Heparin Type IV Collagen Interactions: Equilibrium Binding and Inhibition of Type IV Collagen Self-assembly*

(Received for publication, June 27, 1988)

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Interactions between type IV collagen and heparin were examined under equilibrium conditions with rotary shadowing, solid-phase binding assays, and affinity chromatography. With the technique of rotary shadowing and electron microscopy, heparin appeared as thin, short strands and bound to the following three sites: the NC1 domain, and in the helix, at 100 and 300 nm from the NC1 domain. By solid-phase binding assays the binding of [3H]heparin to solution to type IV collagen immobilized on a solid surface was found to be specific, since it was saturable and could be displaced by an excess of unlabeled heparin. Scatchard analysis indicated three classes of binding sites for heparin-type IV collagen interactions with dissociation constants of 3, 30, and 100 nm, respectively. Furthermore, by the solid-phase binding assays, the binding of tritiated heparin could be competed almost to the same extent by unlabeled heparin and chondroitin sulfate side chains. This finding indicates that chondroitin sulfate should also bind to type IV collagen. By affinity chromatography, [3H]heparin bound to a type IV collagen affinity column and was eluted with a linear salt gradient, with a profile exhibiting three distinct peaks at 0.18, 0.22, and 0.24 M KC1, respectively. This suggested that heparin-type IV collagen binding was of an electrostatic nature. Finally, the effect of the binding of heparin to type IV collagen on the process of self-assembly of this basement membrane glycoprotein was studied by turbidimetry and rotary shadowing. In turbidity experiments, the presence of heparin, even in small concentrations, drastically reduced maximal aggregation of type IV collagen which was prewarmed to 37 °C. By using the morphological approach of rotary shadowing, lateral interactions and network formation by prewarmed type IV collagen were inhibited in the presence of heparin. Thus, the binding of heparin resulted in hindrance of assembly of type IV collagen, a process previously described for interactions between various glycosaminoglycans and interstitial collagens. Such regulation may influence the assembly of basement membranes and possibly modify functions. Furthermore, qualitative and quantitative changes of proteoglycans which occur in certain pathological conditions, such as diabetes mellitus, may alter molecular assembly and possibly permeability functions of several basement membranes.

 Basement membranes are composed of a number of distinctive macromolecules including type IV collagen, laminin, heparan sulfate and chondroitin sulfate proteoglycans, entactin/nidogen, etc. (1). Most of these macromolecules, i.e. type IV collagen, laminin, and heparan sulfate-proteoglycan are known to self-assemble (2–5) and can also bind to each other as well as to other components of basement membranes (1). For example, EHS-derived basement membrane-like heparan sulfate proteoglycan as well as heparin have been reported to bind to type IV collagen via the main noncollagenous, NC1 domain (6, 7). A morphological study indicated that heparan sulfate proteoglycan bound along the length of type IV collagen (8). However, resolution of this heparan sulfate proteoglycan was not adequate and in view of this drawback, the use of only a morphological approach did not allow for a better characterization of the interaction. In a different study, by affinity chromatography heparan sulfate proteoglycan was shown to bind to pepsin-extracted type IV collagen which lacks the noncollagenous NC1 domain (6). Further studies are required to better characterize the binding events between intact type IV collagen and the above mentioned glycosaminoglycans.

 Previous studies revealed that other interstitial collagens, such as collagen types I, II, and III, bind to heparin and a number of proteoglycans via their protein core and side chains (9). In most instances this binding resulted in regulation of assembly of the above-mentioned collagens into fibrils (9–15). We therefore undertook the current study in order to examine and characterize (a) interactions between intact EHS-derived type IV collagen and heparin, several disaccharide units of which resemble those of heparan sulfate proteoglycan although the latter has a lower degree of sulfation, and (b) the possible effects of heparin binding on the process of type IV collagen assembly.

MATERIALS AND METHODS
Heparin (porcine intestinal mucosa, grade I; 15 kDa), chondroitin/dermatan sulfate (porcine skin, type B, 20 kDa) and dextran sulfate (8 kDa), were purchased from Sigma. [3H]Heparan sulfate (0.4 mCi/mg) was purchased from DuPont-New England Nuclear.

Isolation of Type IV Collagen—Type IV collagen was isolated from

*This study was supported by National Institutes of Health Grant DK 39216 and grants from the American Diabetes Association and Juvenile Diabetes Foundation International (to E. C. T.), National Institute of Diabetes and Digestive and Kidney Diseases Grant 39868 and grants from the American Diabetes Association and AHA Minnesota Affiliate (to A. S. C.), and National Institutes of Health Grants AM 07651, CA 29995, CA 21465, and E1 39510 (to L. T. F.).

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The eHS tumor grown subcutaneously in athymic mice, as previously described (16, 17), isolated type IV collagen was used following purification by bath incubation with DEAE-52 (Whatman) in 4 M NaCl, 0.05 Tris-HCl, pH 8.6, containing 1 mM EDTA, 50 μg/ml phosphatase inhibitors, and 0.25 mM sodium fluoride. At the end of the incubation, the suspension was centrifuged to pellet the DEAE beads, and the supernatant was dialyzed against 0.05 M Tris-HCl, pH 7.4, containing 2 M guanidine HCl (ultra-pure grade, Sigma), 2 mM diithiothreitol, and the above-mentioned protease inhibitors (buffer A). Following dialysis, type IV collagen was centrifuged to remove aggregates larger than 50 S, at 40,000 rpm (rotor Ti-70) in a Beckman L8-M ultracentrifuge for 90 min at aliquots of 30 ml, and the supernatant was kept on ice until further use. In several instances, following incubation with DEAE-52, type IV collagen was further purified by gel filtration through a Sephacryl S-400 (Pharmacia LKB Biotechnology Inc.) column (5 × 95 cm). The column was equilibrated in 0.05 M Tris-HCl, pH 7.4, containing 2 mM 2, 2′-dithiothreitol, 1 mM glycine, and the above-mentioned protease inhibitors (buffer B). The purity of ion-exchange and gel filtration-purified type IV collagen was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and as described elsewhere (2, 16, 17). Protein concentration was determined by the method of Waddell (17, 18).

3H-Labeling of Type IV Collagen—Type IV collagen was labeled with [3H]-NaI (Amersham Corp.) by a modification of the lactoperoxidase method (2, 4). Briefly, type IV collagen was dialyzed in buffer B (without NaI) overnight at 4 °C. The next day, type IV collagen was centrifuged at 40,000 rpm for 20 min to remove large aggregates (see above). Approximately 500 μg of protein in 1-2 ml of buffer were mixed with 80 μl of rehydrated lactoperoxidase-glucose oxidase Enzymobeads in H2O (Bio-Rad) in the presence of 0.2 M sodium phosphate buffer, pH 7.0. In sequence, 5 μC [3H]-NaI (Amersham Corp., IMS 300) and 25 μl of a solution containing 1% β-D-glucose were added and the reaction was allowed to proceed for 30–40 min on ice. Following the end of the incubation, 100 μl of 0.1 M diithiothreitol in H2O were added, and labeled type IV collagen was separated from free iodide on a Sephacryl S-400 (Pharmacia G-200) column (1 × 25 cm) equilibrated in buffer B. Aliquots of 5 μl from eluted fractions (see section above) were then quantitated in a gamma-counter (gamma Trac 1193, TM-analytics, Elk Grove Village, IL). The radioactive peak which contained type IV collagen was then collected, dialyzed against buffer A, and stored on ice until further use. Incorporated [3H] in type IV collagen was tested by trichloroacetic acid precipitation and was found to be 90%–95% precipitable. Unlabeled type IV collagen was mixed with [3H]-type IV collagen and the mixture (at a final specific activity of 20,000 cpm/μg) was used to determine the amount of protein coated in 96-well polystyrene plates which were used for binding assays (see below).

Solid-phase Binding Assays—A modification of the method of Skibutz et al. (19) was used. Type IV collagen, in PBS containing 0.02% sodium azide, and precleared of aggregates by centrifugation, was coated in 96-well polystyrene Immulon 1 plates (Dynatech). Aliquots of labeled type IV collagen containing 100 pg/ml type IV collagen were dried overnight by incubation at 29 °C. Under these conditions 1.3 μg of type IV collagen was adsorbed/well, as determined by the use of [125I]-type IV collagen. The concentration of 60 μg/ml was found to be optimal, since it was the minimal concentration required to bind approximately 100% of the plateau value of [125I]-heparin binding (62,000 dpm). As a control, 50 μl of bovine serum albumin (BSA) (tasty acid free, fraction V, ICN Immunobiologicals) was coated/well at 8 μg/ml, a protein concentration equimolar to that of type IV collagen. The next day, 200 μl of a solution containing 2 mg/ml BSA, 68 μg CaCl2 in 0.01 M Tris-HCl buffered at pH 6.8 with 0.01 M HEPES buffer C) were added to each well and incubated for 120 min at 37 °C. This step was used to “block” uncoated sites in the wells. Following incubation, the wells were washed three times with 200 μl of buffer C containing 0.05% Triton X-100. Bound [125I]-heparin was then solubilized by incubation with 100 μl of 0.05 N NaOH and 1% sodium dodecyl sulfate for 30 min at 60 °C and quantitated in a Beckman LS-3801 scintillation counter. Specific [125I]-heparin binding was determined as total binding of [125I]-heparin to type IV collagen minus binding of [125I]-heparin to BSA-coated wells. All experiments were performed at least three to five times in triplicate. The same source and dilution of heparin was previouly shown to behave similarly in binding to unlabeled heparin (19).

Affinity Chromatography—A type IV collagen affinity column was prepared as follows: 10 ml of Affi-Gel-15 (Bio-Rad) was washed with 50 ml of H2O, and the beads were resuspended in buffer C to final concentration of 0.15 M sodium chloride, 1 mM sodium azide, and pH 7.4. The beads were equilibrated in this buffer overnight at 4 °C (3 changes, 20 ml each). Type IV collagen was dialyzed overnight against the same buffer and was cleared of aggregates by centrifugation. Approximately 8.5 mg of type IV collagen was added to the beads and allowed to interact overnight with gentle rocking at 4 °C. The next day, 100 μl of 1 M ethanolamine, pH 7.4, was added to inactivate any remaining reactive sites. Approximately 90% of the type IV collagen which was added coupled to the beads by this procedure. The beads were then packed in a 1.5 × 5-cm Econo-column (Bio-Rad). After packing, the column was washed with 150 ml of 1 mM Tris-HCl, pH 7.0. One mg of unlabeled heparin mixed with 250 pg/ml BSA was then added to the column. Eluted fractions were collected (50 μl/fraction) and 100 μl of each fraction was quantitated in a Beckman LS-3801 scintillation counter.

Rotary Shadowing—Type IV collagen was dialyzed against PBS overnight at 4 °C and was centrifuged to clear aggregates larger than 20 S. 200 μl of the supernatant was mixed with heparin in PBS (final concentrations of ligands were: 150 μg/ml type IV collagen and 50–100 μg/ml heparin). The following permututions were used as controls: (a) type IV collagen alone (150 μg/ml); (b) type IV collagen (150 μg/ml) mixed with BSA (100 μg/ml); and (c) type IV collagen (150 μg/ml) mixed with both BSA and heparin, each at 100 μg/ml. All samples were incubated for 1 h at 37 °C. 75 μl of each sample was then mixed with 20% glycerol in 0.15 M NaCl, 1 M HCO3, pH 7.7 and sprayed on freshly cleaved mica sheets as previously reported (16, 17, 20). Rotary-shadowed replicas were collected on 300-mesh copper grids and examined with a Philips 300 transmission electron microscope operating at 60 kV. Two types of measurements were performed: (a) statistical evaluation of the fields containing individual association networks of type IV collagen was done as previously described (17); (b) a histogram of the distribution of heparin along the length of type IV collagen molecules was constructed from photographic images of complexes at a final magnification × 500,000 (17). In this instance, the distance of a binding event from the NC1 domain was divided by the total length of each fragment (which is equal from the NC1 domain). Three criteria were used for selecting binding events. 1) The whole length of type IV collagen molecules should be clearly visualized. 2) The length of type IV collagen should be 350–450 nm. 3) The binding of heparin to type IV collagen should also be clearly visualized. A similar approach was used previously to determine binding sites of intact laminin to type IV collagen (20). Statistical analysis was performed by testing the goodness of fit of the Poisson distribution (21).

Turbidity Measurements—Type IV collagen was dialyzed against PBS overnight and then cleared of aggregates that were >20 S by centrifugation. Aliquots of 100 μg/ml type IV collagen were incubated with increasing concentrations of heparin in PBS (final heparin concentrations: 10, 100, 200, and 400 μg/ml) at 0 °C. One-ml aliquots of each sample were then incubated for 60 to 90 min in quartz cuvettes at 35 or 37 °C. The following controls were used: (a) type IV collagen (250 μg/ml) incubated in PBS (BSA (250 μg/ml); (b) heparin alone (250 μg/ml) incubated at the same temperature; and (c) temperature was maintained by automatic control with a Peltier III Kinetics system (Beckman). The change of absorbance at 360 nm was followed over time with a Beckman DU-6 spectrophotometer.

All types of experiments, with the exception of affinity chromatography were performed with both ion-exchange- and gel filtration-purified type IV collagen (the affinity column was made only with ion-exchange-purified type IV collagen). Similar results were obtained in most of the experiments. In solid-phase binding assays, gel filtra-
tion-purified type IV collagen was occasionally observed to bind slightly higher amounts of \(^{3}H\)heparin.

**RESULTS**

**Rotary Shadowing**

When examined with this morphological approach, heparin molecules (which are composed of side chains without a protein core) were visualized as thin, linear strands, approximately 2 to 3-nm thick and 15 to 20-nm long (Fig. 1). Heparin molecules were observed to bind to several discrete sites along the length of type IV collagen molecules (Fig. 1). A histogram of the distribution of heparin-type IV collagen-binding events, constructed as described under Materials and Methods, revealed three distinct binding sites. The highest percentage of binding events occurred in the NC1 domain. Two additional sites were evident in the triple helix-rich domain, one at a distance of approximately 100 nm from the NC1 domain and another at 300 nm from the NC1 domain (Fig. 2). These three binding sites were statistically significant when examined by the goodness of fit of the Poisson distribution \((p < 0.001)\).

In a different experiment, type IV collagen was coincubated with heparin following passage of the latter through a type IV collagen affinity column. Heparin that bound to this column was eluted with a linear salt gradient, and three major peaks were obtained (see below). Subsequently, the ionic strength of each peak of heparin was adjusted to 0.15 M NaCl, and a sample of each was incubated with type IV collagen. Binding events were evaluated by rotary shadowing for each peak separately. All peaks of heparin had a similar distribution along the length of type IV collagen in that the majority of binding events were clustered in domain NC1 and at distances of 100 and 300 nm from the NC1 domain (Table I). These data provide evidence that charge heterogeneity of disaccharide units in commercially available heparin preparations did not interfere with the binding of heparin along the length of type IV collagen.

**Solid-phase Binding Assays**

In solid-phase binding assays where type IV collagen was adsorbed onto plastic wells at a concentration of 1.3 µg/well, the binding of increasing concentrations of \(^{3}H\)heparin in solution was found to be saturable (Fig. 3). Although some variability of the percentage of bound \(^{3}H\)heparin was observed between different preparations of type IV collagen, on the average 25-30% of the added \(^{3}H\)heparin bound at the lowest concentrations (which corresponded to 0.1-0.2 ng of \(^{3}H\)heparin/well), and 1-2% of the added \(^{3}H\)heparin bound at the highest concentrations (which corresponded to 5-7 ng of \(^{3}H\)heparin/well). Fitting of the specific binding data from a representative experiment in the Scatchard equation indicated a complex curve fit (Fig. 4) with three apparent classes of binding sites. The three dissociation constants were cal-

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**TABLE I**

Incidence of binding events of various fractions of heparin along the length of type IV collagen (expressed as percentage of the total number of bound heparin molecules examined)

| Distance from the NC1 domain | %     | %     | %     | Total no. of binding events |
|-----------------------------|-------|-------|-------|-----------------------------|
| 0-20 nm                     | 28    | 25    | 18    | 147                         |
| 80-120 nm                   | 31    | 19    | 16    | 146                         |
| 280-320 nm                  | 24    | 21    | 19    | 135                         |
| Total no. of binding events | 29    | 18    | 16    | 115                         |

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Fig. 1. Appearance of type IV collagen-heparin complexes in rotary shadowed images. Examples of heparin molecules (thin, short strands indicated by arrowheads) bound in the area of the main noncollagenous NC1 domain (A, B, and respective diagrammatic representations), at approximately one-fourth of the total length of the type IV collagen molecule (C and D) and at about three-fourths of the total length of type IV collagen (E and F) are depicted. Bar equals 100 nm.

Fig. 2. Histogram of the distribution of heparin binding events along the length of type IV collagen. The whole length of type IV collagen, including the NC1 domain was divided into 20 equal segments. The frequency of binding events, expressed as percentage of total bound heparin molecules, was plotted against each segment. Each of the three peaks was statistically significant \((p < 0.001)\).
Heparin-type IV Collagen Interactions

FIG. 3. Binding of [3H]heparin in solution to type IV collagen coated on 96-well plates. Type IV collagen used for this experiment was gel filtration-purified and the actual amount adsorbed/well was 1.3 mg. Increasing concentrations of [3H]heparin (specific activity = 900,000 dpm/μg) were added to triplicate wells and tritiated heparin was allowed to bind for 2 h by incubating at 37 °C. At the end of the incubation time, unbound [3H]heparin was removed by washing and bound [3H]heparin was assayed by a buffer containing 0.05 N NaOH and 1% sodium dodecyl sulfate. Total dpm of bound tritiated heparin were plotted against total dpm of added [3H]heparin (highest added concentration was 350 ng). Bars represent the standard deviation.

FIG. 4. Scatchard analysis of a typical solid-phase binding experiment in which increasing concentrations of [3H]heparin were added to 1.3 μg of collagen adsorbed on plastic wells. Twenty different concentrations were used in triplicate wells to allow for more accurate quantitation of the binding. Type IV collagen used for this experiment was gel filtration-purified.

Calculated to be, respectively: k_{in} = 3 nM (r = 0.92, p < 0.001), k_{out} = 30 nM (r = 0.97, p < 0.001) and k_{off} = 100 nM (r = 0.97, p < 0.001). Each of these three distinctive affinities may correspond to one of the three binding sites which were observed by rotary shadowing.

**Competition of the Binding of [3H]Heparin Binding to Type IV Collagen by Sulfated Polysaccharides**

Specificity of the binding of [3H]heparin to type IV collagen with the solid-phase approach was tested by competing the binding of [3H]heparin with: (a) unlabeled heparin (15 kDa); (b) chondroitin/dermatan sulfate side chains (20 kDa); and (c) dextran sulfate (8 kDa). In this experiment, various concentrations of each of the above mentioned polysaccharides were added concomitantly in the presence of a constant amount of [3H]heparin to wells coated with 1.3 μg of type IV collagen. Both unlabeled heparin and chondroitin/dermatan sulfate were equally effective in their ability to compete with [3H]heparin for binding to type IV collagen (Fig. 5); 50% of the [3H]heparin bound to type IV collagen was displaced by 1.5 × 10^{-11} M of either heparin or chondroitin/dermatan sulfate, whereas 33 times more dextran sulfate (5 × 10^{-10} M) was required for 50% displacement of bound [3H]heparin. These results suggest significant specificity in the type IV collagen-heparin interactions and also indicate that chondroitin/dermatan sulfate side chains should bind to type IV collagen as well.

In all instances, nonspecific binding of [3H]heparin was less than 100 dpm.

**Binding of [3H]Heparin to Type IV Collagen by Affinity Chromatography**

In this assay, 1 mg of heparin (also containing small amounts of [3H]heparin) was added in 1 ml of Tris-HCl, pH 7.0, to an affinity column containing type IV collagen. Heparin was allowed to interact with type IV collagen by incubation overnight at 4 °C. The next day, a salt gradient (0-0.3 M KCl) was applied to the column, and eluted fractions were tested for radioactivity. Three distinct major peaks of [3H]heparin were obtained at about 0.18, 0.22, and 0.24 M KCl, all eluting at ion strengths higher than physiologic (0.15 M). A fourth minor peak was present at a concentration of 0.26 M KCl (Fig. 6). These data suggest that the interactions between heparin and type IV collagen may be ionic.

Because commercially available heparin preparations usually contain heterogeneous mixtures of disaccharide types, the possibility existed that the major peaks of heparin which were obtained by affinity chromatography represented different disaccharide types which preferentially bound to type IV collagen based on their ionic properties. In order to rule out this possibility, the peak eluting at an ion strength of 0.18 M KCl was randomly selected, adjusted to 1 mM Tris-HCl, pH 7.0, and was tested by a second passage through the type IV collagen affinity column. Bound [3H]heparin was eluted with a linear salt gradient similar to the one mentioned above. In this instance, the profile of elution again revealed three major peaks. The ion strength of each peak from the purified heparin coincided with that obtained from the commercially available mixture of heparin disaccharides (data not shown). Therefore, these data provide evidence for the existence of three major binding sites in type IV collagen for heparin and...
prewarmed to 35 or 37 °C, type IV collagen self-associated occurred, indicating a decrease in type IV collagen self-assembly. The change in absorbance at 360 nm was plotted against time intervals for the duration of the experiment. Type IV collagen was used for turbidimetry following ion-exchange purification. The conductivity and radioactivity of each fraction were quantitated. Total dpm of eluted, tritiated heparin, and ionic strength of the applied KCl gradient (deduced from conductivity measurements) were plotted against collected fractions. Type IV collagen was used to bind to this affinity column following ion-exchange purification.

Thus corroborate the rotary shadowing data and the data obtained by Scatchard analysis of radiolabeled heparin-type IV collagen binding (see above).

Effect of Heparin Binding on the Process of Type IV Collagen Polymerization

Turbidity Measurements—Self-association of type IV collagen at 250 μg/ml in PBS, was determined by the development of turbidity. As described previously (2, 16, 17), when prewarmed to 35 or 37 °C, type IV collagen self-associated and raised turbidity readily, without a lag phase, to a plateau value within 30-40 min (Fig. 7). In the presence of heparin, a dramatic decrease in the development of maximal turbidity occurred, indicating a decrease in type IV collagen self-assembly. At a concentration as low as 10 μg/ml, heparin coincubated with type IV collagen (250 μg/ml) at 37 °C decreased turbidity by 55%. When heparin was present in higher concentrations, it further suppressed the development of turbidity. For example, at 400 μg/ml, heparin caused ≈80% inhibition of the maximal turbidity developed by type IV collagen. Some variability was observed between different preparations of type IV collagen in that occasionally, slightly higher concentrations of heparin were required to produce similar effects (data not shown). The presence of BSA at 100 μg/ml did not have any significant effect on maximal turbidity of type IV collagen. Heparin at 400 μg/ml in PBS, alone did not raise turbidity (data not shown). These observations suggest that the binding of heparin to type IV collagen has an inhibitory effect on the process of type IV collagen self-assembly into a network-like structure.

Rotary Shadowing—Type IV collagen in PBS (150 μg/ml) was incubated in the absence or presence of heparin (100 μg/ml) at 37 °C for 1 h and was then examined for the formation of lateral associations and networks by rotary shadowing and electron microscopy. When incubated alone, type IV collagen has assembled to laterally associated structures in ≈73% of the fields examined, as previously reported (17). The presence of heparin caused a dramatic decrease in network formation by type IV collagen, where networks were present in ≈27% of the fields (Fig. 8). This percentage represents approximately background levels (17). The presence of BSA in either the type IV collagen solutions or the mixtures of type IV collagen and heparin did not have any significant effect on numbers of lateral associations observed (Fig. 8). These observations corroborate the findings by turbidimetry in that they indicate that heparin inhibits the process of self-assembly of type IV collagen.

DISCUSSION

In this study we report the existence of multiple interactions between type IV collagen and heparin. Heparin was selected because it has a disaccharide unit structure which in some instances resembles that of heparan sulfate proteoglycan, a component of basement membranes and cell surfaces (22-24). Though heparin is known to be heterogeneous in charge and size, the appearance by rotary shadowing indicated that the variability in size was not large, as judged by the thickness and length of heparin molecules. To our knowledge, this is the first report visualizing heparin with the technique of rotary shadowing and electron microscopy. Visualization of heparin was important in order to determine the localization of the heparin binding along the length of the rod-like, type IV collagen molecule. This morphological study revealed, following statistical evaluation, the existence of three distinct binding sites for heparin along the length of type IV collagen.
Heparin was previously shown to bind the main noncollagenous NC1 fragment of type IV collagen by affinity chromatography, but pepsin-extracted, placental type IV collagen failed to bind to a heparin-Sepharose affinity column (7). In contrast, we report here that in rotary-shadowed images, intact (not pepsin-extracted) EHS-derived, dimeric type IV collagen bound heparin at two distinct sites along the triple-helical domain of type IV collagen, located at 100 and 300 nm from the NC1 domain, respectively. Species differences and/or different experimental conditions might account for the discrepancy of results. However, it is also possible that pepsin treatment cleaved, at least partially, interruptions of the Gly-X-Y sequence in the helix which are abundant in both the α1- and α2-chains of type IV collagen (the decerphered sequence of the α1-chain contains a total of 21 interruptions (25, 26), and several matching interruptions occur in the known sequence of part of the α2-chain (27)). If one or more of these interruptions were to be involved in the binding of type IV collagen to heparin, then treatment with pepsin could have at least partially impaired this binding. Indeed, ongoing experiments indicate that EHS-derived, pepsin-treated type IV collagen binds heparin only minimally.

The above-mentioned observations indicate that heparin, several disaccharide units of which may resemble those of heparan sulfate proteoglycan, would perhaps require an intact triple helix-rich domain, including the interruptions, for binding. In addition, intact, EHS-derived, heparan sulfate proteoglycan was reported to bind to the triple helical domain (6, 8) and the NC1 domain of type IV collagen as well (6). With the technique of rotary shadowing, Laurie et al. (8) observed that heparan sulfate proteoglycan bound to two distinctive sites along the rod-like part of intact type IV collagen. The distribution of the latter binding in the triple helix-rich domain of type IV collagen, although broader, was roughly similar to the distribution of heparin along the rod-like part of type IV collagen which we reported in this study (Fig. 1 of this report and Fig. 6; Ref. 8). In their study, Laurie et al. did not observe binding of heparan sulfate proteoglycan to the NC1 domain. It is possible that this binding may have been impaired because of conditions of the rotary shadowing technique, i.e. presence of glycerol (2), etc. In a different study, Fujiiwara et al. (6) using affinity chromatography and zonal rate velocity sedimentation demonstrated both the binding of intact heparan sulfate proteoglycan and isolated protein core to the NC1- and triple-helical domains of type IV collagen.

In addition to rotary shadowing, we demonstrated specific binding of heparin to intact type IV collagen by solid-phase binding assays. Scatchard analysis of the binding data indicated three classes of binding sites, which corroborated the rotary shadowing observations. The three distinctive dissociation constants obtained of 3, 30, and 100 nM indicate that these binding sites are of relatively high affinity. Furthermore, specificity of this interaction was tested by competing the binding of [3H]heparin to type IV collagen with different sulfated polysaccharides. With this approach, we obtained evidence that heparin and probably chondroitin/dermatan sulfate side chains bound to intact type IV collagen. About the same concentration of each of these two competitors was required for 50% displacement of [3H]heparin bound to type IV collagen. This observation would suggest that type IV collagen may contain two different classes of binding sites, one of which should preferably bind chondroitin sulfate.

Affinity chromatography indicated that interactions between type IV collagen and heparin are electrostatic in nature, since they were abolished by increasing ionic strength. Similarly, interactions between type IV collagen and heparan sulfate, chondroitin and dermamn sulfate (both side chains and intact proteoglycans) were shown to be charge-mediated (10). Interestingly, three major distinct peaks of type IV collagen-bound tritiated heparin were apparent, each eluting at a different ionic strength. The possibility that different subpopulations of disaccharides in the commercially available heparin mixtures were preferentially eluted because of their charge properties was ruled out by a second passage of one major peak through the type IV collagen affinity column. This charge-selected subpopulation was shown to bind to the same affinity column. The elution profile following application of a linear KCl gradient again contained three major peaks, and the ionic strength of each coincided with that of the original elution profile. Furthermore, each peak of heparin eluted from a type IV affinity column (at 0.18, 0.22, and 0.24 M KCl, respectively) was subsequently shown by rotary shadowing to bind in all three sites along the length of type IV collagen in the NC1 domain and at distances of 100 and 300 nm from the NC1 domain, respectively. Thus by three different approaches, rotary shadowing, solid-phase binding assays, and affinity chromatography, the existence of three distinct classes of binding sites for heparin in type IV collagen was indicated.

The effect of these multiple interactions on the process of self-assembly of type IV collagen was also examined. Previous studies have shown that assembly and fibril formation of at least several interstitial collagens is regulated by heparin, chondroitin sulfate side chains, and by a number of intact proteoglycans, either side chains or the protein core (11). For example, when added before nucleation in lathyritic tropocollagen preparations, chondroitin sulfate, dermatan sulfate, heparin and intact chondroitin sulfate or dermatan sulfate proteoglycan were shown to accelerate fibril formation (11). In contrast, when the same glycosaminoglycans were added to type I collagen after the nucleation phase, they delayed fibril formation (11). Oegema et al. (12) reported that proteoglycan derived from nasal cartilage delayed fibril formation by acid-extracted type I collagen. More recently Vogel et al. (14, 15) have described a small, tendon-derived dermatan sulfate proteoglycan which specifically inhibits fibrillogenesis by type I and II collagen in vitro.

In this study, we report that type IV collagen assembly is also inhibited by one glycosaminoglycan, heparin. Both by turbidimetry and rotary shadowing, the polymerization process of this basement membrane collagen to a complex network was drastically reduced in the presence of heparin. It remains to be substantiated whether intact proteoglycans such as heparan sulfate have similar effects. If this were the case, then a more general picture emerges, that of an effect over self-association of several families of collagens by a variety of proteoglycans, via either their side chains or protein cores or both. The precise molecular mechanisms responsible for specific effects on the assembly of a certain type of collagen are not well understood. In the case of type IV collagen, the binding of heparin in rotary-shadowing images coincided with two of the sites where NC1 domain bound in the helix in order to initiate lateral assembly (at 100 and 300 nm from the NC1 domain, respectively; 16). This observation indicates that at least for steric reasons, if these sites were occupied by heparin (or potentially by related proteoglycans as well), assembly of type IV collagen should be hindered.

Both heparan sulfate and chondroitin sulfate proteoglycan occur in basement membranes (28, 29) and cell surfaces as
well (22-24, 30, 31). Also, at least one cell type, endothelial cells in tissue culture, have been reported to contain heparan sequences in the heparan sulfate chains of their proteoglycan (32). Cell surface-heparan sulfate proteoglycan in human epithelial cells was reported to bind to interstitial collagens (33). One could speculate that interactions between type IV collagen and proteoglycans of either cell surfaces or basement membranes might result in partial inhibition of molecular assembly, depending upon availability of varying components. In different basement membranes, even relatively small quantitative differences in the components could lead to different molecular assembly or function. This mechanism could partially explain basement membrane polymorphism in different tissues (34). Similarly, in certain pathological conditions, such as diabetes, the observed decrease of the amount of available proteoglycans (35-38) could result in a perturbed ultrastructural assembly of newly synthesized components which might be involved in the pathogenesis of abnormal function of diabetic basement membranes.

Acknowledgments—We are indebted to Dr. James White for providing facilities for rotary shadowing, Marcy Krumwiede for excellent technical assistance with the rotary shadowing experiments, Jay Dege for technical assistance with the solid-phase binding assays, Drs. John Norman, Andreas Rosenberg, and Bruce Lester for helping with the Scatchard analysis, Drs. Amy Skubitz and James B. McCarthy for helpful discussions, and Carol El-Ghandour for typing the manuscript.

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