**, 101–106.
6. Ghosh, A., Bandyopadhyay, K., Kole, L. & Das, P.K. (1999) Isolation of a laminin-binding protein from the protozoan parasite *Leishmania donovani* that may mediate cell adhesion. *Biochem. J.* **337**, 551–558.
7. Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R. & Timpl, R. (1990) Identification of the Arg-Gly-Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1. *FEBS Lett.* **262**, 82–86.
8. Ancsin, J.B. & Kisilevsky, R. (1996) Laminin interactions important for basement membrane assembly are promoted by zinc and implicate laminin zinc finger-like sequences. *J. Biol. Chem.* **271**, 6845–6851.
9. Mayer, U., Nischt, R., Poschl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y. & Timpl, R. (1993) A single EGF-like motif of laminin is responsible for high affinity nidogen binding. *EMBO J.* **12**, 1879–1885.
10. Ancsin, J.B. & Kisilevsky, R. (1997) Characterization of high affinity binding between laminin and the acute-phase protein, serum amyloid A. *J. Biol. Chem.* **272**, 406–413.
11. Hynes, R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11–25.
12. Schwartz, M.A. & Ingber, D.E. (1994) Integrating with integrins. *Mol. Biol. Cell* **5**, 389–393.
13. Sarkar, K. & Das, P.K. (1997) Protective effect of neoglycoprotein conjugated muramyl dipeptide against *Leishmania donovani* infection. *J. Immunol.* **158**, 5357–5365.

14. Chakraborty, P. & Das, P.K. (1988) Role of mannose/*N*-acetylglucosamine receptors in blood clearance and cellular attachment of *Leishmania donovani*. *Mol. Biochem. Parasitol.* **28**, 55–62.
15. Kahl, L.P. & McMahon-Pratt, D.J. (1987) Structural and antigenic characterization of a species- and promastigote-specific *Leishmania mexicana amazonensis* membrane protein. *J. Immunol.* **138**, 1587–1595.
16. Hall, D.E., Frazer, K.A., Hann, B.C. & Reichardt, L.F. (1988) Isolation and characterization of a laminin-binding protein from rat and chick muscle. *J. Cell Biol.* **107**, 687–697.
17. Malinoff, H.L. & Wicha, M.S. (1983) Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. *J. Cell Biol.* **96**, 1475–1479.
18. Greenwood, F., Hunter, W. & Glover, J. (1963) The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114–123.
19. Leary, J.J., Brigati, D.J. & Ward, D.C. (1983) Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridised to DNA or RNA immobilized on nitrocellulose: bioblots. *Proc. Natl Acad. Sci. USA* **80**, 4045–4049.
20. Kurz, L.L., Klink, H., Jakob, I., Kuchenbecker, M., Benz, S., Lehmann-Horn, F. & Rudel, R. (1999) Identification of three cysteines as targets for the Zn^{2+} blockade of the human skeletal muscle chloride channel. *J. Biol. Chem.* **274**, 11687–11692.
21. Yamada, K.M. (1991) Adhesive recognition sequences. *J. Biol. Chem.* **266**, 12809–12812.
22. Iwamoto, Y., Robey, F.A., Graf, J., Sasaki, M., Kleinman, H.K., Yamada, Y. & Martin, G.R. (1987) YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. *Science* **238**, 1132–1134.
23. Iwamoto, Y., Graf, J., Sasaki, M., Kleinman, H.K., Greatorex, D.R., Martin, G.R., Robey, F.A. & Yamada, Y. (1988) Synthetic pentapeptide from the B1 chain of laminin promotes B16F10 melanoma cell migration. *J. Cell. Physiol.* **134**, 287–291.
24. Engel, J. (1992) Laminins and other strange proteins. *Biochemistry* **31**, 10643–10651.
25. Grant, D.S., Kinsella, J.L., Fridman, R., Auerbach, R., Piasecki, B.A., Yamada, Y., Zain, M. & Kleinman, H.K. (1992) Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) *in vitro* and induction of angiogenic behaviour *in vivo*. *J. Cell. Physiol.* **153**, 614–625.
26. Hinek, A., Wrenn, D.S., Mecham, R.P. & Borondes, S.H. (1988) The elastin receptor: a galactoside-binding protein. *Science* **239**, 1539–1541.
27. Mecham, R.P., Hinek, A., Griffin, G.L., Senior, R.M. & Liotta, L.A. (1989) The elastin receptor shows structural and functional similarities to the 67-kDa tumor cell laminin receptor. *J. Biol. Chem.* **264**, 16652–16657.
28. Berg, J.M. (1990) Zinc fingers and other metal-binding domains. Elements for interactions between macromolecules. *J. Biol. Chem.* **265**, 6513–6516.
29. Narindrasorasak, S., Lowery, D.E., Altman, R.A., Gonzalez-DeWhitt, P.A., Greenberg, B.D. & Kisilevsky, R. (1992) Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins. *Laboratory Invest.* **67**, 643–652.
30. Nissinen, M., Vuolteenaho, R., Boot-Handford, R., Kallunki, T. & Tryggvason, K. (1991) Primary structure of the human laminin A chain. Limited expression in human tissues. *Biochem. J.* **276**, 369–379.
31. Pikkarainen, T., Eddy, R., Fukushima, Y., Byers, M., Shows, T., Pihlajaniemi, J., Saraste, M. & Tryggvason, K. (1988) Human laminin B1 chain. A multidomain protein with gene (LAMB1) locus in the q22 region of chromosome 7. *J. Biol. Chem.* **262**, 10454–10462.
32. Pikkarainen, T., Kallunki, T. & Tryggvason, K. (1988) Human laminin B2 chain. Comparison of the complete amino acid sequence with the B1 chain reveals variability in sequence homology between different structural domains. *J. Biol. Chem.* **263**, 6751–6758.
33. Sasaki, M. & Yamada, Y. (1987) The laminin B2 chain has a multidomain structure homologous to the B1 chain. *J. Biol. Chem.* **262**, 17111–17117.
34. Sasaki, M., Kleinman, H.K., Huber, H., Deutzmann, R. & Yamada, Y. (1988) Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. *J. Biol. Chem.* **263**, 16536–16544.
35. Kusche-Gullberg, M., Garrison, K., MacKrell, A.J., Fessler, L.I. & Fessler, J.H. (1992) Laminin A chain: expression during *Drosophila* development and genomic sequence. *EMBO J.* **11**, 4519–4527.
36. Montell, D.J. & Goodman, C.S. (1988) *Drosophila* substrate adhesion molecule: sequence of laminin B1 chain reveals domains of homology with mouse. *Cell* **53**, 463–473.
37. Chi, H.C. & Hui, C.F. (1989) Primary structure of the *Drosophila* laminin B2 chain and comparison with human, mouse, and *Drosophila* laminin B1 and B2 chains. *J. Biol. Chem.* **264**, 1543–1550.
38. Mecham, R.P. (1991) Receptors for laminin on mammalian cells. *FASEB J.* **5**, 2538–2546.
39. Graf, J., Iwamoto, M., Sasaki, M., Martin, G.R., Kleinman, H.K., Robey, F.A. & Yamada, Y. (1987) Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell* **48**, 989–996.
40. Clement, B., Segui-Real, B., Savagner, P., Kleinman, H.K. & Yamada, Y. (1990) Hepatocyte attachment to laminin is mediated through multiple receptors. *J. Cell Biol.* **110**, 185–192.
41. Perez, E., Munoz, M.L. & Ortega, A. (1996) *Entamoeba histolytica*: Involvement of $pp125^{FAK}$ in collagen-induced signal transduction. *Exp. Parasitol.* **82**, 164–170.