Laminin Polymerization in Vitro
EVIDENCE FOR A TWO-STEP ASSEMBLY WITH DOMAIN SPECIFICITY*

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Laminin, a major structural glycoprotein of basement membranes, has been found to self-associate in vitro into large polymers. The formation of these complexes can be followed by the development of turbidity upon incubation in neutral phosphate buffer at 21–35 °C and is seen to be time-, concentration-, and temperature-dependent. The polymerization is thermally reversible at 4 °C and the protein can be cycled between a dispersed and an aggregated state by alternating between 4 and 35 °C. Following incubation at 35 °C much of the monomeric laminin, which sediments at 11.4 S, is now seen to sediment at greater than 25 S. Both by turbidimetric and sedimentation analysis, an apparent critical concentration for assembly of about 0.1 mg/ml (10⁻² M) is observed and is interpreted as evidence for a nucleation-propagation polymerization mechanism. The relative paucity of intermediates seen in a size-distribution analysis lends further support for this model. On platinum replicas obtained by rotary shadowing analysis, mostly free monomers are seen in the cold while after incubation at 35 °C, large multimeric aggregates with smaller amounts of oligomers are observed. The interaction between individual molecules appears to be specific because the dimers, trimers, and smaller oligomers are only associated at the terminal globular domains of the laminin molecules. In addition, removal of the globular domains of laminin with proteases results in a disordered and less compact state. A divalent cation dependency for polymerization can be demonstrated and incubation in the presence of EDTA stops the polymerization at an oligomeric intermediate step. Hence overall laminin self-assembly can be divided into at least two steps: an initial temperature-dependent, divalent cation independent step followed by a divalent cation-dependent step.

Laminin, a major intrinsic component of basement membranes (1–3), is a glycoprotein of about one million daltons of molecular mass and is composed of different polypeptide chains of 200,000 and 400,000 daltons (1). In rotary shadowed replicas, as well as in negatively stained preparations, the native protein has the characteristic appearance of an asymmetric cross with globular ends composed of three similar short arms (36 nm length) and one long arm (77 nm length) (4). Evidence has accumulated that laminin binds to other basement membrane components including type IV collagen (5, 6) and proteoheparan sulfate (7). Furthermore, this protein mediates the attachment of adjacent cells to the basement membrane by binding to cell surface receptor(s) (8–10). All of these interactions appear to occur at specific proteolytically defined structural domains which have been identified in laminin: the globular end regions bind to collagen (5, 8), the end of the long arm binds to proteoglycan (11), and the inner aspect of the “cross” (P1 fragment) binds to cells (8).

In this manuscript we report that laminin will self-assemble into large complexes in neutral phosphate buffer when warmed to 21–35 °C in a two-step process with a divalent cation dependency in the second step. Furthermore, we present evidence in support of a topographical specificity for this interaction in which laminin binds to itself by its globular end domains.

MATERIALS AND METHODS

Preparation of Laminin
Laminin was isolated from the Engelbreth-Holm-Swarm tumor grown subcutaneously in athyritic mice based upon the method of Timpl et al. (1). Briefly, the tumor was homogenized and preextracted with 3.4 M NaCl in 50 mM Tris-HCl, pH 7.4, with 50 μg/ml PMSF, 50 μg/ml p-chloromercuribenzoic acid, and 1 mM EDTA. Laminin was then extracted from the residue with 1.7 M NaCl in the same buffer. The protein was then precipitated by dialyzing the salt concentration to 3.4 M and the pellet was resuspended in 2 M urea, 50 mM Tris-HCl, pH 7.4, with 1 mM EDTA, 50 μg/ml PMSF, and 50 μg/ml p-chloromercuribenzoic acid. The solution was then incubated with DEAE-cellulose (Whatman DE52, 1 ml/10 g of wet tissue) previously equilibrated in the same buffer. After 2.5 h at 4 °C, the DEAE-cellulose was removed by centrifugation and the protein chromatographed on a 2.5 × 90-cm agarose A-1.5m column (Bio-Rad) after dialysis into 1 M CaCl₂, 50 mM Tris-HCl, pH 7.4, with PMSF. Laminin, which eluted close to the void volume of the column, was pooled, dialyzed into 0.4 M NaCl, 50 mM Tris-HCl, pH 7.4, with PMSF, concentrated in dialysis bags with Aquacide II-A (Calbiochem-Behring), and stored frozen in liquid nitrogen. Before use, the laminin was thawed, dialyzed against either 0.12 M sodium chloride in 10 mM sodium phosphate, pH 7.4 (neutral phosphate buffer), or 0.13 M sodium chloride in 5 mM Tris-HCl, pH 7.4 (neutral Tris buffer), and the protein was ultracentrifuged in 1-m1 aliquots at 3 °C in a Beckman 65 rotor at 40,000 rpm for 70 min to clear preformed aggregates larger than 15 S.

* The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N',N''-tetraacetate acid.

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Preparation of the P1 Fragment of Laminin

Fragment P1 of laminin was prepared by proteolytic cleavage of isolated \(^{125}\)I-labeled laminin with pepsin, according to Rohde et al. (12). Briefly, laminin isolated from the Engelbreth-Holm-Swarm tumor was mixed with \(^ {125}\)I-labeled laminin and was dialyzed exhaustively against 10% acetic acid, pH 2.0-2.5. Laminin, at 0.7-0.8 mg/ml was then incubated with pepsin (Worthington, 3293 units/mg) at a 1:15 enzyme:substrate ratio for 24 h at 15 °C. The reaction was stopped by addition of pepstatin at twice the concentration of pepsin. Proteolytically cleaved laminin was dialyzed against 1 M CaCl\(_2\), 50 mM Tris-HCl, pH 7.4, with 50 μg/ml PMSF, at -70 °C. Prior to use, each aliquot of P1 was dialyzed against 0.12 M NaCl in 10 mM sodium phosphate, pH 7.4 (neutral phosphate buffer), and ultracentrifuged under conditions which cleared aggregates >15 S (almost none detected).

\(^{125}\)I Iodination of Laminin

Two ml of laminin at a concentration of about 1 mg/ml were dialyzed into 2 M urea, 50 mM Tris-HCl, pH 7.4, centrifuged at 40,000 rpm for 20 min at 3 °C and then the supernatant was mixed with 5 mCi of carrier-free Na\(^ {125}\)I (Amersham Searle), 50 μl of a 1 mg/ml solution of lactoperoxidase (Sigma) in neutral phosphate buffer and 0.1 ml of 0.003% hydrogen peroxyde. The solution was incubated at 22-24 °C for 20 min, placed on ice and then made up to a final concentration of 1 mM dithiothreitol. The solution was purified on a 20-ml Sephadex G-25 (medium) column (Pharmacia) equilibrated in the above buffer to remove the bulk of free iodine. The peak void fractions were pooled and rechromatographed at 4 °C in a 2.5×90 cm agarose 1.5cm column as described above. The pooled peak was then concentrated, dialyzed into 0.4 M NaCl, 50 mM Tris-HCl, pH 7.4, and stored in liquid nitrogen.

Analytical Methods

Turbidity—Laminin solutions were dialyzed at 4 °C into phosphate-buffered saline, and centrifuged in 1-ml aliquots of 40,000 rpm at 3 °C in a 65 rotor (Beckman) for 70 min. The supernatants were placed in prewarmed cuvettes at various temperatures (maintained with a water jacket) and absorbance at 360 nm was followed over time. For thermal reversibility studies the cuvette was maintained at 4 °C and periodically placed in the spectrophotometer (room temperature) for measurements.

Rate Zonal Velocity Sedimentation—Laminin and laminin complexes were evaluated on sucrose gradients as described (13) except that they were centrifuged at 40,000 rpm for 13 h at 3 °C. Determination of Protein Concentration—Protein concentration was determined spectrophotometrically at 700 nm by the method of Lowry et al. (14) using bovine serum albumin (Sigma, fatty acid- and globulin-free) as a protein standard.

Rotary Shadowing—The rotary shadowing method was adapted from the procedure used by Shotton et al. (15). Briefly, laminin samples were diluted into 1 ml of 50% glycerol in 0.15 M ammonium acetate to a final concentration of 5-10 μg/ml and sprayed onto freshly cleaved mica sheets at a distance of about 40 cm. The samples were shadowed at a 6° angle with platinum as described (13).

RESULTS

Sedimentation Analysis—Laminin, following dialysis into neutral phosphate buffer and ultracentrifugation at 3 °C was incubated at 35 °C for 2 h. A turbid solution was seen to develop and the formed complexes were analyzed by rate zonal velocity sedimentation on sucrose gradients (Fig. 1). When maintained in the cold laminin was seen to sediment as a single peak at 11.4 S, a value close to that reported in the literature (4). Except for a small faster migrating shoulder, which may represent dimeric and trimeric complexes, no faster migrating material was seen. On the other hand, following incubation at 35 °C for 2 h, a significant amount of the 11.4 S peak was converted to high molecular weight complexes, greater than about 25 S. Relatively small amounts of intermediate sedimenting material were seen. When the sedimented laminin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, its appearance was identical to the starting material (data not shown).

Turbidity Measurements—The formation of higher molecular weight complexes could be followed by measuring the development of turbidity, a method which has been used to study the polymerization of other extracellular matrix proteins including type I collagen (18) and type IV collagen (13). When laminin, again after ultracentrifugation in neutral phosphate buffer, was incubated at 35 °C, turbidity was seen to increase over time (Fig. 2A) with eventual development of a plateau value. However, an increase in turbidity was not observed with laminin after heat denaturation (Fig. 2). When the time-dependent process was examined more carefully it was seen to consist of a rapid initial small increase in turbidity (within 2 min) from base-line to 7-18% of the maximum value achieved (depending on the concentration) followed by a relative lag in turbidity. This in turn was followed by a major turbidity increase following “pseudo” first order kinetics until plateau was reached. This final rise was pseudo first order.
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**Fig. 2.** Turbidity of native and thermally denatured laminin. Native laminin was precleared by ultracentrifugation, adjusted to 0.2 mg/ml in neutral phosphate buffer, and then divided into 2 aliquots. 1 aliquot was incubated at 90 °C for 5 min. The samples were then warmed to 35 °C in a quartz cuvette. A, the development of turbidity for native (closed circles) and denatured (open circles) laminin was monitored over time. B, thermal reversibility cycle: turbidity of laminin (purified through DEAE-cellulose) was followed with cycling between two temperatures, 35 and 4 °C.

While linear lines were obtained in semilog plots, they were not parallel for different starting concentrations. It was found that the laminin could be repeatedly cycled between a nonturbid and turbid state by alternating between two temperatures, 35 and 4 °C (Fig. 2B). Approximately the same maximal value was achieved each time although the time course for development of turbidity was slightly shorter on the second cycle. This property of thermal reversibility, reflecting alternation between a dispersed and polymeric state, is a property held by many biological reversible polymers such as actin (16), tubulin (17), type I collagen (18), and type IV collagen (13).

Turbidity development appeared to be a concentration-dependent process (Fig. 3A) with virtually no turbidity developing below 0.1 mg/ml, even after extended incubations. It was further found that turbidity decreased over time if the laminin solutions were cooled to 4 °C: the decay of turbidity followed apparent first order kinetics, i.e. the time to reduce the turbidity to half of the initial value (decay half-time) was equal at all concentrations studied.

Although a given preparation of laminin produced the same rate (half-reaction time) and same plateau value at a given concentration in a reproducible fashion, it was found that the rate and maxima varied from preparation to preparation. For example, at 0.6 mg/ml (at 35 °C) the reaction half-time ranged from 10 to 40 min and the plateau value from 0.18 to 0.22. However, all of these preparations, if first ultracentrifuged, gave the same apparent critical concentration as operationally defined, i.e. the intercept of maximal (plateau) turbidity values with zero absorbance when plotted against total protein concentration.

When the plateau value of turbidity was plotted against the total concentration of laminin (Fig. 3A, inset, and Fig. 4) a linear relationship was observed in which the line intersected base-line at about 0.1 mg/ml. Furthermore, if the values at different decay times were plotted similarly, the same intercept value was observed (inset, Fig. 3, reflecting the consequence of first order decay). This intercept value of 0.1 mg/ml is believed to represent a critical concentration for turbid-
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The maximum or plateau value of turbidity at different laminin concentrations was seen to be strongly temperature-dependent at values between 35 and 4 °C (Fig. 3B). The data suggest that the apparent critical condition increases as the temperature is lowered.

Comparison between Sedimentation and Turbidometric Measurements—Changes in turbidity (19) not only can reflect changes in the amount of polymer, but also can reflect changes in size and shape of complexes. In order to determine if the plateau values were reflecting the amount of polymer formed, the turbidity measurements were coupled with those of sedimentation. When the concentration dependence of the amount of maximal turbidity was compared to the amount of formed complexes greater than about 18 S, the two methods were seen to be in good agreement (Fig. 4) with both showing a linear relationship and both lines intercepting at about 0.1 mg/ml, the apparent critical concentration. However, it should be realized that the velocity sedimentation was performed at 3 °C, a temperature which induces thermal reversibility of complex formation. Although much of the polymer would be expected to reach the bottom region of the tube before it would appreciably dissociate, one would predict measuring a higher apparent monomeric concentration than actually exists at 35 °C because of this reversal phenomenon. This would not affect the calculated critical concentration, however, because temperature reversal is seen to follow apparent first order kinetics and regardless of the cold temperature decay time examined one sees the same value of about 0.1 mg/ml (Fig. 3A, inset). Attempts to examine complex formation in warm (35 °C) velocity sedimentation runs proved frustrating because of the tendency of the protein to stick to the centrifuge tubes (various plastics) as well as the development of a significant inhibitory effect due to sucrose at this temperature.

The free monomer concentration above 0.2 mg/ml total concentration was seen in velocity sedimentation runs to be about 0.15 mg/ml (data not shown), about 50% higher than the predicted critical concentration of 0.1 mg/ml as described for theoretical nucleation-propagation reactions (16). While we cannot rule out that a fraction of monomers is defective and incapable of self-associating, another possible explanation for this observation is that the difference is due to a fraction which thermally reverses at the start of the sedimentation run.

Domain Specificity of Interaction—When examined with the technique of rotary shadowing, laminin has the shape of a cross with one long and three short arms, each ending in distinct globular domains (4). When maintained in the cold, purified laminin is seen with this technique mainly in the monomeric state, with occasional dimers and rare trimers interdispersed (Fig. 5A). In contrast, following incubation at 35 °C for 30–60 min at a concentration of 300–500 μg/ml in a neutral phosphate buffer, most of the laminin molecules are found to be associated into large multimeric structures (Fig. 5B, right side). Free monomers, and smaller amounts of oligomeric intermediates are present as well (Fig. 5B, left side). The oligomers, composed of 2 to several monomeric subunits, are frequently linear and appear to be held together

Fig. 4. Comparison between laminin turbidity and amount of sedimentable complex. Laminin, at the indicated concentrations, was incubated at 35 °C until the turbidity measurement reached a plateau. Parallel samples were incubated for 8 h in a 35 °C water bath. 0.2-ml aliquots of the latter samples were analyzed by velocity sedimentation as described. The highest concentration turbidity and sedimentation fraction values were matched to allow comparison for the lower values.

Fig. 5. Rotary shadow platinum replicas of laminin maintained at different temperatures. Laminin, in neutral phosphate buffer at 0.6 mg/ml was either maintained on ice (A) or incubated at 35 °C (B) for 1 h. The sample was then diluted to 5 μg/ml in ammonium acetate/glycerol buffer, sprayed onto mica, and prepared for rotary shadowing. Panel B is a composite to show typical areas of laminin complexes. Bar equals 200 nm.
at sites located within the globular domains of laminin. The specificity of this interaction could most clearly be appreciated in the dimers (Fig. 6) for which globular end-to-end associations, involving both the short and long arms of the laminin cross, were frequently noted. These same end-to-end binding associations could also be identified in many of the oligomeric forms (Fig. 7) consisting of more than two molecules, although interpretation of the images became more difficult as one examined increasingly larger complexes.

Studies with the Proteolytic Fragment P1—In order to further evaluate the apparent domain specificity of the laminin-laminin interaction, the proteolytic laminin fragment P1 ($M_r = 290,000$; Ref. 12), which lacks the globular end regions of the short arms as well as the entire long arm, was studied with respect to assembly. When intact laminin and P1 were incubated separately at 35°C at the same concentration (0.3 mg/ml), only laminin showed an increase in turbidity (Fig. 8A). Further evidence for the lack of self-association of fragment P1 was obtained with velocity sedimentation experiments (Fig. 8B). Intact laminin and P1 were incubated at various concentrations in parallel at 35°C for 6 h. After this incubation 125I-labeled P1 and laminin were separately loaded onto sucrose gradients and centrifuged for 13 h. Fragment P1 migrated as a single narrow peak of about 5 S and did not yield substantial amounts of complexed material even at the highest concentration (0.3 mg/ml), in contrast to intact laminin, which polymerized at concentrations above about 0.1 mg/ml (Fig. 8B).

Fragment P1 at 0.15 mg/ml, a concentration which is approximately equimolar to that of laminin (0.5 mg/ml), can be visualized with the technique of rotary shadowing as three short rods connected at one end as described previously (4). Prior to incubation at higher temperature, P1 is seen exclusively in a monomeric form. Following incubation at the same concentration at 35°C for 30–60 min, P1 remained monomeric (data not shown). When P1 and intact laminin were co-incubated in equimolar concentrations (5 × 10⁻⁷ M) for 30–60 min at 35°C and then examined with the technique of rotary shadowing, laminin was associated to complexes, while P1 did not associate with itself or intact laminin (data not shown). Furthermore, when intact unlabeled laminin (4 × 10⁻⁷ M) was incubated with labeled 125I-P1 (4 × 10⁻⁷ M) and analyzed by velocity sedimentation, the amount of labeled material sedimenting faster than 11.4 S was seen to be 2.5% of the total in the absence, and 4.1% in the presence of intact laminin, supporting the rotary shadow data described above, i.e. P1 will neither polymerize nor bind to intact laminin.

Cation Dependency of Polymerization—When laminin was incubated in the presence of EDTA, turbidity was not seen to develop beyond the initial 1–2-min phase (Fig. 9). However, when laminin was dialyzed first into EDTA followed by dialysis into buffer containing different concentrations of calcium, and then incubated at 35°C, the typical turbidity profile was re-established (Fig. 9). This is interpreted as evidence for a divalent cation dependency. The rate of turbidity increase was apparently greater if calcium was added as a bolus to laminin after several hours incubation with EDTA alone as opposed to dialyzing the calcium into EDTA-treated protein in the cold prior to incubation (Fig. 9, arrow). One possible explanation for the different rates under nonequilibrium conditions could be that the fraction of “nuclei” increased during incubation in the presence of EDTA; however, other explanations are possible. Since EGTA will mimic the EDTA inhibitory effect (data not shown), calcium may be the principal cation involved. However, turbidity reached nearly the same maximum for manganese (85%) although it was less for magnesium (69%) at 0.2 and 1 mM divalent ion concentrations.

Rotary shadowing was used to examine laminin incubated in the presence of EDTA at 35°C. Under this condition large polymers failed to form, even after a long (overnight) incubation period. After 60 min, laminin was seen as a mixture of free monomers and intermediate size oligomers, often linear (Fig. 10A). After many hours (Fig. 10B), the fraction of free monomers was further decreased while the fraction of intermediates increased. While single end-to-end associations predominated, one also could find complexes in which two to three arms of laminin were bound by other laminin molecules. This corresponded to a low level of turbidity. If calcium was added in excess to such a laminin and EDTA mixture, turbidity and large aggregated complexes were observed again as discussed above.

Size Distribution—The mass frequency distribution of the size of laminin complexes before and after incubation was estimated from rotary shadow platinum replicas (Fig. 11). Laminin maintained at 4°C was seen to be about 80% monomeric (on a mass basis) and the remainder of the molecules were dimers to pentamers. It is not clear why even in the cold a small fraction of oligomers is present although the preparation could contain nondissociable components. A dramatic shift in the oligomeric size distribution occurred when laminin was warmed at 35°C. The fraction of monomer was seen to be decreased to about 25% while a new polymeric species emerged as the major component. There was a relative paucity of oligomeric intermediates and therefore this profile could
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be compatible with a cooperative assembly process. However, when laminin was incubated in the presence of EDTA, an increase in oligomers and substantial reduction of large aggregates was confirmed by the mass frequency distribution of the size of laminin complexes (Fig. 11C). Oligomers associated end-to-end were frequently identified and this fraction apparently is enriched.

We believe that the accuracy of the method used to estimate the frequency distribution of the complexes decreases with increasing aggregate size. At low oligomeric number, the size can be confirmed by direct inspection. However, at high oligomer number, the method is probably inaccurate in that molecules can be piled on top of other molecules leading to an underestimation of the amount of polymer. However, even given this difficulty, the basic observation, i.e. that the intermediates are small in amount relative to monomers and polymers, should hold.

The data presented in Figs. 1, 3, 4, and 11 are compatible with a nucleation-propagation mode of polymerization which can be mathematically treated as a two-stage process in which small nuclei serve as an intermediate or growth nidus. Further evidence in support of an assembly process involving at least two stages comes from studies using EDTA (Figs. 9-11). If laminin is incubated in the presence of EDTA, a low and constant level of turbidity is seen (Fig. 9) corresponding to the development of oligomers only (Figs. 10 and 11). On the other hand, if calcium is added to the EDTA in excess, or incubation is done in the absence of EDTA, large complexes can form. Hence the self-assembly process can be divided into two steps: an initial temperature-dependent, divalent cation independent step followed by a divalent cation-dependent step.

**Discussion**

Both, by turbidometric and sedimentation analysis, isolated laminin is seen to form large complexes when incubated at 35 °C in neutral phosphate buffer. This *in vitro* self-association process is seen to be time-, temperature-, and concentration-dependent. Furthermore, it is thermally reversible at 4 °C and it is affected by the concentration of divalent cations.

The observation of an apparent critical concentration for aggregation as well as the relative paucity of intermediates suggested by the sedimentation profile and seen by the rotary shadowing method is more compatible with a model of nucleation-propagation (16, 20), a positively cooperative association in which the initial step or steps are thermodynamically unfavorable (nucleation) as compared to the subsequent growth steps (propagation). This model has been shown to hold for a number of intracellular proteins such as actin (16, 22) or tubulin (16, 17) and it appears to hold for type I collagen as well (23). The alternate model is that of linear or noncooperative association of which tropomyosin (24) and spectrin (21) are examples. In the latter model there is no observed critical concentration and there is a wide distribution of molecular weight species.

Our data suggest that at least a two-step assembly process with three states of laminin can be identified: (a) an initial phase of 0-4 °C in which no turbidity is present and in which free monomers are seen; (b) a 35 °C phase after 1 min of incubation with a very low level of turbidity, maintained in the presence of EDTA, in which frequent oligomers are seen; and (c) a final polymerization state in which substantial turbidity develops and in which a mixture of large aggregates and occasional oligomers are observed.

In this paper we also presented evidence that laminin binds

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**Fig. 7. Laminin oligomers.** Rotary shadow platinum replicas were prepared following incubation of laminin as described in Fig. 5H. Selected replicas of laminin oligomers are shown on the left which reveal end-to-end associations. Interpretive drawing shown on right. Bar equals 100 nm.
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FIG. 8. Comparison of laminin and P1 by turbidity and sedimentation analysis. A, intact laminin (closed circles) and P1 (open circles), both at 0.3 mg/ml in neutral phosphate buffer, were incubated at 35°C and turbidity was followed over time. B, 125I-laminin (closed circles) and 125I-P1 (open circles) were adjusted to various final concentrations in neutral phosphate buffer and incubated at 37°C for several hours. 0.2-ml aliquots were then analyzed by velocity sedimentation on sucrose gradients. Proteins which migrated farther than the main monomeric peak were counted as polymer. The data are expressed as polymer fraction (data from several sedimentation runs).

to itself at specific sites, the globular domains at the ends of the four arms of the cross-shaped molecule. The evidence is 2-fold. First, such associations were identified on images of rotary shadow platinum replicas of many of the oligomeric intermediates. Second, the pepsin-generated P1 fragment, which lacks the end regions as well as the long arm, failed to self-associate or bind to intact laminin under conditions which permitted the polymerization of intact laminin. However, associations could neither be demonstrated nor ruled out in the large aggregates which were formed when laminin was incubated in the absence of EDTA. Given that the assembly process can be divided into two stages, we cannot be sure that the latter complexes are held together only by end-to-end interactions. It is entirely possible that in the second step other interactions take over or even that each laminin subunit undergoes conformational rearrangements.

Although we cannot rule out a more complex assembly process for the second step we currently favor the concept of a two-stage polymerization process in which only end-to-end associations are permitted. Cooperativity might be explained on the basis of the initial association involving only single end-to-end couplings while in the later associations monomers could bind by coupling at two or more sites (i.e. using two or more different types of sites). The two-stage polymerization process is consistent with the observed cooperativity. At low concentrations, the first coupling is rate-limiting, while at high concentrations, the second coupling is rate-limiting.

FIG. 9. Effect of EDTA and calcium on laminin turbidity. Laminin was made up to 10 mM EDTA in 50 mM Tris-Cl, pH 7.4, and 0.4 M NaCl and dialyzed either into 5 mM Tris-Cl, pH 7.4, and 130 mM NaCl with 0.1 mM EDTA, or into 5 mM Tris-Cl, pH 7.4, and 130 mM NaCl with 0.25 or 1.0 mM CaCl2, MgCl2, or MnCl2 overnight in the cold. The samples were then centrifuged as described and adjusted to 0.35 mg/ml. Turbidity was measured at 35°C and is shown for calcium. Laminin in EDTA (open circles); laminin in 0.25 mM CaCl2 (closed circles); laminin in 1 mM CaCl2 (closed triangles). At time indicated by arrow, CaCl2 was added to the sample kept in EDTA to give a final concentration of 1.1 mM.

FIG. 10. Effect of EDTA on appearance of laminin complexes. Laminin was incubated at 35°C at 0.5 mg/ml in 5-10 mM EDTA for either one-half hour (A) or overnight (B), and after dilution into ammonium acetate/glycerol the preparation was sprayed onto mica sheets and was prepared for rotary shadowing. Bar equals 200 nm.
more of the laminin arms). Thus one might visualize the assembly process as an initial unfavorable formation of linear oligomers followed by the more favorable formation of a two-dimensional mesh of laminin molecules.

What would the significance of such a polymerization process be in the context of a basement membrane? If this self-association process is important in vivo, as we suspect it is, then such a polymer might fill a type IV collagen scaffolding (13, 25). The type IV collagen matrix itself presumably is not sufficiently tight (13) to account for the observed sieving properties of, for example, the glomerular basement membrane. The capability of laminin to self-associate into a polymer might provide a means to reduce the pore size of the sieve. Thus, a laminin polymer of a given size (according to the local concentration of the protein and the ionic conditions of the extracellular environment) could possibly associate with the network produced by type IV collagen (13), but also with other components (5, 8, 26-28), and combine to form a pore of a smaller size which is used to restrict macromolecular permeation across basement membranes.

We cannot assume, however, that the various laminin polymer forms seen in vitro are necessarily found in basement membranes. The presence of other components such as heparan sulfate proteoglycan, entactin, and nidogen might conceivably inhibit or otherwise modulate the self-assembly of laminin in a manner perhaps analogous to the control of actin assembly (29). For instance, the second in vitro step could be prevented by interactions of laminin with other macromolecules and thus laminin could conceivably exist in the basement membrane as small oligomers only or as small clusters of molecules. In a previous study, Wewer et al. (30) examined platinum replicas of laminin isolated from placental basement membranes and observed dimers, trimers, and other oligomers quite commonly in the extract. It is interesting that these oligomers are also end-to-end associated. However, the presence of contaminating basement membrane macromolecules such as heparan sulfate proteoglycans was not rigorously excluded and could have accounted for the presence of the laminin complexes. Binding sites for heparan sulfate proteoglycans have been described on the laminin molecule (7). It is conceivable, that multiple binding sites exist on the globular domains of laminin and other macromolecules could serve to stabilize laminin oligomers or polymers.

Given the observation that divalent cation (probably calcium) is needed for the second step of assembly, it is tempting to speculate that this might allow for cellular control of the assembly process by preventing the final self-assembly step until laminin is released into the extracellular space in which the concentration of calcium is high.

With the data at hand we propose that the self-assembly properties studied in vitro and described in this report are intrinsic properties of laminin, that they may be important for basement membrane assembly during development and organogenesis (31), and that they will allow further insight into the organization and function of basement membranes.

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