

A Novel Synthetic Peptide from the B1 Chain of Laminin with Heparin-binding and Cell Adhesion-promoting Activities

Aristidis S. Charonis, Amy P. N. Skubitz, George G. Koliakos, Lorrel A. Reger, Jay Dege, Ann M. Vogel, Robert Wohlhueter, and Leo T. Furcht

Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Abstract. Recent studies using solid-phase-binding assays and electron microscopy suggested the presence of a heparin-binding domain between the inner globule of a lateral short arm and the cross region of laminin. Using the information from the amino acid sequence of the B1 chain of laminin, several peptides were synthesized from areas with a low hydropathy index and a high density of lysines and/or arginines. One of these, peptide F-9 (RYVVLPRVCFEKGMYTVR), which is derived from the inner globular domain of the lateral short arm, demonstrated specific binding to heparin. This was tested in direct solid-phase binding assays by coating the peptide either on nitrocellulose or on polystyrene and in indirect competition assays where the peptide was in solution and either laminin or heparin was immobilized on a solid support. The

binding of [³H]heparin to peptide F-9 was dramatically reduced when heparin but not other glycosaminoglycans other than heparin (dextran sulfate, dermatan sulfate) were used in competition assays. Modification of the free amino groups of peptide F-9 by acetylation abolished its ability to inhibit the binding of [³H]heparin to laminin on polystyrene surfaces. Peptide F-9 promoted the adhesion of various cell lines (melanoma, fibrosarcoma, glioma, pheochromocytoma) and of aortic endothelial cells. Furthermore, when peptide F-9 was present in solution, it inhibited the adhesion of melanoma cells to laminin-coated substrates. These findings suggest that peptide F-9 defines a novel heparin-binding and cell adhesion-promoting site on laminin.

LAMININ is a large (850 kD) basement membrane glycoprotein consisting of three polypeptide chains (B1, B2, A) held together by disulfide bonds (22, 39, 41). By rotary shadowing and electron microscopy, laminin has the shape of an asymmetric cross with three short arms, each with two globular domains near the ends, and one long arm with a large terminal globular domain (6, 22, 39).

Laminin is involved in two general types of interactions. First, it binds to basement membrane macromolecules such as type IV collagen (4, 17), entactin/nidogen (22, 28), heparan sulfate proteoglycan (17), as well as to itself (42). These interactions may be crucial in determining the structure of basement membranes. Second, it has the ability to interact with cell surface molecules, including a 67-kD receptor (19, 21, 38), the CSAT antigen (11), and sulfated glycolipids (29). These interactions with cell surface molecules may influence the adhesion, growth, morphology, and migration (22-24) of various cell types.

Laminin also has the ability to bind to heparin (30). Since macromolecules with heparin-like side chains are present in basement membranes (7, 13, 15, 18, 35) and on cell surfaces (8, 20, 35), this interaction may be very important. Ott et al. (26) have localized a heparin-binding domain on the globule at the distal end of the long arm of laminin. Their studies were performed with laminin fragments derived after various enzymatic digestions. It is possible, however, that other

protease-sensitive, heparin-binding sites are present on the laminin molecule. We have recently localized two additional heparin-binding domains on laminin using monoclonal antibodies (34). One domain was localized to the middle of a short arm while the other domain was localized below the intersection of the cross on the long arm of laminin.

In this study, we synthesized various peptides from the published amino acid sequence of the B1 chain (31). We tested these peptides for heparin-binding properties using various solid-phase assays. One of these peptides, F-9, consistently bound specifically to heparin. This peptide was found to promote the adhesion of various cell lines in solid-phase assays and to compete with laminin when added in solution.

Materials and Methods

Reagents

Laminin was isolated from the Engelbreth-Holm-Swarm (EHS) tumor grown subcutaneously in Swiss Webster mice (Simonsen Laboratories, Inc., Gilroy, CA). The mice were made lathyritic by adding 0.1% beta-amino propionitrile to their water, using previously described techniques (4, 14). Heparin (porcine intestinal mucosa, grade I, 15 kD), dextran sulfate (8 kD), and dermatan sulfate (porcine skin, type B, 20 kD) were all purchased from Sigma Chemical Co. (St. Louis, MO).

Peptide Synthesis and Purification

Peptides were synthesized on a solid-phase support resin according to the technique of Barany and Merrifield (1). Deprotection and release of peptides was achieved by using HF containing 10% anisole for 1 h at 4°C. Peptides were then extracted with ether, dissolved in 10% aqueous acetic acid, filtered to remove the resin, and lyophilized. Peptides were checked for purity by high pressure liquid chromatography, amino acid analysis, and amino acid sequencing.

Peptides were HPLC purified at a semi-preparative scale using a 21.1 × 250 mm C18 reverse phase column and a gradient of acetonitrile in water containing 0.1% (vol/vol) of trifluoroacetic acid. In the experiments, we originally used HPLC-purified peptides and eventually nonHPLC-purified batches in the cases where the biological activity was tested and found to be similar to the HPLC-purified material.

Iodination of Laminin and Peptides

Laminin and peptides (either as shown in Table I or with an addition of one tyrosine at their carboxy terminus) were iodinated by using the chloramine T method (12). Laminin was iodinated using chloramine T at a concentration 1,000 times lower than originally described. After iodination, laminin was purified by gel filtration on a 1 × 25 cm Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ) and subsequently dialyzed against Dulbecco's PBS. Iodinated peptides were purified on SEP-PAK C-18 columns (Waters Associates, Milford, MA) and eluted with 50% acetonitrile in water containing 0.1% (vol/vol) trifluoroacetic acid. Eluted peptides were lyophilized and stored at -70°C.

Modification of Peptide F-9

The free amino groups of peptide F-9 were modified with acetic anhydride (2). 5 mg of peptide F-9 was dissolved in 0.5 ml DMSO, then 1.5 ml of PBS, pH 7.4, was added. Acetic anhydride at a molar ratio to peptide of 1.5:1 was added slowly with stirring on ice. The reaction was allowed to proceed for 30 min at 4°C, while the pH was adjusted to 7.0 with 0.1 M NaOH. After the reaction, the modified peptide was separated from the other reagents on a 1 × 25 cm column of Sephadex G-10 (Pharmacia Fine Chemicals) equilibrated with 25 mM NH₄HCO₃, pH 7.8. Fractions were monitored for peptide content spectrophotometrically at OD₂₈₀. The eluted protein peaks were lyophilized and stored at -70°C. The total yield was 60% of the starting material.

The extent of modification which occurred at the free ε-amino group of the lysine residue was quantitated by use of the enzyme endoproteinase Lys-C (Boehringer-Mannheim Diagnostics, Inc., Indianapolis, IN). This enzyme cleaves specifically at the carboxy group of any intact lysine; however, when the side chain of the lysine is modified, this cleavage does not take place (37). Control and modified peptide F-9 were incubated for 1 h, at 37°C, at an enzyme to substrate ratio of 1:100. Samples were also incubated in the absence of enzyme. At the end of the incubation, an HPLC profile of the modified peptide was compared to the profile of the control peptide and the reduction of the peptide peak was used as a way to quantitate the enzymatic cleavage.

Cells

The cells used in this study included: uv-2237-MM murine fibrosarcoma and K-1735-M4 murine melanoma, kindly provided by Dr. I. J. Fidler (M. D. Anderson Hospital, University of Texas Health Science Center, Houston, TX); bovine aortic endothelial cells isolated from bovine calf thoracic aortas by collagenase digestion were provided by T. Herbst (University of Minnesota, Minneapolis, MN) as described (32); C6 rat glioma cells from the American Type Culture Collection (Rockville, MD) (CCL 107); and PC12 rat pheochromocytoma was kindly provided by Dr. Lloyd Green (New York University, NY). All of the cell lines except the pheochromocytoma cells were maintained in DME (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS), at 37°C in a humidified incubator containing 5% CO₂. In the case of the pheochromocytoma cells, the media used was DME containing 10% FBS and 5% horse sera.

Direct Solid-Phase-binding Assays

The binding of [³H]heparin to the various peptides was measured by direct-binding assays using two different solid supports, nitrocellulose filters and polystyrene plates. For the nitrocellulose dot blot assays, stock solutions of peptides were made at a concentration of 1 mg/ml in 50 mM ammonium

bicarbonate, pH 7.8, and serially diluted 1:1 in the same buffer, producing final concentrations ranging from 1 mg/ml to 1 μg/ml. 100 μl of each concentration was blotted onto a nitrocellulose filter under suction, using a Minifold Microsample Filtration Manifold (Schleicher & Schuell, Keene, NH). Each well was then rinsed with 200 μl of the same buffer three times. The filter was dried overnight at 22°C, then incubated for 2 h with 50,000 dpm/ml of [³H]heparin (0.3 mCi/mg; New England Nuclear, Boston, MA). The filter was then washed four times, 5 min each, with 10 mM Tris-HCl, pH 8.0. Areas where peptides were blotted were then cut out, immersed in Ecolite scintillation fluid (WestChem, San Diego, CA), and counted in a Beckman LS-3801 scintillation counter.

Direct solid-phase-binding assays were also performed in 96-well polystyrene Immulon 1 plates (Dynatech Laboratories, Inc., Alexandria, VA) as previously described (34). Stock solutions of peptides at a maximum concentration of 1 mg/ml were prepared in PBS containing 0.02% NaN₃; peptides were then serially diluted 1:1 in the same buffer, producing final concentrations ranging from 1 mg/ml to 2 μg/ml. 50 μl of each concentration was added to the wells and dried overnight at 29°C. Wells were then treated for 2 h at 37°C with 200 μl of 2 mg/ml BSA (Fatty Acid Free, fraction V, ICN Immunobiologicals, Lisle, IL), in 6 mM phosphate, 100 mM NaCl, 60 μM CaCl₂, pH 6.8 (wash buffer) to minimize nonspecific binding. Next, 50 μl of [³H]heparin (10 μg/ml in the same buffer) was added to each well (50,000 dpm/well) and incubated for 2 h at 37°C. The wells were rinsed three times with wash buffer containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]l-propanesulfonate and then incubated for 30 min at 60°C with 200 μl of 0.5 N NaOH and 1% SDS. The amount of [³H]heparin bound was quantitated as described above.

Inhibition-binding Assays

Inhibition-binding assays were performed by two different techniques: in the first, laminin was immobilized on plastic; in the second, heparin was immobilized on beads.

In the first assay, laminin at 60 μg/ml in PBS containing 0.02% NaN₃ was coated onto 96-well polystyrene plates, using 50 μl per well and dried overnight at 29°C. The wells were then blocked for 2 h at 37°C with 2 mg/ml BSA in wash buffer. Peptides at various dilutions ranging from 0.5 mg/ml to 1 μg/ml in PBS containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]l-propanesulfonate were incubated concomitantly with [³H]heparin (25,000 dpm/well; 5 μg/ml final concentration) for 2 h at 37°C in polypropylene tubes. 50 μL of the mixture was then transferred to the laminin-coated wells and allowed to incubate for an additional 2 h at 37°C. The wells were then rinsed with wash buffer containing 0.05% Triton X-100 and the [³H]heparin which bound was detected as described above.

In the second assay, heparin-Sepharose beads (Pharmacia Fine Chemicals) were equilibrated in 6 mM phosphate, 100 mM NaCl, 60 μM CaCl₂, pH 6.8. 0.5-ml aliquots of beads (packed beads:buffer at 1:1 volume ratio) were incubated in polypropylene tubes at 37°C for 1 h with each peptide at a final concentration of 1 mg/ml. To each tube, 20 μl of radiolabeled laminin (1 μg/ml; 50,000 dpm/20 μl) was added for another hour at 37°C. At the end of this incubation period, the beads were transferred to small columns, rinsed with 10 ml of buffer (flow rate 2 ml/min), air dried, and placed in the gamma counter to measure the radioactivity left on the column. Nonspecific binding was measured as the amount of ¹²⁵I-laminin which bound to plain Sepharose beads. In these experiments, 13.9% of the total laminin counts were found to adhere nonspecifically.

Specificity of Peptide F-9 Binding to Heparin

To determine the importance of the heparin structure for this interaction, 3.0 μg per well of peptide F-9 was dried onto 96-well plates as described above. Wells were blocked for 2 h with 2 mg/ml BSA in wash buffer. To each well, 50 μl of [³H]heparin (50,000 dpm/well) was added concomitantly with various amounts of unlabeled heparin, dextran sulfate, and dermatan sulfate. After incubating for 2 h at 37°C, the wells were washed and the radioactivity was detected as described above.

To determine the importance of the free amino groups of peptide F-9 in its interaction with heparin, we modified peptide F-9 as described above, and then used it in the first type of inhibition assay (laminin-coated plates) exactly as described above.

Cell Adhesion Assays: Direct Binding

Five different cell types were tested for their ability to adhere to substrates coated with laminin and the synthetic peptides in assays similar to those previously described (27). Briefly, mid to late log phase cells were incubated

Table I. Peptides Synthesized from the $\beta 1$ Chain of Laminin

Peptide	Amino acid sequence*	Residue numbers*	Hydropathy index	Number of lysines and arginines
F-9	RYVVLPRPVCFEKGMNYTVR	641-660	-3.9	4
F-11	NIDTTDPEACDKDTGRCLK	960-978	-20.7	3
F-12	VEGVEGPRCDKCTRGY	1,133-1,148	-14.8	3
F-13	ELTNRTHKFLEKAKALKI	1,171-1,188	-12.9	5

* Sasaki et al. (see reference 31).

overnight in DME containing 10% FBS plus either 3 $\mu\text{Ci/ml}$ of [^3H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) for the fibrosarcoma and melanoma cell lines, or 30 $\mu\text{Ci/ml}$ of [^3H]-L-amino acid mixture (240 mCi/ml, ICN Radiochemicals, Irvine, CA) for the glioma and pheochromocytoma cell lines and the endothelial cells. The cells were harvested by gentle trypsinization (two 1-min rinses with 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid) and the trypsinization was terminated by the addition of DME containing 10% FBS. Removal of pheochromocytoma cells from the flasks was achieved by incubating cells for 10 min in Hanks' balanced salt solution (HBSS) without CaCl_2 or MgSO_4 (Sigma Chemical Co.), instead of trypsin. All cells were washed with DME containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma Chemical Co.), pH 7.4, resuspended to 4×10^4 cells/ml in the same buffer containing 2 mg/ml BSA. 100 μl of the cell suspension was placed in wells of polystyrene plates which had been precoated with 1.0 μg of the peptides, laminin, or BSA. The cells were incubated in the wells for 2 h at 37°C, then nonadherent cells were aspirated off and the wells were rinsed three times with 2 mg/ml BSA in DME containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. Adherent cells were solubilized with 200 μl of 0.5 N NaOH and 1% SDS, and quantitated in a scintillation counter.

Cell Adhesion Assays: Competition of Binding

The competition assays concentrated on only the melanoma cell line. They were grown, labeled, and harvested as described above. For 30 min before transfer to laminin-coated 96-well plates (coated as described above) and during a 20-min incubation, various potential inhibitors of cell adhesion were present in solution. These included: laminin or peptides F-9, F-11, F-12, or F-13 at a final concentration of 150 $\mu\text{g/ml}$. Control experiments were done in the absence of inhibitors. Cells were photographed 20 min after they had been transferred to the laminin-coated wells, at a final magnification of 400 \times with a Nikon DIAPHOT inverted microscope and Panatomic X Film ASA32 (Eastman Kodak, Rochester, NY). All experiments described were performed in triplicate and repeated a minimum of two times.

Results

Data obtained by our group in the past using well characterized monoclonal antibodies (33, 34) and direct observations with the technique of rotary shadowing (not shown) strongly suggested that a heparin-binding site on laminin might exist in the area between the inner globule of the lateral short arms and the cross region. To more precisely map this heparin-binding domain, we selected a variety of regions from the published amino acid sequence of the $\beta 1$ chain of laminin, based on two criteria: First, the region was required to have a relatively low (negative) value of hydropathy index (16), based upon the finding that the heparin-binding domain of vitronectin was localized in a region with the most negative hydropathy index (36). To apply this criterion, we constructed the hydropathy index of the full sequence of the $\beta 1$ chain, using the Intelligenetics (Mountain View, CA) computer program with a span setting of 7 amino acids (data not shown). Second, since electrostatic forces may play a major role in the interactions between proteins and heparin, the

regions selected had a relatively high number of the strongly positively charged amino acid residues lysine and arginine.

The amino acid sequences of the peptides which were synthesized and used in this study and their localization along the $\beta 1$ chain are shown in Table I and Fig. 1. Other characteristics of each peptide, including the hydropathy value and the number of arginine and lysine residues present are also listed in Table I.

Binding of [^3H]Heparin to Synthetic Peptides

The direct binding of [^3H]heparin to peptides which had been dried onto nitrocellulose filters was determined (Fig. 2). The actual amount of each peptide that was retained on the filter was quantitated by using iodinated peptides, as described in the Materials and Methods. Peptide F-9 was the only peptide to which [^3H]heparin bound. Similar experiments using polystyrene plates as a surface to which peptides were dried gave similar results (not shown).

Since these assays were performed with the peptides im-

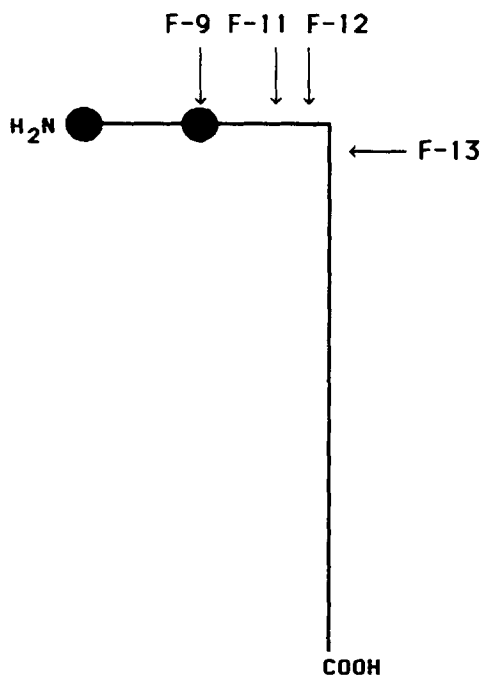


Figure 1. Diagrammatic model of the $\beta 1$ chain of laminin indicating the location of the synthesized peptides. The $\beta 1$ chain of laminin is drawn according to Sasaki et al. (31) and indicates the approximate location of peptides F-9, F-11, F-12, and F-13 which were synthesized and used in this study.

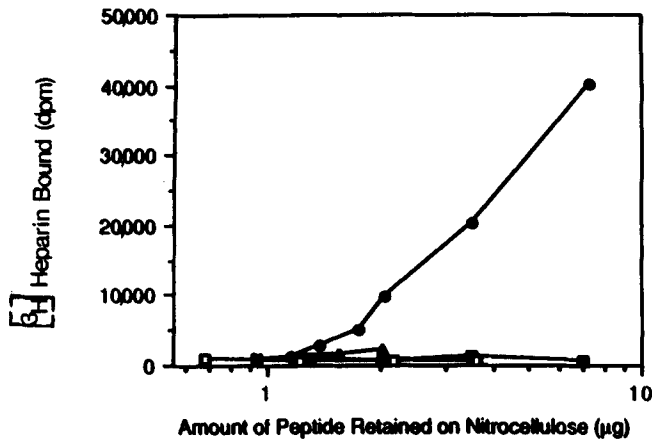


Figure 2. Binding of heparin to laminin peptides dried onto nitrocellulose. 100 µl of peptide solutions at various concentrations (starting from 500 µg/ml) were blotted on nitrocellulose. Then, the filter was incubated with [³H]heparin for 2 h, as described in Materials and Methods. The actual amount of peptide retained on the filter was calculated in experiments using iodinated peptides with SEM < 5%. F-9 (●), F-11 (■), F-12 (△), and F-13 (□).

mobilized on a solid matrix, we were concerned that immobilization might affect the biological or functional properties of the peptides. Therefore, an inhibition assay was designed in which the binding of [³H]heparin to laminin coated on plastic was monitored in the presence of peptides in solution. Only peptide F-9 was able to inhibit [³H]heparin binding to laminin (Fig. 3); confirming the results of the direct-binding assays. This inhibition could be observed at concentrations as low as 10 µg/ml and reached 50% inhibition at 60 µg/ml. As a control, the ¹²⁵I-labeled peptides were added to wells coated with laminin; no binding between laminin and any of the peptides was observed (data not shown). This finding ruled out the possibility that inhibition of heparin-laminin interaction was due to masking of heparin-binding sites on laminin by the peptides.

Since laminin was adsorbed onto plastic in these inhibition

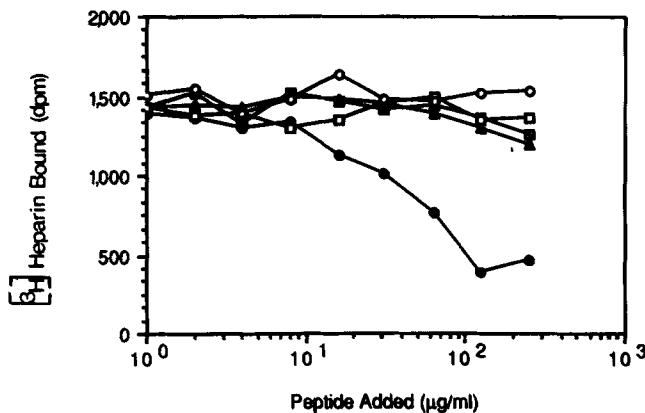


Figure 3. Inhibition of the binding of heparin to laminin by synthetic peptides. Peptides at various concentrations were incubated with [³H]heparin for 2 h and then the mixture was transferred to laminin-coated polystyrene wells for 2 h as described in Materials and Methods, with SEM < 5%. F-9 (●), F-11 (■), F-12 (△), F-13 (□), and modified (acetylated) F-9 (○).

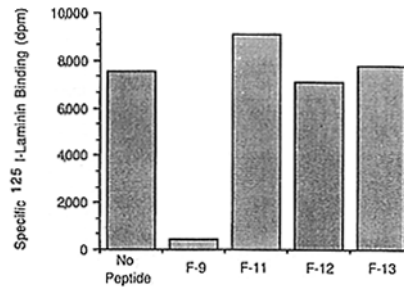


Figure 4. Inhibition of laminin-heparin interaction by synthetic peptides. Heparin-Sepharose beads were incubated for 1 h with various peptides at a final concentration of 1 mg/ml. Then, a small amount of radioactive laminin was added and the mixture was further incubated for another hour. At the end of the final incubation, beads were washed and counted, with SEM < 5%.

assays, it is possible that it was not present in its native state. Therefore, another assay was conducted to further examine the interaction of heparin with peptide F-9 and laminin in an experimental system where both laminin and peptide F-9 would be in solution. Heparin-Sepharose beads were pre-incubated with various peptides at 1 mg/ml, a standard amount of radiolabeled laminin was then added, and the amount of laminin bound was quantitated as described under Materials and Methods. As expected, peptide F-9 was the only peptide which competed for the binding of laminin to heparin (Fig. 4).

The three different experimental approaches described above indicate that peptide F-9 binds to heparin under conditions in which both molecules are in solution or either one of them is immobilized on a solid support.

Specificity of the Interaction Between Peptide F-9 and Heparin

To understand the structural features of peptide F-9 and heparin that are crucial for their interaction, structurally altered molecules were then tested for functional activity.

Heparin is composed of a number of negatively charged groups, in particular, sulfate groups. Two other sulfated

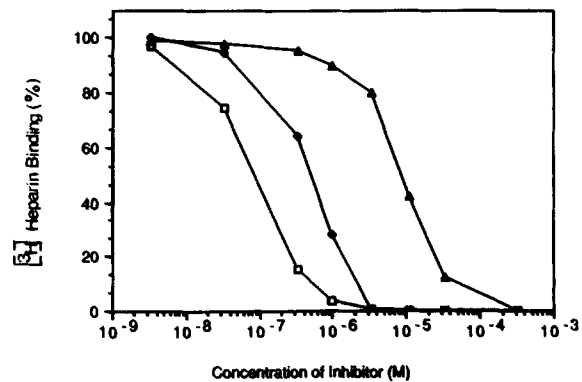


Figure 5. Competition of [³H]heparin-binding to peptide F-9 by sulfated polysaccharides. Wells coated with 3 µg of peptide F-9 were incubated for 2 h with [³H]heparin and various concentrations of unlabeled heparin (□), dextran sulfate (◆) and dermatan sulfate (△), with SEM < 5%.

Table II. Direct Binding of Various Cell Lines to Laminin- and F-9-coated Dishes

Cell line	Cells adhered	
	Laminin*	F-9†
	%	%
Murine melanoma (M4)	28	30
Murine fibrosarcoma (MM)	52	64
Rat pheochromocytoma (PC12)	34	60
Rat glioma (C6)	50	59
Bovine aortic endothelial cells (BAEC)	18	23

* Final amount retained on the well: 700 ng.

† Final amount retained on the well: 69 ng.

polysaccharides with a different structure of the repeated disaccharide unit, dermatan sulfate and dextran sulfate, were tested with heparin in competition-binding experiments. In this assay, peptide F-9 was dried onto polystyrene plates at 3 µg/well. Under these conditions, 150 ng of peptide F-9 is adsorbed and retained on the plastic well. The direct binding of a standard amount of [³H]heparin to the coated peptide was competed off by various concentrations of unlabeled heparin, dermatan sulfate, and dextran sulfate (Fig. 5). A 50% inhibition of binding of [³H]heparin was achieved with $\sim 9 \times 10^{-8}$ M unlabeled heparin, while seven times more dextran sulfate (6×10^{-7} M) or 110 times more dermatan sulfate (1×10^{-5} M) was required for similar levels of inhibition. These results suggest that although the negative charges of the polysaccharides might play an important role in mediating the binding, other structural features of heparin might have a crucial contribution as well.

Peptide F-9 was then chemically modified to determine whether free amino groups are important for heparin-binding activity. This peptide contains two free amino groups: an α -amino group present on the first arginine residue and an ϵ -amino group present on the lysine residue. In situ in the B1 chain, only the ϵ -amino group of lysine is present because the α -amino group of arginine is involved in the formation of a peptide bond. The importance of the free amino groups in heparin-binding was determined by modification with acetic anhydride, a chemical which reacts specifically with free amino groups (2, 37).

After treatment with acetic anhydride, the extent of modification of the ϵ -amino group of lysine was assessed by treatment with endoproteinase Lys-C. This enzyme cleaves at the carboxy terminal of intact lysine residues but fails to do so when the ϵ -amino group is modified (37). Both intact and modified peptide F-9 were digested as described under Materials and Methods, and the disappearance of the peptide peak on the HPLC profile was measured. In the case of the intact peptide, the peak totally disappeared after 60 min digestion. Under identical conditions, the modified peptide peak was reduced only by 27%. We concluded that over 70% of the ϵ -amino groups of lysines have been successfully modified.

The ability of modified peptide F-9 to bind heparin in solution was measured using laminin dried onto plastic. As shown in Fig. 3, modified peptide F-9 was no longer able to compete for the binding of heparin to laminin. These results suggest that the free amino groups of peptide F-9 and/or their

Table III. Inhibition of Melanoma Cell Adhesion on Laminin-coated Wells by Various Inhibitors in Solution

Competitor*	Inhibition of melanoma cell adhesion	
	%	
None	0	
Laminin	63	
F-9	49	
F-11	0	
F-12	5	
F-13	9	

* Final concentration: 150 µg/ml.

environment may be crucial areas for the heparin-binding property of this laminin fragment.

Effect of Peptide F-9 on Cell Adhesion

Because many cells may exhibit heparin-like structures on their surfaces (8, 20, 25, 35), we investigated the role of our synthetic peptides in the phenomenon of cell adhesion. The results described so far made us put specific emphasis on the activity of peptide F-9.

In the first series of experiments, polystyrene plates were coated with either laminin or peptide F-9. The total amount of each protein dried down was 1 µg. Under these conditions 700 ng of laminin and 69 ng of peptide F-9 remain attached to the solid support during the course of the experiment, as calculated by using radiolabeled proteins. After coating and blocking the plates, radiolabeled cells were incubated in the wells for 2 h, as described in Materials and Methods. The number of cells that adhered in each well was quantitated as a percentage of cell adhesion and was calculated by comparing the radioactivity remaining in the well at the end of the experiment with the total radioactivity added to the well. The results shown in Table II indicate that peptide F-9 promotes the adhesion of all of the cell lines examined at a level comparable to intact laminin. Despite the fact that under our experimental conditions the molarity of the peptide is higher than that of laminin, it is still remarkable that such a small fragment of laminin (less than 1/300th of its molecular mass) is so potent in promoting cell adhesion. Under the same experimental conditions, the other peptides and BSA did not promote or only slightly promoted cell adhesion (data not shown).

We then concentrated on one particular cell line, the melanoma cells, to further study cell adhesion. In this series of experiments, melanoma cells were preincubated with laminin or one of the peptides, then added to laminin-coated wells in the presence of these potential inhibitors. The results shown in Table III demonstrate that laminin was able to inhibit melanoma cell adhesion by 63%, peptide F-9 created a 49% inhibition, whereas the other peptides did not produce any substantial inhibition. These results suggest that peptide F-9 is capable of regulating cell adhesion not only while adsorbed on plastic, but also when present in solution.

The effect of peptide F-9 on the morphology of melanoma cells during adhesion was examined with a photomicroscope during the course of the previously described experiment. As seen in Fig. 6, cells incubated with peptide F-9 in solution were mostly rounded and only a few of them appeared at-

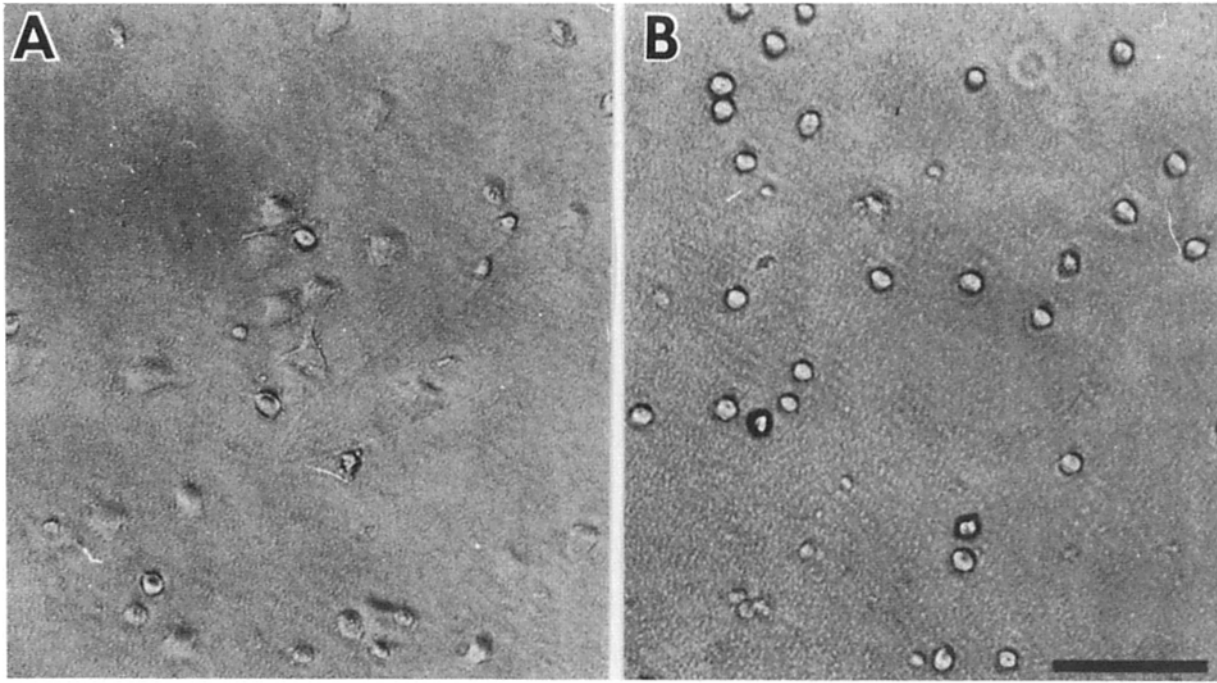


Figure 6. Inhibition of cell adhesion by peptide F-9 in solution. Melanoma cells were harvested with light trypsinization, washed, and incubated for 30 min in 150 $\mu\text{g/ml}$ of inhibitors (laminin or the various peptides). The cell suspensions were then placed in laminin-coated polystyrene wells at a density of 4×10^4 cells/ml, and photographed after 20 min. *A* is in the absence of inhibitor. The same cell morphology was observed in the presence of peptides F-11, F-12, and F-13. *B* is in the presence of peptide F-9. The same cell morphology was observed in the presence of laminin.

tached to the laminin-coated substrate (Fig. 6 *B*), whereas absence of peptide F-9 allowed the vast majority of cells to be well attached to the substrate (Fig. 6 *A*). This microscopic observation confirms our previous finding and further suggests a role of peptide F-9 in melanoma cell adhesion.

Discussion

In this study, a synthetic peptide 20 amino acids long from the B1 chain of laminin has been shown to exhibit heparin-binding activity and to promote the adhesion of various cell lines.

Our previous work has demonstrated that, apart from the heparin-binding domain on the globule of the long arm originally described by Ott et al. (26), other heparin-binding domains exist on the laminin molecule (34). One of them was suggested to be localized to the lateral short arm between the inner globule and the cross region (34). Rotary-shadowing observations also supported this finding, by demonstrating a high frequency of heparin binding at or near the inner globule of the lateral short arm (unpublished observations). According to the model of the B1 chain structure by Sasaki et al. (31), peptide F-9 belongs to the amino acid sequences that form the inner globule of the lateral short arm of the B1 chain.

The data in this report suggest that the binding of heparin to peptide F-9 is specific and is not due exclusively to ionic interactions, since other peptides with higher charge densities fail to bind heparin. The factors that determine whether an amino acid sequence will bind to heparin are not fully known. Our findings indicate that neither a very low hy-

dropathy value nor a high density of positively charged amino acid residues are absolute prerequisites. The peptides synthesized in this study fulfilled both of these criteria, yet only one of them exhibited binding to heparin. Perhaps the exact conformation of each peptide is the crucial factor. The distance that charged amino acid residues are spaced apart may also be an important parameter. Additionally, the structural conformation of the glycosaminoglycan chain might be crucial. The type and amount of hexosamine and uronic acid, the type of bond they form with each other or with the next disaccharide unit (1 \rightarrow 3 or 1 \rightarrow 4), and the degree of sulfation could all be determining factors in their binding to specific amino acid sequences. These factors could actually confer some specificity in the binding of each type of glycosaminoglycan to different polypeptides.

The finding that modification of the free amino groups of peptide F-9 affects heparin binding suggests that at least two residues (the first arginine and the lysine) and/or their environment might be crucial for this interaction. Further work is in progress in order to understand the minimal structural requirements for the heparin-binding and cell-binding activities of peptide F-9.

In these studies, heparin was used as a prototype for a proteoglycan molecule. Although free heparin may not exist as a bona fide basement membrane component, many heparin-like domains are present in the environment of laminin. These include glycosaminoglycan side chains of basement membrane proteoglycans (7, 13, 15, 18, 35) and of cell surface associated proteoglycans of various cell types associated with basement membranes (8, 20, 35). It has recently been reported that an endothelial cell-derived heparan sulfate pro-

teoglycan may contain certain heparin-like stretches in regions of its side chains (25). Therefore, the heparin-binding sites on laminin may be crucial not only for the structural arrangement of basement membrane components but also for regulating cell adhesion to laminin.

Laminin may contain multiple cell adhesion promoting domains and some of them may be cell-type specific. Teranova et al. have proposed that a cell-binding site for human breast carcinoma cells exists on a chymotrypsin fragment of laminin that was thought to consist of the inner nonglobular regions of the three short arms (38). Timpl et al. have suggested that proteolytically derived fragments P1, E5, and E6 represent three distinct laminin domains that promote hepatocyte adhesion (40). The P1 fragment is thought to contain the nonglobular regions of the three short arms next to the cross. Fragments E5 and E6 are possibly derived from the globules of the short arm opposite to the long arm of laminin. Goodman et al. have recently provided evidence that human glioblastoma cells adhere to laminin via two different binding sites, one site located on fragment E 1-4 which consists of the three short arms, and another site located on fragment E8 which consists of the major part of the long arm, including its terminal globule (9). We have observed that a domain in the upper third of the long arm of laminin might be important in mediating the adhesion of murine melanoma cells (33).

It would be of interest to more precisely localize the cell adhesion domains of laminin to specific amino acid sequences. Recently, the full sequence of the B1 chain of laminin was published (31). Graf et al. have provided evidence that the nonapeptide CDPGYIGSR from the B1 chain promotes the attachment of HT-1080 human fibrosarcoma cells and Chinese hamster ovary cells (10). In this report, we provide the sequence of an additional domain from the B1 chain of laminin that promotes the adhesion of murine melanoma, murine fibrosarcoma, rat glioma, rat pheochromocytoma, and bovine aortic endothelial cells. Peptide F-9 has the ability to bind to heparin and to promote the adhesion of various cell lines. These findings suggest that cell surface-associated heparin-like molecules may act as receptors for these laminin domains. However, it cannot be ruled out that domains from the CSAT/integrin complex (11), other macromolecules, or sulfated glycolipids (29) might participate in the binding to this laminin domain.

Peptide F-9 contains the sequence Asn-Tyr-Thr which is a possible acceptor site of N-linked oligosaccharide moieties close to its carboxyl terminal. It is not yet known whether and to what extent laminin is glycosylated in this region and to what extent this post-translational modification could affect the heparin-binding properties of this fragment. If this is the case, differential glycosylation patterns leading to different functional properties of the laminin molecule could be possible.

This novel heparin-binding and cell adhesion-promoting region is localized on the inner globule of the lateral short arm of laminin formed by the B1 chain. This site is relatively close to the cell-binding site described by Graf et al. (10) and possibly close to two other important binding sites on the lateral short arms of laminin for entactin/nidogen (24, 28) and for type IV collagen (38) (if either one or both of them are localized on the B1 chain). Such close arrangement of interactive sites for basement membrane macromolecules and

cell surfaces might be crucial for the structural diversity of basement membranes (3).

It has been suggested that during development (5) and in certain tissues (14) there are different levels of mRNAs coding for the three laminin chains. If the levels of mRNA reflect the actual amount of each chain present in the tissue and are not due to differential message turnover, then the possibility exists that different forms of laminin could be present. In the kidney, the message for the B1 chain has been detected in 13-fold excess of the message for the A chain (15). If laminin is mainly present in this tissue in a form partially or totally lacking the A-chain, then the heparin-binding domain localized on the globule of the long arm will be absent. In this case, the newly described heparin-binding domain on the B1 chain of laminin could play a major role in the organization of kidney basement membranes.

We would like to thank Dr. Jim McCarthy for helpful discussions and suggestions; Dr. Bianca Conti-Tronconi for her help in the synthesis of the peptides and many critical suggestions; Dr. John Alegre for providing us with the hydropathy plot of the B1 chain; Marcie Krumwiede, Jay E. Dege, Ann M. Vogel, Katya Litkewitsch, Kristi Swenson, Selene Swanson, and Dan Mickelson for excellent technical assistance; Drs. Keith Skubitz and Effie Tsilibary for helpful discussions and critical review of the manuscript; and Carol Ghandour for typing the manuscript.

This study was supported by research grants from National Institute of Diabetes, Digestive and Kidney Diseases/National Institutes of Health (NIH) No. 39868, American Diabetes Association, American Heart Association, Minnesota Affiliate, Minnesota Medical Foundation, and Viking Children's Fund to A. S. Charonis; NRSA No. F32-CA08065-01 from National Cancer Institute (NCI)/NIH and an American Cancer Society postdoctoral fellowship PF No. 2885 to A. P. N. Skubitz; and NIH AM-07651, CA-29995, CA-21463, and EI-39510 to L. T. Furcht. L. T. Furcht is the recipient of an Allen-Pardee Professorship.

Received for publication 3 September 1987, and in revised form 19 May 1988.

References

1. Barany, G., and R. B. Merrifield. 1980. Solid-phase peptide synthesis. In *The Peptides*. E. Gross and J. Meienhofer, editors. Academic Press. New York. 1-284.
2. Brownlee, M., H. Vlassara, and A. Cerami. 1983. Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. *Diabetes*. 32:680-684.
3. Charonis, A. S., E. C. Tsilibary, T. Saku, and H. Furthmayr. 1986. Inhibition of laminin self-assembly and interaction with type IV collagen by antibodies to the terminal domain of the long arm. *J. Cell Biol.* 103: 1689-1697.
4. Charonis, A. S., E. C. Tsilibary, P. D. Yurchenco, and H. Furthmayr. 1985. Binding of laminin to type IV collagen, a morphological study. *J. Cell Biol.* 100:1848-1853.
5. Cooper, A. R., and H. A. MacQueen. 1983. Subunits of laminin are differentially synthesized in mouse eggs and early embryos. *Dev. Biol.* 96:467-471.
6. Engel, J., E. Odermatt, A. Engel, J. A. Madri, H. Furthmayr, H. Rohde, and R. Timpl. 1981. Shapes, domain organization and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. *J. Mol. Biol.* 150:97-120.
7. Fujiwara, S., H. Wiedemann, R. Timpl, A. Lustig, and J. Engel. 1984. Structure and interactions of heparan sulfate proteoglycan from a mouse tumor basement membrane. *Eur. J. Biochem.* 143:145-157.
8. Gallagher, J. T., L. Malcon, and W. P. Steward. 1986. Structure and function of heparin sulfate proteoglycans. *Biochem. J.* 236:313-325.
9. Goodman, S. L., R. Deutzmann, and K. von der Mark. 1987. Two distinct cell binding domains in laminin can independently promote nonneuronal cell adhesion and spreading. *J. Cell Biol.* 105:589-598.
10. Graf, J., Y. Iwamoto, M. Sasaki, G. R. Martin, H. K. Kleinman, F. A. Robey, and Y. Yamada. 1987. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell*. 48:989-996.
11. Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The

- cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134-2144.
12. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)* 194:495-496.
 13. Kanwar, Y. S., V. C. Hascall, and M. G. Farquhar. 1981. Partial characterization of newly synthesized proteoglycans isolated from the glomerular basement membrane. *J. Cell Biol.* 90:527-532.
 14. Kleinman, H. K., I. Ebihara, P. D. Killen, M. Sasaki, F. B. Cannon, Y. Yamada, and G. Martin. 1987. Genes for basement membrane proteins are coordinately expressed in differentiating F9 cells but not in normal adult murine tissues. *Dev. Biol.* 122:373-378.
 15. Kleinman, H. K., M. L. McGarvey, L. A. Liotta, P. A. Robey, K. Trygvason, and G. R. Martin. 1982. Isolation and characterization of type IV procollagen, laminin, and heparin sulfate proteoglycan from the EHS sarcoma. *Biochemistry*. 21:6188-6193.
 16. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.
 17. Laurie, G. W., J. T. Bing, H. K. Kleinman, J. R. Hassel, M. Aumailley, G. R. Martin, and R. J. Feldman. 1986. Localization of binding sites for laminin, heparan sulfate proteoglycan and fibronectin on basement membrane (type IV) collagen. *J. Mol. Biol.* 189:205-216.
 18. Ledbetter, S. R., L. W. Fisher, and J. R. Hassel. 1987. Domain structure of the basement membrane heparan sulfate proteoglycan. *Biochemistry*. 26:988-995.
 19. Lesot, H., U. Kuhl, and K. von der Mark. 1983. Isolation of a laminin binding protein from muscle cell membranes. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:861-865.
 20. Lindahl, U., and M. Höök. 1978. Glycosaminoglycans and their binding to biological molecules. *Annu. Rev. Biochem.* 47:385-417.
 21. Malinoff, H., and M. S. Wicha. 1983. Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. *J. Cell Biol.* 96:1475-1479.
 22. Martin, G. R., and R. Timpl. 1987. Laminin and other basement membrane components. In *Ann. Rev. Cell Biol.* G. E. Palade, B. M. Alberts, and J. A. Spudich, editors. Vol. 3. Annual Reviews Inc., Palo Alto, CA. 57-85.
 23. McCarthy, J. B., and L. T. Furcht. 1984. Laminin and fibronectin promote the haptotactic migration of B16 mouse melanoma cells in vitro. *J. Cell Biol.* 98:1474-1480.
 24. McCarthy, J. B., S. L. Palm, and L. T. Furcht. 1983. Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. *J. Cell Biol.* 97:772-777.
 25. Nader, H. B., C. P. Dietrich, V. Buonassisi, and P. Colburn. 1987. Heparin sequences in the heparan sulfate chains of an endothelial cell proteoglycan. *Proc. Natl. Acad. Sci.* 84:3565-3569.
 26. Ott, V. E., E. Odermatt, J. Engel, H. Furthmayr, and R. Timpl. 1982. Protease resistance and conformation of laminin. *Eur. J. Biochem.* 123:63-72.
 27. Palm, S. L., and L. T. Furcht. 1983. Production of laminin and fibronectin by Schwannoma cells: Cell-protein interactions in vitro and protein localization in peripheral nerve in vivo. *J. Cell Biol.* 96:1218-1226.
 28. Paulsson, M., M. Aumailley, R. Deutzmann, R. Timpl, K. Beck, and J. Engel. 1987. Laminin-nidogen complex. Extraction with chelating agents and structural characterization. *Eur. J. Biochem.* 166:11-19.
 29. Robert, D. D., C. N. Rao, J. L. Magnoni, S. L. Spitalnik, L. A. Liotta, and V. Ginsburg. 1985. Laminin binds specifically to sulfated glycolipids. *Proc. Natl. Acad. Sci. USA.* 82:1306-1310.
 30. Sakashita, S., E. Engvall, and E. Ruoslahti. 1980. Basement membrane glycoprotein laminin binds to heparin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 116:243-246.
 31. Sasaki, M., S. Kato, K. Kohno, G. R. Martin, and Y. Yamada. 1987. Sequence of cDNA encoding the laminin B1 chain reveals a multidomain protein containing cysteine-rich repeats. *Proc. Natl. Acad. Sci. USA.* 84:935-939.
 32. Schwartz, S. M. 1978. Selection and characterization of bovine aortic endothelial cells. *In Vitro*. 19:966-980.
 33. Skubitz, A. P. N., A. S. Charonis, E. C. Tsilibary, and L. T. Furcht. 1987. Localization of a tumor cell adhesion domain of laminin by a monoclonal antibody. *Exp. Cell Res.* 173:349-360.
 34. Skubitz, A. P. N., J. B. McCarthy, A. S. Charonis, and L. T. Furcht. 1988. Localization of three distinct heparin-binding domains of laminin by monoclonal antibodies. *J. Biol. Chem.* 263:4861-4868.
 35. Stow, J. L., and M. G. Farquhar. 1987. Distinctive populations of basement membrane and cell membrane heparan sulfate proteoglycans are produced by cultured cell lines. *J. Cell Biol.* 105:529-539.
 36. Suzuki, S., A. Oldberg, E. G. Hayman, M. D. Pierschbacher, and E. Ruoslahti. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2519-2524.
 37. Tarr, G. E. 1986. Manual Edman Sequencing System (Chapter 6). In *Methods of protein microcharacterization*. J. E. Shively, editor. Humana Press. Clifton, New Jersey. 155-194.
 38. Terranova, V. P., C. N. Rao, T. Kalebic, I. M. Margulies, and L. A. Liotta. 1983. Laminin receptor on human breast carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:444-448.
 39. Timpl, R., and M. Dziadek. 1986. Structure, development and molecular pathology of basement membranes. *Int. Rev. Exper. Path.* 29:1-112.
 40. Timpl, R., S. Johansson, V. van Delden, I. Ober-Baumer, and M. Höök. 1983. Characterization of protease resistant fragments of laminin mediating attachment and spreading of rat hepatocytes. *J. Biol. Chem.* 258:8922-8927.
 41. Timpl, R., H. Rohde, P. G. Robey, J. M. Foidart, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933-9937.
 42. Yurchenco, P. D., E. C. Tsilibary, A. S. Charonis, and H. Furthmayr. 1985. Laminin polymerization in vitro. Evidence for a two-step assembly with domain specificity. *J. Biol. Chem.* 260:7636-7644.