Self-assembly of Laminin Induced by Acidic pH*

(Received for publication, May 6, 1999, and in revised form, September 21, 1999)

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The supramolecular architecture of the basement membrane is provided by two enmeshed networks of collagen IV and laminin. The laminin network is maintained exclusively by interactions among individual laminin molecules and does not depend on the presence of other extracellular matrix components. Laminin polymers can be obtained in vitro either in solution or in association with the surface of bilayers containing acidic lipids. In this work, we have tested the hypothesis that the negative charges present on acidic lipids establish an acid microenvironment that is directly responsible for inducing laminin aggregation. Using light-scattering measurements, we show that laminin does not aggregate on vesicles of neutral lipids, whereas instantaneous aggregation occurs to progressively greater extents as the proportion of acidic phospholipids in the vesicles is increased. Aggregation of laminin induced by vesicles containing acidic phospholipids occurs very rapidly, so that maximal aggregation for each condition is reached within 1 min after laminin dilution. Aggregation depends on the presence of Ca\(^{2+}\) ions, is reversed by increasing ionic strength, and can be detected at laminin concentrations as low as 6 nM. In addition, we show that, in the absence of vesicles, acidification of the bulk solution can also induce laminin self-polymerization through a process that exhibits the same properties as lipid-induced polymerization. The fact that there is a correspondence between the processes of self-polymerization of laminin in acidic medium and in neutral medium but in the presence of vesicles containing negatively charged lipids leads us to propose that the microenvironment of an acidic surface may trigger the assembly of laminin networks. In vitro, such an acidic microenvironment would be provided by negatively charged sialic acid and sulfate groups present in the glycocalyx surrounding the cells.

Laminin, a large extracellular glycoprotein (850 kDa), is the major component of basement membranes (1, 2). Actually a family of structurally homologous isoforms (3–5), laminins present a cruciform shape composed of three different polypeptide chains that are held together by coiled-coil interactions and disulfide bonds (6–8). Laminins affect multiple biological functions, including cell adhesion (9), proliferation (10, 11), migration (12), differentiation (13), neurite outgrowth (14, 15), and tumor metastasis (16, 17). These functions depend directly on the supramolecular organization of the basement membrane, which is ultimately determined by the interactions between laminin molecules and between laminin and other macromolecules.

It has been postulated that the overall scaffold of the basement membrane is formed by two independently assembled networks of collagen IV and laminin, bridged by other extracellular matrix components such as entactin (18, 19). Large three-dimensional laminin networks have been obtained in vitro through a Ca\(^{2+}\)-dependent self-polymerization process, which is strictly dependent on a critical laminin concentration of 60 nM (20). Biochemical and ultrastructural analyses of polymers obtained in vitro revealed properties similar to those of laminin networks produced by two different tumor models, the Engelbreth-Holm-Swarm tumor and cultured embryonic carcinoma cells (19).

In 1991, Kalb and Engel (21) showed that laminin polymerized in vitro upon binding to planar lipid bilayers and that lipid-induced polymerization occurred below the critical concentration reported previously for aggregation in solution (20). Polymerization was more effectively induced by bilayers containing negatively charged sulfatides than by bilayers of neutral phospholipids, which led the authors to propose that the density of negative charges on the surface could be critical for laminin binding and self-assembly (21).

In this work, we have investigated the correlation between the density of negative charges on lipid surfaces and the extent of laminin polymerization as well as a possible mechanism by which negative lipids might affect polymerization. Using light-scattering and fluorescence measurements, we show that an increase in the ratio of acidic to neutral phospholipids leads to an increase in laminin polymerization and that the effect of negative lipid surfaces can be mimicked by acidification of the solution phase. We propose that a surface-restricted acidic environment provided by the negative lipids might serve as a substrate for self-assembly at low concentrations of laminin.

EXPERIMENTAL PROCEDURES

Materials—Natural mouse laminin isolated from Engelbreth-Holm-Swarm tumor was purchased from Life Technologies, Inc. TNS was from Molecular Probes, Inc. (Eugene, OR), phosphatidylcholine was from Avanti Polar (Alabaster, AL), phosphatidylserine and Triton X-100 were from Sigma, and hepel silane was from Amersham Pharcma Biotech (Uppsala, Sweden). All other reagents were of analytical grade.

Light-scattering Measurements—Light scattering was measured on an ISSPC-PCI spectrofluorometer (ISS Inc., Champaign, IL) at 35 °C. The wavelength of the incident light was fixed at 400 nm, and scattered light was collected at 90° between 350 and 450 nm. In all experiments, aggregation was initiated by diluting laminin from a stock solution to the indicated concentrations in either 20 mM sodium acetate, pH 4, or 20 mM Tris-HCl, pH 7, to a final volume of 600 µl. Laminin stock solutions (1 mg/ml) were kept at 4 °C until dilution into assay media previously warmed to 35 °C in the sample compartment of the instrument. Under these conditions, i.e. at 4 °C, aggregates are not expected...

* 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.
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to form in stock solutions until the exact time when dilution in pre-warmed buffer takes place (20). The absence of previously formed aggregates in laminin stock solutions was confirmed by the low values of light scattering obtained when laminin was diluted at pH 7.0 (see Fig. 4, dotted line). After the laminin addition, samples were gently mixed, and measurements were taken immediately. The whole procedure took approximately 1 min, which was the minimum time for light-scattering measurement after laminin dilution. Data were corrected by subtracting appropriate blanks containing buffer only or buffer plus variable amounts of phospholipid vesicles. Prior to use, cuvettes were pretreated with heparin solution to avoid adherence of laminin to the quartz surface.

Preparation of Liposomes—Chloroform solutions of phospholipids, either pure or in mixtures of varying molar ratios of PS to PC, were evaporated under nitrogen, and the lipid films formed were resuspended in 20 mM Tris-HCl buffer, pH 7.5, to a final lipid concentration of 1 mM. The suspensions were vortexed vigorously for approximately 5 min. Small unilamellar vesicles were formed by sonicing the turbid suspensions using a Branson sonifier (Sonic Power Company, Danbury, CT) equipped with a titanium microtip probe. Sonication was carried out in an ice bath, alternating cycles of 20 s at 50% full power with 60-s resting intervals until a transparent solution was obtained (usually eight cycles). After sonication, vesicles were centrifuged for 4 min in an Eppendorf microfuge in order to remove titanium released from the probe. Vesicle suspensions were added to buffer before dilution of laminin so as to give a final phospholipid concentration of 0.1 mM.

Fluorescence Measurements—Laminin was diluted to 60 nM in 20 mM of either sodium acetate, pH 4, or Tris-HCl, pH 7, and titrated with increasing concentrations of TNS (using 5 or 0.5 mM stock solutions in a 1:1 mixture of dimethylformamide and water). Fluorescence intensities were measured immediately after each addition of TNS on an ISSPC-PCI spectrofluorometer, using excitation at 385 nm and recording emission between 350 and 480 nm. Fluorescence data were corrected by subtracting blanks containing buffer plus varying amounts of TNS. Additionally, spectra were corrected for dilution.

RESULTS

Effect of Acidic Phospholipids on Laminin Aggregation—In order to determine whether the extent of lipid-induced polymerization of laminin depended on the density of negative charges present on the lipid surfaces, we compared laminin aggregation promoted by small lamellar phospholipid vesicles containing increasing ratios of negative (PS) to neutral (PC) phospholipids (Fig. 1). Aggregation was followed by measuring light scattered at 90°, which correlates with the size of particles in solution (22). Maximal light scattering (maximal laminin aggregation) was obtained when vesicles contained only PS. This value dropped by 90% when vesicles containing only PC were assayed (Fig. 1A). When light-scattering values obtained at 400 nm were plotted against the PS:PC ratio (Fig. 1B), a gradual decrease was observed, suggesting that the extent of aggregation could be related to the density of negative charges on the vesicles. Interestingly, aggregation was complete within 1 min, which is the time necessary to add laminin to the solution containing the vesicles, mix, and carry out the light-scattering measurement. Thus, the rate of aggregation is much faster than that reported for aggregation in solution, which occurs in a time frame of several hours (20).

It is known that laminin binds to a variety of lipids, including phospholipids such as PC or negatively charged phosphatidyl-glycerol (21, 23). If laminin bound simultaneously to more than one vesicle, it could cross-link several of these vesicles, giving rise to a multivalent network involving the two species (laminin molecules and vesicles). Such networks would also lead to an increase in light scattering, like that observed upon laminin addition in Fig. 1. In order to show that the increase in light scattering induced by the addition of laminin to the vesicle suspension was not due to vesicle aggregation but, instead, to the formation of aggregates involving laminin molecules only, we have investigated how solubilization of vesicles after the laminin addition would affect preformed aggregates.

Fig. 2 shows that the addition of 0.1% of the nonionic detergent Triton X-100 was sufficient to totally disrupt PS vesicles (compare first and second bars). The addition of laminin to PS vesicles in the absence of detergent led to an increase in light-scattering intensity of ~800 arbitrary units (compare first and third bars in Fig. 2). When Triton was added to preformed aggregates (PS vesicles plus laminin), light scattering decreased by only 50% (compare third and fourth bars in Fig. 2). The remaining intensity, i.e. the light-scattering intensity of the detergent-resistant aggregates, was approximately 800 arbitrary units, which is similar to the increase in light scattering observed when laminin was added to the vesicles. This result shows that the high molecular order aggregates that are formed when laminin is added to PS vesicles and are responsible for the increase in light scattering are preserved even after solubilization of the vesicles, which indicates that they do not contain vesicles but involve polymerization of laminin molecules only.

Influence of Ca 2+ and Ionic Strength on Lipid-induced Polymerization of Laminin—Kalb and Engel (21) reported that laminin aggregates promoted by sulfatide bilayers were disrupted by the addition of EDTA or by increasing the ionic strength, which suggests that those aggregates were stabilized by divalent cations and by electrostatic interactions. Fig. 3A shows that the addition of 5 mM EDTA to aggregates formed in the presence of PS vesicles completely reversed aggregation,
reducing light-scattering to the value found for vesicles alone. When EDTA was present in the medium before the addition of laminin, formation of aggregates was 70% inhibited (Fig. 3B). It is interesting to note that these experiments were carried out in the presence of contaminant Ca\(^{2+}\) (10\(^{-7}\) M) and that the addition of 1 mM CaCl\(_2\) did not lead to any additional increase in light scattering (not shown).

The addition of increasing concentrations of NaCl to aggregates obtained in the presence of PS vesicles led to a decrease in light scattering, indicating disruption of laminin aggregates (Fig. 4). It is noteworthy that at 150 mM NaCl more than 90% of light scattering still remained, indicating that aggregates formed in these conditions may occur at physiological ionic strength. The addition of 2 mM NaCl to aggregates obtained in the presence of vesicles of all phospholipid compositions assayed here (see Fig. 1) led to complete reversal of aggregation (data not shown).

**Effect of pH on Laminin Aggregation in Solution**—Laminin aggregation on lipid vesicles was proportional to the relative content of negatively charged phospholipids (PS) in the vesicles. This observation raised the possibility that the driving force for lipid-induced aggregation could be the electrostatic surface potential associated with the negative charges of the phospholipids. Nevertheless, a second interpretation for the above data would be that the stimulus triggering laminin polymerization was simply its binding to the lipid surface, with the negative charges being responsible only for increasing the affinity of laminin binding. A negative electrostatic potential, however, leads to a reduction of the pH in the vicinity of the membrane (24). Thus, if the electrostatic potential contributes to lipid-induced aggregation of laminin, one could expect acidification of the bulk solution to mimic the effect of PS vesicles in promoting laminin polymerization. To test this hypothesis, we investigated whether, in the absence of lipid surfaces, reductive pH could induce formation of laminin aggregates with properties similar to those reported for polymers obtained under acidic conditions (Fig. 5). A shows light-scattering records obtained immediately after dilution of laminin to 60 nM in buffer at pH 7 (solid line) or pH 4 (dotted line). Records shown were corrected by subtracting records obtained for buffer only.

This result shows that acidification can also induce instantaneous aggregation of laminin *in vitro*.

**Effects of Ca\(^{2+}\) and Ionic Strength on Acid-induced Aggregation of Laminin**—In order to investigate whether the aggregates formed under acidic conditions presented properties similar to those obtained in the presence of acidic phospholipids, we evaluated the sensitivity of acid-induced laminin aggregates to EDTA and to increasing ionic strength. EDTA added to preformed aggregates promoted a decrease of 94% in light scattering (Fig. 7A), whereas the addition of EDTA to the medium prior to laminin dilution reduced formation of aggregates by 96% (Fig. 7B). An interesting finding was that aggregation of laminin was not observed in the presence of EDTA at any pH in the range of 2–8. The fact that aggregation strictly depends on the presence of divalent cations rules out the pos-
Laminin samples were diluted to 60 nM in 20 mM acetate buffer. Pro- 
tective buffer alone (left), buffer plus 60 nM laminin (center), or laminin in 
buffer plus 5 mM EDTA (right). In A, EDTA was added to preformed 
aggregates. B shows light scattering of acetate buffer plus 5 mM EDTA 
(left) and of 60 nM laminin added to buffer in the presence of EDTA 
(right). S.E. values result from three separate determinations.

FIG. 6. Polymerization of laminin in solution is pH-dependent. 
Laminin samples were diluted to 60 nM in 20 mM acetate buffer. Pro- 
gressive alkalization was carried out by adding increasing amounts of 
0.1 M Tris-HCl, pH 10. The pH values shown on the abscissa were 
measured with a pH meter in a separate sample containing buffer only.

FIG. 7. The addition of EDTA reverses laminin aggregates 
formed at acidic pH. A, light-scattering intensities obtained for acet- 
ate buffer alone (left), buffer plus 60 nM laminin (center), or laminin in 
buffer plus 5 mM EDTA (right). In A, EDTA was added to preformed 
aggregates. B shows light scattering of acetate buffer plus 5 mM EDTA 
(left) and of 60 nM laminin added to buffer in the presence of EDTA 
(right). S.E. values result from three separate determinations.

Effect of pH on Laminin Conformation—Formation of large 
protein aggregates such as laminin polymers reported here 
could be associated with protein denaturation. In order to in- 
vestigate the possibility that acid-induced aggregation of lami-
nin resulted from a denaturation process, we examined binding 
to laminin of the conformation-sensitive fluorescent probe 
TNS. A nonfluorescent molecule when free in solution, TNS 
binds to hydrophobic pockets in proteins, and binding is accom- 
ppanied by an increase in its fluorescence (25). At neutral pH, 
TNS did not bind to laminin (Fig. 11, open circles). When pH 
was decreased to 4, TNS bound to laminin as revealed by a 
fluorescence increase of 800-fold for a saturating concentration 
of the probe (open squares). When laminin was previously de- 
naturated by preincubation with 2 or 5 mM urea, TNS binding at 
acidic pH was abolished (closed circles and squares, respecti- 
vely). These results demonstrate that laminin does not denature 
at acidic pH but instead undergoes a conformational change 
upon acidification that preserves its tertiary structure and also 
favors its fast polymerization in vitro.

DISCUSSION

In this work, we report that acidification induces instantane- 
ous in vitro self-assembly of laminin at concentrations below 
60 nM, previously shown to be the critical concentration nec- 
cessary for assembly in solution at neutral pH (20). In addition, we 
show that the aggregation triggered by acid pH in solution, 
which does not involve laminin denaturation or isoelectric pre- 
cipitation, exhibits properties similar to those observed when 
self-assembly occurs at neutral pH but on the surface of vesi- 
cles containing acidic phospholipids, namely (a) very fast ki- 
etics, (b) lack of a detectable minimum concentration for ag-
Laminin was diluted to various final concentrations ranging from 6 to 60 nM. The inset shows light-scattering records obtained at pH 4 (upper line) and 7 (bottom line) at 6 nM laminin, where aggregation is not clear in the main figure. Data were corrected by subtracting blanks. Results shown correspond to the average of three separate experiments, with the S.E. values being smaller than the size of the symbols used.

Fig. 9. Acid-induced polymerization occurs at low laminin concentrations. Light-scattering intensities were measured after laminin was diluted in 20 mM of either Tris-HCl buffer, pH 7 (C) or acetate buffer, pH 4 (B) or Tris-HCl buffer, pH 7 (C) to various final concentrations ranging from 6 to 60 nM. The inset shows light-scattering records obtained at pH 4 (upper line) and 7 (bottom line) at 6 nM laminin, where aggregation is not clear in the main figure. Data were corrected by subtracting blanks. Results shown correspond to the average of three separate experiments, with the S.E. values being smaller than the size of the symbols used.

Fig. 10. Polymerization of laminin on PS vesicles occurs at low laminin concentrations. Light-scattering intensities were measured after laminin was diluted to various final concentrations in 20 mM Tris-HCl, pH 7, containing PS vesicles (0.1 mM phospholipids). The inset shows light-scattering records obtained for vesicles only (bottom line) and after the addition of 6 nM laminin (upper line). Data were corrected by subtracting blanks containing vesicles only. S.E. values result from three separate determinations.

Fig. 11. Acidification promotes conformational changes in laminin. Laminin was diluted to 60 nM in 20 mM of either Tris-HCl, pH 7 (C) or acetate buffer, pH 4 ([square]), [circle], in the absence ([square], [circle]) or in the presence of 2 mM ([square]) or 5 mM ([circle]) urea. TNS was added to the indicated concentrations, and fluorescence spectra were recorded. Data shown correspond to the fluorescence intensities obtained at the peak of TNS fluorescence (443 nm) corrected for dilution after subtraction of blanks containing appropriate amounts of TNS in the absence of laminin. Data correspond to averages of at least two separate experiments, with S.E. values smaller than the symbols used.

addition of EDTA could prevent fusion but not reverse it. The second possibility, that laminin could be cross-linking PS vesicles, is also ruled out by the finding that solubilization of vesicles did not reverse the laminin-induced increase in light scattering (Fig. 2). Thus, the most likely interpretation is that PS vesicles trigger laminin aggregation without becoming an essential component of the aggregates.

It is well established that laminin aggregation in solution at neutral pH takes place only at protein concentrations above 60 nM (18, 20). The lack of a critical concentration for laminin aggregation, as shown here, had been reported previously for laminin polymerization onto sulfatide bilayers (21), where it was proposed that, upon binding to the surface of the bilayer, laminin would reach a higher effective concentration. In the present study, however, aggregation at low laminin concentrations was also obtained in solution upon acidification. The latter observation leads us to conclude that the contribution of the lipid surfaces is not restricted to a concentration effect, resulting from cumulative binding of laminin to the lipids, but that it may also involve conformational transitions in laminin, triggered by the contact with the lipid surface.

The observed parallel between the polymerization of laminin induced by acid and that induced by negatively charged lipids suggests that the electrostatic surface potential associated with the negative lipids may be the driving force for laminin polymerization. In fact, the predictable capacity of a negatively charged lipid surface to create a microenvironment of localized lower pH has been experimentally demonstrated using model vesicles of phosphatidylglycerol. The effective surface pH on these vesicles was determined to be approximately 2 units lower than the pH in bulk solution (24).

The biological events modulated by laminin are classically mediated by protein-protein or protein-carbohydrate interactions, involving a variety of membrane-associated molecules ranging from protein receptors of the integrin family (27, 28) or α-distroglycan (29–31) to heparan sulfate proteoglycans such as perlecan (32). In addition, adhesive properties of laminin have been shown to involve binding of the basement membrane protein to glycolipids on the cell surface. For instance, the capacity of laminin to agglutinate erythrocytes is known to involve its specific binding to sulfated glycolipids (33, 34). Furthermore, in order to promote neurite outgrowth, one of its best characterized properties, laminin binds to sialic acid-contain-
ing gangliosides present on the neuronal membrane (35).

The microenvironment of the plasma membrane surface is occupied by the glyocalyx, the carbohydrate-rich region covering the cell surface. Membrane-associated glycoproteins as well as gangliosides possess negatively charged residues of sialic acid. In addition, sulfate groups of the glycosaminoglycan moiety of proteoglycans may also contribute to the formation of an electrostatic potential, capable of reducing the local pH on the cell surface. We propose that a negative surface potential generated on the cell surface by the combined effect of all negatively charged molecules present in the glyocalyx is responsible for triggering laminin aggregation.

A series of previous proposals appearing in the literature provide background support for our hypothesis. First, a negative electrostatic potential has been proposed to play a role in the modulation of the interaction between granulocyte-macrophage colony stimulating factor and cell-associated glycosaminoglycans (36). Second, the electrostatic potential generated by sialic acid residues in gangliosides has been identified as being responsible for cell-substrate interactions mediated by fibronectin and laminin (37). Finally, negatively charged sulfate groups have been shown to mediate the stimulatory effect of glycosaminoglycans on laminin aggregation (38).

Microstructural analysis of laminin aggregates formed in solution at neutral pH revealed that, under these conditions, self-assembly leads to large three-dimensional aggregates. The properties of these aggregates are similar to those found in collagen-free basement membranes of embryonic carcinoma cells and in the collagen-rich basement membranes of Engelbreth-Holm-Swarm tumors (19). In both cases, however, laminin is present at micromolar concentrations (18), which, considering the dimensions of the extended lammin molecule (39), which, considering the dimensions of the extended lammin molecule (39), which, considering the dimensions of the extended lammin molecule (39), which, considering the dimensions of the extended lammin molecule (39), indicates that laminin deposits more likely correspond to sheet-like structures than to random three-dimensional aggregates.

Kalb and Engel (21) proposed that the plasma membrane of epithelial cells may function as a surface to orient laminin self-assembly into a two-dimensional arrangement. To their model we add the observation that self-assembly of laminin at low concentrations may be driven by a negative electrostatic potential arising from negative charges on membrane-associated molecules. This model acquires particular interest if we consider the time course of the physiological events, where laminin is the first extracellular matrix protein to be secreted (41, 42) and must therefore initiate its self-assembly process at low concentrations and in the absence of other basement membrane elements.

Acknowledgements—We thank Dr. Martha M. Sorenson for critical reading of this manuscript and Dr. Sérgio T. Ferreira for the use of laboratory facilities.

REFERENCES
