Artificial Laminin Polymers Assembled in Acidic pH Mimic Basement Membrane Organization* §

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Natural laminin matrices are formed on cell membranes by a cooperative process involving laminin self-polymerization and binding to cognate cellular receptors. In a cell-free system, laminin can self-polymerize, given that a minimal critical concentration is achieved. We have previously described that pH acidification renders self-polymerization independent of protein concentration. Here we studied the ultrastructure of acid-induced laminin polymers using electron and atomic force microscopies. Polymers presented the overall appearance of natural matrices and could be described as homogeneous polygonal sheets, presenting struts of 21 ± 5 and 86 ± 3 nm of height, which approximately correspond to the sizes of the short and the long arms of the molecule, respectively. The addition of fragment E3 (the distal two domains of the long arm) did not affect the polymerization in solution nor the formation of adsorbed matrices. On the other hand, the addition of fragment E1', which contains two intact short arms, completely disrupted polymerization. These results indicate that acid-induced polymers, like natural ones, involve only interactions between the short arms. The electrostatic surface map of laminin α1 LG4–5 shows that acidification renders the distal end in the long arms exclusively positive, precluding homophilic interactions between them. Therefore, acidification reproduces in vitro, and at a physiological protein concentration, what receptor interaction does in the cellular context, namely, it prevents the long arm from disturbing formation of the homogeneous matrix involving the short arms only. We propose that acid-induced polymers are the best tool to study cellular response to laminin in the future.

Laminin is a self-polymerizing extracellular matrix protein implicated in survival, proliferation, migration, differentiation, and apoptosis of a variety of cell types (1, 2). In particular, laminin plays a pivotal role in the instruction of neural cells during both tissue development and regeneration (3, 4). It is well accepted that laminin signaling depends on its self-polymerization (1), which naturally occurs on cell membranes as long as its longest arm interacts with a membrane receptor, be it a protein, such as a β1-containing integrin or dystroglycan, or a glycolipid, such as sulfatide (5). Laminin polymerization and its anchorage have been proposed to cooperate for the formation of a supramolecular network, which have unequivocally been demonstrated to involve only interactions between the LN domains at the distal ends of each short arm (5, 6). Such network consists of a regular lattice, whose struts correspond to the size of laminin short arms (7), both in matrices assembled in vitro by tumors as well as in artificial matrices thermally gelled at 3.5 mg/ml.

Because laminin has an important role in determining cell fate, it is of major scientific and biotechnological interest to produce artificial laminin matrices that can mimic the polymers produced by cells in vivo. Artificial self-polymerization of laminin has been obtained in vitro either by maintaining the protein above a critical concentration (8) or by providing a surface of negative lipids to assist polymerization (9). More recently, we have found that diluting laminin in acidic pH led to instantaneous solution polymerization, whereas such polymers adsorbed on coverslips could induce neuritogenesis and cell migration out of explants of newborn rat brains (10, 11). When such explants were plated onto laminin adsorbed in neutral pH, laminin in EDTA, or poly-lysine, there was absolutely no migration or axonal growth. Immunolabeling of the matrices produced at each pH with anti-laminin antibodies revealed major morphological differences between them, whereas the acidic one seemed more flat and organized, which could account for its particular signaling properties to neural cells (11).

Although the molecular organization of the flat laminin mesh in basement membranes seems to be stable, there is plenty of evidence supporting the notion that the supramolecular organization of laminin can change throughout development and among tissues. In the nervous system, for instance, it has been demonstrated that laminin goes from cell-associated to scattered extracellular matrix deposits or from a punctuated pattern to small or large sheet-like aggregates in developing brain (4, 12). Cells in culture can also assemble different networks as demonstrated for midbrain and cortical neural cells (13, 14) or Schwann cells (15). In addition, we have previously described that the laminin matrix synthesized by embryonic astrocytes (which efficiently supports neurite outgrowth)
resembles the artificial matrix assembled upon laminin acidification because it is flat, organized, and closely associated to the cell surface (14).

Here we investigated at the molecular level the structural properties of acidic laminin polymers obtained in vitro at low protein concentration (10–50 μg/ml). We evaluated their ultrastructure and stability toward laminin fragments and concluded that such polymers are sheet-like matrices formed by interactions among the terminal ends of the short arms, whereas the long ones are left free at an orthogonal plane. Such polymers better correspond to the natural matrices characterized so far and should be considered as a model of artificial laminin substrate in further cell biology studies, as well as in possible therapeutic usage of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Natural mouse laminin 111 isolated from Engelbreth-Holm-Swarm tumor was purchased from Invitrogen, and Repel Silane was from Amersham Biosciences. N-Pro pyl gallate, rabbit anti-laminin (diluted 1:30), sheep anti-rabbit IgG, and Cy3-conjugated antibody (diluted 1:800) were from Sigma-Aldrich. Laminin fragments E1’ and E3 were a generous gift of Dr. Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ).

**Electron Microscopy**—Laminin was diluted to a final concentration of 50 μg/ml in 20 mM sodium acetate, pH 4.0, containing 1 mM CaCl₂. For freeze etching method the solution was pipetted on a Balzers support disk, rapidly frozen by impact against a copper block, cooled by liquid nitrogen to −196 °C, fractured, and etched for 20 min by raising the temperature to −100 °C. After this, the specimens were rotary-shadowed with platinum at 15 °C and further coated with carbon at 90 °C, digested in sodium hypochlorite (NaClO₃) for 30 min, and collected on 200-mesh copper grids. For negative staining 5 μl of the laminin solution (50 μg/ml) was deposited on a Formvar-coated copper grid. The solution was partially removed with a filter paper, and 5 μl of uranyl acetate (2%) was added. After 1 min, the solution was completely dried with a filter paper. The specimens were examined in a Zeiss EM 900 transmission electron microscope operated at 80 kV.

**Atomic Force Microscopy**—Laminin was diluted to 50 μg/ml in acetate buffer and pipetted onto the surface of a newly cleaved mica, fixed for 2 h with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h, rinsed three times in acetate buffer, dehydrated through an ethanol series, and critical point dried (Balzers CPD 050). The samples were examined in a MFP-3D atomic force microscopy (Asylum Research, Santa Barbara, CA). The images were acquired in air using intermittent contact mode. Cantilever elastic constants were obtained by the thermal noise method. For intermittent contact mode, tetrahedral-shaped cantilevers (AC240TS, Olympus, Tokyo, Japan; nominal spring constant 2 N/m) were used at a low scan rate (0.5 Hz). Phase images were acquired with 512 × 512 pixels of resolution, and image processing was performed in IGOR-PRO (Wavemetrics, Portland, OR) using a MFP-3D template developed by Asylum Research.

**Light Scattering Measurements**—Light Scattering was measured in a QuantaMaster spectrofluorometer (Photon Technol-ogy International, Lawrenceville, NJ) at 37 °C. The wavelength of the incident light was fixed at 400 nm, and scattered light was collected at 90 °C between 350 and 450 nm. Laminin self-polymerization was initiated by diluting the protein from a stock solution either to a final concentration of 10 μg/ml in 20 mM sodium acetate (pH 4.0) or to a final concentration of 200 μg/ml in 20 mM Tris-HCl (pH 7.0), both containing 1 mM CaCl₂ (final volume, 800 μl). The fluorescence cuvette was previously treated with Repel Silane to avoid protein binding to the quartz walls. The assay was performed in the presence or in the absence of the laminin E1’ or E3 fragments. Laminin stock solutions (~1 mg/ml) were kept at 4 °C until dilution into assay medium previously warmed to 37 °C in the sample compartment of the instrument. Under these conditions, i.e. at 4 °C, aggregates are not expected to form in stock solutions until the exact time when dilution in prewarmed buffer takes place (8). After laminin addition, the samples were gently mixed, and measurements were taken within ~1 min. The data were corrected by subtracting appropriate blanks containing buffer only.

**Immunostaining**—Laminin matrices were obtained by diluting the stock solution to 50 μg/ml in prewarmed (37 °C) 0.1 M either acetate (pH 4.0) or Tris (pH 7.0) buffers with 1 mM CaCl₂. One hundred microliters of the diluted solutions were dropped onto glass coverslips and incubated for 12 h at room temperature. For immunostaining, coverslips were fixed with 4% paraformaldehyde for 3 min and rinsed three times with 0.1 M phosphate-buffered saline (PBS). The matrices were blocked with 5% bovine serum albumin in PBS for 30 min and subsequently incubated overnight at 4 °C with rabbit anti-laminin antibody diluted in blocking solution. The matrices were then washed three times with PBS and incubated with anti-rabbit, Cy3-conjugated antibody for 2 h, at room temperature. After being washed three times with PBS and once in water, the coverslips were mounted in N-propyl-gallate and visualized using a fluorescence microscope (Zeiss Axioplan).

**Electrostatic Surface Potential of the LG4−5 Domains of Laminins α1 and α2**—The surface potential was calculated by using the Adaptive Poisson-Boltzmann Solver software package (16), and images were generated using PyMol 0.97 (17). The atomic coordinates for laminin α-chains 1 and 2 were obtained from the Protein Data Bank entry 2J4D for α1 and 1DYK for α2 (18, 19). The calculations were performed by assigning either a neutral or a positive charge for His residues. A probe sphere of 1.4 Å radius was used to generate the solvent-accessible surface. The dielectric constants used for protein and solvent were 20 and 80, respectively, and the system temperature was set to 310 K.

**RESULTS**

**Analysis of the Acidic Laminin Matrix by Electron Microscopy**—To gain information about the architecture of the polymeric matrix obtained in acidic pH, we studied its ultrastructure by the freeze etching technique. The protein was diluted to a final concentration of 50 μg/ml in acetate buffer (pH 4.0) and immediately dropped onto a support disk. It is

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The abbreviation used is: PBS, phosphate-buffered saline.
Acidic Laminin Matrices

![Image](image.png)

FIGURE 1. Analysis of the acid-induced laminin polymers by electron microscopy reveals organized polygonal arrays. Laminin was diluted to 50 μg/ml in acetate buffer (pH 4) and immediately processed for freeze etching (A) or for negative staining (B). Electron micrographs are contrast reversed to provide the sensation of depth necessary for better visualization of the three-dimensional nature of the polymers. The arrow indicates the apparent transition between a single (left) to a double-layered (right) polymer. The scale bars represent 100 nm in A and 200 nm in B.

It is important to note that acid-induced laminin polymerization occurs instantaneously upon dilution (10); therefore, in this experimental set-up, the polymers are formed in solution and are decanted already in the associated state. Analysis of the polymeric matrix revealed a planar structure displaying irregular polygons as shown in Fig. 1A. The left third (see arrow) of the image seems to correspond to a single layer of the sheet-like polymer, whereas the right two-thirds, where the polygons are smaller, suggest the superposition of more than one polygonal sheet.

The size of the struts in the hexagonal-shaped matrix previously characterized in basement membrane extracts and in heat-gelled pure laminin was between 30 and 40 nm (7, 20). This exactly corresponds to the size of the short arms in the laminin molecule, known to interact to form the hexagons observed in natural matrices (6). Although the sizes of the struts in Fig. 1A were in this same range, their thickness prevented precise measurements using this technique.

We next analyzed the ultrastructure of the acidic polymers by negative staining. Observation of Fig. 1B reveals a very organized mesh-like array, composed of polygons of an approximate hexagonal shape. Given the three-dimensional nature of the polymeric matrix, the superposed struts, instead of excluding the dye, actually worked as substrates for the uranyl because it percolated down through the mesh. This resulted in the protein appearing as the stained structure (white in the contrast reversed electron micrograph). The sides of the hexagons corresponding to the struts of the mesh were quantitatively analyzed by measuring 100 struts randomly chosen. The average size found was 21 ± 5 nm, which approximately corresponds to the size of laminin short arm, similarly to previously described for natural and heat-gelled laminin matrices (7, 20). Together freeze etching and negative staining indicate that acid-induced polymers are planar and present a repetitive structure compatible with a model where all intermolecular interactions are restricted to the single plane defined by the short arms.

Analysis of Acidic Laminin Matrices by Atomic Force Microscopy—Acid-induced laminin polymers were further evaluated by atomic force microscopy. The initial analysis of a squared window of 0.25 μm² using conventional topography scanning showed a regular but poorly contrasted image, indicative of a flat surface (Fig. 2A). A second image was generated by analyzing the phase delay undergone by the cantilever (Fig. 2C). Phase imaging is extremely sensitive to the elastic and adhesive properties of the specimens present on the surface (21), leading to a pronounced increase in contrast. A homogenous image containing polygons similar to those found in electron microscopy was seen using phase mode. Because laminin is much more adhesive and elastic than the mica substrate, the delay in phase amplified the subtle changes in heights probed by the topographic image (Fig. 2, compare A and C).

To gain information on the height of the laminin layer, we have intentionally searched for random interruptions of the matrix in which the cantilever could be introduced to probe the distance to the underlying surface. Three of such regions were analyzed, providing an average depth of 86 ± 3 nm. This corresponds to the size of the laminin long arm, which therefore would not participate in the hexagonal mesh but instead would be projected to an orthogonal plane relative to it.

Effect of Laminin Fragments on Laminin Polymerization in Solution—to confirm the lack of contribution of the long arm to the formation of the acid-induced laminin network, we have investigated whether addition of the E3 fragment, which contains the distal two domains of the long arm, would interfere with laminin polymerization in solution. The experiment was carried out at 10 μg/ml, a concentration at which no polymerization is observed at neutral pH (8). Polymerization was monitored as the increase in light scattering in solution at 400 nm. Fig. 3A shows the intensities of light scattering obtained upon laminin dilution in acidic pH in the absence or in the presence of a 20-fold molar excess of the fragments. In the presence of...
which confirms that interactions with the long arm are not involved in the acidic polymers.

Solution polymerization was also investigated in neutral buffer (Fig. 3B). At 10 μg/ml no increase in light scattering was observed even after 2 h of incubation, and the addition of EDTA did not lead to decrease of the initial intensity (data not shown). When the concentration of laminin was changed to 200 μg/ml, light scattering was progressively increased, reaching a maximal enhancement of 4.5-fold in within 2 h. Ten millimolar EDTA were added to disrupt aggregates, which led to an instantaneous drop in light scattering down to the initial intensity at time zero. When polymerization was essayed in a 20-fold molar excess of fragment E3, only 50% increase in light scattering was observed, which indicates that, although polymerization was still seen in the presence of E3, the maximal level of aggregation was affected by the fragment. The latter result is in contrast with previously reported data in the literature showing that the E3 fragment did not interfere with laminin polymerization in neutral pH (22). This can be explained by the fact that we followed aggregation by measuring light scattering, which not only takes into account the aggregated state but also will respond to the final sizes of the aggregates formed. On the other hand, sedimentation of the polymers by centrifugation, as used before, establishes a cut-off between aggregated and nonaggregated states and could be not sufficiently sensitive to detect eventual changes in specific aggregated species.

**Effect of Laminin Fragments on Laminin Matrices Deposited on Coverslip**—Although laminin aggregates could not be detected in neutral pH at 50 μg/ml, we have previously demonstrated that deposits of the aggregated protein became visible when the laminin solution was incubated over the surface of a glass coverslip (11, 14). Such deposits were characterized by immunostaining and shown to be morphologically different from others obtained in acidic pH. An analogous comparison between laminin aggregates obtained in distinct pHs could not be performed at the ultrastructural level because polymers obtained in neutral pH were visible as amorphous aggregates in electron microscopy, both using negative staining or freeze etching (data not shown). A visible network of laminin under neutral pH has only been reported at an extremely high protein concentration (3.5 mg/ml) (7).

Fragments E1’ and E3 were tested for their ability of interfering with the formation of laminin matrices adsorbed on coverslips at each pH (Fig. 4). Laminin alone formed flat and organized polymers in acidic pH, whereas in neutral pH the polymers were bulky and discontinuous. Fragment E1’ clearly disrupted both polymers, whereas E3 affected only the deposits obtained in neutral pH. It is interesting to note that although E3 clearly disrupts the large aggregates typical of laminin deposited in neutral pH, small aggregates remained on the matrix, which indicates that, although E3 interferes with the final polymerized state, it still preserved some level of protein aggregation.

**Comparison between the Electrostatic Surface Potentials of the LG4–5 Domains of Laminin at Neutral and Acidic pH**—We finally asked the question about why acidic buffer would stabilize sheet-like polymers. A recent paper has described the crystallographic structure of LG4–5, the two most distal domains
Acidic Laminin Matrices

![Image of pH 4 and pH 7 matrices with E1' and E3](image)

**FIGURE 4. Fragments E1’ and E3 selectively interfere with the formation of adsorbed laminin matrices.** Laminin matrices were formed by adsorption of the protein (60 nm) on the surface of glass coverslips in the absence (top panel) or in the presence of fragments E1’ or E3 (1.2 μm; middle and bottom panels, respectively). Although E1’ completely disrupted aggregates, E3 did not interfere with acid-induced polymerization but decreases the sizes of aggregates formed in neutral buffer. The scale bar corresponds to 50 μm.

in the long arm of laminin α1. We have used the PyMol software to analyze the electrostatic surface of the protein at pHs 7 and 4. Fig. 5 shows that in neutral pH negatively charged groups (red) are clustered in a predominantly positive (blue) surface. This would allow for some degree of electrostatic interaction between two of these domains. Indeed, a significant proportion of dimers formed by the association between the distal ends of two long arms have been observed among spontaneously formed laminin dimers detected using rotatory shadowing (8). On the other hand, when the pH is decreased to 4, the surface becomes totally positive, which would completely prevent interactions between the two distal ends of the long arm of laminin 111. Interestingly, this would not be the case for laminin 211, where the long arm is contributed by the α2 chain. In this case, alternated negatively and positively charged clusters are seen in both neutral and acidic pH (Fig. 5). Accordingly, acidification of pH produced only a modest increase in the light scattering of laminin 211 (not shown).

**DISCUSSION**

Since the seminal work of Yurchenco and co-workers in 1985 (8), demonstrating that laminin could self-polymerize in the absence of a cell membrane or any other cellular component, it became established that all information necessary for the formation of laminin polymers was contained within the isolated protein. In the following years, the same group additionally described how exactly polymers were formed on the cell surface, establishing sulfatides as important receptors for the initiation of self-assembly on biological membrane (5). In the naturally assembled laminin network only the short arms interact, giving rise to a continuous hexagonal sheet, whereas the long arm remains in contact with a laminin receptor, i.e. connecting the polymer to the cell surface.

The fact that laminin could self-polymerize in a cell-free system opened the possibility of using artificial polymers as substrates to study the cellular response to the underlying matrix. In this context, it has been assumed that the artificial polymers described back in 1985 properly corresponded to the polymers produced by cells *in vivo*. Natural polymers, however, had been morphologically characterized, whereas the structure of the polymers produced by *in vitro* self-polymerization was not available for comparison. Moreover, rotatory shadowing studies had shown that the distal ends of the long arms interacted with each other, whereas such interaction did not occur in the natural laminin mesh. Here we showed that laminin polymerized in acidic buffer in a concentration as low as 60 nm (50 μg/ml) produced a continuous polygonal network, contributed only by the short arms. We also showed that upon acidification the interaction between the distal ends of the long arms is hampered, given that the surface charge of the LG4–5 domains becomes completely positive. Our interpretation is that acidification impairs bridging of two long arms, stabilizing the interaction among short arms only. An equivalent blockage of the long arm is not necessary in a natural situation when the laminin molecule binds to a cell receptor that recognizes its distal end such as α5β1, α6β1, and αcβ1 integrins, dystroglycan, or sulfatides. In this case, the interaction with the receptor will occupy the long arm and impair it from participating in the laminin polymer. On the other hand, in neutral solution, the long arms would be free to interact with each other and would disturb the formation of the homogeneous polymer involving the three short arms only.

In this work, we used several direct and indirect methodological tools to demonstrate that acid-induced laminin polymers are structurally similar to natural polymers produced *in vivo* by cells. Negative staining and freeze etching followed by electron microscopy or by atomic force microscopy showed a clear correspondence between the morphology of matrices produced by Engelbreth-Holm-Swarm and mouse embryonic carcinoma cell tumors and acid-induced ones. Additionally, the use of laminin fragments confirmed that, in the acid-induced polymer, as in the natural one, fragment E3 does not interfere with polymerization neither in solution nor in large aggregates used for cell cultivation. Curiously, the data reported here using light scattering measurements revealed that E3 partially disassembles polymers formed in neutral pH at high protein concentration. Moreover, the presence of E3 led to the disruption of the very large aggregates observed in the matrices adsorbed in neutral pH (Fig. 4). These results taken as a whole point to the interpretation that the interactions between the distal ends of the long arms that seem to occur in neutral buffer actually work as bridges, cross-linking small stretches of the sheet-like polygonal polymer. Such interactions would impair the formation of a continuous mesh that can be distinguished from smaller polymers by the light scattering technique,
which can discriminate between protein aggregates of different sizes.

The fact that acid-induced laminin polymers, as opposed to laminin polymers obtained in neutral pH, reproduce in vitro the structural properties of the natural laminin matrix opens new research perspectives and suggests that previous data obtained in functional studies using laminin polymerized in neutral buffer need to be interpreted with more care and indeed may require revision. Acid-induced polymers can now be used in vitro to study their signaling properties to overlaying cells, as well as in vivo to study the role of laminin in biological phenomena related to the embryonic development and in tissue regeneration. It is interesting to note that although laminin has clearly been pointed out as an instructive substrate for both the development and regeneration of the nervous system, little benefit has been associated to the use of laminin in spinal cord regeneration. In this regard, we have recently demonstrated that, contrary to laminin in neutral buffer, treatment with laminin polymerized in pH 4 significantly improves open field locomotion of rats submitted to experimental spinal cord injury.6

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