Minimal Sequence in Heparin/Heparan Sulfate Required for Binding of Basic Fibroblast Growth Factor*

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Experiments based on interaction in free solution between basic fibroblast growth factor (FGF-2) and saccharides related to heparin/heparan sulfate showed that the growth factor binds to heparin and to selectively glucosaminyl 6-O-desulfated heparin but poorly to iduronosyl 2-O-desulfated heparin. 2-O-sulfate groups thus are essential to the interaction, whereas 6-O-sulfates are not required nor do they interfere with FGF-2 binding. Comparison of various bound/nonbound oligosaccharides implicated a minimal pentasaccharide sequence for FGF-2 binding, with the structure: -hexuronic acid–glucosamine N-sulfate–hexuronic acid–glucosamine N-sulfate–iduronic acid 2-O-sulfate— (reducing terminus to the right). Such (overlapping) sequences are abundant in heparin, albeit heavily obscured by irrelevant O-sulfate groups, and occur also in heparan sulfate, with or without additional O-sulfates.

Most of the biological activities known to be associated with proteoglycans are due to interactions between the negatively charged glycosaminoglycan chains and various proteins (Jackson et al., 1991; Kjellen and Lindahl, 1991). Such interactions range from highly specific, "lock-and-key" type binding, as described for the antithrombin-binding region in heparin (Lindahl et al., 1984), to relatively nonspecific, co-operative electrostatic association. A group of proteins that has attracted particular interest in recent years is the heparin-binding growth factors (Burgess and Maciag, 1989; Klagsbrun, 1990), of which the best characterized members are the acidic and basic (FGF-2) fibroblast growth factors. Neither the mode of binding between these proteins end heparin or acidic heparan sulfate (HS) nor the functional implications of the interactions have been well understood.

Recent studies have indicated an important role for HS proteoglycans in mediating functional responses to fibroblast growth factors. HS proteoglycan binds FGF-2 at the cell surface and in the extracellular matrix, thus providing a storage form of the growth factor, which is relatively protected against proteolytic degradation (Saksela et al., 1988). The factor may be released in active form either by addition of heparin or through mobilization by enzymatic cleavage of the HS carrier (Naparstek et al., 1984; Ishai-Michaeli et al., 1990; Vlodavsky et al., 1991a). Furthermore, cells that are lacking HS proteoglycan or produce undersulfated HS are unable to respond to added FGF-2, unless supplemented with exogenous HS or heparin. It has been proposed that the polysaccharides induce a conformational change in FGF-2, which is a prerequisite to efficient binding of the growth factor to its high affinity receptor at the cell surface (Yayon et al., 1991; Rapraeger et al., 1991). The present study has been aimed at defining the minimal saccharide structure required for binding of heparin/HS to FGF-2.

**EXPERIMENTAL PROCEDURES**

**Polysaccharides**—Heparin from pig intestinal mucosa (stage 14, Inolix Pharmaceutical Division, Park Forest South, IL) was purified as described (Lindahl et al., 1965). Two samples of selectively O-desulfated heparin were given by A. Naggi and G. Grazioli, Institute G. Ronzoni, Milan, Italy. One sample was generated by preferential 6-O-desulfation (treatment with dimethyl sulfoxide, 10% water at 110°C for 5 h) (Nagasawa et al., 1977) along with N-desulfation of the starting material, followed by re-N-sulfation; compositional analysis of the product (see below) indicated non-O-sulfated –HexA–GlcN–X (39%) and –IdoA(2-O-SO3)–GlcN–X (46%) (X = (N-sulfate or (N-acetyl) as the predominant disaccharide units (Fig. 5A)). Because ~70% of the disaccharide units of the unmodified heparin would contain IdoA(2-O-SO3) residues (Kutsche et al., 1990), about one-third of the 2-O-sulfate groups must have been lost along with the 6-O-sulfates. The other sample was obtained by selective 2-O-desulfation under alkaline conditions (Jaseja et al., 1998) and contained 21% non-O-sulfated –HexA–GlcN–X and ~70% –IdoA–GlcN–(6-O-SO3)–disaccharide units (Fig. 5B).

A low sulfated HS preparation (~0.5 sulfate group/disaccharide unit), isolated from human aorta (Iverius, 1971), was provided by W. Murphy, University of Monash, Melbourne, Australia. HS from bovine kidney was given by K. Sugahara, Women’s College of Pharmacy, Kobe, Japan. The preparations of chondroitin sulfate (bovine cartilage) and dermanatan sulfate (pig intestinal mucosa) were as described (Lyckavek et al., 1991).

**Oligosaccharides**—Even numbered heparin oligosaccharides were generated by partial deaminative cleavage of the polysaccharide with nitric acid (pH 1.5; cleavage at N-sulfated GlcN units) (Shively and Conrad, 1976a), essentially as described (Pejler et al., 1988), and the resulting 2,5-anhydro-D-mannose residues were reduced with NaBH4 (The Radiochemical Center, Amersham, U.K.). The labeled oligosaccharides (~0.6 × 10⁶ dpm 3H/nmol) were separated by repeated gel chromatography on Sephadex G-50 (superfine, 1 × 190 cm column, eluted with 11 mM NaCl) into even numbered species, from 4- to 14- saccharides (designated H-4 to H-14), homogeneous with respect to molecular size (Lane et al., 1984); in addition, a labeled fraction estimated to contain largely 20- to 24-saccharides was recovered. The selectively 6-O-desulfated heparin was treated in a similar way to yield 4- to 12-saccharides (~2 × 10⁶ dpm 3H/nmol) and a fraction of larger oligosaccharides. The isolated and desalted oligosaccharides were subjected to mild acid treatment (25 mM H2SO4, 80°C, 30 min),
were concentrated and desalted by passage through columns (PD-10; 2.5 Sigma units) of heparinase I (Sigma) from Flavobacterium heparinum in 200 \mu l of 5 mM phosphate buffer, 200 mM NaCl, 0.5 mg/ml bovine serum albumin, pH 7.0. After 10 min at 30 °C the digest was heated at 100 °C for 1 min and centrifuged, and the supernatant was fractionated by gel chromatography on Sephadex G-50, as indicated above. The hexa- and octasaccharide fractions, indicated by distinct peaks corresponding to 16 and 14%, respectively, of the \(^3H\) applied to the column, were recovered and desalted by passage through PD-10 columns (Pharmacia). The nonreducing terminal, 4,5-unsubstituted hexuronic acid units were eliminated by treatment with 40 \mu l (final volume) of 10 \mu M mercuric acetate in 130 mM sodium acetate, pH 5.0, for 30 min at room temperature (Ludwigs et al., 1987; Kusche et al., 1988). The samples were adjusted to 0.5 ml with 5 mM NaCl and then were desalted by successive passage through PD-10 columns equilibrated with 1 M NaCl and water; the high salt separation step was found to be necessary to prevent removal of mercuric ions from the samples. The predicted pentas- and heptasaccharide structures of the products (H-5 and H-7) were confirmed by analytical gel chromatography on Sephadex G-50, against even numbered oligosaccharide standards (data not shown).

Hexa- and octasaccharides (HS-4 to HS-14) from HS (human aorta) followed a different strategy, involving cleavage at sites occupied by N-acetylated GlcN units. A sample (5 mg) of HS was deacetylated (Guo and Conrad, 1989) by treatment with 1 ml of hydrazine, containing 30% (v/v) water and 1% (w/v) hydrazine sulfate, at 100 °C for 4 h. After repeated evaporation in the presence of triethylamine the mixture was passed through Sephadex G-15, lyophilized, and deaminated with nitric acid (pH 3.9; cleavage at N-unsubstituted GlcN units; Shively and Conrad, 1976a). The resulting oligosaccharides were radiolabeled by reduction with NaB\(_3\)H\(_4\) and separated by gel chromatography as described in the legend to Fig. 2. In addition, oligosaccharides, resolated after mild acid treatment as described above, had a specific radioactivity of \(\approx 0.6 \times 10^5 \text{ dpm} \text{H}^{3}\)/nmol. Octasaccharide was converted into heptasaccharide (HS-7) by digestion with \(\beta\)-d-glucuronidase as described in the legend to Table II.

Molar concentrations of oligosaccharides were determined on the basis of hexuronic acid contents, as measured by the carbazole reaction (Bitter and Muir, 1962).

Interaction between Saccharides and FGF-2—Human recombinant FGF-2, purified from yeast cells (Zymogenetics, Inc.) as described by Olwin and Haaschka (1989), was given by Dr. B.B. Olwin (University of Wisconsin-Madison, Madison, Wisconsin). For analytical scale experiments FGF-2 was incubated at room temperature for 2 h with the appropriate saccharide samples (see the text) in 300 \mu l (unless otherwise stated) of 50 mM Tris-HCl, pH 7.4, containing NaCl at various concentrations and 0.5 mg of bovine serum albumin/ml. The growth factor, along with any bound carbohydrate, was recovered by quick passage of the samples through nitrocellulose filters (Sartorius, pore size 0.45 \mu m; 25 mm diameter), which had been placed onto a 10-well vacuum-assisted manifold filtration apparatus. The filters were prewashed with 2 x 5 ml of 50 mM Tris-HCl, 130 mM NaCl, pH 7.4, before application of the samples, which were immediately followed by another 2 x 5 ml of the same buffer; each washing step was completed within 5 sec. Protein-bound radioactivity was determined after submersion of the filters in 2 ml of 3 M NaCl for 30 min; 1 ml of the eluate was mixed with 0.5 ml of H\(_2\)O and 3.5 ml of OptiPhase scintillation mixture (Pharmacia LKB Biotechnology Inc.) and counted in a Beckman LS 6000C scintillation spectrometer. No residual radioactivity could be detected on the filters.

In preparative experiments, incubation mixtures of various volumes were passed through larger filters (Sartorius; 38 mm diameter); the number of filters used for each incubation was adjusted so that the amount of bovine serum albumin (0.3-0.5 mg/mg oligosaccharide mixture) never exceeded 100 \mu g/cm\(^2\) of filter. Nonbound material was recovered after washing (2 x 20 ml) as described above. Protein-bound saccharides, eluted from the filters with 3 ml of 3 M NaCl, were concentrated and desalted by passage through columns (PD-10; Pharmacia LKB Biotechnology G-25).

Analytical Methods—Compositional analysis of heparin or HS oligosaccharides involved degradation of samples with nitric acid (pH 1.5) followed by reduction of the resulting di- and tetrasaccharides with NaB\(_3\)H\(_4\). The procedure of Kusche et al. (1990) was scaled down so that 20-100 \times 10\(^3\) dpm of end group-labeled oligosaccharide (\(\approx 30-170\) pmol, corresponding to \(\approx 80-400\) ng of a heparin-derived octasaccharide) was deaminated and reduced with 25-50 \mu M NaB\(_3\)H\(_4\). The resulting hexa-[\(^3H\)]Man\(_8\) disaccharide fractions were isolated and analyzed further by anion-exchange HPLC (Bienkowski and Conrad, 1985), as described in more detail in the legend to Fig. 5. Due to the poor separation of the non-O-sulfated disaccharides in this procedure, GlcA-[\(^3H\)]Man\(_8\) and IdoA-[\(^3H\)]Man\(_8\) were resolved by descending paper chromatography (ethyl acetate/acetic acid/H\(_2\)O, 5:3:1) and quantified by liquid scintillation counting of the paper strips. The proportion of labeled hexa-[\(^3H\)]GlcNac-Glc[\(^1\]H] Man\(_8\) tetrasaccharides in relation to total disaccharides was determined by gel chromatography (Sephadex G-15) following mild acid treatment as described above. Di- and tetrasaccharide sequences occupying the reducing terminal position were identified in separate experiments, utilizing the \(^3H\) label introduced upon initial labeling of the 2,5-anhydromannose end group in the intact oligosaccharide. For compositional analysis of full sized glycosaminoglycans (aorta HS and partially O-desulfated heparins) the compounds were N-deacetylated by hydrazinolysis (4 h) as described above and then subjected to deamination first at pH 1.5 (cleavage at N-sulfated GlcN units) and then at pH 3.9 (cleavage at N-unsubstituted GlcN units) according to Riesenfeld et al. (1982), followed by reduction of the disaccharide products with NaB\(_3\)H\(_4\).

High-voltage paper electrophoresis (40 V/cm) was conducted on Whatman No. 3MM paper in 1.6 M formic acid (pH 1.7). Additional separation methods (gel chromatography and anion-exchange HPLC of oligosaccharides) are described in the legends to the figures.

RESULTS

Binding to FGF-2 of \(^3H\)Heparin along with Unlabeled Glycosaminoglycans—The ability of selected polysaccharide preparations to displace \(^3H\)-labeled heparin from FGF-2 was investigated, using a nitrocellulose filter disc procedure (see “Experimental Procedures”). The labeled ligand consisted of fragments >18 monosaccharide units, recovered after partial deaminative cleavage of pig mucosal heparin and reduction of the products with NaB\(_3\)H\(_4\). Binding of this fragment to FGF-2 was efficiently precluded in the presence of intact, unlabeled heparin, 50% inhibition of binding occurring at a heparin concentration of \(\approx 0.2 \mu g/ml\) (30 ng added per 150 \mu l of incubation mixture) (Fig. 1). The displacing ability of selectively 6-O-desulfated heparin was only slightly lower than that of the intact, unlabeled parent heparin.

![Fig. 1. Competitive binding of \(^3H\)-labeled heparin and unlabeled polysaccharides to FGF-2. incubation mixtures containing 150 \mu l of 50 mM Tris-HCl (pH 7.4), 130 mM NaCl, 0.42 \mu g of FGF-2 (160 nM), 15 \times 10^5 dpm of \(^3H\)-labeled heparin fragments (\(\approx 20\) to 24-saccharides; \(\approx 170\) nm), and various amounts of unlabeled polysaccharides.](image)
that of the intact polysaccharide, whereas 2-O-desulfated heparin was about two orders of magnitude less potent. This finding suggests that the IdoA 2-O-sulfate group (see Table I for abbreviations) is important to the interaction with FGF-2, whereas the GlcN 6-O-sulfate group is relatively unimportant. The two preparations of HS tested, from human aorta and bovine kidney, showed displacing activity, albeit appreciably lower than those of native or 6-O-desulfated heparin (Fig. 1). Chondroitin and dermatan sulfate were inactive. Binding to FGF-2 of 1H-labeled HS Oligosaccharides—Heparan sulfate from human aorta was N-desacetylated by hydrazinolysis, and the product was degraded by treatment with nitric acid at pH 3.9, followed by reduction with NaB\textsubscript{3}H\textsubscript{4} (see “Experimental Procedures”). Under these conditions N-unsubstituted GlcN units are deaminated, along with cleavage of the glucosaminidic linkages, whereas N-sulfated GlcN units remain intact (Shively and Conrad, 1976a). The expected labeled products were GlcA-[1-\textsuperscript{3}H]aMan\textsubscript{R} disaccharides (derived from (–GlcNAc)–(GlcA–GlcNAc)\textsubscript{R} sequences) and GlcA-(GlcNSO\textsubscript{3})-HexA\textsubscript{R} disaccharides (derived from (–GlcNAc)–(GlcA–GlcNAc)\textsubscript{R}–GlcNAc sequences). The HexA units in the nonreducing terminal position of di- and oligo-saccharides had been bound (at C4) to N-acetylated GlcN units in the intact polysaccharide and would therefore retain D-gluc0 configuration (Jacobson et al., 1984). Gel chromatography of these components afforded a predominant disaccharide fraction preceded by smaller peaks of 4- to 12-saccharides, the size of each peak decreasing with increasing M\textsubscript{o} of the oligosaccharide (Fig. 2). Assigning each purified oligosaccharide pool (Fig. 2, insert) for FGF-2 binding showed increased proportions of protein-bound label with increasing M\textsubscript{o} of the oligosaccharide and with decreasing ionic strength of the incubation medium, the smallest species showing appreciable binding being octasaccharides (Fig. 3A). Preparative affinity partition of the labeled octasaccharide fraction was conducted at physiological ionic strength (130 mM NaCl) and a molar FGF-2/octasaccharide ratio of 4/1, as described in the legend to Fig. 4. The resulting FGF-2-bound fraction amounted to 14% of the added radioactivity. Repeated challenge of nonbound octasaccharides with FGF-2 failed to produce any significant additional protein-bound labeled saccharide, indicating that the pool of FGF-2-binding species had been depleted (see the legend to Fig. 4).

The fractions of FGF-2-bound and -nonbound octasaccharides were separated further by anion-exchange HPLC on Mono-Q. Components bound to FGF-2 were preferentially derived from the more sulfated subfractions of the total octasaccharides (Fig. 4A). Fractions of FGF-2-bound (B1-B5) and -nonbound (NB1-NB5) HS octasaccharide, separated by anion-exchange HPLC (Fig. 4A), were subjected to compositional analysis based on deamination with nitric acid, reduction with NaB\textsubscript{3}H\textsubscript{4} and identification of the resulting HexA-[1-\textsuperscript{3}H]aMan\textsubscript{R} disaccharides (Fig. 5 and Table I). While 14% of the disaccharides derived from the aorta HS starting

### Table I

**Compositional analysis of heparin/heparan sulfate oligosaccharides**

| Oligosaccharide fraction | Tetrasaccharides | Disaccharides |
|--------------------------|------------------|---------------|
|                          | GlcA-aMan\textsubscript{R} | [idoA-aMan\textsubscript{R} (2-O-SO\textsubscript{3})-aMan\textsubscript{R}] | GlcA(2-O-SO\textsubscript{3})-aMan\textsubscript{R} | idoA-aMan\textsubscript{R} (6-O-SO\textsubscript{3})-aMan\textsubscript{R} | idoA(2-O-SO\textsubscript{3})-aMan\textsubscript{R} | idoA(2-O-SO\textsubscript{3})-aMan\textsubscript{R} |
| Heparan sulfate octasaccharides |                  |               |
| Unfractionated            | 0.90 (0.30) | [2.34 (0.56)] | ND (ND) | 0.14 (0.02) | 0.25 (0.09) | 0.39 (0.03) | 0.01 (ND) |
| FGF-2-bound fraction      |                  |               |
| B1                       | 0.14 (0.14) | 1.34 (0.99) | 0.76 (0.43) | 0.13 (ND) | 0.03 (ND) | 0.21 (0.15) | 1.36 (0.19) | 0.02 (ND) |
| B2                       | 0.03 (0.03) | 1.53 (0.25) | 0.96 (0.60) | 0.08 (ND) | 0.08 (0.03) | 0.12 (0.07) | 1.02 (0.02) | 0.17 (ND) |
| B3                       | 0.13 (0.13) | 1.63 (0.22) | 0.54 (0.33) | 0.04 (ND) | 0.12 (0.03) | 0.28 (0.16) | 1.17 (0.13) | 0.10 (ND) |
| B4                       | 0.11 (0.11) | 1.50 (0.10) | 0.59 (0.40) | ND (ND) | 0.29 (0.06) | 0.32 (0.29) | 1.06 (0.04) | 0.14 (ND) |
| B5                       | 0.11 (0.11) | [1.69 (0.40)] | ND (ND) | 0.38 (0.10) | 0.51 (0.33) | 0.93 (0.06) | 0.39 (ND) |
| FGF-2-nonbound fraction  |                  |               |
| B1                       | 0.48 (ND) | [3.52] | ND (ND) | ND (ND) | ND (ND) | ND (ND) |
| NB2                      | 1.27 (0.46) | [2.45 (0.48)] | ND (ND) | ND (ND) | 0.03 (0.03) | 0.25 (0.03) | ND (ND) |
| NB3                      | 0.87 (0.21) | [2.87 (0.79)] | ND (ND) | ND (ND) | 0.02 (ND) | 0.24 (ND) | ND (ND) |
| NB4                      | 0.69 (0.21) | 1.68 (0.20) | 0.74 (0.42) | ND (ND) | 0.07 (0.03) | 0.14 (0.06) | 0.71 (0.03) | ND (ND) |
| NB5                      | 0.50 (0.20) | 1.58 (0.17) | 0.51 (0.28) | ND (ND) | 0.24 (0.07) | 0.44 (0.25) | 0.55 (0.03) | 0.21 (ND) |
| Heparin sulfate hexa-     |                |                   | | | | | |
| saccharide               |                  |               |
| Heparin hexasaccharide    |                  |               |
| 6-O-Desulfated heparin    |                  |               |
| octasaccharide            |                  |               |
| FGF-2-bound fraction      |                  |               |
| B1                       | 0.56 | [2.05] | ND (ND) | ND (ND) | 1.22 | 0.17 |
| B5                       | ND (1.24) | 0.25 | 0.27 | 1.53 | 0.73 |
| FGF-2-nonbound fraction  |                  |               |
| NB1                      | 1.56 | [1.94] | ND (ND) | ND (ND) | 0.41 | 0.09 |
| NB4                      | 0.80 | [2.05] | ND (ND) | 0.24 | 0.26 | 0.53 | 0.12 |

* Moles of disaccharide units/mol of oligosaccharide. Values in parentheses indicate the fractions of the respective HexA-aMan\textsubscript{R} disaccharide or HexA-GlcNAc-GlcA-aMan\textsubscript{R} tetrasaccharide sequences that occupy reducing terminal position in the corresponding intact oligosaccharides. The disaccharides were quantified after separation by anion-exchange HPLC or paper chromatography. Abbreviations: HexA, unspecified hexuronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine); aMan\textsubscript{R}, 2-acetamido-2-deoxy-D-glucose.
FIG. 2. Preparation of oligosaccharides from aorta HS. Human aorta HS was N-deacetylated, cleaved by treatment with nitrous acid (pH 3.9), and reduced with NaBH₄ as described under "Experimental Procedures." The resulting labeled oligosaccharides were fractionated on a column (1 × 150 cm; Vₑ, 40 ml; Vₘ, 100 ml) of Bio-Gel P-10, eluted with 1 M NaCl at a rate of 1 ml/h. Eluent fractions were analyzed for radioactivity. The number of monosaccharide units/oligosaccharide is indicated above each peak. The insert shows a blow-up of the part of the chromatogram corresponding to the larger oligosaccharides. These were pooled as indicated by the vertical lines; each pool was concentrated and rerun through the same column to obtain size-homogeneous oligosaccharides. The final fractions were desalted by passage through Sephadex G-15.

FIG. 3. Binding of HS/heparin oligosaccharides to FGF-2. Labeled oligosaccharides were incubated with 5 μg of FGF-2 (930 nM) under standard conditions (final volume, 300 μl) in the presence of 130 mM (●), 250 mM (○), 500 mM (■), or 1 M (□) NaCl. The oligosaccharides were derived from (A) aorta HS (10 × 10⁶ dpm ³H; ~60 nM); (B) heparin (10 × 10⁶ dpm; ~60 nM); (C) 6-0-desulfated heparin (30 × 10⁶ dpm; ~56 nM). The amounts of ³H associated with FGF-2 were determined by the nitrocellulose filtration procedure described in "Experimental Procedures." The values given are the means of duplicate assays and are expressed as per cent of the radioactivity added to the incubations.

FIG. 4. Anion exchange chromatography of octasaccharides separated with regard to affinity for FGF-2. A, octasaccharides (8 × 10⁶ dpm ³H; ~440 nM) derived from aorta HS were incubated with FGF-2 (1.0 mg; 1.9 μM) under standard conditions (130 mM NaCl) in a final volume of 30 ml. After separation with regard to affinity for FGF-2 (see "Experimental Procedures") the resulting bound (●, 14% of added radioactivity) and nonbound (○, 86%) fractions were desalted and separately applied to a Mono Q column (HR 5/5; Pharmacia), equilibrated with 50 mM sodium acetate, pH 4.0, and 0.1 M NaCl. The column was eluted at a flow rate of 0.5 ml/min, using a 80-ml linear gradient extending from 0.1-6 M NaCl (indicated by the dashed line) in the same buffer. Effluent fractions of 1.5 ml were analyzed for radioactivity; the chromatograms presented are normalized to equal amounts of radioactivity. Fractions were combined as indicated and desalted by gel chromatography. The depletion of FGF-2-binding species from fractions NB4 and NB5 (not bound to FGF-2) was ascertained in analytical incubations with the growth factor, at a FGF-2/octasaccharide molar ratio of ~32/1 (i.e. 8-fold higher than that employed for the preparative affinity separation). The resulting FGF-2-bound saccharides derived from either sample amounted to <1% of the added radioactivity. B, octasaccharides (3.2 × 10⁶ dpm ³H; ~530 nM) derived from 6-0-desulfated heparin were incubated with FGF-2 (50 μg; 930 nM) under standard conditions (130 mM NaCl) in a final volume of 30 ml and separated into FGF-2-bound (●, 13% of added radioactivity) and nonbound (○, 87%) fractions. Two successive reincubations of nonbound fraction with similar additions of FGF-2 removed another 3.8 and 1.1% of protein-bound radioactivity. The chromatograms shown refer to octasaccharides initially bound to FGF-2 and to those remaining in the final nonbound fraction after the 3 consecutive incubations. The HPLC was performed as described under A, except for the slightly different salt gradient (from 0.2 to 0.7 M NaCl).

material appeared as mono-O-sulfated IdoA(2-OSO₃)-aMan₂ (most of the disaccharides being nonsulfated) (see Fig. 5C), the various FGF-2-bound HS octasaccharide fractions gave higher yields of IdoA(2-OSO₃)-aMan₃ (see analysis of fraction B2 in Fig. 5D) in the range from 24 to 34% of total disaccharides. In fact, the parent native IdoA(2-OSO₃)-GlcNSO₃-structure is the only O-sulfated disaccharide constituent approaching or exceeding 1 mol/mol of each FGF-2-bound octasaccharide fraction (a value of 1.0 in Table I corresponds to 25% of the total disaccharide units). Other O-sulfated disaccharide units generally increased in quantity in fractions of increasing charge density but were consistently present in less than, equimolar amounts. The IdoA(2-OSO₃)-GlcNSO₃-sequence was the most abundant O-sulfated structure also in the various fractions of FGF-2-nonbound octasaccharide, amounting to at most 0.7 mol/octasaccharide (fraction NB4).
occur in a structural context not conducive to FGF-2 binding. FGF-2-binding species (see legend to Fig. 4), these units must have been derived predominantly from one of the two internal disaccharide units.

Experiments were therefore undertaken to locate the IdoA(2-OSO3) residue. In the latter fractions, depleted of B2; NB4, NB5; Fig. 4A), these GlcNAc units were not restricted to a single position within the octasaccharides.

Hydrazinolysis; these GlcNAc units were not restricted to a single position within the octasaccharides. Identifications of the reducing terminal disaccharide units, using the 3H label initially introduced upon reduction of the intact octasaccharides (see "Experimental Procedures"), showed a variety of sulfated and nonsulfated structures but minor proportions only of IdoA(2-OSO3) residues; this finding applied to FGF-2-bound and -nonbound octasaccharide fractions alike (Table I). The nonreducing terminal disaccharide unit would be predicted to contain GlcA but no IdoA(2-OSO3) units (Jacobsson et al., 1984), as verified by digestion with β-D-glucuronidase (see the generation of HS-7 in Table II). The IdoA(2-OSO3)-aMan8 disaccharide must thus have been derived predominantly from one of the two internal disaccharide units.

To distinguish between the two alternative positions, two fractions (B2 and B4) of FGF-2-bound and two fractions (NB4 and NB5) of FGF-2-nonbound octasaccharide were digested with heparinase I from F. heparinum (see legend to Fig. 6), and the products were analyzed by gel chromatography. Both fractions B2 and B4 were partially (37 and 33%, respectively) cleaved to labeled tetrasaccharide (Fig. 6A), whereas fractions NB4 and NB5 were not affected to any significant extent (Fig. 6B). In a separate experiment with 5 times more concentrated enzyme, complete digestion fraction B2 yielded 67% tetrasaccharide (data not shown). Because the bacterial heparinase I cleaves glucosaminic bonds in -GlcNS03-, IdoA(2-OSO3)-sequences (Linhardt et al., 1990), these results indicate that at least two-thirds of the FGF-2-bound fractions have a basic GlcA-GlcNS03-HexA-GlcNS03-IdoA(2-OSO3)-GlcNS03-HexA-aMan4n structure (HS-8 (+) in Fig. 7). In FGF-2-nonbound fractions the IdoA(2-OSO3) residue would be shifted one disaccharide unit toward the nonreducing terminus (HS-8 (-) in Fig. 7), apparently inaccessible to the enzyme. In accord with these proposals, about half of the molecules in the HS hexasaccharide fraction, virtually unable to bind FGF-2 (Fig. 4A), were found to contain an IdoA(2-OSO3) residue that was located almost exclusively within the single internal disaccharide unit (Table I and Fig. 7). The extension of the binding region was further defined by the finding that digestion of the FGF-2-binding HS-8 subspecies with β-D-glucuronidase (yielding HS-7) essentially abolished the binding (Table II, Fig. 7). The nonreducing terminal GlcA-

![FIG. 5. Anion-exchange HPLC of sulfated and nonsulfated HS-5, 23902](https://example.com/fig5.png)

Table II: Even and odd numbered oligosaccharides in FGF-2 binding

| Oligosaccharide* | FGF-2 binding* |
|-----------------|----------------|
|                 | % of added     |
| HS-8            | 61             |
| HS-7            | 42             |
| H-8             | 53             |
| H-7             | 40             |
| H-6             | 33             |
| H-5             | 12             |
| H-4             | 1              |

*The preparation of oligosaccharides is described under "Experimental Procedures." The HS-8 preparation used in the experiment had been recovered after previous binding to FGF-2, but had not been fractionated by anion-exchange chromatography. To generate HS-7 this material was incubated with 0.2 mg of bovine liver β-D-glucuronidase (Sigma) and 1 mg of bovine serum albumin in 0.5 ml of 50 mM sodium acetate, pH 5.0. After ~13 h of incubation at 37°C another 0.1 mg of enzyme was added. Incubation was continued for a total of 18 h. The mixture was heated at 100°C for 2 min and analyzed by gel chromatography on Sephadex G-50 (see "Experimental Procedures"), which showed a single peak with an elution position intermediate to those of HS-8 and HS-6 (data not shown). This material was desalted by passage through Sephadex G-15 before further analysis.*

2 Analytical scale control experiments indicated that the N-acetyl groups were quantitatively eliminated following more extensive hydrazinolysis (data not shown).
Binding Site for FGF-2 in Heparin/Heparan Sulfate

Binding of Heparin-derived Oligosaccharides to FGF-2—Pig mucosal heparin was depolymerized by limited deamination

of N-sulfated GlcN units. The oligosaccharides formed were end group labeled by reduction with NaB₃H₄, separated by gel chromatography, and tested for the ability to bind FGF-2. The degree of binding was found to increase with increasing molecular size of the oligosaccharides, approaching a level of 70% binding for large fragments (~20- to 24-saccharides) (Fig. 3B). No significant proportion of added tetrasaccharides but as much as 30% of the hexasaccharide fraction bound to the growth factor at physiological ionic strength. Exhaustive deamination converted the bound hexasaccharide almost exclusively into the di-O-sulfated disaccharide IdoA(2-OSO₄)₆-aManR(6-OSO₄)(5) (Fig. 5E and Table I). Odd numbered heparin oligosaccharides were generated through a combination of limited deaminative cleavage, limited lyase digestion, and elimination of nonreducing terminal, 4,5-unsaturated hexuronic acid units by treatment with mercuric acetate (see “Experimental Procedures”). Testing a series of even and odd numbered heparin oligosaccharides showed that the conspicuous loss of FGF-2 binding, going from H-6 to H-4, was largely manifest already at the pentasaccharide (H-5) stage (Table II; see Fig. 7 for structures).

Partially O-desulfated heparin, essentially depleted of 6-O-sulfate groups but retaining ~two-thirds of the 2-O-sulfate groups of the native compound (see “Experimental Procedures”), was depolymerized in a similar manner. The smallest molecules showing significant binding to FGF-2, albeit at a relatively low level, were again hexasaccharides (Fig. 3C). The octasaccharide fraction (13% binding to FGF-2 at physiological ion strength) was separated by preparative affinity partition on nitrocellulose discs, and the resulting FGF-2-bound and -nonbound fractions were subjected to anion-exchange HPLC. The elution profiles (Fig. 4B) were essentially similar to those of the corresponding fractions derived from aorta HS octasaccharide (Fig. 4A). Also the compositional analysis of the resulting subfractions gave results strikingly similar to those obtained with the corresponding HS octasaccharide fractions (Table I). The least anionic FGF-2-bound fraction (B1) thus contained ~2 non-O-sulfated, N-

![Fig. 6. Heparinase digestion of HS octasaccharides. A, components B2 (●) and B4 (○) of the FGF-2-bound fraction; B, components NB4 (■) and NB5 (□) of the FGF-2-nonbound fraction. Samples (30 x 10⁶ dpm ³H; ~0.05 nmol) of each octasaccharide fraction were incubated at 30 °C in 30 µl of 5 mM phosphate, 200 mM NaCl, pH 7.0, with ~3 mIU (1.9 Sigma units) of heparinase I (Sigma); similar additions of enzyme in 30 µl of buffer were repeated after 24 and 48 h of incubation. After a total incubation period of 72 h the reactions were terminated by heating at 100 °C for 1 min, and the samples were applied, along with 200 µg of unlabeled carrier oligosaccharides (heparin deamination products), to a column (1 X 190 cm) of Sephadex G-50 (Vo, 60 ml; V, 150 ml) equilibrated with 1 M NaCl. Effluent fractions of ~1 ml were collected at a rate of 3 ml/h and analyzed for radioactivity. The numbers above the peaks refer to the number of monosaccharide units/molecule.

![Fig. 7. FGF-2-binding properties of heparin (H) and heparan sulfate (HS) oligosaccharides. The structures have been aligned with regard to the single IdoA(2-OSO₄) unit (within the solid box) that is postulated to be an essential component of the FGF-2 binding pentasaccharide sequence (within the dashed boxes). For additional information see the text.]
sulfated disaccharide units, and 1.2 \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) unit/octasaccharide molecule.

**DISCUSSION**

The binding of heparin and heparin-derived oligosaccharides to FGF-2 (Bashkin et al., 1989; Ishii-Michaeli et al., 1992) presumably reflects the ability of HS, in the extracellular matrix and at cell surfaces, to provide storage of the growth factor and promote the interaction of the factor with its high affinity receptor (Vlodavsky et al., 1991a, 1991b; Yayon et al., 1991; Rapraeger et al., 1991). FGF-2-binding structures within HS molecules were recently defined through affinity-chromatographic separation, of oligosaccharides generated by heparitinase digestion of HS. Characterization of FGF-2-bound and -nonbound oligosaccharides implicated the \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) sequence in growth factor binding, and it was suggested that such sequences may be clustered to form a specific binding region. Turnbull et al. (1992) proposed a FGF-2 binding tetradecasaccharide containing 5 consecutive \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) disaccharide units, whereas Hrabuch et al. (1992) favored 3 such units.

In the present study we attempted to define the minimal structure in heparin or HS that is required for binding of FGF-2. Heparin is generally considered to bind FGF-2 at least as strongly as any HS preparation. Partial, selective O-desulfation of heparin, involving essentially complete elimination of GlcN 6-O-sulfate groups but only limited release of IdoA 2-O-sulfate groups, yielded a product that bound FGF-2 almost as avidly as the unmodified heparin (Fig. 1). This product, as expected, contains a high proportion (46% of the total disaccharide units) of the N-sulfated, mono-O-sulfated \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) disaccharide sequence implicated in FGF-2 binding to HS, in accord with the proposal that the IdoA 2-O-sulfate group is important to this interaction. Conversely, the GlcN 6-O-sulfate group does not seem to contribute to, or interfere with, FGF-2 binding.

The smallest possible, least sulfated HS oligosaccharides capable of FGF-2 binding were octasaccharides, which contained essentially all N-sulfated GlcN units, a single IdoA 2-O-sulfate residue, but generally less than one GlcN 6-O-sulfate group/molecule. Remarkably, other octasaccharides of similar overall composition were unable to bind the growth factor. The difference in binding properties could be rationalized in terms of the position of the IdoA (2-OSO_3) unit, as illustrated in Fig. 7. Alignment of the two HS octasaccharide sequences with regard to this particular unit shows a nonreducing terminal disaccharide extension in the FGF-2 binding octasaccharide (HS-8) that is lacking in the nonbinding species (HS-8 (-)), hence is conceivably part of the binding region. The actual extent of the binding region was defined through the FGF-2-binding properties of a number of related oligosaccharides derived from heparin or HS (Fig. 7). In particular, (i) the heavily O-sulfated heparin hexasaccharide (H-6) bound FGF-2 whereas the pentasaccharide derivative (H-5) showed greatly reduced and the homologous tetrasaccharide (H-4) no binding, (ii) a HS hexasaccharide containing a single, internal IdoA (2-OSO_3) unit did not appreciably bind the growth factor, and (iii) digestion of HS octasaccharide with \(\beta\)-glucuronidase abolished FGF-2 binding. Together, these results implicate the pentasaccharide sequence (surrounded by dashed boxes in Fig. 7). \(-\text{HexA}-\text{GlcNSO}_3-\text{HexA}-\text{GlcNSO}_3-\text{IdoA}(2-\text{OSO}_3)-\). The nonreducing terminal HexA unit may apparently be either GlcNA (as in the FGF-2-bound HS-octasaccharide) or IdoA (in the heparin hexasaccharide), and the data available are compatible with similar variability also for the internal HexA unit. Judging from the structural characteristics of the FGF-2 binding HS-8 fractions (see "Results"), the two GlcN units within the binding region are invariably N-sulfated. A role in FGF-2 binding for the remaining N-sulfated GlcN unit (outside the dashed box of HS-8 (+) in Fig. 7) appears unlikely, in view of the markedly different structural properties of the corresponding (terminal \(\alpha\text{Man}(6\text{-OSO}_3)\)) unit in the (FGF-2 binding) hexasaccharide, H-6. O-Sulfate groups other than the single IdoA 2-O-sulfate residue at the reducing end of the pentasaccharide sequence do not appear to be essential, although our results do not exclude that some of them may contribute to FGF-2 binding. In fact, model building (not shown) suggested that the nonreducing terminal 6-O-sulfate group of the pentasaccharide H-5 (Fig. 7) could be sterically accommodated to substitute for the negatively charged terminal carboxyl group of the intact FGF-2-binding sequence, in accord with the residual binding ability displayed by this compound.

In view of the relatively modest activity of the aorta HS in the competitive binding assay (Fig. 1), it might be argued that the pentasaccharide sequence represents only part of the actual binding region and that efficient binding of heparin or HS to FGF-2 would in fact require a more extensive clustering of consecutive \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) units. However, the apparent affinity of a polysaccharide preparation in such an assay will depend not only on the strength of the interaction at an isolated binding site but also on the abundance of such sites (see e.g. binding of thrombin to heparin (Olson et al., 1991) and hyaluronan to liver endothelial cells (Laurent et al., 1986)). The pentasaccharide motif implicated in FGF-2 binding is obviously scarce in the aorta HS; calculations based on the overall yields of fragments \(\geq\) octasaccharides (Fig. 2) and on reasonable assumptions regarding the proportion of FGF-2-binding species (Fig. 3) suggest that less than every other HS chain \((M_r \approx 50 \times 10^6, \text{estimated by gel chromatography, data not shown})\) will carry the FGF-2-binding region. Heparin, on the other hand, will be replete with this structure, albeit heavily obscured by 6-O-sulfate groups, as will the selectively 6-O-desulfated heparin. In particular, the properties of the latter compound are well in accord with the postulated structure/function relationship. This modified polysaccharide binds FGF-2 almost as avidly as native heparin (Fig. 1); the difference may well be accounted for by the partial loss of IdoA 2-O-sulfate groups occurring along with GlcN 6-O-desulfation (see "Experimental Procedures"). Yet the low-O-sulfated FGF-2 binding octasaccharides obtained following partial depolymerization of the 6-O-desulfated heparin showed a composition similar to that of the corresponding fraction derived, at much lower yield, from the aorta HS (Table I). Both octasaccharides thus contained a single \(-\text{IdoA}(2-\text{OSO}_3)-\text{GlcNSO}_3\) sequence in addition to other N-sulfated disaccharide units. It therefore seems reasonable to conclude that the low sulfated pentasaccharide sequence does indeed represent a fundamental site for FGF-2 binding. While the number of sulfate groups actually interacting with FGF-2 is unknown, it is noteworthy that two sulfate ions were found at defined sites in FGF-2 crystals (Eriksson et al., 1991; Zhang et al., 1991).

Recent studies by other groups (Ornitz et al., 1992; Ornitz and Leder, 1992; Ishihara et al., 1993) have indicated that the polysaccharide sequence required to elicit a biological response of added growth factor in cultured cells is longer than that needed to simply bind the factor. Evidence presented in the accompanying paper (Guimond et al., 1993) demonstrates that a dodecasaccharide represents the minimal structure required to promote binding of FGF-2 to its high affinity receptor in Swiss 3T3 cells and to induce a mitogenic response.
It is proposed that this dodecasaccharide sequence encompasses a pentasaccharide site, as defined in the present report, that interacts with the growth factor and, in addition, a second site that binds to the receptor. The dodecasaccharide structure thus serves to bridge the two proteins.

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