THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 50, Issue of December 12, pp. 31525–31532, 1997
Printed in U.S.A.

Self-assembly of Laminin Isoforms*

(Received for publication, April 11, 1997, and in revised form, October 1, 1997)

Yi-Shan Cheng‡, Marie-France Champliaud§, Robert E. Burgeson§, M. Peter Marinkovich¶‖, and Peter D. Yurchenco‡**

From the ‡Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, the ¶Division of Dermatology, Palo Alto Veterans Affairs Health Care System, Palo Alto, California 94061, the ‖Department of Dermatology, Stanford University School of Medicine, Stanford, California 94305, and the §Cutaneous Biology Research Center, Massachusetts General Hospital-East, Harvard Medical School, Charlestown, Massachusetts 02129

The α, β, and γ subunits of basement membrane laminins can combine into different heterotrimeric molecules with either three full short arms (*e.g.* laminins-1–4), or molecules containing one (laminins-6–9) or more (laminin-5) short arm truncations. Laminin-1 (α1β1γ1), self-assembles through a calcium-dependent thermal gelation requiring binding interactions between N-terminal short arm domains, forming a meshwork polymer thought to contribute to basement membrane architecture (Yurchenco, P. D., and Cheng, Y. S. (1993) *J. Biol. Chem.* 268, 17286–17299). However, it has been unclear whether other isoforms share this property, and if so, which ones. To begin to address this, we evaluated laminin-2 (α2β1γ1), laminin-4 (α2β2γ1), laminin-5 (α3Aβ3γ2), and laminin-6 (α3Aβ1γ1). The first two isoforms were found to self-aggregate in a concentration- and temperature-dependent manner and a close self-assembly relationship among laminins-1, -2, and -4 were demonstrated by: (*a*) polymerization of all three proteins was inhibited by EDTA and laminin-1 short arm fragments, (*b*) polymerization of laminin-1 was inhibited by fragments of laminins-2 and -4, (*c*) laminin-2 and, to a lesser degree, laminin-4, even well below their own critical concentration, co-aggregated with laminin-1, evidence for co-polymerization. Laminin-5, on the other hand, neither polymerized nor co-polymerized with laminin-1. Laminin-6 failed to co-aggregate with laminin-1 at all concentrations evaluated, evidence for a lack of a related self-assembly activity. The data support the hypothesis that all three short arms are required for self-assembly and suggest that the short arm domain structure of laminin isoforms affect their architecture-forming properties in basement membranes.

Basement membranes are animal extracellular matrices that appear early in development and that are present in nearly all tissues. They act as supportive architecture for communities of cells, providing differentiation and migration information that are transmitted through cell receptors. These matrices consist of one or more members of the laminin family, one or more members of the type IV collagen family, entactin/nidogen, and smaller amounts of other components such as perlecan (heparan sulfate proteoglycan). From studies of the extracellular matrix components originally discovered in the EHS[1] matrix and elsewhere, a model for the assembly and architecture of an idealized basement membrane scaffolding has been proposed (1, 2). Central to this model are the binding interactions of "classical" laminin, type IV collagen, and entactin/nidogen in which three groups of interactions play key roles: type IV collagen polymerization, laminin polymerization, and the stabilizing connections of entactin/nidogen. The type IV collagen network consists of triple helical monomers joined together through lateral, N-terminal, and C-terminal interactions (3, 4). The N-terminal (7S) and C-terminal (NC-1) bonds become stabilized by reducible and irreducible covalent cross-links. The laminin polymer is self-assembled from monomers in a calcium-dependent, reversible, and cooperative heat-gelation with a measured critical concentration of assembly of 70–140 nM (5, 6). It has been found, based on biochemical and electron microscopic studies conducted with laminin-1 and laminin-1 fragments, that all three short arms are involved in polymerization and furthermore all three appear to be essential for assembly (7, 8). Entactin/nidogen can form a stable noncovalent bridge between laminin and type IV collagen and also bind to perlecan and the fibulins (2). The resulting architecture, as proposed, is that of a double polymer network bearing stabilizing entactin/nidogen cross-links.

However, the different laminin and type IV collagen family isoforms recently identified may provide a molecular basis for distinct architectural variations that in turn create functional diversity. Naturally occurring and targeted mutations in the various chains of these two families illustrate functional differences. For example, mutations in the chains of laminin-5 cause skin blistering, with a loss of epidermal-dermal anchorage at hemidesmosome sites in diverse tissues. A null mutation in the β2 chain of laminin, present in glomerulus, leads to profound renal failure despite apparent compensation by the β1 chain. Mutations that affect the α2 chain of laminin-2 can cause a muscular dystrophy despite partial replacement of this chain by the α1 chain (9–11). Two questions arise. First, how universal is the laminin self-assembly model? Second, if there are differences in self-assembly properties among different members of the laminin and collagen-IV families, what are these differences and can they explain basement membrane structural and functional heterogeneity? In the case of the laminins, we already recognize five α, three β, and two γ subunits that can combine into at least eleven heterotrimeric molecules (12–

* This work was supported by National Institutes of Health Grants R01-DK36425 (to Y. S. C. and P. D. Y.), R01-AR 35689 (to M. F. C. and R. E. B.), and P01-AR-44012 (to M. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. Pathology & Laboratory Medicine, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: 732-235-4674; Fax: 732-235-4825; E-mail: yurchenc@rwja.umdnj.edu.

[1] The abbreviations used are: EHS, Engelbreth-Holm-Swarm; HMB, *p*-hydroxymecuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

16). At the level of domain structure, one can define three groups: laminins that contain a complete complement of domains (laminins-1, -2, -3, -4, -10, -11), laminin which lacks domains in all three short arms (laminin-5 containing the common α3A chain splice variant), and laminins which lack an α chain short arm but which retain full-sized β and γ chains (laminins-6, -7, -8, and -9). The three-short arm hypothesis for laminin assembly predicts that only the "full-sized" laminins that have three short arms would be able to readily polymerize.

In this study we have evaluated the capacity of two full-sized laminin isoforms, a laminin isoform bearing extensive short arm truncations, and a laminin isoform bearing a truncation of a single short arm, to self-assemble. In developing approaches to study some laminin isoforms, we needed to overcome the problem that unlike laminin-1, the other forms of laminin can be obtained in only modest (laminins-2, -4, and -5) or only very small (laminin-6) amounts. A solution to this difficulty was accomplished by developing a specific laminin co-polymerization assay which exploits the abundance of laminin-1, using it to drive the specific polymerization of other laminin isoforms. From the analysis we found that only full-size laminins were capable of laminin-1 type self-assembly.

MATERIALS AND METHODS

Proteins and Protein Purification—(*a*) Laminin-1, either free or complexed with entactin/nidogen, and its fragments E4 and E1′ were purified from mouse EHS tumor as described previously (17). 85–95% of protein was typically capable of polymerization (determined from slope of a plot of total *versus* polymer concentration). (*b*) Fresh normal term human placentas, stored initially on ice, were obtained 1–3 h after delivery from the Medical Center at Princeton (Princeton, NJ) or the St. Peter's Medical Center (New Brunswick, NJ). After removal of blood samples to screen for hepatitis antigens and human immunodeficiency virus, the tissue was cut up into small pieces, frozen in liquid nitrogen, and stored at −80 °C until use. Laminin-2 and laminin-4 were isolated from human placenta by ion-exchange, gel filtration, and heparin affinity chromatography as follows: ~800 g of human placenta was homogenized and washed in a total of 5 liters of 50 mM Tris-HCl, 100 mM NaCl pH 7.4 (TBS) containing 1% Triton X-100, 10 μg/ml *p*-hydroxymecuribenzoic acid (HMB), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM diisopropyl fluorophosphate. The suspended tissue was washed by several rounds of centrifugation (10,000 rpm for 30 min at 4 °C) and re-suspension. The pelleted material was then re-suspended in 400 ml of TBS containing 2 mM $CaCl_2$, 10 μg/ml HMB, 0.5 mM diisopropyl fluorophosphate, and 0.5 mM PMSF and incubated with 50 μg/ml bacterial collagenase (Worthington, CLSPA grade) at 37 °C for 20 h. The incubation mixture was adjusted to 10 mM EDTA and stirred for 6–8 h on ice followed by centrifugation (20,000 rpm, 30 min, 4 °C). The supernatant was precipitated with 40% saturated $(NH_4)_2SO_4$, centrifuged, and the pellet resuspended in 100 ml of TBS containing 2 mM EDTA, 10 μg/ml HMB, and 0.5 mM PMSF. The soluble sample was then passed down a column of Sepharose CL-6B (Pharmacia Biotech Inc.; 5 cm × 95 cm) equilibrated in TBS, 2 mM EDTA, 0.5 mM PMSF, 10 μg/ml HMB in the cold. The void peak was collected and loaded onto a DEAE-Sephacel column (Pharmacia, 5 × 11 cm) equilibrated in the same buffer and eluted with a linear 0–0.8 M NaCl gradient in a total volume of 2 liters. The first eluted peak was pooled, concentrated in dialysis bags with Aquacide (Calbiochem, La Jolla, CA), and the protein was then bound to an HPLC TSKgel DEAE-5PW column (5 cm × 0.5 cm inner diameter, glass; Toso-Haas, Philadelphia, PA) equilibrated in 50 mM Tris-HCl, pH 8.0, with 2 mM EDTA, at room temperature (~25 °C) and eluted with a shallow linear 0–0.5 M NaCl gradient. The first eluted peak contained a mixture of laminins-2 and -4 with the leading edge of the broad peak enriched in laminin-4. The central peak and leading edge fractions were separately purified by binding to an HPLC heparin-5PW column (0.5 cm inner diameter × 5 cm, glass; Toso-Haas) equilibrated in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA and eluted with a linear 0–1 M NaCl gradient. The main peak from each was collected, concentrated, and stored in TBS. Laminin-2 was purified from the laminin-2/4 mixture by adjusting the sample to 0.3 M $(NH_4)_2SO_4$ and passing the sample through an fast protein liquid chromatography phenyl-Superose HR5/5 column (Pharmacia, 0.5 cm inner diameter × 5 cm) equilibrated in TBS containing 0.3 M $(NH_4)_2SO_4$ and 0.1 mM EDTA. The unbound protein was laminin-2. The fraction of the laminin-2/4 preparation capable of self-aggregation was found to vary from ~40 to over 60%. An increase in the fraction of active protein (used with preparations of low activity) was achieved by subjecting the preparation of laminin-2/4 to a polymerization-depolymerization cycle (*i.e.* heat-gelled and the sedimented gel subsequently re-solubilized in 10 mM EDTA on ice overnight). The remaining supernatant protein was intact as judged by SDS-PAGE, had the same elastase digest pattern by SDS-PAGE, but was largely inactive in both the assembly and co-assembly assays (described ahead) and may have corresponded to a denatured fraction that accumulated in the placenta prior to cooling and extraction. The recovered amounts of isolated laminin-2 and laminin-4 were too small to evaluate in direct polymerization assays and instead were evaluated by co-polymerization. (*c*) Laminin-5 was purified from conditioned SCC-25 culture medium by monoclonal antibody purification as described previously (18). (*d*) Laminin-6 was obtained using conditioned medium from SV-40 immortalized keratinocytes lacking the γ2 chain (LSV) and that secrete laminin-6 but not laminin-5 (19). Two-hundred ml of conditioned serum-free keratinocyte medium from these cells was precipitated with ammonium sulfate (30% saturation), dissolved in 1 to 2 ml of TBS, and dialyzed against the same buffer.

Antibodies—Polyclonal antibody specific for the laminin α2G domain (C-terminal globule) was prepared by immunizing a New Zealand White rabbit with recombinant human laminin α2G domain generated with the baculovirus system in Sf9 cells and purified as described (20, 21). The antibody, purified by protein A affinity chromatography, reacted in enzyme-linked immunosorbent assays, and in Western immunoblots, with the G domain of laminins containing the α2 chain, but not the α1 chain. The antibody was also evaluated by tissue immunofluorescence with frozen sections of human skeletal muscle and kidney, reacting in a highly restricted fashion with the peri-myotubular basement membranes and kidney. A rabbit polyclonal antibody (anti-kalinin, as described in Marinkovich *et al*. (27) specific for human laminin-5 and laminin-6 and reacting with α3 chain but poorly with the chains of mouse laminin-1 was used to detect laminin-6.

Protein Determinations, SDS-Polyacrylamide Gel Electrophoresis, Protein Immunoblotting, Gel Densitometry, and Phosphorimaging—(*a*) Protein in solution was determined by absorbance at 280 nm as described (22). Laminin-2 and laminin-4 concentrations were determined in Coomassie Blue-stained acrylamide gels by gel densitometry in comparison to laminin-1 standards. (*b*) SDS-PAGE was carried out in 3.5–12% or 3–5% linear gradient gels as described (7) and stained with Coomassie Brilliant Blue R-250. (*c*) Electrophoretic transfer of proteins onto 0.2 μm nitrocellulose membranes (Schleicher & Schuell) was performed by Western immunoblot (23). Immunoblots were prepared by incubating nitrocellulose membranes with either 10 μg/ml rabbit anti-laminin α2 followed by ^{125}I-protein A (2.5×10^6 cpm/ml) or by incubating the membranes with a 1/100 dilution of rabbit anti-kalinin antiserum containing 50 μg/ml elastase-generated laminin-1 fragments (to block residual laminin-1 staining seen at high laminin-1 concentrations), followed by a 1/40,000 dilution of anti-rabbit IgG coupled to alkaline-phosphatase (Sigma). ^{125}I-Protein A reacting bands were visualized by fluorography, while alkaline phosphatase conjugate-IgG-treated membranes were visualized after treatment with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine and *p*-nitro blue tetrazolium chloride using a 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine-*p*-nitro blue tetrazolium chloride color development kit obtained from Bio-Rad and processed according to the manufacturer's instructions. (*d*) Gel densitometry and phosphorimaging: Coomassie Blue-stained acrylamide gels were scanned with a GS-670 gel densitometer (Bio-Rad) and evaluated with accompanying "Molecular Analyst" (version 1.1) software. Gel bands were quantitated against a known laminin-1 standard after subtraction of background gel levels and linearity in the range to be studied was initially established with known concentrations of laminin-1. Radiolabeled bands were detected after exposure of immunoblots to either an HS or CS phosphor screen (Bio-Rad) with a GS-363 molecular imager (Bio-Rad) and quantitated as volumes (sum of pixel densities over the area of the band) using the above software.

Polymerization Assays—Aliquots of laminin in polymerization buffer (TBS with 1 mM $CaCl_2$, 0.1% Triton X-100), at various concentrations, were incubated at 37 °C for 3 h in 0.5-ml Eppendorf tubes. The samples were then centrifuged (10,500 rpm, 15 min) and the supernatant and pelleted fractions solubilized in Laemmli solubilizing buffer under reducing conditions. For co-polymerization assays, laminin isoform was incubated with 0–0.5 mg/ml of laminin-1 in polymerization buffer in 50-μl aliquots. The incubation procedures were identical as in the polymerization assay. The fractions were analyzed by SDS-PAGE and either stained with Coomassie Blue or, after electroblotting onto nitro-

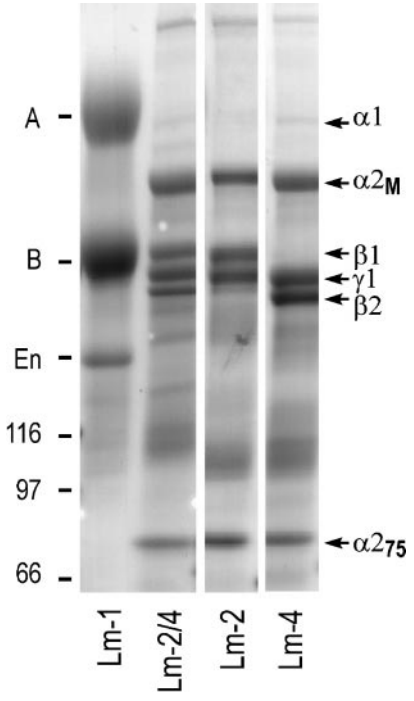

FIG. 1. **Laminin-2 and laminin-4 isoforms.** Laminin isoform fractions extracted from human placenta with EDTA/collagenase and purified as described were analyzed by 3.5–12% SDS-acrylamide gradient gel electrophoresis under reducing conditions and stained with Coomassie Blue. The lanes shown, from *left* to *right*, are EHS laminin-1/nidogen, a laminin-2/laminin-4 mixture, laminin-2 ($\alpha2\beta1\gamma1$ chains), and laminin-4 ($\alpha2\beta2\gamma1$ chains). The *A* ($\alpha1$), *B* ($\beta1/\gamma1$), and entactin/nidogen (*En*) bands of the EHS complex are indicated on the *left*. The $\alpha2$ chain, when subjected to SDS-denaturation/reduction, separates into a larger N-terminal moiety of ~300 kDa (designated $\alpha2_m$) and a smaller C-terminal moiety of ~75 kDa (designated $\alpha2_{75}$). Under native conditions, they remain associated with the $\beta1/2$ and $\gamma1$ chains.

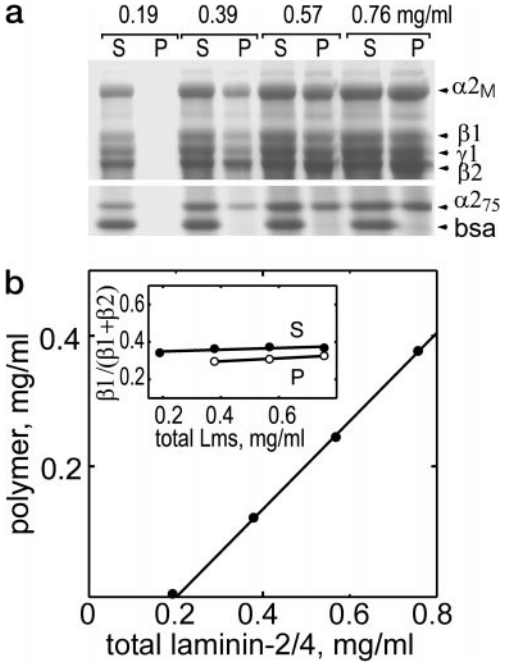

FIG. 2. **Polymerization of laminin-2 and laminin-4.** Laminin-2/4 (mixed fraction, following purification through an aggregation/dissociation cycle) was incubated in polymerization buffer at 37 °C in 50-μl aliquots at increasing concentrations and then centrifuged in Eppendorf tubes to sediment the polymer. The amount of laminin in each fraction was analyzed by SDS-PAGE (*a*, Coomassie Blue-stained) and quantitated by gel densitometry (*b*). Laminins-2 and -4 as indicated by the respective $\beta1$ and $\beta2$ chain partitioning between the supernatant (*S*) and pellet (*P*) fractions (*inset*), polymerized nearly equivalently. A critical concentration of 0.14 mg/ml (200 nM) was calculated for laminin-2/4 from the mathematical product of the slope (0.7) and *x* intercept (0.2 mg/ml) and compares with 70–140 nM for laminin-1. The activity of the preparation was judged from the slope to be 70%.

cellulose membranes, incubated with appropriate antibody and ^{125}I-protein A or, in the case of laminin-6 co-polymerization, with antibody followed by alkaline phosphatase detection. A small aliquot of bovine serum albumin (BSA), which does not bind to laminin, was added to most co-polymerization incubation mixtures to determine the level of nonspecific protein adhesion to the tube (<1%) and the level of nonspecific trapping of protein in the laminin polymer pellet (generally 1-~7% in the range of 0.05–0.5 mg/ml laminin-1). Protein A, which does not bind to laminin, was used to measure nonspecific sticking and trapping for determination of laminin-6 co-aggregation (several bands present in the serum-free conditioned medium migrated in the region of BSA, making it difficult to quantitate).

RESULTS

Aggregation of Laminin-2/4—The laminin-2/4 (Fig. 1) purified from human placenta as described was found to have similar purity compared with an earlier method but with substantially higher yields (24). The first and major fraction (several milligrams per placenta) consisted of a mixture of laminin-2 and laminin-4 ($\alpha2$, $\beta1$, $\gamma1$, $\beta2$ chains). The second fraction consisted of laminin-2 ($\alpha2$, $\beta1$, $\gamma1$ chains) and the third fraction consisted of laminin-4 ($\alpha2$, $\beta2$, $\gamma1$ chains). The latter two fractions could only be obtained in limited (micrograms to milligrams) amounts for study. The $\alpha2$ chain had the characteristic ~300 kDa "M" band and 75–80 kDa C-terminal band (25). The two fragments co-purified through all steps of purification and remained associated under native conditions. The $\beta1$, $\gamma1$, and $\beta2$ bands migrated at 210, 200, and 190 kDa. The fraction of laminin-2 in the laminin-2/4 purified fraction, as judged by the ratio of $\beta1$ to $\beta2$ chain, varied from ~0.35 to 0.5 in different preparations. The laminin-2/4 and laminin-4 fractions were examined by rotary shadow electron microscopy and were found to be indistinguishable in appearance from laminin-1 (data not shown).

The laminin-2/4 fraction was evaluated for its ability to polymerize under standard conditions (neutral isotonic salt buffer, 37 °C, 1 mM calcium) using an aggregation-sedimentation assay. When purified laminin-2/4, after a recycling purification step (see methods), was incubated at different protein concentrations, it was found to polymerize with a higher slope (Fig. 2; slope = 0.7) and compared with slopes ranging from ~0.8 to 0.95 for different preparations of EHS laminin. The corrected critical concentration, reflecting a cooperative nucleation-propagation type of self-assembly, was calculated to be 200 nM, or 1.5–2 times the value for EHS laminin.[2] The fraction of laminin-2 and laminin-4 (determined from the ratio of $\beta1$ to $\beta2$ chain in Coomassie Blue-stained gels) varied little at different concentrations, indicating that the two isoforms aggregated nearly equivalently.

We also evaluated laminin-2/4 preparations prior to the recycling enrichment step (data not shown). These preparations aggregated in a concentration-dependent manner, and from the slopes it was estimated that ~40->60% of the unfractionated protein was active. We have examined the polymerizing and poorly polymerizing fractions separated by recycling and identified no obvious size or elastase-fragment differences by SDS-PAGE or immunoblotting with anti-$\alpha2$G, anti-$\beta2$, or anti-$\beta1$ antibodies. The differences, more subtle in nature, might be due to partial inactivation of this conformationally sensitive

[2] Calculated from the product of the slope and *x* axis intercept given that the predicted slope for a pure polymerizing protein that follows nucleation-propagation thermodynamics is one. Thus 70% of the laminin-2/4 preparation is active.

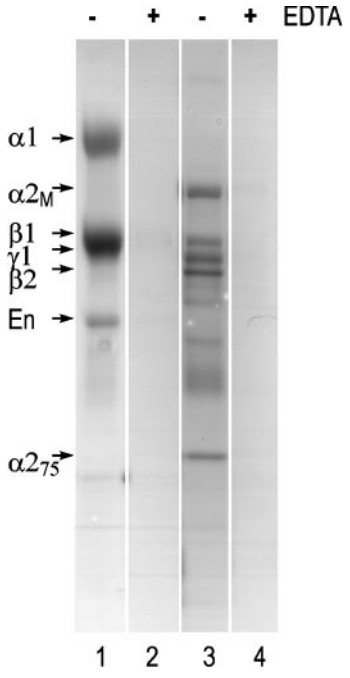

FIG. 3. **Divalent cation dependence of laminin-2/4 polymerization.** Laminin-2/4 was incubated at 37 °C in TBS in the presence of 1 mM calcium (polymerization buffer) or 10 mM EDTA and the pelleted material analyzed by SDS-PAGE under reducing conditions. Treatment with EDTA (+), compared with controls in 1 mM calcium (−), inhibited polymerization of both laminin-1 (*lanes 1* and *2*) and laminin-2/4 (*lanes 3* and *4*). *En*, entactin/nidogen.

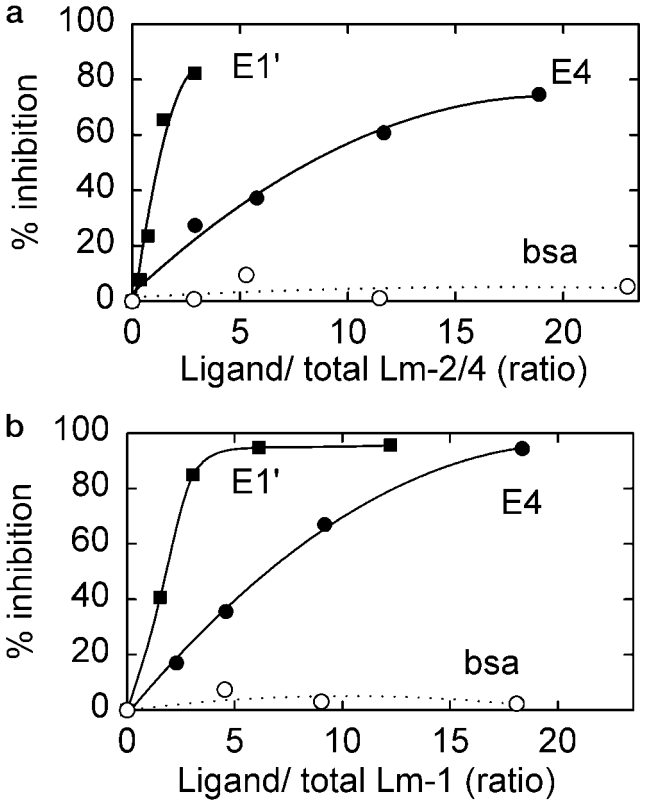

FIG. 4. **Inhibition of laminin-2/4 polymerization with laminin-1 fragments.** It has been shown previously that laminin-1 fragments E1′ (short arm complex containing N-terminal domains of the α and γ chains) and E4 (short arm fragment containing domains VI and V of the β chain) specifically inhibit laminin-1 polymerization. Laminin-2/4 (*upper panel*, 0.87 mg/ml total laminin, 0% inhibition corresponding to 0.25 mg/ml polymer) and laminin-1 (*lower panel*, 0.25 mg/ml total laminin, 0% inhibition corresponding to 0.1 mg/ml polymer) were incubated in the presence of increasing concentrations of BSA, E1′, or E4. The laminin fragments inhibited the formation of laminin-2/4 and laminin-1 polymers, and therefore, it was interpreted as evidence that the different laminins were self-assembled by a related mechanism.

protein during purification or possibly reflect differences of post-translational modification (*e.g.* carbohydrate) or splicing variations in the self-assembly domains.

Specific Inhibition of Laminin Polymerization—It has been shown previously that laminin polymerization is dependent on the presence of divalent cation (in particular calcium), which appears to bind to domain VI of the γ1 chain (7). Laminin-1 polymerization can be specifically inhibited with either an unfractionated elastase digest of laminin-1 or with purified laminin fragments E4 and E1′ which contain the N-terminal short arms (7). Laminin-1 (as control), and a mixture of laminin-2 and laminin-4, were incubated in TBS containing either 1 mM calcium or 10 mM EDTA and evaluated for aggregation as described (Fig. 3). EDTA was found to inhibit the aggregation of all three laminins. Laminin-1 and laminins-2 and -4 were also co-incubated in increasing concentrations of fragments E4 and E1′. The two fragments, in contrast to an albumin control, inhibited the polymerization of all these laminins (Fig. 4). The inhibition activity achieved with fragment E1′, which contains the α1 and γ1 short arms, was significantly greater than that achieved with fragment E4, which contains part of the β1 short arm, as reported previously (7).

Treatment of the laminin-2/4 mixture with elastase converted the laminin to a mixture of fragments whose sizes, as determined under reducing SDS-PAGE, were 210, 120, 70, 61, 55, 49, 32, and 20 kDa (Fig. 5*a*). When an elastase digest of laminin-2/4 was co-incubated with a fixed concentration of laminin-1, the fraction of aggregated laminin-1 was progressively decreased as the fragment concentration was increased (Fig. 5*b*). The possibility that residual protease activity (digestion was inhibited with PMSF and diisopropyl fluorophosphate) degraded the laminin-1 could be ruled out, because the products were analyzed by SDS-PAGE with no degradation of laminin-1 detected. Thus it was concluded that the elastase fragments of one polymerizing laminin will inhibit the aggregation of a polymerization-competent isoform.

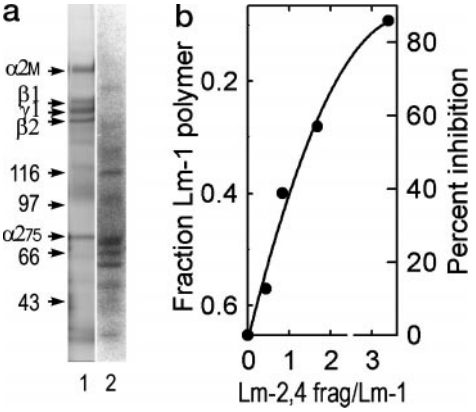

FIG. 5. **Effect of elastase-digested laminin-2/4 on the polymerization of laminin-1.** *a*, Coomassie Blue-stained gel (SDS-PAGE, reducing conditions). Laminin-2/4 (*lane 1*), treated with pancreatic elastase, was cleaved into a series of fragments (*lane 2*). *b*, aliquots of elastase-fragmented laminin-2/4 were incubated with a fixed concentration of laminin-1 (0.25 mg/ml, 85% active) in polymerization buffer at 37 °C, centrifuged to separate aggregated from soluble laminin, and analyzed by SDS-PAGE (Coomassie Blue-stained gels). Fragmented laminin-2/4 was observed to progressively inhibit laminin-1 polymerization.

Co-polymerization of Laminin-1, Laminin-2, and Laminin-4—If two or more laminins use a shared mechanism and binding sites to form a polymer, it is predicted that they will

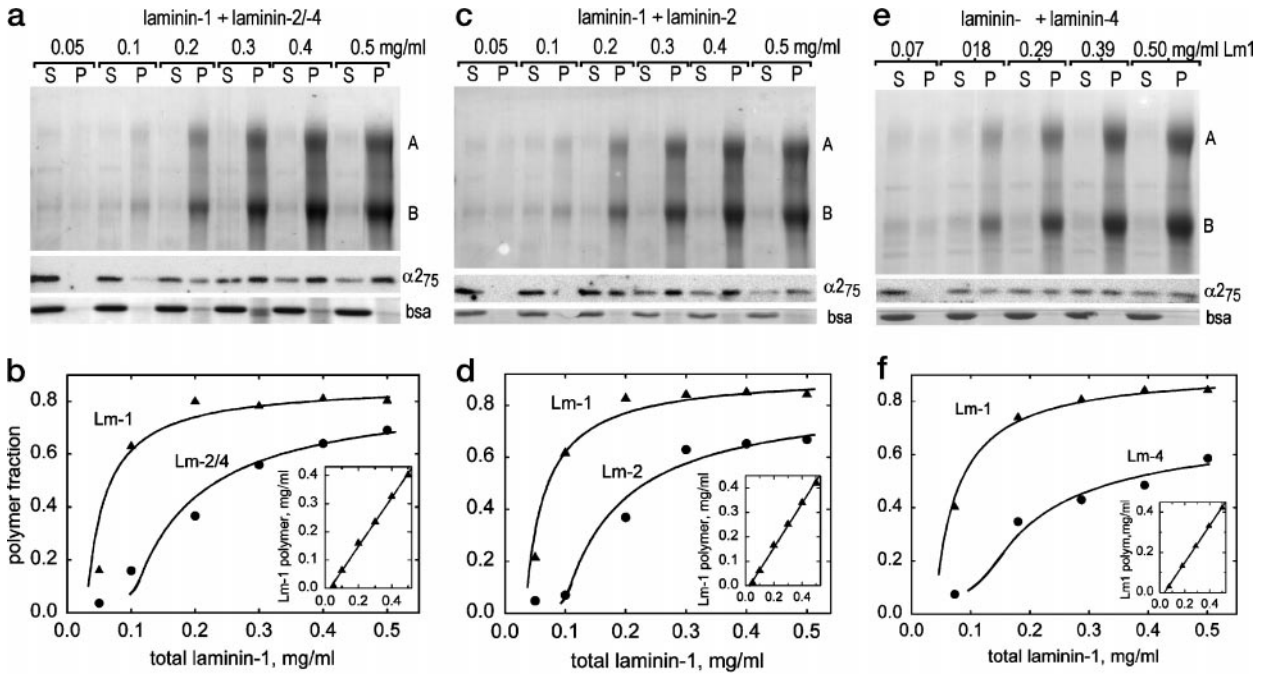

FIG. 6. **Co-assembly of laminin-2 and laminin-4 with laminin-1.** Aliquots of laminin-2/4 (15 μg/ml, constant; *a* and *b*), laminin-2 (15 μg/ml, constant; *c* and *d*) and laminin-4 (12 μg/ml, constant; *e* and *f*) were co-incubated in duplicate 50 μl (*a–d*) or 40 μl (*e* and *f*) aliquots with increasing concentrations of laminin-1 at 37 °C for 3 h in TBS containing 1 mM calcium chloride and sedimented to separate supernatant (*S*) from pelleted (*P*) fractions. One set of fractionated aliquots was analyzed by SDS-PAGE with the laminin-1 A chain volumes quantitated by gel densitometry to determine the polymer fraction. The *upper frames* in *a, c*, and *e* show the Coomassie Blue (*CB*)-stained laminin-1 A (α1) and B (β1, γ1) chains. The second set of aliquots, following SDS-PAGE, was immunoblotted onto nitrocellulose, incubated with polyclonal α2G domain-specific antibody, and detected with radioiodinated protein A to determine the amount of α2–75-kDa band in the polymer fraction (*middle frames* of *a, c*, and *e*). Carrier BSA was detected by Amido Black staining and used to determine the degree of nonspecific protein trapping by the laminin pellet (lower frames). *b, d*, and *f*, quantitation of laminin-1 (gel densitometry) and laminin-2/4 (α2–75 phosphorimaging) fractions. A plot of laminin-1 polymer concentration is shown in the *insets* for each. Note the progressive increase in the fraction of laminin-2/4, laminin-2, and laminin-4 following fraction of laminin-1 polymer.

co-polymerize so long as the sum of all concentrations, and regardless of molar ratios, exceeds the critical concentration. It should therefore be possible to use laminin-1 to drive the self-assembly of other polymerization-competent laminins and to test for its ability to self-assemble with a mechanism similar to laminin-1. If the test protein binding sites are identical to that of laminin-1, the concentration dependence of its polymerization (a plot of fraction polymer *versus* total laminin concentration) would be expected to be coincident with that of laminin-1. If, on the other hand, the test protein activity is similar but not identical to that of laminin-1, then it may be expected that the polymerization profiles would not be coincident. To test this hypothesis, and to develop an assay to evaluate other laminins available only in very small amounts, we co-incubated laminin-1 at different concentrations (0.05–0.5 mg/ml range) with laminin-2/4, laminin-2, or laminin-4 at low fixed concentrations (<0.1 mg/ml) as shown in Fig. 6. Laminin-1 was chosen as the "driving" component. Following co-incubation, the supernatant and pellet fractions were analyzed by SDS-PAGE in combination with immunoblotting of the associated α2–75 G-domain. Quantitation was achieved by volume integration of radioactive α2–75 G-domain peak as detected by phosphorimaging, subtracting any nonspecific precipitation determined from fraction (usually ≤7%) of albumin in the pellet. In the first set of experiments, the laminin-2/4 mixture, previously shown to independently polymerize, was evaluated (Fig. 6, *a* and *b*). As the laminin-1 concentration was increased, a progressively larger fraction of the laminin-2/4 sedimented as measured by the distribution of α2–75 in the supernatant and pelleted fractions. Purified laminin-2 and laminin-4 were then evaluated for co-polymerization with laminin-1 (Fig. 6, *c–f*). The concentration dependence of aggregation for the three test laminins were not coincident with that of laminin-1. In particular, higher concentrations of total laminin was required to achieve comparable polymer fractions as compared with laminin-1. Furthermore, the profiles of laminin-2/4 and laminin-2 following that of laminin-1 more closely than that of laminin-4. These data suggest (*a*) that the polymerization interaction is similar, but not identical for the different laminins, and (*b*) that laminin-4 does not co-polymerize with laminin-1 as well as laminin-2 or a mixture of laminin-2 and laminin-4. Given laminin-4 shares only one short arm (γ1) in common with laminin-1, while laminin-2 shares two short arms (β1 and γ1) in common with laminin-1, it may be that the degree of co-polymerization among full-sized laminins is dependent on the number of short arms shared in common.

Characterization of Co-polymerization—We considered the possibility that laminin co-polymerization might be sensitive to the ratios between the "driving" laminin and the test laminin and that the degree of co-polymerization might decrease as molar equivalence between laminins was approached. We therefore evaluated laminin-2/4 co-polymerization with laminin-1 over a wide range of laminin-2/4 concentrations using a fixed laminin-1 concentration (0.25 mg/ml, below maximal co-polymerization), shown in Fig. 7. The fraction of laminin-2/4 polymer remained nearly constant from that of a minor fraction to molar equivalency (as molar equivalency is exceeded, the fraction would be expected to increase). The data suggest that the degree of co-polymerization is relatively insensitive to the concentration of the test laminin isoform when laminin-1 is used to drive the reaction. Furthermore, the co-aggregation assay provided a method to test laminins in small amounts for their ability to interact with laminin-1 through its polymer-forming bonds.

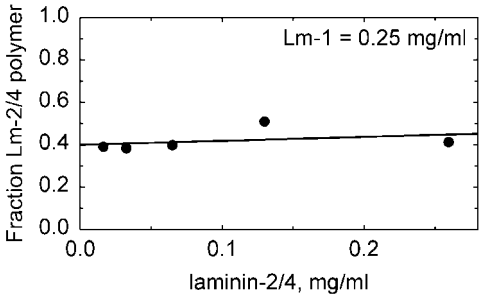

FIG. 7. **Laminins-2 and -4 co-aggregation with laminin-1 is independent of the concentration of the species held below its critical concentration.** Laminin-2/4 was serially diluted, co-incubated with a fixed (0.25 mg/ml) concentration of laminin-1 (0.7 polymer fraction) at 37 °C for 3 h and centrifuged to sediment polymer. The aliquots were separated by SDS-PAGE under reducing conditions, transferred by immunoblotting to nitrocellulose, and then incubated with laminin α2G antibody followed by radioiodinated protein A. The Western blot of the α2–75-kDa band is shown with the BSA trapping control. Note that the polymer fraction of laminin-2/4 was little changed at these widely different concentrations. Antibody could not detect the α2G domain below the lowest concentration shown.

The possibility that laminin-2 and -4 co-aggregated with laminin-1 through a nonpolymerization bond was also considered. The three laminins were co-incubated on ice and in the presence of EDTA, conditions that inhibit polymerization but that do not block most typical protein-protein interactions (Fig. 8). Laminin-2 and -4 were then immunoprecipitated with antibody specific for the α2 chain, and the chains present were detected by immunoblotting with α2G- and laminin-1-specific antibodies. Laminin-2/4 was found to be completely separated from laminin-1 under these conditions. Other evidence to support true co-polymerization is the finding of a cooperative[3] calcium-dependent laminin-2/4 polymerization in the absence of laminin-1 and the finding of the inhibition of laminin-2/4 polymerization with laminin-1 fragments as well as EDTA.

Evaluation of Laminin-5 Self-assembly—Laminin-5 (α3Aβ3γ2) is a rod-like laminin that lacks short arms. A purified preparation of laminin-5 obtained from SCC-25 conditioned medium was evaluated for both polymerization as an isolated protein (Fig. 9) and for co-aggregation with laminin-1 (Fig. 10). In these experiments, supernatant and pellet fractions of laminin-1 and laminin-5 were analyzed in Coomassie Blue-stained gels. Laminin-5 was detected and quantitated by the uniquely positioned bands under reducing conditions. No self-aggregation was detected at concentrations as high as 1 mg/ml, and no co-aggregation with laminin-1 was observed. Thus laminin-5, *un*like laminins-2 and -4, lacks the ability to polymerize or co-polymerize through the bonds used by laminin-1.

Evaluation of Laminin-6 Co-assembly with Laminin-1—Laminin-6 (α3Aβ1γ1) shares the β and γ chains in common with laminin-1; however, it lacks an α chain short arm, giving it a Y-shaped appearance. We evaluated the ability of this laminin, as obtained from an ammonium sulfate serum-free conditioned keratinocyte medium fraction, to co-polymerize with laminin-1. Laminin-6 was found not to co-assemble with laminin-1 (Fig. 11). This behavior was similar to that of laminin-5 and different from that of laminin-2 and laminin-4.

Because the laminin-6 study was conducted with conditioned medium enriched in laminin-6 rather than purified protein, we first evaluated the effect of conditioned medium from LSV cells (produce laminin-6) and SCC-25 cells (produce mostly laminin-5) on laminin-1 and laminin-2/4 co-assembly (data not shown). The critical concentration of the laminin-1 preparation

[3] As evidenced by a measurable critical concentration.

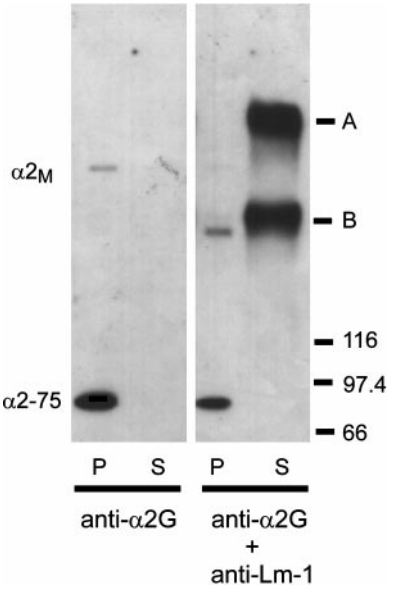

FIG. 8. **Laminin-2/4 immunoprecipitation in the presence of laminin-1.** Laminin-2/4 was mixed with laminin-1 in molar excess in TBS, 1 mM calcium (polymerization buffer, 25 μl), adjusted to 5 mM EDTA and placed on ice (nonpolymerization conditions). Three hours later rabbit anti-laminin α2G domain antibody (10 μg) was added and incubated at 5 °C overnight. The antibody was then precipitated with protein A-Sepharose-4B beads and centrifuged into supernatant (*S*) and pellet (*P*) fractions. Bound protein was released from the beads in Laemmli solubilizing buffer and analyzed by Western blotting following SDS-PAGE, first with anti-α2G antibody (*left frame*), and then, by re-probing, with anti-EHS laminin antibody (*right frame*). Anti-α2 laminin antibody precipitated laminin-2/4 and left laminin-1 in solution under nonpolymerizing conditions.

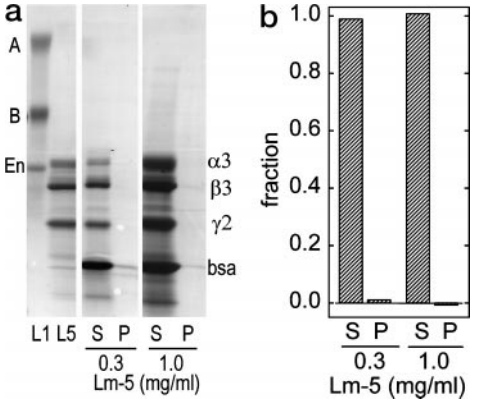

FIG. 9. **Lack of laminin-5 self-assembly.** Purified laminin-5 (*L5*) was incubated in polymerization buffer at 0.3 and 1 mg/ml, at 37 °C for 3 h, centrifuged to separate self-aggregated protein, and analyzed in Coomassie Blue-stained gels (*a*) under reducing conditions. Quantitation of supernatant (*S*) and pelleted (*P*) polymer fractions from gel densitometry tracings shown in *b*. No self-assembly was detected.

increased from 0.05 to 0.1 mg/ml (with same slope), suggesting a partial reduction in self-assembly activity unrelated to the presence of laminin-6 (since it also occurred with the laminin-5 medium). Co-polymerization of added laminin-2/4 was readily detected in conditioned medium, with the relationship with laminin-1 preserved. Thus, despite a medium affect on laminin-1, co-polymerization could readily be detected when present.

DISCUSSION

Earlier studies have shown that laminin-1 reversibly self-assembles into a lattice-like polymer and that this polymer is present in the basement membranes of embryonal carcinoma cells, EHS tumor, and mouse placenta (1, 6). The assembly

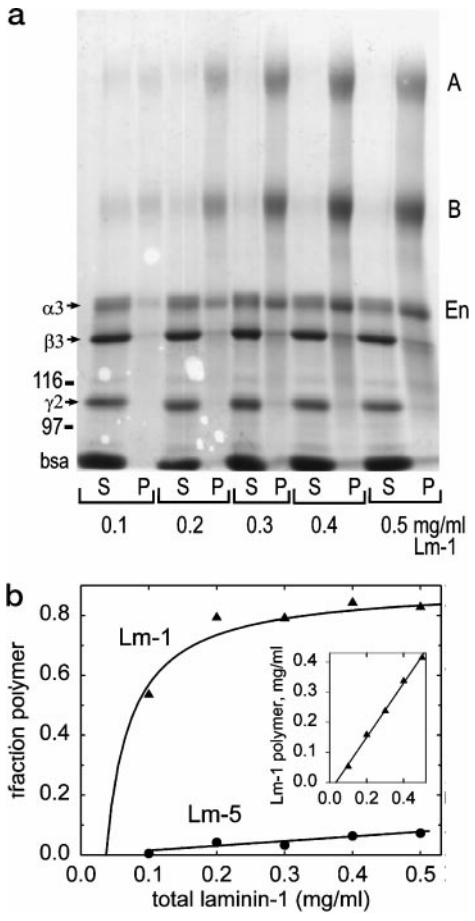

FIG. 10. **Lack of co-assembly of laminin-5 with laminin-1.** Laminin-5 (0.2 mg/ml) was incubated at 37 °C for 3 h with increasing concentrations of laminin-1 in polymerization buffer and centrifuged to separate polymer from free protein as described previously. *a*, the supernatant and pellets fractions were analyzed by SDS-PAGE under reducing conditions after staining with Coomassie Blue. The α3, β3, and γ2 bands of the laminin-5 preparation are seen to remain in the supernatant fraction, even at the highest laminin-1 concentration. *b*, gel densitometry was used to quantitate the distribution of laminin-1 and the β3 band of laminin-5 as a function of laminin-1 concentration.

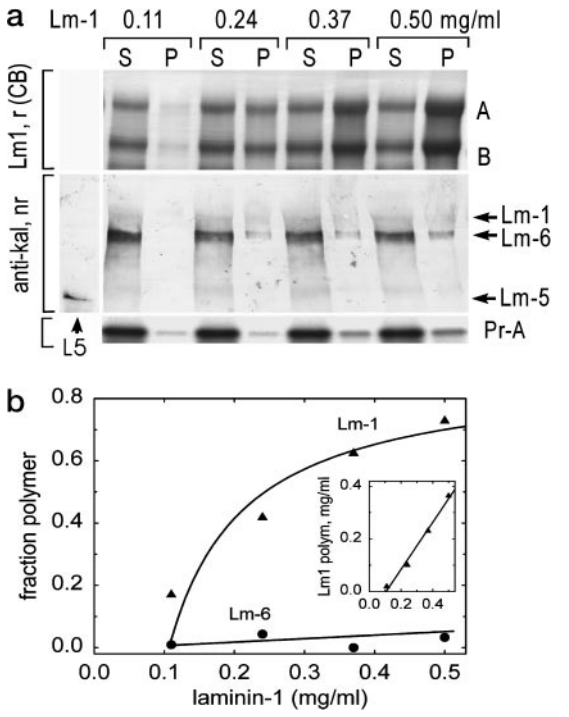

FIG. 11. **Lack of laminin-6 co-assembly with laminin-1.** Laminin-6 enriched (serum-free) conditioned medium from immortalized γ2-null keratinocytes, after concentration and dialysis into polymerization buffer, was mixed with the indicated concentrations of laminin-1, incubated at 37 °C for 3 h, and centrifuged to sediment polymer. Supernatant (*S*) and polymer (*P*) fractions were split into equal aliquots and analyzed by both reducing (*r*) and nonreducing (*nr*) SDS-PAGE. The reduced gel was stained with Coomassie Blue (*CB*) and used to follow laminin-1 polymerization (*upper frame, a*). An immunoblot was prepared from the nonreduced gel with laminin-6 detected with anti-kalinin antiserum (*anti-kal, middle frame, a*). The faster migration of a laminin-5 standard (*L5*) is shown in the *far left lane*. A direct phosphorimage of radioiodinated protein-A (*Pr-A, lower frame, a*), which migrates near the front, was used to measure nonspecific sticking and trapping of protein in the laminin-1 pellet. The fraction of laminin-1 and laminin-6 in the polymer fraction was determined by densitometry and plotted as shown in *b*. Little or no laminin-6 co-sedimented with laminin under polymer-forming conditions.

process is a cooperative heat-gelation in which divalent cation, in particular calcium, is required. Given evidence that type IV collagen separately polymerizes using N-terminal, C-terminal, and lateral associations into a covalently stabilized network (3, 4), and that entactin/nidogen binds firmly to both the laminin γ1 chain and to the triple helix of type IV collagen (26), a model for the assembly and structure of an idealized basement membrane has been described (reviewed in Refs. 1 and 26). The model, however, is limited in that it only considers the classical basement membrane components, *i.e.* those first identified in the EHS tumors and several cultured cell lines. We have now characterized four laminin isoforms with respect to their ability to form a network polymer in a manner similar to laminin-1.

Laminin-2 and laminin-4, both possessing three full short arms, were found to polymerize in a time-, concentration- and temperature-dependent manner. Self-assembly was cooperative with an apparent critical concentration of 0.2 μM, about twice the value (0.07 - 0.14 μM) observed for laminin-1. This self-assembly appeared to be closely related to that found for laminin-1 for several reasons. First, all polymerizations were inhibited by EDTA and N-terminal laminin-1 fragments E4 and E1′, the latter two previously shown to be specific inhibitors of laminin-1 self-assembly (6, 7). Second, laminin-2/4 fragments inhibited laminin-1 polymerization. Third, when laminin-1 was maintained above its critical concentration, and one or both of the laminin isoforms were maintained above or below their critical concentration, co-aggregation was observed. This co-aggregation occurred with isolated laminin-2 and laminin-4, but was better between laminin-1 and -2 compared with laminin-1 and -4. A possible explanation for the difference is that laminin-1 shares two chains in common with laminin-2 but only one chain in common with laminin-4. Thus the three laminins co-polymerize and, when expressed together, can form a composite network using similar bonds between the different isoforms. Since laminin-2 and laminin-4 have α2β1γ1 and α2β2γ1 chain compositions, respectively, one can deduce that laminin-3, which has an α1β2γ1 chain composition, also polymerizes. In contrast to the full-sized isoforms, laminin-5 (α3Aβ3γ2), a rod-like molecule whose short arms lack most of their domains, was found not to polymerize at concentrations at or below 1 mg/ml, nor to co-polymerize with laminin-1. Furthermore, laminin-6 (α3Aβ1γ1), a Y-shaped laminin with two short arms, did not co-polymerize with laminin-1 and therefore, given the association between polymerization and co-polymerization, probably does not self-assemble in a manner similar to laminin-1. It is even possible, although it could not be evaluated in this study, that at higher concentrations of laminin-6 one would observe an inhibitory effect of the isoform on laminin-1, in a manner analogous to the double short arm structure E1′ (7).

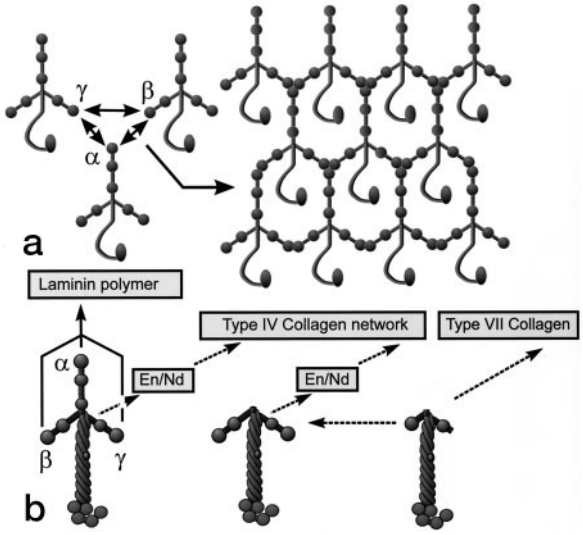

FIG. 12. **Model for the matrix assembly of laminin isoforms.** *a*, three-arm interaction model of laminin polymerization in which the N-terminal regions of all three arms form a bond. The model, first proposed by Yurchenco and Cheng (7), is supported by the findings of this study. *b*, laminins containing all three short arms interact to form a polymer network while a laminin bearing one or more truncations in its short arms does not. Data from other studies indicate that laminin-5, a nonpolymerizing laminin, will not bind to entactin/nidogen and retains a connection to the rest of a basement membrane through laminins-6 and -7.

The self-assembly behavior of these isoforms fits a prediction of a three-arm interaction model which holds that the polymer is formed by the joining of the ends of the three short arms to create a lattice-like array (6, 7). The failure of laminin-5, which lacks most of the short arm domains (although does possess one domain 6 homologue in the β3 chain), to self-assemble is consistent with the above hypothesis. A more exacting test of the model, however, was provided by laminin-6. This laminin, like laminins-7, -8, and -9, and unlike laminins-1, -2, -4, has two rather than three full short arms and would be expected either not to polymerize/co-polymerize or to polymerize/co-polymerize poorly. The inability of laminin-6 to co-polymerize with laminin-1 argues that the three-arm assembly hypothesis is correct. Note that this non-co-polymerizing laminin shares two short arms (β1, γ1) in common with laminin-1, in contrast to the co-polymerizing laminin-2, which also shares two short arms (β1, γ1), and even the co-polymerizing laminin-4, which shares only one short arm (γ1) in common with laminin-1. Thus presence of an α chain short arm appears to be critical for laminin self-assembly. Recent studies[4] reveal that laminin-2 isolated from the dystrophic dy^{2J} mouse, which only lacks α2-domain VI, co-polymerizes poorly with laminin-1. This domain is of course absent in laminin-6 and suggests that loss of only the N-terminal globule of the α chain adversely affects self-assembly.

The co-polymerization assay has proved to be a useful tool in the current study to evaluate the self-assembly properties of other isoforms in amounts below their critical concentrations. The property of co-polymerization furthermore suggests that where a given basement membrane contains a mixture of two or more polymerizing laminins, these laminins will form a cooperative network unless specifically prevented by other immobilizing bonds. Laminins-1, -2, and -4 share this property (Fig. 12). Laminin-5 can form a disulfide-stabilized bond to laminin-6 or laminin-7 (27, 28) but cannot bind to entactin/nidogen (29). Laminins-6 and -7, on the other hand, can bind to entactin/nidogen (and indirectly to the type IV collagen network), since they possess a γ1 chain. It appears that laminin-5 forms a cable-like structure connecting the hemidesmosome of the epidermis with the underlying dermis through type VII collagen and links this cabling through the rest of the basement membrane through laminins-6 and -7 (30). Finally, it is possible that laminins-5, -6, and -7 have other as yet undiscovered matrix binding interactions. It is conceivable that laminins lacking critical short arms domain could form large oligomeric or polymeric complexes using a completely different set of bonds compared with laminin-1. However, it is difficult to imagine how this would occur given the homology of the remaining short arm domains with those present in laminin-1 that are found not to participate in any detectable self-assembly.

Acknowledgments—We thank the assistance of the Office of Research Administration, Palo Alto Veterans Affairs Health Care System, Palo Alto, CA. We also thank Dr. Patricia Rouselle (Center National de la Recherche Scientifique, Lyon, France) for providing us with several aliquots of laminin-5-specific antibodies. Finally, we are grateful to the nursing staff at the Medical Center at Princeton and Dr. Susan Shen-Schwartz at St. Peter's Medical Center (New Brunswick, NJ) for their assistance in providing placental tissue.

[4] H. Colognato and P. D. Yurchenco, unpublished results.

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