

Drosophila Laminin: Characterization and Localization

L. I. Fessler, A. G. Campbell, K. G. Duncan, and J. H. Fessler

Molecular Biology Institute and Department of Biology,
University of California at Los Angeles, Los Angeles, California 90024-1570

Abstract. *Drosophila* laminin was isolated from the medium of *Drosophila* K_c cell cultures. It was purified by velocity sedimentation, gel filtration, and chromatography. *Drosophila* laminin is a disulfide-linked molecule consisting of three chains with apparent molecular masses of 400, 215, and 185 kD. In electron micrographs, it has the cross-shaped appearance with globular domains characteristic of vertebrate laminin with closely similar dimensions. The amino acid composition and lectin-binding properties of *Drosophila* laminin are given.

Polyclonal antibodies to *Drosophila* laminin were prepared and their specificity was established. In developing embryos immunofluorescence staining was

detected between 6 and 8 h of development; and in sections of 8–9-h and older embryos immunostaining was seen at sites where basement membranes are present surrounding internal organs, muscles, underlying the hypodermal epithelium, and in the nervous system. Basement membrane staining was also seen in larva and adults. Cells from *Drosophila* embryos dissociated at the cellular blastoderm stage were grown in culture and some specific, differentiated cells synthesized laminin after several hours of culture as shown by immunofluorescence. The significance of the evolutionary conservation of the structure of this basement membrane component is discussed.

THE extracellular protein laminin is a component of vertebrate basement membranes (7, 44). It is a multidomain protein which facilitates growth and migration of cultured cells (22, 23, 42, 43) and promotes the extension of growth cones by neurites (4, 12, 15, 19, 25). The fly *Drosophila melanogaster* provides a unique experimental system for investigating the control of expression and functioning of gene products during development. To learn more about the assembly of basement membranes, and the roles of these structures in development, we initiated a search for their component materials in *Drosophila* (18). Here we report the isolation and characterization of a molecule with properties of a *Drosophila* laminin, and the production of antibodies which enable us to study the early appearance and distribution of this material during *Drosophila* embryogenesis.

Molecules of vertebrate laminin have a characteristic, unique appearance of a cross in the electron microscope after spraying onto a flat surface and rotary metal shadowing (14). After reduction, three polypeptide chains have been obtained from vertebrate laminin: A, B1, and B2. The glycosylated chains migrate in SDS-PAGE with apparent molecular masses of 400, 220, and 210 kD, respectively. A model of the molecule has been suggested in which the carboxyl portions of one copy of each chain participate in a coiled-coil α -helix in the long arm of the cross, while each short arm of the cross is made up of only one chain. The model is supported by biophysical measurements and a rapidly progressing knowledge of the amino acid and nucleotide coding sequences of the three chains (3, 32, 38, 48).

The primary sources of vertebrate laminin have been ex-

tra-embryonic tissues, especially Reichert's membrane, parietal endoderm cells, and tumors derived from them (21, 31, 44). Antibodies made against mouse laminin stain basement membranes of various vertebrates, suggesting conservation of antigenic determinants. However, such antibodies either did not stain *Drosophila* tissues and cell cultures, or did so only extremely weakly. As we had found that several *Drosophila* cell lines secrete a basement membrane collagen (18, 28), we searched for *Drosophila* laminin in conditioned cell culture media.

Materials and Methods

Cell Culture Conditions

The *Drosophila melanogaster* K_c cell line, originally derived by Echaliier and Ohanessian (10), was maintained as spinner cultures in D-20 medium (11) at 22°C. The maximum cell density of these cultures was $\sim 7 \times 10^6$ cells per ml. The cells were transferred to roller bottles at a density of $\sim 3 \times 10^6$ cells per ml and were grown to a density of $\sim 1 \times 10^7$ cells per ml in D-20 medium. For radioisotope labeling, the K_c cells were sedimented gently in a centrifuge (International Equipment Co., Boston, MA) at 1,200 rpm for 2 min, washed twice with phosphate-buffered salt solution (containing 1.7 mM CaCl₂ and 6.4 mM MgCl₂) and suspended in DME supplemented with 50 μ g/ml ascorbic acid and devoid of the amino acids added as label. The cultures were labeled with 12.5 μ Ci/ml each of [³H]5-proline and [³H]2,3-leucine (Schwarz-Mann, Boston, MA) or [³⁵S]sulfate (New England Nuclear, Boston, MA) for 20 h.

Isolation and Purification of Laminin

The medium was clarified by centrifugation in a Sorvall G3 rotor (DuPont Co., Wilmington, DE) at 5,000 rpm for 15 min, and the following inhibitors

were added: 20 mM EDTA, 5 mM *N*-ethylmaleimide, and 0.5 mM PMSF, and the pH was adjusted to pH 6.5 to 7. The proteins were precipitated at 4°C with $(\text{NH}_4)_2\text{SO}_4$ at 45% saturation, and the precipitate was collected in the Sorvall G3 rotor at 8,000 rpm for 30 min. The precipitate was dissolved in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 (buffer A) containing 20 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 mM PMSF or diisofluorophosphate. The sample was dialyzed against buffer A and clarified in the Sorvall SS34 rotor for 30 min at 10,000 rpm at 4°C.

Velocity Sedimentation and Electrophoretic Analysis

The mixed proteins were separated on a 5–20% sucrose gradient containing buffer A with a 60% sucrose pad in an SW60 or SW41 rotor (Beckman Instruments, Palo Alto, CA) at 4°C. Purified molecules were sedimented on 5–20% sucrose gradients containing buffer A with 2 M urea and 0.1% Triton X-100, pH 7.5, and the sedimentation coefficients were calculated as described (16). Fractions were collected and electrophoretically analyzed on SDS polyacrylamide slab gels (24) or on 1.5% agarose gels. The agarose (type I; Sigma Chemical Co., St. Louis, MO) was dissolved in 0.19 M Tris-HCl, pH 8.8, and 0.25% SDS and 100 ml were used to cast a 12.5×15 cm gel. For electrophoresis, the horizontal slab gel was submerged in 0.19 M Tris-glycine, pH 8.3, and 0.2% SDS. The gels were stained with Coomassie Blue dye. A mixture of rabbit myosin and its oligomers were used as high molecular mass markers (gifts from Dr. E. Reisler, University of California at Los Angeles). Other molecular weight markers—myosin, phosphorylase B, BSA, and ovalbumin—were purchased from Bethesda Research Laboratories (Bethesda, MD). Mouse laminin was prepared from the medium of Pf-HR9 cell cultures as described (2).

Gel Filtration

Separation of proteins on an A-50 m agarose (100–200 mesh, Bio-Rad Laboratories) column $47 \text{ cm} \times 1.5 \text{ cm}$ was carried out at 4°C using 1 M urea, 0.3 M sucrose, 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 7.5 buffer. The separations were monitored by electrophoresis of the proteins, either nonreduced or reduced, on SDS 4.5% polyacrylamide slab gels, followed by Coomassie Blue staining and densitometric measurement using a Quick Scan R & D densitometer (Helena Laboratories, Beaumont, TX).

DEAE-cellulose (Whatman DE52) Chromatography

DEAE-cellulose chromatography was carried out at 4°C using 2 M urea, 0.03 M Tris-HCl, 0.1% Triton X-100, pH 7.8 buffer, and a linear NaCl gradient (0–0.5 M) to elute the proteins.

Phenylboronate Agarose Matrix Gel

Samples were applied to phenylboronate agarose matrix gel (PBA-10; Amicon Corp., Danvers, MA) at 4°C in 0.02 M HEPES, 0.15 M NaCl, 0.05% Triton X-100, pH 8.0 buffer, and eluted with 1 M urea in 0.1 M Tris-HCl, pH 7.2, 0.15 M NaCl, 0.1% Triton X-100, 0.1 M sorbitol buffer.

Heparin-Sepharose Column Absorption

Laminin dissolved in PBS (pH 7.4), and 0.1% Triton X-100 was first passed over Sepharose C1-4B (Pharmacia Fine Chemicals, Piscataway, NJ) and then over heparin-Sepharose (Pharmacia Fine Chemicals) at 4°C, and the bound protein was eluted with 1 M NaCl in the above buffer.

Binding to Lectin Column

Bandeirea simplicifolia B₄ isolectin immobilized on Sepharose 4B was kindly given to us by Dr. I. J. Goldstein. Unlabeled *Drosophila* laminin and [³H]leucine-labeled mouse laminin dissolved in 0.3 M NaCl, 0.1 mM CaCl₂, 0.1 M sodium phosphate buffer, pH 7.2, 0.02% NaN₃ were passed over the columns and bound material was eluted with 10 mg/ml 1-O-methyl- α -D-galactopyranoside (Sigma Chemical Co.) (35, 41). Aliquots of the effluent and eluate fractions were counted for radioactivity and analyzed by SDS 4.5% PAGE, stained with Coomassie Blue, and quantitated by densitometry. *Drosophila* laminin in PBS bound to wheat germ agglutinin-Sepharose (Sigma Chemical Co.). The bound laminin could be eluted with 0.07% SDS in PBS, but not with 0.5 M *N*-acetylglucosamine (Sigma Chemical Co.) plus 1% Triton X-100 or 1% deoxycholate.

Electron Microscopy

Electron microscopy samples were sprayed onto mica and rotary shadowed, and measurements were made as described (2).

Amino Acid Analysis

Amino acid analysis of purified laminin was carried out as described (30, 33).

Preparation of Anti-laminin IgG

Laminin purified by velocity sedimentation, gel filtration on A-50 m agarose, and DEAE chromatography was subjected to electrophoresis without reduction on an SDS 4% polyacrylamide slab gel and stained with Coomassie Blue. The laminin band was cut out and used for immunization of rabbits. Antiserum was precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 35% saturation, and the IgG precipitate was dissolved in PBS and dialyzed against this buffer. Purified laminin was linked to CNBr-activated Sepharose C1-4B (Pharmacia Fine Chemicals). The laminin-specific IgG was bound to this column and then eluted with 0.1 M acetic acid, neutralized immediately with NaOH, and dialyzed against PBS. The ELISA was as follows: highly purified laminin ($\sim 10 \mu\text{g}/50 \mu\text{l}$) dissolved in PBS was bound to test plates at 22°C for 12 h, the plates were washed with PBS containing 0.05% Tween, and the antiserum or purified antibodies dissolved in PBS containing 0.05% Tween were added at 7°C for 16 h. After rinsing, Protein A-alkaline phosphatase (Miles Laboratories, Inc., Elkhart, IN) was added for 3 h at 22°C. After rinsing, the phosphatase activity was assayed using *p*-nitrophenylphosphate (Sigma Chemical Co.) dissolved in 0.05 M Tris-HCl, pH 9.2, and 0.01 M MgCl₂. The color reaction was quantitated spectrophotometrically. Antibodies to mouse laminin were a gift from Dr. I. J. Goldstein (University of Michigan, Ann Arbor, MI) and Dr. R. Timpl (Max-Planck-Institute for Biochemistry, Martinsried, FRG). For controls, we used preimmune serum and anti-*Drosophila* laminin serum which had been reacted first with an excess of *Drosophila* laminin bound to microtiter plates.

Western Blot (46)

The proteins were separated by SDS 4.5% PAGE. Representative lanes were stained with Coomassie Blue. The proteins in the unstained gel were blotted onto nitrocellulose at 100 V for 6 h. The nitrocellulose was blocked with 5% FCS (heat inactivated), 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.25% gelatin (Sigma Chemical Co.), 0.05% Triton X-100, and 0.02% Thimerosal (Sigma Chemical Co.) at room temperature for 3 h. The blot was then reacted with antiserum, affinity-purified antibodies, or preimmune serum diluted with the above buffer at 4°C overnight. The blot was washed for 1–2 h with three changes of the above buffer and two changes of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Triton X-100, and then reacted with anti-rabbit IgG linked to horseradish peroxidase 1:1,000 (Cappel Laboratories, Malvern, PA) for 1 h at room temperature, washed as above, and reacted with 0.5 mg/ml 4-chloro-1-Naphthol and 0.025% H₂O₂ in the dark.

Immunofluorescence Staining

After a 3-h precollection period, *Drosophila* embryos were collected from population cages kept at 25°C over 1-h periods and incubation of the embryos was continued at 25°C. The embryos were washed and frozen in Tissue-Tek O.C.T. Compound (Miles Laboratories, Inc.) and frozen rapidly in liquid nitrogen. Sections of 4.5 μm were cut on a cryostat (South London Electrical Equipment Co., London, England). The sections were mounted on freshly prepared polylysine- (Sigma Chemical Co.) coated slides and were fixed with freshly prepared 2% paraformaldehyde in PBS for 2 min. The slides were washed in PBS-2% glycine two times for 5 min and then they were incubated with 0.04 mg/ml hyaluronidase (Serva Fine Biochemicals, Inc., Garden City Park, NY) in PBS for 30 min at 37°C, washed three times with PBS-2% glycine for 5 min, and incubated with 30% goat serum for 20 min at room temperature, and washed twice with PBS-2% glycine for 5 min. The sections were then incubated with the appropriate dilution of antibodies or preimmune serum at 4°C overnight, and washed in PBS-1% goat serum and then PBS-2% glycine three times for 5 min. Then fluorescein-coupled goat anti-rabbit IgG (Miles Laboratories, Inc.) was added for 1 h at room temperature in the dark. The slides were washed three times for 5 min in PBS-2% glycine. The sections were mounted in Gelvitol Poly-

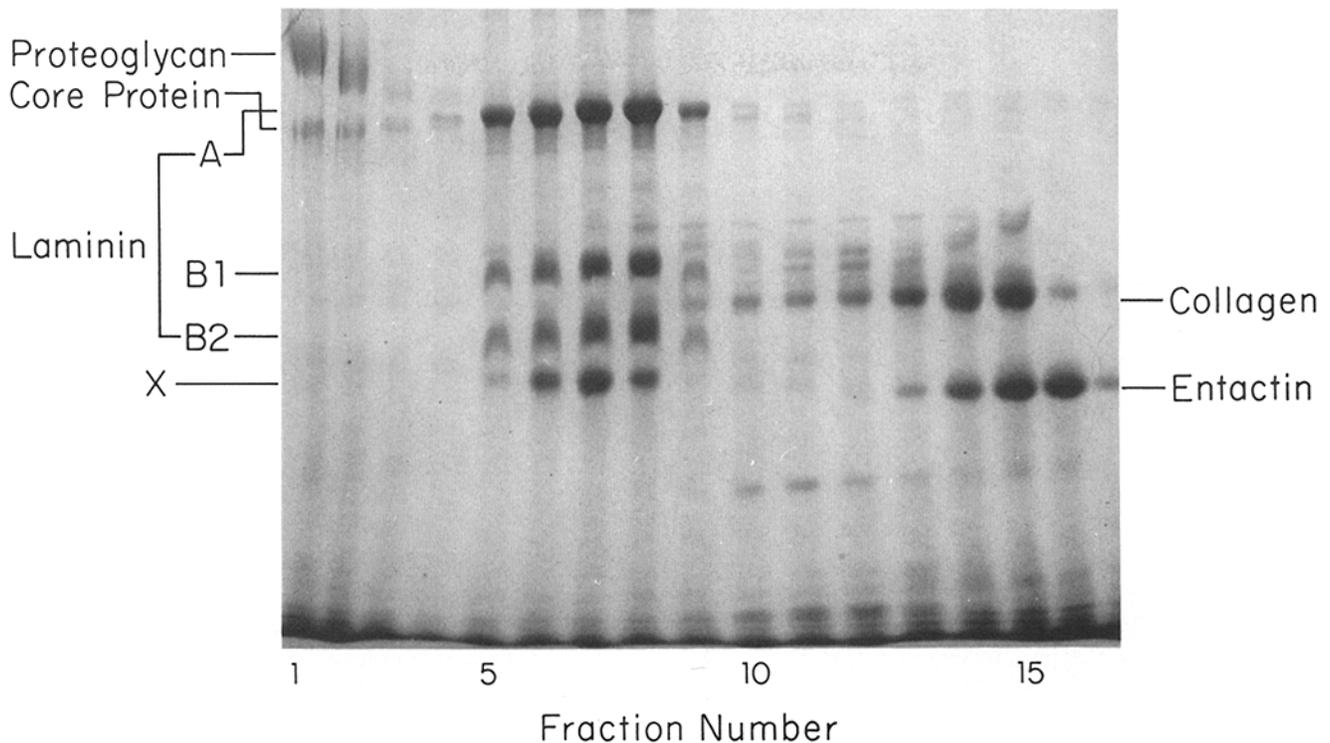


Figure 1. Separation of medium proteins by velocity sedimentation. The proteins precipitated from the culture medium with $(\text{NH}_4)_2\text{SO}_4$ were dissolved in buffer A plus inhibitors, dialyzed against buffer A, and sedimented on a sucrose gradient in an SW41 rotor (Beckman Instruments, Inc.) at 4°C at 38,000 rpm for 21 h. 20 fractions were collected; an aliquot of each fraction was reduced and subjected to electrophoresis on a 4.5% polyacrylamide slab gel. The gel was stained with Coomassie Blue and the electrophoretogram is shown. Sedimentation was from right to left.

vinyl alcohol (Monsanto Co., St. Louis, MO) (37). Whole imaginal discs were isolated from third instar larva, treated with PBS, 0.5% NP-40, 5% FCS, and incubated with primary antibody for 1 h, washed three times with PBS, 0.5% NP-40, and reacted with secondary antibody as above. *Drosophila* primary cell cultures were prepared as described by Seecof (39) and immunofluorescence was developed as described above for sections.

For all these procedures, sections or cells were reacted in parallel with fractions at the same concentrations of immune serum or affinity-purified IgG and preimmune serum from the same rabbit to assure specificity of the immune reactions. Another control included staining with the second antibody alone. In all cases, the preimmune serum and the controls showed only very weak overall nonspecific staining. The sections stained either with immune or preimmune serum or IgG were photographed with the same exposure times, and prints were made under standard conditions. Sections treated with immune serum, as shown in Fig. 8, gave clear images, while sections treated with preimmune serum gave blank prints under these standard conditions.

Results

Drosophila K_c cells were grown to high density in suspension cultures in roller bottles. Several high molecular mass proteins were secreted into the medium. After concentration of the proteins by precipitation with $(\text{NH}_4)_2\text{SO}_4$, native *Drosophila* laminin was initially separated from many of the other components by velocity sedimentation on a sucrose gradient. Aliquots of individual sedimentation fractions were analyzed by SDS-PAGE, either before or after reduction. The electrophoretogram of the reduced proteins is shown in Fig. 1. *Drosophila* laminin gave rise to three pep-

tides on reduction and was a major component of sedimentation fractions 5–8. Proteoglycans sedimented to the bottom of the gradient, while most of the collagen IV and a glycoprotein resembling entactin sedimented more slowly. A disulfide-linked component X, however, cosedimented with laminin, and smaller molecular mass materials were spread throughout the sucrose gradient.¹

When the sedimentation fractions rich in laminin and component X were passed through an agarose A-50 m gel filtration column in 1 M urea, 0.3 M sucrose buffer, the two materials were separated, as is shown in Fig. 2. For comparison, the collagen IV and entactin which sedimented more slowly (fractions 14–16) were also separated on the same column, and their elution position is indicated on the same plot of Fig. 2. Unlabeled *Drosophila* and [^3H]leucine-labeled mouse laminin coeluted from this column. The laminin was then bound to DEAE-cellulose. The column was eluted with an NaCl gradient in 2 M urea buffer and laminin was released as a sharp peak by ~ 0.2 M NaCl (not shown). DEAE-cellulose columns were useful both for concentrating

1. The identification of these proteins has been reported (18) and characterizations are given in separate manuscripts. Proteoglycan: A. G. Campbell, Ph.D. Thesis, (University of California at Los Angeles), and manuscript submitted to *J. Biol. Chem.* Collagen IV: G. Lunstrum, Ph.D. Thesis (University of California at Los Angeles). Blumberg et al. (7); and manuscripts in preparation. Entactin: manuscript in preparation. Component X: A. G. Campbell, Ph.D. Thesis, (University of California at Los Angeles).

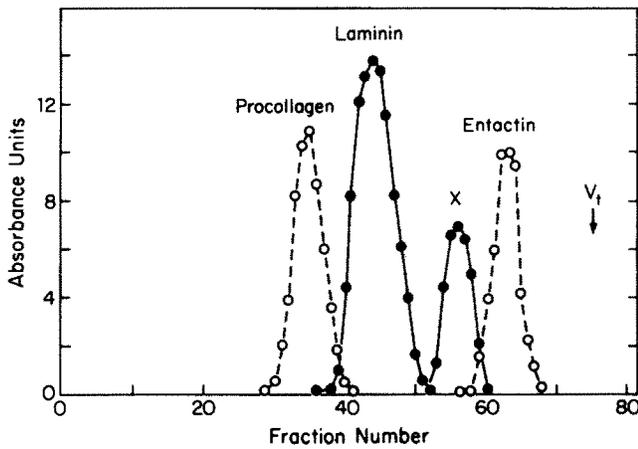


Figure 2. Separation of secreted proteins by gel filtration. A composite of the separation of the components of two sedimentation peaks is shown. Laminin and component X (—●—); procollagen monomers and entactin (- - - - -○- - - - -). The proteins of fractions 6–8 (Fig. 1) were passed over an agarose A-50 m column (1.5 × 47 cm) and 80 1-ml fractions were collected. Then the mixture in fractions 14–16 (Fig. 1) were separated on the same column. Aliquots of each fraction were subjected to electrophoresis and densitometric measurements of the indicated materials were made and the elution profile for procollagen monomers (○), laminin (●), component X (●), and entactin (○) are plotted.

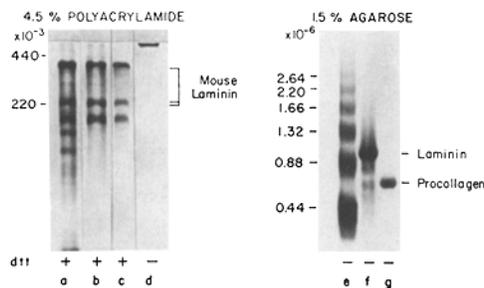


Figure 3. Electrophoretogram of *Drosophila* laminin. (Left) SDS 4.5% polyacrylamide slab gel electrophoretogram. Impure laminin isolated by velocity sedimentation is shown in reduced form in lane *a*. This fraction was passed over an A-50 m agarose column and the peak fractions were pooled and concentrated (Fig. 2, fractions 42–46), and this sample was subjected to electrophoresis in reduced form (lane *b*). This material was then chromatographed on a DEAE-cellulose column and the peak fraction was subjected to electrophoresis in reduced form in lane *c* and nonreduced form in lane *d*. Marker rabbit myosin and reduced mouse laminin were subjected to electrophoresis in adjacent lanes, and the position of these bands is indicated. (Right) SDS 1.5% agarose slab gel electrophoretogram. Nonreduced laminin (partially purified, lane *f*), *Drosophila* collagen (lane *g*), and marker rabbit myosin polymers (lane *e*) were subjected to electrophoresis. The Coomassie Blue-stained gels are shown.

laminin and as a further purification step. However, when the initial mixture of secreted proteins was directly applied to such a column, without prior velocity sedimentation separation, there was poor resolution of most components, except collagen IV.

Fig. 3 shows a comparison of the electrophoretic mobilities of *Drosophila* and mouse laminins. Before reduction, each comigrated as a single electrophoretic band on an SDS 4.5% polyacrylamide gel. Electrophoresis on an SDS 1.5%

Table I. Amino Acid Composition of Laminin

	Residues/1,000	
	<i>Drosophila</i>	Mouse*
Asp	101	109
Thr	60	58
Ser	92	77
Glu	138	122
Pro	41	59
Gly	107	93
Ala	71	76
Cys/2	32	30
Val	48	48
Met	11	14
Ile	28	42
Leu	64	92
Tyr	28	27
Phe	26	31
Lys	45	52
His	28	24
Arg	46	50

* See reference 44.

Drosophila laminin was purified by velocity sedimentation, A-50 m agarose gel filtration, and DEAE-cellulose chromatography. The amino acid analysis was performed by Dr. R. B. Burgeson.

agarose gel, together with a marker ladder of oligomerically linked myosin molecules, showed that before reduction *Drosophila* laminin migrated slightly more slowly than the 880-kD dimer of myosin (Fig. 3, *e* and *f*). The electrophoretic mobilities of the component chains set free by reduction of *Drosophila* and mouse laminins are slightly different (Fig. 3 *c*). The apparent molecular masses of the *Drosophila* laminin chains were 400, 215, and 185 kD relative to the molecular mass standards: myosin, phosphorylase B, and BSA.

When unlabeled *Drosophila* laminin and [³H]leucine-labeled mouse laminin were mixed and then sedimented at 4°C on a buffered sucrose gradient containing 2 M urea, quantitative electrophoretic analysis of the sedimentation fractions showed that *Drosophila* laminin sedimented 1.05 times as fast as mouse laminin. The partial specific volumes of the two materials were assumed to be the same. If this is taken as 0.73 ml/g sedimentation coefficients of 10.2 S resulted for mouse laminin and 10.7 S for *Drosophila* laminin.

The amino acid composition of *Drosophila* and mouse laminin are closely similar, as shown in Table I.

An electron micrograph and diagram of *Drosophila* laminin sprayed unto mica and rotary shadowed is shown in Fig. 4. The measurements of the arms and distribution of the globular domains is not very different from those found for mouse laminin (14). The globular domains on the long arm of *Drosophila* laminin were usually distinct, while mouse laminin frequently showed one curled-over globular domain.

Drosophila laminin is glycosylated (Table II) and bound to phenylboronate agarose affinity columns. All three chains of laminin were stained with the periodic acid Schiff staining procedure. Unlike mouse laminin, *Drosophila* laminin has a very low level of sulfation of these sugar moieties, as was observed when cell cultures were labeled with [³⁵S]sulfate (not shown). The A chain of *Drosophila* laminin was slightly sulfated, and the [³⁵S]sulfate incorporation was very weak

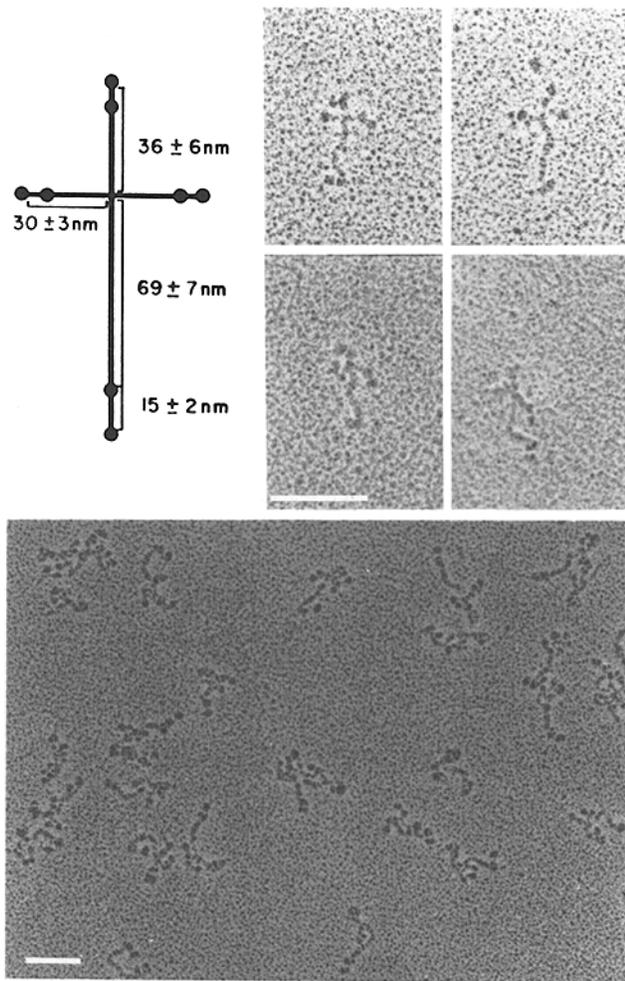


Figure 4. Electron micrograph and diagram and length measurements of the arms of laminin. The electron micrographs show cross-like molecules with four arms as depicted diagrammatically. The molecules were measured, exclusive of the end knobs. The number of molecules examined for each dimension were 42 (long arm) 24, (medium arm) and 43 (short arms). Electron micrographs of individual laminin molecules examined after rotary shadowing and the field of molecules are shown. Bar, 100 nm.

Table II. Binding of Laminin to Different Substrates

	% Unbound	% Bound
Phenylboronate agarose matrix gel		
Drosophila laminin	0	100
Bandeiraea simplicifolia B ₄ isolectin Sepharose		
Drosophila laminin	100	0
Mouse laminin	5	95
Drosophila laminin*	100	0
Mouse laminin*	2	98
Wheat germ agglutinin Sepharose		
Drosophila laminin	0	100
Heparin Sepharose		
Drosophila laminin	0	100

* In a mixture of Drosophila and mouse laminins, Drosophila laminin was present in 10-fold the concentration (~100 µg/ml) compared with mouse laminin (5–10 µg/ml). Drosophila laminin was unlabeled and mouse laminin was labeled with [³H]leucine.

Culture Medium + Protease Inhibitor
 Precipitation of Proteins with (NH₄)₂SO₄ at 45% saturation
 Partial Separation of Proteins on a Sucrose Gradient by Velocity Sedimentation
 Gel Filtration on A-50 m agarose
 DEAE-Cellulose Chromatography
 Heparin-Sepharose Affinity Chromatography
 Electrophoresis on an SDS-Polyacrylamide Gel
 Immunization of Rabbits with Acrylamide Gel Fragments
 Antibody Purification: Laminin-Sepharose Affinity Column

Figure 5. Purification scheme.

as compared with the sulfation of Drosophila entactin or proteoglycan. Mouse laminin has terminal α-D-galactopyranoside groups and binds to Bandeiraea simplicifolia B₄ isolectin immobilized on Sepharose 4B, facilitating purification of mouse laminin (35, 41). Drosophila laminin, however, lacks these groups and did not bind to this lectin. Drosophila laminin bound tightly to wheat germ agglutinin-Sepharose. Elution was achieved only with 0.07% SDS in PBS and not with 0.5 M N-acetylglucosamine plus detergents. Drosophila laminin bound to heparin-Sepharose columns. Since proteoglycan did not bind to this column, traces of this material, which could contaminate laminin, were removed by including a heparin-Sepharose column step in the purification procedure. In the concentrated mixture of secreted proteins, laminin is partially associated with the proteoglycan.

To ascertain that the laminin and the other medium components were secreted, biosynthetic products of the K_c cells, the cultures were incubated in the presence of [³H]proline and [³H]leucine for 20 h and the proteins that had been secreted into the medium were separated by velocity sedimentation and electrophoresis, as above. The most prominently labeled proteins seen on an SDS 4.5% polyacrylamide slab gel were laminin, component X, collagen IV, a high molecular mass proteoglycan and a 155-kD glycoprotein resembling entactin, two unidentified larger proteins, and a number of proteins of lower molecular masses (not shown).

Antibodies were raised in rabbits against Drosophila laminin by injection of nonreduced purified laminin which had been subjected to electrophoresis on an SDS 4% polyacrylamide gel. The scheme for purification of laminin is outlined in Fig. 5. The IgG was affinity purified on a laminin-Sepharose column. The affinity-purified IgG fraction specifically immunoprecipitated native [³H]leucine and [³H]proline-labeled Drosophila laminin, which was shown to consist of A, B1, and B2 chains by electrophoresis of the reduced immune precipitated material. The preimmune serum did not precipitate labeled proteins.

The antibody titer was assayed by ELISA using purified Drosophila laminin as antigen (Fig. 6). The antibodies against Drosophila laminin were removed from the antisera when these were incubated with Drosophila laminin which had been adsorbed on microtiter wells. After this treatment, both ELISAs for laminin and immunostaining of either sections or whole mounts of Drosophila embryos were negative. Some antisera raised against mouse laminin did not cross react with Drosophila laminin, but one anti-mouse laminin antiserum (kindly donated by Dr. R. Timpl) showed weak cross-reactivity (Fig. 6). The cross reacting antibodies of this antiserum bound to Drosophila laminin that had been ad-

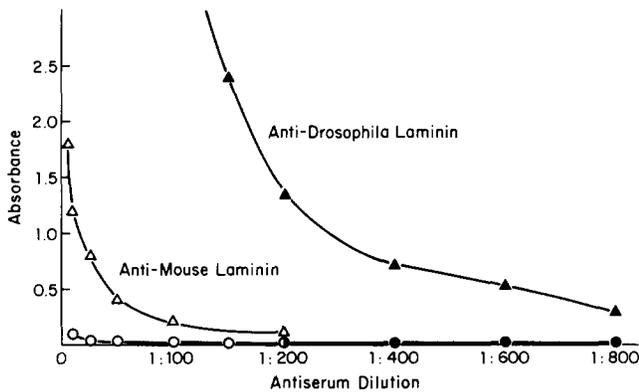


Figure 6. ELISA of antisera to laminin. Dilutions of antisera against *Drosophila* laminin (\blacktriangle) or mouse laminin (\triangle) were reacted with purified *Drosophila* laminin immobilized on microtiter plates. The antisera at the indicated dilutions were reacted with *Drosophila* laminin immobilized on microtiter plates and the treated sera were analyzed as above to determine the level of the residual titers in anti-*Drosophila* laminin (\bullet) and anti-mouse laminin (\circ) sera. Preimmune serum gave values superimposed at the baseline over a dilution range of 1:10 to 1:500 (not plotted).

sorbed onto microtiter wells, as subsequently no reaction of the remaining antiserum was found by ELISAs. The ELISA with preimmune serum gave baseline values. The affinity-purified IgG to *Drosophila* laminin reacted both with non-reduced laminin which had high affinity for the antibodies, and with the reduced laminin A, B1, and B2 chains on a Western blot (Fig. 7). A mixture of secreted proteins and proteins of a cell lysate showed reaction only with the laminin chains and not with other proteins. The laminin A, B1, and B2 chains seen in the cell lysate were present as individual chains, which had not yet been assembled into the final disulfide-linked laminin molecule, while all of the secreted laminin in the medium was present as disulfide-linked

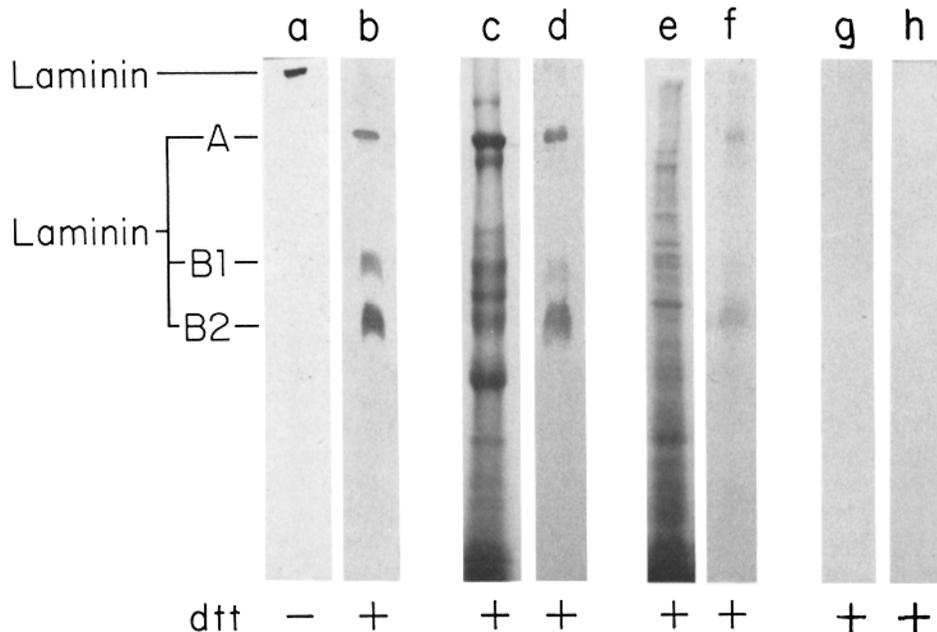


Figure 7. Western blot of medium and cell proteins with *Drosophila* anti-laminin antibodies. Purified laminin (*a* and *b*), the total medium proteins concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (*c*, *d* and *g*), and an SDS cell lysate (*e*, *f* and *h*) were subjected to electrophoresis on SDS 4.5% polyacrylamide gels. The proteins in lanes *c* and *e* were stained with Coomassie Blue. The proteins in the other lanes were blotted onto nitrocellulose and a Western blot was developed with affinity-purified anti-laminin antibodies (1:500; lanes *a*, *b*, *d* and *f*) or with preimmune serum (1:500; lanes *g* and *h*), and anti-rabbit IgG linked to horseradish peroxidase (1:1,000).

molecules consisting of A, B1, and B2 chains. Some crude antisera, as opposed to affinity-purified IgG, showed a weak cross reactivity with *Drosophila* proteoglycans on Western blots. Preadsorption of the antisera on immobilized, purified proteoglycans abolished any cross reactivity. The preimmune serum did not react.

Immunofluorescence staining of *Drosophila* embryo sections showed staining in the locations around internal organs, underlying the hypodermal epithelium and around muscles where basement membranes have been seen by electron microscopy (1). Weak immunostaining of the basement membranes was first detected in sections of embryos at ~ 6 to 8 h of development, while staining was very distinct between 8 and 9 h of development and appeared to increase thereafter. The first detection of laminin in *Drosophila* embryos is thus later than in mouse embryos (8, 9, 27) where laminin was seen after only a few cell divisions. An example of the staining of a section of an 18-h *Drosophila* embryo with affinity-purified antibodies is shown in Fig. 8 A. Examination of whole mounts of *Drosophila* embryos ranging in age from 6 to 18 h confirmed the time sequence observed on sections (not shown). Detailed localization of laminin and other basement membrane proteins in whole mounts of *Drosophila* embryos in internal organs and in the nervous system were observed and will be reported separately. In sections of *Drosophila* larva and adults, staining with these antibodies was seen in locations where basement membranes are present (not shown) (1, 36). Isolated imaginal discs were treated with antibodies in the presence of detergent and the basement membranes surrounding the discs were maintained and stained with antibodies to laminin (Fig. 8 C).

When dispersed cells derived from 3-4-h *Drosophila* embryos were placed into tissue culture, the cells differentiated (39, 40). Examination of these cultures over a 30-h period showed that some cell clusters synthesized basement membrane components which were detected by immunostaining with antibodies to laminin at 20 h (Fig. 8 D). The same type

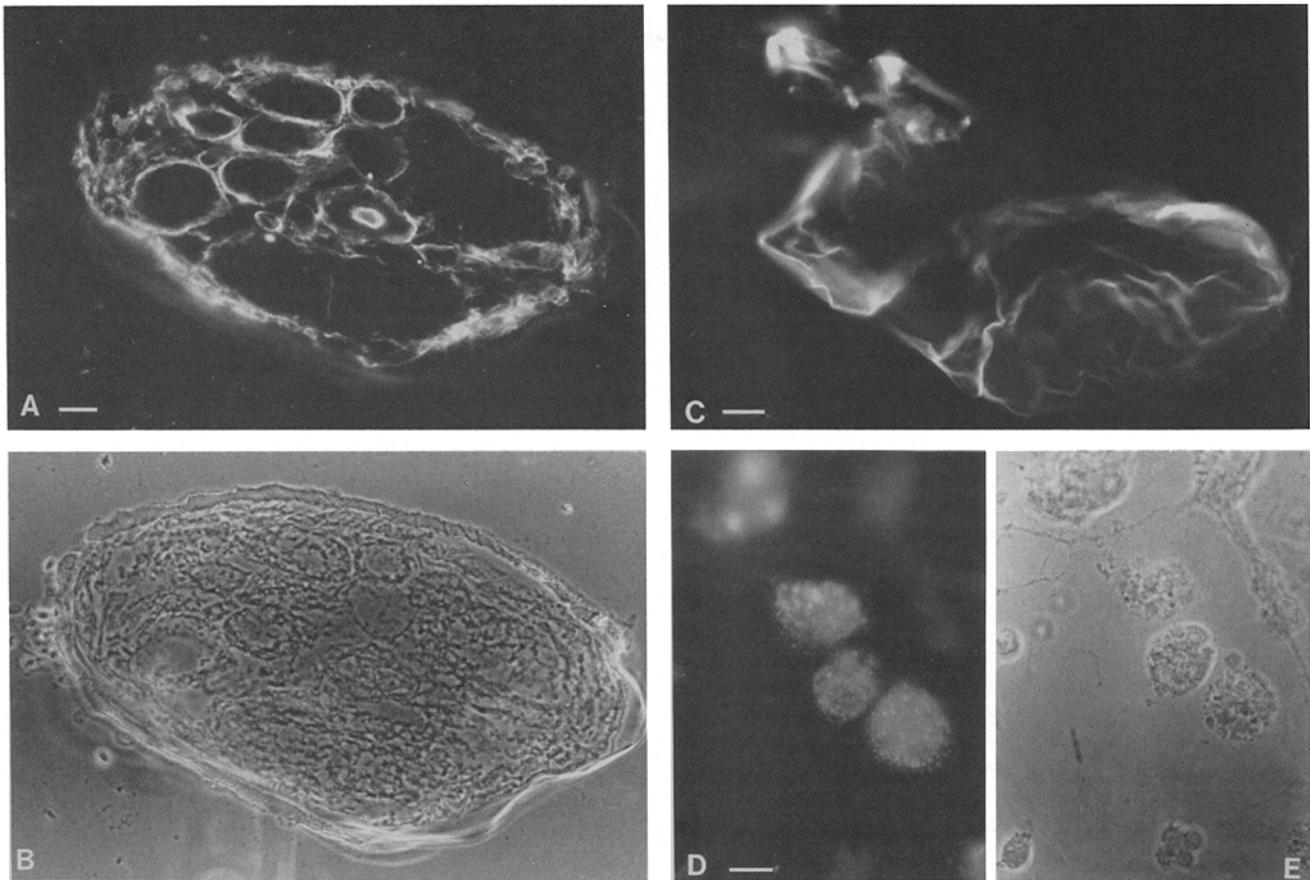


Figure 8. Immunofluorescence staining. (A) A *Drosophila* 18-h embryo section was stained with affinity-purified anti-laminin IgG and fluorescein-conjugated goat anti-rabbit IgG. (B) Represents the appearance of the section by phase microscopy. (C) An isolated, unfixed *Drosophila* imaginal disc treated with PBS, NP-40 was stained with antiserum to laminin and goat anti-rabbit IgG-fluorescein. (D) Primary *Drosophila* cells grown in culture for 20 h were stained with affinity-purified anti-laminin IgG and goat anti-rabbit IgG-fluorescein. (E) Shows the same cells as seen by phase microscopy. Staining with preimmune serum gave only an overall very weak, nonspecific staining reaction in all cases (not shown). Under standard photographic exposure conditions, the preimmune serum stains gave blank prints. Bars: (A) 50 μ m; (D and C) 25 μ m.

of cell also showed positive staining with antibodies to *Drosophila* collagen (not shown). Thus, these separated cells differentiated independently and did not require induction by associated, adjacent cell layers.

In all immunofluorescence staining analyses, preimmune serum showed no specific staining patterns, and antiserum preadsorbed on laminin also gave negative staining patterns.

Discussion

This glycoprotein is so similar to vertebrate laminin that we consider it to be *Drosophila* laminin. The principal difference is that while the B1 and B2 chains of vertebrate laminin have nearly the same electrophoretic mobilities, those of *Drosophila* differ. In current models for vertebrate laminins, the B1 and B2 chains contribute equally to the trimeric molecular structure (3, 32, 38, 48). Unless the different electrophoretic mobilities of *Drosophila* B1 and B2 laminin chains are entirely due to some large differences of glycosylation, the present results indicate that at least in *Drosophila* these two chains are not quite equivalent. In addition, there are small differences between *Drosophila* and mouse laminins in

the lengths of the short arms of the cross shape, and this could be related both to the electrophoretic mobilities of the B chains and the slightly larger apparent sedimentation coefficient of the *Drosophila* protein.

The interaction of vertebrate laminin with cells is evident in its growth-promoting and chemotactic properties (22, 42, 43), and particularly in the facilitation of neurite growth cone extension (4, 12, 15, 19, 25). Cell receptors for vertebrate laminin have been identified (29, 34, 47), and proteolytic fragmentation of mouse laminin demonstrated separate domains for binding to cells and to other basement membrane matrix components such as collagen IV, proteoglycan, and entactin (43). The multidomain structure of vertebrate laminin is also suggested by its electron microscopic appearance (14). Our finding of a closely similar appearance of *Drosophila* laminin suggests that the various functional domains are conserved. We have initiated a study of its interaction with cells and with other identified *Drosophila* basement membrane components: collagen IV, proteoglycan, and entactin.

The electron microscopic appearances of vertebrate and insect basement membranes are similar (1, 36), and have so far not provided an understanding of the molecular substructure

tures. Although laminin has been located to vertebrate basement membranes by immunoelectron microscopy (26), its general occurrence in basement membranes was demonstrated by optical immunomicroscopy. With this technique, we have found laminin in those places where basement membranes occur in *Drosophila* embryos, larvae and adults; e.g., around the gut and muscles, underlying the hypodermal epithelium and enveloping imaginal discs, and around organs such as the brain and ventral nerve cord. As described elsewhere, laminin, together with the other basement membrane components, appears in a sheath that not only surrounds the developing *Drosophila* ventral nerve cord but also penetrates it at segmental intervals (17). Thus, laminin is a common component of diverse basement membranes in *Drosophila* as well as in vertebrates.

The synthesis of laminin by *Drosophila* cell clusters, after they have differentiated in culture, is in contrast to the formation of basement membranes during the interaction of cell layers in the developing vertebrate embryo (13). This indicates that some of the *Drosophila* blastoderm cells are determined to make laminin. Antibodies titrated for equal avidity detected laminin and entactin simultaneously during *Drosophila* organogenesis, and soon thereafter *Drosophila* basement membrane collagen IV and proteoglycan were seen. This suggests that although individual *Drosophila* cells initiate laminin production in culture, basement membrane components are coordinately secreted in the embryo.

The limitations of antibodies prevent us from detecting both the first secretion of laminin and the thinnest initial deposits during tissue organization, particularly during the formation of the nerve cord. The potential role of laminin in *Drosophila* neural development remains to be determined, and to assist this we are characterizing its genes at the recombinant DNA level.

The similarity of *Drosophila* and mouse laminins has implications for the nature of laminin and of basement membranes. The body construction of insects differs radically from vertebrates; not only are there the contrasts of exo- and endoskeletons, but also insect organs are bathed directly in hemolymph, without intervening blood vessels. Thus, it is not evident, a priori, that some extracellular matrix components should have been conserved during divergent evolution. It seems that the specialized, condensed extracellular matrix that constitutes basement membrane may serve particular functions in relation to the cells with which it is closely associated (5, 20), and the necessity of these functions may constrain the evolution of laminin and related molecules.

Recently, it was found that the carboxyl domains of another basement membrane macromolecule, collagen IV, are extraordinarily alike in *Drosophila*, mouse, and man (6). As these carboxyl ends are specialized to form hexameric junctions between pairs of collagen IV molecules, they are an integral part of the network of collagen which forms the molecular scaffold of basement membranes (45, 49). The use of these intermolecular junctions probably governed their evolutionary conservation. Presumably, the critical entities for conservation of such molecules are the binding sites, yet these are likely to account for only a small part of the total mass of such a large molecule as laminin. The striking maintenance of the molecular shape and size of laminin suggests

that in addition to the binding sites, the spacing between the interactive units is also important.

We thank Drs. R. Timpl and I. J. Goldstein for antibodies to mouse laminin, and Dr. Goldstein for the Bandeirea simplicifolia B₄ isolectin-Sepharose. We thank Dr. R. B. Burgess for the amino acid analyses and Dr. Carolyn Buzin for introducing us to the preparation of cell cultures from *Drosophila* embryos. We thank Dr. Walter Gehring for a most enjoyable and instructive visit in his laboratory, and Dr. J. Wirz for showing us the immunostaining techniques for *Drosophila* embryo whole mounts.

This research was supported by a United States Public Health Service research grant (No. AGO2128), Fogarty International Fellowship 1-F06-TWO1108-01 to J. H. Fessler, and a USPHS training grant (No. GM7386) to K. G. Duncan.

Received for publication 25 March 1987, and in revised form 9 July 1987.

References

- Ashurst, D. E. 1982. The structure and development of insect connective tissue, basement membranes. In *Insect Ultrastructure*. R. C. King, and H. Akai, editors. Plenum Publishing Corp., New York. 338-340.
- Bächinger, H. P., L. I. Fessler, and J. H. Fessler. 1982. Mouse procollagen IV: characterization and supramolecular association. *J. Biol. Chem.* 257: 9796-9803.
- Barlow, D. P., N. M. Green, M. Kurkinen, and B. L. M. Hogan. 1984. Sequencing of laminin B chain cDNAs reveals C-terminal regions of coiled-coil alpha-helix. *EMBO (Eur. Mol. Biol. Org.) J.* 3:2355-2362.
- Baron van Evercooren, A., H. K. Kleinman, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. DuBois-Dalcq. 1982. Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* 8:179-193.
- Bissel, M. J., H. G. Hall, and G. Parry. 1982. How does the extracellular matrix direct gene expression. *J. Theor. Biol.* 99:31-68.
- Blumberg, B., A. J. MacKrell, P. F. Olson, M. Kurkinen, J. M. Monson, J. E. Natzle, and J. H. Fessler. 1987. Basement membrane procollagen IV and its specialized carboxyl domain are conserved in *Drosophila*, mouse and human. *J. Biol. Chem.* 262:5947-5950.
- Chung, A. E., R. Jaffe, I. L. Freeman, J. P. Vergnes, J. E. Braginski, and B. Carlin. 1979. Properties of a basement membrane-related glycoprotein synthesized in culture by a mouse embryonal carcinoma-derived cell line. *Cell.* 16:277-281.
- 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.
- Dziadek, M., and R. Timpl. 1985. Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. *Dev. Biol.* 111:372-382.
- Echalier, G., and A. Ohanessian. 1969. Isolament en cultures in vitro de lignees cellulaires diploides de *Drosophila melanogaster*. *C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat.* 268:1771-1773.
- Echalier, G., and A. Ohanessian. 1970. In vitro culture of *Drosophila melanogaster* embryonic cells. *In Vitro* 6:162-172.
- Edgar, D., R. Timpl, and H. Thoenen. 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO (Eur. Mol. Biol. Org.) J.* 3:1463-1468.
- Eklblom, P. A., K. Alitalo, A. Vaheri, R. Timpl, and L. Saxen. 1980. Induction of a basement membrane glycoprotein in embryonic kidney: possible role of laminin in morphogenesis. *Proc. Natl. Acad. Sci. USA.* 77: 485-489.
- Engel, J., E. Odermatt, A. Engel, J. A. Madri, H. Furthmayr, H. Rhode, and R. Timpl. 1981. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. *J. Mol. Biol.* 150:97-120.
- Engvall, E., G. E. Davis, K. Dickerson, E. Ruoslahti, S. Varon, and M. Mantorpe. 1986. Mapping of domains in human laminin using monoclonal antibodies: localization of the neurite-promoting site. *J. Cell Biol.* 103:2457-2465.
- Fessler, L. I., and J. H. Fessler. 1974. Protein assembly of procollagen and effects of hydroxylation. *J. Biol. Chem.* 249:7637-7646.
- Fessler, L. I., A. G. Campbell, B. Blumberg, A. MacKrell, and J. H. Fessler. 1987. Basement membrane synthesis in *Drosophila* embryos. *J. Cell. Biochem.* 11A:287.
- Fessler, J. H., G. Lunstrum, K. G. Duncan, A. G. Campbell, R. Sterne, H. P. Bächinger, and L. I. Fessler. 1984. Evolutionary constancy of basement membrane components. In *The Role of Extracellular Matrix in Development*. R. Treilstad, editor. Alan R. Liss, Inc., NY. 207-219.
- Hammerback, J. A., S. L. Palm, L. T. Furcht, and P. C. Letourneau. 1985. Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. *J. Neurosci. Res.* 13:213-220.

20. Hay, E. D. 1981. *Cell Biology of the Extracellular Matrix*. Plenum Publishing Corp., New York. 379-409.
21. Hogan, B. L. M., D. P. Barlow, and M. Kurkinen. 1984. Reichert's membrane as a model for studying the biosynthesis and assembly of basement membrane components. *Ciba Found. Symp.* 108:60-74.
22. Kleinman, H. K., F. B. Cannon, G. W. Laurie, J. R. Hassell, M. Aumailley, V. P. Terranova, G. R. Martin, and M. DuBois-Dalcq. 1985. Biological activities of laminin. In *The Role of Extracellular Matrix in Development*. R. L. Trelstad, editor. Alan R. Liss, Inc., NY. 123-143.
23. Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473-485.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
25. Lander, A. D., D. K. Fujii, and L. F. Reichardt. 1985. Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. *Proc. Natl. Acad. Sci. USA.* 82:2183-2187.
26. Laurie, G. W., C. P. Leblond, S. Inoue, G. R. Martin, and A. Chung. 1984. Fine structure of the glomerular basement membrane and immunolocalization of five basement membrane components to the lamina densa (basal lamina) and its extensions in both glomeruli and tubules of the rat kidney. *Am. J. Anat.* 169:463-481.
27. Leivo, I., A. Vaheri, R. Timpl, and J. Wartiovaara. 1980. Appearance and distribution of collagen and laminin in the early mouse embryo. *Dev. Biol.* 76:100-114.
28. Lunstrum, G. P., and J. H. Fessler. 1980. Cell adhesion in invertebrates: a potential basement membrane collagen of *Drosophila melanogaster*. *J. Supramol. Struct.* 4:183.
29. 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.
30. Moore, S., D. H. Spackman, and W. H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins. *Anal. Chem.* 30:1185-1190.
31. Oshima, R., and E. Linney. 1980. Identification of murine extra-embryonic endodermal cells by reaction with teratocarcinoma basement membrane antiserum. *Exp. Cell Res.* 126:485-490.
32. Paulsson, M., R. Deutzmann, R. Timpl, D. Dalzoppo, E. Odermatt, and J. Engel. 1985. Evidence for coiled-coil alpha-helical regions in the long arm of laminin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:309-316.
33. Piez, K. A., E. A. Eigner, and M. S. Lewis. 1963. The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry.* 2:58-66.
34. Rao, C. N., S. H. Barsky, V. P. Terranova, and L. A. Liotta. 1983. Isolation of a tumor cell laminin receptor. *Biochem. Biophys. Res. Commun.* 111:804-808.
35. Rao, C. N., I. J. Goldstein, and L. A. Liotta. 1983. Lectin-binding domains on laminin. *Arch. Biochem. Biophys.* 227:118-124.
36. Rizki, T. M., and R. M. Rizki. 1984. The cellular defense system of *Drosophila melanogaster*: basement membranes as a factor for recognition of self. In *Insect Ultrastructure*. R. C. King, and H. Akai, editors. Plenum Press, Inc., New York and London. 579-604.
37. Rodriguez, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. *Virology.* 12:316-317.
38. Sasaki, M., S. Kato, K. Kohno, G. R. Martin, and Y. Yamada. 1987. Sequencing of cDNA encoding the laminin B1 chain reveals a multi-domain protein containing cysteine rich repeats. *Proc. Natl. Acad. Sci. USA.* 84:935-939.
39. Seecof, R. L. 1979. Preparation of cell cultures from *Drosophila melanogaster* embryos. *Tissue Culture Association Manual*. Vol. 5. pp. 1019-1022.
40. Seecof, R. L., N. Alleaume, R. L. Teplitz, and I. Gerson. 1971. Differentiation of neurons and myocytes in cell cultures made from *Drosophila gastrulae*. *Exp. Cell Res.* 69:161-173.
41. Shibata, S., B. P. Peters, D. D. Roberts, I. J. Goldstein, and L. A. Liotta. 1982. Isolation of laminin by affinity chromatography on immobilized griffonia simplifolia I lectin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 142:194-198.
42. Terranova, V. P., D. Rohrbach, and G. R. Martin. 1980. Role of laminin in the attachment of PAM212 cells to basement membrane collagen. *Cell.* 22:719-726.
43. Timpl, R., J. Engel, and G. R. Martin. 1983. Laminin—a multifunctional protein of basement membranes. *Trends Biochem. Sci.* 8:207-209.
44. Timpl, R., H. Rohde, P. Gehron, F. Robey, S. I. Rennard, J. M. Foidart, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membrane. *J. Biol. Chem.* 254:9933-9937.
45. Timpl, R., H. Wiedemann, V. van Delden, H. Furthmayr, and K. Kühn. 1981. A network model for the organization of type IV collagen molecules in basement membranes. *Eur. J. Biochem.* 120:203-211.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
47. von der Mark, K., and Köhl, U. 1985. Laminin and its receptor. *Biochem. Biophys. Acta.* 823:147-160.
48. Yamada, Y., M. Sasaki, K. Kohno, H. K. Kleinman, S. Kato, and G. Martin. 1985. A novel structure for the protein and gene for the mouse laminin B1 chain. In *Basement Membranes*. S. Shibata, editor. Elsevier Science Publishers, Amsterdam. 139-146.
49. Yurchenko, P. D., and H. Furthmayr. 1984. Self-assembly of basement membrane collagen. *Biochemistry.* 23:1839-1850.