THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 33, Issue of August 17, pp. 30744–30752, 2001

Printed in U.S.A.

Sequence Analysis of Heparan Sulfate Epitopes with Graded Affinities for Fibroblast Growth Factors 1 and 2*

Received for publication, March 23, 2001
Published, JBC Papers in Press, June 13, 2001, DOI 10.1074/jbc.M102628200

Johan Kreuger‡, Markku Salmivirta‡§¶, Luisa Sturiale‖, Guillermo Giménez-Gallego, and Ulf Lindahl‡ ‡‡**

*From the ‡Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden, the §Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, FIN-20521, Finland, ¶BioTie Therapies Corp., FIN-20520 Turku, Finland, ‖G. Ronzoni Institute for Chemical and Biochemical Research, via G. Colombo 81-20133 Milan, Italy, and the **Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Cientilicas) Velázquez 144, 28006 Madrid, Spain*

Proteins that belong to the fibroblast growth factor (FGF) family regulate proliferation, migration, and differentiation of many cell types. Several FGFs, including the prototype factors FGF-1 and FGF-2, depend on interactions with heparan sulfate (HS) proteoglycans for activity. We have assessed tissue-derived HS fragments for binding to FGF-1 and FGF-2 to identify the authentic saccharide motifs required for interactions. Sequence information on a range of *N*-sulfated HS octasaccharides spanning from low to high affinity for FGF-1 was obtained. All octasaccharides with high affinity for FGF-1 (≥0.5 M NaCl required for elution) contained an internal IdoUA(2-OSO_3)-$GlcNSO_3$(6-OSO_3)-IdoUA(2-OSO_3)-trisaccharide motif. Octasaccharides with a higher overall degree of sulfation but lacking the specific trisaccharide motif showed lower affinity for FGF-1. FGF-2 was shown to bind to a mono-*O*-sulfated HS 6-mer carrying a single internal IdoUA(2-OSO_3)-unit. However, a di-O-sulfated -IdoUA(2-OSO_3)-$GlcNSO_3$-IdoUA(2-OSO_3)-trisaccharide sequence within a HS 8-mer gave stronger binding. These findings show that not only the number but also the positions of individual sulfate groups determine affinity of HS for FGFs. Our findings support the notion that FGF-dependent processes can be modulated *in vivo* by regulated expression of distinct HS sequences.

Heparan sulfate (HS)[1] proteoglycans are present on the surface of all adherent mammalian cells as well as in the extracellular matrix. The biological functions of the structurally diverse HS chains are mediated through binding to a variety of proteins, including enzymes, enzyme inhibitors, cytokines/growth factors, and extracellular matrix molecules (1–4). More than 100 proteins have been reported to interact with the HS moiety of HS proteoglycan or with heparin, a more highly sulfated related polysaccharide. The best studied example of such binding is that of antithrombin, which interacts with a specific pentasaccharide sequence that carries several sulfate groups in critical positions (5). A question of current interest is whether other proteins also bind specifically to distinct saccharide epitopes. Although many proteins bind the highly sulfated heparin structure, it has been proposed that the same ligands may selectively interact with cognate HS species containing more sparsely distributed sulfate groups (2). Research of the last decade has indeed identified a multitude of physiologically and pathophysiologically important processes that depend on HS/protein interactions (1, 5–7).

The structural diversity of HS is generated during biosynthesis of the polysaccharide (2, 3, 6). A polymer of alternating D-glucuronic acid (GlcUA) and GlcNAc units, joined in $[GlcUA\beta1,4GlcNAc\alpha1,4]_n$ structure, is modified through *N*-deacetylation/*N*-sulfation of GlcNAc units, C-5 epimerization of GlcUA to L-iduronic acid (IdoUA), and *O*-sulfation at various locations (C-2 of IdoUA and GlcUA and C-3 and C-6 of GlcN units). The modification reactions are generally incomplete, thus generating the diverse distribution of *N*-substituents, GlcUA/IdoUA units, and sulfate groups typical for HS. Although the mechanisms in control of polymer modification are not fully understood, it is clear that the number of distinct saccharide epitopes actually expressed will be restricted because of the substrate specificities of the enzymes involved. Much of the structural variability within HS chains resides in the contiguous *N*-sulfated (NS) regions, which are interspersed by essentially unmodified *N*-acetylated sequences and by alternating *N*-acetylated and NS disaccharide units (7–9). "Heparin," in this context, may be considered an unusually extended and highly *O*-sulfated NS domain.

Compositional analysis of HS preparations from different tissues (7, 10, 11) as well as immunohistochemical evidence (12, 13) point to differential regulation of HS biosynthesis, which may be modulated in normal development and aging (14, 15) and perturbed in disease (16). It is believed that subtle changes in HS structure may result in altered interaction with proteins, and such effects have recently been demonstrated (15, 17, 18). Although we thus assume that many proteins recognize distinct HS epitopes, the minimal requirements, in terms of HS

* This work was supported by European Comission Grants QLK-CT-1999.00536 (Program "Biologically Active Novel Glycosaminoglycans") and BIO4-CT98-0538 (Program "Heparan Sulfate Sequencing Demonstration"), Medical Research Council Grant K96-03P,K99-03X,2309, Swedish Cancer Society Grant 3919-B97, and by funds from the Finnish Cancer Union, the Sigrid Juselius Foundation, and Polysackaridforskning AB. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Dept. of Medical Biochemistry and Microbiology, P.O. Box 582, S-75123 Uppsala, Sweden. Tel.: 46-18-471-4196; Fax: 46-18-471-4209; E-mail: ulf.lindahl@imbim.uu.se.

[1] The abbreviations used are: HS, heparan sulfate; $[^3H]aMan_R$, 2,5-anhydro-D-[1-^{3}H]mannitol; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GlcUA, D-glucuronic acid; GlcN6Sase, glucosamine-6-sulfatase; $GlcNSO_3$, N-sulfoglucosamine; HexA, hexuronic acid; IdoUA, L-iduronic acid; IdoUAase, α-L-iduronidase; IdoA2Sase, iduronate-2-sulfatase, MALDI-TOF, matrix assisted laser desorption ionization-time of flight; $pHNO_2$, partial cleavage with HNO_2; NS, *N*-sulfated.

structure, for interaction with a given protein have still been defined only for antithrombin. Moreover, we note that whereas the antithrombin-binding sequence features a "rare" component, the 3-*O*-sulfated GlcN unit (5), it seems likely that most other proteins interact with structures made up of the commonly occurring disaccharide units (19).

More than 20 members of the FGF family have been identified, and most of these growth factors bind heparin/HS. Experiments with the prototype species, acidic FGF (FGF-1) and basic FGF (FGF-2), using target cells deficient in HS biosynthesis have shown that the growth factors depend on cell surface HS for their mitogenic activity (20–24). The HS-deficient cells turn responsive to growth factor upon addition of exogenous heparin (that is normally contained in intracellular granules of the mast cell, thus unable to interact with extracellular growth factors). Experiments using selectively desulfated heparin preparations pointed to distinct *O*-sulfate requirements for interactions with FGF-1 (both IdoUA 2-*O*-sulfate and GlcN 6-*O*-sulfate groups) and FGF-2 (2-*O*-sulfate only) (22, 23, 25, 26), in accord with results of compositional analysis of affinity-fractionated HS oligomers (18, 25, 27, 28). Moreover, selected preparations of native HS were found to differ in their ability to promote FGF-1- and FGF-2-induced biological responses (17). However, most information so far available regarding the molecular aspects of FGF/polysaccharide interactions derives from crystallographic analysis of growth factors complexed with fully sulfated heparin oligosaccharides, with or without FGF receptor (FGFR) ectodomains (29–32). Intriguingly, the patterns revealed by these studies were highly diverse with respect to orientation, contact sites, and even stoichiometry of the interacting species. Nevertheless, binding of FGF to saccharide sequences spanning six monosaccharide units or less was a common feature of all models, presumably essential to receptor activation and intracellular signaling. The HS proteoglycan thus is ascribed a co-receptor function in which the HS chain interacts with the growth factor and, in most models postulated, also with the FGF tyrosine kinase receptor. The HS domain required to span growth factor and receptor extends beyond the sequence committed to growth factor binding alone (22, 31, 32). A recent study from our laboratory identifies HS sequences interacting with one of the FGFR species (33).

Contrary to heparin oligosaccharides, abundantly available, an oligosaccharide derived from authentic HS and selected for ability to interact with a given protein will usually be obtained in minute, often subnanomol, quantities. The methods for sequence analysis of such samples have only recently been developed (34–36). In the present study, we have applied one of these procedures to a series of HS oligosaccharides isolated from pig mucosal HS and fractionated with regard to affinity for FGF-1. A minimal "binding motif" is identified, with three *O*-sulfate groups in fixed positions, although additional *O*-sulfation may increase the affinity depending on position. Using similar experimental protocol and scope we also reassess the interaction between HS and FGF-2.

EXPERIMENTAL PROCEDURES

Materials—HS from pig intestinal mucosa was a gift from G. van Dedem (Diosynth, Oss, The Netherlands). Oligosaccharides corresponding to the NS domains, thus fully *N*-sulfated, were isolated from this preparation as described previously (18). Briefly, HS was *N*-deacetylated by hydrazinolysis and subsequently subjected to high pH deamination to eliminate all *N*-unsubstituted disaccharide units. The products, following reduction with NaB^3H_4 (64 Ci/mmol; Amersham Pharmacia Biotech) had the general structure (*O*-sulfate groups not included) GlcUA-[$GlcNSO_3$-HexA]$_n$-[1-^{3}H]aMan$_R$. The labeled oligosaccharides were separated with regard to size by gel chromatography on a column (1 × 190 cm) of Bio-Gel P10 (Bio-Rad) in 0.2 M NH_4HCO_3, at a flow rate of 2 ml/h and pooled according to the elution positions of known heparin oligosaccharide standards (see Fig. 1). To achieve size homogeneity, each pool was concentrated and rerun on the same column. The pooled oligosaccharide fractions were analyzed for hexuronic acid using the *meta*-hydroxydiphenyl method (37) with GlcUA as standard. Saccharide concentration was calculated assuming a hexuronic acid content of 40%. HS from human aorta was isolated as described (15) and processed similar to the intestinal HS to yield *N*-sulfated oligosaccharides. Recombinant human FGF-1 and FGF-2 were produced in a bacterial expression system as described (38).

Affinity and Anion-exchange Chromatography—Recombinant FGF-1 (1.5 mg) or FGF-2 (1 mg) was incubated with 1 ml of CH-Sepharose-4B (Amersham Pharmacia Biotech) in 40 mM $NaHCO_3$, 0.36 M NaCl, pH 8, at 4 °C for 4 h. The immobilization was carried out in the presence of heparin at 5-fold molar excess over FGF to protect the saccharide binding sites of the FGFs. To prevent coupling of heparin to the matrix, the heparin preparation used had previously been cleaved with HNO_2 at pH 3.9 followed by recovery of the high molecular weight product by gel filtration. The conjugated materials were transferred to small plastic columns (10 ml; Bio-Rad) and washed with 2 M NaCl to remove the protecting heparin before the binding studies. Generally, HS fragments were applied to the FGF columns in 0.5 ml of 0.14 M NaCl, 50 mM Tris-HCl, pH 7.4. The columns were kept for 30 min at 4 °C and eluted with a stepwise gradient (total volume, 70 ml) ranging from 0.14 to 2 M NaCl (buffered as above) (for FGF-1) or with a linear gradient (total volume, 60 ml) from 0 to 1.5 M NaCl (for FGF-2). Effluent fractions of 1 ml were collected, analyzed for radioactivity and pooled as indicated under "Results."

Pooled oligosaccharide fractions were desalted on PD-10 columns (Amersham Pharmacia Biotech) and further resolved by anion-exchange chromatography on a 4 × 250-mm Propac PA-1 column (Dionex, Surrey, UK) eluted with a linear gradient (total volume, 100 ml) from 0 to 1.5 M NaCl in H_2O adjusted to pH 3 with HCl. Saccharide fractions of similar charge density were pooled, desalted, and dried in a centrifugal evaporator.

Sequence Analysis—Sequence analysis was performed essentially as described previously (33, 36). *N*-Sulfated, [1-^{3}H]aMan$_R$ end group-labeled oligosaccharides (mostly octasaccharides, occasionally hexasaccharides) were subjected to partial cleavage with nitrous acid ($pHNO_2$) by incubation with 2–4 mM $NaNO_2$ in 20–40 mM HCl in a total volume of 20 μl on ice. The precise reaction conditions were adjusted as required to yield appropriate (ideally equimolar) ratios of labeled di-, tetra-, and hexasaccharide degradation products (monitored by pilot runs on the Propac PA-1 column). The deamination reaction was terminated at various time points (typically 15, 30, 45, 60, and 75 min) by transferring 4-μl aliquots at 10 μl of 200 mM sodium acetate, pH 6. The pooled resultant fragment mixtures were split into 4-μl aliquots and subjected to enzyme digestion. The enzymes used were iduronate-2-sulfatase (IdoUA2Sase), α-L-iduronidase (IdoUAase), and glucosamine-6-sulfatase (GlcN6Sase), purchased from Oxford GlycoSciences (Abingdon, UK). The substrate specificities of the enzymes were confirmed using known heparin di- and tetrasaccharides as substrates. Each reaction contained 1 milliunit of IdoUA2Sase, 1 milliunit of IdoUAase, and 0.2 milliunit of GlcN6Sase, as single additions or in combination as indicated in a total volume of 25 μl of 50 mM sodium acetate, pH 5.0, containing 0.1 mg/ml bovine serum albumin. The samples were incubated at 37 °C for 16 h according to the manufacturer's instructions. Generally, 15–20 × 10^3 dpm ^{3}H or ~3–4 pmol of oligosaccharide was used in each incubation, although occasionally as little as 2000 dpm was successfully analyzed. The sequences were interpreted by comparing the Propac anion-exchange elution patterns of $pHNO_2$ mixtures before and after enzyme treatment. The elution positions of the intact saccharides were ascertained to enable sequence readout. Propac runs with gradients up to 1 M NaCl (in H_2O adjusted to pH 3 with HCl) were analyzed on-line using a radioactivity flow detector (Radiomatic 500 TR, Packard, CT) with background subtraction set to 40 dpm. The samples run with gradients exceeding 1 M NaCl were collected in 1-ml fractions and analyzed for radioactivity in a liquid scintillation counter.

Mass Spectrometry—The molecular mass of HS oligosaccharides was determined by MALDI-TOF mass spectrometry, essentially as described (35). Briefly, oligosaccharides (3–5 pmol) were mixed with 10 pmol of basic peptide (Arg-Gly)$_{19}$-Arg (Sigma-Genosys Ltd.), required to enhance ionization of the saccharide, in 4 μl of matrix solution (caffeic acid (Aldrich), 12 mg/ml, in 50% aqueous acetonitrile (Fluka)). Aliquots (1–2 μl) of the sample-matrix mix were deposited on a stainless steel chip, dried, and analyzed in a Bruker Biflex III MALDI-TOF instrument (delayed extraction, mass gate set to 2000 Da). Mass spectra were calibrated with cytochrome *c* (Sigma) and adrenocorticotropic hormone (Sigma).

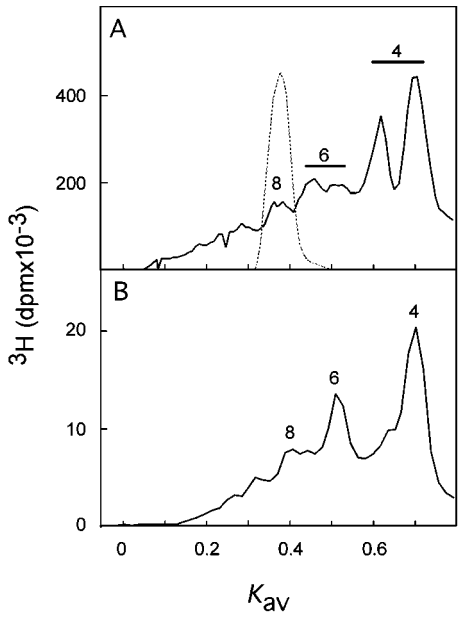

FIG. 1. **Gel chromatography of HS oligosaccharides derived from *N*-sulfated domains of HS chains.** HS samples from pig intestinal mucosa (*A*) and human aorta (*B*) were *N*-deacetylated and cleaved with nitrous acid (pH 3.9), and the products were reduced with NaB^3H_4. The resultant mixtures of ^{3}H-labeled even-numbered oligosaccharides were separated according to size (indicated *above* the *peaks* as the number of monosaccharide units/molecule) on a Bio-Gel P-10 column (see "Experimental Procedures"). Octa- and hexasaccharides were pooled and rerun on Bio-Gel P-10 to generate size-homogeneous fractions (rerun shown for 8-mers in *A*, *dotted line*) for affinity fractionation.

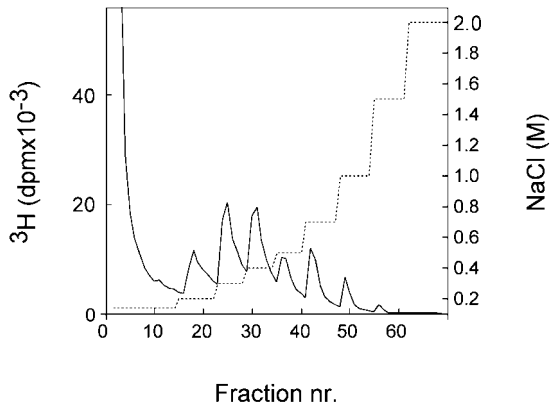

FIG. 2. **Affinity chromatography of HS octasaccharides on immobilized FGF-1.** ^{3}H-Labeled octasaccharides isolated from pig intestinal mucosa HS were applied to a FGF-1 affinity column equilibrated with 0.14 M NaCl in 50 mM Tris-HCl, pH 7.4. Bound saccharides (60×10^6 dpm, 25% of the applied material) were eluted with a stepwise gradient of NaCl (*dashed line*) and collected in 1-ml fractions that were analyzed for radioactivity.

RESULTS

Sequence Analysis of FGF-1-Binding HS Octasaccharides—Affinity and Charge Fractionation of Octasaccharides - Oligosaccharides corresponding to NS domains were obtained from pig intestinal mucosa HS as described under "Experimental Procedures" and were reduced with NaB^3H_4 to introduce a terminal [1-^{3}H]aMan$_R$ label. This tag was used to monitor fractionation of intact oligosaccharides with regard to bioaffinity and charge density but also to provide the essential reference point in sequence analysis (36). An octasaccharide fraction (specific radioactivity, 4.4×10^3 dpm/pmol) was recovered by gel chromatography (Fig. 1*A*) and applied to affinity fractionation on immobilized FGF-1. The selection of octasaccharides was based on our previous survey of oligosaccharides from HS, 8-mers being the smallest species capable of significant binding to FGF-1 (18).[2] Approximately 25% of the ^{3}H-labeled octasaccharides derived from intestinal HS bound to the FGF-1 column at physiological ionic strength. Initial attempts at elution using a linear salt gradient gave no apparent resolution of components, and a protocol of stepwise elution was therefore adopted (Fig. 2). Several affinity fractions (in the following referred to by the NaCl concentration required for their elution) were recovered and further separated by anion-exchange chromatography (Fig. 3). Contrary to the octasaccharide starting material, which has a large number of poorly resolved peaks (Fig. 3*A*), the fractions recovered by salt elution from the FGF-1 column showed a limited number, usually two or three, of distinct major peaks. The relative elution positions of these components suggested that they differ from each other with regard to single sulfate groups. A general correlation between charge and FGF-1 affinity is discerned (Fig. 3, *C–H*). On the other hand, the weakly FGF-1 binding octasaccharides in the 0.2 M NaCl fraction (Fig. 3*C*) contain components more highly sulfated than some of the strong binders in the 0.5 M NaCl fraction (Fig. 3*F*). The predicted molecular weight of recovered octasaccharides was confirmed by MALDI-TOF mass spectrometry (Table I), as illustrated in Fig. 4 for the components of the 0.2 M NaCl fraction emerging after 58 min from the Propac column (Fig. 3*C*). The M_r of 1912 calculated for the predominant species (Fig. 4) corresponds to that of a HexA-[GlcNSO$_3$-HexA]$_3$-aMan$_R$ octasaccharide with four *O*-sulfate groups.

In addition, to confirm that saccharides used for sequence analysis did not contain any *N*-unsubstituted or *N*-acetylated glucosamines, two octasaccharide fractions (0.5 M NaCl for 60 min in Fig. 3*F* and 0.7 M NaCl for 60 min in Fig. 3*G*) were subjected to a second round of complete hydrazinolysis followed by treatment with nitrous acid at pH 3.9. Subsequent analysis by anion-exchange chromatography showed that the fragments were unaffected by the treatment, indicating complete *N*-sulfation (data not shown). The components of individual peaks were recovered, desalted, and subjected to sequence analysis. Sequencing revealed that each of the charge-homogeneous fractions contained one to three major components (see below).

Strong FGF-1 Binders —For sequence analysis, charge-homogeneous oligosaccharides were subjected to partial deaminative cleavage, yielding a series of labeled, even-numbered fragments with nonreducing-terminal HexA-GlcNSO$_3$-disaccharide units, with or without *O*-sulfate groups. To elucidate the substitution patterns these fragments were digested with IdoUA2Sase alone, IdoUA2Sase + IdoUAase, or IdoUA2Sase + IdoUAase + GlcN6Sase, and the effects were assessed by anion-exchange high perfomance liquid chromatography (see "Experimental Procedures"). In some cases**,** the results provided direct sequence readout for the parent oligosaccharide. In other instances fragment analysis revealed heterogeneity within the parent fraction, because of variable distribution of *O*-sulfate groups. Notably, sequences of mixed oligosaccharides could often be resolved by inference through a combination of fragment structures to match the known number of *O*-sulfate groups of the parent compounds. Whenever such deduction was unfeasible, the partial sequence information obtained was used to outline potential alternative parent oligosaccharide structures. All sequence information obtained in the study has been compiled in Fig. 10.

"Strong binders" were arbitrarily defined as saccharides eluted from the immobilized FGF-1 by NaCl at ≥0.5 M concentration.

[2] Because of the design of the cleavage process required to generate oligosaccharides, the actual protein binding site may be expected to occupy only part of the saccharide (43).

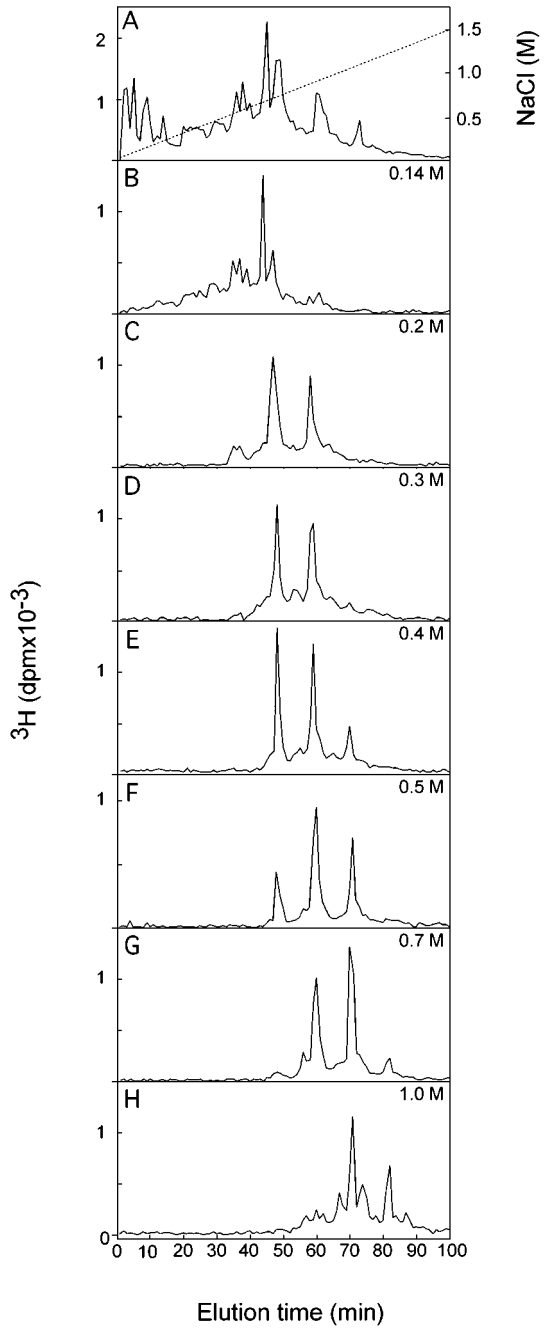

FIG. 3. **Ion-exchange chromatography of HS octasaccharides fractionated on immobilized FGF-1.** Affinity fractions generated as shown in Fig. 2 were subjected to anion-exchange chromatography on a Propac PA-1 column, eluted with a linear gradient of NaCl. *A*, octasaccharide mixture before FGF-1 fractionation. *B*, flow-through fraction collected at 0.14 M NaCl. *C–H* show saccharides eluted from the FGF-1 column at the NaCl concentrations indicated in each *upper right corner*.

The least sulfated components within each affinity class were of particular interest, because they would presumably express the minimal structural requirements for such interaction. Octasaccharides displaced from FGF-1 with 0.5 M NaCl gave rise to three major peaks on anion-exchange chromatography (Fig. 3*F*). The elution position of the least retarded peak (at 48 min) indicated three *O*-sulfate groups. Notably, octasaccharides with the same net charge (but with different *O*-sulfate distribution) occurred in affinity fractions displaced already at physiological ionic strength from the FGF-1 column (Fig. 3*B*). Partial depolymerization of the 48-min fraction with nitrous acid (Fig. 5*C*) generated two major fragments in addition to a peak that retained the original elution

TABLE I
Mass determination of oligosaccharides by MALDI-TOF MS

Saccharide fraction[a]	Number of *O*-sulfate groups	*m/z*	
		Predicted[b]	Detected
FGF-1 0.2 M for 47 min (*8a*, *b*, *c*)	3	1832	1830
FGF-1 0.2 M for 58 min (*8d*, *e*)	4	1912	1912
FGF-1 0.5 M for 48 min (*8f*)	3	1832	1831
FGF-1 0.5 M for 60 min (*8g*, *h*, *i*)	4	1912	1912
FGF-1 0.7 M for 60 min (*8i*)	4	1912	1912
FGF-1 1.0 M for 71 min (*8j*, *k*)	5	1992	1991
FGF-2 0.4 M (*8l*)	2	1752	[c]
FGF-2 0.7 M (*8m*)	2	1752	1751
FGF-2 0.3 M (*6a*)	1	1255	1253

[a] Saccharide fractions are identified by the FGF species used as affinity matrix, the concentration of NaCl required for their elution, and (FGF-1 binding saccharides only) their retention time on subsequent anion-exchange chromatography (Fig. 3). Designations within parentheses refer to the sequences identified (Fig. 10).

[b] Predicted *m/z* values are based on saccharide structures as shown in Fig. 10, including protonation of carboxyl and sulfate groups.

[c] Amounts insufficient for analysis.

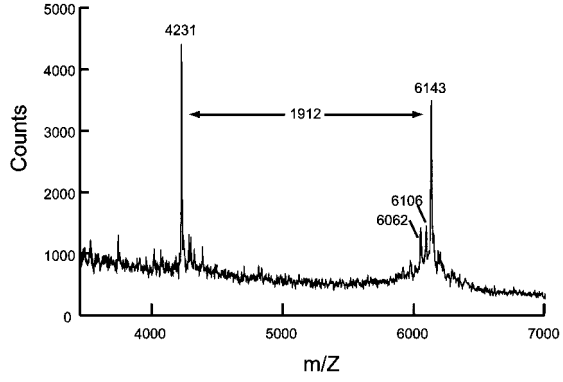

FIG. 4. **MALDI-TOF mass spectrometry of HS octasaccharides.** Mass determination of heptasulfated HS octasaccharides (mix of *8d* and *8e*) is shown. The saccharides observed are 1:1 molar complexes with the basic peptide $(Arg\text{-}Gly)_{19}\text{-}Arg$. The recorded *m/z* value of the saccharides (1912) represents the difference in recorded mass between the peptide-saccharide complex (6143) and the peptide alone (4231). This value corresponds to a fully *N*-sulfated and protonated HS octamer carrying four *O*-sulfate groups. The results of similar analysis of the other HS oligosaccharides studied are shown in Table I.

position of the parent octasaccharide (Fig. 5*B*). The intact octasaccharide will hereafter be denoted *8f* (see Fig. 10*A* for complete notation system). The two smaller fragments appeared at the positions of a nonsulfated disaccharide (*2f*) and a mono-*O*-sulfated (*N*-sulfated) tetrasaccharide (*4f*).[3] In addition, a hexasaccharide (*6f*) was coeluted with the remaining parent octasaccharide, as revealed by the shift in elution position induced by subsequent IdoUA2Sase digestion (Fig. 5*D*). The elution properties of *6f* relative to the *8f* starting material indicate that the nonreducing terminal disaccharide released by nitrous acid treatment was devoid of *O*-sulfate, thus defining units 1 and 2 of the intact octasaccharide (Fig. 5*A*).[4,5] The peak shift of *6f* upon

[3] Partially and completely *O*-desulfated heparin oligosaccharides with known sulfate contents were used throughout the study as reference compounds (J. Kreuger and U. Lindahl, unpublished data).

[4] Unit 1 is invariably GlcUA in all oligosaccharides generated by the *N*-deacetylation/deamination process, because the -GlcNAc-GlcUA- sequence (cleaved in this process) is not a substrate for the C-5 epimerase that converts GlcUA to IdoUA during heparin/HS biosynthesis (44).

[5] Dependent on the charge density of the saccharide analyzed, removal of terminal IdoUA monosaccharide or GlcUA-$GlcNSO_3$ disaccharide units variously affected the elution position in anion-exchange chromatograms. Generally, parental fragments eluted before 50 min were shifted to the left, and those emerging between 50 and 70 min were only slightly affected, whereas components appearing after 70 min were shifted to the right.

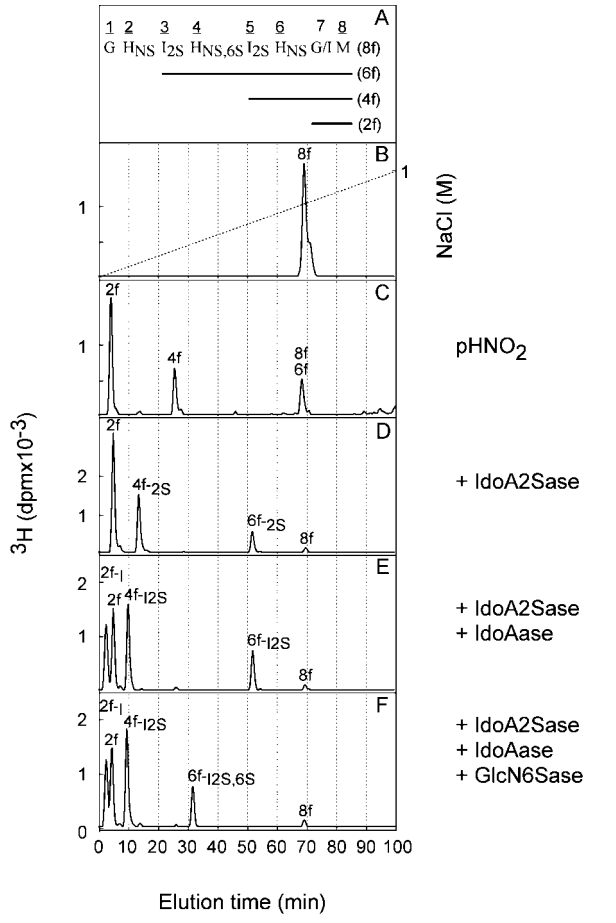

FIG. 5. **Sequence analysis of octasaccharides eluted from FGF-1 with 0.5 M NaCl.** The octasaccharide species analyzed (*8f*) contains three *O*-sulfate groups (see Fig. 3*F*, peak at 48 min). *A* shows the deduced structure of *8f* and of the corresponding cleavage products generated by $pHNO_2$ (*6f*, *4f*, and *2f*), all carrying a ^3H label at the reducing end. *G*, GlcUA; *I*, IdoUA; I_{2S}, IdoUA(2-OSO_3); H_{NS}, GlcNSO_3; $H_{NS,6S}$, GlcNSO_3(6-OSO_3); *M*, [1-^{3}H]aMan$_R$. *B–F* show analytical Propac ion-exchange chromatograms of intact *8f* (*B*) and of fragments obtained by $pHNO_2$-degradation, either without further treatment (*C*) or following digestion with (*D*) IdoUA2Sase, (*E*) IdoUA2Sase + IdoUAase, or (*F*) IdoUA2Sase + IdoUAase + GlcN6Sase. The effects of enzyme digestion on individual components are indicated in subtractive fashion; for example, peak 6f$_{-I2S,6S}$ represents the product generated by successive release of a 2-*O*-sulfate group, an IdoUA unit, and a 6-*O*-sulfate group from hexasaccharide *6f*. The salt gradient (*dotted line*) used to elute the Propac column is shown in *B*.

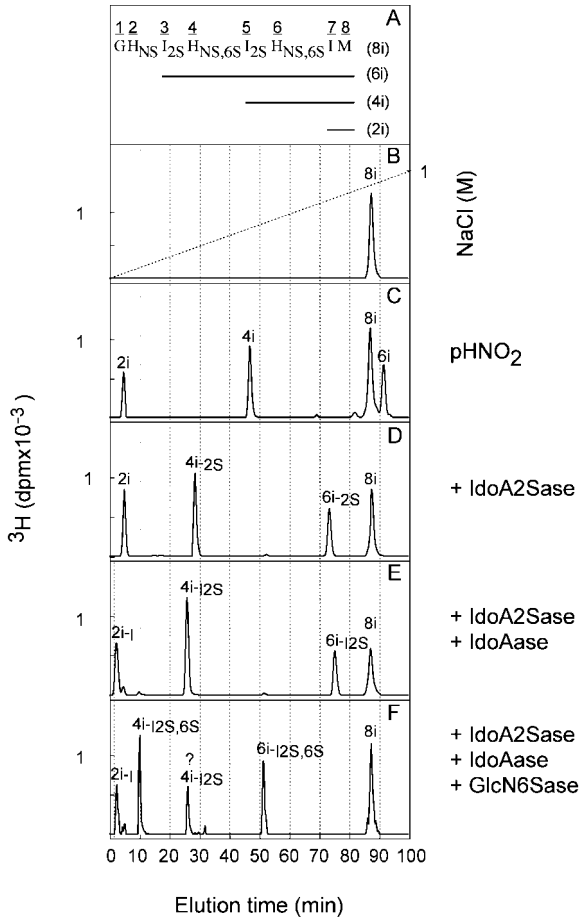

FIG. 6. **Sequence analysis of octasaccharides eluted from FGF-1 with 0.7 M NaCl.** The octasaccharide species analyzed (*8i*) contains four *O*-sulfate groups (see Fig. 3*G*, peak at 60 min). *A* shows the deduced structure of intact *8i* and of the corresponding cleavage products generated by $pHNO_2$ (*6i*, *4i*, and *2i*). *B–F* are analogous to the corresponding sections of Fig. 5.

digestion with IdoUA2Sase reflects the loss of a 2-*O*-sulfate group from terminal IdoUA, and the additional shift following treatment with IdoUAase and GlcN6Sase indicates the release of IdoUA and a GlcN 6-*O*-sulfate group. Units 3 and 4 of *8f* were thus defined as -IdoUA(2-OSO_3)-GlcNSO_3(6-OSO_3)-. The single *O*-sulfate group of tetrasaccharide *4f* was released by IdoUA2Sase (Fig. 5*D*), indicating an -IdoUA(2-OSO_3)-GlcNSO_3 structure for units 5 and 6 (Fig. 5*A*). The nonsulfated disaccharide *2f* was partially cleaved by IdoUAase, suggesting the presence of both GlcUA and IdoUA at position 7. The overall structure of *8f* (Figs. 5*A* and 10*A*) is characterized by an internal tri-*O*-sulfated trisaccharide sequence (units 3–5), -IdoUA(2-OSO_3)-GlcNSO_3(6-OSO_3)-IdoUA(2-OSO_3)-, that is recognized as an FGF-1-binding motif.

Sequence analysis of octasaccharides in the same affinity class, *i.e.* the 0.5 M NaCl fraction, but with one additional *O*-sulfate group (peak at 60 min in Fig. 3*F*; altogether four *O*-sulfate groups/molecule) presented more complex patterns. Partial depolymerization with nitrous acid generated six ^{3}H-labeled subpeaks (data not shown) that could be ascribed to three differentially sulfated octasaccharides, *8g*, *8h*, and *8i* (see Fig. 10*A*). Two of these peaks represented hexasaccharides containing three and four *O*-sulfate groups, respectively, both with a terminal IdoUA(2-OSO_3)-GlcNSO_3(6-OSO_3)- structure (units 3–4). Also, all tetrasaccharides contained terminal IdoUA(2-OSO_3)- (unit 5). Thus all octasaccharides express the FGF-1-binding motif with three *O*-sulfate groups defined above. The positions of the fourth *O*-sulfate group were deduced as follows. One was located on unit 8 (in octasaccharide *8g*; see Fig. 10*A*), as indicated by the identification of the labeled 6-*O*-sulfated disaccharide, IdoUA-aMan$_R$(6-OSO_3) (see below). The octasaccharide (8 h) yielding a tri-*O*-sulfated labeled hexasaccharide upon partial deamination must contain an additional *O*-sulfate group on units 1 and 2, presumably a 6-*O*-sulfate group. Finally, a third octasaccharide (*8i*) carried a 6-*O*-sulfate group on unit 6, as evidenced by the identification of a terminal IdoUA(2-OSO_3)-GlcNSO_3(6-OSO_3)- sequence in one of the tetrasaccharides.

The less sulfated component of the 0.7 M NaCl affinity fraction (60-min peak in Fig. 3*G*) was an essentially homogeneous octasaccharide with four *O*-sulfate groups, similar to *8i* previously identified along with *8g* and *8h* in the 0.5 M NaCl fraction. This structure apparently binds stronger to FGF-1 than either *8g* or *8h*. Its identification is shown in Fig. 6. Notably, fragment *4i* was repeatedly found to be only partially degraded by GlcN6Sase (Fig. 6*F*), suggesting that a minor fraction ($<1/3$) may carry a 3-*O*-sulfate group instead of a 6-*O*-sulfate group on *unit 6*.

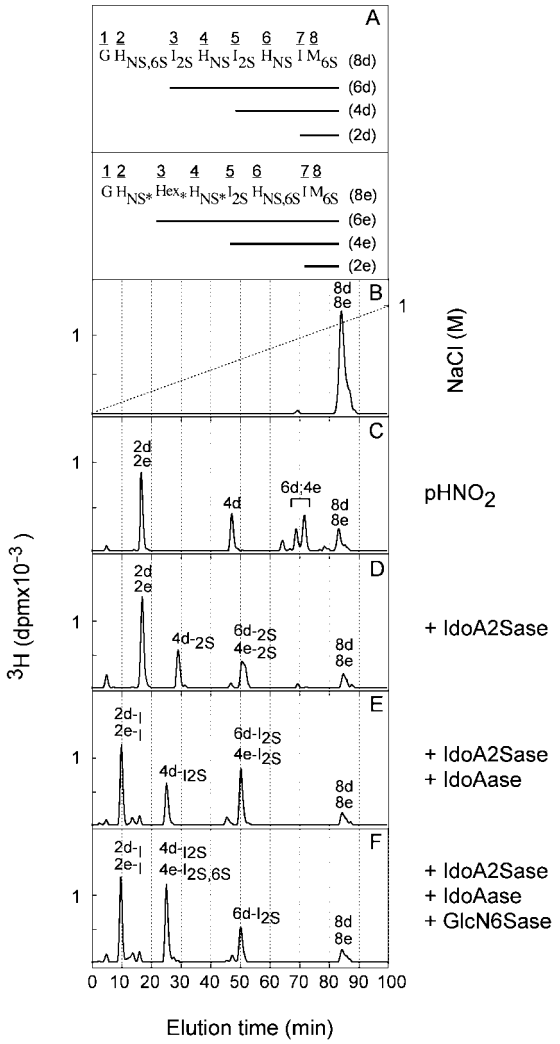

FIG. 7. **Sequence analysis of octasaccharides eluted from FGF-1 with 0.2 M NaCl.** The octasaccharide species analyzed (mixture of *8d* and *8e*) contain four *O*-sulfate groups (see Fig. 3*C,* peak at 58 min). *A* shows the deduced structures of intact *8d* and *8e* and of the corresponding cleavage products generated by $pHNO_2$. *B–F* are analogous to the corresponding sections of Fig. 5. The *peaks* at 68 and 72 min in *C* represent hexasaccharide *6d* and tetrasaccharide *4e*, respectively (indicated by the *horizontal bracket*), according to the elution positions of known *N*-sulfated, tri-*O*-sulfated tetra- and hexasaccharide reference compounds.[3] Because the positions of *O*-sulfate groups in the reference compounds differ from those deduced from the sample oligosaccharides, the assignment of each individual component is uncertain. Note that no hexasaccharide *6e* was detected; hence one of the *O*-sulfate groups in *8e* could be located at any of the positions marked with an *asterisk*.

Sequence analysis of octasaccharides in the 71-min peak of the 1.0 M NaCl fraction yielded two penta-*O*-sulfated structures (*8j* and *8k* in Fig. 10*A*). Both octasaccharides exhibit the FGF-1-binding motif and one additional 6-*O*-sulfate group on unit 6, similar to *8i*. In addition, *8j* and *8k* carry 6-*O*-sulfate groups on units 2 and 8, respectively (sequence analysis not shown).

Weak FGF-1 Binders—We also analyzed octasaccharides having relatively low affinity for FGF-1 that were eluted from the affinity matrix with 0.2 M NaCl. This fraction gave rise to two major peaks on Propac anion-exchange chromatography, the more retarded one (at 58 min in Fig. 3*C*) corresponding to an *N*-sulfated octasaccharide with four *O*-sulfate groups. Notably, octasaccharides with the same net charge were found in affinity fractions displaced with up to 0.7 M NaCl from the FGF-1 column (Fig. 3), and we therefore primarily aimed at characterizing this high sulfated, low affinity material. Partial depolymerization with nitrous acid generated four major fragments (Fig. 7*C*). One of these products, hexasaccharide *6d*, emerged well before the parent octasaccharide, indicating that it had been formed by release of an *O*-sulfated nonreducing terminal disaccharide (units 1 and 2). This hexasaccharide lost a sulfate group on treatment with IdoUA2Sase (Fig. 7*D*) but was not further affected by GlcN6Sase (along with IdoUAase digestion) (Fig. 7, *E* and *F*), thus identifying units 3 and 4 of octasaccharide *8d* as IdoUA(2-OSO_3)-GlcNSO_3. The two *O*-sulfate groups required to add up to the four *O*-sulfates of the parent octasaccharide were found in the tetrasaccharide *4d*, which was likewise attacked by IdoUA2Sase but resistant to GlcN6Sase (Fig. 7, *D–F*). Therefore, one of these groups was at C-2 of unit 5, whereas unit 6 was devoid of 6-sulfate. Because IdoUA-aMan_R(6-OSO_3) was the only labeled disaccharide present in significant amounts,[6] the fourth *O*-sulfate group of octasaccharide *8d* would be located at unit 8 (see Fig. 10*A*).

An additional tetrasaccharide, *4e*, carrying three *O*-sulfate groups was detected among the deamination products of *8d* (Fig. 7*C*), suggesting that the original octasaccharide fraction was heterogeneous. Exoenzyme digestion of this tetrasaccharide released both a 2-*O*-sulfate and a 6-*O*-sulfate group (along with IdoUA) identifying a di-*O*-sulfated unit 5 and 6 disaccharide sequence (Fig. 7, *D–F*). The third *O*-sulfate group of *4e* would be located on unit 8. No fragment *6e* was detected, either because it remained hidden under the initial octasaccharide or because the linkage between units 2 and 3 in octasaccharide *8e* was extensively cleaved in the deamination reaction; hence the fourth *O*-sulfate group of *8e*, within the unit 2–4 trisaccharide structure cannot be definitely allocated. Importantly, however, no single insertion of *O*-sulfate at any of the three positions available would complete the FGF-1-binding motif characteristic of the strong binders (see Fig. 10*A*). Likewise, this motif was lacking in all of three octasaccharides (*8a*, *8b*, and *8c*; identification not shown), each containing three *O*-sulfate groups, also eluted from the FGF-1 column with 0.2 M NaCl (see Fig. 10*A*).

Sequence Analysis of FGF-2-Binding HS Oligosaccharides—Three previous studies of interactions between heparin/HS and FGF-2, based on compositional analysis of FGF-2-bound oligosaccharides (25) and on crystallography (29, 32), implicated distinct albeit related minimal binding sequences (see Fig. 10*C*). A common conclusion of these and other studies (26, 27) was the essential role of IdoUA 2-*O*-sulfate residues and the relative unimportance of GlcN 6-*O*-sulfate groups. We decided to reinvestigate this matter using the novel sequencing method and HS from human aorta as starting material. This polysaccharide species is distinguished by its relatively low 6-*O*-sulfate content (9, 15) and would therefore be expected to provide oligosaccharides with the minimal structural features required for FGF-2 binding. Initial experiments were done with hexasaccharides, isolated from NS domains (Fig. 1*B*), and radiolabeled as before, mainly because heparin hexasaccharides had been used in some of the previous studies. Approximately 50% of the added HS hexamer was retained by the FGF-2 column and subsequently released during elution with NaCl (linear gradient, 0–1.0 M), as an essentially single peak at ~0.3 M NaCl concentration (data not shown). Ion-exchange chromatography of this material resolved two major components, carrying one and two *O*-sulfate groups/hexamer. Sequence analysis of the minimally sulfated component (not shown) gave hexasaccharide *6a* in Fig. 10*C*. Notably, this structure does not match any of the sequences previously implicated, possibly because a hep-

[6] The peak at 17 min in Fig. 7 (*C* and *D*) corresponds to an IdoUA-aMan_R(6-OSO_3) disaccharide standard, and that at 10 min in Fig. 7 (*E* and *F*) corresponds to aMan_R(6-OSO_3) monosaccharide (which is not a substrate for GlcN6Sase; Fig. 7*F*).

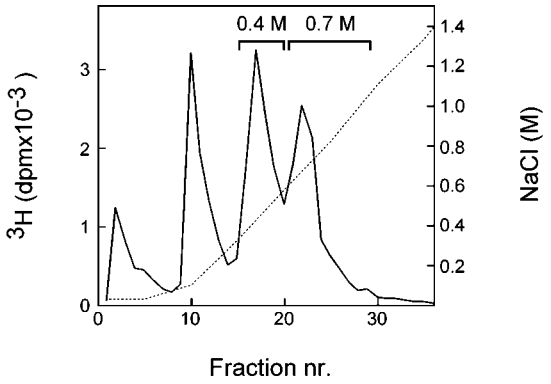

FIG. 8. **Affinity chromatography of HS octasaccharides on immobilized FGF-2.** ^{3}H-Labeled octasaccharides isolated from human aortic HS were applied to a FGF-2 column. Bound saccharides (1.3×10^6 dpm, 60% of the applied material) were eluted with a linear gradient of NaCl (*dotted line*). Fractions of 1 ml were collected, analyzed for radioactivity, and pooled in 0.4 and 0.7 M fractions as indicated.

arin hexasaccharide is more extensively 2-*O*-sulfated than a HS NS domain of similar size (8, 9). We therefore turned to HS octamers from the same tissue source.

Gradient elution of aortic HS octamers bound to the FGF-2 column yielded a series of fairly distinct peaks (Fig. 8) (contrary to the intestinal HS octamers on the FGF-1 column). Fractions corresponding to the peaks emerging at 0.4 and 0.7 M NaCl were recovered and subjected to anion-exchange chromatography. Octamers with two *O*-sulfate groups were isolated from both affinity fractions and sequenced. Two components, *8l* and *8m*, both from the 0.4 M NaCl fraction, were identified (sequence analysis not shown) (see Fig. 10*C*). The di-*O*-sulfated fraction from the high affinity pool (0.7 M NaCl eluate) contained octasaccharide *8m* without any significant admixture (sequence analysis in Fig. 9). We conclude that *8m* has higher affinity for FGF-2 than *8l*, and that the presence of *8m* in the 0.4 M NaCl fraction reflects the incomplete separation of peaks upon affinity chromatography (Fig. 8).

DISCUSSION

Compelling evidence from many research groups implicate HS proteoglycans with a "co-receptor" function in FGF signaling (see the Introduction). The precise role of the HS chain is still somewhat unclear, because it appears to interact not only with the growth factor but also with the receptor protein. Nevertheless, recent findings suggest that subtle modulation of HS structure may alter cellular responsiveness to FGFs (17, 24, 39). Analysis of such modulation has so far been hampered by lack of methodology, in particular the inability to determine the fine structure, *i.e.* sequence of sulfated HS domains involved in protein binding. A recently developed method for sequence analysis has been applied to the characterization of HS domains interacting with FGF-1 and FGF-2. These applications show that heparin/HS-derived oligosaccharides in the low pmol range are amenable to sequence analysis. The novel sequencing technology, including the recent mass spectrometry-based approaches (35), will greatly reinforce studies of the structure/function relations of HS.

The structural requirements for interaction of HS with FGF-2 are relatively simple, a single IdoUA 2-O-sulfate group appropriately located in an NS domain being sufficient for appreciable affinity and an additional 2-*O*-sulfate group being sufficient for strong binding (Fig. 10*C*). Accordingly, most of the octamers tested, even from the low sulfated aortic HS, bound the FGF-2 column (Fig. 8). The ability to bind FGF-2 thus would seem to be constitutively expressed by most HS species. By contrast, the more complex sequences required for high affinity (≥0.5 M NaCl)

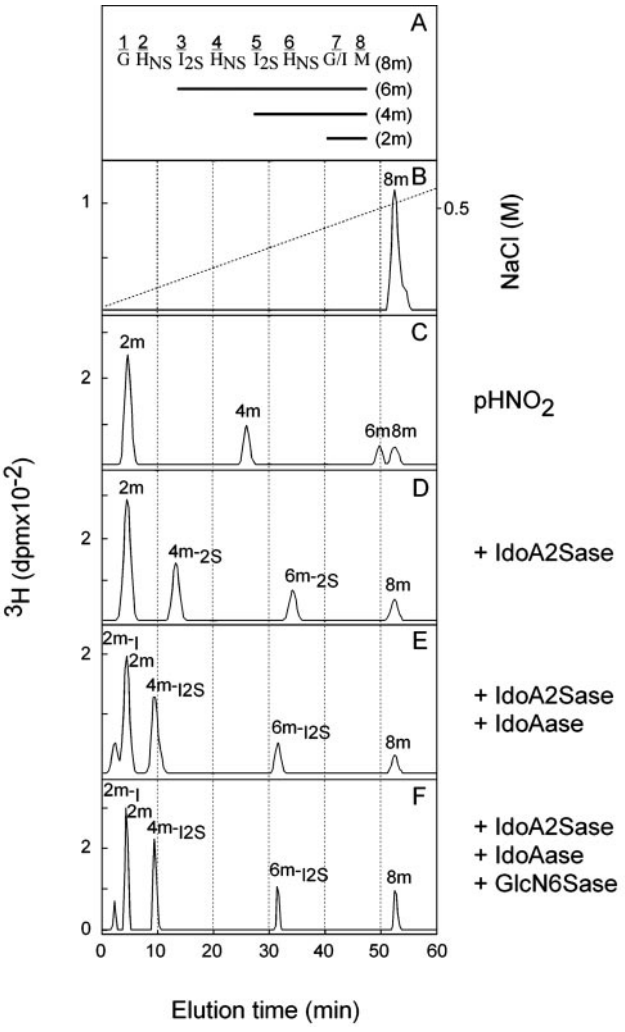

FIG. 9. **Sequence analysis of octasaccharide eluted from FGF-2 with 0.7 M NaCl.** The octasaccharide species analyzed (*8m*) contains two O-sulfate groups. *A* shows the deduced structures of intact *8m* and of the corresponding cleavage products generated by $pHNO_2$. *B–F* are analogous to the corresponding sections of Fig. 5.

interaction with FGF-1 occur in only ~5% of all isolated *N*-sulfated octamers from (the more highly sulfated) intestinal mucosa HS (Fig. 2). Such binding was found to require a characteristic tri-*O*-sulfated trisaccharide motif, minimally expressed in octasaccharide *8f,* that could not be substituted for by other structures containing a larger number of sulfate groups within the same octamer framework (Fig. 10*A*) (the relative importance of individual *N*-sulfate groups is not assessed in the present study). The subtle specificity of the interaction is illustrated by comparison of structures *8b* (low affinity, 0.2 M NaCl) and *8f* (high affinity, 0.5 M NaCl), which differ by the location of a single 6-*O*-sulfate group. Binding strength could be further increased by additional 6-*O*-sulfation, but only given the presence of the basic binding trisaccharide motif. By contrast, this motif contributed less to FGFR-4 binding than a larger number of more sparsely distributed *O*-sulfate groups (Fig. 10*B*) (33). These findings point to the importance of regulation in HS biosynthesis, particularly regarding the distribution of 6-*O*-sulfate groups and add to the significance of recent studies of biosynthetic 6-*O*-sulfation patterning in HS domains (8, 9).

Our results relating oligosaccharide structure to affinity for, in particular, FGF-1 suggest that HS sequences may be tailored to bind protein ligands with graded strength. At present we can only speculate over the functional meaning of such an arrangement. It should be emphasized that the HS chains serving as

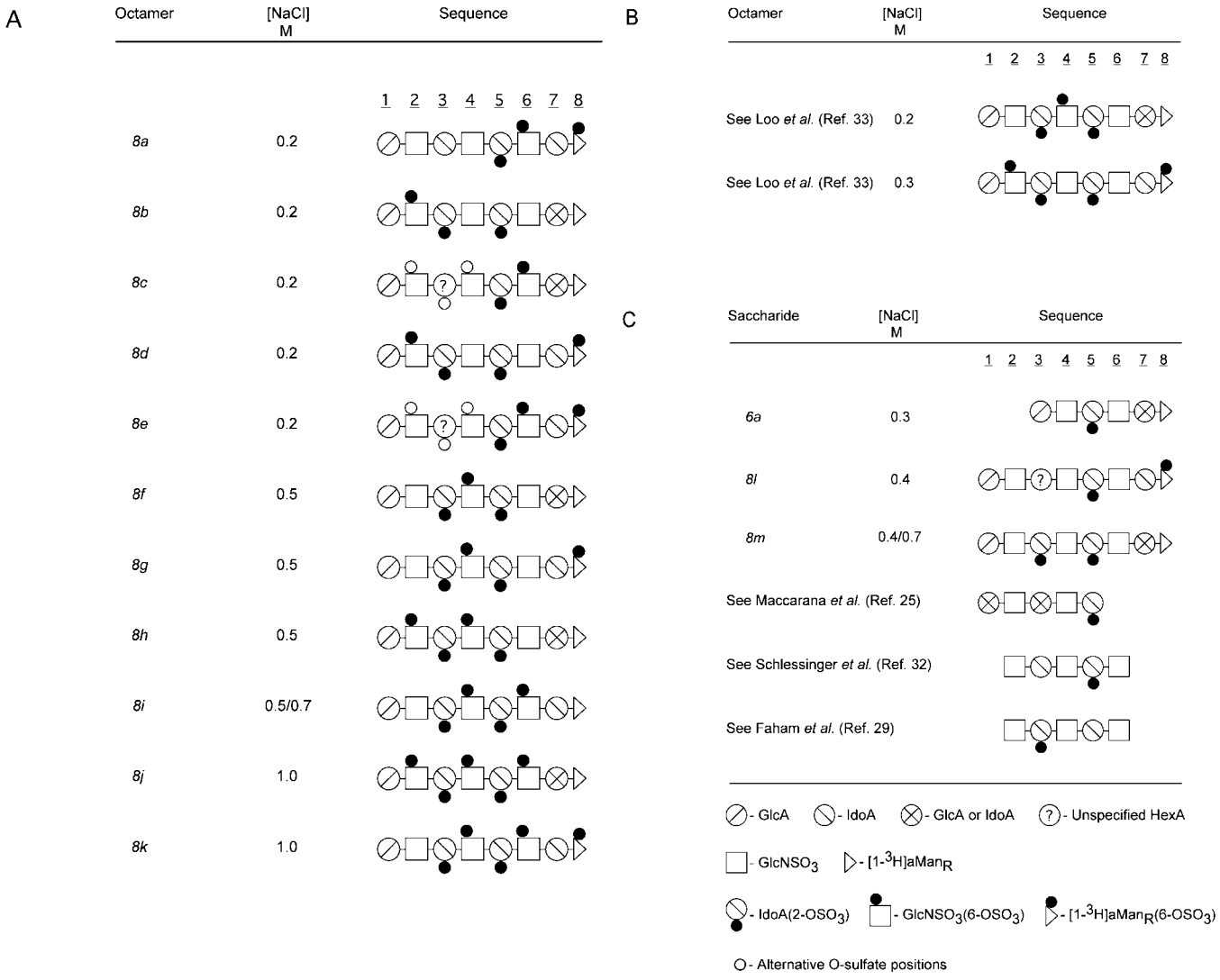

FIG. 10. **Heparan sulfate structures interacting with FGF-1, FGF-2, and FGFR-4.** The NaCl concentration required to displace each oligosaccharide species from its affinity matrix is indicated. *A*, HS octasaccharides fractionated on immobilized FGF-1. *B*, HS octasaccharides fractionated on immobilized FGFR-4 ectodomain as shown by Loo *et al.* (33). *C*, HS hexa- and octasaccharides fractionated on immobilized FGF-2. Structures previously implicated in FGF-2 binding are shown for comparison and have been aligned to maximal fit with structure *8m*.[7] The contribution of individual *N*-sulfate groups to growth factor binding has not been considered.

starting material for the preparation of labeled oligosaccharides were derived from complex tissues (intestinal mucosa and vascular wall), presumably from a variety of proteoglycan species variously located at cell surfaces and in the extracellular matrix. We have no information regarding the distribution of differentially sulfated NS domains between these proteoglycans, nor do we know whether all of these domains, only selected subpopulations, or yet other domains with mixed acetyl and sulfate *N*-substituents (not evaluated in the present study) are accessible to functional interactions with different FGFs in the intact tissues. Moreover, the HS/FGF-1 interaction may fulfill various biological purposes. Importantly, modulation of the binding of FGF to HS may regulate FGF-FGFR complex formation, receptor dimerization, and activation. However, the interaction may also serve to protect FGF against proteolysis (40), control growth factor distribution in tissues (41), and capture growth factors for HS-mediated "facilitated diffusion" toward molecular encounters at the cell surface (42).

The first attempt at defining the minimal structural requirements for HS interacting with FGF-2 (25) implicated a pentasaccharide sequence with three hexuronic acid units and two *N*-sulfated GlcN residues, the reducing-terminal IdoUA unit being 2-*O*-sulfated (Fig. 10*C*). Lacking methods at the time for direct sequencing, the binding structure was deduced from the compositional analysis of oligosaccharides from different sources, including partially *O*-desulfated heparin. This structure was recovered within one of the FGF-2-binding HS octamers (*8l*) identified in the present work. The IdoUA 2-*O*-sulfate group on unit 5 was found also in octasaccharide *8m*, which bound FGF-2 with higher affinity. The increase in affinity was likely due to the additional 2-*O*-sulfate residue on unit 3. These structures may be compared with the binding motif deduced from crystallographic analysis, by Faham *et al.* (29) of a complex between FGF-2 and a fully sulfated heparin hexasaccharide. This motif included the 2-*O*-sulfate group on unit 3 but not the one on unit 5. Yet another FGF-2-binding motif was put forth by Schlessinger *et al.* (32) in their study of a FGF-2/FGFR-1/heparin 10-mer complex (Fig. 10*C*). Notably, although this motif again differed from that deduced by Faham *et al.* (29), both structures are covered by HS octasaccharide *8m*.[7] Also, variable sets of sulfate groups in a heparin 10-mer could

[7] According to Schlessinger *et al.* (32) 6-*O*-sulfate groups on units 2 and 6 contribute weakly to the interaction.

contribute to FGF-1 binding, as shown by DiGabriele *et al.* (30). These observations suggest that the interaction potential of HS sequences may not be readily deduced from crystal data involving heparin (used as a substitute for the authentic HS provided at the cell surface). Similar concern potentially applies also to the recent crystallographic analyses of more complex interaction systems, involving growth factors, extracellular receptor domains, and heparin oligomers (31, 32). We anticipate that interaction studies, by crystallography as well as other methods, will be refined through the future availability of synthetic homogeneous oligosaccharides that express the minimal structural features required for interactions with growth factors and receptors.

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