Specificity in the Interactions of Extracellular Matrix Proteins with Subpopulations of the Glycosaminoglycan Heparin†

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ABSTRACT: Many extracellular matrix glycoproteins—including laminin, fibronectin, thrombospondin, type I collagen, and other collagens—bind the glycosaminoglycan heparin, yet little is known about the functional significance of these interactions. It is also not known if heparin-binding extracellular matrix proteins recognize distinct structural elements in heparin, nor whether all extracellular matrix proteins recognize the same or different aspects of heparin structure. If extracellular matrix proteins each recognize *distinct* features of heparin, such specificity could be of importance in vivo, where structurally distinct heparan sulfate species occur. To investigate specificity in the binding between extracellular matrix proteins and heparin, the method of affinity coelectrophoresis (ACE) was used [Lee, M. K., & Lander, A. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2768–2772]. Low M_r (~6 kDa) ^{125}I-heparin was fractionated by electrophoresis through agarose gel lanes containing extracellular matrix proteins at various concentrations; from heparin migration patterns, binding affinities were calculated. The results indicate that fibronectin, type I collagen, and laminin—but not thrombospondin—each fractionate heparin into subpopulations that differ substantially in binding affinity. From ACE gels containing either fibronectin, type I collagen, or laminin, fractions of heparin were isolated that represent the 25% of molecules most strongly bound and the 25% least strongly bound by each of these proteins. Subsequent ACE analysis of these six fractions showed that (1) for each of fibronectin, type I collagen, and laminin, strongly- and weakly-binding heparin subfractions differ ~5–30-fold in K_d; (2) heparin that binds strongly to any one of fibronectin, type I collagen, or laminin also binds strongly to the other two; (3) heparin that binds weakly to any one of fibronectin, type I collagen, or laminin, also binds weakly to the other two; (4) heparin subfractions that differ greatly in affinity for fibronectin, type I collagen, and laminin show little difference in K_d for thrombospondin or for the heparin-binding growth factor basic fibroblast growth factor (bFGF); (5) neither heterogeneity in molecular charge [as measured by diethylaminoethyl (DEAE) chromatography] nor size nor the presence or absence of antithrombin III recognition sequences can account for the selective binding of heparin subpopulations to fibronectin, type I collagen, and laminin. These results suggest that structural elements within heparin can confer preferential binding to extracellular matrix proteins. Sensitivity of some, but not all, extracellular matrix proteins to these structural features suggests that similar features, if present in heparan sulfates or other glycosaminoglycans, may be physiologically relevant in vivo.

Heparin-binding proteins comprise a diverse group of molecules that perform important roles in extracellular matrix structure and function, cell adhesion, growth, and differentiation [see Ruoslahti (1988) and Jackson et al. (1991) for reviews]. The normal ligands of most heparin-binding proteins are believed to be heparan sulfate chains found on extracellular matrix and cell-surface proteoglycans. Heparan sulfates, like other glycosaminoglycans, consist of a linear backbone of uronic acid and amino sugar disaccharide subunits, of varying lengths and with varying patterns of complex modifications, such as epimerization and N- and O-sulfation.

Features of protein structure that control heparin binding have been partially elucidated by studying the heparin-binding properties of protein fragments, synthetic peptides, and proteins modified chemically or by mutagenesis [e.g., see Rosenberg and Damus (1973), Yamada et al. (1980), Baird et al. (1988), Harper and Lobb (1988), Yabkowitz et al. (1989), and Lawler et al. (1992)]. Such studies have led to the identification of putative heparin-binding consensus sequences (Cardin & Weintraub, 1989; Jackson et al., 1991). In contrast to what has been learned about the role of protein structure in protein–glycosaminoglycan binding, relatively little is known about the role of glycosaminoglycan structure. Some studies have identified relationships between crude features of heparin structure (e.g., polymer length, degree of sulfation) and relative affinity for proteins [e.g., see Ogamo et al. (1985) and Lindahl and Hook (1978) for reviews], but only in one case has the structure of a protein-binding site been elucidated: The plasma protein antithrombin III binds to a site on heparin consisting of a distinct pentasaccharide sequence containing an unusual modification, a 3-*O*-sulfate on the central glucosamine, as well as several other critical *N*- and *O*-sulfates on the other saccharides [see Marcum and Rosenberg (1989) for review]. The specificity of antithrombin III for this binding site is such that heparin molecules lacking it are bound with approximately 1000-fold lower affinity (Jordan et al., 1979).

Although the heparin-binding site(s) on antithrombin III are representative of typical heparin-binding consensus sequences (Blackburn & Sibley, 1980; Blackburn et al., 1984), it is unknown whether the strong carbohydrate sequence specificity exhibited by antithrombin III is typical of heparin-

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binding proteins or is an exception. Emerging evidence that the glycosaminoglycan sequences found in heparan sulfate proteoglycans are not random but vary in cell-type-specific ways (Turnbull & Gallagher, 1990; Kato et al., 1991) has emphasized the importance of determining the role, if any, of glycosaminoglycan sequences in controlling protein–proteoglycan interactions.

Recently, the technique of affinity coelectrophoresis (ACE)[1] was introduced (Lee & Lander, 1991) as a convenient method to study protein–glycosaminoglycan interactions. By use of this technique, proteins including bFGF and antithrombin III were found to display heparin-binding properties (affinity, selectivity) consistent with previous studies. However, ACE analysis of heparin binding to fibronectin revealed the existence of subpopulations of heparin that were bound with different affinities by fibronectin. Further analysis of these glycosaminoglycan subpopulations showed that they did not differ substantially from each other in molecular weight or apparent charge, suggesting that aspects of carbohydrate sequence might account for their differences in binding affinity for fibronectin.

In this report, we have studied the binding of heparin to three other heparin-binding extracellular matrix proteins, laminin, type I collagen, and thrombospondin, as well as to fibronectin. As in previous experiments, heparin was size-selected. Low M_r ($\leq$ 6000) heparin chains were used to minimize the occurrence of multivalent binding, i.e., the binding of many protein molecules to a single heparin chain, which can complicate the analysis of binding affinity (Lim et al., 1991). To minimize possible effects on heparin's binding properties, the labeling group used (tyramine) was of the minimum size that could be radioiodinated and was coupled exclusively to the reducing end of the polysaccharide chains. The results indicate that fibronectin, laminin, and type I collagen all bind selectively to subpopulations of heparin, while neither thrombospondin nor the growth factor bFGF exhibits such selectivity. Experiments to address whether fibronectin, type I collagen, and laminin select for molecules on the basis of specific carbohydrate sequences or less specific biochemical features (e.g., net charge) are described.

MATERIALS AND METHODS

Materials. Low melting point agarose (Seaplaque) and GelBond were from FMC Bioproducts, heparin (Grade I; porcine intestinal mucosa) was from Sigma, colominic acid (M_r ~10 000, from *Escherichia coli*) was from Calbiochem, bovine serum albumin (crystalline) was from ICN, and human plasma fibronectin was from the New York Blood Center (New York). Type I collagen was isolated from rat tail tendon (San Antonio et al., 1992), thrombospondin was prepared from human platelets (Lawler et al., 1985), laminin was isolated from the Engelbreth–Holm–Swarm sarcoma (Kleinman et al., 1982; Timpl et al., 1982), bFGF was purified from bovine brain (Lobb & Fett, 1984), and antithrombin III was the generous gift of Dr. Robert D. Rosenberg of the Massachusetts Institute of Technology. Proteins were stored at −85 °C, except for type I collagen, which was dissolved in 0.5 N acetic acid and stored at 4 °C.

Heparin was substituted with tyramine using a modification of a published technique (Lee et al., 1991). Briefly, heparin (9 mg) was dissolved in 750 μL of a solution of tyramine (5% w/v) in formamide and heated to 80 °C for 1 h. Sodium cyanoborohydride (1 mg) was added, and the sample was incubated at room temperature overnight. It was then diluted with 9 volumes of distilled water and dialyzed exhaustively against distilled water, using M_r = 1000 cutoff dialysis tubing (Spectrapor). The sample was concentrated by evaporation, and the heparin concentration was determined by measuring uronic acid (Dische, 1947) and the tyramine content by OD_{278}. It was determined that about 75% of heparin chains were tyramine end-labeled. Tyramine-heparin was radioiodinated to a specific activity of about 30 000 cpm/ng using glass tubes coated with Iodogen (Pierce). The ^{125}I-tyramine-heparin (^{125}I-Tyr-heparin) was fractionated by gel filtration on Sephadex G-100 (Lee & Lander, 1991), and the last 11% of the radioactivity to elute ($0.57 < K_{av} < 0.76$) was pooled as the low M_r fraction. This fraction has been previously shown to contain heparin chains of $M_r \leq 6000$ (Laurent et al., 1978; Jordan et al., 1979; Rosenberg et al., 1979). ^{125}I-Tyr-heparin was radioprotected by the addition of crystalline bovine serum albumin (to ~0.25 mg/mL) and stored at −80 °C.

Electrophoretic Analysis of Binding. ACE was carried out as previously described (Lee & Lander, 1991; Lim et al., 1991). Briefly, a 1% low melting point agarose solution in 50 mM sodium MOPSO (pH 7.0)/125 mM sodium acetate/0.5% CHAPS electrophoresis buffer was poured hot onto a piece of GelBond fitted within a Plexiglas gel casting tray, in which a Teflon comb and strip were positioned. After the agarose solidified, the comb and strip were removed, leaving a 4-mm-thick gel containing nine parallel 4- × 45-mm rectangular wells and a single 66- × 1-mm slot 2 mm away from the tops of each of the wells. Before electrophoresis, protein samples were thawed and insolubles removed by centrifugation (13000*g*, 1 min). Fibronectin, laminin, and thrombospondin samples were serially diluted into electrophoresis buffer, before being mixed with an equal volume of molten 2% agarose in electrophoresis buffer at 37 °C just before introduction into wells of ACE gels (see below). Type I collagen samples were first serially diluted into 0.5 N acetic acid and then neutralized with an equal volume of 0.5 N NaOH. The samples were then rapidly mixed first with an equal volume of 100 mM MOPSO, pH 7.0, and then with the 2% agarose solution, followed by pipetting into the wells of ACE gels. This latter procedure was employed to avoid type I collagen gel formation prior to pouring of the protein–agarose wells. Laminin samples included 0.5 mM sodium EDTA within the protein solutions to inhibit aggregate formation, and for the thrombospondin gels, all samples and electrophoresis solutions included 0.5 mM calcium acetate to maintain native protein conformation (Lawler & Simons, 1983).

After the gel was submerged under electrophoresis buffer and the sample slot was loaded with 150 μL of ^{125}I-Tyr-heparin in electrophoresis buffer containing 5% sucrose and tracking dyes (Lee & Lander, 1991), electrophoresis was conducted at 80 V for 3 h. Buffer was recirculated, and a flow of cold tap water through the coolant ports of the apparatus was used to maintain buffer temperature at 20–25 °C. After electrophoresis, gels were air-dried and subjected to autoradiography or analysis by phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Preparative Electrophoretic Fractionation of Heparin. In some experiments ^{125}I-Tyr-heparin was subjected to electrophoresis through agarose containing a single concentration of protein to isolate strongly- and weakly-binding heparin populations. Briefly, 1% low melting point agarose in electrophoresis buffer (see above) was poured hot onto a piece

[1] Abbreviations: bFGF, basic fibroblast growth factor; ACE, affinity coelectrophoresis; DEAE, diethylaminoethyl; -W, weakly retarded heparin fraction; -S, strongly retarded heparin fraction; CL, collagen; FN, fibronectin; LN, laminin; TS, thrombospondin; K_d, equilibrium dissociation constant; ^{125}I-Tyr-heparin, ^{125}I-tyramine-heparin; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; CHAPS; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

of gelbond fitted within a Plexiglas gel casting tray, in which a 4- × 7-cm plastic block and a Teflon strip were positioned so that after the agarose solidified, removal of the block and strip left a 4- × 7-cm well with a 66- × 1-mm slot positioned 2 mm away from and parallel to one of the short edges of the 4- × 7-cm well. Proteins were prepared and mixed with agarose as described above to a final agarose concentration of 1%, and 10-mL samples were loaded into the 4- × 7-cm well and allowed to gel. Gels were submerged under electrophoresis buffer, ^{125}I-Tyr-heparin was loaded into the 66- × 1-mm slot, and electrophoresis was carried out as detailed above. Afterward, gels were removed and affixed to a surface marked with a millimeter grid, with the electrophoretic origin at top. Regions of the gel to the left and right of the 4 × 7 cm block as well as the 3 mm of the block itself that were immediately adjacent to the left and right edges were cut away and discarded. The resultant 3.4- × 7-cm gel was then sectioned into 3-mm segments, perpendicular to the direction of electrophoresis. The amount of ^{125}I-Tyr-heparin in each segment was determined with a γ counter, and after the fractions were melted in a boiling water bath, appropriate fractions were pooled (see Results), divided into aliquots, and stored at −20 °C. To prepare these samples for subsequent electrophoretic analysis, they were melted at 70 °C for 20 min, brought to 6 M urea by the addition of solid urea (urea blocks gelation of the agarose and may also minimize renaturation of proteins also present in the samples) and 0.05% bromophenol blue, and loaded directly into ACE gels as detailed in the previous section (since urea does not migrate in the electrophoretic field, its removal from samples prior to electrophoresis is not required).

Ion-Exchange Chromatography of Heparin. DEAE-Spectragel (Spectrum Scientific Co.) was rinsed in 1.0 M Tris-HCl (pH 7.5) and then equilibrated in buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, and 0.1 M NaCl). Heparin samples were loaded in 1.0 mL of the same buffer onto 0.15 mL of DEAE-Spectragel in disposable columns (Bio-Rad), and unbound material was eluted with 3 mL of running buffer. Elution of heparin was effected by stepwise passage of 150-μL aliquots of the same buffer containing increasing amounts of NaCl, to a final concentration of 2.0 M.

Data Analysis. Heparin mobility within ACE gels was measured using a phosphorimager (Molecular Dynamics) by scanning each protein lane and determining relative radioactivity content per 88-μm pixel along the length of the lane. Heparin mobility in each lane was taken as the pixel position that subdivided these curves into halves of equal area. The heparin retardation coefficient R was calculated for each lane as the heparin mobility shift in that lane divided by the mobility of heparin in a protein-free lane [i.e., $R = (M_0 - M)/M_0$, where M_0 is the mobility of free heparin and M is heparin's mobility through protein). Under appropriate experimental conditions, R is proportional to the fractional saturation of heparin by protein, so that values of the equilibrium binding constant may be determined from the relationship between R and protein concentration (Lee & Lander, 1991; Lim et al., 1991). Data were analyzed graphically, by curve-fitting to the equation $R = R_\infty/(1 + K_d/[\text{protein}])$ (Lim et al., 1991). Nonlinear least-squares fits were calculated using the Kaleidagraph software package (Synergy Software, Reading, PA). In some cases, the data were better fit by the equation $R = R_\infty/(1 + K_d/[\text{protein}]^n)$, where n equals either 2 or 3, suggesting positive cooperativity in binding (Lim et al., 1991). In these cases, reported values of apparent K_d are actually the nth root of the calculated values, so as still to indicate the protein concentration at which binding is half-maximal.

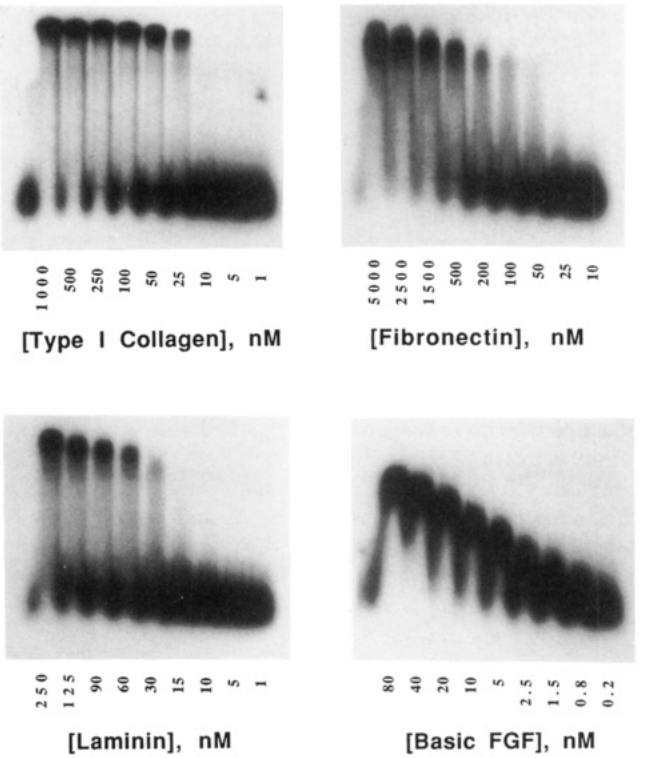

FIGURE 1: Evidence for heterogeneity in binding of heparin to fibronectin, laminin, and type I collagen but not to bFGF. Affinity coelectrophoresis (ACE) gels were constructed that contained the proteins indicated at the concentrations shown. Low M_r ^{125}I-Tyr-heparin was loaded into the sample slot (located at the top in each photograph) and electrophoresis was conducted with the anode at bottom. Heparin migration was visualized by autoradiography. The series of peaks that are seen represent heparin that has migrated within protein-containing lanes and has been retarded in its migration (e.g., see type I collagen gel lanes at concentrations of ≥25 nM protein). Valleys occur between each lane because protein-free agarose (in which heparin migrates unimpeded) separates each protein-containing lane. From such electrophoretograms, the dissociation constant (K_d) can be estimated from the protein concentration at which the heparin is half-shifted from being fully mobile to maximally retarded (Lee & Lander, 1991).

Although a choice of n greater than 1 sometimes improved the fit of curves to data, derived values of apparent K_d were not strongly dependent on the choice of n.

RESULTS

Heterogeneity in the Binding of Heparin to Extracellular Matrix Proteins. In a previous study (Lee & Lander, 1991) it was reported that fibronectin appears to bind selectively to subpopulations of low M_r heparin. This behavior was revealed by ACE experiments in which fronts of migrating heparin that passed through lanes containing particular concentrations of fibronectin became broadly smeared, suggesting that, at particular fibronectin concentrations, some heparin molecules were bound weakly and others were bound strongly. In the present study, ACE analysis of the binding of low M_r ^{125}I-Tyr-heparin to type I collagen, laminin, thrombospondin, and bFGF was carried out. As was observed before with fibronectin, laminin demonstrated significant smearing of the heparin migration front along the full length of some protein-containing lanes in electrophoretograms (Figure 1), suggesting heterogeneity in protein–glycosaminoglycan binding. Heterogeneity in protein-heparin binding was also indicated in ACE gels containing type I collagen (Figure 1), in which, at certain protein concentrations (e.g., 50 nM), the heparin migration front was apparently fractionated into at least two populations: one which was significantly retarded and re-

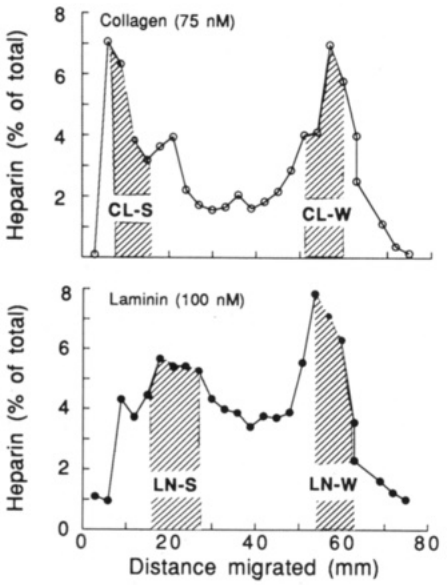

FIGURE 2: Isolation of heparin subpopulations that bind type I collagen and laminin weakly and strongly. Low M_r ^{125}I-Tyr-heparin was fractionated by electrophoresis through agarose gels containing a single protein, either type I collagen or laminin, at a concentration near the apparent average K_d for heparin (cf. Figure 1). Afterward, gels were sectioned perpendicular to the direction of electrophoresis, and the heparin content per slice was determined. Fractions containing the most retarded 25% of the heparin [designated by the suffix -S attached to a two-letter abbreviation for the protein used for fractionation, i.e., strongly retarded type I collagen (CL-S) and laminin (LN-S)] and the least retarded 25% of the heparin [similarly designated by the suffix -W, i.e., weakly retarded type I collagen (CL-W) and laminin (LN-W)] are indicated by shading and were pooled.

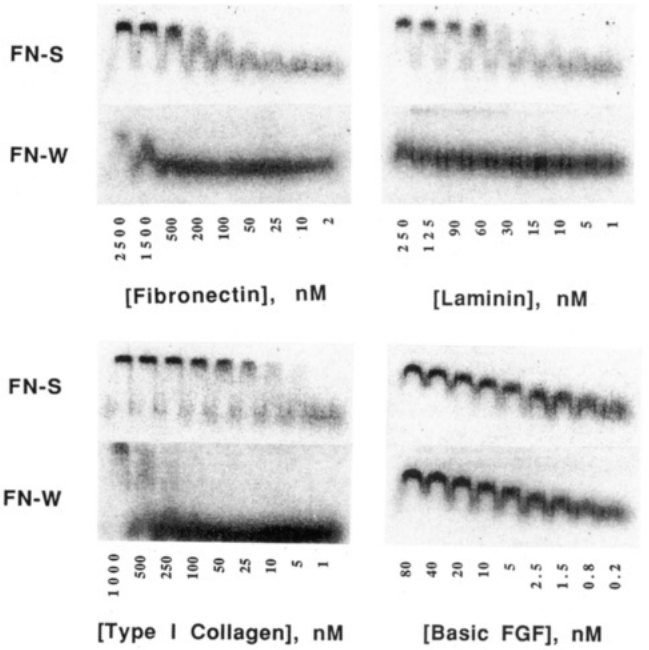

FIGURE 3: ACE analysis of the interactions between heparin subpopulations and heparin-binding proteins. Heparin subpopulations isolated as described in Figure 2 were tested using ACE for binding to fibronectin, laminin, type I collagen, and bFGF (see legend to Figure 1). Images of heparin migration patterns within ACE gels were obtained using a phosphorimager (Molecular Dynamics). The electrophoretograms shown are from experiments in which the heparin subpopulations that bound fibronectin strongly (FN-S) and weakly (FN-W) were analyzed for their binding to fibronectin, laminin, type I collagen, and bFGF.

mained at the top of the gel and the other which was highly mobile and migrated with unbound heparin, toward the bottom of the gel. In contrast, electrophoretic retardation, but little smearing or heparin fractionation along the length of protein lanes, was observed when heparin was subjected to electrophoresis in the presence of thrombospondin (not shown) or bFGF (Figure 1). In addition, a non-glycosaminoglycan polyanionic polysaccharide (colominic acid, M_r ~10 000), labeled with tyramine and radioiodinated in the same manner as heparin, failed to show any electrophoretic interaction, either retardation or smearing, with type I collagen or bFGF (unpublished observations).

Isolated Heparin Subpopulations Display Large Differences in Affinity for Fibronectin, Type I Collagen, and Laminin but Not bFGF or Thrombospondin. If broad smearing or fractionation of the heparin migration front in ACE gels represents true binding heterogeneity, it should be possible to recover strongly- and weakly-retarded heparin fractions from such gels and demonstrate that these two fractions represent pools that differ substantially in affinity. To demonstrate that this is the case, heparin was fractionated by electrophoresis through gels containing a single agarose well in which protein (laminin, type I collagen, or fibronectin) was present at a concentration near the apparent average K_d of protein–heparin binding, as previously determined by ACE (e.g., see Figure 1). After electrophoresis, gels were cut into sections perpendicular to the direction of electrophoresis, and the amount of radioactivity in each fraction was measured.

Typically, heparin fractionated into either two major peaks (Figure 2) or a broad smear. Fractions were pooled representing (approximately) the leading 25% of the heparin (most weakly retarded) and the trailing 25% of the heparin (most strongly retarded). The most extreme fractions (i.e., the fractions that were most strongly or weakly retarded) were discarded when the amount of labeled material they contained was very low and would undesirably dilute the pooled material (discarding the most extreme fractions would only be expected to slightly reduce the degree of affinity difference observed between strongly- and weakly-retarded fractions).

Heparin subpopulations isolated in this way were then tested for binding to laminin, type I collagen, fibronectin, bFGF, and thrombospondin. For the experiment shown (Figure 3), a gel containing fibronectin (500 nM) was used to isolate the subpopulations representing the 25% of heparin molecules most strongly retarded by fibronectin (designated fibronectin-S, where -S signifies strongly retarded) and the 25% of heparin molecules most weakly retarded by fibronectin (designated fibronectin-W, where -W signifies weakly retarded). It can be observed by visual inspection of the electrophoretograms (Figure 3) that fibronectin-S binds with higher affinity than fibronectin-W to fibronectin, laminin, and type I collagen, whereas bFGF and thrombospondin (not shown) bind both heparin subpopulations with similar affinity.

The binding of heparin populations that were strongly and weakly retarded by type I collagen and laminin (similarly designated type I collagen-S and -W and laminin-S and -W) to the same test proteins was also studied. In each case, mean heparin mobility was measured within each protein lane, and retardation coefficients (R) were calculated and plotted against protein concentration. The results are shown in Figure 4 and summarized in Table I. When the differences in affinity that each protein exhibits for each heparin subpopulation were compared, it was found that fibronectin, laminin, and type I collagen show large (~5–30-fold) differences in affinity for strongly-bound heparins vs weakly-bound heparins, whereas bFGF and thrombospondin show very small (≤2.3-fold) differences (Table I, ratios). In addition, for each protein, some differences were observed in the ratios of affinities of -S and -W fractions that were isolated using fibronectin, type

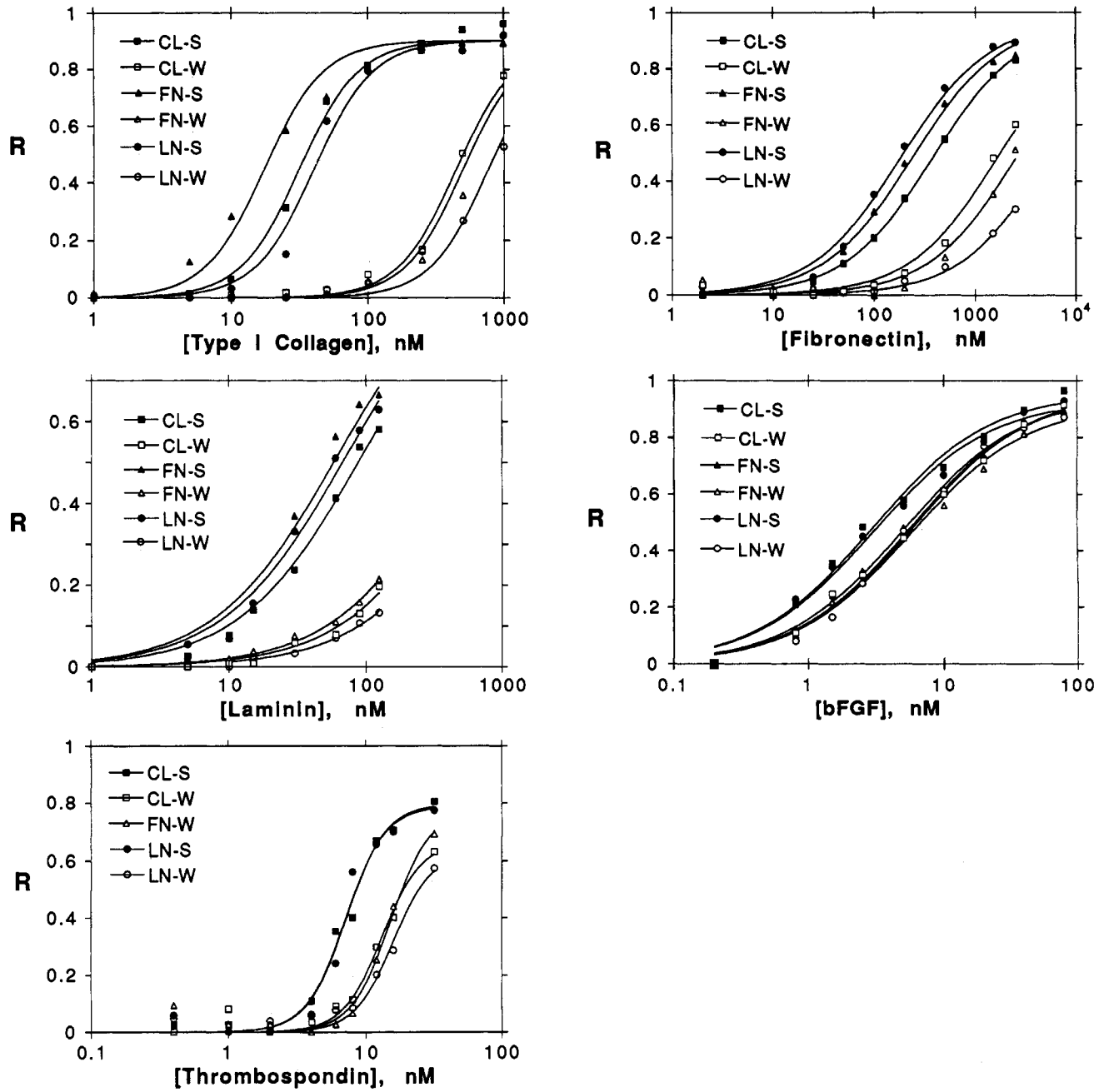

FIGURE 4: Calculation of affinities of heparin subpopulations for heparin-binding proteins. Heparin subpopulations isolated as shown in Figure 2 were subjected to ACE analysis as illustrated in Figure 3. Heparin retardation coefficients (R) within each protein-containing lane were determined (see Materials and Methods) and are plotted against protein concentration. Each heparin subpopulation is referred to by an abbreviation, as previously described (see legend to Figure 2). Symbols: type I collagen-S, filled squares; fibronectin-S, filled triangles; laminin-S, filled circles; type I collagen-W, open squares; fibronectin-W, open triangles; laminin-W, open circles. Smooth curves represent nonlinear least-squares fits to the equation $R = R_\infty/(1 + K_d/[\text{protein}]_n)$. For bFGF, fibronectin, and laminin, $n = 1$; for type I collagen, $n = 2$; for thrombospondin, $n = 3$ (see Materials and Methods). R_∞ was either taken as a free variable (for bFGF and thrombospondin) or was fixed at a value that well fit the family of curves obtained using each protein (0.97 for fibronectin, 0.902 for type I collagen, and 1.0 for laminin). Derived values of R_∞ for bFGF and thrombospondin varied from 0.93 to 0.96 and from 0.64 to 0.80, respectively.

I collagen, and laminin. For example, type I collagen-S and type I collagen-W differed 14-fold in affinity for type I collagen, while fibronectin-S and fibronectin-W differed 28-fold in affinity for type I collagen. Given the potential for variability in the selection of heparin subpopulations by electrophoresis, the degree of precision with which heparin mobility was measured, and the potential for error in the curve-fitting process, we doubt that such differences are statistically significant. In agreement with this view, the direction and magnitude of these small variations are not consistent from one experiment to another (not shown).

Relationship between Heparin Size, Charge (Retention by DEAE), and Protein Affinity. Previous work suggested that the heparin molecules in fibronectin-S are only slightly larger than those in fibronectin-W (Lee & Lander, 1991). In agreement with this finding, fractionation of heparin into subpopulations by type I collagen was found to depend little on heparin M_r: When heparin was size-selected to exclude both the smallest 11.6% and the largest 12.3% of molecules ("medium M_r ^{125}I-Tyr-heparin") and analyzed by ACE for binding to type I collagen and bFGF, it produced electrophoretic patterns very similar to those obtained with low M_r ^{125}I-Tyr-heparin. Specifically, the apparent K_ds obtained were similar (for medium M_r heparin, 11 nM for bFGF and 111 nM for type I collagen; for low M_r heparin, 5 nM for bFGF and 179 nM for type I collagen) and the degree of smearing

Table I: Apparent Affinities of Heparin Subpopulations Isolated on the Basis of Strength of Protein Binding[a]

	K_d (nM)			K_d (nM)			K_d (nM)		
	CL-S	CL-W	ratio	FN-S	FN-W	ratio	LN-S	LN-W	ratio
CL	32	450	14	18	510	28	41	780	19
FN	380	1700	4.5	240	2600	11	190	5300	28
LN	88	570	6.5	58	460	8	67	800	12
bFGF	2.9	5.6	1.9	5	5.6	1.1	3	5.8	1.9
TS	7.2	13	1.8		15		7.1	16	2.3

[a] K_d values are derived from the data shown in Figure 4. Abbreviations are type I collagen (CL), fibronectin (FN), laminin (LN), basic FGF (bFGF), and thrombospondin (TS); -S and -W are defined in legend to Figure 2.

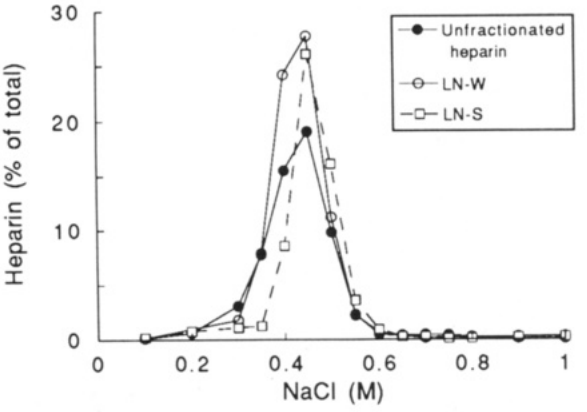

FIGURE 5: DEAE chromatography of heparin. Unfractionated low M_r ^{125}I-Tyr-heparin and heparin subpopulations isolated by ACE as fractions that bound strongly or weakly to laminin (Figure 2) were chromatographed on DEAE-Spectragel. Heparin was eluted with a step gradient of NaCl concentrations, and the heparin content of column fractions was determined.

of the electrophoretic pattern at type I collagen concentrations near K_d was also similar [e.g., in lanes containing a type I collagen concentration of 100 nM, the distribution of heparin along the lane could be characterized by a nearly identical variance for low versus medium M_r heparin (data not shown)].

It is possible that differences in overall charge or charge density of heparin molecules (reflecting degree of sulfation), rather than heparin size, might underlie the differences in heparin–protein affinity that were observed in this study (Ogamo et al., 1985; Sudhalter et al., 1989). Charge differences among heparin molecules are most readily revealed as differences in retention by anion-exchange matrices, such as DEAE. To examine the relationship between heparin retention by DEAE and protein affinity, two types of experiments were done.

First, previously isolated heparin fractions were adsorbed to DEAE columns and eluted with a stepwise NaCl gradient. Elution profiles of heparin fractions that differ substantially in protein affinity (e.g., laminin-S vs laminin-W or type I collagen-S vs type I collagen-W) were compared with each other and with the elution profile of unfractionated low M_r heparin. The results with laminin-S and laminin-W are shown in Figure 5. The data indicate that, on average, the molecules in laminin-S are slightly more strongly retained by DEAE than the molecules in laminin-W, although there is substantial overlap in the behavior of the two fractions.

In the second set of experiments, an unfractionated sample of low M_r heparin was first fractionated into pools on the basis of DEAE retention, and then each pool was tested for protein affinity. When fractions representing material eluted by stepwise increases of 0.05 M NaCl were tested for binding to fibronectin and type I collagen (Figures 6 and 7), an approximately linear relationship between apparent K_d and DEAE retention was observed (Figure 8A).

The data in Figures 6 and 7 indicate that the most extreme DEAE fractions exhibit a wide range of protein affinities,

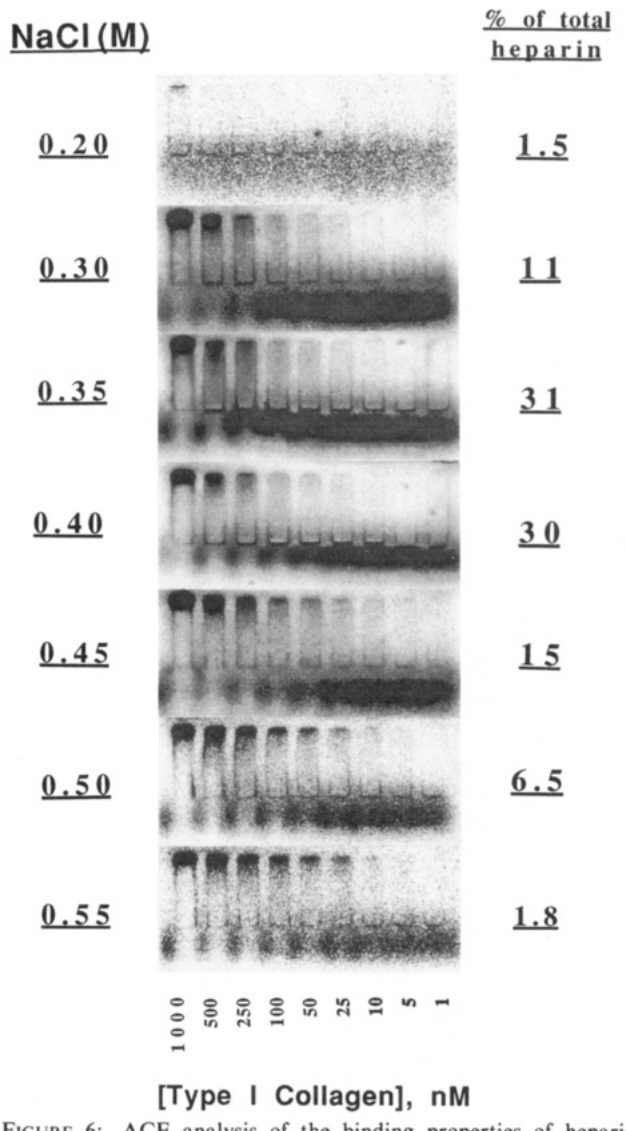

FIGURE 6: ACE analysis of the binding properties of heparin subpopulations isolated by DEAE chromatography. Low M_r ^{125}I-Tyr-heparin was fractionated by DEAE chromatography into subpopulations eluting at 0.2, 0.3, 0.35, 0.4, 0.45, 0.5, and 0.55 M NaCl. Fractions were diluted in electrophoresis buffer and tested for their binding to type I collagen or fibronectin (not shown) by ACE. Images of heparin migration patterns within gels were obtained using a phosphorimager (Molecular Dynamics).

comparable to the range exhibited by heparin fractions that had been isolated by protein affinity (cf. Table I). However, the extreme fractions isolated by DEAE chromatography represent only a small part of the initial low M_r heparin sample (Figure 8B), whereas the fractions isolated by protein affinity each represent approximately 25% of the total. Were a comparison to be made between the 25% of heparin most strongly bound by DEAE and the 25% most weakly bound by DEAE (i.e., DEAE-S and DEAE-W), the difference between the protein affinities exhibited by these fractions would not

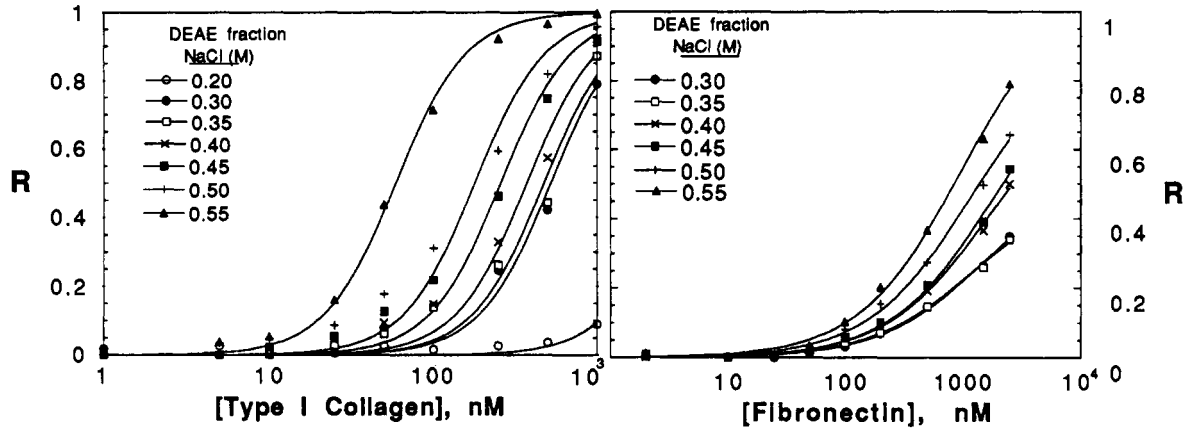

FIGURE 7: Calculation of affinities of heparin subpopulations, isolated by DEAE chromatography, for heparin-binding proteins. Data from Figure 6 and from measurements of binding of the same heparin fractions to fibronectin (not shown) were analyzed graphically to obtain measurements of the values of K_d's. Retardation coefficients (R) within each protein-containing lane were determined (see Materials and Methods) and are plotted against protein concentration. Each heparin subpopulation is referred to by the concentration of NaCl that was required to elute it from a DEAE column. Smooth curves represent nonlinear least-squares fits to the equation $R = R_\infty/(1 + K_d/[\text{protein}]^n)$. For fibronectin, $n = 1$; for type I collagen, $n = 2$ (see Materials and Methods). Values of R_∞ were fixed at 0.97 for fibronectin and 1.0 for type I collagen.

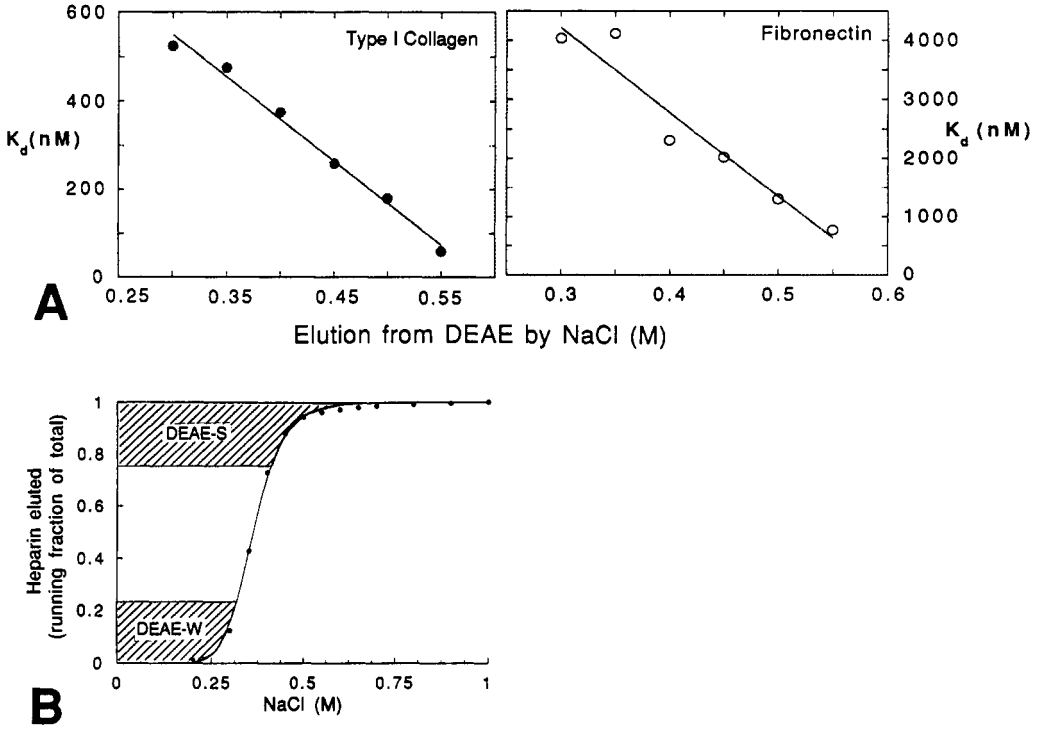

FIGURE 8: Relationship between binding of heparin to DEAE and heparin–protein affinity. Affinity constants derived from heparin–protein binding curves (Figure 7) are shown in panel A as a function of heparin retention by DEAE. Values on the abscissa are given as the NaCl concentration at which heparin fractions eluted from DEAE-Spectragel. The data could be approximated by the linear equations $f(x) = 1119 - 1900x$ for binding to type I collagen (correlation coefficient = 0.995) and $f(x) = 8513 - 14326x$ for binding to fibronectin (correlation coefficient = 0.965), where x stands for the NaCl concentration at which heparin subpopulations were eluted. From these data, it is possible to determine the average K_d that would be exhibited by any heparin subpopulation eluting from DEAE between any two given NaCl concentrations, provided that the data are appropriately weighted to reflect the relative amounts of heparin that elute at different salt concentrations. This information is shown in panel B, where the profile of heparin elution from DEAE as a function of NaCl concentration has been plotted as a running total. Hatched areas indicate the 25% of heparin most strongly bound (DEAE-S) and least strongly bound (DEAE-W) by the ion-exchange matrix and the NaCl concentration ranges required to elute them. The data have been arbitrarily fit to the equation $g(x) = 1/[1 + (1.074 \times 10^{-4})\, x^{-9}]$. The predicted value of K_d for a heparin subpopulation eluting from DEAE between any two NaCl concentrations, x_1 and x_2, would therefore be given by the formula $K_d = [\int_{x_1}^{x_2} f(x)\, dg(x)]/g(x_2) - g(x_1)$, which calculates an average value of the function $f(x)$, weighted according to $g(x)$.

be substantial. To calculate these values precisely, the data in Figure 8 were first fit to equations. The protein affinity of any heparin fraction eluted within a certain range of salt concentrations was then calculated using a formula that weights the contributions of all heparin species according to their abundance (see legend to Figure 8). The results (Table II) confirm that fractions DEAE-S and DEAE-W would differ only about 2.5-fold in protein affinity.

Relationship between Protein Affinity and the Presence or Absence of Antithrombin III Recognition Sequences. The best-known example of a protein that displays selectivity for subpopulations of heparin is antithrombin III. This protein binds with high affinity only to heparin chains that contain a particular carbohydrate sequence; such chains comprise about a third of most heparin preparations [see Marcum and Rosenberg (1989) for review]. To determine whether type I

Table II: Comparison between Protein Affinities Exhibited by Heparin Subpopulations Isolated by DEAE Chromatography and Protein Affinities Exhibited by Heparin Subpopulations Isolated on the Basis of Strength of Protein Binding[a]

	heparin fraction			heparin fraction		
	25% most strongly bound to DEAE	25% least strongly bound to DEAE	ratio	25% most strongly bound to protein	25% least strongly bound to protein	ratio
K_d for CL	240	570	2.4	32	450	14
K_d for FN	1860	4400	2.4	240	2570	11

[a] Values for heparin fractions representing the 25% most strongly and least strongly bound to DEAE-Spectragel was calculated as described in the legend to Figure 8. Values for heparin fractions representing the 25% most strongly and least strongly bound to proteins come from the determination made for type I collagen–S and type I collagen-W binding to type I collagen and for fibronectin-S and fibronectin-W binding to fibronectin, as reported in Table I.

collagen, laminin, and fibronectin recognize the same structural elements in heparin as antithrombin III, the binding of type I collagen-S and type I collagen-W to antithrombin III was analyzed by ACE. Using this method, heparin populations with high and low affinity for antithrombin III are cleanly separated from each other (Lee & Lander, 1991), and the relative amounts in each population may be determined. When unfractionated low M_r ^{125}I-Tyr-heparin was tested in this way, 36% of the material was found to display high-affinity binding. In contrast, 55% of type I collagen-S and 8% of type I collagen-W heparin subpopulations showed high-affinity binding to antithrombin III (data not shown).

DISCUSSION

We have used the method of affinity coelectrophoresis to compare the affinity and selectivity of heparin binding to five proteins: fibronectin, laminin, type I collagen, thrombospondin, and bFGF. Low M_r heparin displayed evidence of considerable heterogeneity in its binding affinity for type I collagen, laminin, and fibronectin but little heterogeneity in binding to thrombospondin and bFGF. Isolation of strongly- and weakly-binding fractions of heparin from gels containing laminin, fibronectin, or type I collagen indicated that heparin molecules bound strongly by any one of these proteins are bound strongly by the other two. Likewise, heparin molecules bound weakly by any one of the three are bound weakly by the other two.[2]

To assess the relationship between heterogeneity in the protein affinities of heparin molecules and heterogeneity in heparin's negative charge, the protein affinities of heparin molecules fractionated by a DEAE matrix were compared with those of heparin fractionated by electrophoresis through type I collagen, laminin, and fibronectin. Whereas these proteins fractionated heparin into populations each containing 25% of total low M_r heparin and differing ~5–30-fold in affinity (Table I), populations selected by DEAE as being the 25% most strongly and least strongly bound differ by <2.5-fold in affinity (Figures 6–8 and Table II). Conversely, populations of heparin selected as being the 25% most strongly and least strongly bound by laminin and type I collagen were found to differ only slightly in their binding to DEAE (Figure 5). Thus, a positive correlation exists between retention by DEAE and protein affinity, but the correlation is a relatively weak one.

A small correlation was also found between affinity of heparin molecules for antithrombin III and affinity for type I collagen. Whereas antithrombin III-binding heparin molecules represent about a third of unfractionated low M_r heparin, they are somewhat more abundant (by about 50%) in the 25% of heparin molecules that bind most tightly to type I collagen.

Taken together, the data allow several conclusions to be drawn about the structural basis for selectivity in the binding of heparin subpopulations to extracellular matrix proteins:

(1) Type I Collagen, Laminin, and Fibronectin Can Distinguish among Structural Features in Heparin. It is unlikely that these features represent mere variations in net molecular charge, since the correlation between DEAE binding and protein affinity of heparin molecules is a weak one. In addition, previous work (Lee & Lander, 1991) and data presented here both argue that variation in heparin chain length is also not the basis for selectivity in fibronectin binding to heparin. Potentially, type I collagen, laminin, and fibronectin may recognize specific carbohydrate sequences, as does antithrombin III. They do not, however, recognize the antithrombin III recognition sequence itself, since only about half of the molecules binding most tightly to type I collagen contain that sequence. The specificity displayed by type I collagen, laminin, and fibronectin also differs from that displayed by antithrombin III in that heparin molecules bound most tightly by these extracellular matrix proteins differ only 5–30-fold in affinity from those bound least tightly; with antithrombin III, the difference between strongly- and weakly-binding fractions is about 1000-fold (Jordan et al., 1979). The impression that type I collagen, laminin, and fibronectin are, in some way, less discriminating than antithrombin III may be misleading, however, since the heparin subpopulations that were isolated in this study were each selected to contain 25% of the heparin that was present in the starting material. If heparin consists of molecules displaying a continuum of affinities, it is likely that the most extreme molecules (e.g., the top and bottom 5%) vary in affinity by considerably more than the 5–30-fold reported here.

(2) Thrombospondin and bFGF Show Little Preference in Their Binding to Low M_r Heparin Molecules. This result does not imply that these proteins are incapable of recognizing and distinguishing among structural features in heparin. Indeed, recent studies imply that bFGF can preferentially bind certain short heparan sulfate-derived oligosaccharides (Turnbull et al., 1992). What can be concluded, however, is that all, or nearly all, of the low M_r heparin molecules used in this study contain the structural features required for high-affinity binding to thrombospondin and bFGF. In contrast, only some of the molecules in low M_r heparin contain the

[2] In a previous study (Lee & Lander, 1991), heparin was labeled by mild CNBr activation, derivatization with fluoresceinamine, and subsequent radioiodination (Smith & Knauer, 1987). Interestingly, it was found that the ^{125}I-Tyr-heparin used in the present study consistently bound with a 3–10-fold lower affinity to all heparin-binding proteins tested than did ^{125}I-fluoresceinamine-heparin (unpublished observations). This result suggests that iodofluorescein moieties may interact nonspecifically with proteins and stabilize labeled heparin–protein interactions. Nonetheless, parallel experiments conducted with ^{125}I-fluoresceinamine-heparin revealed essentially the same specificities and ranges of affinities as those reported here (unpublished observations). Thus, we think it highly unlikely that either method of heparin labeling creates or influences the heterogeneity in binding affinity that we have observed.

structural features required for highest affinity binding to type I collagen, laminin, and fibronectin.

(3) Although the Basis for High-Affinity Binding to Type I Collagen, Laminin, and Fibronectin is neither Net Charge nor Chain Length, There Is Some Positive Correlation between Both Properties and Binding Affinity for These Proteins. Although it is possible that these gross structural features play some role in high-affinity binding, it is also possible that these correlations are a statistical artifact. For example, it is to be expected that any carbohydrate sequence will be more prevalent among longer than average chains. Likewise, any carbohydrate sequence containing a greater than average number of charged groups is expected to be more prevalent among chains that are more charged than average. Such correlations have, in fact, been observed with respect to the distribution of antithrombin III recognition sequences among heparin chains (Laurent et al., 1978), a fact that could also explain why the presence of antithrombin III binding sequences also correlates weakly with type I collagen affinity (see above).

Whether the selectivity displayed by type I collagen, laminin, and fibronectin for subpopulations of heparin molecules can be traced directly to short oligosaccharide sequences (as has been done with antithrombin III) remains to be determined by direct sequence analysis of strongly- and weakly-binding heparin molecules and fragments thereof. The present study provides the first evidence that such sequences exist for at least some extracellular matrix proteins. Previous studies—in which the relationship between properties such as net charge and binding affinity was not dealt with in a quantitative way—failed to establish this point, leading some to assert that sequence recognition by extracellular matrix molecules does not occur (Ruoslahti, 1988).

An important consideration is that the present study was carried out using heparin, whereas the biologically relevant ligands for laminin, fibronectin, and type I collagen are likely to be heparan sulfates. Because the steps in the biosynthesis of heparin and heparan sulfate are apparently the same, it is believed that all of the carbohydrate sequences found in heparin can, at least in principle, occur in heparan sulfates. It is this fact that justifies the use of heparin as a tool to look for carbohydrate binding specificities that may be of in vivo relevance. Nevertheless, the relative abundance of particular sequences in heparin and heparan sulfates is likely to be substantially different, owing to differences in the levels and arrangements of carbohydrate modifications. Accordingly, the question of how discriminating laminin, fibronectin, and type I collagen are in their binding preferences in vivo should ideally be posed with heparan sulfates, or preferably with intact heparan sulfate proteoglycans. Preliminary studies (Herndon & Lander, 1992; Sanderson et al., 1992) suggest that at least as much selectivity occurs in the interactions of these proteins with heparan sulfate proteoglycans as is reported here for heparin.

Presumably, both the affinities and the selectivities for heparin chains that were exhibited by the heparin-binding proteins used in this study are determined by information encoded in protein structure. All of the proteins studied here (with the exception of type I collagen, for which heparin-binding sequences have not yet been identified) contain more than one putative heparin-binding site encoded in primary protein sequence(s) [see Jackson et al. (1991) for review]. Furthermore, four of the proteins are composed of two or more subunits, and some are capable of forming higher-order structures (e.g., aggregates or fibrils). Thus, in addition to the heparin-binding domains present within single polypeptide subunits, protein tertiary and quaternary structure may also control protein–glycosaminoglycan affinity and selectivity. This view is consistent with the observation that high-affinity heparin or heparan sulfate binding by type I collagen requires the native triple-helical conformation and possibly the presence of fibrils (Stamatoglou & Keller, 1982; Koda et al., 1985; Keller et al., 1986; San Antonio et al., 1992).

The results of the present study suggest that specific sequences in *both* glycosaminoglycans and glycosaminoglycan-binding proteins account for the specificity in glycosaminoglycan–protein interactions. The extent to which such specificity is used in vivo depends upon the relationship between the tissue locations of glycosaminoglycan sequences and the expression patterns of sequence-specific glycosaminoglycan-binding proteins. In the case of antithrombin III, evidence suggests that antithrombin III-binding heparan sulfates are enriched at tissue locations (e.g., the blood vessel wall) where antithrombin III is expected to act (Agostini et al., 1990). Whether a similar correspondence exists for fibronectin, laminin, or type I collagen remains to be seen.

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REFERENCES

Agostini, A. I., Watkins, S. C., Slayter, H. S., Youssoufian, H., & Rosenberg, R. D. (1990) *J. Cell Biol. 111*, 1293–1304.
Baird, A., Schubert, D., Ling, N., & Guillemin, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2324–2328.
Blackburn, M. N., & Sibley, C. C. (1980) *J. Biol. Chem. 255*, 824–826.
Blackburn, M. N., Smith, R. L., Carson, J., & Sibley, C. C. (1984) *J. Biol. Chem. 259*, 939–941.
Cardin, A. D., & Weintraub, H. J. R. (1989) *Arteriosclerosis 9*, 21–32.
Dische, Z. (1947) *J. Biol. Chem. 167*, 189–192.
Harper, J. W., & Lobb, R. R. (1988) *Biochemistry 27*, 671–678.
Herndon, M. E., & Lander, A. D. (1992) *Mol. Biol. Cell 1*, 373a.
Jackson, R. L., Busch, S. J., & Cardin, A. D. (1991) *Physiol. Rev. 71*, 481–539.
Jordan, R., Beeler, D., & Rosenberg, R. (1979) *J. Biol. Chem. 254*, 2902–2913.
Kato, M., Turnbull, J., Hooper, K., Gallagher, J., & Bernfield, M. (1991) *J. Cell Biol. 115*, 729 (abstr.).
Keller, K. M., Keller, J. M., & Kuhn, K. (1986) *Biochim. Biophys. Acta 882*, 1–5.
Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., & Martin, G. (1982) *Biochemistry 21*, 6188–6193.
Koda, J. E., Rapraeger, A., & Bernfield, M. (1985) *J. Biol. Chem. 260*, 8157–8162.
Laurent, T. C., Tengblad, A., Thunberg, L., Hook, M., & Lindahl, U. (1978) *Biochem. J. 175*, 691–701.
Lawler, J., & Simons, E. (1983) *J. Biol. Chem. 258*, 12098–12101.
Lawler, J., Derick, L., Connolly, J., Chen, J., & Chao, F. (1985) *J. Biol. Chem. 260*, 3762–3772.
Lawler, J., Ferro, P., & Duquette, M. (1992) *Biochemistry 31*, 1173–1180.
Lee, K. B., Al-Hakim, A., Loganathan, D., & Linhardt, R. J. (1991) *Carbohydr. Res. 214*, 155–168.
Lee, M. K., & Lander, A. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2768–2772.
Lim, W. A., Sauer, R. T., & Lander, A. D. (1991) *Methods Enzymol. 208*, 196–210.

Lindahl, U., & Hook, M. (1978) *Annu. Rev. Biochem. 47*, 385–417.
Lobb, R. R., & Fett, J. W. (1984) *Biochemistry 23*, 6295–6299.
Marcum, J., & Rosenberg, R. (1989) Heparin and Related Polysaccharides, *Ann. N.Y. Acad. Sci. 556*, 81–94.
Ogamo, A., Nagai, A., & Nagasawa, K. (1985) *Biochim. Biophys. Acta 841*, 30–41.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490–6505.
Rosenberg, R. D., Jordan, R. E., Favreau, L. V., & Lam, L. H. (1979) *Biochem. Biophys. Res. Commun. 86*, 1319–1324.
Ruoslahti, E. (1988) *Annu. Rev. Cell Biol. 4*, 229–255.
San Antonio, J. D., Lander, A. D., Wright, T. C., & Karnovsky, M. J. (1992) *J. Cell Physiol. 150*, 8–16.
Sanderson, R. D., Sneed, T. B., Maes, L. A., Sullivan, G. L., & Lander, A. D. (1992) *J. Immunol. 148*, 3902–3911.
Smith, J. W., & Knauer, D. J. (1987) *Anal. Biochem. 160*, 105–114.
Stamatoglou, S. C., & Keller, J. M. (1982) *Biochim. Biophys. Acta 719*, 90–97.
Sudhalter, J., Folkman, J., Svahn, C. M., Bergendal, K., & D'Amore, P. A. (1989) *J. Biol. Chem. 264*, 6892–6897.
Timpl, R., Rohde, H. Risteli, L., Ott, U., Robey, P. G., & Martin, G. R. (1982) *Methods Enzymol. 82*, 831–838.
Turnbull, J., & Gallagher, J. (1990) *Biochem. J. 265*, 715–724.
Turnbull, J., Fernig, D. G., Ke, Y., Wilkinson, M. C., & Gallagher, J. T. (1992) *J. Biol. Chem. 267*, 10337–10341.
Yabkowitz, R., Lowe, J. B., & Dixit, V. M. (1989) *J. Biol. Chem. 264*, 10888–10896.
Yamada, K. M., Kennedy, D. W., Kimata, K., & Pratt, R. M. (1980) *J. Biol. Chem. 255*, 6055–6063.