Characterization of Heparin and Heparan Sulfate Domains
Binding to the Long Splice Variant of Platelet-derived Growth Factor A Chain

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Platelet-derived growth factors (PDGFs) are homo- or heterodimers of two related polypeptides, known as A and B chains. The A chain exists as two splice variants due to the alternative usage of exons 6 (PDGF-AL, longer) and 7 (PDGF-AS, shorter). Exon 6 encodes an 18-amino acid sequence rich in basic amino acid residues, which has been implicated as a cell retention signal. Several lines of evidence indicate that the retention is due to binding of PDGF-AL to glycosaminoglycans, especially to heparan sulfate. We have analyzed the saccharide domains of smooth muscle cell-derived heparan sulfate involved in this interaction. Furthermore, we have employed selectively modified heparin oligosaccharides to elucidate the dependence of the binding on different sulfate groups and on fragment length. The shortest PDGF-AL binding domain consists of 6–8 monosaccharide units. Studies using selectively desulfated heparins and heparin fragments suggest that N-, 2-O-, and 6-O-sulfate groups all contribute to the interaction. Structural comparison of heparan sulfate oligosaccharides separated by affinity chromatography on immobilized PDGF-AL showed that the bound pool was enriched in -IdceA(2-OSO3)-GlcNSO3(6-OSO3)-disaccharide units. Furthermore, analogous separation of a partially O-desulfated heparin decamer preparation, using a highly selective nitrocellulose filter-trapping system, yielded a PDGF-AL-bound fraction in which more than half of the disaccharide units had the structure -IdceA(2-OSO3)-GlcNSO3(6-OSO3). Our results suggest that the interaction between PDGF-AL and heparin/heparan sulfate is mediated via N-sulfated saccharide domains containing both 2-O- and 6-O-sulfate groups.

Platelet-derived growth factors (PDGFs)¹ are dimeric polypeptides that regulate the proliferation and differentiation of smooth muscle cells, fibroblasts, and other cells of mesenchymal origin (for review, see Heldin and Westermark (1)). PDGFs occur as homo- or heterodimers of two distinct but closely related, A and B, peptide chains. Cellular responses to PDGF are mediated via cell surface α and β tyrosine kinase receptors that form homo- or heterodimers upon ligand binding. The PDGF-A chain appears as two splice variants due to the alternative usage of exons 6 and 7 in the gene. The longer variant (PDGF-AL) contains an 18-amino acid polybasic sequence encoded by exon 6 toward its carboxyl terminus that is replaced by a Glu-Val-Arg sequence, encoded by exon 7, in the shorter splice variant (PDGF-AS) (2, 3). A similar, but not identical, polybasic sequence is found in the PDGF-B propeptide chain, also encoded by exon 6. As a result of carboxy-terminal proteolytic processing, this sequence is absent in shorter, “mature” forms of PDGF-B. No differences in receptor binding, or in the cellular responses elicited thereafter, have been found between differentially spliced/processed forms of PDGF. Instead, the presence or absence of an exon 6-encoded sequence seems to regulate the secretion of PDGF from its producer cells. Studies on the PDGF secretion in transfected COS or Chinese hamster ovary cells expressing different PDGF constructs show that only PDGF-AS is effectively secreted to the culture medium, whereas the forms containing an exon 6-encoded sequence (i.e., PDGF-AL and “immature” PDGF-B) remain associated with the producer cells or with the extracellular matrix (4–6). A mutational analysis of the PDGF-AL retention motif has demonstrated a critical role for a repeat of basic amino acid residues for the retention function. Replacement of 2 or 3 out of 7 basic residues with alanine thus resulted in the secretion of the mutated PDGF-AL form to the culture medium in COS cells (7).

The retention of the PDGF species containing exon 6-encoded sequences is at least in part related to the ability of these sequences to bind glycosaminoglycans, particularly heparan sulfate (HS). HS, generally in the form of HS proteoglycans (for reviews, see Refs. 8–11), is present at most cell surfaces as well as in basal laminae and other extracellular matrices. The exon 6-encoded peptide in PDGF-AL has been shown to bind heparin and HS (12–14). Furthermore, PDGF-AL can be released from cells by heparitinase treatment (7) as well as by addition of exogenous heparin (5). Together, the current data suggest that HS may be involved in the regulation of secretion, storage, and possibly also receptor binding of PDGF-AL.

Heparin and the structurally related but less sulfated polysaccharide, HS, are known to bind a number of proteins, such as acid; SAX, strong anion exchange; deca, decamer.
as peptide growth factors, extracellular matrix components, enzymes, enzyme inhibitors, and others (for review, see Refs. 8, 9, and 15). These interactions are generally considered to be electrostatic in nature, involving basic amino acid residues in the protein component and negatively charged groups in the polysaccharide. The interactions with different proteins differ in degree of specificity with regard to carbohydrate structure. Some proteins, such as fibronectin and platelet factor 4 (16), were claimed to bind heparin and HS in a nonspecific fashion, i.e., their binding could not be attributed to certain sulfate groups within a sequence of defined size but rather increased with increasing net charge of the saccharide. Other proteins, such as antithrombin (reviewed in Bourin and Lindahl (17)), fibroblast growth factor 2 (FGF-2) (18–20), and hepatocyte growth factor (HGF) (21, 22) bind in a more specific fashion. The antithrombin-binding heparin/HS sequence, for example, is a pentasaccharide containing a rare GlcNSO$_3$(3-OSO$_3$) unit, whereas a defined IdeA(2-OSO$_3$) unit is essential for binding of FGF-2. Some proteins may recognize more complex multismain sequences in HS, as shown in a recent report describing the interferon-γ-binding HS structure that encompasses two terminal highly sulfated domains separated by a N-acetylated domain (23). The current study was undertaken in order to characterize the structural requirements for the interaction between heparin/HS and PDGF-A$_L$.

MATERIALS AND METHODS

Glycosaminoglycan Preparations—Heparin from pig intestinal mucosa (stage 14, Inoxel Pharmaceutical Division, Park Forest South, IL) was purified as described previously (24) and used either unlabeled or radiolabeled by $^3H$-acylating free amino groups (25) (specific activity, $-$0.34 $\times$ 10$^5$ dpm/nmol of disaccharide). Similarly purified bovine heparin was used in the preparation of partially desulfated material (see Table I). N-Desulfation was carried out by Me$_2$SO treatment as described elsewhere (26). Selective 2-O-desulfation was performed at pH 12.5 by lyophilization (27) and preferential 6-O-desulfation in Me$_2$SO:methanol (9:1) at 93 °C for 2 h (28). O-Desulfated (simultaneously N-desulfated) heparin preparations were N-terminated (29) and N-desulfated preparations N-acetylated (30) as described earlier. Even-numbered $^3H$-labeled heparin oligosaccharides were generated by partial deaminative cleavage of bovine heparin by nitrous acid at pH 1.5, followed by the reduction of the products with NaBH$_4$ (31). The preferentially 6-O-desulfated heparin was partially dephosphorylated by limited deaminative cleavage at pH 3.9 (32), N-terminated, $^3H$-labeled, and a decasaccharide fraction (Pref. 6-O-DS deca-A) recovered. Alternatively, a similar fragment (Pref. 6-O-DS deca-B) was generated from heparin that had been treated with Me$_2$SO:methanol (9:1) at 80 °C for 2 h. Finally, an analogous decasaccharide fraction (2-O-DS deca) was recovered from the 2-O-desulfated heparin derivative. Additional glycosaminoglycan preparations used included bovine kidney and aorta HSs (gifts from Dr. Anders Malmström, University of Lund). Samples of each polysaccharide were $^3H$-acylabeled as described previously (25) and used for binding analysis.

Preparation of HS from Smooth Muscle Cells—Human arterial smooth muscle cells (hASMC) derived from the inner media of uterine arteries were prepared and maintained as described previously (34). Cells were labeled metabolically with $^{14}$C-uracil at 8M urea and conditioned labeling medium were brought to 8M urea (see Table I). The mixture was passed through a nitrocellulose filter (Whatman Inc., Clifton, NJ) HPLC column eluted with a stepwise gradient of 0.1% Triton X-100 and 0.05M sodium acetate, pH 4.5. Fractions corresponding to proteoglycans were pooled, dialyzed against water, and used for binding analysis. To purify HS, the pooled material ($-$4 $\times$ 10$^6$ dpm) was first treated with 1 unit of chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan) for 4 h at 37 °C. Subsequently, HS chains were liberated from their protein cores by alkaline β-elimination (treatment with 0.5 M NaOH at 4 °C overnight). Free HS chains were recovered using DEAE-chromatography. Deaminative cleavage with nitrous acid at pH 3.9 resulted in quantitative degradation of the purified material, as demonstated by gel chromatography on Superose 12 (Pharmacia Biotech Inc.), indicating that pure HS had been obtained (not shown).

Preparation of HS Fragments—hASMC HS was deacylated by treatment in hyaluronic hydrate (Fluka Chemie AG, Buchs, Switzerland) containing 30% water (containing 1% (w/v) hyaluronic acid sulfate (Merck, Darmstadt, FRG) at 100 °C for 5 h (33). Deacylated HS was dialyzed, dried, and treated with 0.5 ml of nitric acid reagent at pH 3.9, thus effecting a deaminative cleavage at the N-unsubstituted GlcNS residues generated by deacylation (32). The resulting fragment mixture was desalted by passage through a Sephadex G-15 column (1 × 60 cm) in 0.2 M NH$_4$HCO$_3$, dried, and used in PDGF-A$_L$ affinity chromatography as described below.

PDGF Binding Experiments—All binding assays were performed using recombinant PDGF-A$_L$, produced in an Escherichia coli expression system (14). Monomeric ligand was used in the preparation of the PDGF-A$_L$ affinity column (see below); in all other experiments, dimeric PDGF-A$_L$ was used. Binding between PDGF-A$_L$ and GAGs was assayed using a previously documented in-solution assay (16). PDGF-A$_L$, along with radiolabeled GAGs (amounts as specified in the text or figure legends), were incubated in 200 µl of phosphate-buffered saline containing 0.1% bovine serum albumin at room temperature for 60 min, after which the mixture was passed through a nitrocellulose filter (Sartorius, diameter 25 mm, pore size 0.45 µm) using a vacuum-assembled suction apparatus. The filters were then rapidly washed twice with 2 ml of phosphate-buffered saline. Protein-bound, together with any protein-bound GAGs, were recovered from the filter. Protein-bound GAGs were then removed from the filter with 2 ml of 1 M NaCl and quantified in a liquid scintillation counter. In preparative binding experiments (20 µg of PDGF-A$_L$), the bound and unbound GAGs were recovered from the filter-bound and nonbound fractions, respectively, and desalted by passage through a column (1 × 60 cm) of Sephadex G-15 in NH$_4$HCO$_3$ or by dialysis against water, and subjected to structural analysis (see below).

Other binding studies were performed using a PDGF-A$_L$ affinity column. Recombinant monomeric PDGF-A$_L$ (7.5 µg) was mixed with an equimolar amount of heparin and immobilized to a 3.0-ml column of CH-Sepharose CLAB (Pharmacia) according to the instructions of the manufacturer. Following equilibration of the column with 0.15 M NaCl containing 50 mM Tris (pH 7.4), radiolabeled GAGs were applied to the column, which was then eluted with a linear gradient (total volume, 40 ml) of 0.15 to 2.0 M NaCl in 50 mM Tris, pH 7.4. Fractions were collected and analyzed for radioactivity and for NaCl concentration (using conductometry). In preparative experiments, the bound and unbound fractions were recovered and analyzed as described below.

Structural Analysis of Oligosaccharides—$^{35}$S-labeled HASMC HS fragments separated by affinity chromatography on the PDGF-A$_L$, matrix were subject to gel chromatography on a Superdex 30 fast performance liquid chromatography column (1.6 × 60 cm; Pharmacia). The column was run in 0.2 M NH$_4$HCO$_3$ and calibrated using $^3H$-labeled heparin oligosaccharides of known size. One-ml fractions were collected and analyzed for radioactivity. Size-selected fractions were recovered for rechromatography on the PDGF-A$_L$ affinity column or for structural analysis (see below).

The disaccharide composition of oligosaccharide fractions separated with regard to affinity for PDGF-A$_L$, was assessed by analysis of HexA$\text{Man}$_{3-5} disaccharides generated by deaminative cleavage (31). Treatment with nitric acid at pH 1.5 cleaves glucosaminidic linkages of N-sulfated GlcN units, which are converted to 2,5-anhydroaminosug residues (32). The deamination products of $^{35}$S-labeled, N-sulfated HS fragments were reduced with NaBH$_4$, to yield terminal aMan$_5$ units, and the resultant disaccharides were recovered by passage through a Sephadex G-15 column (1 × 190 cm) in 0.2 M NH$_4$HCO$_3$. The disaccharides were identified using a Partisil-10 SAX (4.6 × 250 mm; Whatman Inc., Clifton, NJ) HPLC column eluted with a stepwise gradient of KH$_2$PO$_4$ at a flow rate of 1 ml/min (31). One-ml fractions were collected and analyzed for radioactivity in a liquid scintillation counter. PDGF-A$_L$ bound and unbound fractions derived from Pref. 6-O-DS deca-B were similarly analyzed for disaccharide composition with the exception that radiolabel was introduced after the deaminative cleavage by reduction of the products with NaBH$_4$; moreover, the radioactivity of the HPLC effluent was determined with a radioactivity flow detector.

RESULTS

Interaction of PDGF-A$_L$, with Glycosaminoglycans—The binding of radiolabeled GAGs to PDGF-A$_L$, was initially studied using an assay based on trapping of GAG-protein complexes on
a nitrocellulose filter (see “Materials and Methods”). Heparin and bovine kidney HS bound to PDGF-A_L in a dose-dependent and saturable manner (data not shown). We also studied the binding of radiolabeled chondroitin 4-sulfate and dermatan sulfate to PDGF-A_L and observed that these GAGs showed 7–10 times less binding compared to heparin (not shown). These results were in accord with previous findings that chondroitin sulfate and dermatan sulfate are 5 times less effective than heparin in displacing PDGF-A_L bound to heparin (14).

Additional studies were conducted using PDGF-A_L affinity chromatography. Radiolabeled saccharides were allowed to bind to the affinity matrix at physiological ionic strength, and any bound material was subsequently eluted from the column with a linear salt gradient. Native, full-length heparin showed the highest apparent affinity among the saccharides tested; 1.3 M NaCl being required for its elution from the column. HS from hASMCs was eluted at 0.9 M NaCl, whereas bovine aorta HS, with a lower degree of sulfation (35), was eluted at 0.6 M NaCl (Fig. 1).

Effect of Saccharide Fragment Length—The size of the smallest heparin fragment retaining the ability to bind PDGF-A_L was determined using 3H-labeled, even-numbered heparin oligomers of different lengths (see “Materials and Methods”). Size-defined fragments were tested for PDGF-A_L binding in filter-trapping and affinity chromatography systems. For filter-trapping, a relatively large amount (20 μg) of PDGF-A_L was used in each incubation. Under these experimental conditions, no binding of tetra- or hexasaccharides was detected, whereas about 20% of the added heparin octasaccharide was bound. Decameric fragments showed about 50% and larger fragments maximal binding (i.e. 70–80% of the added saccharide) (Fig. 2A). These findings suggest that the actual PDGF-A_L binding region is contained within a heparin octasaccharide sequence; presumably, the apparent higher affinities of the larger oligosaccharides reflect the occurrence of multiple, overlapping binding regions. Affinity chromatography on immobilized PDGF-A_L showed no significant binding of heparin tetramer. Hexamer was eluted at ~0.4 M NaCl, whereas octa- and decamers required ~0.6 and ~0.7 M NaCl for elution, respectively (Fig. 2B). These results thus agree with those of the filter-trapping assay in that octasaccharide showed more avid binding than hexasaccharide. However, even the latter fragment retained significant affinity for the PDGF-A_L matrix, whereas no binding could be demonstrated in the filter-trapping system. While the reason for this discrepancy is unknown, it is noted that the two procedures differ drastically with regard to the relative amounts of the interacting components as well as in their mode of interaction.

To obtain HS oligosaccharides for PDGF-A_L binding studies, metabolically 35S-labeled hASMC HS was deacylated by hydrazinolysis and treated with nitrous acid at pH 3.9. This procedure will degrade any previously N-acetylated regions in the polysaccharide while the N-sulfated sequences remain intact. When the products of such cleavage were passed through the PDGF-A_L affinity column, two distinct peaks were observed (Fig. 3A). The first peak emerged from the column during the
initial wash with Tris-buffered saline, and represented material unbound in 0.15 M NaCl, whereas a fraction of bound material was eluted between 0.25 and 0.7 M NaCl. The bound and unbound pools were passed through a Superdex 30 gel chromatography column and the elution positions of HS fragments were compared with those of standard heparin oligosaccharides (Fig. 3B). The bound pool consisted mainly of octasaccharides and larger fragments, whereas the unbound pool contained tetra- and disaccharides as its major constituents. However, both pools contained some material that emerged between the elution positions of octa- and tetrasaccharide standards. Rechromatography on the PDGF-A_L affinity column of this fraction, recovered from the pool of initially bound material, resulted in >90% rebinding, whereas the corresponding previously unbound HS oligosaccharides showed no binding (not shown). These two oligomer pools were subjected to compositional analyses, as described below.

**Binding of Selectively Desulfated Saccharides to PDGF-A_L**—The role of sulfate substituents in binding was assessed using the filter-trapping assay and unlabeled, selectively desulfated heparin preparations (Table I) as inhibitors of the interaction between fully sulfated [\(^3^H\)]heparin and PDGF-A_L. At a concentration of 5 \(\mu\)g/ml, N-desulfated, 2-O-desulfated, or preferentially 6-O-desulfated heparin inhibited the binding between fully sulfated \([\(^3^H\)]\)heparin and PDGF-A_L by 48, 45, and 35%, respectively (Fig. 4A). With 5 \(\mu\)g/ml of fully sulfated unlabeled heparin, the inhibition was more than 95% (Fig. 4A); 50% inhibition was achieved at 0.22 \(\mu\)g/ml (not shown). These results suggest that N-, 2-O-, and 6-O-sulfate groups all participate in the heparin-PDGF-A_L interaction.

The ability of desulfated heparin derivatives to interact with PDGF-A_L was further examined by direct binding assays. For these studies, selectively 2-O-desulfated and preferentially 6-O-desulfated heparin decamers (containing terminal \([\(^3^H\)]\)a-Man_R units) were used, and their binding was compared to that of a fully sulfated heparin decamer. In the filter-trapping procedure, only 1–2% of each of the selectively desulfated decasaccharides were bound to the protein, when added at concentrations resulting in binding of 10–20% of the fully sulfated decamer (not shown). When the same preparations were applied to PDGF-A_L affinity chromatography, fully sulfated heparin decamer was eluted at \(\sim 0.7 \text{ M NaCl}\) while 2-O-desulfated and preferentially 6-O-desulfated heparin decamers were eluted at \(\sim 0.4\) and \(\sim 0.5 \text{ M NaCl}\), respectively (Fig. 4B). These direct binding experiments thus suggest, in accordance with the inhibition assays, that the interaction is sensitive to the selective removal of either 2-O- or 6-O-sulfate groups. It is noted that the 6-O-desulfated decamer bound slightly better to immobilized PDGF-A_L than the 2-O-desulfated decamer (Fig. 4B), in spite of a lower overall negative charge. The charge difference is due to the loss of about one-third of the 2-O-sulfate groups during the 6-O-desulfation procedure (see Table I). These findings thus suggest that whereas 2-O-sulfate and 6-O-sulfate groups are both required for the binding of PDGF-A_L, the contribution of the former substituent is larger.

**Disaccharide Composition of PDGF-A_L Bound and Unbound**

| Preparation | HexA-aMan_R | GlcUA(2-OSO_3)-aMan_R | GlcUA-aMan_R | IdxA-aMan_R | IdxA(2-OSO_3)-aMan_R | IdxA(2-OSO_3)-aMan_R |
|-------------|-------------|-----------------------|-------------|-------------|---------------------|---------------------|
| Native heparin | 3 | 1 | 6 | 6 | 7 | 77 |
| N-Desulfated, N-acetylated heparin | 1 | 5 | 18 | 4 | 10 | 62 |
| 2-O-Desulfated heparin | 11 | ND^b | 5 | 83 | 1 | ND |
| Preferentially 6-O-desulfated heparin | 30 | ND^b | 2 | 7 | 61 | ND |
| 2-O-DS decamers | 14 | ND^b | 4 | 80 | 2 | ND |
| Preferentially 6-O-DS decamers-A | 37 | ND^b | 1 | 5 | 55 | 2 |
| Preferentially 6-O-DS decamers-B | 40 | ND^b | 2 | 6 | 48 | 4 |

^a Amount of disaccharide as mole percent of total disaccharides. All of the preparations are derived from bovine lung heparin. The preparation of oligosaccharides and the various desulfation treatments are described under “Materials and Methods.” For disaccharide compositional analysis, samples were treated with nitrous acid at pH 1.5, or for N-desulfated/N-acetylated heparin, at pH 3.9 (before N-acetylation), and the products were reduced with NaB\(^3\)H\(_4\). Disaccharides were then isolated by gel filtration and separated by anion-exchange HPLC as described under “Materials and Methods.”

^b ND, not detected.
Oligosaccharides—$^{35}$S-Labeled, N-sulfated HS oligosaccharides were separated with regard to affinity for PDGF-$\alpha_1$, as described above (Fig. 3), and the disaccharide composition of the resultant bound and unbound hexa/octasaccharide fractions was analyzed. Disaccharides were obtained by deaminative cleavage and analyzed by anion-exchange HPLC. As shown in Fig. 5, the PDGF-$\alpha_1$-bound pool yielded a major peak corresponding to -IdceA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)- disaccharide units, whereas disaccharides derived from the unbound pool were practically devoid of this component. Both fractions contained -IdceA(2-OSO$_3$)-GlcNSO$_3$- units. Since data from other experiments indicate that both 2-O- and 6-O-sulfate groups contribute to binding, different interpretations of this result are conceivable. The -IdceA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)- disaccharide unit accounts for a major proportion of the total 6-O-sulfate groups in hASMC HS and thus could fulfill the requirement for such groups, whereas the 2-O-sulfate groups, also implicated in binding, might be located elsewhere in the binding site. Alternatively, the result may reflect a more specific need for the -IdceA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)- disaccharide unit. In an attempt to differentiate between these alternatives, we employed a heparin-derived, partially 2-O- and 6-O-desulfated decamer (Pref. 6-O-DS deca-B) in the filter-trapping assay. The disaccharide composition of this decamer (Table I) resembles that of PDGF-$\alpha_1$ binding N-sulfated HS oligosaccharides (Fig. 5A), but shows a lower proportion of the di-O-sulfated -IdceA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)- unit. This decamer was shown to bind in dose-dependent and saturable fashion to PDGF-$\alpha_1$ albeit much less efficiently than the fully sulfated decamer (Fig. 6). Only 1% of added Pref. 6-O-DS deca-B was bound to PDGF-$\alpha_1$, under experimental conditions resulting in 20% binding of the fully sulfated heparin decamer. On PDGF-$\alpha_1$ affinity chromatography, the Pref. 6-O-DS deca-B preparation was eluted with $-0.5$ M NaCl (not shown), while the fully sulfated heparin decamer appeared at $-0.7$ M NaCl (Fig. 4B). Pref. 6-O-DS deca-B was subjected to preparative affinity separation using the filter-trapping system and the PDGF-$\alpha_1$ bound and unbound pools were recovered. Anion-exchange HPLC of disaccharides obtained by deaminative cleavage of these pools, indicated that the bound fraction was highly enriched in the -IdceA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)- disaccharide unit (Fig. 7A). This trisulfated disaccharide sequence thus accounted for 13% of the O-sulfated disaccharide, and for at least half of the total disaccharide units (the exact proportion is somewhat uncertain, due to the occurrence of unidentified $^3$H-containing contaminants in the break-through fraction of the anion-exchange HPLC run; Fig. 7A). By contrast, the same structure accounted for $-5$% of the unbound fraction, the major O-sulfated disaccharide constituent being -IdceA(2-OSO$_3$)-GlcNSO$_3$- (Fig. 7B).
In the current study, we have elucidated some structural features in heparin/HS important for binding to PDGF-A<sub>L</sub>. The minimal heparin fragments capable of binding to PDGF-A<sub>L</sub> in the filter-trapping assay were identified as octasaccharides, whereas affinity chromatography on immobilized PDGF-A<sub>L</sub> suggested hexamers as the shortest binding fragments. The observed minimal lengths probably correspond to shorter (5–7-mer) oligosaccharides in native heparin/HS, assuming that the 2,5-anhydromannitol residues at the reducing termini of the saccharides used in this study do not contribute to the binding. The somewhat discrepant results regarding the minimal fragment length required for the interaction may conceivably be ascribed to inherent differences between the two techniques employed, as noted under “Results.” Alternatively, this finding might also reflect a difference in the heparin-binding requirements between monomeric (used in the affinity chromatography) and dimeric PDGF-A<sub>L</sub> (used in the filter-trapping assay). Notably, studies with low molecular weight heparin and PDGF-A<sub>L</sub> pointed to a high affinity interaction involving two heparin-binding sites in a dimeric PDGF-A<sub>L</sub> molecule (14).

Compositional analysis of PDGF-A<sub>L</sub>-bound and unbound HS-oligosaccharides, separated by affinity chromatography, revealed that only the bound pool contained significant amounts of -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) disaccharide units, whereas both pools contained -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>- units. The observed enrichment of -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) units could indicate a specific role for such units, but might also reflect the need for both 2-O- and 6-O-sulfate groups in a less strictly defined structural context. A more selective approach, employing a partially desulfated heparin decamer and the filter-trapping method resulted in a bound saccharide pool, which represented only 1% of the total saccharides added. The content of -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) disaccharide units was ~20-fold higher for the bound fraction than for the unbound one (>50% versus <5%) of all disaccharides, respectively; see Fig. 7). Since both -GlcUA/IdceA-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) units and -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>- units occurred in the unbound decamer, these data implicate the -IdceA(2-OSO<sub>3</sub>)-Glc-NSO<sub>3</sub>(6-OSO<sub>3</sub>) structure as such, rather than further separated 2-O- and 6-O-sulfate groups, in the binding. However, our data do not exclude the possibility that oligosaccharides substituted with 2-O- and 6-O-sulfate groups in other positions could also bind PDGF-A<sub>L</sub> presumptively with lower affinity. In fact, such HS oligosaccharides probably occurred in the PDGF-A<sub>L</sub>-bound pool, eluted as a broad peak at 0.25–0.7 M NaCl (Fig. 3A). Attempts to fractionate hASMC HS oligosaccharides by eluting the affinity column in a stepwise manner, or by using the filter-trapping procedure, yielded pools of higher apparent affinity, but in insufficient amounts for structural analysis.

Several heparin- and HS-binding proteins may be expressed by the same cell types or may colocalize in tissues. This applies to FGFs and PDGFs, that have been implicated in various aspects of embryonic development, in wound healing, as well as in the pathological cell proliferation and matrix production seen in diseases such as atherosclerosis and cancer. The minimal structures proposed in this study to be required for binding of heparin/HS to PDGF-A<sub>L</sub> differ from sequences reported to bind FGF-2 (18–20), in which IdceA(2-OSO<sub>3</sub>) units are essential, whereas 6-O-sulfate groups do not appear to contribute to binding. Such groups may, however, play a critical role in the suggested binding of HS to FGF receptors and in the subsequent receptor activation, attributed to a dodecasaccharide fragment containing both 2-O- and 6-O-sulfate groups (36). Thus PDGF-A<sub>L</sub> and the FGF-2/FGF receptor complex may be recognized by structurally overlapping HS sequences. HGF is another HS-binding growth factor which shares some functional properties with PDGFs. Two recent studies that have addressed the structural requirements in heparin/HS for HGF binding suggest the involvement of -GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) (21) or -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) (22) units within an ~8-mer saccharide sequence. Moreover, the enzyme lipoprotein lipase, occurring at the surface of vascular endothelia, is also recognized by -IdceA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>)-rich HS structures (37). It is obvious that the binding requirements for lipoprotein lipase, HGF, and PDGF-A<sub>L</sub>, can be effectively, albeit nonselectively, satisfied by heavily sulfated heparin-like sequences of sufficient length. While such sequences may indeed
represent the optimal binding region for lipoprotein lipase (37), they would appear to be “oversulfated” with regard to the minimal needs for HGF or PDGF-A\(_B\) binding. Instead, the minimal recognition domains for these proteins may be more selectively expressed in HS, which generally shows low abundance of -Idc\(\text{A}(2\text{-OSO}_3)\)GlcNS\(\text{O}_3\)(6\text{-OSO}_3)\) disaccharide units. Under such circumstances, the number and location of -Idc\(\text{A}(2\text{-OSO}_3)\)GlcNS\(\text{O}_3\)(6\text{-OSO}_3)\) units, as well as the sequences flanking such units, are likely to critically affect the binding of PDGF-AL to its signaling receptor. In fact, chlorate- treated many extracellular matrices, and it cannot be excluded that other sulfated GAGs than HS contribute to the retention of PDGF-A\(_B\) in matrix as well, albeit via low affinity interactions. Moreover, it is possible that highly sulfated members of the chondroitin/dermatan sulfate family, such as chondroitin sulfate E, show higher affinity toward PDGF-A\(_B\) than the preparations employed in the present study and in studies by other investigators (12–14).

The exon-6-encoded sequence in PDGF-A\(_B\) is highly conserved between mammalian species and Xenopus laevis (12, 42). Such basic sequences are also found in propeptide forms of PDGF-B as well as in vascular endothelial growth factor (43). Interestingly, the basic sequence found in vascular endothelial growth factor is also encoded by exon 6 and its occurrence in the polypeptide is regulated by alternative mRNA splicing (42). It is not known whether phenomena other than heparin/HS binding can be attributed to these polypeptide sequences. Furthermore, PDGF-A\(_B\) that lacks the exon-6-encoded sequence, also binds heparin, albeit with low affinity (13), suggesting that additional heparin/HS binding sites may be present in PDGF-A\(_B\) as well. Further studies are clearly needed to assess the heparin/HS binding properties of the various PDGF-isoforms and related proteins, and to elucidate the biological consequences of these interactions.

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