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Interaction of Laminin with *Entamoeba histolytica* Cysteine Proteinases and Its Effect on Amebic Pathogenesis

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The *Entamoeba histolytica* 27-kDa cysteine proteinases exhibit striking binding specificities for immobilized laminin over other components of the extracellular matrix, such as collagen and fibronectin. Inactivation of the proteinase with the active-site inhibitor L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane abolishes laminin binding by the enzyme, and conversely, laminin inhibits cleavage of a fluorogenic dipeptide substrate of the amebic cysteine proteinase, suggesting that the substrate binding pocket of the enzyme is involved in the binding of laminin. The addition of laminin but not fibronectin or collagen to *E. histolytica* trophozoites significantly reduces amebic liver abscess formation in severe combined immunodeficient mice, further supporting the hypothesis that *E. histolytica* cysteine proteinases play an important role in amebic pathogenesis. The specific interaction of amebic proteinases with laminin may be exploited in designing new inhibitors of these enzymes.

The protozoan parasite *Entamoeba histolytica* is the causative agent of human amebiasis. Invasive disease is characterized by ulcerations of the intestinal wall and in some cases by invasion through the wall and dissemination to the liver, resulting in the clinical syndromes of amebic dysentery and liver abscess, respectively (8). Ultrastructural studies of experimental *E. histolytica* infection revealed the degeneration of epithelial cells adjacent to invading trophozoites and the penetration of trophozoites into the lamina propria through the basement membrane (16). The interaction of amebic factors with components of the extracellular matrix potentially plays an important role in the penetration of trophozoites through the intestinal mucosa. In order to identify *E. histolytica* proteins that interact with components of the extracellular matrix, amebic lysates were fractionated over laminin-Sepharose, fibronectin-Sepharose, and collagen-Sepharose. We report here that *E. histolytica* 27-kDa cysteine proteinases exhibit striking binding specificities for immobilized laminin over immobilized fibronectin or collagen. Furthermore, the coinjection of laminin but not fibronectin or collagen with *E. histolytica* trophozoites greatly reduces liver abscess formation in severe combined immunodeficient (SCID) mice.

MATERIALS AND METHODS

Materials. Laminin isolated from Engelbreth-Holm-Swarm sarcoma was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and was generously provided by Hynda Kleinman. Bovine fibronectin was purchased from Calbiochem (San Diego, Calif.). Calf skin type I collagen coupled to Sepharose (1 mg of beads per ml) was generously provided by Samuel Santoro.

Cells. *E. histolytica* HM1-IMSS trophozoites were obtained from the American Type Culture Collection (Rockville, Md.). The amebas were grown in BI-S-33 as previously described (3).

Ameba radiolabeling. Cultures (72 h) of amebic trophozoites were metabolically labeled with ^{35}S *trans* label (85% methionine, 15% cysteine, 50 μCi/ml; ICN) in methionine-free minimal essential medium-alpha, supplemented with 0.1% bovine serum albumin (BSA)–5.7 mM cysteine for 4 h at 35°C. The cells were harvested by chilling and low-speed centrifugation, washed with phosphate-buffered saline (PBS), and solubilized. The solubilization buffer consisted of 1% Nonidet P-40, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM phenylmethylsulfonyl fluoride, and 5 μM L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) in 10 mM Tris-Cl buffer, pH 8.0. The cell extracts were clarified after centrifugation at 10,000 × *g* for 5 min.

Affinity chromatography. Engelbreth-Holm-Swarm laminin or fibronectin was conjugated to Sepharose CN 4B (Pharmacia LKB Biotechnology Inc.) at a ratio of 1 mg of protein to 1 ml of Sepharose according to the manufacturer's instructions. Under these conditions, 90% coupling was achieved, as monitored by the A_{280} of the protein solution after coupling. Aliquots of detergent extracts of amebic trophozoites (0.5 ml, containing 1×10^6 to 1.5×10^6 cells) were incubated with an equivalent volume of laminin-Sepharose overnight at 4°C with gentle agitation. The beads were washed with 10 volumes of buffer A (0.1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0), 10 volumes of buffer B (0.1% Nonidet P-40, 500 mM NaCl, 10 mM Tris-HCl, pH 8.0), and 5 volumes of buffer C (50 mM Tris-Cl, pH 6.8) as described by Woo et al. (21). Proteins bound to laminin-Sepharose were eluted with Laemmli sample buffer or with 4 M urea. In some experiments, 0.1 M *N*-acetyllactosamine or heparin (Sigma Chemical Co., St. Louis, Mo.) (5 mg/ml) was added to the laminin-Sepharose incubations. In order to inactivate the cysteine proteinase, trophozoite lysates were incubated with 500 μM E-64 (1) at 25°C for 20 min.

Protein purification and microsequencing analysis. Proteins bound to laminin-Sepharose were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, electroblotted onto polyvinyldene difluoride, stained with Coomassie blue, excised, and subjected to microsequencing as described previously (9).

Solid-phase binding assay of ^{125}I-labeled affinity-purified *E. histolytica* cysteine assay. Laminin affinity-purified *E. histolytica* cysteine proteinase (10 μg) was iodinated by incubation of 1 mCi of Na^{125}I in the presence of two Iodobeads (Pierce) according to the manufacturer's recommendations. The labeled cysteine proteinase was further purified by gel filtration with a Superose-12 column in PBS–2 mM dithiothreitol. Laminin, fibronectin, collagen, or BSA (8 μg) in PBS was applied to 96-well polystyrene radioimmunoassay wells overnight at room temperature. The plates were washed and blocked for 1 h with 0.5% BSA. The wells were incubated at 4°C with labeled *E. histolytica* cysteine proteinase (20 ng, 1,000 cpm/ng) and were aspirated, and the plates were washed three times with 0.5% BSA. The wells were excised and counted in a gamma counter. Each experiment was carried out in quadruplicate.

Assays for proteolytic activity. Protease activity was assessed by gelatin substrate gel electrophoresis as described previously (12). Protease activity was also assayed by monitoring cleavage of a fluorogenic substrate, Boc-arginine-arginine-4-amino-7-methylcoumarin (ZRR-AMC), as described previously (12). A 1-μl aliquot of laminin affinity-purified cysteine proteinase in 4 M urea was added directly to the reaction mixture.

Effect of laminin on amebic liver abscess formation in SCID mice. SCID mice were inoculated with 10^6 *E. histolytica* trophozoites in either 100 μl of BI-S-33 medium, 100 μl of BI-S-33 plus 20 μg of laminin, 100 μl of BI-S-33 plus 20 μg of fibronectin, or 100 μl of BI-S-33 plus 20 μg of collagen as described previously (14). After 48 h, SCID mice were sacrificed, their livers were removed and inspected for the presence of amebic liver abscesses, abscesses and livers were weighed, and the percentage of liver abscessed was calculated (14).

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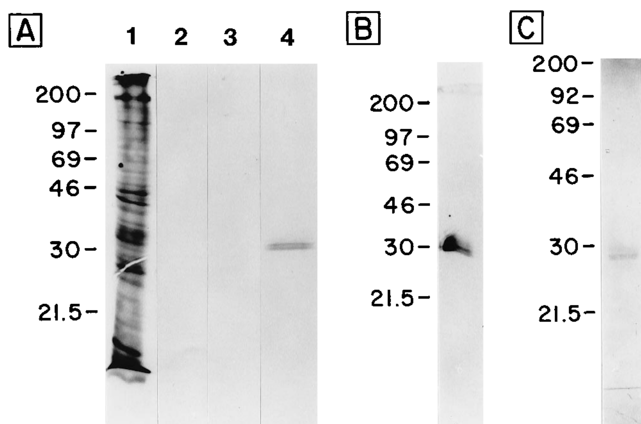

FIG. 1. Laminin-Sepharose chromatography of *E. histolytica* extracts and conditioned media. Detergent extracts of trophozoites were incubated in the presence of laminin-Sepharose, fibronectin-Sepharose, or collagen-Sepharose and analyzed by gel electrophoresis (for details, see Materials and Methods). (A) Fluorogram of gel from [^{35}S]methionine-labeled trophozoites. Total lysate is shown in lane 1. Fractions eluted with 4 M urea after binding to collagen-Sepharose (lane 2), fibronectin-Sepharose (lane 3), and laminin-Sepharose (lane 4) are also shown. (B) [^{35}S]methionine-labeled conditioned media incubated with laminin-Sepharose. (C) Coomassie blue-stained unlabeled cysteine protease eluted from laminin-Sepharose. Molecular weight standards (in thousands) are shown in the margins.

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Laminin Binding Protein        APKAVDERKEGKVTPIRDQGN
                                 ES  W  K

Eh-CPp1                        APKAVDWRKKGKVTPIRDQGN

Eh-CPp2                        APESVDWRKEGKVTPIRDQGN
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FIG. 2. Comparison of the N-terminal sequence of the 27-kDa laminin-binding proteins with the amino acid sequences of two *E. histolytica* cysteine proteases. At positions 3, 4, 7, and 10, two amino acid residues were detected. The sequences of Eh-CPp1 and EH-CPp2 are derived from the work of Tannich et al. (18).

RESULTS AND DISCUSSION

Detergent extracts of [^{35}S]methionine-radiolabeled *E. histolytica* trophozoites were incubated in the presence of laminin-Sepharose. The beads were washed with 0.5 M NaCl, and the bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 1A, two closely spaced protein bands estimated to be approximately 27 kDa in molecular mass were observed. These were the only two bands detected which withstood a high-concentration salt wash of the column. Under these conditions, binding to neither fibronectin-Sepharose nor collagen-Sepharose (Fig. 1A) was observed. Binding was not inhibited by the addition of 0.1 M *N*-acetyllactosamine or heparin (5 mg/ml), indicating that binding was not lectin mediated or heparin dependent, as reported for several other nonintegrin laminin-binding proteins (5, 10, 15, 22). As shown in Fig. 1B, the *E. histolytica* laminin-binding protein can also be isolated from conditioned culture media. Coomassie blue staining of the affinity-purified protein fraction, shown in Fig. 1C, confirmed that the 27-kDa laminin-binding proteins were the predominant species retained on laminin-Sepharose after the high-concentration salt wash.

To further characterize the 27-kDa laminin-binding proteins, the affinity-purified proteins were subjected to microsequence analysis. The two protein bands could not be easily separated on the polyvinylidene difluoride membrane. The N-terminal sequence obtained from affinity-purified protein, shown in Fig. 2, consequently revealed heterogeneity in the sample. Comparison of the sequence with the deduced amino acid sequence of two genes encoding homologous *E. histolytica* 27-kDa neutral cysteine proteinases, recently cloned by Tannich and coworkers (17, 18), suggests that the laminin-Sepharose affinity-purified fraction includes both cysteine proteinases.

The levels of binding of ^{125}I-labeled affinity-purified *E. histolytica* cysteine proteinase to laminin, fibronectin, collagen, and BSA adsorbed to the wells of a polystyrene radioimmunoassay plate were measured (Table 1). The addition of 50 μg of soluble laminin to each well inhibited binding of the labeled cysteine proteinase by 95% ± 2% (mean ± standard deviation; *n* = 2). Thus, the *E. histolytica* cysteine proteinases appear to exhibit a relative binding specificity for immobilized laminin compared with those for other proteins which also serve as substrates for these enzymes (6, 7, 13). The 27-kDa *E. histolytica* cysteine proteinases belong to the papain superfamily (17, 18). ^{125}I-labeled papain, a cysteine proteinase homologous to the amebic cysteine proteases, does not exhibit increased binding to immobilized laminin compared with those of fibronectin, collagen, and BSA in this assay (Table 1).

The proteinase activity of the affinity-purified protein was assessed by gelatin substrate gel electrophoresis. As shown in Fig. 3, several clear bands, ranging from 30 to 69 kDa in molecular mass, were observed, indicating that the affinity-purified protein had proteinase activity. When the [^{35}S]methionine-labeled affinity-purified material was subjected to electrophoresis through a gelatin substrate gel, several higher-molecular-mass bands were observed, corresponding to the bands of clearing (Fig. 3). That the cysteine protease has a larger apparent molecular mass when subjected to electrophoresis through a gelatin substrate gel under nonreducing conditions has been reported by a number of investigators (6, 7).

E-64 is an inhibitor of cysteine proteinases which forms a covalent link between the sulfur of the active-site cysteine and the C-2 atom of the inhibitor (1). Although the inclusion of the active-site protease inhibitor E-64 at concentrations as high as 0.5 mM in lysis buffer at 4°C fails to inhibit binding of the proteases to laminin (data not shown), preincubation of the lysates at room temperature with 0.5 mM E-64 for 20 min abolishes binding to laminin (Fig. 4). The temperature dependence for the inhibitory effect of E-64 may reflect the relatively low affinity of E-64 relative to laminin for the substrate pocket. In the papain–E-64 complex, the inhibitor interacts with the S subsites on the enzyme and causes a slight widening of the active-site cleft (19), so that E-64 could also have an allosteric effect on laminin binding.

Hydrolysis of the fluorogenic peptide substrate ZRR-AMC by 0.5 U of laminin affinity-purified *E. histolytica* cysteine proteinase is inhibited 93% ± 4% (mean ± standard deviation; *n* = 4) by the addition of 0.1 mg of laminin per ml. In contrast,

TABLE 1. Binding of ^{125}I-labeled affinity-purified *E. histolytica* cysteine proteinase and papain[a]

Binding to:	cpm (mean ± SD) of bound:	
	Cysteine proteinase	Papain
Laminin	1,900 ± 200	100 ± 20
Fibronectin	70 ± 10	150 ± 20
Collagen	70 ± 20	60 ± 20
BSA	40 ± 20	50 ± 10

[a] Each experiment was carried out in quadruplicate wells as described in Materials and Methods. The values shown represent the means ± standard deviations of two separate experiments.

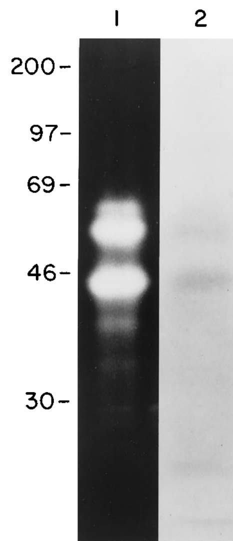

FIG. 3. Gelatin substrate gel electrophoresis of proteinase activity of the 27-kDa laminin-binding proteins. Lane 1, Coomassie blue-stained gelatin substrate gel of ^{35}S-labeled 27-kDa affinity-purified laminin-binding protein. Lane 2, fluorogram of the gelatin substrate gel of ^{35}S-labeled 27-kDa affinity-purified laminin-binding protein. Molecular weight standards (in thousands) are noted in the margin.

the addition of 0.1 mg of collagen per ml has no significant effect on hydrolysis of the fluorogenic peptide substrate by the laminin affinity-purified *E. histolytica* cysteine proteinase. These results also suggest that the substrate binding pocket of the enzyme is involved in the binding of laminin.

E. histolytica cysteine proteinases appear to play important roles in the pathogenesis of invasive amebiasis. The degrees of inflammation and necrosis produced by different strains of *E. histolytica* correlate with proteinase activity in animal models (4, 11, 12). Inhibition of amebic protease activity markedly reduces the inflammatory lesions resulting from highly virulent

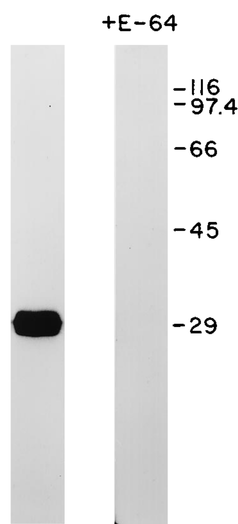

FIG. 4. Inhibition of laminin binding by inactivation of the cysteine proteinase with E-64. Shown are fluorograms of laminin-Sepharose affinity-purified fractions from detergent lysates of [^{35}S]methionine-labeled trophozoites incubated in the presence (+) or absence (blank) of 0.5 mM E-64 prior to incubation with laminin-Sepharose. Molecular weight standards (in thousands) are indicated in the margin.

TABLE 2. Inhibition of amebic liver abscess formation in SCID mice by administration of laminin with *E. histolytica* trophozoites

Inhibitor	n^a	% liver abscessed (mean ± SD)	P^b
Control medium	12	24 ± 16	
Laminin	19	4 ± 7	<0.001
Fibronectin	10	15 ± 6	0.124
Collagen	10	17 ± 9	0.25

[a] *n*, number of SCID mice in each group. The results represent the totals from three separate experiments for control and laminin groups and two experiments which included the fibronectin and collagen groups.

[b] *P* values are derived from the two-tailed *t* test for comparisons between each of the listed inhibitors and the control group for percentages of liver abscessed. The mean abscess size for SCID mice to which laminin was administered as an inhibitor was also significantly smaller compared with that when fibronectin ($P < 0.001$) and collagen ($P < 0.001$) were used as inhibitors.

E. histolytica (11). We have previously reported immunohistochemical studies of tissue sections of a SCID mouse amebic liver abscess which indicate that significant quantities of extracellular *E. histolytica* cysteine proteinase are present within the amebic liver abscess and have shown that treatment of *E. histolytica* trophozoites with the cysteine proteinase inhibitor E-64 blocked or greatly reduced liver abscess formation in SCID mice (14). We have shown that *E. histolytica* cysteine proteinases bind to laminin tightly and that laminin inhibits the cleavage of other substrates. In order to examine the effect of laminin on amebic pathogenesis in vivo, we examined whether the coinjection of trophozoites with laminin would alter amebic liver abscess formation (Table 2). SCID mice were injected with 10^6 trophozoites in 100 μl of BI-S-33 (serum-free) medium alone or medium containing 20 μg of either laminin, fibronectin, or collagen. The addition of laminin, fibronectin, or collagen did not affect the viability (90 to 95% by trypan blue exclusion), nor did it visibly affect the motility of the trophozoites in the inoculum prepared as described after incubation at 37°C for up to 4 h. All 12 control animals had amebic liver abscesses, with a mean of 24% ± 16% (mean ± standard deviation) of the total liver abscessed. The mean abscess size for the 19 mice receiving trophozoites treated with laminin was 4% ± 7%, with 9 animals having no detectable abscesses. Thus, the amebic liver abscesses were significantly smaller in mice inoculated with trophozoites that had been coinjected with laminin than those in the control animals ($P < 0.001$). In contrast, the amebic liver abscesses in mice inoculated with trophozoites that had been coinjected with fibronectin or with collagen were not significantly smaller than those in the control animals (Table 2).

The effect of laminin compared with the effects of fibronectin and collagen on amebic liver abscess formation in vivo thus appears to correlate with the tight binding of *E. histolytica* cysteine proteinases to immobilized laminin compared with their binding to immobilized fibronectin and collagen observed in vitro. The results are consistent with the hypothesis that the *E. histolytica* cysteine proteinases play important roles in the development of amebic liver abscess in the SCID mouse model and with the inhibitory effects of laminin on amebic cysteine proteinase activity. However laminin is a large (~900-kDa) multidomain complex that exhibits multiple biological activities, including cell adhesion and cell migration (2), and one cannot exclude the possibility that the in vivo effects of laminin on amebic liver abscess formation are due to interactions of laminin with the trophozoite other than inhibition of proteolytic activity. It is interesting that in an animal model of a

fungal pathogen, *Paracoccidioides brasiliensis*, which binds to laminin via a surface glycoprotein receptor, the coinjection of the organisms with 20 μg of laminin enhances pathogenicity (20).

In summary, the *E. histolytica* cysteine proteinases exhibit striking binding specificities for immobilized laminin over other components of the extracellular matrix. Treatment of amebic trophozoites with laminin at the time of their inoculation into SCID mice results in significantly decreased liver abscess size, which may be secondary to the inhibition of the amebic cysteine proteinases. The structural basis for the interaction of the amebic cysteine proteinases with laminin may be potentially exploited in designing specific inhibitors of these enzymes.

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