Inhibition of Laminin Self-Assembly and Interaction with Type IV Collagen by Antibodies to the Terminal Domain of the Long Arm

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Abstract. Laminin is a major glycoprotein of the basement membrane. Although its precise localization and orientation within this structure is unknown, it is presumably anchored to other macromolecules such as type IV collagen or proteoheparan sulfate. In vitro, laminin has the ability to self-assemble and to bind to type IV collagen molecules at distinct sites. To identify more precisely the domains of the complex, cross-shaped laminin molecule that are involved in these interactions, images of laminin–laminin dimers and laminin–type IV collagen complexes obtained by the rotary shadowing method were analyzed. We observed that the complex domain at the end of the long arm of laminin is predominantly involved in these interactions. By using Fab fragments of antibodies specific for a peptide fragment derived from this complex domain, it is shown that laminin self-assembly is inhibited in their presence, as measured by turbidity and by electron microscopy. In addition, these antibodies inhibit the specific interaction of laminin with type IV collagen. These data suggest that the complex domain at the end of the long arm of laminin contains binding sites of potential importance for the assembly of basement membranes.

LAMININ is a large and complex extracellular glycoprotein found in association with basement membranes (2, 31). It has a molecular weight of ~1,000,000 and in rotary shadow images it usually appears as a cross-shaped molecule with three short arms and one long arm. Each short arm contains two globular regions and the long arm ends at a larger and complex domain (8).

There is evidence to suggest that laminin plays a key role during development: it is the first known extracellular matrix molecule which is produced by the mouse embryo (20), and it promotes attachment and spreading of epithelial cells (13, 29) and neurite outgrowth (6, 23) in vitro. In adult tissues, laminin is localized exclusively in basement membranes (3, 18). It interacts with certain cell surfaces by binding to plasma membrane receptors (21, 28). Laminin has also been suggested to interact in vitro with heparin (26), basement membrane proteoheparan sulfate (10), entactin/nidogen (4), and type IV collagen (1, 14), and to self-assemble and form dimers, oligomers, and polymers in a concentration-, temperature-, and divalent cation–dependent manner with nucleation–propagation kinetics (33).

However, it is still unknown how laminin is anchored in the basement membrane and which domains of the molecule are involved in this function. To more precisely localize the domain or domains important for the self-assembly process and the interaction with type IV collagen, we have prepared antibodies to a peptide fragment (E3) originating from the distinct domain at the end of the long arm (24). By using these antibodies and the technique of rotary shadowing (27), we provide evidence that the complex terminal domain of the long arm of laminin is mediating both the self association of laminin and its binding to type IV collagen in vitro.

Materials and Methods

Isolation Procedures

Laminin, type IV collagen, and entactin/nidogen were extracted from Engelbreth-Holm-Swarm (EHS)[1] tumors grown subcutaneously in mice rendered lathyritic by addition of 0.25% β-aminoproprionitrile fumarate to the drinking water.

Laminin was extracted and purified to homogeneity according to Timpl et al. (31), and was stored at −196°C. Type IV collagen was isolated according to the method of Kleinman et al. (15), and stored at 0°C. A detailed description of these protocols is published elsewhere (1).

Entactin/nidogen was isolated from a 2 M guanidine HCl extract of EHS tumor by using a DEAE-Sephacel column equilibrated in 7 M urea, 0.13 M Tris-HCl, pH 7.5, 50 μg/ml *p*-phenyl methylsulfonyl fluoride, 8 mM *N*-ethylmaleimide, 1 mM EDTA. The protein was eluted with a linear NaCl gradient (0–1 M) and was further purified by gel filtration on a Sepharose CL-6B column in 6 M guanidine HCl, 50 mM Tris-HCl, pH 7.5, 50 μg/ml *p*-phenyl methylsulfonyl fluoride, 8 mM *N*-ethylmaleimide, 1 mM EDTA. The final preparation was analyzed by gel electrophoresis and rotary

1. *Abbreviation used in this paper*: EHS, Engelbreth-Holm-Swarm.

shadowing. It contained multiple entactin/nidogen fragments (50–130 kD) in addition to the intact 150-kD form (data not shown).

Fragment E3 was generated from purified laminin according to the method of Ott et al. (24) with some modifications. Laminin was dialyzed against 0.2 M NH_4HCO_3, pH 7.9, and incubated at 500 μg/ml with elastase (Worthington Biochemical Corp., Freehold, NJ) at an enzyme-to-substrate ratio of 1:100 for 30 min at 4°C, followed by incubation at 20°C for 30 min, and finally at 37°C for 6 h. The reaction was stopped by adding *p*-phenyl methylsulfonyl fluoride at a final concentration of 50 μg/ml at pH 7.9. The proteolytic digest was then applied to a heparin–Sepharose column equilibrated with the same buffer. The column was washed extensively with the same buffer and bound material was eluted with 0.5 M NaCl. The eluted protein peak was concentrated with Aquacide II-A (Calbiochem-Behring Corp., La Jolla, CA), it was dialyzed against 0.2 M NH_4HCO_3, pH 8.5, and further purified on a Sephacryl S-300 column (2.5 × 100 cm) equilibrated with the same buffer. The peak containing fragment E3 was concentrated, it was dialyzed against phosphate-buffered saline (PBS) and stored at −20°C. Amino acid composition was determined after hydrolysis with 6 N HCl (18 h, 110°C), on a Durrum amino acid analyzer. PAGE was performed on a 10% gel in 2% SDS and 2% 2-mercaptoethanol, according to Laemmli (16).

Immunochemical Methods

Antibodies against BSA were raised in rabbits by three intracutaneous (5 mg with complete Freund's adjuvant) alternating with three intraperitoneal injections (10 mg) spaced 2 wk apart. The IgG fraction of the BSA antiserum was prepared by 50% $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography (9). Antibodies against fragment E3 of laminin were prepared by three intracutaneous injections of 0.1 mg with complete Freund's adjuvant, spaced 2 wk apart. The IgG fraction was purified by binding to and elution from two successive laminin–Sepharose columns. Laminin for the first affinity column was produced as originally described (31). Laminin for the second affinity column was prepared by gel permeation chromatography in 6 M guanidine-HCl to remove contaminating entactin/nidogen (4). The purity of the laminin peak was tested by immunoblotting (see below).

Transfer of laminin to nitrocellulose paper from 5% Laemmli gel was performed according to described techniques (32). Staining of nitrocellulose strips was done with india ink, according to the technique of Hancock and Tsang (12). Nonspecific binding sites on blots were blocked by incubation for 2 h at 45°C in PBS/3% BSA/1% Ficoll/1% polyvinylpyrrolidine followed by 30 min in PBS/0.1% BSA and by 60 min on goat serum. The primary antibody was either affinity-purified anti-E3 IgG or normal rabbit IgG at a concentration of 1.4 μg/ml in PBS/0.1% BSA; strips were incubated in this solution at 37°C for 60 min. They were then washed in PBS/0.1% Tween and incubated for 60 min at 37°C with goat anti-rabbit Ig's coupled to alkaline phosphatase (Tago Inc., Burlingame, CA) at a 1:1,000 dilution. Alkaline phosphatase was detected as described by Leary et al. (19).

Fab fragments were generated from anti-E3 and anti-BSA antibodies according to published procedures (5). Briefly, purified antibodies were dialyzed against 100 mM sodium phosphate, 100 mM cysteine, 2 mM EDTA, pH 7.2, and digested with papain (Sigma Chemical Co., St. Louis, MO) at an enzyme-to-substrate ratio of 1:100 for 16 h at 37°C. The digests were cooled to 4°C, they were dialyzed against PBS, and passed over a laminin–Sepharose (for anti-E3) or a BSA–Sepharose (for anti-BSA) column, both equilibrated with 0.15 M NaCl. After elution, the pH was raised to 6.0 and Fab solutions were dialyzed against 0.2 M NH_4HCO_3, pH 8.5. Fab fragments were then concentrated and purified further by gel filtration on a Sephacryl S-300 column (25 × 100 cm).

Determination of Protein Concentration

Protein concentration was determined colorimetrically at 700 nm according to the method of Lowry et al. (22) using BSA (Sigma Chemical Co.; fatty acid and globulin free) as a protein standard. In the case of type IV collagen, protein concentration was determined by amino acid analysis.

Turbidity Measurements

Turbidity measurements were performed in a Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). The samples were placed in prewarmed cuvettes and the temperature was maintained at 35°C in a water-jacketed chamber throughout the experiment. The change of absorbance at 360 nm was followed with time.

Table I. Involvement of the Globular Region of the Long Arm of Laminin in Dimer Formation and in Binding at Specific Sites on Type IV Collagen

Dimer formation	Laminin dimers with: Long to long arms	Laminin dimers with: All other permutations
	%	%
Expected frequency if random	6.2 (1/16)	93.8 (15/16)
Observed frequency		
Laminin (n = 68)	35.3	64.7
Laminin + 10 mM EDTA (n = 54)	59.3	40.7
Binding at specific sites on type IV collagen	**Laminin dimers with: Long arm**	**Laminin dimers with: Short arm**
	%	%
Expected frequency if random	25 (1/4)	75 (3/4)
Observed frequency (n = 50)	46	54

Rotary Shadowing

The technique originally described by Shotton et al. (27) was used. Final protein concentration was 5–10 μg/ml in 0.1 M ammonium acetate (pH 6.6) or ammonium bicarbonate (pH 8.0), or in 0.1 M ammonium bicarbonate adjusted to pH 7.4. All samples contained 50% glycerol. 1 ml final volume was sprayed onto freshly cleaved mica sheets from a distance of 40 cm. Samples were dried under vacuum and replicated with platinum and carbon in a Balzers apparatus. Grids carrying the replicas were examined in a Phillips 300 electron microscope operating at 60 kV. The size of laminin polymers and the binding site of laminin on type IV collagen were analyzed with a digitizing measuring tablet attachment on a Zeiss videoplan computer by using a (Y)1 Videoplan program. In the case of laminin binding to type IV collagen, the results from these measurements were used to construct histograms in which the relative frequency of binding was plotted against the length of type IV collagen molecules, divided into 20 equal segments. Statistical analysis of histograms was performed by the goodness of fit of the Poisson distribution (34).

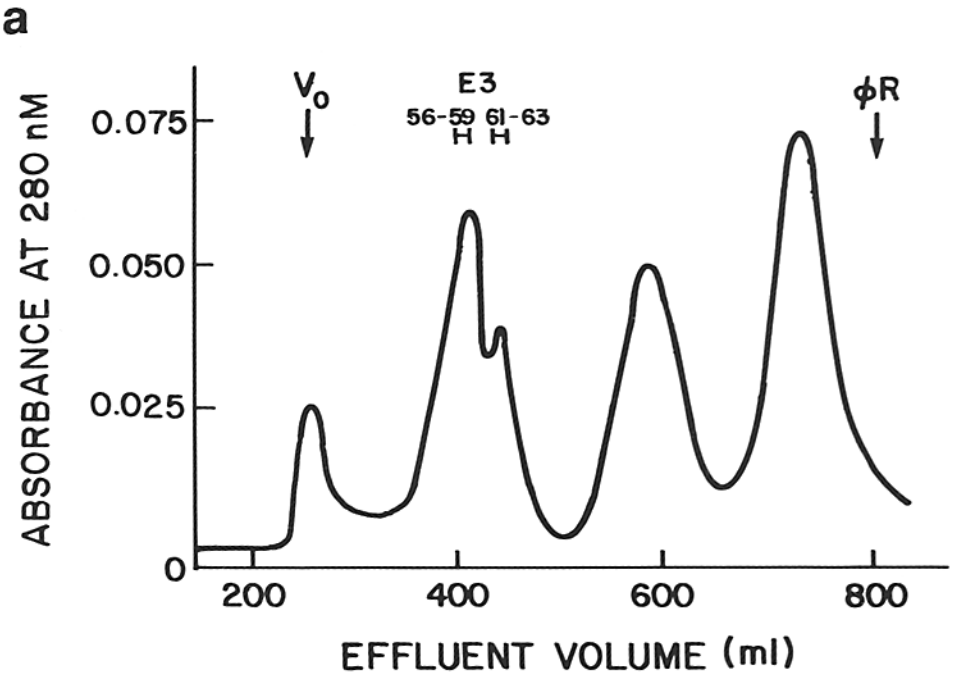
Results

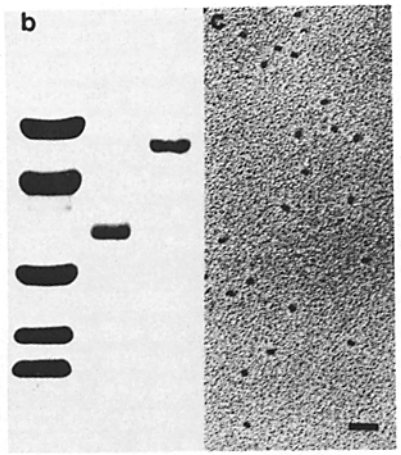
Laminin has the ability to self assemble under certain conditions. When a solution of laminin is incubated at 35°C, above a critical concentration of 0.1 mg/ml, small oligomers are formed in the absence of divalent cations. In the presence of low concentration of Ca^{++}, these oligomers polymerize further to form large complexes which are difficult to analyze directly with regard to location of binding sites, size, and detailed organization by current electron microscopic technology (33). In earlier studies, we have observed with the technique of rotary shadowing that the terminal domains of laminin arms are involved in the formation of at least dimers and small oligomers (33) and also, that they bind to distinct sites on type IV collagen molecules (1).

In the present study, we have examined images of laminin dimers obtained by the rotary shadowing method and calculated the relative percentage of dimers which form by interaction between the globular domains on the long arms of two laminin molecules (long-to-long arm dimers), between the globular domains of two short arms (short-to-short arm dimers), and between the globular domains of the long and one short arm (long-to-short arm dimers). The experiments were performed after incubation of laminin (300 μg/ml) for 6 h in PBS or in PBS containing 10 mM EDTA (600 μg/ml) for 12 h. If we assume that the end-globular domains of all

four arms of laminin have equal affinity for each other, then we would expect a long-to-long arm laminin dimer to be a relatively rare event (1 out of 16). As shown in Table I, the fraction of long-to-long arm dimers is however enriched considerably, since it is sixfold higher than expected when laminin is incubated in the absence of EDTA and it becomes 10-fold higher than expected in the presence of EDTA (Table I). Similarly, the globular domain of the long arm appears to be of importance for laminin–type IV collagen interactions, since the frequency of binding of this domain of laminin at specific sites along the length of type IV collagen molecules is about twice higher than one would expect (Table I). If the globules of all four arms of laminin had similar affinities for binding to type IV collagen, then one would have expected the globule of the long arm to be involved in the formation of only 25% of all complexes. The data of Table I suggest that for the first step of laminin self-assembly (dimer formation) as well as for the binding of laminin to type IV collagen, the terminal complex domain of the long arm is important.

This domain appears to have a molecular weight of ~150,000, as indicated by measurements from rotary shadow replicas, negatively stained and unstained laminin molecules (8), and scanning transmission electron microscopy (7), and it occasionally exhibits a subunit structure of either two or three small globules (25). Isolation of this domain in an intact form has not been possible thus far. However, Ott et al. (24) have described the isolation of fragment E3 of laminin, which is thought to originate from the terminal domain of the long arm. After digestion with elastase and purification with a heparin–Sepharose affinity column, a final purification step by gel filtration was used to obtain a series of distinct peaks (Fig. 1 *a*). The E3 peak (fractions 56–58) eluted at a ratio of E3 to phenol red of 0.508. SDS PAGE under reducing conditions showed that the material contained a single peptide with a molecular weight of 60,000 (Fig. 1 *b*, *right lane*). Rotary shadow appearance (Fig. 1 *c*) and amino acid analysis of this peptide fragment (Fig. 1 *d*) proved to be similar to the published data (24).





d

AMINO ACID ANALYSIS OF FX 56-58 (E3)

	RESIDUES/1000 RESIDUES		
ASP	89	MET	11
THR	62	ILE	31
SER	79	LEU	99
GLU	102	TYR	41
PRO	57	PHE	33
GLY	91	HIS	41
ALA	82	LYS	39
CYS	11	ARG	62
VAL	68		

Figure 1. Isolation and characterization of fragment E3. (*a*) Profile of gel permeation chromatography on a Sephacryl S-300 column (100 × 2.5 cm) after elastase digestion and elution from a heparin–Sepharose column. Fractions 56–58 (E3) and 61–63 were pooled. (*b*) 10% Laemmli gel with 2% SDS and 2% 2-mercaptoethanol. (*Left lane*) Molecular weight standards, from top to bottom: BSA (66,000); ovalbumin (45,000); trypsinogen (24,000); *b*-lactoglobulin (18,400); lysozyme (14,300). (*Middle lane*) Fraction 61–63. (*Right lane*) Fraction 56–58 (E3). (*c*) Rotary shadow image of fraction 56–58. Bar, 50 nm. (*d*) Amino acid analysis of fraction 56–58.

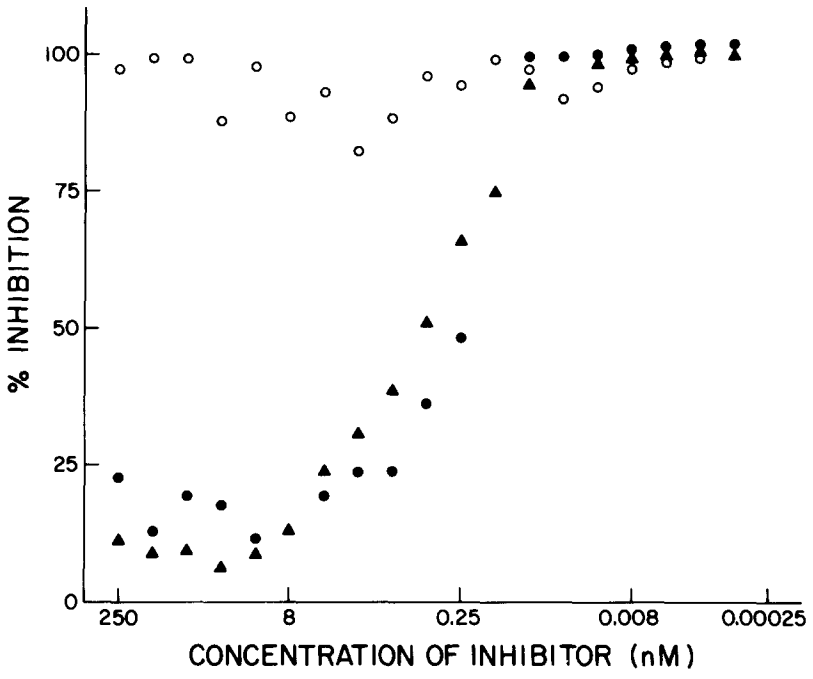


Figure 2. Competition ELISA for intact laminin, and P1 and E3 fragments of laminin. ELISA plates were coated overnight at 4°C with 100 μg laminin per well. Concentration of the affinity-purified anti-E3 used is 300 μg/ml (2 nM). Molarity range of competing molecules is from 250 to 1,100 nM. ○, P1 fragment of laminin; ▲, E3 fragment of laminin; ●, intact laminin.

The other peak (fractions 61–63) that eluted close to E3 in gel filtration (Fig. 1 *a*), was observed to contain a single 35,000-mol-wt band (Fig. 1 *b*, *middle lane*). Antibodies against this polypeptide cross-reacted extensively with E3 (data not shown), suggesting that the 35,000-mol-wt fragment could have been derived from E3.

Antibodies were prepared against fragment E3 as described in Materials and Methods and they were purified by affinity chromatography. After the first laminin–Sepharose affinity column, the eluted antibodies reacted in immunoblots with the 400,000-mol-wt laminin band, but some reactivity with lower molecular weight bands was also observed. These bands were characterized as fragments of entactin/nidogen by immunoblotting, with antibodies generously supplied by Dr. R. Timpl (Munich, Germany). This finding suggested that not only laminin purified by the originally described methods contained fragments of entactin/nidogen, but also that the antibodies raised against E3 were contaminated with antibodies to this protein. To remove these contaminating antibodies, a second laminin–Sepharose column was used. The laminin used to construct this column was tested and found free of any contamination with entactin/nidogen, as determined by immunoblotting (data not shown).

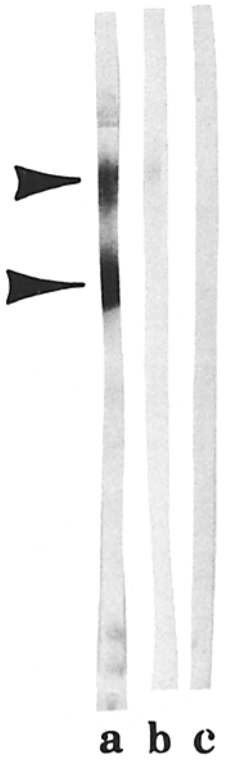


Figure 3. (Lane *a*) Nitrocellulose stained after the transfer with India ink. (Lane *b*) Staining with anti-E3 Igs (1.4 μg/ml). (Lane *c*) Staining with normal rabbit Igs (1.4 μg/ml). Arrowheads indicate the positions of 400,000- and 200,000-mol-wt bands of laminin.

For the characterization of the purified E3 antibodies, we used solid-phase assays, immunoblotting, and electron microscopy.

In solid-phase assays, intact laminin and fragment E3 competed equally well with the binding of E3 antibodies (2 nM) to laminin-coated plates (100 ng/well) with a 50% inhibition at 0.5–0.25 nM, whereas fragment P1 did not show any competition (Fig. 2). In similar assays, no inhibition was observed with type IV collagen and EHS-derived proteoheparan sulfate (data not shown). These results, combined with the electron microscopic characterization (see below and see Fig. 4) and with the findings of Timpl et al. (30) that E3 antibodies do not cross-react with fragments E4, E5, and E6 of laminin, suggest that fragment E3 may represent a peptide derived only from the terminal complex domain of the long arm of laminin.

In immunoblots, the purified E3 antibody reacted with the 400,000-mol-wt band of laminin, but not with the 200,000-mol-wt band(s) (Fig. 3 *b*). Very faint reactivity was observed with some lower molecular weight bands (Fig. 3 *b*), which we have not characterized further; we believe that these may represent degradation products from the 400,000-mol-wt

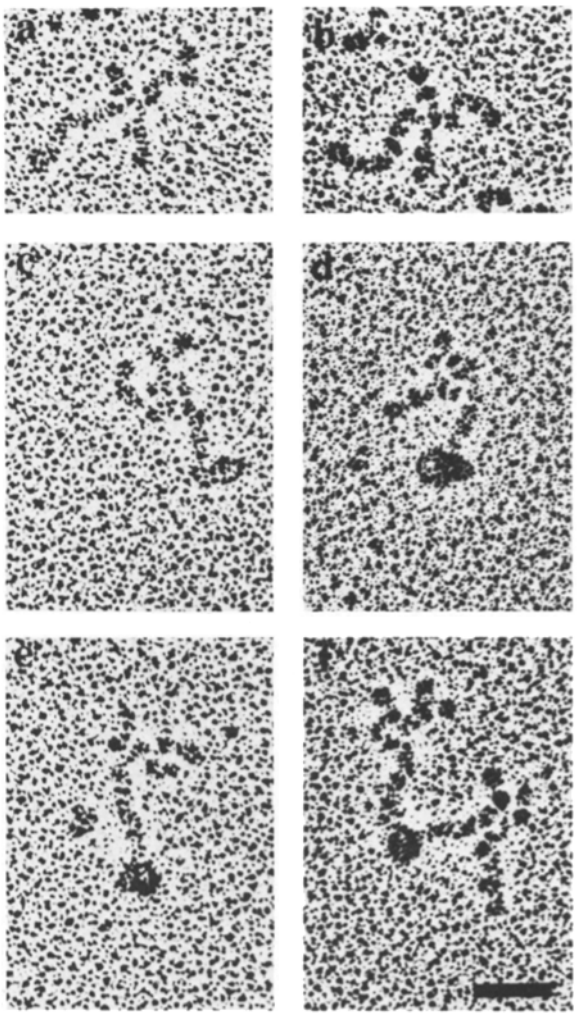


Figure 4. Rotary shadow images of laminin (*a* and *b*) and laminin interacting with anti-E3 (*c–f*). Bar, 50 nm.

band. The antibody did not react with entactin/nidogen but it reacted strongly with fragment E3 (data not shown).

To localize fragment E3 to intact laminin molecules, the antibodies were incubated at a 1:1 molar ratio with laminin at 4°C for 12 h, followed by incubation at 20°C for 1 h. On the metal replicas, the antibodies had a globular appearance and were observed to bind almost exclusively (29 out of 30 laminin–antibody complexes) at the complex terminal domain of the long arm of laminin (Fig. 4), indicating that fragment E3 originates from that portion of the molecule. Anti-BSA IgGs, used as a control, were found to associate infrequently and randomly with the laminin molecule (data not shown).

We have taken advantage of these antibodies to determine the role of the domain E3 or the entire terminal complex domain for laminin self-assembly and for the interaction of laminin with type IV collagen. Fab fragments were prepared to avoid precipitation and the formation of large aggregates of laminin. Anti-BSA Fab fragments were used as a control for these experiments. Fab fragments from both anti-E3 and anti-BSA antibodies, when examined by PAGE in the presence of SDS, were migrating as a single band with an apparent molecular weight of 50,000 under nonreducing conditions, or an apparent molecular weight of 25,000 under reducing conditions (data not shown), suggesting the absence of uncleaved IgG.

We first examined the effect of anti-E3 Fab fragments on dimer formation. Laminin (250 μg/ml) was incubated at 35°C for 60 min in a neutral phosphate buffer containing 10 mM EDTA, in the presence of 10-fold molar excess of either anti-E3 or anti-BSA Fab fragments (125 μg/ml), and the specimens were examined with the technique of rotary shadowing. In the presence of anti-BSA Fab fragments, the number of long-to-long arm dimers was 51.4% of the total number of dimers (n = 72), a result similar to that obtained in control laminin specimens (incubated in the absence of any additional protein) (cf. Table I). In the presence of anti-E3 Fab fragment, the long-to-long arm dimers represented only 22.4% of total dimers observed in the sample (n = 69).

In addition, we examined the effect of anti-E3 Fab fragments on the formation of large complexes of laminin which occurs in the presence of divalent cations. Laminin at 250 μg/ml was co-incubated for different time periods with either anti-BSA or anti-E3 Fab fragments at 125 μg/ml in PBS at 35°C and the change of turbidity was measured at 360 nm. Fab fragments incubated alone gave only minimal turbidity change. Laminin incubated in the presence of anti-E3 Fab fragments showed substantially reduced turbidity when compared to laminin alone or to laminin incubated in the presence of anti-BSA Fab fragments (Fig. 5), suggesting that the size of laminin complexes was decreased.

Samples were also analyzed after a 60-min incubation with the technique of rotary shadowing, as described in Materials and Methods, and the data were analyzed on prints taken at a final magnification of 87,000. Laminin oligomers up to pentamers could be identified directly from the prints. The numbers of laminin molecules participating in polymers larger than pentamers was calculated by measuring the surface area of the polymer and then dividing this number by the average surface area of laminin monomers. The results from these measurements are shown in Fig. 6. Incubation of laminin in the presence of anti-E3 Fab (Fig. 6 *B*) reduced the amount of laminin which forms large complexes more than threefold (71%), as compared to that observed in the presence of anti-BSA Fab (Fig. 6 *A*). From the data of Fig. 5, it can be seen that at the 60-min time interval turbidity was also reduced by more than threefold (77%) in the presence of anti-E3 Fab. Thus the results obtained with the two different techniques correlate well.

In further studies, the effect of blocking the E3 domain was explored to determine its importance for the binding of laminin to type IV collagen. Laminin (50 μg/ml) was incubated with type IV collagen (100 μg/ml), at 35°C for 60 min in the presence of 25 μg/ml anti-E3 Fab or anti-BSA Fab (10-fold molar excess compared to laminin). At the end of the incubation time, the samples were examined with the technique of rotary shadowing. Laminin and type IV collagen formed distinct complexes, as described previously (1). To be able to compare the frequency of complex formation in the presence of either anti-E3 or anti-BSA Fab, we determined the number of complexes on prints (final magnification 87,000×), and from these we calculated the relative frequency of complex formation (occupancy) as the percentage of non-aggregated type IV collagen molecules which were associated with laminin. We observed that the occupancy in the presence

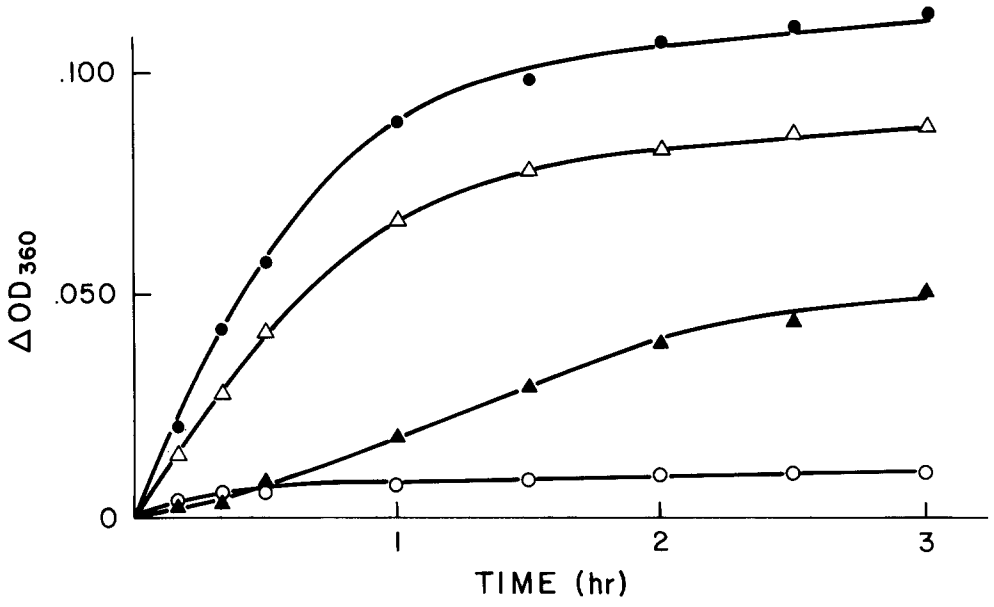


Figure 5. Laminin at 250 μg/ml was incubated at 35°C in PBS either alone (△) or in the presence of anti-BSA Fab (●) or anti-E3 Fab (▲). The presence of anti-E3 Fab considerably suppressed the development of turbidity. Anti-BSA Fab alone (○) did not contribute in any turbidity changes. Concentration of Fab in all cases was 125 μg/ml.

of anti-E3 Fab was 10.3% (n = 824), while in the presence of anti-BSA Fab it was 15.6% (n = 865).

We then examined whether the binding of laminin occurred at random or at preferred sites along the length of type IV collagen. This was done by measuring the distance of binding of laminin to type IV collagen molecules from the carboxy-terminal, NCl domain of type IV collagen on prints at a final magnification of 404,000 and then expressing this value as a ratio by dividing it with the total measured length of the molecule. The criteria used for selection of appropriate complexes have been described in detail elsewhere (1). The results of these measurements are shown in Fig. 7. In the presence of anti-BSA Fab (Fig. 7 *A*), laminin demonstrated preferential binding to two sites along the length of

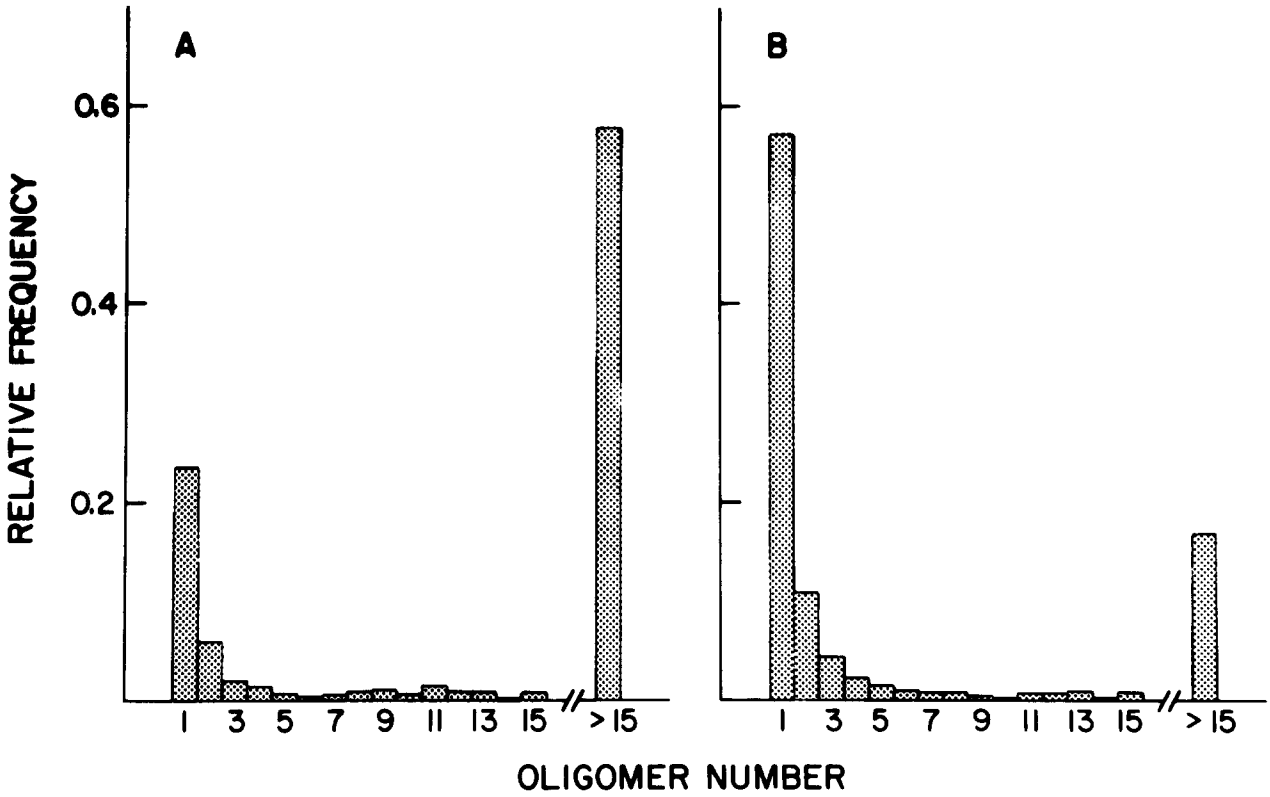


Figure 6. Laminin (250 μg/ml) was incubated for 60 min at 35°C with Fabs (125 μg/ml), then rotary shadowed, and the relative mass frequency distribution of laminin molecules in every dimer was calculated in the case of anti-BSA Fab (*A*) or anti-E3 Fab (*B*). Anti-E3 Fab created a nearly fourfold decrease of the mass of laminin participating in polymeric forms.

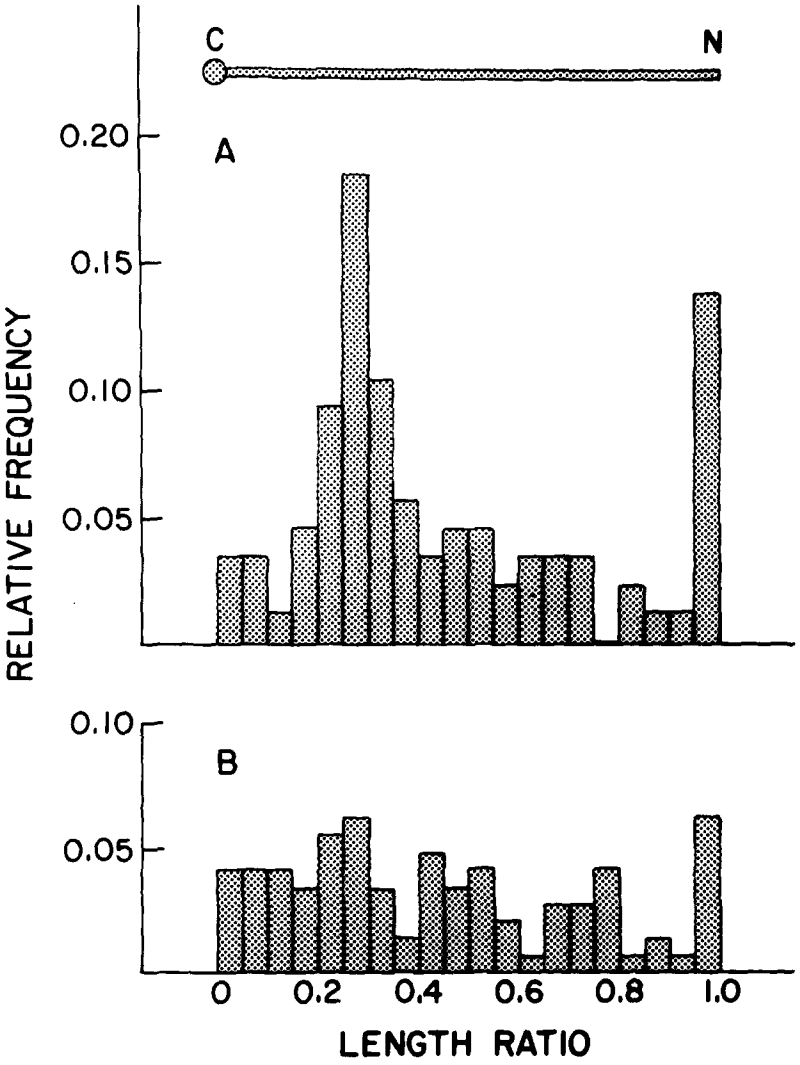


Figure 7. Histogram of the relative frequency distribution of laminin binding along the length of type IV collagen (divided in 20 segments) shown diagrammatically on top (COOH terminal [*C*]; NH_2 terminal [*N*]). Distribution of binding in the presence of anti-BSA Fab (*A*) and anti-E3 Fab (*B*). Statistical analysis demonstrates that the distribution in A is nonrandom ($P < 0.01$) and that in *B* is highly random ($0.5 < P < 0.25$).

type IV collagen: one at about one-fourth to one-third of the length of the molecule measured from domain NCl and the other at a site close to the amino terminus. Statistical analysis of the histogram demonstrated that this distribution is nonrandom, at a highly significant level ($P < 0.01$). These data are similar to those reported previously (1). In the presence of anti-E3 Fab fragments (Fig. 7 *B*), however, the binding of laminin was distributed at random over the length of type IV collagen ($0.5 < P < 0.25$), indicating that the specific binding of laminin to type IV collagen was effectively blocked.

Discussion

In this report we provide direct evidence that fragment E3 is part of the terminal complex domain of the long arm of laminin and we present data which indicate that this fragment contains sites that mediate two important laminin properties in vitro: self-assembly and binding to type IV collagen.

It has been suggested previously (33) that laminin under certain conditions self-assembles in a step-wise fashion: in a divalent cation-independent first step, monomeric molecules interact with each other to form dimers and small oligomers which, in a second, divalent cation-dependent step, polymerize further to form large aggregates in vitro. When examined on metal shadow casts, self-assembled laminin shows numerous long-to-long arm dimers, indicating a preference for the formation of this type of dimer during the initial step of laminin polymerization. In the presence of anti-E3 Fab fragments, the number of long-to-long arm dimers decreases to less than half of that observed in control specimens.

The anti-E3 Fab fragments are also responsible for a marked decrease in the number of large laminin complexes which formed upon incubation at 35°C. These effects indicate substantial blocking of the sites of laminin which are involved in the formation of long-to-long arm dimers and they also indicate that these dimers may represent important intermediates in the formation of the larger polymers. The incomplete inhibition of both steps may be due to the presence of dimers already in existence prior to incubation at higher temperatures, as has been shown previously (Fig. 11 in reference 33). These dimers may not dissociate in the presence of the antibodies and they may thus serve as nuclei for polymerization. However, incomplete inhibition could be due to the presence of another binding site, not related to E3, on the terminal complex domain of the long arm. Fragment E3 may not contain the binding site for dimer assembly itself, but

may only be close to it; the observed decrease of long-to-long arm dimers could thus be due to steric hindrance caused by the antibodies. Immunochemical data indicate that the terminal complex domain of the long arm of laminin possibly contains two copies of fragment E3 (25). Incomplete saturation of the antibody binding sites in the presence of even 10-fold molar excess of Fab may thus account for the partial inhibition observed in the case of laminin self-assembly.

We have demonstrated recently that laminin binds specifically to type IV collagen at two sites (1). In the present study, we provide evidence that the terminal complex domain of the long arm of laminin is involved in these interactions.

Terranova et al. (28) have previously presented data indicating that the short arms of laminin are exclusively involved in the binding to type IV collagen. Different types of assays—cell binding and filter binding methods—as well as incomplete enzymatic digestions may account for the discrepancy with our data. On the other hand, Laurie et al. (17) have examined the binding of laminin to type IV collagen with the rotary shadowing technique by procedures similar to ours. They interpreted their findings to indicate that the binding involves almost exclusively short arms of laminin. There are important differences, however, in the way the electron microscopic data were analyzed. First, in their study, most of the complexes analyzed involve laminin molecules collapsed on or crossing over type IV collagen molecules; in our study only those complexes were selected for analysis which contained both molecules well spread and in which laminin was associated via the terminal domain of one of its arms. Other types of complexes were not included since they could not be evaluated in relation to specific binding or superimposition. Secondly, the data by Laurie et al. (17, 18) were not analyzed for the domain of laminin which mediates the binding at the specific binding site on type IV collagen. Such data would be important to check since ~50% (1) or even 70% (17) of the binding events between laminin and type IV collagen can be considered as nonspecific. In contrast, the direct measurements (Table I) and indirect experiments using Fab fragments (Fig. 7) strongly suggest that the terminal complex domain of the long arm of laminin contains a binding site for type IV collagen.

The complex domain at the end of the long arm of laminin has a molecular weight of ~150,000 (8). Recent studies with negative staining have indicated that this domain may consist of three subdomains (8). It has not been clearly established whether the three subdomains are similar or whether only one or two of them contain E3 (25). To definitely answer this question, the amino acid sequence of this region needs to be determined.

A number of observations indicate that the terminal domain of the long arm of laminin has multifunctional properties. In addition to the data presented in this report, the binding of laminin to heparin appears to be mediated by domain E3, since isolated fragment E3 binds avidly to a heparin column (>98%). This interaction appears to be sensitive to salt, because an increase of NaCl from 50 to 200 mM decreases E3 bound to heparin by 60–90% (24). Furthermore, rotary shadow images of laminin incubated in the presence of EHS-derived proteoheparan sulfate show the latter to be bound preferentially to this region of the long arm of laminin (17). In addition, fragment E8 of laminin, which contains the terminal globule and a portion of the long arm (6), was shown to promote both neuronal survival and neurite outgrowth. Both of these effects were inhibited by anti-E3 antibodies (6).

If the terminal domain of the long arm of laminin contains binding sites for all of these different ligands, the question arises which of these interactions are important for assembly in vivo? What factors determine, for instance, whether a laminin molecule interacts with another laminin molecule, as opposed to type IV collagen or proteoheparan sulfate? There are no answers to these questions at the present time. We also do not know the factors that favor certain interactions and therefore are responsible for the final structural organization of the basement membrane. The multiplicity of "choices" for interactions could be a common feature of all the basement membrane components, since type IV collagen or proteoheparan sulfate also interact with a number of different ligands (11). It is tempting to speculate, for example, that the amount of type IV collagen, entactin/nidogen, or proteoheparan sulfate present in the microenvironment into which laminin is secreted regulates the extent of the self-assembly observed in vitro with pure laminin, because these macromolecules, by interacting with laminin, can reduce the amount of "free laminin" below its critical concentration for polymerization. This effect would be similar to the inhibition of laminin self-assembly observed in the presence of Fab fragments. It is conceivable that the microenvironment at the basal surface of a polarized cell, with its varying local concentrations of the different components, crucially determines the outcome of these multiple interactions and therefore the final organization of the basement membrane. If this speculation is correct, then one would predict that basement membranes, although built with the same building blocks, can vary in their structural organization and that the determining factor for such variability should be attributed to the cell and the control of its secretory activity.

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