Powell, J. S., Berkner, K. L., Lebo, R. V., & Adamson, J. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6465-6469.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487-491.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger, F., Coulson, A. R., Hong, G. F., Hill, D. F., & Petersen, G. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 729-773.
Suzuki, K., Dahlback, B., & Stenflo, J. (1982) *J. Biol. Chem. 257*, 6556-6564.
Takahashi, N., Ortel, T. L., & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 390-394.
Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., et al. (1984) *Nature 312*, 342-347.
Toole, J. J., Pittman, D. D., Orr, E. C., Murtha, P., Wasley, L. C., & Kaufman, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5939-5942.
Tracy, P. B., & Mann, K. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2380-2384.
Tucker, M. M., Foster, W. B., Katzmann, J. A., & Mann, K. G. (1983) *J. Biol. Chem. 258*, 1210-1214.
Vehar, G. A., Keyt, B., Eaton, D. L., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M., & Capon, D. J. (1984) *Nature 312*, 337-342.
Viskup, R. W., Tracy, P. B., & Mann, K. G. (1987) *Blood 69*, 1188-1195.
Wigler, M., Pellicer, A., Silverstein, S., & Axel, R. (1978) *Cell 14*, 725-731.

Articles

Identification and Characterization of a 43-Kilodalton Laminin Fragment from the "A" Chain (Long Arm) with High-Affinity Heparin Binding and Mammary Epithelial Cell Adhesion-Spreading Activities†

C. N. Rao* and Nicholas A. Kefalides

Connective Tissue Research Institute and Department of Medicine, University of Pennsylvania and University City Science Center, 3624 Market Street, Philadelphia, Pennsylvania 19104

Received February 6, 1990; Revised Manuscript Received April 18, 1990

ABSTRACT: A recently described procedure of reduction and carboxymethylation followed by heparin-Sepharose chromatography [Arumugham et al. (1988) *Connect. Tissue Res. 18*, 135-147] was used to characterize high-affinity heparin binding fragments of the laminin "A" chain. Two laminin fragments of M_r 53K and 43K selectively bound to the heparin-Sepharose column from the chymotrypsin digest of laminin, indicating that these fragments originate from the "A" chain. Without reduction and carboxymethylation but in the presence of 2.0 M urea, the heparin-Sepharose-bound material from the chymotrypsin laminin digest contains all the attachment-promoting activity for normal mouse mammary epithelial cells. The reduced 200-kDa intact three short arm fragment, fragments of M_r 70K-160K obtained either from laminin or from the reduced 200-kDa three short arm fragment, and the 53-kDa heparin binding fragment were all inactive in promoting the adhesion of mouse mammary epithelial cells. The mammary epithelial cell adhesion and spreading properties of laminin are associated with the high-affinity heparin binding 43-kDa fragment. The mammary epithelial cells attach to the 43-kDa fragment substrate and synthesize laminin, collagen type IV, and desmoplankins I and II as are the cells attached to laminin substrate and to the cells grown on tissue culture dishes. The biologically active 43-kDa fragment is generated from laminin, but not from the three short arm fragment. These results suggest that normal mouse mammary epithelial cells interact with laminin through a single site which is present in the 43-kDa heparin binding fragment located on the long arm of the "A" chain.

The glycoprotein laminin (M_r 850K) is the most abundant component of specialized extracellular matrices known as basement membranes (Chung et al., 1979; Timpl et al., 1979). Laminin consists of three chains designated "A" (M_r 400K), "B_1" (M_r 220K), and "B_2" (M_r 200K); all three have been cloned and sequenced to full length (Sasaki & Yamada, 1987; Sasaki et al., 1987, 1988; Pikkarainen et al., 1987). By rotary-shadowing electron microscopy, laminin has the shape of an asymmetric cross with one long arm and three short arms (Engel et al., 1981). Laminin is synthesized by a variety of cell types which include endothelial, epithelial, muscle, and schwann cells and is involved in numerous biological functions (Kleinman et al., 1985; Liotta et al., 1986; Martin & Timpl, 1986; Timpl, 1989; Panayotou et al., 1989). By interaction with components of basement membrane macromolecules such

† This work was supported by NIH Grants AR 20553, HL 29492, and AR 07490.
* Address correspondence to this author at Cancer Diagnostic Inc., 2101 E. Jefferson St., Suite 310, Rockville, MD 20852.

0006-2960/90/0429-6768$02.50/0

as collagen type IV (Woodley et al., 1983; Rao et al., 1985; Charonis et al., 1985; Laurie et al., 1986) and heparan sulfate proteoglycans (Sakashita et al., 1980; Delrosso et al., 1981) as well as with itself (Yurchenko et al., 1985), laminin plays a crucial role in determining the structure of basement membranes. Laminin also has the ability to interact with a variety of cell types through cell surface molecules, such as the 67-kDa laminin receptor (Rao et al., 1983, 1989; Terranova et al., 1983; Barsky et al., 1984; Malinoff & Wicha, 1983; Lesot et al., 1983; Graf et al., 1987a,b; Sugrue, 1987), the integrin-related proteins (Horwitz et al., 1985; Gehlsen et al., 1988; Tamoselli et al., 1988), and sulfated glycolipids (Roberts et al., 1985). Through these interactions, laminin promotes cell adhesion, growth, morphology, differentiation, migration, agglutination, and neurite outgrowth and metastasis.

To identify and characterize domains with biological activity, the laminin molecule has been fragmented with proteolytic enzymes, and the isolated fragments have been used in functional assays. These studies report the following: (1) The E8 fragment (M_r 280K), encompassing half of the long arm including the large globule, binds to heparin (Ott et al., 1982), promotes cell adhesion and spreading, and facilitates neurite outgrowth (Edgar et al., 1984; Engvall et al., 1987; Dillner et al., 1988; Nurcombe et al., 1989). (2) Fragment E5 (30K–50K) originated from short arms promotes attachment and spreading of rat hepatocytes (Timpl et al., 1983). (3) The binding to the tumor cells occurs at the intersection of the three short arms (M_r 300K–400K); this region does not bind to type IV collagen (Terranova et al., 1983). By use of monoclonal antibodies, four distinct heparin binding sites and a cell attachment promoting site near the intersection of the short arms but on the long arm for highly metastatic melanoma cell line K-1735 M4 (Skubitz et al., 1987, 1988) were reported. In a recent approach, the sequence information of all three chains was used to make synthetic peptides which may promote adhesion and migration of a variety of cell types and for binding to heparin in solid phase binding and competition assays. Three synthetic peptides designated YIGSR, PA 22-2, and F-9 were reported to be biologically active in promoting cell adhesion, chemotaxis, and neurite outgrowth and for binding to heparin (Graf et al., 1987a,b; Charonis et al., 1988; Sephel et al., 1989; Ken-Ichiro-Tashiro et al., 1989). Taken together, these approaches led to the identification of at least four different sites on the laminin molecule for binding to cells and to heparan sulfate proteoglycan.

In the present study, we used proteolytic enzymes to obtain laminin fragments and report that normal mouse mammary epithelial cells interact with laminin through a single site which is located in a 43-kDa fragment. The laminin 43-kDa fragment originates from the long arm of the "A" chain and also contains a high-affinity heparin binding site.

Materials and Methods

Materials

Reagents were purchased from the following companies: Dulbecco's mofidied Eagle's medium (DMEM), penicillin–streptomycin (5000 units/mL), and fetal bovine serum from Grand Island Bilogicals, Grand Island, NY; 10× MEM (Eagle's) with Earle's salts and without glucose, methionine, and sodium bicarbonate from Hazelton-Duthcland Inc., Denver, PA; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Research Organics Inc., Cleveland, OH; phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, hirudin, chymotrypsin, L-glutamine, Coomassie blue R, Hanks balanced salt solution (HBSS), and bovine serum albumin (BSA), RIA grade fraction V, from Sigma Chemical Co., St. Louis, MO; antidesmoplankins I and II and aprotinin from Boehringer Mannheim Biochemicals, Indianapolis, IN; prestained high molecular weight protein standards from Bethesda Research Laboratories, Gaithersburg, MD; 2-mercaptoethanol, acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylenediamine (TEMED), ammonium persulfate, and protein silver staining and assay kits from Bio-Rad Laboratories, Richmond, CA; 35-mm bacteriological petri dishes, Falcon catalog no 1008, from Fisher Scientific, Philadelphia, PA; heparin–Sephorose CL 6B, protein A–Sepharose, PD-10 Sephadex G 25 disposable columns, and Mono Q ion-exchange column from Pharmacia-LKB Bio-technology Inc., Piscataway, NJ; ^{35}S trans label from ICN Radio Chemicals, Irvine, CA; liquid scintillation fluid, formula 989, from NEN Research Products, Boston, MA; autofluor from National Diagnostics, Inc., Manville, NJ; human α-thrombin was a gift from Dr. John Feton, New York State Department of Health, Albany, NY; polyclonal antiserum to mouse laminin was a gift from Dr. Lance A. Liotta, National Cancer Institute, Bethesda, MD.

Methods

Cell Culture. Normal mouse mammary epithelial cells frozen at passage 9 were purchased from American Tissue Culture Collection Center, Rockville, MD (Owens et al., 1974a,b). Cells were routinely cultured in DMEM containing 10% FBS, 4.5 g/L D-glucose, 10 mM glutamine, 15 mM HEPES, and 50 units/mL penicillin–streptomycin on tissue culture plastic dishes at 37 °C, in an atmosphere of 95% air and 5% carbon dioxide.

Protein Purification. Laminin was isolated from Engelbreth Holm Swarm (EHS) tumor by using a procedure described previously (Timpl et al., 1979) and stored frozen (−70 °C) at a concentration of 1 mg/mL in phosphate-buffered saline (PBS).

Enzyme Digestion. Laminin was digested with human α-thrombin or chymotrypsin at a 1:25 enzyme to protein ratio, as described previously (Rao et al., 1982a,b). The activity of chymotrypsin was stopped by the addition of 0.2 M PMSF to a final concentration of 2 mM. Two units of hirudin were added to stop the activity of α-thrombin which was used to digest 1 mg of laminin. In a sequential digestion experiment, 1 mg of 600 kDa, three short arm fragment as first purified by anion-exchange fast protein liquid chromatography (FPLC) was used for digestion by chymotrypsin as described above.

Heparin–Sepharose Chromatography. Reduction and carboxymethylation followed by heparin–Sepharose chromatography of laminin and laminin digests was performed according to a previously described procedure (Arumugham et al., 1988). To obtain a 2 M urea/heparin-bound and unbound fractions, the chymotrypsin digest of native laminin was dialyzed at 4 °C against 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 2.0 M urea (washing buffer). The sample was loaded on a 5 × 0.5 cm heparin–Sepharose column which was preequilibrated with washing buffer. The unbound material was collected in washing buffer, and the material bound to the column was eluted with washing buffer containing 1.0 M NaCl. The bound and unbound fractions were dialyzed against 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl and separately assayed for cell attachment activity. The polypeptides contained in the bound and unbound fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Anion-Exchange FPLC. A Mono Q (HR 5/5) anion-exchange column attached to the FPLC system was used to

fractionate laminin fragments from enzyme digests. One-milligram reaction mixtures were diluted to 4.0 mL with 15 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 2.0 M urea (buffer A) and further dialyzed at 4 °C against 1 L of buffer A. The contents in the dialysis tubing were cleared by centrifugation in a microfuge at 4 °C for 10 min at 12000 rpm. The clear supernatant was applied to a Mono Q column which was preequilibrated with buffer A. The unbound laminin fragments were collected in five 1-mL fractions. The elution of the bound laminin fragments was achieved with 30 mL of a linear concentration gradient in which the NaCl concentration of buffer A was raised to 1.15 M. A flow rate of 1.0 mL/min was maintained, and 1.0-mL fractions were collected. Protein-containing bound and unbound fractions were stored at −70 °C.

Cell Attachment and Spreading. Quantitation of the mammary epithelial cells attached to substratum-bound laminin and laminin fragments was achieved following the reported procedure of Turner et al. (1987). Plastic petri dishes (35 mm, Falcon 1008) were coated with 2.0 mL of 0.05 M sodium carbonate buffer, pH 9.6, containing increasing amounts of proteins to be assayed for cell attachment activity. The coating was terminated after incubating the dishes overnight at 37 °C under a humidified atmosphere. The dishes were washed with PBS, 3 times and each time with 1.0 mL. Just prior to each assay, a total of 2.0 mL of attachment medium (HBSS containing 2.38 g/L HEPES/20 mg/mL BSA, pH 7.4, and without sodium bicarbonate) and antibodies to be tested were added and left at room temperature for 30 min. The cells used for the attachment assays were labeled with [^{35}S]methionine (0.25 μCi/mL) in growth medium for 16–20 h. During the labeling period, the cells are at about 80% confluency. By this way, the cells are usually labeled to a specific activity of 0.25–0.35 cpm/cell. The cells were detached with 0.1% trypsin solution and washed successively with 10-mL volmes of growth medium, attachment medium without BSA, and attachment medium. The cells are routinely checked for viability by trypan blue exclusion, and usually greater than 90% are viable. After the last wash, the cell pellet is suspended in attachment medium. One hundred microliters of this cell suspension containing (1.0–2.0) × 10^6 cells was seeded to each dish, swirled for 5 s, and incubated at 37 °C for 1 h under humidified atmosphere. The unattached cells were removed with three 1.0-mL washes, each wash at 100 rpm for 30 s. The attached cells were lysed for 30 min in 1.0 mL per dish of 1% Triton X-100 in PBS with constant rotation. The extracts were transferred to scintillation vials for counting, and the percent attachment is defined as (radioactivity extracted from attached cells)/(radioactivity in cells added to assay) × 100. Generally, variation between samples in different assays was less than 5%. The radioactivity attached to blank dishes was less than 0.2%. In preliminary experiments, we observed a linear relationship between the cell number and radioactivity (not shown).

To follow the cell spreading, after removal of the unattached cells, incubation was continued at 37 °C in HBSS. The spreading was monitored by observing the cells during a period of 6 h in a phase-contrast microscope set at 25×. Pictures were taken with Kodak plus X pan film. Since the cell attachment and spreading were studied in 6 h, sterile conditions and solutions were not used.

Metabolic Labeling and Immunoprecipitation. The labeling of cells with [^{35}S]methionine was performed under sterile conditions. Mouse mammary epithelial cells (1 × 10^6 cells/well) suspended in complete growth medium were allowed to attach to three tissue culture plastic wells (35 mm, Costar) for 90 min. Cells were also attached to laminin substrate and active fraction substrate in triplicate Falcon 1008 dishes at 37 °C for 1 h. After removal of unattached cells, attached cells were preincubated for 15 min with methionine-free MEM containing 0.1% glucose, 0.06% L-glutamine, 0.05% sodium bicarbonate, and 15 mM HEPES. Parallel experiments conducted with radiolabeled cells indicated that an approximately similar number of cells attached in all three groups. The cells were labeled with [^{35}S]methionine at a concentration of 100 μCi/mL in the medium described above for 2 h at 37 °C. The cells were extensively washed with PBS and lysed in PBS containing 1% Nonidet P-40 and the protease inhibitors PMSF (2 mM), NEM (10 mM), aprotinin (0.5%) and EDTA (2 mM). The cell lysates were clarified by centrifugation at 12000 rpm for 10 min at 4 °C. Portions of 200 μL of cell lysates (from a total of 1.5 mL) were diluted to 1.0 mL with lysis buffer and incubated with appropriate amounts of primary antibodies to laminin, type IV collagen, and desmoplankins I and II. After overnight incubation at 4 °C, 2 μL of secondary antibody is added where necessary and incubated for 1 h at room temperature. The antigen-antibody complexes were adsorbed to 100 μL of a 25% protein A–Sepharose suspension following 1 h of mixing at room temperature. The Sepharose beads were pelleted by centrifugation in a microfuge and washed as described previously (Rao et al., 1989). The protein A–Sepharose pellets were suspended in 100 μL of electrophoresis sample buffer (0.062 M Tris-HCl, p.H 6.8, 2% SDS, 24% urea, 10% glycerol, and 0.002% bromophenol blue) containing 5% 2-mercaptoethanol and boiled for 3 min. The proteins in the supernatants were fractionated on 7% SDS–PAGE and visualized by fluorography.

Analytical Procedures. The amount of the protein bound to the petri dishes was assayed in the following manner: The solution at the end of the incubation was recovered and concentrated, and the protein was assayed with Bradford's reagent (Bradford, 1976) in a microassay procedure with BSA as the standard. SDS–PAGE in the presence of 0.5 M urea on 5, 7, or 12% separating gels was carried out according to Laemmli (1970); 2× electrophoresis sample buffer containing 5% 2-mercaptoethanol was used to apply the proteins for separation on polyacrylamide gels. Silver staining of the proteins was achieved by using the reagent kit and the company's instruction manual (see Materials). For detection of the labeled polypeptides, gels were fixed for 1 h in 10% trichloroacetic acid/50% methanol, washed for 15 min in distilled water, impregnated with Autofluor for 2 h, dried, and exposed to Kodak XAR-5 film.

RESULTS

Characterization of High-Affinity Heparin Binding Laminin "A"-Chain Fragments. Arumugham et al. (1988) have shown that the 400-kDa (A) chain but not the 200-kDa (B) chains of reduced and alkylated laminin bound to the heparin–Sepharose affinity matrix in the presence of 6.0 M urea. In the present study, this procedure was used to identify the "A"-chain fragments from the chymotrypsin digest of laminin (Rao et al., 1982b; Palm et al., 1985). The fragments generated from laminin by 4-h chymotrypsin digestion are subjected to reduction and carboxymethylation and heparin–Sepharose chromatography. The chromatographic profile is shown in Figure 1A. Two peaks were detected: the unbound protein representing 75% of the total protein and the bound protein containing the remaining 25%. The unbound and bound polypeptides were analyzed by SDS–PAGE and silver staining. The unbound material contained polypeptides mi-

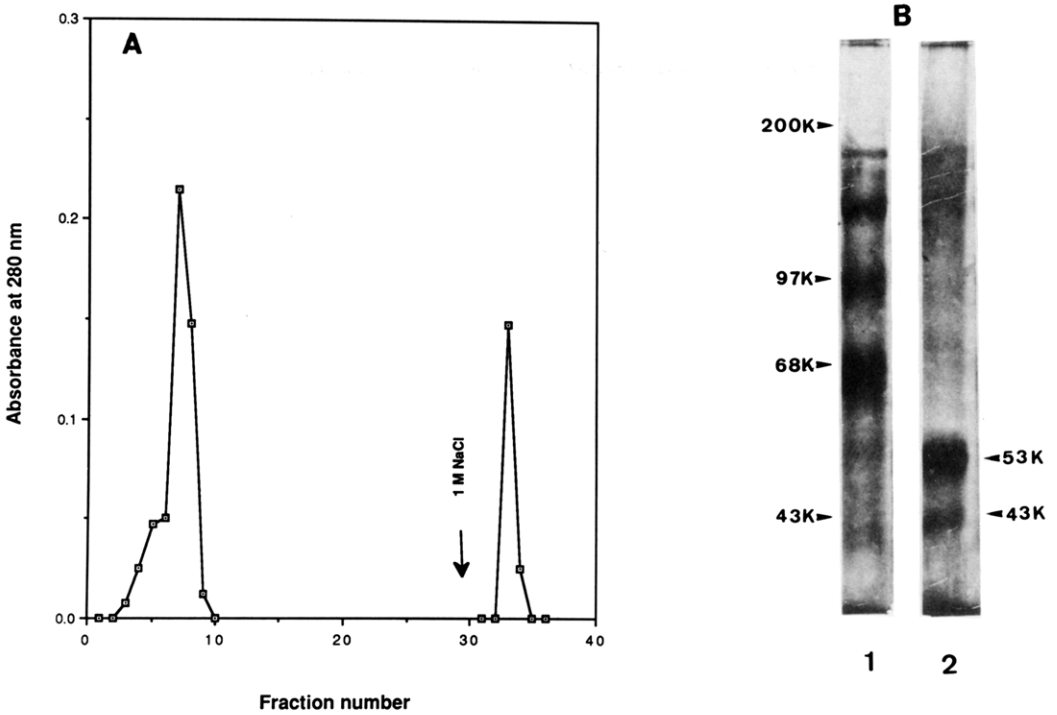

FIGURE 1: Heparin–Sepharose affinity chromatography of reduced and alkylated chymotrypsin-digested laminin polypeptides. 1.5 mg of protein in 3 mL of 10 mM Tris-HCl, 6.0 M urea, and 5 mM EDTA, pH 7.4, was applied to a 5 × 0.5 cm heparin–Sepharose column equilibrated in the buffer at 22 °C. The unbound material was removed with 10 mL of starting buffer (fractions 1–10). The column was washed with 20 mL of 50 mM Tris-HCl, pH 7.4, and 6.0 M urea (fractions 10–30). Bound polypeptides were then eluted in 5.0 mL of 5.0 mM Tris-HCl, pH 7.4, 6.0 M urea, and 1.0 M NaCl (fractions 30–35). Fractions of 1.0 mL were collected and their relative protein concentrations determined by measuring the absorbance at 280 nm. The chromatographic profile is shown in panel A. SDS–PAGE of heparin–Sepharose-unbound and -bound polypeptides on a 7% separating gel is shown in panel B: lane 1, heparin-unbound fraction; lane 2, heparin-bound fraction. Only 53- and 43-kDa fragments bound to the heparin–Sepharose column and appeared in the column-bound fraction.

grating at 160, 130, 110, 90, and 70 kDa (Figure 1B, lane 1). The bound material contained two polypeptides, 53 and 43 kDa (Figure 1B, lane 2); very little of these fragments showed up in the unbound material. These observations indicate that the heparin binding activity of the "A" chain is associated with the 53- and 43-kDa polypeptides.

Cell Attachment and High-Affinity Heparin Binding Activities Are Colocalized. Reduced and carboxymethylated laminin was tested to promote the adhesion of mouse mammary epithelial cells. As shown in Figure 4 (◊), the cell attachment promoting activity of laminin is lost during reduction and carboxymethylation. In the presence study, we attempted to separate the 53- and 43-kDa fragments by heparin–Sepharose chromatography in the presence of 2 M urea and without reduction and carboxymethylation. Fractionation of the chymotrypsin digest of laminin on heparin–Sepharose affinity matrix in the presence of 2.0 M urea yielded two fractions; 40% of the protein applied eluted in the unbound fraction, and the remaining protein (60%) is recovered in the bound fraction after elution with 1.0 M NaCl (data not shown). The unbound and bound materials were analyzed by SDS–PAGE and silver staining as shown in Figure 2, lanes 1 and 2, respectively. The bound fraction (lane 2) contained all the chymotrypsin laminin fragments which include the "A"-chain heparin binding 53- and 43-kDa polypeptides and the larger molecular weight 70K–160K "B"-chain fragments. In contrast, the unbound material (lane 1) did not contain 53- and 43-kDa polypeptides, but the remaining large "B"-chain fragments (M_r 70K–160K) were present. These fragments (70–160 kDa) did not bind to heparin–Sepharose in the presence of 6.0 M urea (Figure 1B), thus indicating that their binding to heparin was of low affinity. The cell attachment activity of the total, unbound, and bound laminin fragments was measured and is represented in Table I. The heparin-bound material contained all the cell attachment promoting activity while in the unbound material this activity was absent. These results indicate that the cell attachment promoting activity of laminin may be associated with the high-affinity heparin binding 53- and 43-kDa fragments.

Table I: Mammary Epithelial Cell Attachment Activity of Heparin–Sepharose-Bound and Unbound Fractions[a]

substratum	concn (μg/mL)	% of attached cells
laminin	5	34
heparin–Sepharose-bound fraction	5	50
heparin–Sepharose-unbound fraction	5	0.2

[a] See Materials and Methods for details of heparin–Sepharose column chromatography in the presence of 2 M urea and cell attachment activity of the column unbound and bound protein fractions. Values are the average of triplicate dishes.

Identification and Characterization of Laminin Fragment Promoting Mammary Epithelial Cell Attachment and Spreading. The laminin fragment promoting the attachment activity of normal mouse mammary epithelial cells is partially purified by anion-exchange chromatography. Human α-thrombin and chymotrypsin laminin digests and chymotrypsin digest of the purified reduced 200-kDa three short arm laminin fragment (without reduction M_r 600K) were separated by Mono Q column chromatography. From all the chromatograms, only two peaks were detected; the first peak representing the unbound material came off the column with buffer A, and a second peak representing the bound material was eluted with 0.25 M NaCl in buffer A (data not shown). The proteins present in each peak were examined by 7% SDS–PAGE and shown in Figure 3. The reduced 200-kDa laminin fragment, generated by digestion of laminin with α-thrombin, was detected in the bound material (Figure 3A, lane 2) while

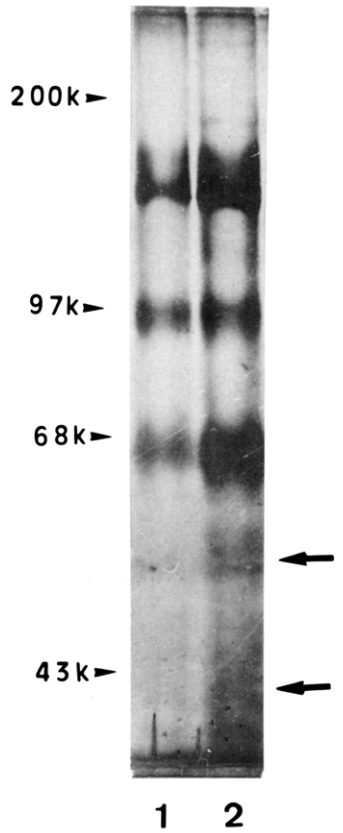

FIGURE 2: SDS–PAGE of reduced heparin–Sepharose-unbound and -bound polypeptides from digests of laminin by chymotrypsin in the presence of 2.0 M urea. The proteins were separated on a 7% separating gel and identified by silver staining. Lane 1, heparin-unbound fraction; lane 2, heparin-bound fraction. The arrows designate the polypeptides (M_r 53K and 43K) specifically eluted in the bound fraction.

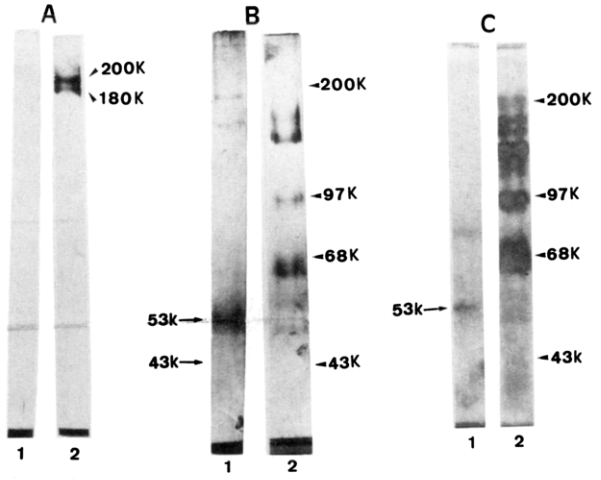

FIGURE 3: SDS–PAGE of anion-exchange column unbound and bound polypeptides of enzyme laminin digests. Samples were diluted in 2× electrophoresis sample buffer containing 5% 2-mercaptoethanol and separated on a 7% acrylamide gel, and proteins were identified by silver staining. Panel A: lane 1, unbound column fraction from α-thrombin digest of laminin; lane 2, bound column fraction from α-thrombin digest of laminin. Panel B: lane 1, unbound column fraction from chymotrypsin digest of laminin; lane 2, bound column fraction from chrymotrypsin digest of laminin. Panel C: lane 1, unbound column fraction from chymotrypsin digest of reduced 200-kDa laminin fragment; lane 2, bound column fraction from chymotrypsin digest of reduced 200-kDa laminin fragment.

the unbound material showed no polypeptides detectable on 7% gels (Figure 3A, lane 1). The reduced 200-kDa laminin fragment clearly resolved into two bands, one at 200 kDa and the other at 180 kDa, on 7% acrylamide gels (Figure 3A, lane 2). However, throughout the text, this complex has been referred to as the reduced 200-kDa laminin fragment. Multiple fragments with molecular weights ranging from 70 to 160K were detected in the bound material obtained by digestion of laminin with chymotrypsin (Figure 3B, lane 2). In the unbound fraction, a major 53-kDa band and a minor 43-kDa band were observed (Figure 3B, lane 1). Sometimes, as was shown in Figure 3B, lane 1, two very minor large fragments, 160 and 130 kDa, were also seen. These two large fragments primarily were found in the bound material (Figure 3B, lane 2). The reduced 200-kDa laminin fragment was purified in PBS and subjected to chymotrypsin digestion, and the digest was processed by anion-exchange column chromatography. The digestion products from the unbound and bound factions were separated by 7% SDS–PAGE and shown in Figure 3C, lanes 1 and 2, respectively. The fragments detected in the bound material (Figure 3C, lane 2) are nearly identical with those observed in the bound material obtained from laminin digested with chymotrypsin (Figure 3B, lane 2). On the other hand, only the 53-kDa fragment is present in the column-unbound fraction from the chymotrypsin digest of the reduced 200-kDa three short arm laminin fragment (Figure 3C, lane 1). Increasing quantities of proteins from each of the above six peaks were tested for their ability to promote the adhesion of mouse mammary epithelial cells, and the data are shown in Figure 4 (unpublished observations from our laboratory indicate that 70% of the protein from the bound fraction and 62% of the protein from the unbound fractions adsorbed onto the plastic dishes). Striking differences were observed between the materials from the six peaks in their ability to promote mammary epithelial cell adhesion. The

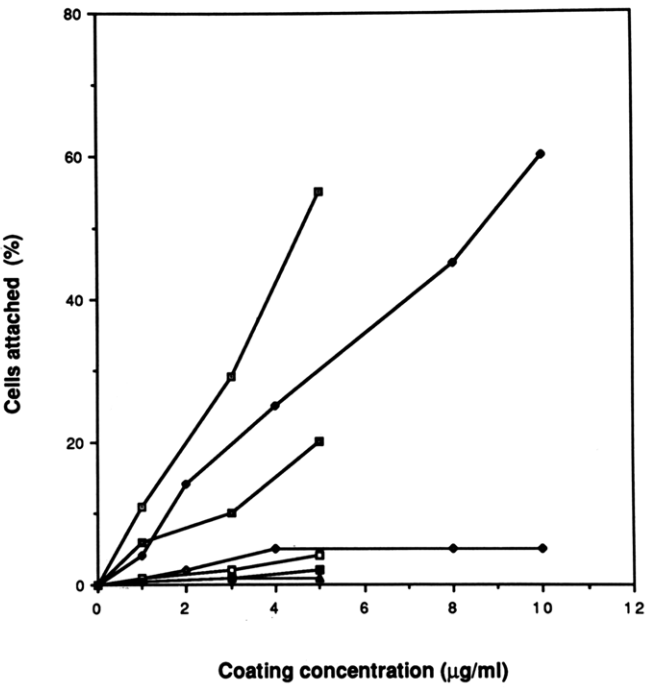

FIGURE 4: Cell binding activity of laminin fragments. Laminin fragments were partially purified by chromatography on a Mono Q column. Plastic petri dishes were coated with the indicated amounts of fragments in unbound (◈) and bound (◇) fractions from the chymotrypsin digest of laminin; unbound (◘) and bound fractions (▲) from the α-thrombin digest of laminin and the unbound (■) fraction from the chymotrypsin digest of reduced 200-kDa laminin fragment were incubated with radiolabeled mouse mammary epithelial cells (1.5×10^6 cells) for 60 min. Laminin (●) and reduced and alkylated laminin (♦) were also included in the experiment. The unattached cells were removed, and the attached cells were quantitated as described under Methods. The values are the average of triplicate samples.

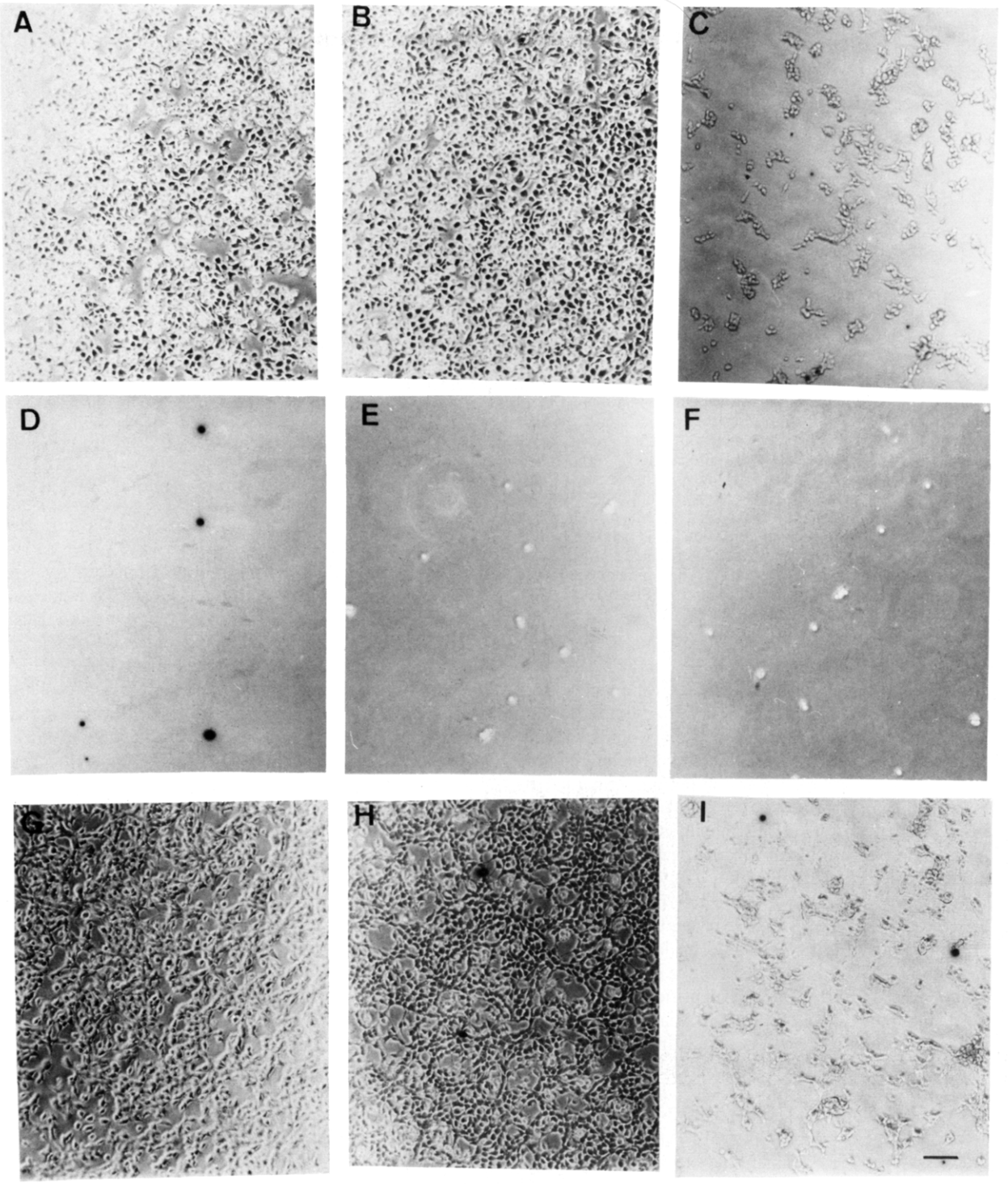

FIGURE 5: Adhesion and spreading of normal mouse mammary epithelial cells on laminin and on laminin fragments. Mouse mammary epithelial cells were allowed to attach to plastic-bound laminin (A, 12 μg/mL), the unbound column fraction of the chymotrypsin digest of laminin (B, 5 μg/mL), the unbound column fraction of the α-thrombin laminin digest (C, 2 μg/mL), the bound column fraction of the α-thrombin of digest of laminin (D, 5 μg/mL), the bound column fraction of the chymotrypsin digest of laminin (E, 5 μg/mL), and the unbound column fraction of the chymotrypsin digest of the reduced 200-kDa laminin fragment digest for 60 min. The unattached cells were removed as described under Methods. The attached cells were incubated at 37 °C in HBSS and photographed at 25× after 1 h (A–F) and after 6 h (G–I). Bar represents 100 nm.

bound materials representing the 600-kDa, three short arm fragment, 70–160-kDa fragments from the chymotrypsin digest of laminin, or the reduced 200-kDa laminin fragment were not active to promote mammary epithelial cell adhesion, beyond background levels even at 10 μg/mL concentration [Figure 4, (▲) and (□), respectively]. The unbound material containing the 53-kDa fragment originated from the reduced 200-kDa three short arm structure was also not active in biological assays [Figure 4 (■)]. The cell attachment promoting activity was found to be associated with laminin fragments present in unbound materials obtained from the α-thrombin digest of laminin and from chymotrypsin-digested laminin [Figure 4, (◈) and (◘), respectively]. The cells attached to the two unbound active fractions were followed for spreading, and the data are shown in Figure 5. Mammary epithelial cells attached to laminin substrate spread and attain

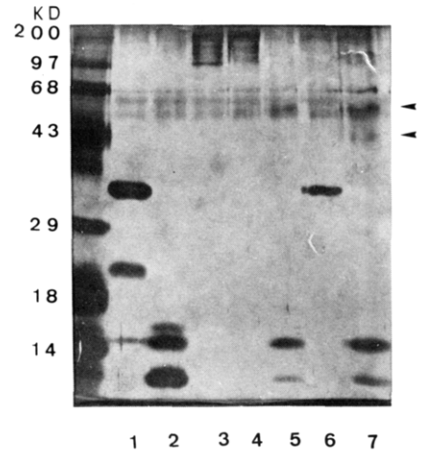

FIGURE 6: 12% SDS–PAGE of cell attachment active and inactive laminin fragments. Proteins were reduced with 2-mercaptoethanol and identified by the silver staining procedure as described under Materials and Methods. Prestained molecular mass standards are shown to the right. Lane 1, α-thrombin enzyme (4 μg); lane 2, chrymotrypsin enzyme (4 μg); lane 2, chymotrypsin enzyme (4 μg); lane 3, laminin (3 μg); lane 4, reduced 200-kDa laminin fragment; lane 5, unbound column fraction from the chymotrypsin digest of reduced 200-kDa laminin fragment; lane 6, unbound column fraction from the α-thrombin digest of laminin; lane 7, unbound column fraction from the chymotrypsin digest of laminin. The top arrow points to the 53-kDa polypeptide present in lanes 5 and 7. The bottom arrow points to the 43-kDa polypeptide present only lane 7.

a polygonal shape (Figure 5A,G), similar to the cells grown in serum (not shown). Cells attached to active material from the chymotrypsin digest of laminin also spread and become polygonal, a morphology typical of mammary epithelial cells (Figure 5B,H). In contrast, cells attached to the unbound fraction from the α-thrombin digest of laminin remained attached and did not spread (Figure 5C,I). Very few cells attached to the reduced 200-kDa laminin fragment, the bound column fraction from the chymotrypsin digest of laminin, and the unbound column fraction from the chymotrypsin digest of the reduced 200-kDa three short arm fragment (Figure 5D,E,F, respectively). Since the size of the biologically active laminin fragments was suspected to be less than 53 kDa (Figure 2) and the cell attachment active material from α-thrombin-digested laminin was not identified on a 7% acrylamide gel (Figure 3A, lane 1), these materials were separated on a 12% acrylamide gel and are shown in Figure 6. The material promoting attachment and spreading of mammary epithelial cells contained the 53-, 43-, 15-, and 12-kDa polypeptides Figure 6, lane 7). The 15- and 12-kDa polypeptides appeared to be the enzyme chymotrypsin as these two peptides comigrated with peptides present in the enzyme (Figure 6, lane 2). Chymotrypsin (10 μg) when bound to plastic did not promote epithelial cell adhesion beyond background levels (not shown). The inactive unbound material generated from the reduced 200-kDa three short arm fragment (Figure 6, lane 5) contained the 53-kDa peptide and the two peptides belonging to the enzyme chymotrypsin. Thus, the cell attachment and spreading activities of the unbound material from the crymotrypsin digest of laminin could be associated with the 43-kDa polypeptide. The unbound material from the α-thrombin digest of laminin which promoted cell attachment but not spreading contains a major 39-kDa polypeptide (Figure 6, lane 6). Figure 6, lane 1, shows the α-thrombin enzyme used in the present study. The 39-kDa polypeptide present in the unbound material (Figure 6, lane 6) comigrated with the enzyme α-thrombin (Figure 6, lane 1). No other fragments were detected in this material. Substratum bound α-thrombin

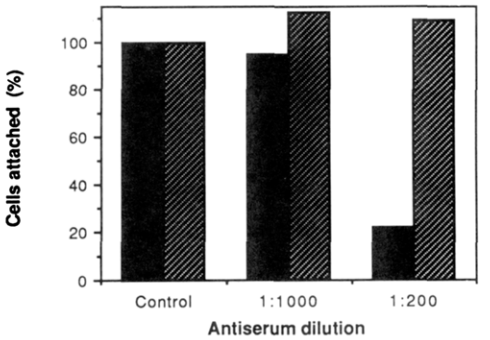

FIGURE 7: Anti-laminin inhibits cell attachment to laminin but not to the 43K fragment. Plastic petri dishes were coated with 1 μg of laminin (solid bar) or with the unbound column fraction containing the 43-kDa polypeptide (hatched bar), as described under Methods. Anti-laminin diluted in attachment medium was added to the appropriate dishes at two different dilutions and incubated for 1 at 22 °C. Radiolabeled mouse mammary epithelial cells were then seeded and incubated for 1 h at 37 °C. The unattached cells were removed, and the attached cells were counted as described under Methods. Control, without antiserum.

significantly (40% of the cells added), promoted the attachment of mammary epithelial cells, and did not cause cell spreading (data not shown). These observations suggest that the cell attachment but not the spreading activity associated with the unbound material to the Mono Q column present in α-thrombin-digested laminin may be attributed to the presence of the α-thrombin enzyme itself. From densitometric scanning, the 43-kDa polypeptide represents about 10% of the material present in the active fraction, which is sufficient to cause attachment and spreading activities equivalent to 10 μg of laminin for mouse mammary epithelial cells. We also fractionated 2 mg of undigested laminin by ion-exchange chromatography. The unbound material to the anion-exchange column from intact laminin had little cell attachment activity (data not shown). These results suggest that the active 43-kDa fragment is not a contaminant in laminin preparations but is generated from laminin by digestion with chymotrypsin.

Polyclonal Antiserum to Laminin Inhibits Attachment of Cells to Laminin but Not to the 43-kDa Fragment. Figure 7 represents data on the ability of the polyclonal antiserum to laminin to interfere with the adhesion of mammary epithelial cells to substratum-bound laminin (solid bar) or the 43-kDa fragment (hatched bar). Preimmune serum failed to inhibit cell adhesion to both laminin and the fragment (data not shown). Immune serum, when diluted to 1:1000, did not inhibit cell adhesion to either laminin or the fragment. At 1:200 dilution, the laminin antiserum blocked the attachment of cells to laminin substrate but not to the fragment substrate.

Synthesis of Laminin, Collagen Type IV, and Desmoplankins I and II by Mouse Mammary Epithelial Cells Attached to Laminin and the 43-kDa Fragment Substrates. We compared the metabolic activity and synthesis of selected proteins by mammary epithelial cells attached to laminin and to the 43-kDa fragment substrates. Cells grown on tissue culture dishes in the presence of growth medium during a 2-h period served as the control. The experiment was done under conditions in which about 80% of the 1×10^6 cells added attached to laminin and fragment substrates. The attached cells are pulse-labeled with radioactive methionine as described under Methods. The total trichloroacetic acid precipitable radioactivity present in cell lysates did not significantly differ among cells attached to laminin and the 43-kDa fragment and to tissue culture plastic in the presence of serum (data not shown). The synthesis of laminin, collagen type IV, and desmoplankins by mammary epithelial cells attached to la-

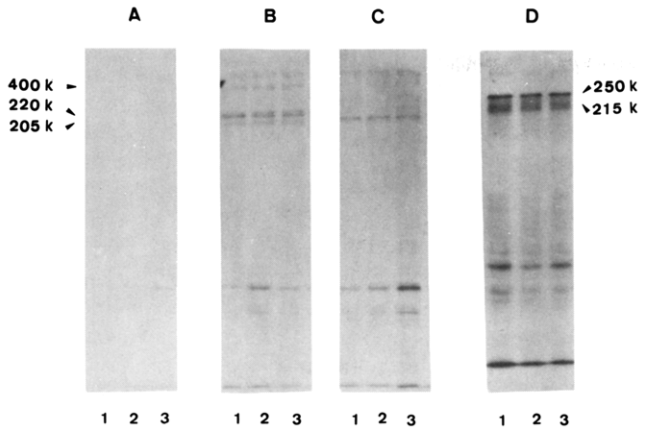

FIGURE 8: Immunoprecipitation of [^{35}S]methionine-labeled cell lysates. Cell lysates were incubated with normal rabbit serum (panel A), anti-laminin (panel B), anti-collagen type IV (panel C), and Mab anti-desmoplankins I and II (panel D). Immunoprecipitates were prepared as described under Methods. The immunoprecipitates were dissolved in reducing electrophoresis sample buffer and analyzed by 7% SDS–PAGE and fluorography. In all the panels: lane 1, cells attached to tissue culture plastic in serum; lane 2, cells attached to laminin substratum; lane 3, cells attached to 43-kDa laminin fragment.

minin and the 43-kDa fragment substrates was analyzed by immunoprecipitation as shown in Figure 8. The lysate from the cells attached to fragment substrate contained laminin (lane 3, panel B), collagen type IV (lane 3, panel C), and desmoplankins I and II (lane 3, panel D) in quantities comparable to lysates from cells attached to laminin substrate (lane 2 in panels B–D, respectively) and to the cells grown on tissue culture dishes in the presence of serum (lane 1 in panels B–D, respectively). Preimmune serum failed to precipitate any of the proteins examined in this study (panel A). These results demonstrate that mouse mammary epithelial cells attached to the 43-kDa fragment substrate are metabolically active and synthesize laminin, type IV collagen, and desmoplankins at a rate equivalent to the cells attached to laminin substrate.

Discussion

In the present study, we report on the identification of two high-affinity heparin binding sites located in the "A" chain of laminin. One site is present within a 53-kDa fragment possibly originating from the short arm of the "A" chain. The second site was contained within a 43-kDa fragment which is located within the long arm of the "A" chain. We also report in this study that mouse mammary epithelial cell attachment and spreading activities of laminin are associated with the 43-kDa fragment on a molar basis. Thus, these cells interact with substratum-bound laminin through a single site which is present in the 43-kDa fragment from the long arm of the "A" chain. Proteolytic enzymes were used to obtain laminin fragments, and the latter were tested in a variety of functional assays. We chose the enzymes α-thrombin and chymotrypsin because the structures of the large fragments generated from laminin were characterized before (Rao et al., 1982a,b, 1983; Palm et al., 1985). Localization of the high-affinity heparin binding sites in the 53- and 43-kDa fragments was based on the data obtained by following the procedure of reduction and alkylation of laminin described by Arumugham et al. (1988). In this procedure, only the "A" chain of laminin binds to heparin–Sepharose in the presence of 6 M urea. Since the reduced and alkylated 53- and 43-kDa fragments bound to heparin–Sepharose in the presence of 8 M urea, we suggest that these two fragments originate from the "A" chain of laminin. On the other hand, 70–160-kDa fragments originated from the "B" subunits (Rao et al., 1983) bind to heparin–Sepharose in the presence of 2 M but not 6 M urea. These data suggest and confirm that multiple heparin binding sites exist on the laminin molecule (Skubitz et al., 1988) but with different affinities. We provide evidence here that the heparin binding sites of the laminin "A" chain are of higher affinity as compared to those present on the "B" chains.

It has been reported that mouse mammary epithelial cells interact with laminin in the matrigel and show an increase in the synthesis of milk protein β-casein (Li et al., 1987). Thus, this cell line is a good candidate to study and characterize the cell binding fragment(s) of laminin. The identification of the 43-kDa fragment as the active cell binding fragment was based on two lines of evidences. The 43-kDa fragment is present in the 2 M urea–heparin-bound fraction which efficiently promoted the attachment of mouse mammary epithelial cells. The cell attachment activity and the 43-kDa fragment are absent in the 2 M urea–heparin-unbound fraction. The 2 M urea–heparin-unbound fraction consists of fragments of 70–160-kDa which originate from the "B" subunits (Rao et al., 1983). The second line of evidence comes from the observations that the reduced 200-kDa intact three short arm fragment was inactive in promoting the attachment of mouse mammary epithelial cells. Digestion of the intact three short arm fragment with chymotrypsin did not generate the 43-kDa fragment but generates the inactive high-affinity heparin binding 53-kDa fragment. On the other hand, digestion of laminin with chymotrypsin generates the active cell binding 43-kDa fragment. On the basis of these observations, we proposed that the active 43-kDa fragment is present in the long arm of the "A" chain. Polyclonal antibodies to laminin did not block the adhesion of cells to the 43-kDa laminin fragment but blocked cell adhesion to whole laminin. Aumailley et al. (1987) have made similar observations when examining the cell adhesion to a long arm fragment E8, suggesting that the "adhering of cells to this fragment is not inhibited by laminin antiserum. Since the "A" chain is poorly antigenic and the 43-kDa fragment as shown in this study is also located in the long arm, there may not be enough blocking antibodies present in the laminin antiserum to inhibit cell adhesion to the fragment. The inhibiting effect of polyclonal antibodies on cell adhesion to laminin is probably due to steric hindrance.

Attachment of cells to laminin causes cell spreading (Kleinman et al., 1985). Similarly, mammary epithelial cells attached to the 43-kDa fragment substrate spread and showed morphology typical of epithelial cells within 6 h after attachment. These observations allow us to conclude that the 43-kDa high-affinity heparin binding fragment, originated from the long arm of the "A" chain, promotes mammary epithelial cell adhesion and spreading as efficiently as the intact protein. Furthermore, mammary epithelial cells after attaching to the 43-kDa fragment responded in a way similar to those of cells attached to laminin by synthesizing three important epithelial cell proteins, laminin, type IV collagen, and desmoplankins I and II. Thus, the fragment probably conveyed signals similar to those conveyed by laminin to induce the mammary epithelial cells to spread and to synthesize laminin, type IV collagen, and desmoplankins. These three proteins have been reported to play a major role in the maintenance of proper epithelial cell morphology and growth (Farquhar & Palade, 1963; Hay, 1981).

Goodman et al. (1987) have reported the existence of two distinct cell binding domains in laminin which can independently promote nonneuronal cell adhesion and spreading. Laminin fragment E8 promotes cell adhesion and spreading of secondary murine myoblasts while E8 and the three short arm

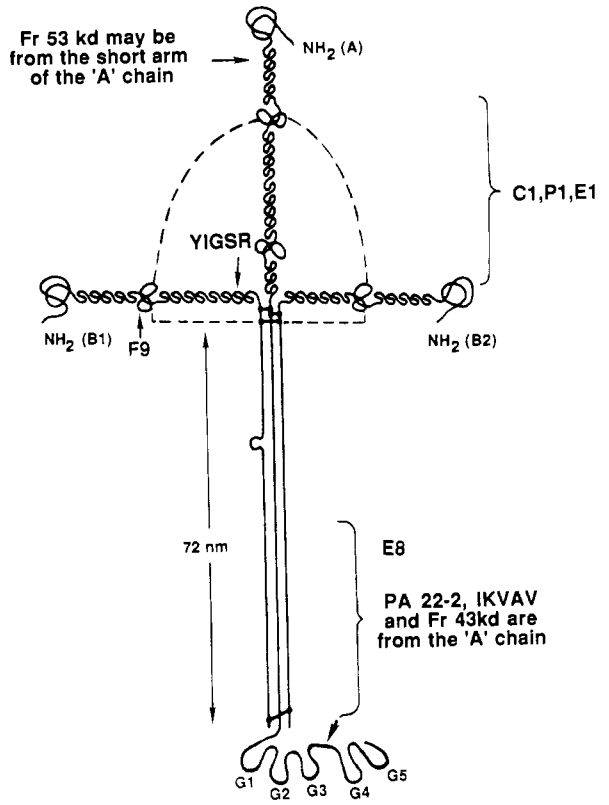

FIGURE 9: Schematic model of laminin and location of cell attachment and heparin binding active sites. Diagrammatic representation of the laminin molecule is adopted from Bruch et al. (1989). Cell binding sites YIGSR (Graf et al., 1987a,b), C1, P1, and E1 (Timpl et al., 1983; Terranova et al., 1984), F-9 (Charonis et al., 1988), E8 (Edgar et al., 1984; Aumailley et al., 1987) and PA 22-2 and IKVAV (Ken-Ichiro Tashiro et al., 1989) were reported previously. The 53-kDa heparin binding fragment of the short arm of the "A" chain and the 43-kDa cell binding, heparin binding fragment of the long arm of the "A" chain are from this study. The origin of the 53-kDa high-affinity heparin binding fragment is tentatively placed in the short arm belonging to the "A" chain. This was because this fragment can be generated from the reduced 200-kDa three short arm fragment. Sequencing of the 53-kDa fragment should be helpful to suggest its location on the "A" chain.

fragments (E1–4) both promote adhesion and spreading of Rugli human glioblastoma and HeLa cells. A site within the three short arms was reported to promote cell adhesion and chemotaxis (Graf et al., 1987a,b) and mediate binding of cells to type IV collagen (Rao et al., 1982a,b; Terranova et al., 1983). Several recent reports indicated the presence of an active cell binding site and a neurite outgrowth promoting site within the long arm of the "A" chain (Skubitz et al., 1987; Dillner et al., 1988; Nurcombe et al., 1989; Sephel et al., 1989; Ken-Ichiro Tashiro et al., 1989). The present study clearly demonstrates the location of the cell binding site for mouse mammary epithelial cells within the long arm of the "A" chain (Figure 9). Thus, it can be stated that mouse mammary epithelial cells, just like the secondary myoblasts, interact with the laminin through a single site present in the long arm. Also, recent immunohistochemical studies at the ultrastructural level indicate that epitopes present in the end of the long arm are exposed to the membrane receptors on adjacent cells (Abrahmson et al., 1989; Schittny et al., 1989). These studies also favor the location of the cell binding site on the long arm of the "A" chain. The 43-kDa cell binding laminin fragment characterized in this study also contains a high-affinity heparin binding site. On the basis of heparin binding activity of the 43-kDa fragment, and the finding that it is not present within the intact three short arm laminin fragment, we would like to place this fragment as a part of the active E8 fragment (M_r 280K) characterized by others (Aumailley et al., 1987; Nurcombe et al., 1989). It has been reported that fragment E8 from the long arm consists of domain 1 sequences from all three chains and large portions of domain 6 (large globule) (Paulsson et al., 1985, 1987). The 43-kDa active fragment, just like the E8 fragment, extensively promoted neurite outgrowth (Rao and Kefalides, submitted for publication). Unlike the E8 fragment, the 43-kDa fragment originates from the "A" chain only. Recently, a synthetic peptide of 19 amino acids from the carboxy-terminal end of the "A" chain and within the E8 fragment was reported to promote neurite outgrowth in PC 12 cells and attachment and cell migration of B16 melanoma cells (Sephel et al., 1989; Ken-Ichiro Tashiro et al., 1989). At present, we do not know if this sequence is present within the 43-kDa "A"-chain fragment. In conclusion, we have identified and characterized an active fragment of M_r 43K from the "A" chain of laminin which contains some of the biological activities of the intact molecule.

ACKNOWLEDGMENTS

We thank Drs. Lance A. Liotta and Mark E. Sobel for helpful discussions. We thank Drs. Edward J. Macarak and Panela S. Howard for help with the phase contrast microscope and Zahra Ziae for critically reading the manuscript. We also thank Margarette J. Wills for her expert preparation of the manuscript.

REFERENCES

Abrahamson, D. R., Irwin, M. H., St. John, P. L., Perry, W. W., Accavitti, M. A., Heck, L. W., & Couchman, J. R. (1989) *J. Cell Biol. 109*, 2477–2492.

Arumugham, R. G., Trumbroe, M., Thomas, T., Mukhlouf, S., & Tanzer, M. L. (1988) *Connect. Tissue Res. 18*, 135–147.

Aumailley, M., Nurcombe, V., Egdar, D., Paulson, M., & Timpl, R. (1987) *J. Biol. Chem. 262*, 11532–11538.

Barsky, S. H., Rao, C. N., Williams, J. E., & Liotta, L. A. (1984) *J. Clin. Invest. 74*, 843–848.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–252.

Bruch, M., Landwehr, R., & Engel, J. (1989) *Eur. J. Biochem. 185*, 271–279.

Carlin, B. E., Durkin, M. E., Bender, B., Jaffe, R., & Chung, A. E. (1983) *J. Biol. Chem. 258*, 7729–7737.

Charonis, A. S., Tsilibary, E. C., Saku, T., & Furthmayr, H. (1985) *J. Cell Biol. 100*, 1848–1853.

Charonis, S., Skubitz, A. P. N., Koliakos, G. G., Reger, L. A., Dege, J., Vogel, A. M., Wohlheuter, R., & Furcht, L. T. (1988) *J. Cell Biol. 107*, 1253–1260.

Chung, A. E., Jaffe, R., Freeman, I. L., Vergnes, J. P., Braginski, J. E., & Carlin, B. (1979) *Cell 16*, 277–281.

Chung, A. E., Jaffe, R., Bender, B., Lewis, M., & Dunkin, M. (1983) *Lab. Invest. 49*, 576–581.

Delrosso, M., Cappelleti, R., Viti, M., Vannucchi, S., & Chiarugi, V. (1981) *Biochem. J. 199*, 699–704.

Dillner, L., Dickerson, K., Manthrope, M., & Ruoslahti, E. (1988) *Exp. Cell Res. 177*, 186–198.

Edgar, D., Timpl, R., & Thoenen, H. (1984) *EMBO J. 3*, 1463–1468.

Engel, J., Odermantt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., & Timpl, R. (1981) *J. Mol. Biol. 150*, 97–120.

Engvall, E., Davis, G. E., Dickerson, K., Ruoslahti, E., Varon, S., & Manthrope, M. (1986) *J. Cell. Biol. 103*, 2457–2466.

Farquhar, M. G., & Palade, G. E. (1963) *J. Cell Biol. 17*, 375–412.

Gehlsen, K. R., Dillner, L., Engvall, E., & Ruoslahti, E. (1988) *Science 241*, 1228–1229.
Goodman, S. L., Deutzmann, R., & Vondermark, K. (1987) *J. Cell Biol. 105*, 589–598.
Graf, J., Iamoto, Y., Sasaki, M., Martin, G. R., Kleinman, H. K., Robey, F. G., & Yamada, Y. (1987a) *Cell 48*, 989–996.
Graf, J., Ogle, R. C., Robey, F. G., Sasaki, M., Martin, G. R., Yamada, Y., & Kleinman, H. K. (1987b) *Biochemistry 26*, 6896–6900.
Hay, E. D. (1981) *J. Cell Biol. 91*, 205S–223S.
Horowitz, A., Duggan, K., Greggs, R., Decker, C., & Buck, C. A. (1985) *J. Cell Biol. 101*, 2134–2144.
Kleinman, H. K., Cannon, F. B., Laurie, G. W., Hassell, J. R., Aumilley, M., Terranova, V. P., Martin, G. R., & Dubois Dalcq, M. (1985) *J. Cell. Biochem. 27*, 317–325.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. R., & Feldmann, J. R. (1986) *J. Mol. Biol. 189*, 205–216.
Lesot, H., Kuhl, U., & Vondermark, K. (1986) *EMBO. J. 2*, 861–865.
Li, L. M., Aggeler, F., Farson, D. A., Hatier, C., Gassell, J. R., & Bissell, M. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 136–140.
Liotta, L. A., Goldfarb, R. H., & Terranova, V. P. (1981) *Thromb. Res. 21*, 663–673.
Liotta, L. A., Rao, C. N., & Wewer, U. M. (1986) *Annu. Rev. Biochem. 55*, 1037–1057.
Malinoff, H. L., & Wicha, M. S. (1983) *J. Cell Biol. 96*, 1475–1579.
Martin, G. R., & Timpl, R. (1987) *Annu. Rev. Cell Biol. 3*, 57–85.
Nurcombe, V., Aumailley, M., Timpl, R., & Edgar, D. (1989) *Eur. J. Biochem. 80* 9–14.
Ott, U., Odermatt, E., Engel, J., Furthmayr, H., & Timpl, R. (1982) *Eur. J. Biochem. 123*, 63–72.
Owens, R. B. (1974a) *J. Natl. Cancer Inst. 52*, 1375–1378.
Owens, R. B. (1974b) *J. Natl. Cancer Inst. 53*, 261–269.
Palm, S. L., McCarthy, J. B., & Furcht, L. T. (1985) *Biochemistry 24*, 7753–7760.
Panayotou, U. G., End, P., Aumailley, M., Timpl, R., & Engel, J. (1989) *Cell 56*, 93–101.
Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., & Engel, J. (1987) *Eur. J. Biochem. 166*, 11–19.
Pikkarainen, T., Eddy, R., Fukishima, Y., Byers, M., Showsit, T., Pihlanjaniemi, T., Saraste, M., & Tryggvason, K. (1987) *J. Biol. Chem. 262*, 10154–10462.
Rao, C. N., Margulies, I. M. K., Tralka, T. S., Terranova, V. P., Madri, J. A., & Liotta, L. A. (1982a) *J. Biol. Chem. 257*, 9740–9744.
Rao, C. N., Marguiles, I. M. K., Goldfarb, R. H., Madri, J. A., Woodley, D. T., & Liotta, L. A. (1982b) *Arch. Biochem. Biophys. 219*, 65–70.
Rao, C. N., Barsky, S. H., Terranova, V. P., & Liotta, L. A. (1983) *Biochem. Biophys. Res. Commun. 111*, 804–808.
Rao, C. N., Margulies, I. M. K., & Liotta, L. A. (1985) *Biochem. Biophys. Res. Commun. 128*, 45–52.
Rao, C. N., Castronova, V., Schmitt, C., Wewer, U. M., Claysmith, A. P., Liotta, L. A., & Sobel, M. (1989) *Biochemistry 28*, 7476–7486.
Roberts, D. D., Rao, C. N., Magnoni, J. L., Spitalnik, S. L., Liotta, L. A., & Ginsburg, V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1306–1310.
Sakashita, S., Engvall, E., & Ruoslahti, E. (1980) *FEBS Lett. 116*, 243–246.
Sasaki, M., & Yamada, Y. (1987) *J. Biol. Chem. 62*, 17111–17117.
Sasaki, M., Kato, S., Kohno, K., Martin, G. R., & Yamada, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 935–939.
Schittny, J. C., Timpl, R., & Engel, J. (1988) *J. Cell Biol. 107*, 1599–1610.
Sephel, G. C., Tashiro, K. I., Sasaki, M., Greatorex, D., Martin, G. R., Yamada, Y., & Kleinman, H. K. (1989) *Biochem. Biophys. Res. Commun. 162*, 821–829.
Skubitz, A. P. N., Charonis, A. S., Tsilibary, E. C., & Furcht, L. T. (1987) *Exp. Cell Res. 173*, 349–360.
Skubitz, A. P. N., McCarthy, J. B., Charonis, A. S., & Furcht, L. T. (1988) *J. Biol. Chem. 263*, 4861–4868.
Sugrue, S. (1988) *Differentiation 38*, 169–176.
Tashiro, K. I., Sephel, G. C., Weeks, B., Sasaki, M., Martin, G. R., Kleinman, H. K., & Yamada, Y. (1989) *J. Biol. Chem. 264*, 16176–16182.
Terranova, V. P., Rao, C. N., Kalabec, T., Margulies, I. M. K., & Liotta, L. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 844–848.
Timpl, R. (1989) *Eur. J. Biochem. 180*, 487–502.
Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., & Martin, G. R. (1979) *J. Biol. Chem. 254*, 9933–9937.
Timpl, R., Johansson, S., Delden, V. V., Oberbaumer, I., & Hook, M. (1982) *J. Biol. Chem. 257*, 8922–8927.
Tomaselli, K. J., Damsky, C. H., & Reichardt, L. F. (1988) *J. Cell Biol. 107*, 1241–1252.
Turner, D. C., Flier, L. A., & Carbonetto, S. (1987) *Dev. Biol. 121*, 510–525.
Woodley, D. T., Rao, C. N., Hassell, J. R., Liotta, L. A., Martin, G. R., & Kleinman, H. K. (1983) *Biochim. Biophys. Acta 761*, 278–283.
Yurchenko, P. D., Tsilibary, E. C., Charonis, A. S., & Furthmayr, H. (1985) *J. Biol. Chem. 260*, 7636–7644.