Isolation of a Cell Surface Receptor Protein for Laminin from Murine Fibrosarcoma Cells

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ABSTRACT We used affinity chromatography to isolate a specific laminin-binding protein from murine fibrosarcoma cells. These cells bind exogenous laminin to their surface with high affinity ($K_d = 2 \times 10^{-9}$M for laminin) with ~5×10^4 sites per cell. Laminin affinity chromatography of [^{35}S]methionine-labeled cell extracts produced two distinct proteins. One was identified as Type IV (basement membrane) collagen based on its migration pattern on SDS gels and bacterial collagenase sensitivity. The other protein, which migrates as a single band or closely spaced doublet on reduced SDS gels, has a reduced molecular weight of 69,000. Using a nitrocellulose filter disk assay, we found that the latter protein specifically bound ^{125}I-laminin with the same high affinity ($K_d = 2 \times 10^{-9}$M for laminin) as did intact fibrosarcoma cells. By iodinating intact cells, we demonstrated that this laminin-binding protein is on the cell surface. We conclude that this protein with reduced molecular weight of 69,000 is a subunit or component of a larger cell surface receptor protein for laminin in this fibrosarcoma model. This laminin receptor may mediate the interaction of the cell with its extracellular matrix.

The interaction of cells with their extracellular matrix is important for their growth, differentiation, and locomotion (1). There is increasing evidence that in many cases this cell-matrix interaction is mediated by so-called "attachment proteins" of which laminin and fibronectin are examples (2, 3). In vivo, laminin is located in the lamina lucida of basement membranes, interposed between a cell's basal surface and the supporting matrix of type IV (basement membrane) collagen (4). In vitro studies have shown that laminin can mediate the attachment of a wide variety of epithelial and endothelial cells to type IV collagen (5, 6). In addition, there is evidence that some tumor cells can use laminin to attach to type IV collagen in vitro (6, 7). Although Surgue and Hay (8) have shown that laminin, when added to corneal epithelial cells in vitro, can cause orientation of the cells' cytoskeleton, it is not clear by what mechanism this takes place. Since this interaction does not require cellular protein synthesis, it has been postulated that laminin may bind directly to the cell surface via a receptor.

Recently, we described (9) a cell line derived from a murine fibrosarcoma that is capable of specifically binding exogenous laminin to its surface in vitro. We now report the isolation of a cell surface laminin-binding protein from these cells. We studied the binding kinetics of this protein to laminin by immobilizing it on nitrocellulose filter disks. We find that the isolated protein retains the same high affinity for laminin as the intact cells. We conclude that this protein with a reduced molecular weight of 69,000 is a specific cell surface receptor for laminin.

MATERIALS AND METHODS

Cells and Substrates: The Np subline derived from a 3-methylcholanthrene-induced murine fibrosarcoma has been well characterized (10, 11). The cells were maintained in culture as previously described (10). Laminin was prepared from the EHS (Engelbreth-Holm-Swarm) sarcoma maintained in C57BL mice as described (12). The purity of laminin used in these experiments was verified by gel electrophoresis.

Radioactive Labeling of Cellular Proteins: Np cells were labeled in log phase of growth with [^{35}S]methionine, using 50 μCi/ml (1,300 Ci/mmol) of culture media (New England Nuclear, Boston, MA), and maintained for an additional 48 h before harvesting. Culture medium was removed, and 3×10^6 cells were harvested in 10 ml of a solution containing 0.5 M NaCl, 50 mm Tris, pH 8.3, 50 μg/ml *N*-ethylmaleamide (Sigma Chemical Co., St. Louis, MO), 50 μg/ml phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and either 1.0% Triton X-100 (Sigma Chemical Co.) or 1.0% Nonidet P-40 (Sigma Chemical Co.) (either detergent gave identical results). The cells were then sonicated and extracted with stirring at 4°C overnight. The extract was centrifuged at 3,000 rpm for 15 min and the supernatant dialyzed against 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3, with protease inhibitors as described above, at 4°C for 16 h.

Laminin Affinity Chromatography: Laminin affinity chromatography was performed on a column of laminin coupled to Sepharose 4B (Sigma Chemical Co.). Procedures were carried out at 20°C. Samples prepared as described above were applied to the column, which had a total bed volume of 6 ml with 2 mg of laminin bound to Sepharose 4B. The column was then washed with 20 ml of 0.5 M NaCl, 0.1 M $NaHCO_3$ pH 8.3, until [^{35}S] counts returned to baseline. The column was then eluted consecutively with 8 ml of either 6 M Urea or 0.1 M glycine-HCl, pH 2.4, and fractions containing eluted [^{35}S]methionine were pooled. Material eluted with glycine-HCl was neutralized to pH 7.4 with Tris buffer.

Electrophoretic Analysis: PAGE in the presence of SDS with or without reduction was carried out according to the method of Laemmli (13) using 5 or 10% acrylymide. Unlabeled molecular weight standards were obtained from

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Sigma Chemical Co. (St. Louis, MO). ^{14}C molecular weight standards were obtained from Amersham Corp. (Arlington Heights, IL). Collagenase digestion of samples was performed with purified bacterial collagenase form III (Advanced Biofactures, Lynbrook, NY) by method of Peterkofsky and Digelman (14).

Fluorography: Fluorography was carried out at −70°C using En^{3}Hance (New England Nuclear, Boston, MA) with film exposed for 96 h (14).

Laminin-binding Assay of Affinity Purified Proteins: 6-mm disks of nitrocellulose paper (0.45 μm, Schleicher & Schuell, Keene, NH) were used to immobilize proteins that had been eluted from the laminin affinity column. Indicated amounts of protein (determined by method of Lowry [15]) were spotted on each disk in a total volume of 10 μl. These disks were then placed in a microtiter well and incubated at 37°C in a solution of 3% bovine serum albumin (BSA) (Sigma Chemical Co.) for 1 h in order to occupy unbound sites on the disk. The disks were rinsed three times in 3% BSA and then used in laminin-binding assays. Laminin was iodinated by the lactoperoxidase method as previously described (16; Malinoff, H. L., P. McCoy, J. Varani, and M. S. Wicha, manuscript submitted for publication) to high specific activity (4.0 μCi/μg). In each microtiter well containing the nitrocellulose filter disk, a measured amount of ^{125}I-laminin was added with or without a 100-fold excess of unlabeled laminin in a final reaction volume of 50 μl. Inclusion of 10% fetal calf serum (FCS) in the assay had no effect on binding. The disks were then incubated for varying times at 20°C. At the end of the incubation period the disks were washed three times for 1 h each in a solution of 3% BSA. The disks were then counted in a gamma counter. Specifically bound laminin represented the total radioactivity bound (^{125}I-laminin) minus counts per minute of ^{125}I-laminin bound in the presence of 100-fold excess unlabeled laminin. Specific binding represented ~70% of total counts bound. There was no detectable specific binding of laminin to disks which had been treated with 3% BSA alone.

Surface Labeling of Np Cells with [^{125}I]: Confluent cultures of Np cells were harvested by brief trypsinization. Cells were then incubated in fresh culture media supplemented with 10% FCS at 37°C for 1 h to allow regeneration of cell surface receptors (Malinoff, H. L., P. McCoy, J. Varani, and M. S. Wicha, manuscript submitted for publication). 4 × 10^6 cells were then centrifuged, washed in phosphate-buffered saline (PBS) three times, pH 7.4, and incubated with 2 mCi of NaI^{125} (New England Nuclear), glucose oxidase, 300 U/ml (Sigma Chemical Co.), lactoperoxidase, 5 U/ml (Sigma Chemical Co.), and

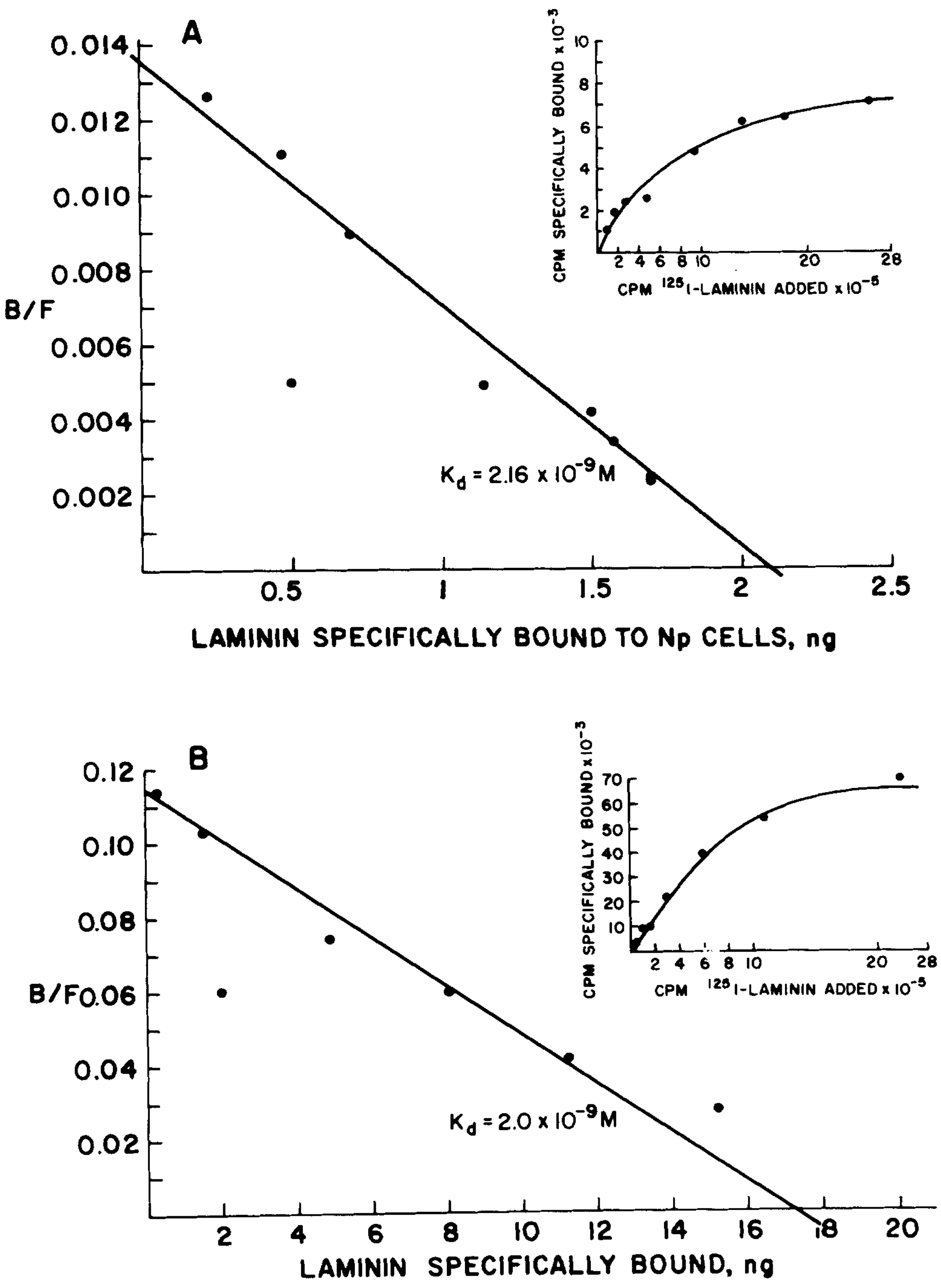

FIGURE 1 Scatchard analysis of laminin binding to (*A*) intact fibrosarcoma cells (10^5 cells/assay) and (*B*) isolated laminin receptor. 1 μg of protein in 10 μl was spotted on nitrocellulose disks. Binding assays in *A* and *B* were performed at 20°C as described in Materials and Methods. Both systems display linear kinetics consistent with a single class of high affinity receptors. *Insets* show concentration dependence of specific laminin binding.

2 mM glucose in PBS, pH 7.4, in a total volume of 750 μl (16, 17). Incubation was carried out on ice for 45 min at which time the cells were centrifuged, washed three times with PBS, and then extracted as above with detergent (see the section, Affinity Chromatography).

RESULTS

Binding of Laminin to Intact Murine Fibrosarcoma Cells

Incubation of the Np subline of murine fibrosarcoma cells with various amounts of ^{125}I-laminin (4 μCi/μg) with and without 100-fold excess of unlabeled laminin revealed that laminin specifically bound to these cells in a time and concentration dependent manner (9; Malinoff, H. L., P. McCoy, J. Varani, and M. S. Wicha, manuscript submitted for publication). Scatchard analysis (18) (Fig. 1) reveals linear binding kinetics with K_d of 2.1×10^{-9} M for laminin, and 5×10^4 binding sites per Np cell. These data are consistent with a single class of high-affinity binding sites for laminin.

Purification of Laminin-binding Proteins by Affinity Chromatography

We used affinity chromatography as a method of purifying laminin-binding proteins from Np cells. When total Np cell protein was labeled with [^{35}S]methionine, extracted with detergents, and subjected to laminin affinity chromatography, two separate peaks were obtained by consecutive elution with 0.1 M glycine HCl, pH 2.4 (Peak I), and 6 M urea (Peak II) (Fig. 2 *B*). Reversal of the order of elution gave an equivalent pattern (Fig. 2 *A*), suggesting that at least two separate [^{35}S]methionine-labeled proteins were bound to the column. No such peaks were obtained when cell extracts were applied to a column of albumin linked to Sepharose 4B.

When reduced and unreduced samples of each [^{35}S]methionine-labeled peak were subjected to SDS PAGE, the fluorogram in Fig. 3 was obtained. Peak II, when reduced, co-

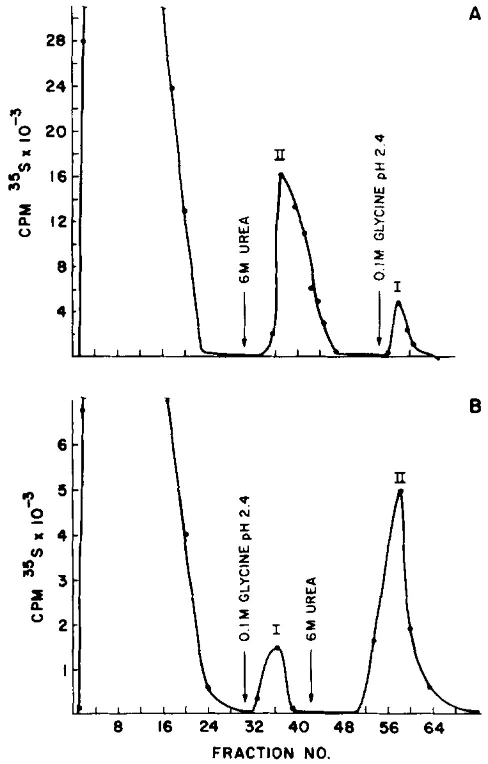

FIGURE 2 Elution profiles of laminin affinity column chromatography. In each experiment, cellular protein was labeled with [^{35}S]-methionine, extracted, and dialized as in Materials and Methods before being applied to a laminin affinity column. By varying the order of elution with urea and glycine-HCl, pH 2.4 (*A* and *B*), two separate peaks of the same relative size were obtained.

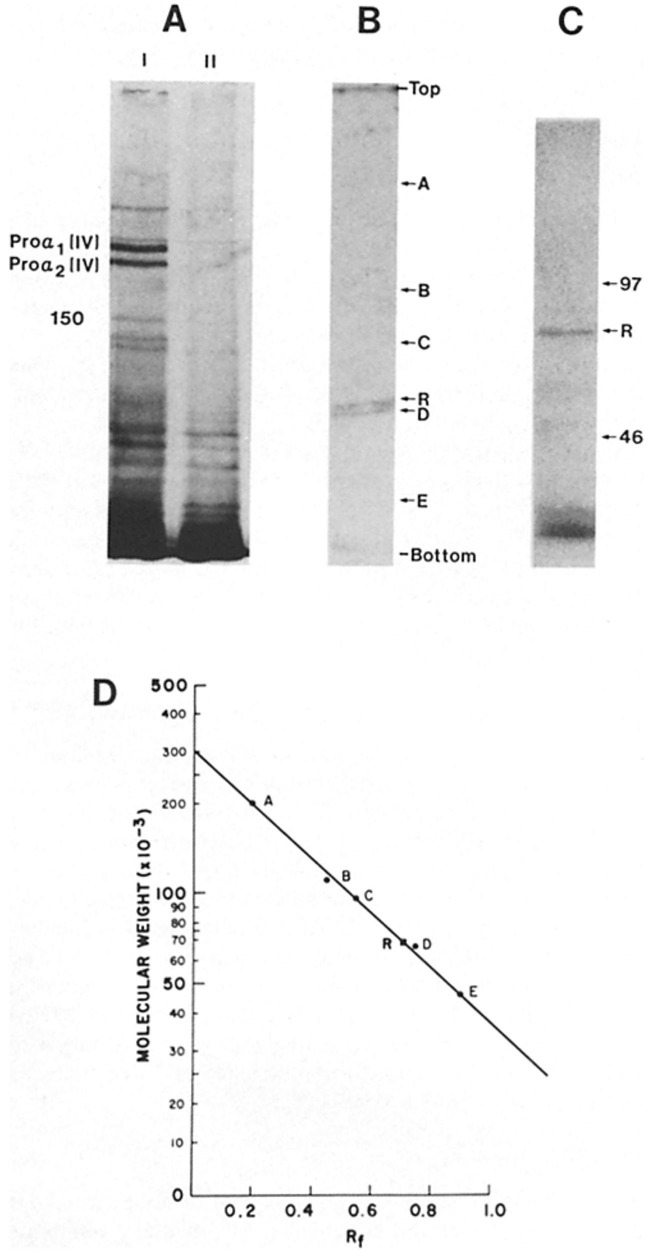

FIGURE 3 Analysis of proteins eluted from laminin affinity column on 5% polyacrylamide gels (*A*, *B*, and *D*) or 10% gels (*C*). (*A*) Peak II without (I) and with (II) bacterial collagenase digestion prior to electrophoresis. (See Materials and Methods.) (*B*) Peak I when analyzed and compared to known standards migrates as a single band (or doublet) at 69,000 mol wt. This protein was bacterial collagenase insensitive (data not shown). Some material remained at the top of some reduced gels. (*C*) Analysis of ^{125}I-labeled surface protein eluted from laminin affinity column with glycine-HCl, pH 2.4. (*D*) Semi-log plot of molecular weight standards showing calculated molecular weight of 69,000 for reduced laminin receptor. Protein standards are: (*A*) myosin, 200,000; (*B*) β-galactosidase, 116,000; (*C*) phosphorylase *b*, 96,000; (*D*) BSA, 66,000; (*E*) ovalbumin, 45,000. *R* indicates migration of reduced receptor compared to these standards.

migrates with purified type IV collagen, which Np cells are known to synthesize (Malinoff, H. L., P. McCoy, J. Varani, and M. S. Wicha, manuscript submitted for publication), and indeed is sensitive to purified bacterial collagenase (Fig. 3*A*). Peak I, upon reduction and SDS PAGE, produces a single band (or closely spaced doublet) with a molecular weight of 69,000 when compared to known standards (Fig. 3*B* and *D*). The unreduced sample of Peak I does not penetrate the gel. This protein is bacterial collagenase insensitive (data not shown).

Assay for Laminin Binding to Affinity Purified Proteins

The proteins contained in both peaks eluted from the laminin affinity column were tested for their ability to specifically bind exogenous laminin using the nitrocellulose filter disk assay described in Materials and Methods. We found that the material eluted in Peak II type (IV collagen) specifically bound ^{125}I-laminin only at high laminin concentrations (Table I). This indicates a relatively low binding affinity between laminin and type IV collagen in this assay.

In contrast, the protein in Peak I specifically bound ^{125}I-laminin in a time- and concentration-dependent manner with high affinity (Fig. 1*B* and Table I). Binding was maximum by 45 min at 20°C. Scatchard analysis (18) (Fig. 1*B*) reveals a K_d for laminin of $\sim 2 \times 10^{-9}$ M, which is similar to that of laminin binding to the surface of intact Np cells (Fig. 1*B*). From these data, we conclude that the protein in Peak I is the receptor for laminin in our fibrosarcoma model.

Surface Labeling of Fibrosarcoma Cells with ^{125}I

To determine whether the proteins isolated from the laminin affinity column were present on the cell surface, intact cells were subjected to labeling with ^{125}I by the lactoperoxidase method. After labeling, cells were noted to be intact and viable by phase-contrast microscopy and by trypan blue exclusion. The cells were centrifuged and washed to remove free iodide, following which they were sonicated and extracted with detergent. Laminin affinity chromatography again produced two separate peaks when the column was eluted consecutively with urea and glycine. Urea eluted only a small amount of labeled protein. Glycine-HCl eluted a larger peak that co-migrated with the [^{35}S]methionine-labeled protein in Fig. 3 when reduced and subjected to SDS PAGE (Fig. 3*C*).

DISCUSSION

The glycoprotein laminin is a component of the extracellular matrix of epithelial and endothelial cells in many vertebrate systems, including man (4). Immunohistochemical studies have shown that laminin is located in the lamina lucida of basement membranes, interposed between a cell's basal surface and the lamina densa, which contains type IV collagen (2, 4). The precise biologic role of laminin remains undetermined, but in vitro studies have shown that laminin readily mediates the attachment of a wide variety of cells to type IV collagen (2, 6, 7, 9). Other in vitro studies have shown that both normal and malignant cells express laminin on their surface or have the ability to bind exogenous laminin (9, 20, 21, 22). These data have led to the hypothesis that laminin may act as an "attachment protein" for some types of normal and malignant cells in vivo (7, 21). While the in vitro binding of laminin to type IV collagen has been well described, little is known about the interaction of laminin with the cell surface.

TABLE I

Result of ^{125}I-Laminin Binding to Proteins Eluted from Laminin Affinity Column

^{125}I Laminin added	Laminin specific bound	
	Peak II (type IV collagen)	Peak I (laminin receptor)
(ng)	*(cpm)*	
50	0	14,150 ± 1,725
500	9,250 ± 750	73,150 ± 3,570

Proteins were spotted on nitrocellulous discs (1 µg of Peak I, 10 µg of Peak II) and then used in laminin-binding assays (see Materials and Methods). Results represent the mean of triplicate experiments with standard deviation as noted.

We have previously examined the binding of laminin to the surface of cells derived from a murine fibrosarcoma (9). We found that these cells bind exogenous laminin to their surface with high affinity. We now report the isolation of a cell surface protein from this cell line that binds laminin with the same high affinity as intact cells as determined by nitrocellulose filter disk assay. This receptor shows no cross reactivity with type IV collagen, fibronectin, or albumin. When reduced, the receptor migrates as a single band, or closely spaced doublet at 69,000 mol wt on SDS gels; it does not penetrate gels when unreduced. We conclude that this band is a subunit or a component of a larger cell surface receptor for laminin in this murine fibrosarcoma model. We recently found the presence of sulfate in this isolated protein (H. L. Malinoff and M. S. Wicha, unpublished observations). The presence of laminin receptors is not restricted to malignant cells, as we have found that laminin specifically binds to normal rat mammary epithelium (Malinoff and Wicha, unpublished observations) and mouse peritoneal macrophages (23). We are now in the process of characterizing these cell surface laminin receptors and determining their function.

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