Heparin Modulation of Laminin Polymerization*

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Previously, it has been shown that laminin will self-assemble by a two-step calcium-dependent process using end-domain interactions (Yurchenco, P. D., Tiel library, E. C., Charonis, A. S., and Furthmayr, H. 1985) J. Biol. Chem. 260, 7636–7644). We now find that heparin, at low concentrations, modifies this polymerization by driving the equilibrium further toward aggregation, by producing a denser polymer, and by inducing aggregation in the absence of calcium. This effect on self-assembly is specific in that it is observed with heparin but not with several heparan sulfates or other glycosaminoglycans; it correlates with affinity and depends on the degree of polysaccharide sulfation. Heparin binds to laminin in a calcium-dependent manner with a single class of interaction (K_D = 118 ± 18 nM) and with a binding capacity of one heparin for two laminins. We find the long arm globule (E3) is the only laminin domain which exhibits substantial heparin binding: heparin binds E3 with an affinity (K_D = 94 ± 12 nM) and calcium dependence similar to that for intact laminin. These data strongly suggest that heparin modifies laminin assembly by binding to pairs of long arm globular domains. As a result the polymer may be stabilized at domain E3 and laminin inter-domain interactions induced or modified. We further postulate that heparins may act in vivo as specific regulators of the structure and functions of basement membranes by both altering the laminin matrix and by displacing weakly binding heparan sulfates.

Basement membranes serve as versatile barriers with sieving and cell regulatory functions. While sharing common structural elements, these extracellular matrices must be able to adapt to different physiological demands. For example, in inflammation, microvessels and their basement membranes increase their permeability to macromolecules and accommodate large increase in the flux of serum proteins. Such fluctuating needs may, at least in part, be met by secreted macromolecules such as glycosaminoglycans which serve as specific regulators of matrix structure and function.

Laminin (M_r = 800,000), a major intrinsic basement membrane glycoprotein, is composed of three polypeptide chains in which three short arms and one long arm extend out from a common vertex. Electron micrographs reveal a peripheral pair of globular domains on each short arm and a single larger globular domain at the end of the long arm (1). The N-terminal moieties of the component B1, B2, and A chains form the three short arms and join to form the long arm and A-chain terminal globule (2). Usually laminin is tightly complexed (3) to entactin/nidogen (4) in the vicinity of the intersection of the three short arms. Laminin will self-assemble in vitro (5) to form large polymers by a mechanism of nucleation-propagation. Polymerization can be divided into two steps: a temperature-dependent oligomer forming step followed by a divalent cation-dependent aggregation step (5). Half-maximal aggregation step is achieved at about 10 μM Ca^2+ with one to two calcium interactions of sufficient affinity to account for the development of conformational changes which confer polymerization activity (6, 7). While it is not clear whether calcium plays a regulatory role, or serves simply to constitutively maintain laminin in a functional conformation in vivo, the calcium phenomenon has aided in the dissection of the complex assembly process as well as to provide evidence for the physiological relevance of self-assembly (6).

In low angle rotary shadowed Pt/C replicas examined in the electron microscope, end-to-end associations involving both long and short arms have been seen in the dimers and many of the oligomers (5) and more recently, using high angle shadowing of freeze-etched laminin polymers (8) it has been possible to visualize the polymer in three-dimensions as a geometric array of short struts with lengths similar to those of the laminin arms meeting at vertices. Biochemical evidence has accumulated that terminal interactions of the arms are crucial for self-assembly and may be the only interactions involved in laminin polymerization (7, 9, 10). These interactions may be essential for establishing the three-dimensional architecture of basement membranes.

Laminin, in addition to binding to itself, binds heparin (11, 12) and heparan sulfate (12, 13), the latter a moiety of basement membrane and cell surface proteoglycans. While the function of glycosaminoglycan binding has been unclear, it has been thought that the role of this binding is structural with anchoring of both cell surface and intrinsic basement membrane heparan sulfate to laminin. The C terminal globule of the long arm contains a major heparin binding site (14) although other binding sites have also been proposed for the short arm regions of laminin (15, 16). In this study, we have examined the nature of the interaction of glycosaminoglycans with laminin and describe a modulating effect that heparin exerts on the self-assembly of laminin. This effect is dependent upon heparin binding and is specific both with respect to glycosaminoglycan and site of interaction on laminin. We further discuss the possibility that a heparin interaction mechanism may operate in vivo to regulate such basement membrane functions as sieving and structural support in different physiological state.

MATERIALS AND METHODS

Preparation of Macromolecules and Their Fragments

Laminin—Purification steps were carried out at 0–5 °C. Laminin was purified from lathyritic EHS tumor as the laminin-entactin

The abbreviations used are: EHS, Engelbreth-Holm-Swarm; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfite; HSPG, heparan sulfate proteoglycan.

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complex based on the method of Paulsson (6). Briefly, about 250 g of frozen tumor was homogenized in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4, containing 0.5 mM diisopropyl fluorophosphate, 0.5 mM PMSE, and 10 µg/ml p-hydroxymercuribenzoic acid. After washing the pellet by centrifugation, laminin/entactin was twice extracted with 1 liter of the above buffer containing 10 mM EDTA and centrifuged to remove insoluble residue. Aliquots (80–100 ml) of combined extract were chromatographed on a Sephadex G-400 (Pharmacia, Sweden) column (5 × 95 cm) in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 0.5 mM PMSE, 10 µg/ml p-hydroxymercuribenzoic acid. The first and major peak was then pooled and loaded onto a DEAE-Sephacel (Pharmacia) column (5 × 10 cm) equilibrated in 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 10 µg/ml p-hydroxymercuribenzoic acid, and 0.5 M NaCl. After collection of a 20 ml unbound fraction, the laminin/entactin, the column was subjected to a linear 0–0.8 M NaCl gradient (total 500 ml). The unbound and first bound peaks were separately pooled. Although both laminin/entactin fractions had similar aggregation properties, for this study we used the unbound fraction when evaluating intact protein. The unbound and bound fractions were combined and used for the generation of defined proteolytic fragments, described below.

Fragments E3, E8, E4, and E1° of Laminin—The heparin-binding domain derived from the globule of the long arm (E3), a fragment containing two short arms and a portion of the third (E1°), a fragment containing the N-terminal globule of the B1 chain (E4), and a fragment representing the distal long arm moiety and adjacent globule moiety (E8) were all generated proteolytically with elastase and purified by a modification of the methods of Ott et al. (14) and Paulsson (6). Laminin/entactin, at 1–2 mg/ml in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, and 10 µg/ml p-hydroxymercuribenzoic acid, was digested with a 1:200 substrate ratio with porcine elastase (Serva, N.Y., 101 units/mg) at 4 °C for 1 h followed by 25 °C for 23 h. After addition of 1 mM PMSF, 80-ml aliquots of digested protein were concentrated to 5–7 mg/ml in a 0–0.5 M NaCl gradient in the same buffer. Fragments El', E8, and E3, the protein solution was adjusted to 0.1 mg/ml with dextran sulfate (5 kDa) prior to mixing with activated beads. Remaining coupling activity was quenched with 1% ethanolamine in the same buffer in the cold.

Preparation of Protein Affinity Columns—Septarose 4B (Pharmacia) was activated with CNBr (0.1 mg/ml beads) and, following washing, incubated with an equal volume of protein (1–2 mg/ml) in 0.2 M sodium bicarbonate in the cold overnight. For laminin fragment E3, the protein solution was adjusted to 0.1 mg/ml with dextran sulfate (5 kDa) prior to mixing with activated beads. Remaining coupling activity was quenched with 1% ethanolamine in the same buffer in the cold.

Analytical Methods

Turbidity—Laminin/entactin solutions (1–2.5 mg/ml) were dialyzed on ice into degassed TBS containing either 1 mM CaCl2 (TBS/Ca) or 2 mM EDTA (TBS/EDTA) and centrifuged at 4°C in 1.5-ml aliquots at 40,000 rpm at 2°C in a Type 65 rotor (Beckman) for 70 min. The supernatants were collected, assayed for protein concentration, and diluted in the above buffers as required. Protein aliquots (100 µl) were then placed in a temperature-equilibrated 1-ml quartz cuvettes (maintained at 35 or 10°C in a thermostatic heater). Absorbance was measured at 1-min intervals in an automated double beam spectrophotometer (Perkin-Elmer model Lambda-4B) using a buffer blank. For turbidity-time plots, lag times were operationally defined as the time between the start of the incubation and the time axis intercept of a line drawn through the maximal slope of increase. These numbers were distinguished from the rapid growth phase expressed as the time to achieve half-maximal turbidity minus the lag time.

Sedimentation of Laminin and Laminin-Heparin Aggregates—For quantitation of laminin polymer formed, aggregated protein was separated from monomers and small oligomers by the microcentrifugation technique of Paulsson (6). Briefly, 1-ml volumes of laminin solutions, before or after incubation at various temperatures, were centrifuged in polyethylene Eppendorf tubes at 10,000 rpm for 15 min (with removal of aggregated material from the supernatant as described by absence turbidity). The supernatants were separated from the small protein pellets, and protein (and radioactivity when required) was measured in supernatants and pellets. Aggregated protein was calculated by subtracting supernatant protein from total protein present in the tubes prior to sedimentation. Sedimented protein polymer volumes were determined by subtracting tube mass from mass of tube plus pellet.

Zonal Velocity Sedimentation—The interactions of heparin with laminin fragments were evaluated on 5–20% (w/v) linear sucrose gradients in TBS/Ca or TBS/EDTA containing 0.1% Triton X-100 using either an SW40-Ti rotor (Beckman) or a TH-641 rotor (Sorvall) set at 10,000 rpm at 2°C in a Type 65 rotor (Beckman). 50 µl of a 1 mg/ml solution of lactoperoxidase (Sigma), and 50 µl of 0.033% 123Iodine (ICN), 20 µl of a 1 mg/ml solution of lactoperoxidase (Sigma), and 50 µl of 0.033% 123Iodine (ICN) were added to the supernatant solution and mixed. The samples were subjected to a linear 0–0.8 M NaCl gradient (total 500 ml). The unbound and first bound peaks were then rechromatographed in the cold in separate 0.6 × 50-cm Sepharose CL-6B columns.

Gelation Point and Gel Breakpoint—Laminin, concentrated to 6–8

3982 Heparin Modulation of Laminin Polymerization

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Heparin Modulation of Laminin Polymerization

mg/ml) in TBS/Ca on ice, was adjusted to 0.25 mg/ml incremental steps of concentration ranging from 0.15 to 5.5 mg/ml in 0.5 ml aliquots. The aliquots were placed in 24-well tissue culture wells and incubated in a moist chamber at 37 °C. After 2 hr, the plates were tilted at a 45° angle, and the liquid in each well was allowed to settle. The gelation concentration was defined as the lowest protein concentration in which the solution had completely solidified (did not flow). The shear-force (P x s) required to break a laminin gel was determined in a temperature-controlled cone-plate viscometer (Brookfield, MA, model RVTDCD) using a CP51 cone and a preset motor drive speed of 0.5 rpm. The protein, with or without heparin, was incubated for 1 h at 35 °C in the instrument prior to the initiation of the motor and application of shear force. The motor was then activated and axial force progressively transmitted to the cone and polymer. The shear force developed for gel disruption was measured by the instrument.

Determination of Protein Concentration and SDS-Polyacrylamide Gel Electrophoresis—Protein in solution was determined either by absorbance (6) at 280 nm (for protein cleared of turbid material) or by a modification of the method of Lowry (22) as described (5). Protein containing Triton X-100 for calorimetric determination was first cleared by centrifugation before placing in cuvettes. Laminin, at concentration of 1 mg/ml as determined by amino acid analysis (Protein Chemistry Laboratory Service, Yale University School of Medicine, New Haven, CT), corresponded to a Lowry-determined concentration of 1.12 mg/ml (against a bovine serum albumin standard) and an absorbance of 0.76 at 280 nm. From the sum of published (2,23-25) amino acid residue molecular mass for the three chains of laminin (710.1 kDa = 196.9 + 177.5 + 335.7 kDa) and entactin/nidogen (124.7 kDa) it can be calculated that the protein molecular mass of this complex is 834.8 kDa, and the total molecular mass, given 12% carbohydrate, 950 kDa. For fragment E3, 1 mg/ml by amino acid analysis corresponded to 1.35 mg/ml by Lowry determination.

This fragment is also 98% protein (2,26) and the same correction factor was used. Laminin/entactin and E3 values were expressed corrected to amino acid analysis values while the other fragment values were used uncorrected. SDS-polyacrylamide gel electrophoresis was accomplished using 3.5 to 10% or 3.5 to 12% linear gradient gels as described (17) and stained with Coomassie Brilliant Blue R-250.

Determination of Radioactivity—Aliquots of [3H]-labeled samples were adjusted to 0.5 ml with water and mixed with 5 ml of Hydrofluor (National Diagnostics, NJ) scintillation fluid and radioactivity quantitated in a scintillation counter (Beckman model LS-233). Protein pellets were solubilized in a small volume of SDS sample buffer prior to addition of scintillation fluid. Samples captured on nitrocellulose (0.45 µm, Schleicher & Schuell, NH) filters (6 x 6 mm) were wetted with 50 µl water and dissolved in 5 ml of Filtron-X (National Diagnostics) scintillation fluid.

Counting efficiency was found to be 91% with respect to aqueous samples determined with Hydrofluor using an open tritium channel. Aqueous counting efficiency was 0.28 for fragments (1 ml or less) of [125I]-labeled protein was determined using a gamma counter (LKB model 1271). Counting efficiency for [125I]-label was found to be 0.735. Samples containing both isotopes (with tritium maintained in 7-10-fold excess) were counted in a gamma counter and scintillation counter and iodine counts were multiplied by 0.826 (gamma/scintillation cpm) and subtracted from tritium counts for each fraction.

Heparin Binding to Laminin Fragments—[3H]Heparin (142 units/mg; 0.43 mCi/mg) was incubated in 0.1-ml aliquots in TBS/Ca or TBS/EDTA with protein in a temperature-controlled water bath (model RTE-210, Neslab, NH) at 35 °C in Eppendorf tubes. The protein and bound heparin were captured on a nitrocellulose membrane (Schleicher & Schuell, 0.45 µm) fitted in a 96-well vacuum manifold. After pipetting of each sample into a well, each well was washed with 0.2 ml of buffer under vacuum (wash time less than 2 s). The nitrocellulose sheet was then cut into individual squares (corresponding to the wells) for scintillation counting. More than 95% protein was captured on these filters as determined with radiolabeled protein fragments.

Fitting of Binding Data—Plots of moles of heparin bound/mol of µmolec (B) versus free heparin (F) were computer-fitted (Enzfitter program) by non-linear regression to the ligand binding equation B = C x F/(K_D + F) where K_D is the dissociation constant and C the molar binding capacity.

Electron Microscopy—Macromolecular samples in 60% glycerol, 0.40% 15 m ammonium bicarbonate were sprayed onto freshly cleaved mica and rotary shadowed at an 8° angle with 0.6-0.8 nm Pt/C in a Balzers BAF 301 or 500K freeze-etch/fracture unit. Replicas were examined in a Phillips 420EM at 60 kV with a 30-µm objective aperture.

RESULTS

Effect of Heparin on the Rate and Extent of Laminin Polymerization—In this study we have used laminin complexed (6) to entactin (nidogen). We found results were more reproducible with respect to quantitation of polymer formed using the complex and attribute this to protein purification without the use of chaotropic agents. We further note that entactin/nidogen does not appear to be required for laminin self-assembly or modification by heparin. Laminin, dissociated from entactin/nidogen in 2 M guanidine HCl and purified by gel filtration, exhibits both calcium- and heparin-dependent alterations of aggregation as measured by turbidity (data not shown).

When laminin/entactin was incubated at 35 °C with increasing concentrations of heparin, a biphasic alteration of maximum turbidity and amount of polymer was observed as a function of heparin concentration (Fig. 1). The rise in slope was steep from 0 to 5 µg/ml, becoming less steep above this concentration with a turbidity/polymer maximum achieved at 25-50 µg/ml. At higher concentrations of heparin (50-200 µg/ml), reflecting an 8-31 molar ratio range of aggregation as measured by turbidity (data not shown).

Previously it has been shown that there is a linear relationship between turbidity and amount of laminin polymer formed (6). A plot of the ratio of the two has a slope near zero. However, at increasing concentrations of heparin the turbid-
Protein concentrations substantially higher than the critical concentration for gelation of laminin were found to quantitatively increase the amount of polymer formed. A graph (Fig. 3A) of different concentrations of laminin incubated with different fixed ratios of heparin for several hours could be plotted as a series of parallel lines, one for each heparin/protein ratio, and each with a slope value (0.9) close to one. The x axis intercept (panel A of Fig. 3) gave a critical concentration, or concentration at and below which no polymer forms, for each different heparin/protein mass ratio and different absolute protein concentrations. Panel C, rate relationships for the rapid growth phase plotted as the time to achieve half-maximal turbidity minus lag time at different heparin/protein mass ratios and different absolute protein concentrations.

FIG. 2. Effect of heparin on rate of laminin polymerization. Laminin/entactin, in TBS/Ca, was incubated at 35 °C for 1 h. Panel A, turbidity as a function of incubation time (0-30 min shown out of total 60 min assay) plotted for laminin in absence and presence of heparin. Plot shown for protein at 0.4 mg/ml (and similarly evaluated in a range of 0.1 to 0.5 mg/ml protein for preparation of plots in panels B and C). Panel B, reaction lag time as a function of different heparin/protein mass ratios and different absolute protein concentrations. Panel C, rate relationships for the rapid growth phase plotted as the time to achieve half-maximal turbidity minus lag time at different heparin/protein mass ratios and different absolute protein concentrations.

FIG. 3. Effect of heparin on critical concentration of laminin polymerization. Laminin/entactin, in TBS/Ca, was incubated at 35 °C for 2-3 h (high-low conc.) at the indicated protein concentrations with heparin at different heparin/protein mass ratios. In parallel turbidity assays plateau was reached in 1-2 h. The concentration of polymerized protein was determined by sedimentation and sedimentation coefficients determined from the x axis intercept of fitted lines shown in panel A. Laminin/entactin, in TBS/Ca, was mixed at the start of incubation at 35 °C for 1 h. Panel A, turbidity as a function of incubation time (0-30 min shown out of total 60 min assay) plotted for laminin in absence and presence of heparin. Plot shown for protein at 0.4 mg/ml (and similarly evaluated in a range of 0.1 to 0.5 mg/ml protein for preparation of plots in panels B and C). Panel B, reaction lag time as a function of different heparin/protein mass ratios and different absolute protein concentrations. Panel C, rate relationships for the rapid growth phase plotted as the time to achieve half-maximal turbidity minus lag time at different heparin/protein mass ratios and different absolute protein concentrations.

FIG. 4. Effect of heparin on thermal gelation of laminin. Panel A, minimal concentration for gelation. Aliquots (0.25 ml) of laminin/entactin in TBS/Ca were prepared at concentrations ranging from 1.42 to 7.52 mg/ml in 0.24 mg/ml intervals and mixed with the indicated amounts of heparin. The aliquots were then pipetted into 1-ml capacity plastic tissue culture wells and incubated at 37 °C in a moist chamber. The minimal protein concentration at and below which no polymer forms, for each different heparin/protein ratio. This critical concentration was observed to decrease from a value of 50 µg/ml protein (60 nM) to a minimal value of 12 µg/ml (14 nM) when heparin was incubated in molar excess (2:1 molar ratio) with respect to protein (Fig. 3B). Effect of Heparin on the Thermal Gelation of Laminin—At protein concentrations substantially higher than the critical concentration, laminin/entactin (and pure laminin) incubated at 35 °C, not only polymerized, but underwent a distinctive sol-to-gel transition as well. The gel which formed was clear and maintained its shape in a tube when completely inverted. When placed on ice, the gel converted back to a liquid state. Thus, the temperature-dependent sol-gel interconversion paralleled (at higher concentration) the thermal reversible aggregation described for laminin earlier (5). The minimal protein concentration for gelation of laminin was found to be 1.7 mg/ml protein (i.e. 1 ml of buffer required a minimum of 2 µM laminin/entactin complex to form a gel). We interpreted this physical property as follows: laminin polymers have relatively fixed maximal intermolecular spacing. At concentrations below the gel point, a suspension of polymer aggregates form while at concentrations at and above the gel point the suspended laminin aggregates become so close to each other they contact and fuse to form a continuous polymer.

The sol-to-gel transition point was observed to change when protein was incubated in the presence of heparin (Fig. 4A). At 20 µg/ml heparin, 3 mg/ml of protein was required for gelation, and at 40 µg/ml heparin, 3.5 mg/ml of protein was required. At a concentration above the minimum required for gelation in laminin with heparin, the shear-force required to disrupt a heparin-laminin gel (at 20 µg/ml protein) was found to be one-half as great as that required to disrupt a pure laminin gel (Fig. 4A, inset). Thus, for a constant protein concentration, the addition of heparin renders the polymer more fragile. The seemingly paradoxical finding that heparin drives assembly toward completion but raises the minimal protein concentration for gelation could be explained given the above described model for protein packing. Heparin decreases the average center-to-center molecular spacing producing a denser geometry. A protein gel at fixed concentration would form a sol with heparin because there would now be insufficient protein subunits to fill the fixed volume as a continuous polymer at the new required density. This interpretation is supported by the observation (Fig. 4B) that laminin polymer aggregates formed below the gel-point became increasingly dense as increasing heparin concentrations. While the force of centrifugation used to sediment the aggregates (15,000 × g) led to a severalfold compression of the
polymer, a definite and continuous relationship was noted between polymer mass and volume such that as heparin was increased, polymer mass increased while polymer volume decreased (nearly 2-fold over the range studied). The effect was specific and dependent on glycosaminoglycan binding (see ahead) since EHS heparan sulfate (from low density HSPG), a related polyanionic macromolecule, had essentially no detectable effect on polymer density under the same conditions (plot not shown).

Calcium Dependence of Heparin Effect—The second step of laminin self-assembly, in which large aggregates form, is calcium-dependent and blocked at the oligomer step in EDTA (5, 6). While laminin aggregation occurred in the presence of calcium and was inhibited in the presence of EDTA as expected (Fig. 5), heparin was found to induce aggregation even in the presence of EDTA at 35 °C (although at a level reduced compared with that in calcium). This effect was temperature dependent and, at these concentrations, essentially absent in the cold.

Heparin Binding to Laminin—When 125I-labeled laminin/entactin complex was passed down a heparin-Sepharose CL-4B affinity column, a small fraction of radioactivity passed through the column in TBS and the remainder (96%) bound to the column was eluted with 1 M NaCl. In order to determine equilibrium conditions for heparin binding to laminin/entactin, [3H]heparin (2 and 20 μg/ml) was incubated with laminin/entactin complex (0.36 mg/ml protein) at 35 °C in TBS/Ca. One-ml aliquots incubated between 0 and 90 min were sedimented in the cold. The fraction of precipitated protein reached a plateau by 20 min, and the heparin/protein ratio reached a plateau within 5 min (data not shown). The amount of heparin which bound the laminin/entactin complex (0.36 mg/ml) in a polymer was investigated at apparent equilibrium (1 h) in both the presence and absence of calcium. Protein was allowed to polymerize in the presence of heparin (0.45–49 μg/ml). A typical binding curve is shown in Fig. 6. From these data, dissociation constants (Kd = 135 nM in calcium and 507 nM in EDTA) and binding capacities (n = 0.46 in calcium and 0.42 in EDTA) were determined by non-linear regression fitting of the data using the Enzfitter computer program (standard errors, 7% and 12% for Kd values in presence and absence of calcium respectively; 2 and 5% for binding capacities in the presence and absence of calcium, respectively). Non-linear regression fitting of data from additional separate assays (in calcium) yielded an average dissociation constant of 118 ± 18 nM (n = 3 where separate values were 100, 135, and 119 nM) and an average heparin/laminin molar binding capacity of 0.52 ± 0.06 (n = 3 where separate values were 0.58, 0.46, and 0.53). The Scatchard transforms of the data could be superimposed by transforms of the fitted lines (see example in Fig. 6, inset), and all plots were best fitted as a single class of binding interaction. In the presence of EDTA, heparin binding (Fig. 6) was observed to be substantially decreased (Kd = 507 nM) with a similar binding capacity (0.42). Thus, heparin binding itself is calcium-dependent.

Relative Binding Affinities of Glycosaminoglycans for Laminin—The relative ability of selected glycosaminoglycans to prevent labeled heparin from binding laminin was examined (Fig. 7). On a mass basis, a 10-fold higher concentration of low molecular mass heparin (4–6 kDa) was required to produce 50% inhibition while about 100-fold more d-N-sulfated heparin, basement membrane heparan sulfate, and bovine kidney heparan sulfate were required to produce 50% inhibition. From this it was concluded that heparin binds laminin with substantially greater affinity as compared with the other polysaccharides tested. Furthermore, there also appears to be a heparin size dependence for binding.

Specificity of Heparin Modulation of Laminin Self-assembly—Various polysaccharides were incubated with laminin/entactin (Fig. 8) at a condition (glycosaminoglycans at 10 μg/ml; protein at 0.4 mg/ml) in which one observes a near-maximal effect with heparin (Figs. 1 and 3). Heparin, derived from different sources, produced a substantial increase in turbidity. When heparin cleaved to about one-third its parent size (4–6 kDa) was incubated with protein, a greatly diminished enhancement was observed. Removal of the N-sulfates from heparin (heparin is both N-sulfated and O-sulfated) abolished the modifying influence of heparin. Structurally related heparan sulfate, derived either from the EHS basement membrane (low density form) or from a more general bovine kidney source, had no modulating influence. EHS heparan sulfate from high density HSPG had little effect. Chondroitin sulfates B (dermatan sulfate) and C and hyaluronic acid had no effect. On the other hand, dextran sulfates produced a pronounced enhancement of turbidity. In a separate series of turbidity-time experiments carried out with...
FIG. 7. Inhibition of heparin binding to laminin polymer by other related glycosaminoglycans. Laminin/entactin (0.36 mg/ml) in TBS/Ca incubated with labeled heparin (1.5 µg/ml) the half-saturation binding determined from data of Fig. 5 in 1-ml aliquots alone and with progressively higher concentrations of unlabeled heparin (closed circles), low molecular mass (4-6 kDa) heparin (open squares), heparan sulfate from EHS low density HSPG (solid triangles), de-N-sulfated heparan (open circles), and bovine kidney heparan sulfate (open squares). Percent inhibition was determined from bound radioactivity for a given concentration of unlabeled glycosaminoglycan (GAG) divided by bound radioactivity for [3H]heparin in the absence of unlabeled glycosaminoglycan × 100.

FIG. 8. Specificity of heparin effect on laminin polymerization. Turbidity time plot of laminin/entactin (0.36 mg/ml) in TBS/Ca incubated with various polysaccharides (10 µg/ml) at 35°C for 1 h with absorbance determinations at 1-min intervals. Symbols: Lm, laminin/entactin; Hep (p.i.), heparin, 15 kDa, pig intestinal mucosa; Hep (b.l.), heparin, bovine lung, Hep(4-6kDa), low molecular mass (4-6 kDa) heparin; Hep(dNS), de-N-sulfated heparin; Ld.HS/EHS, heparan sulfate (50 kDa) isolated from EHS low density HSPG; h.d.HS/EHS, heparan sulfate isolated from EHS high density HSPG; HS(b.k.), bovine kidney heparan sulfate; HA, hyaluronic acid; DS(5kDa), high molecular mass (500 kDa) dextran sulfate; DS(5kDa), low molecular mass (5 kDa) dextran sulfate; CS-B, chondroitin sulfate B; CS-C, chondroitin sulfate C.

polysaccharide at 10 µg/ml and protein at 0.25 mg/ml (plots not shown), dextran was found to have no effect on polymerization compared with dextran sulfates. In comparing the relative affinities (Fig. 7) to turbidity differences (Fig. 8) for parent heparin, low M, heparin, and heparan sulfate from EHS tumor and bovine kidney, it was noted that the greater the affinity, the greater the increase in aggregation. Thus, the relative abilities of these glycosaminoglycans to enhance laminin aggregation are similar to their relative abilities to bind laminin. Furthermore, these depend on the degree of sulfation as well as size.

Antithrombin III, a serum anticoagulant, can specifically bind heparins which contain, in the presence of other sulfates, a 3-O-sulfate at glucosamine in a pentasaccharide unit (27). Heparin could be substantially inhibited (65% at 100 µg/ml with antithrombin III with plateau value approached) with respect to its enhancement of laminin aggregation by coincubation with antithrombin III (data not shown). Sixty % of heparin was found to bind an antithrombin-Sepharose CL-4B affinity column in TBS, and the degree of inhibition corresponds to the fraction of heparin capable of binding antithrombin. It was further found that essentially all heparin can bind a laminin fragment E3 affinity column (2.8 ml) in 0.1 M ammonium bicarbonate (plots not shown). It thus appears that laminin does not discriminate between the two classes of heparin.

Contributions of Laminin Domains to Heparin Modulating Effect—In order to understand the sites and site-specific affinities, stoichiometries and calcium dependence of heparin interaction, we evaluated the binding of heparin to defined laminin fragments (Fig. 9A, inset). From the amino-terminal sequence of fragment E3 (26) and the total sequence of the A chain of mouse laminin (2) it can be deduced that E3 starts at residue number 2666 and, given its molecular mass (50-kDa total; 44-kDa protein), extends to or near the C terminus, representing the distal moiety (globular subdomains G4 and G5 (21) of the long arm globular domain (Fig. 9A, inset). This fragment has the appearance of 7-8-nm diameter globules in rotary shadowed Pt/C replicas and migrates as a 50 kDa band in SDS-polyacrylamide gels. The large (elastase-derived) short arm domain fragment E1 (6, 7) is observed in rotary shadowed Pt/C replicas as a Y-shaped particle with two short (each ~37 nm long) arms, each with a pair of globules and a third shorter arm segment (~20 nm), the latter usually lacking the inner globular domain. When analyzed by SDS-polyacrylamide gel electrophoresis it migrates as a doublet (460, 420 kDa) non-reduced, and this fragment, as prepared, is very similar to that reported by Paulsson et al. (6, 7). Fragment E8 represents the distal moiety of the long arm and adjacent proximal portion of the terminal globule. It has the appearance of a thin rod (~40 nm) attached to a globule and migrates in nonreduced gels as two bands (~140 and ~80 kDa). Fragment E4 is the N-terminal end of the B1 chain of laminin. This fragment, which binds laminin and inhibits polymerization (28), migrates as a single band (70 kDa) by SDS-polyacrylamide gel electrophoresis and has the appearance of a small globule with an adjacent short stem in Pt/C replicas.

Initial studies were carried out by affinity chromatography on heparin columns, a method we find can detect weak interactions. While fragments E3, E1', and E8 could bind heparin affinity columns, the latter two eluted at lower ionic strength (0.2 M) compared with E3 (0.3 M). We found no detectable binding for fragments E1', E4, and E8 (Fig. 9A) by incubation in solution and analysis on nitrocellulose filters (or by ultracentrifugation with E1'; data not shown). In contrast, binding could readily be detected and quantitated between heparin and fragment E3 (Fig. 9A and B). The time course of [3H]heparin (2.65 × 10^6 cpm/µg) binding to fragment E3 in solution was evaluated. Following incubation of radiolabeled heparin (3 µg/ml) with E3 (0.075 mg/ml protein; 1.7 µM) in TBS/Ca and TBS/EDTA, the amount of heparin bound to protein was determined after trapping of the protein on nitrocellulose after incubation at 35°C at intervals between 1 and 60 min and maximal binding was achieved in less than 5 min. Radiolabeled heparin was then incubated under apparent
equilibrium conditions (30 min) in solution with purified fragment E3 (Fig. 9, A and B) in the presence and absence of calcium. Binding of heparin to protein was found to reach saturation and the data could best be fitted as a single class of noncooperative binding interaction. From five separate determinations an average dissociation constant of $9.4 \pm 1.2 \text{nM}$ ($89, 79, 112, 93, \text{and} 95 \text{nM}$) was calculated in the presence of calcium, similar to that for intact laminin. Furthermore, a severalfold reduction in affinity ($K_d = 225 \text{nM}$) was observed in the presence of EDTA. However, the molar binding capacity (0.35 heparin/dimeric E3) estimated by this method was lower than that expected (one heparin for each dimer) and determined by other means (see below).

Fragment E3-heparin incubation mixtures were analyzed by zonal velocity sedimentation (Fig. 9C). A single faster moving species containing nearly all of the protein and a fraction of the heparin was identified and the molar ratio of this species was determined (from specific radioisotope ratio of peaks) to be 0.54/E3 monomer (i.e. one heparin/E3 dimer, see below). When analyzed in gradients containing heparin (plot not shown) the S value of protein (relative to standards) increased from 3.1 (without heparin) to 4.2 (with heparin). The molar ratio could be confirmed by a determination of the molecular weight of the complex by equilibrium centrifugation. The effective reduced molecular weights ($\epsilon$) of E3 and E3-heparin were found to be 1.88 and 2.43, respectively. From these values, and partial specific volumes (see "Materials and Methods"), average molecular masses of 106 kDa (E3) and 119 kDa (E3-heparin) were calculated. Thus, fragment E3 existed on average as a dimer at this concentration at physiological pH and ionic strength. Furthermore, the molecular mass increase (13 kDa) is consistent with the addition of one heparin/E3 dimer.

**Topographical Distribution of Heparin on Laminin/Entactin—**Heparin, as visualized in low angle Pt/C replicas (Fig. 10A) appeared as short thin filaments. When laminin/entactin was incubated with heparin in the cold and the complexes analyzed in the electron microscope (Fig. 10B), it could be seen that heparin decorates the protein cross in a nonrandom distribution, interacting preferentially at the globule of the long arm of laminin. The contour lengths of heparin molecular forms were measured and plotted as a histogram (Fig. 11A). A length of $21.4 \pm 8.1 \text{ nm}$ (average $\pm$ S.D., $n = 267$) was determined. Given an average $M_r$ of 15,000 and 500/disaccharide, we estimate 30 disaccharides, or 0.7 nm/disccharide, similar to that observed earlier (13). The topographical sites on laminin decorated by heparin were analyzed by arbitrarily dividing laminin into eight regions and scoring the apparent interactions (Fig. 11B). The major fraction (0.7) of associations were located at the long arm globule of laminin: the remaining minor fraction (0.3) was distributed at various locations, each with a low frequency. We interpret the long arm globular domain interactions as significant. Given the number of observed interactions (46), the other decorated sites could either reflect nonspecific associations or weak binding interactions.

**DISCUSSION**

**Effect of Heparin on Laminin Assembly—**Heparin has a biphasic effect on laminin self-assembly. At high ($> 6 \text{ \mu M}$) heparin concentrations, laminin aggregation is inhibited. We have focused on the effects of lower concentrations (0-4 $\text{ \mu M}$) where heparin binding alters the thermodynamics of laminin polymerization such that assembly occurs at a faster rate, equilibrium is pushed further toward completion, and a denser polymer is formed. At maximal effective heparin concentrations, laminin is almost completely converted to an aggregated state with about a 2-fold increase in density. If the critical concentration is a true measure of the propagation constant for laminin, as it is for other protein polymerizations (29), then heparin decreases this constant. We have further seen that at protein concentrations (above 2 $\text{ \mu M}$) approaching those in basement membranes (about 13 $\text{ \mu M}$ in the EHS matrix) laminin polymerization leads to the formation of a gel. Assuming the laminin gel is essentially a homogenous polymer, we can interpret the heparin alteration of the gelation point as a glycosaminoglycan-induced decrease in the spacing between protromers. From the protein molarity at the sol-to-gel transition point we can calculate an average inter-laminin distance of 90 nm in the absence of heparin. In the presence of heparin the minimal protein molarity required for sol-to-gel conversion increases, and a denser polymer is formed. From a minimal gelation concentration in heparin (4...
Heparin Modulation of Laminin Polymerization

FIG. 10. Electron micrographs of heparin and heparin incubated with laminin. Panel A, heparin (15 kDa; 10 μg/ml) rotary shadowed with Pt/C. Note very thin short filaments (white arrowheads indicate examples), ranging from less than 10 to greater than 40 nm. Panel B, heparin (20 μg/ml) was incubated with laminin/entactin (0.4 mg/ml) in 0.15 M ammonium bicarbonate, 2 mM EDTA at 10 °C for 1 h (these conditions, which permit reduced heparin binding, inhibit laminin aggregation), diluted 10-fold into glycerol/ammonium bicarbonate, and rotary replicated with Pt/C. Of 1009 molecular forms examined with the electron microscope, 4% were aggregated; of the remainder, nearly all (95%) were monomers with occasional dimers. Heparin associations (small arrows) of these molecules could be identified in about 5% of molecular forms, and 16 examples are shown.

μM), we would calculate this average distance decreases to 74 nm for the highest amount of heparin evaluated.

Laminin aggregation requires the presence of divalent cation, in particular calcium. Heparin, however, induces an aggregation even in the absence of divalent cation. Heparin will bind laminin in both calcium or EDTA, the latter state producing an interaction of lower but sufficient affinity to alter protein self-assembly. The heparin/EDTA effect suggests that heparin either activates calcium-dependent binding sites of laminin (otherwise inactive in EDTA) or induces new laminin-laminin interactions (bypassing the calcium-dependent step). While it is not clear whether laminin uses all of the same protein self-assembly interaction sites in the presence of heparin, we know that heparin binds the long arm globule (see below), and this implies that heparin-induced aggregation interactions are mediated through this domain.

Heparin Binding to Laminin—Heparin is a repeating disaccharide of glucuronic/Iduronic acid and N-acetyl glucosamine with both O- and N-sulfate substitutions (about two disaccharide unit). With an average of about 15,000 daltons in our preparations, it possesses about 30 similar disaccharide repeats measuring an average of 21 nm in length. Heparin is found to bind laminin in calcium with a $K_d$ of 118 ± 18 nM. The heparin/laminin molar ratio at saturation of 1:2 indicates that heparin binds to pairs of laminin molecules, possibly acting as a bridge. This idea is not surprising given the length of heparin, its repeating disaccharide structure, and the smaller (about 8 nm) size of a long arm globule of laminin.

Laminin Sites for Heparin Binding—Plots of heparin binding to intact laminin are best fitted for a single class of binding interaction and this implies a single site of interaction. We have confirmed, and furthermore quantitated, earlier observations (14) that heparin binds the globule of the long arm. Since the affinity and calcium dependence of E3 is quite similar to that for intact laminin, we conclude that the distal moiety of the long arm globule is that binding site. It has
recently been proposed (15, 16) that there are additional heparin-binding sites on the short arms of laminin. Our data, taken in conjunction with published data (14, 30, 31), indicate that other sites on the short arms or long arm (E8) are of substantially lower affinity as compared with the long arm globule. It has previously been noted by heparin affinity chromatography (14, 30, 31) that fragments E4 (N-terminal globule and stem of B1 chain short arm), E1 (short arm complex without globular domains), and E10 (proximal globular domain of B1 chain short arm lying between E4 and E1') will not bind heparin. By direct binding assays and electron microscopy of heparin interactions with intact laminin, we confirm these earlier findings and extend them to include the short arm complex with globules, fragment E1'. While fragments E1' and E8 (distal end of long arm without major heparin binding region) can bind heparin columns at ionic strengths significantly lower than for E3, both a quantitative binding assay and electron microscopy fail to demonstrate these interactions. We therefore doubt that other heparin-binding sites are relevant for laminin polymerization at aggregation-enhancing concentrations and further expect that heparin can bind these other sites only at high, and possibly non-physiological, glycosaminoglycan concentrations.

**Specificity of Glycosaminoglycan Interactions**—The heparin interaction with laminin is specific. Of the polysaccharides tested, only heparin and dextran sulfate have significant activity. Chondroitin sulfate C, dermatan sulfate, hyaluronic acid, and several heparan sulfates are inactive. Activity correlates with relative affinity and both affinity and activity correlate with the degree of sulfation. 1) Bovine kidney heparan sulfate, while quite similar to heparin, has a lower degree of sulfation (average of one versus two sulfates/disaccharide, respectively) and has little relative binding (about 100-fold less on a molar basis) and no measurable aggregating activity. 2) De-N-sulfation eliminates heparin activity. 3) While highly sulfated dextran sulfate (a polysaccharide with two to three sulfates/sugar residue (32)) which, like heparin, is also an anticoagulant and inhibitor of smooth muscle proliferation (33, 34) has a potent effect on aggregation: non-sulfated dextran does not. Size also appears to play a role. Heparin cleaved to one-third its parent size has little relative binding and little aggregating activity. Like bovine kidney heparan sulfate, the heparan sulfate purified from the low density HSPGs from the EHS tumor matrix has little binding activity and no effect on laminin aggregation. It has been found (27) that 70% of this particular disaccharide of this glycosaminoglycan are non-sulfated, 20–25% monosulfated, and 5% disulfated. Most of the sulfates are N-linked, and the molecule does not bind antithrombin III. In contrast, heparan sulfate derived from the embryonic Reichert's membrane is more heavily sulfated (minimum of two sulfates/disaccharide) and binds antithrombin III (27). Thus, there appears to be variability of basement membrane heparan sulfation, and we would predict that the most highly sulfated forms can probably bind laminin and alter its self-assembly.

**Molecular Models**—The molecular mechanism for the effect of heparin on laminin polymerization, while only partially understood at present, can be divided into two parts (Fig. 12). 1) The data show that heparin specifically and selectively binds pairs of laminin long arm globules and that this binding alters the thermodynamics of assembly. The simplest model is that heparin stabilizes the interaction between pairs of C-terminal globular domains by cross-linking. The laminin binding capacity of one heparin for every two laminins, the single heparin-binding site, the length (21 nm) and repeating structure of heparin, the reduced effect with short heparin, all are consistent with this interpretation. However, we have yet to demonstrate a prediction of this model, i.e. that heparin directly modifies the isolated C-terminal domain monomer-dimer equilibrium. As attractive as this model is, we can conceive of an alternate more complex model which needs to be considered. In this model heparin binding to C-terminal domains dimerizes results in conformational changes ("allosteric" effect) that lead to higher affinity interactions with other laminin domains. Data consistent with this second interpretation is the finding that in the presence of EDTA,
membranes. Heparin, because it binds more avidly to laminin as compared to other glycosaminoglycans, has the inherent ability to displace more weakly binding glycosaminoglycans from the long arm globule. The relative ratio of glycosaminoglycans, as discussed below, would then determine the relative occupancy on laminin.

A Hypothesis for Regulation of Basement Membrane Structure in Vivo—Heparin has been shown to act as an extracellular modulator of a number of physiological processes. In addition to its function as an anticoagulant, heparin inhibits the proliferation of smooth muscle cells (33), alters the secretion of smooth muscle cells below, would then determine the relative occupancy on basement membranes. On the other hand, it is released by the cells that normally produce heparan sulfate chains. A lo-20-fold increase in heparin concentration could act directly on the newly forming basement membrane. We can distinguish a separate effect of heparin binding to laminin (Fig. 12B). Heparin, glycosaminoglycans, has the inherent ability to displace more highly sulfated heparan sulfates, laminin supramolecular architecture in vitro—Heparin has been shown to act as an extracellular modulator of a number of physiological processes. In addition to its function as an anticoagulant, heparin inhibits the proliferation of smooth muscle cells (33), alters the secretion of smooth muscle cells below, would then determine the relative occupancy on laminin.

In short, intrinsic basement membrane structure and function could be changed by a transient rise in concentration of a single extrinsic glycosaminoglycan. While heparin can be implicated in alterations of basement membrane structure and functions (36, 41, 42), no direct cause and effect have yet been established. The findings here described how heparin, in vitro, can act as a specific regulator of laminin supramolecular assembly and structure. This raises the interesting possibility that such heparin mechanisms may exist in vivo and may be of importance in a number of physiological events. Furthermore, these findings suggest in molecular terms, how such mechanisms might operate.

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