

Laminin—A Glycoprotein from Basement Membranes*

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We have isolated a large noncollagenous glycoprotein, laminin, from a mouse tumor that produces basement membrane. The protein consists of at least two polypeptide chains ($M_r = 220,000$ and $M_r = 440,000$) joined to each other by disulfide bonds. Laminin and type IV collagen are major constituents of the tumor. Laminin is distinctly different from fibronectin, another component of basement membranes, in amino acid composition and immunological reactivity. Pepsin digestion of laminin releases a large, cystine-rich fragment which retains most of the antigenicity of the original protein. Immunological studies using purified antibody against laminin show that it is produced by a variety of cultured cells. In addition, these antibodies react with the basement membranes of normal tissues, suggesting that this protein or an immunologically related protein is a constituent of the basement membranes of these tissues.

Basement membranes are extracellular structures containing both collagenous and noncollagenous glycoproteins. The collagenous component is known as type IV collagen and is found only in basement membranes. The characterization of this collagen is still in progress but it appears to resemble the procollagens of interstitial connective tissue. The characterization of the noncollagenous glycoproteins has been attempted, but has been hampered by the low solubility of these components as well as by difficulties in obtaining significant quantities of basement membranes for chemical studies (1, 2). However, the current model for basement membrane proposes that the noncollagenous glycoproteins interact with the collagenous proteins to generate this extracellular matrix (2). Kefalides (2) noted earlier that glycoproteins, including some larger than a $M_r = 200,000$, could be extracted from glomerular basement membranes with 8 M urea. These may have included laminin, although this has not been shown.

We recently have described (3) a transplantable mouse tumor, the EHS sarcoma, which produces an extracellular matrix of basement membrane. The tumor, exclusive of its capsule, is a convenient source for isolating the collagenous component because type IV collagen accounts for 5 to 10% of the tumor protein (4, 5). Very little type IV collagen is extracted from the tumor with neutral buffers but it is extracted with dilute acetic acid from tumors grown in lathyritic mice. Neutral buffers, however, extract substantial amounts of non-

collagenous proteins. A major constituent of these extracts was isolated and found to be a high molecular weight noncollagenous glycoprotein. This protein, which we have named laminin, is a major component of the tumor matrix and has been localized to basement membranes in normal tissues by immunological techniques.

EXPERIMENTAL PROCEDURES

Purification of Proteins—The tumor was grown either subcutaneously or intramuscularly in normal or lathyritic mice (C57Bl strain) essentially as described before (3). When the tumors approached 2 to 4 cm in diameter, they were harvested, homogenized in 3.4 M NaCl, 0.05 M Tris-HCl, pH 7.4, at 4°C and centrifuged to remove serum and cellular proteins. The insoluble residue was then homogenized in a 10- to 20-fold volume (w/v) of 0.5 M NaCl, 0.05 M Tris-HCl, pH 7.4, and extracted with stirring two times overnight at 4°C. All extractions were carried out in the presence of the protease inhibitors *p*-hydroxymercuribenzoate (50 $\mu\text{g/ml}$) and phenylmethanesulfonyl fluoride (50 $\mu\text{g/ml}$). The 0.5 M NaCl extract was found to contain laminin and other glycoproteins and only small amounts of collagenous protein as estimated from the hydroxyproline content when analyzed.

The small amounts of type IV collagen in the extract were removed from these solutions by increasing the salt content to 1.7 M and centrifuging the solution in a Sorvall centrifuge at 10,000 rpm. About 200 to 400 ml of the supernatant fluid containing laminin was dialyzed against 2 M urea, 0.05 M Tris-HCl, pH 8.6, and passed over a column of DEAE-cellulose (2.5 \times 25 cm) equilibrated with the same solvent at 4° or 15°C. Some 50 to 60% of the proteins in the solution, including fibronectin, are bound to the DEAE-cellulose under these conditions. Unbound material was found to include laminin. For molecular sieve chromatography, this pool was concentrated by ultrafiltration (Amicon, Diaflo, filter XM 100A) with a slight loss of material due to precipitation of the protein on the filter. The concentrated sample was passed over an agarose A-1.5 m column (3.5 \times 140 cm) equilibrated, and eluted with 1 M CaCl₂, 0.05 M Tris-HCl, pH 7.4. Laminin appeared in the void volume of the column and was either lyophilized following dialysis against 0.05% acetic acid or was dialyzed against 0.4 M NaCl, 0.05 M Tris-HCl, pH 7.4, concentrated by ultrafiltration to about 1 mg/ml, and stored at 4°C. In general, higher aggregates barely penetrating a 3.5% acrylamide gel on electrophoresis were observed in lyophilized preparations of the protein.

Type IV collagen was purified from an acetic acid extract of the mouse tumor as described previously (4). Human α_2 -macroglobulin and plasma fibronectin were a gift of Dr. U. Becker, Behringwerke, Marburg, Germany. For the isolation of fibronectin from normal mouse serum, the serum was passed over a column of collagen $\alpha 1(I)$ chain coupled to Sepharose and eluted with 0.2 M arginine-HCl (6).

Chemical and Protease Treatment Modifications—Proteins were reduced with 0.02 M dithioerythritol in 8 M urea and alkylated with ethyleneimine (7). Reagents were usually removed by dialysis against 0.05% acetic acid or 0.2 M ammonium bicarbonate, pH 7.9. For digestion with pepsin, a solution of laminin (0.5 to 1 mg/ml) was dialyzed in the cold against 10% acetic acid, pH 2.5. After adding pepsin at an enzyme/substrate ratio of 1:50, the mixture was incubated for 24 h at 15°C and the reaction terminated by lyophilization.

Analytical Methods—Amino acid composition was determined on a Durrum D-500 analyzer after hydrolyzing the samples in 6 N HCl

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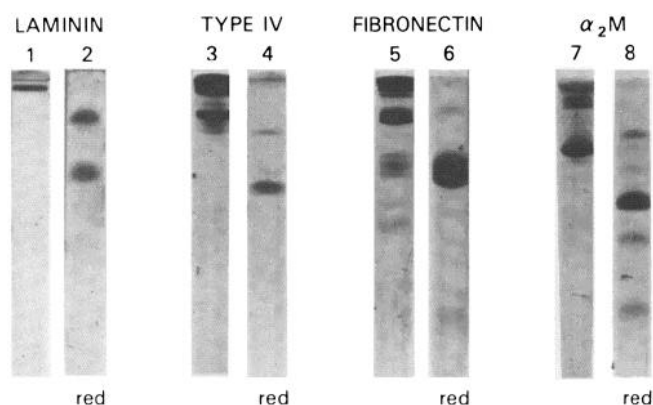


FIG. 1. SDS-polyacrylamide gel electrophoresis. Laminin, Type IV collagen, fibronectin, and α_2 -macroglobulin (α_2 M) were electrophoresed on 3.5% acrylamide gels in the presence of 2% SDS. Samples of these proteins electrophoresed without reduction of disulfide bonds are pictured in the odd-numbered gels, and samples that were reduced and alkylated prior to electrophoresis are pictured in the even-numbered gels.

for 24 h at 110°C under N_2 . The values obtained for threonine and serine were multiplied by the factors 1.08 and 1.21, respectively, to adjust for their degradation during acid hydrolysis.

Polyacrylamide gel electrophoresis followed a previously described procedure (8). Usually, the concentration of sodium dodecyl sulfate was increased to 2% in the gels, which gave narrower bands.

Immunological Methods—Antisera against laminin were prepared in rabbits by subcutaneous injection of 1 mg of protein dissolved in 0.5 ml of 0.05 M Tris-HCl, pH 7.4, in 0.4 M NaCl, and mixed with an equal volume of complete Freund's adjuvant. A similar injection was made 4 weeks later and the animals were bled 3 to 5 weeks after the second injection. Antibodies were purified from the antisera by affinity chromatography (9) on an adsorbent of antigen bound to cyanogen bromide-activated Sepharose 4B (10) and cross-adsorbed on a type IV collagen affinity column. Purified rabbit antibodies to human fibronectin were those used in a previous study (11).

Antigens were labeled with ^{125}I (0.5 mCi/25 μ g of antigen) using the chloramine-T method (12). The binding of labeled antigen to antibody and the inhibition of this reaction by nonlabeled antigen was determined as described previously (13). These reactions were carried out in phosphate-buffered saline, pH 7.2, containing 0.04% Tween 20 to reduce nonspecific binding of labeled antigen below 10% (14).

The extent to which antibodies prepared to purified laminin or fibronectin reacted with each protein was measured by ELISA.¹ Purified goat antirabbit immunoglobulin (Miles Laboratories) was conjugated with alkaline phosphatase or horseradish peroxidase. Flat-bottomed microtiter plates (Cooke Laboratory) were coated with 100 or 200 ng of purified antigen as previously described (15). Various amounts of fibronectin or laminin were incubated with different dilutions of affinity-purified antibody for 2 h at room temperature in a volume of 0.22 ml. Subsequently, 0.2 ml of each sample was transferred to an antigen-coated well and incubated for an additional 2 h. Goat anti-rabbit immunoglobulin with covalently attached enzyme (either peroxidase or alkaline phosphatase) was added, incubated for 2.5 h, and then washed. The appropriate substrate (Sigma substrate No. 104, Sigma Chemical Co., or *o*-phenylenediamine) was added to each plate for a timed interval. The reaction was terminated with 0.05 ml of 8 N H_2SO_4 or 3 N NaOH and the optical density of the supernatant fluid was measured.

Quantitation of Laminin and Type IV Collagen—Individual tumors (6 to 14 g wet of weight) were harvested from nonlathyratic mice and, after removal of the capsules, were extracted with 3.4 M NaCl followed by 0.5 M NaCl as described above. The residue was further extracted with 0.5 M acetic acid (4) followed by pepsin digestion in 0.5 M acetic acid (5) and reduction and alkylation in 8 M urea (see above). Prior to analysis, the extracts, except the pepsin digest, were dialyzed against 0.2 M ammonium bicarbonate, pH 7.9, to remove free amino

acids and reagents. Total protein was determined in hydrolyzed aliquots of the extracts with an amino acid analyzer. Type IV collagen was determined from the content of 4-hydroxyproline and hydroxylysine assuming the composition of purified acid-soluble type IV collagen reported previously (4). All the extracts, including a hydrolysate prepared from the whole tumor, showed a ratio of 4-hydroxyproline to hydroxylysine close to 0.40, which is the ratio found in soluble type IV collagen (4). The content of laminin was determined by radioimmunoassay using native laminin or a pepsin digest of laminin as reference inhibitors. The content of laminin in the extract prepared by reduction and alkylation was determined using labeled and nonlabeled reduced laminin as test antigen and reference inhibitor, respectively.

RESULTS

Attention was first directed to laminin when neutral salt extracts of the EHS sarcoma produced precipitates when warmed to room temperature. Examination of lyophilized material by electrophoresis showed that the material barely penetrated the gel until disulfide bonds were reduced. The purification of type IV collagen was modified so that contaminating protein could be removed and laminin isolated in a nonaggregating state. The current procedure for isolating this

TABLE I
Amino acid composition

	Laminin	Laminin P1	Human ^a fi-bronectin	Chick ^b fi-bronectin	Type IV ^c collagen
Half-Cys	30	35	26	18	7
3-Hyp					6
4-Hyp					104
Asp	109	113	92	94	53
Thr	58	56	97	100	34
Ser	77	81	68	74	57
Glu	122	106	116	118	102
Pro	53	63	76	77	65
Gly	93	118	80	105	280
Ala	76	47	43	48	44
Val	48	40	81	66	20
Met	14	6	11	13	13
Ile	42	20	44	37	25
Leu	92	54	57	55	52
Tyr	27	28	45	37	9
Phe	31	30	27	19	29
His	24	33	21	16	11
Hyl	2	2			37
Lys	52	30	36	34	11
Arg	50	38	52	53	32
Trp	n.d. ^d	n.d. ^d	28	n.d. ^d	n.d. ^d
Total	1000	1000	1000	1000	1000

^a From Ref. 18.

^b From Ref. 19.

^c Ref. 4.

^d n.d., Not determined.

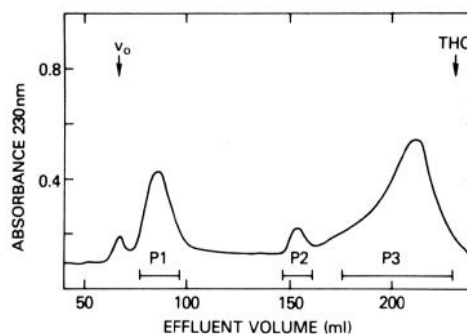


FIG. 2. Separation of a pepsin digest of laminin on an Agrose A-1.5 column (1.8 \times 120 cm) equilibrated in 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 7.4. THO denotes the elution position of tritiated water and V_0 , the void volume of the column.

¹ The abbreviation used is: ELISA, enzyme linked immuno sorbent assay.

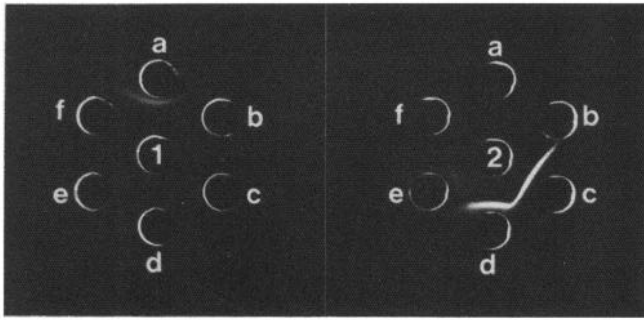


FIG. 3. Immunodiffusion of rabbit anti-laminin (1) and rabbit antifibronectin (2). Antibodies raised against laminin (1) react with purified laminin in the native state (a) but not when the material is reduced (b). No reaction was observed with human fibronectin in either the reduced (c) or native (d) state. In addition, no reaction was observed with whole mouse serum (e) or with isolated type IV collagen (f). Antibodies raised against fibronectin reacted positively with fibronectin in both the reduced (c) and native (d) state. In addition, partial reaction with whole mouse serum was observed indicating species cross-reactivity between human and mouse fibronectin (21).

protein involves removing serum and cellular proteins with high NaCl washes. Laminin is extracted from the tumor with 0.5 M NaCl at neutral pH. Traces of type IV collagen in the extract are removed by precipitation with 1.7 M NaCl. Passage of the extract over DEAE-cellulose separates fibronectin, which binds to the column, from 50 to 70% of the laminin which does not bind to the column. An apparently homogeneous protein is obtained by molecular sieve chromatography. Laminin migrates essentially as a single high molecular weight band on sodium dodecyl sulfate electrophoresis (Fig. 1). When reduced and alkylated, two bands are observed. The faster moving band has a molecular weight of approximately 220,000, whereas the slower moving band appears to be about twice that size. Lyophilized samples appear to contain higher aggregates than material that is stored in solution. Fibronectin from human serum migrates after reduction almost entirely as a $M_r = 220,000$ component (16). Reduced type IV collagen contains two slightly faster migrating bands which are not well resolved in the gels used here and, in addition, some oligomeric components. The components resolved by the electrophoresis of α_2 -macroglobulin (17) are also distinct from laminin both before ($M_r = 340,000$) and after ($M_r = 185,000$) reduction and alkylation.

The amino acid composition of laminin is shown in Table I along with the compositions obtained from type IV tumor collagen (4) and fibronectin from chick fibroblasts (18) and human serum (19). Laminin lacks both 3- and 4-hydroxyproline, but contains a small amount of hydroxylysine possibly representing a small amount of contamination with type IV collagen. The compositions of type IV collagen and laminin are strikingly different. The compositions of laminin and fibronectin also differ significantly, particularly in threonine, proline, alanine, valine, leucine, and lysine contents. Laminin is a glycoprotein, since it is stained intensely after electrophoresis by the periodic acid-Schiff procedure (not shown).

Treatment of laminin with pepsin produced several fragments which could be partially separated on agarose A-1.5 (Fig. 2). A large fragment, P1, which accounts for 25% of the protein in the digest, appeared shortly after the void volume of the column and migrated on electrophoresis as a broad single band with a mobility intermediate between those of the two bands of reduced laminin. The fragment, P1, showed a composition similar to that of laminin except that it contained 4 times as much cysteine and less hydrophobic amino acids (Table I). Similar large fragments rich in cysteine can also be

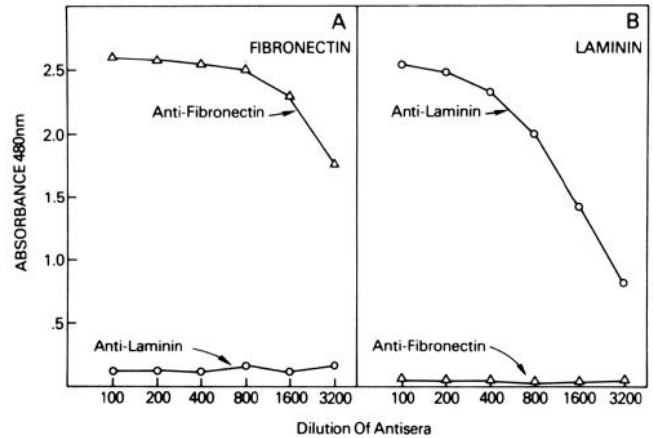


FIG. 4. Binding of purified antibodies to fibronectin and laminin in the ELISA assay. The ELISA assay was performed as described in the text. Antilaminin does not bind to fibronectin-coated wells at any dilution of antisera (A). Conversely, antifibronectin does not bind to laminin-coated wells (B).

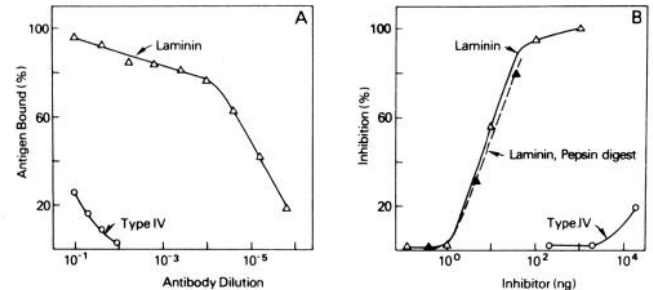


FIG. 5. Reaction of purified antibodies to laminin in radioimmunoassay. A, Binding of ^{125}I -labeled laminin and type IV collagen. B, Inhibition of the reaction with labeled laminin. Inhibitors were laminin (Δ — Δ), pepsin-digested laminin (\blacktriangle — \blacktriangle) and type IV collagen (\circ — \circ). An inhibition curve resembling those of the whole pepsin digest was observed with the purified laminin fragment P1.

prepared from laminin by treatment with trypsin or cyanogen bromide.² This indicates that almost all of the disulfide bonds are located in a segment comprising about one quarter of the original protein.

Further evidence that laminin is distinct from fibronectin was obtained by comparing the reaction of these proteins with antibodies prepared against each one. As shown in Fig. 3, a single precipitin line was observed between laminin and antibody to laminin. No reaction occurred between this antiserum and purified fibronectin or with serum. Antibody to fibronectin produced a single precipitin band with fibronectin and a line of partial identity with serum. The reaction with serum is expected since serum contains cold-insoluble globulin which is closely related or identical to fibronectin. This could be demonstrated by binding the mouse serum antigen to a collagen adsorbent and displacement of the antigen from the adsorbent by arginine (6).

The antibodies prepared against laminin and fibronectin were tested against these purified proteins by ELISA assays (Fig. 4). Little or no cross-reaction was observed. Purified antibody to laminin reacted with laminin but not fibronectin, and the antibody to fibronectin reacted with this protein and not the other. Possible reactions between antibody to type IV collagen and laminin were also studied by radioimmunoassay (Fig. 5). These studies showed that little or no cross-reaction occurred between these proteins and the antibodies prepared to them, providing that the antibodies were purified by affinity

² H. Rohde and R. Timpl, unpublished results.

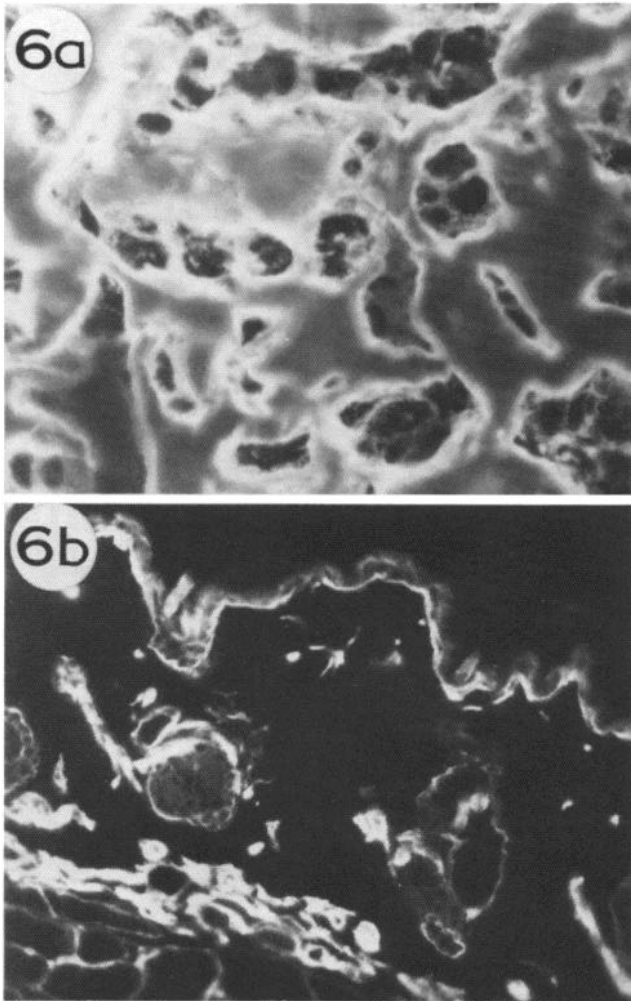


FIG. 6. Staining of histological sections of the EHS sarcoma (A) and mouse skin (B) with rabbit antibodies to laminin. The antibodies were purified as described in "Experimental Procedures" and used in indirect immunofluorescence. The tumor matrix stains intensely with these antibodies whereas the cells do not. The basement membranes of the dermis, at the epidermal-dermal junction, around glands and small capillaries, and in deeper layers around muscle bundles, are also stained by these antibodies, indicating that laminin is a component of these structures.

chromatography. Pepsin treatment of laminin did not destroy its antigenicity (Fig. 5) and most of the activity was retained in the large fragment P1, (not shown).

The distribution of laminin in the tumor was investigated using immunofluorescence techniques. As shown in Fig. 6A, the tumor matrix stained intensely when exposed to antibody to laminin while the cells in the tumor showed a much less intense stain. In contrast to the pattern of staining with antibodies against type IV collagen (4), antilaminin antibodies did not stain the tumor matrix evenly. Rather, those portions of the matrix closest to the cells stained more intensely than the more distant portions. It is possible that laminin is concentrated in this region or that the protein is present throughout the matrix but masked to the antibody by other proteins in the central portions of the matrix.

The possibility that laminin occurred in normal tissue was also investigated by immunofluorescence. Intense staining of authentic basement membranes was observed in sections of skin (Fig. 6B), kidney, and placenta. These studies suggest that authentic basement membranes contain laminin or an immunologically related protein.

TABLE II
Quantitation of protein, laminin, and type IV collagen in various extracts of the EHS sarcoma grown in nonlathyritic mice

Extract	Total protein ^a	Type IV ^a collagen	Laminin ^b
(mg/g wet weight; mean \pm S.D.)			
3.4 M NaCl	16.9 ^c	0.05 ^c	0.04 ^c
0.5 M NaCl	15.1 \pm 2.8	0.23 \pm 0.06	10.6 \pm 1.4
0.5 M acetic acid	1.4 \pm 0.8	0.21 \pm 0.13	0.08 \pm 0.05
Acetic acid and pepsin	10.4 \pm 0.8	3.0 \pm 0.5	1.6 \pm 1.2
0.02 M Dithioerythritol in 8 M urea	4.2 \pm 0.3	1.0 \pm 0.1	0.05 \pm 0.03
Insoluble	0.3 \pm 0.1	<0.01	n.d. ^e
Total tumor	64.0 \pm 10.1	4.9 \pm 1.0	n.d.
Sum column 1-6	48.3	4.4	12.4
Sum column 2-6 ^d	31.4	4.4	12.3

^a By amino acid analysis.

^b By radioimmunoassay.

^c Single tumor.

^d Considered to resemble mainly the extracellular matrix of the tumor.

^e n.d., Not defined.

The amounts of laminin and of type IV collagen in various extracts of the tumor were estimated by immunological assay (laminin) and amino acid analysis (collagen) (Table II). Most of the laminin was removed by the 0.5 M NaCl extract of the tumor. Digestion of the matrix with pepsin, or denaturing agents used with reducing agents, dissolved the largest amount of type IV collagen. The total amount of laminin present in the tumor was greater than the amount of type IV collagen. However, different methods were used to estimate these proteins and the results may not be exactly comparable. However, it is likely that roughly similar levels of these proteins occur in the matrix of the tumor.

The production of laminin and type IV collagen by various cell lines was also studied by indirect immunofluorescence and ELISA assays. Fibroblasts, including CHO and strains from human skin, were not found to produce either protein. In contrast, certain epithelial cell lines including 3T3, PYS and rat breast epithelium, corneal epithelium, liver cell line ARL6, and myoblasts produced both laminin and type IV collagen.

DISCUSSION

We have been studying the matrix proteins extracted from a tumor, the EHS sarcoma, which produces a matrix of basement membrane. Previously, we have extracted a collagenous protein from the tumor which resembles in composition the collagens present in authentic basement membranes of normal tissues. Antibody prepared against this collagen labeled the basement membranes present in normal tissues suggesting that they contain a similar or identical protein (4, 5).

Here, we report isolating a second protein from the tumor which also appears to be a component of the tumor matrix. This protein, which we have named laminin, is a high molecular weight protein joined with disulfide bonds. After reduction of disulfide bonds, we observe two components estimated to have $M_r = 220,000$ and $M_r = 440,000$. It is not known if these components are related as, for example, monomer and dimer. If they are monomer-dimer they are joined by a covalent bond other than a disulfide bond. The unreduced component migrates with the characteristics expected for a tetramer of the $M_r = 220,000$ chain or of a molecule composed of

one or two $M_r = 220,000$ and an $M_r = 440,000$ component.

The purification scheme for laminin allows ready separation from other components of the tumor, particularly the collagenous protein and fibronectin. Quantitation of laminin in complex mixtures was achieved by immunological assays using either radioimmunoassay or ELISA. In these assays, it was found necessary to purify the antibody by affinity columns prepared from the immunizing antigen to remove contaminating antibodies. Both laminin and type IV collagen are highly antigenic and traces of laminin are often present in preparations of type IV collagen. No cross-reaction was observed between laminin, fibronectin, and type IV collagen using purified antibodies to these proteins.

Antibody to laminin was used in indirect immunofluorescence studies to localize the protein. As expected, the tumor matrix, particularly those regions closest to the cells, stained intensely for laminin. In addition, the antibody to laminin stained a variety of basement membranes in normal tissues including those in placenta, kidney, and skin. Further studies are needed at the level of resolution of the electron microscope to localize the protein to one or another region of the basement membrane. However, the staining of authentic basement membrane with antibody to laminin indicates that laminin or an immunologically related protein is a component of the basement membranes of normal tissues.

Several cell lines were found to synthesize laminin. These lines included PYS and FCL, which grow *in vivo* into tumors with a basement membrane matrix, and 3T3, an apparently normal cell strain. Fibroblasts did not produce detectable amounts of laminin. GP-2, a recently described glycoprotein isolated from a teratocarcinoma by preparative electrophoresis under denaturing conditions (20), appears to be a subunit of laminin, as determined by ELISA.

At present, it is not clear what role laminin plays in basement membranes. However, where we have measured type IV collagen and laminin we have found them to occur in similar amounts. If indeed laminin is located along with type IV collagen, it may be that it fulfills a structural role.

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