

Biosynthetic Oligosaccharide Libraries for Identification of Protein-binding Heparan Sulfate Motifs

EXPLORING THE STRUCTURAL DIVERSITY BY SCREENING FOR FIBROBLAST GROWTH FACTOR (FGF) 1 AND FGF2 BINDING*

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Heparan sulfate is crucial for vital reactions in the body because of its ability to bind various proteins. The identification of protein-binding heparan sulfate sequences is essential to our understanding of heparan sulfate biology and raises the possibility to develop drugs against diseases such as cancer and inflammatory conditions. We present proof-of-principle that *in vitro* generated heparan sulfate oligosaccharide libraries can be used to explore interactions between heparan sulfate and proteins, and that the libraries expand the available heparan sulfate sequence space. Oligosaccharide libraries mimicking highly 6-*O*-sulfated domains of heparan sulfate were constructed by enzymatic *O*-sulfation of *O*-desulfated, end-group ³H-labeled heparin octasaccharides. Acceptor oligosaccharides that were 6-*O*-desulfated but only partially 2-*O*-desulfated yielded oligosaccharide arrays with increased ratio of iduronyl 2-*O*-sulfate/glucosaminyl 6-*O*-sulfate. The products were probed by affinity chromatography on immobilized growth factors, fibroblast growth factor-1 (FGF1) and FGF2, followed by sequence analysis of trapped oligosaccharides. An *N*-sulfated octasaccharide, devoid of 2-*O*-sulfate but with three 6-*O*-sulfate groups, was unexpectedly found to bind FGF1 as well as FGF2 at physiological ionic strength. However, a single 2-*O*-sulfate group in the absence of 6-*O*-sulfation gave higher affinity for FGF2. FGF1 binding was also augmented by 2-*O*-sulfation, preferentially in combination with an adjacent upstream 6-*O*-sulfate group. These results demonstrate the potential of the enzymatically generated oligosaccharide libraries.

Heparan sulfate (HS)¹ is a sulfated polysaccharide found in the extracellular matrix and on the surface of most cells as part

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¹ The abbreviations used are: HS, heparan sulfate; aMan_n, 2,5-anhydro-D-mannitol; FGF1, fibroblast growth factor-1; FGF2, fibroblast growth factor-2; GlcUA, β-D-glucuronate; GlcNAc, *N*-acetyl-α-D-glucosamine; GlcNS, *N*-sulfo-α-D-glucosamine; GlcNS6S, *N*,6-*O*-disulfo-α-D-glucosamine; HexUA, hexuronate; IdoUA, α-L-iduronate; IdoUA2S, 2-*O*-sulfo-α-L-iduronate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; MALDI, matrix-assisted laser desorption ionization; HPLC, high performance liquid chromatography.

of proteoglycans. HS binds to a large number of proteins and thus influences a variety of biological processes (1–3). Because of its postulated roles in various phenomena of clinical importance, such as wound healing, inflammation, tumor growth, and accompanying metastasis, there is increasing interest in the potential generation of drugs that may selectively interfere with HS-protein interactions. Alternatively, drugs could substitute for endogenous HS in agonist mode. Nevertheless, the anticoagulant action of heparin, based on interaction with the protease inhibitor antithrombin (4), represents the only significant clinical application of HS-related saccharides so far. A major obstacle to further drug development is the lack of information regarding protein-binding epitopes in HS chains, as well as means of generating tailored saccharides.

The HS carbohydrate backbone consists of alternating hexuronate (HexUA) and α-D-glucosamine monosaccharide units (2, 5). The HexUA can be either β-D-glucuronate (GlcUA) or α-L-iduronate (IdoUA). The amino group of the α-D-glucosamine is usually acetylated (GlcNAc) or sulfated (GlcNS), but it may also be unsubstituted. Within and adjacent to *N*-sulfated domains, 2-hydroxyl groups of IdoUA (IdoUA2S) or, less commonly, GlcUA, and 6-hydroxyl groups of GlcNS (GlcNS6S) or GlcNAc may be sulfated. Additionally, 3-*O*-sulfate groups can occur on GlcNS units. These modifications give rise to a large variety of different motifs along the HS chain, which usually consists of 50–400 monosaccharide units. Considering the heterogeneity of naturally occurring HS, the possibility of different proteins interacting with distinct HS epitopes is apparent. Indeed, following the development of HS sequencing methods (6, 7) protein-specific HS motifs have been elucidated (8, 9).

Attempts to identify protein-binding HS motifs have so far relied on HS isolated from tissues (10). Owing to the structural variability of HS molecules from different cells (5, 11, 12), there is an impending risk that a HS epitope assigned to interaction with a given protein *in vivo* may be poorly represented in a tissue-derived HS pool used for screening *in vitro*.

We have therefore taken a novel approach, generating HS libraries *in vitro*, that are based on enzymatic *O*-sulfation of *N*-sulfated precursors. The potential of the library saccharides to functionally mimic HS domains was verified by their selec-

samine; GlcNS, *N*-sulfo-α-D-glucosamine; GlcNS6S, *N*,6-*O*-disulfo-α-D-glucosamine; HexUA, hexuronate; IdoUA, α-L-iduronate; IdoUA2S, 2-*O*-sulfo-α-L-iduronate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; MALDI, matrix-assisted laser desorption ionization; HPLC, high performance liquid chromatography.

tive interactions with fibroblast growth factor-1 (FGF1) and FGF2.

EXPERIMENTAL PROCEDURES

Chromatography Methods—Separation of oligosaccharides of different size was performed by size-exclusion chromatography at a flow rate of 2 ml/h on a column (10 × 1500 mm) of Bio-Gel P-10 (Bio-Rad) equilibrated with 0.5 M NH₄HCO₃. Oligosaccharide solutions were desalted by size-exclusion chromatography using PD-10 columns (Sephadex G-25) (Amersham Biosciences) equilibrated with H₂O. Anion-exchange HPLC of oligosaccharides was performed using a ProPac PA1 column (Dionex, Surrey, UK), equilibrated with H₂O, or with a NaCl solution, adjusted to pH 3 by addition of HCl. In analytical scale experiments, the column was connected to a 500 TR Flow Scintillation Analyzer (Packard, Meriden, CT). Samples were loaded, and NaCl gradients (10 mM/ml) were used to elute the oligosaccharides at a flow rate of 1 ml/min. Unless otherwise indicated in legends to figures, the gradients started at [NaCl] = 0 (see “Results”).

Preparation of O-Desulfated Heparin Octasaccharides—The procedure employed in library generation is outlined in Scheme 1. Completely or partially O-desulfated heparin from bovine lung (Upjohn) was used to generate oligosaccharide substrates for library synthesis. 6-O-Sulfate and some 2-O-sulfate groups were removed by incubating 50 mg of heparin pyridinium salt with 2.5 ml of dimethyl sulfoxide containing 10% (v/v) methanol at 100 °C for 16 h (13). Part of the product (10 mg), was completely 2-O-desulfated by dissolution in 10 ml of 30 mM NaOH, pH 12.5, followed by lyophilization (14). The N/O-desulfated heparins were re-N-sulfated by incubation with 4.5 ml of 80 mM Na₂CO₃, 50 mM sulfur trioxide trimethylamine complex at 55 °C for 24 h (15). The 6-O- and partially 2-O-desulfated preparation contained 42% -IdoUA2S-GlcNS- disaccharide units, but no detectable 6-O-sulfated components, as verified by disaccharide composition analysis (HNO₂, pH 1.5, NaB³H₄ procedure; Ref. 16) (data not shown).

To obtain oligosaccharides, O-desulfated, re-N-sulfated heparin preparations (8 mg of completely O-desulfated heparin in 800 μl and 1 mg of completely 6-O-desulfated, partially 2-O-desulfated heparin in 250 μl, respectively) were partially cleaved by treatment with 2.5 mM NaNO₂, 70 mM H₂SO₄, pH 1.5 (17), on ice for 3 h. Reaction mixtures were adjusted to pH 8 by addition of 4 M NaOH, and oligosaccharides were then reduced with excess NaB³H₄ (5 mCi, ~60 Ci/mmol, Amersham Biosciences), yielding a ³H-labeled 2,5-anhydro-D-mannitol (aMan_R) residue at the reducing end. Labeled oligosaccharides were separated with regard to size on the Bio-Gel P-10 column, and fractions corresponding to octasaccharides were recovered for enzymatic O-sulfation.

Anion-exchange HPLC (ProPac PA1 column) of the extensively O-desulfated octasaccharide gave a predominant peak at the elution position expected for a fully N-sulfated 8-mer, devoid of O-sulfate groups (see “Results” and *top panel* in Fig. 1). Partial cleavage at GlcNS residues (HNO₂, pH 1.5) (6, 8) yielded distinct peaks at positions corresponding to di-, tetra-, hexa-, and octasaccharides with the predicted number of N-sulfate groups (data not shown). Separate digestions of these oligosaccharide products with the α-iduronidase and β-glucuronidase confirmed that most (>90%) of the hexuronates were IdoUA units, as expected for a heparin-derived material (data not shown). By contrast, the partially 2-O-sulfated octasaccharide preparation was highly heterogeneous, and contained components ranging from zero to four O-sulfate groups per molecule. The elution pattern from the ProPac PA1 column was consistent with a stochastic mode of 2-O-desulfation (see “Results” and *top panel* in Fig. 3). This assumption was verified by sequence analysis (see below) of purified 8-mers, which revealed 2-O-sulfate groups in all potential positions (data not shown).

Enzymatic O-Sulfation of Octasaccharides—O-Sulfation of oligosaccharides was catalyzed by O-sulfotransferases from solubilized mouse mastocytoma microsomal fraction (18, 19) and by recombinant HS 2-O-sulfotransferase from human embryonic kidney 293 (293-HEK) cell lysates (20). The two enzyme preparations contained ~30 and ~20 mg of protein/ml, respectively, as judged by absorbance measurements. O-Sulfation reactions contained 3'-phosphoadenosine 5'-phosphosulfate (PAPS) at the concentrations indicated, 3–30 μM oligosaccharide (~300 cpm ³H/pmol), 10 mM MnCl₂, 5 mM CaCl₂, 10 mM MgCl₂, 3.5 μM NaF, 0.3% (v/v) Triton X-100, 50 mM Hepes, pH 7.4, and 10–50% (v/v) microsomal fraction or 293-HEK cell lysate. The reactions were incubated at 37 °C for 4 h. Two oligosaccharide libraries were generated.

Library 1—Completely O-desulfated octasaccharide, 50% of the final library, was incubated with 293-HEK cell lysate and 1 mM PAPS. Half of this reaction mixture was fortified with microsomal O-sulfotrans-

ferase and 5 mM PAPS and incubated for another 4 h. The remaining 50% was incubated with microsomal O-sulfotransferase and 5 mM PAPS only. All reaction products (2 × 10⁷ cpm ³H) were then pooled to give Library 1.

Library 2—6-O-Desulfated and partially 2-O-desulfated octasaccharide (5 × 10⁶ cpm ³H) was incubated with mastocytoma microsomal O-sulfotransferase and 5 mM PAPS.

Before use, both libraries were adsorbed on DEAE-Sephacel (Amersham Biosciences), equilibrated with H₂O, pH 3, to remove proteins, and then eluted with 1 M NaCl. The libraries were desalted by passage through PD-10 columns.

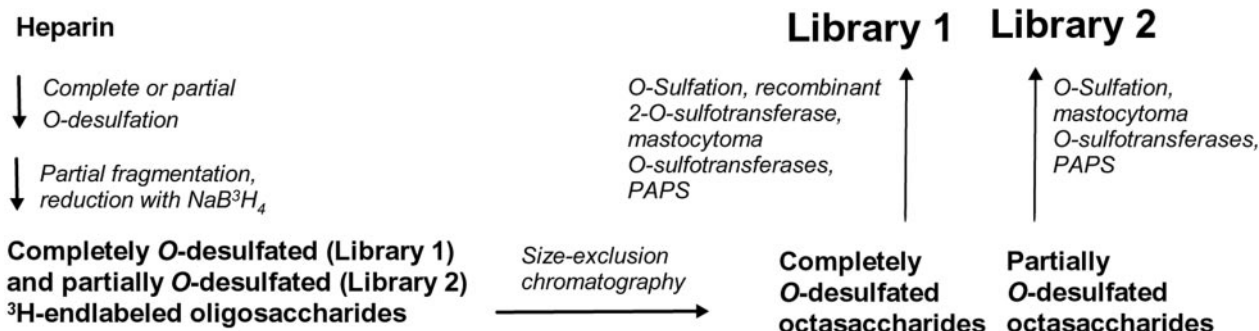
Affinity Chromatography—Preparations of recombinant FGFs were as described (21). Affinity columns were made by coupling primary amines in the proteins (1 mg) to activated esters in Activated CH Sepharose 4B gel matrix (1 g, dry weight), in a total volume of 4 ml according to the instructions of the manufacturer (Amersham Biosciences). To protect lysine residues involved in binding of HS, FGF-Sepharose conjugates were prepared in the presence of heparin (5 mg), which had been treated with HNO₂ at pH 3.9 to eliminate N-unsubstituted glucosamine residues (17). The FGF columns were equilibrated with 50 mM Tris/HCl, pH 7.4, and the respective octasaccharide library (~2 × 10⁶ cpm) was loaded. After washing the columns with buffer, bound octasaccharides were eluted using a stepwise NaCl gradient in 50 mM Tris/HCl, pH 7.4.

Characterization of Affinity-fractionated Oligosaccharides—Octasaccharides were separated further by anion-exchange HPLC on the ProPac PA1 column. Molecular weights of oligosaccharides were determined by MALDI mass spectrometry as described (22, 23) with slight modifications. Oligosaccharides (10 pmol) were directly dissolved in 4 μl of the matrix solution (12 mg of caffeic acid/ml in 50% aqueous acetonitrile) containing 20 pmol/ml basic peptide (Arg-Gly)₁₉-Arg (Sigma-Genosys Ltd.). The peptide-oligosaccharide sample (1 μl) was then deposited on the stainless steel MALDI plate and dried at room temperature. Further sample purification was performed *in situ* on the MALDI target as described (24). Mass spectrometry measurements were performed on a Bruker Biflex III MALDI time-of-flight instrument (delayed extraction, mass gate set to 2000 Da) calibrated with cytochrome *c* and adrenocorticotrophic hormone.

Purified and partially purified octasaccharides were subjected to ³H-end label sequence analysis, based on a combination of chemical and exoenzymatic degradation steps (6–8). The enzymes used were from Oxford GlycoSciences (Abingdon, UK), except for glucosamine 6-sulfatase that was a gift from J. Hopwood (Adelaide, Australia). Briefly, oligosaccharides were subjected to partial deaminative cleavage at the sites of GlcNS units, and the products were separately digested with (i) iduronate 2-sulfatase, (ii) a mixture of iduronate 2-sulfatase and α-iduronidase, and (iii) a mixture of iduronate 2-sulfatase, α-iduronidase, and glucosamine 6-sulfatase (*cf.* “Results” and Fig. 4). The deamination and enzyme digestion products were analyzed on the ProPac PA1 column.

RESULTS

Characterization of Octasaccharide Libraries—Oligosaccharide libraries were obtained by subjecting variously O-desulfated, ³H-end-labeled heparin octasaccharides to enzymatic O-sulfation (Scheme 1). Preliminary experiments with completely O-desulfated substrate, mastocytoma enzymes, and 5 mM PAPS resulted in appreciable O-sulfation of the fragments. Anion-exchange chromatography of the products showed non-, mono-, di-, and tri-O-sulfated major components in varying proportions depending on the precise conditions, whereas more highly sulfated species occurred in smaller amounts (data not shown). The sulfation afforded by the sulfotransferases was critically dependent on the PAPS concentration, as judged by the number and positions of peaks obtained on anion exchange chromatography; however, higher PAPS concentration than 5 mM did not further increase the degree of sulfation. Release of the terminal, ³H-labeled disaccharide units by HNO₂, pH 1.5, cleavage yielded nonsulfated disaccharide only, as shown by anion-exchange HPLC, indicating that the IdoUA-aMan_R sequence was not recognized as substrate for O-sulfation. Sequence analyses of isolated octasaccharides (not shown) indicated an average GlcNS6S:IdoUA2S ratio of ≥10:1. To increase the relative amount of 2-O-sulfate groups, the octasaccharide substrate was



SCHEME 1

first exposed to recombinant 2-O-sulfotransferase expressed in 293-HEK cells (20), and the product was then further incubated with mastocytoma microsomal O-sulfotransferases, as described under "Experimental Procedures," to generate Library 1 (Fig. 1). Whereas the use of 2-O-sulfotransferase thus increased the 2-O-sulfate content (with ~ 0.5 2-O-sulfate groups per octamer in the library), the octasaccharides provided by Library 1 still showed a higher 6-O-/2-O-sulfate ratio than commonly observed in N-sulfated domains of tissue-derived HS (8). Because many HS-protein interactions involve 2-O-sulfate groups (25), we aimed at further increasing the degree of 2-O-sulfation by using a completely 6-O-/2-O-desulfated, but only partially 2-O-desulfated, heparin octasaccharide preparation as substrate for the microsomal O-sulfotransferases. Again, extensive 6-O-sulfation of the fragments was obtained, resulting in a highly heterogeneous assembly of octasaccharides (Library 2; Fig. 3).

Probing the Oligosaccharide Libraries for FGF Ligands—The octasaccharides of Library 1 were probed for FGF2 binding using affinity chromatography on immobilized growth factor, and the resultant fractions of ^3H -labeled octasaccharides were separated further by anion-exchange HPLC (Fig. 1). Most of the octasaccharides, including those without O-sulfate groups, were retained by FGF2 in 50 mM Tris/HCl, pH 7.4. Adding 0.15 M NaCl (to attain $I = 0.20$) released most of the oligosaccharides from the column. The affinity fractions eluted between $I = 0.25$ and $I = 0.55$ contained distinct octasaccharides that apparently had been selected for. There was no simple relation between FGF2 affinity and oligosaccharide charge density, as illustrated by the occurrence of tri/tetra-O-sulfated, and mono-O-sulfated species in the $I = 0.25$ and $I = 0.45$ fractions, respectively (Fig. 1). Most of the oligosaccharides retained at higher ionic strengths were absent in the $I = 0.20$ fraction, demonstrating that the FGF2 column was not overloaded. Notably, the four major octasaccharide fragments of Library 1 were poorly retained by the FGF2 column at $I > 0.20$, and none remained bound at $I > 0.25$. However, the major tri-O-sulfated component **a** appeared in the $I = 0.25$ m fraction. To ascertain that this octasaccharide was indeed bound to FGF2 at physiological ionic strength (see "Discussion"), purified component **a** was reappplied onto the FGF2 column and eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris/HCl, pH 7.4. The octasaccharide emerged with a peak elution position corresponding to $I \sim 0.3$, thus at higher than physiological ionic strength (data not shown).

Library 1 was also subjected to selection against immobilized FGF1 (Fig. 2). At least one of the octasaccharides retained on the FGF2 column bound also to FGF1 with appreciable affinity (component **c** in Figs. 1 and 2). However, most (if not all) of the other octasaccharides in the FGF1 affinity fractions differed from those in corresponding fractions from the FGF2 selection, reflecting the different requirements for O-sulfate groups in growth factor binding.

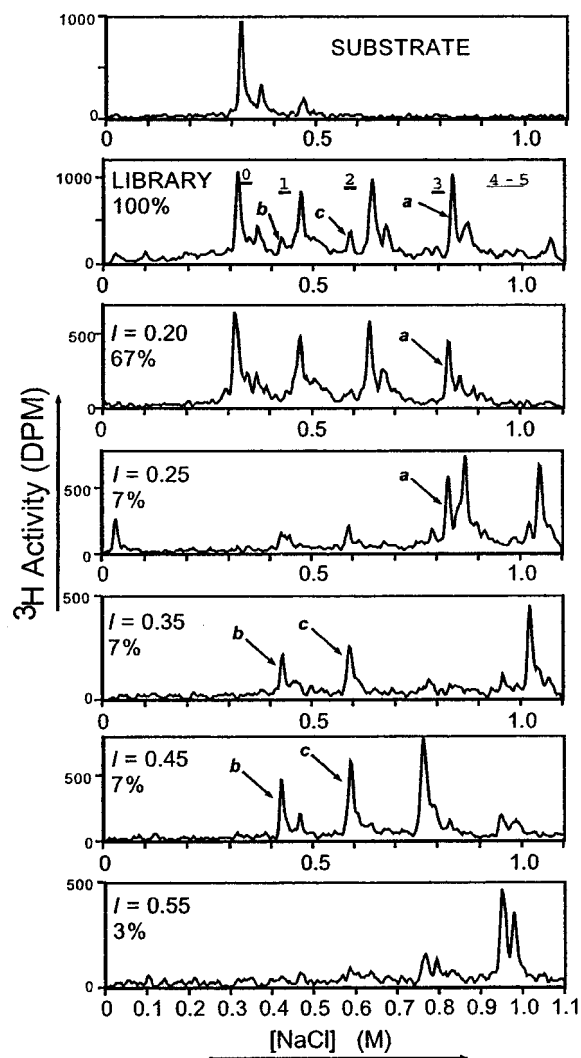


FIG. 1. Anion-exchange chromatography of octasaccharide substrate, complete Library 1, and subfractions separated with regard to affinity for FGF2 (see "Experimental Procedures"). The fragments were eluted from the FGF2 column at the ionic strengths and yields indicated. The approximate number of O-sulfate groups in octasaccharides appearing at the different elution positions are indicated in the LIBRARY panel (underlined). The octasaccharides **a**, **b**, and **c**, marked by arrows, were subjected to HNO_2 /exoenzyme sequence analysis.

Library 2, obtained through enzymatic O-sulfation of partially 2-O-desulfated, exhaustively 6-O-desulfated heparin octamer, was loaded onto the FGF1 affinity column. Again, distinct octasaccharide species emerged at the different ionic strengths used to develop the column (Fig. 3). As seen for

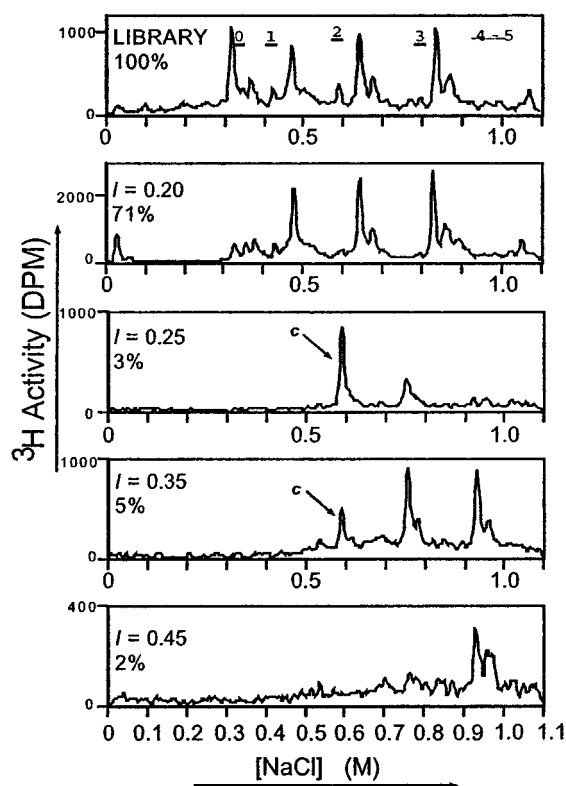


FIG. 2. Anion-exchange chromatography of octasaccharides in Library 1 and subfractions separated with regard to affinity for FGF1 (see "Experimental Procedures"). The fragments were eluted from the FGF1 column at the ionic strengths and yields indicated. For additional information, see Fig. 1. The octasaccharide *c*, marked by an arrow, is identical to *c* in Fig. 1.

Library 1 with FGF1 as probe (Fig. 2), as well as previously for native HS *N*-sulfated domains (8), the fractions showing highest affinity toward FGF1 contained predominantly highly sulfated oligosaccharides. However, selected octasaccharides of lower charge density remained bound to the growth factor at relatively high ionic strength, e.g. fragments *e* and *g* in Fig. 3, indicating selectivity in binding also to FGF1.

Sequence Analysis of Affinity-fractionated Octasaccharides—Following purification on the ProPac PA1 column of the affinity-fractionated octamers, several oligosaccharides were sequenced using the strategy developed by Vivès *et al.* (7) (see "Experimental Procedures"). Fig. 4 shows an example of such analysis, of the fragments making up peak *g* in Fig. 3. Notably, the method enables concomitant analysis of mixtures of two (or even three) components, provided that their degree of sulfation is known (8). The occurrence of two components in fraction *g* was indicated by the identification of two distinct disaccharides, IdoUA-³H]aMan_R and IdoUA2S-³H]aMan_R, following exhaustive deaminative cleavage of the end group-labeled parent fraction (data not shown). Accordingly, exoenzyme digestions of the products obtained by partial deamination revealed two distinct tetrasaccharides and also two hexasaccharides (Fig. 4). All structures deduced are shown in Fig. 5. No GlcUA residues were found, except at the nonreducing terminus (unit 1) of fragment *c*. The relative molecular masses of three octasaccharides were determined as 1831 (*a*), 1672 (*b*), and 1754 (*c*), respectively, by MALDI mass spectrometry analysis, in agreement with the results of the sequencing.

DISCUSSION

In vitro generated libraries are important tools in protein and nucleic acid biochemistry. For example, libraries of RNA,

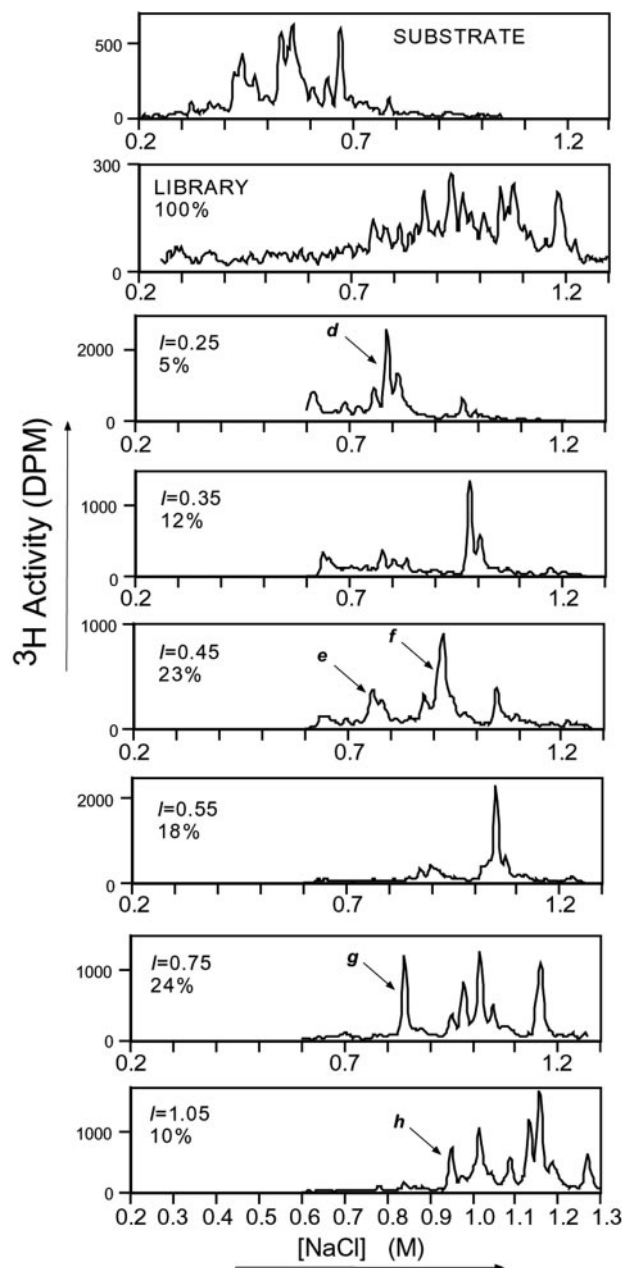


FIG. 3. Anion-exchange chromatography of octasaccharide substrate, complete Library 2, and subfractions separated with regard to affinity for FGF1 (see "Experimental Procedures"). Before loading the sample, the column was equilibrated with a NaCl solution of the same concentration as the starting concentration of the gradient (0.2 M for the substrate octasaccharides, 0.25 M for the complete library, and 0.6 M for the affinity-separated fractions). Library 2 was obtained by enzymatic O-sulfation of extensively 6-O-desulfated, partially 2-O-desulfated heparin octamer (SUBSTRATE panel). The fragments were eluted from the FGF1 column at the ionic strengths and yields indicated. The octasaccharides *d*, *e*, *f*, *g*, and *h*, marked by arrows, were subjected to HNO₂/exoenzyme sequence analysis.

obtained by a combination of organic synthesis and RNA polymerase-catalyzed reactions, have been used to develop new catalytic RNA molecules (e.g. Ref. 26) with impact on the theories of the origin of life (27). Enzyme libraries can be created by changing the corresponding genes in stochastic manner using, e.g., DNA shuffling (28). By expressing the variant genes, enzymes with improved or novel properties can be obtained (29, 30). Glycosaminoglycans constitute a large class of macromolecules of increasingly recognized biological significance. Their functional roles are largely expressed through interac-

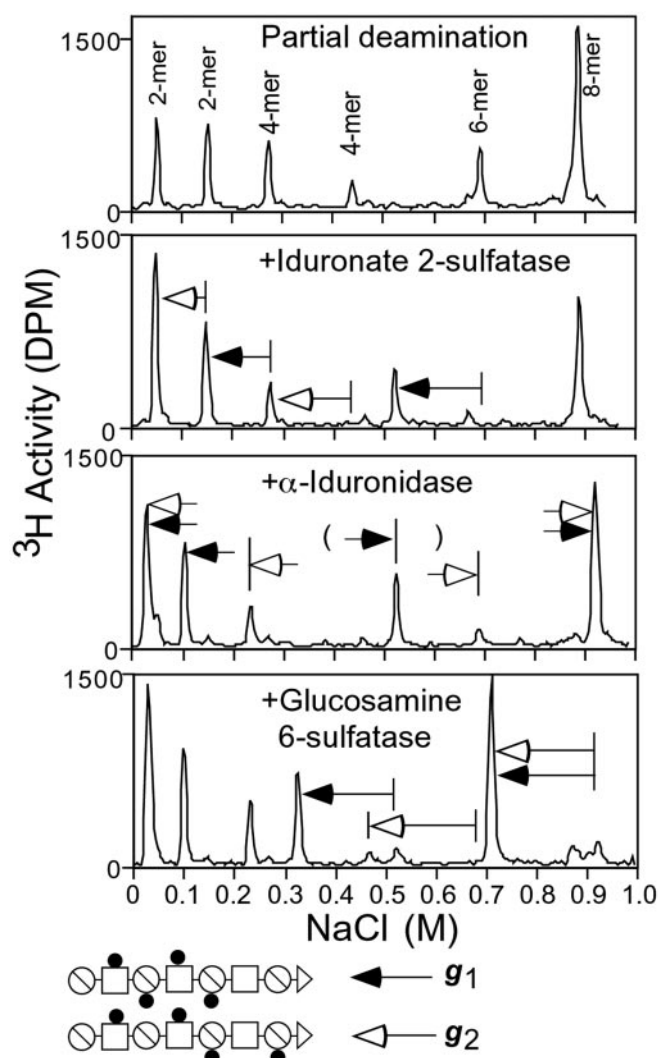


FIG. 4. Sequence analysis (HNO_2 /exoenzyme procedure) of the two octasaccharides of peak *g* (see Fig. 3). The sample was first subjected to partial deaminative cleavage (6 mM NaNO_2 , 20 mM HCl, incubated at room temperature; aliquots withdrawn at different time points during 1–30 min were adjusted to pH 6 and pooled), and then to digestions with either iduronate 2-sulfatase alone (10 microunits/ μl), iduronate 2-sulfatase and α -iduronidase (10 microunits/ μl), or iduronate 2-sulfatase, α -iduronidase glucosamine 6-sulfatase (0.1–0.5 microunits/ μl), as indicated in the panels. The top panel shows the anion-exchange HPLC of end group-labeled oligomers obtained by partial deamination of the parent octasaccharide fraction. The arrows indicate the shifts in elution position induced by the different enzymatic treatments; black arrows, fragments derived from *g*₁; hollow arrows, fragments derived from *g*₂ (see Fig. 5 for details on the structures). See Refs. 7 and 8 for further details.

tions with proteins, and studies of recent years indicate that many such interactions depend critically on the fine structures of distinct saccharide domains. Attempts to define structure/function relationships for these polymers would benefit from the use of *in vitro* generated libraries. Although purely synthetic saccharide libraries are emerging (31, 32), this field is still in its infancy.

Our present findings demonstrate that studies of complex HS-protein interactions are indeed amenable to a library approach, using a combined chemical and enzymatic strategy to generate diversity. This approach has several advantages over studies involving oligosaccharides derived from authentic HS. The starting point for library generation is *N*-sulfated but *O*-desulfated heparin oligosaccharides, relatively monodisperse with regard to molecular size compared with the hetero-

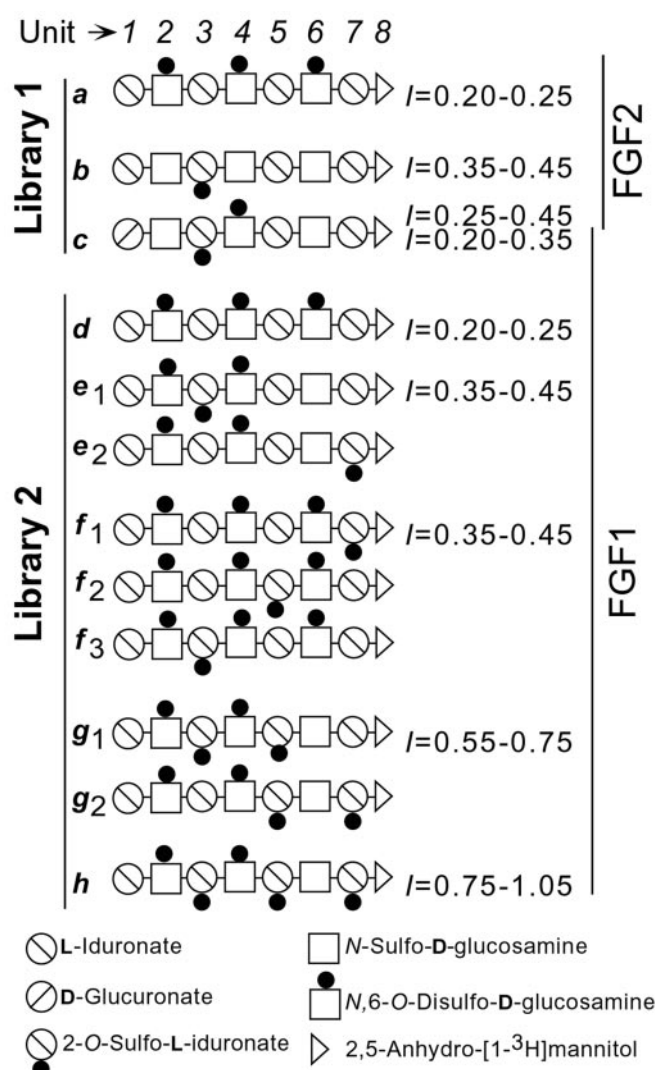


FIG. 5. Structures of *in vitro* generated HS-like octasaccharides recovered from immobilized FGF2 (see Fig. 1) and FGF1 (see Figs. 2 and 3). The fragments were eluted from the respective columns at the ionic strengths indicated. The structures were deduced as described in Fig. 4 and “Experimental Procedures” and in Refs. 7 and 8. Fractions *e*, *f*, and *g* contained more than one oligosaccharide.

ogeneous fractions recovered after partial depolymerization of native HS. The products generated by enzymatic *O*-sulfation mimic *N*-sulfated domains from HS chains in their ability to bind FGF1 and FGF2. However, the *in vitro* generated HS-like oligosaccharides may reveal motifs that are expressed *in vivo*, but in such small amounts that they cannot be isolated from tissue-derived HS. Libraries of the types described thus expand the experimentally available HS sequence space, as shown through generation of highly 6-*O*-sulfated oligosaccharides devoid of 2-*O*-sulfate groups. Although 6-*O*-sulfation occurs adjacent to a preformed 2-*O*-sulfate group, the reverse order of events is prohibited because of the substrate specificities of *O*-sulfotransferases involved (18, 33). This restriction is reflected by the excessive 6-*O*-sulfation observed following incubation of *O*-desulfated heparin oligomers with mixed *O*-sulfotransferases from mastocytoma tissue. The problem was partly alleviated by incubating the substrate with recombinant 2-*O*-sulfotransferase before further incubation with mixed *O*-sulfotransferases (Library 1). To achieve more extensive 2-*O*-sulfation, however, partially 2-*O*-desulfated, exhaustively 6-*O*-desulfated octamers were employed as substrate (Library 2).

Sequence analysis of affinity-captured oligosaccharide spe-

cies is an essential feature of our library strategy. This aim was achieved by enzymatic incorporation of unlabeled sulfate groups into [³H]end group-labeled oligosaccharide components, the radiolabel serving as the reference point required for alignment of exoenzyme-digested fragments (7, 8). Very recently, Wu *et al.* (34) reported a somewhat similar project, also based on enzymatic incorporation of sulfate groups into heparin-derived oligosaccharides. These experiments aimed primarily at defining the types of O-sulfate groups required for interaction with a given protein. However, the protocol employed did not involve isolation of protein-binding oligosaccharide species. Moreover, oligosaccharides were radiolabeled by use of ³⁵S-labeled PAPS as sulfate donor, and thus were not readily amenable to sequence analysis.

The pending availability of all the biosynthetic enzymes as heterologously expressed proteins will lead to further opportunities of expanding, as well as tailoring, HS libraries. In theory, it should be possible to generate all 128 conceivable 2-O-sulfated (four positions) and 6-O-sulfated (three positions) IdoUA-containing octasaccharides by fine-tuning of conditions and substrates. Further diversity of the oligosaccharides, with regard to the hexuronate, may be created by treatment of O-desulfated heparin ([4IdoUA α 1-4GlcNS1 α -]_n) or of chemically N-sulfated capsular polysaccharide from *Escherichia coli* K5 ([4GlcUA β 1-4GlcNS α 1-]_n), with GlcUA C5-epimerase, that catalyzes reversible C5 inversion of the HexUA units (35). "Composite" libraries may be produced by mixing products obtained by the separate approaches outlined. Future engineering of the biosynthetic enzymes may allow random or directed synthesis of enzyme-generated libraries *in vitro*, that cover the entire potential sequence space of a HS oligosaccharide.

Although only a few of these options have been exploited so far, the applications of the oligosaccharide libraries to FGF binding demonstrate the potential of the approach, as novel aspects of FGF-HS interactions were revealed. The FGF1 and FGF2 targets were selected because octasaccharides derived from N-sulfated domains of authentic HS have been separated with regard to affinity for these growth factors and sequenced (8). These HS oligomers represented primarily a section of the sequence space dominated by 2-O-sulfate groups, and affinity fractionation of such fragments against FGF2 highlighted 2-O-sulfate (along with N-sulfate) groups as essential for strong binding. 6-O-Sulfate groups on the other hand were considered insignificant (8, 36–40). Accordingly, the single 2-O-sulfate group on unit 3 confers appreciable affinity of library fragment **b** for FGF2. Nevertheless, fragment **a**, with three 6-O-sulfates but no 2-O-sulfate group, binds FGF2 at physiological ionic strength (Figs. 1 and 5). A sulfation pattern similar to that of fragment **a** is quite unusual in authentic HS, but may explain why murine cells deficient in 2-O-sulfotransferase could rescue FGF2-dependent receptor signaling by increasing 6-O-sulfation of HS (41).

Previous studies showed that both 2-O- and 6-O-sulfate groups are important for the interaction of HS with FGF1, and an -IdoUA2S-GlcNS6S-IdoUA2S- trisaccharide motif comprising units 3–5 was implicated with high affinity binding. A shift of the 6-O-sulfate group from unit 4 to unit 2 leads to appreciable loss of affinity (8). The specific trisaccharide motif was also identified in the high affinity library fragments **g**₁ and **h**. However, determinants for FGF1 binding could be further qualified through the present library approach. For example, comparison of **g**₁ and **g**₂ shows that the -IdoUA2S-GlcNS6S-IdoUA2S- motif is not obligatory for strong binding. Addition of a 2-O-sulfate group on unit 3 of **g**₂ or unit 7 of **g**₁ yields

fragment **h**. Hence, for fragment **h**, these 2-O-sulfate groups contribute about equally to the affinity. Further, the structure/affinity relations for fractions **e**₁, **e**₂ and **f**₁, **f**₂, **f**₃ suggest that octasaccharides with two or three adjacent 6-O-sulfate groups, will gain affinity for FGF1 (*cf.* fragment **d**) by the presence of a single 2-O-sulfate group regardless of its position. Still, the combined effects of the 2-O-substituents are reflected by the high affinities of fragments **g**₁, **g**₂, and **h** (Fig. 5). These findings again emphasize the expanded sequence space of the *in vitro* generated libraries that may be exploited to probe the functional roles of different motifs and individual residues.

In HS biosynthesis 2-O-sulfation generally precedes 6-O-sulfation, and the distribution of the latter substituents appears to be quite strictly regulated (12, 42). Thus, whereas excessively 6-O-sulfated sequences such as that displayed by fragment **a** are rarely seen in nonmanipulated systems, 2-O- and 6-O-sulfated structures of **g** and **h** type may be detected in authentic HS (8, 42). The physiological significance of the high affinity for FGF1 (and other growth factors) expressed by these structures, as compared with weaker binders, remains unclear. Conceivably, a gradient of HS motifs with increasing affinities may serve to capture and direct a growth factor molecule toward the site of interaction with its tyrosine kinase receptor target at the cell surface (43). However, we cannot exclude that high affinity binding may sequester growth factors at sites on HS chains out of reach for receptor interaction. Productive interaction appears to depend on binding of not only FGF but also its receptor to the same HS chain (25). It will therefore be important to isolate and structurally characterize HS domains capable of accommodating both proteins in a ternary complex.

In conclusion, the oligosaccharide libraries obtained in the current work present a novel tool to explore protein-HS interactions. Other potential applications include the analysis of substrate specificity for biosynthetic enzymes such as sulfotransferases. Finally, the technique may be useful in guiding future enzyme engineering toward catalysts that can generate tailored HS oligosaccharides, for use as reagents in research or even as drugs.

REFERENCES

- Sasisekharan, R., and Venkataraman, G. (2000) *Curr. Opin. Chem. Biol.* **4**, 626–631
- Esko, J. D., and Lindahl, U. (2001) *J. Clin. Invest.* **108**, 169–173
- Varki, A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 543–545
- Bourin, M.-C., and Lindahl, U. (1993) *Biochem. J.* **289**, 313–330
- Lindahl, U., Kusche-Gullberg, M., and Kjell n, L. (1998) *J. Biol. Chem.* **273**, 24979–24982
- Turnbull, J. E., Hopwood, J. J., and Gallagher, J. T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2698–2703
- Vives, R. R., Pye, D. A., Salmivirta, M., Hopwood, J. J., Lindahl, U., and Gallagher, J. T. (1999) *Biochem. J.* **339**, 767–773
- Kreuger, J., Salmivirta, M., Sturiale, L., Gim nez-Gallego, G., and Lindahl, U. (2001) *J. Biol. Chem.* **276**, 30744–30752
- Loo, B.-M., Kreuger, J., Jalkanen, M., Lindahl, U., and Salmivirta, M. (2001) *J. Biol. Chem.* **276**, 16868–16876
- Gallagher, J. T. (2001) *J. Clin. Invest.* **108**, 357–361
- Brickman, Y. G., Ford, M. D., Gallagher, J. T., Nurcombe, V., Bartlett, P. F., and Turnbull, J. E. (1998) *J. Biol. Chem.* **273**, 4350–4359
- Safaiyan, F., Lindahl, U., and Salmivirta, M. (2000) *Biochemistry* **39**, 10823–10830
- Nagasawa, K., Inoue, Y., and Kamata, T. (1977) *Carbohydr. Res.* **58**, 47–55
- Jaseja, M., Rej, R. N., Sauriol, F., and Perlin, A. S. (1989) *Can. J. Chem.* **67**, 1449–1456
- Levy, L., and Petracek, F. J. (1962) *Proc. Soc. Exp. Biol. Med.* **190**, 901–905
- Pejler, G., B ckstr m, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S., and Timpl, R. (1987) *J. Biol. Chem.* **262**, 5036–5043
- Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
- Jacobsson, I., and Lindahl, U. (1980) *J. Biol. Chem.* **255**, 5094–5100
- Kusche, M., Hannesson, H. H., and Lindahl, U. (1991) *Biochem. J.* **275**, 151–158
- Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2000) *Biochem. J.* **346**, 463–468
- Zazo, M., Lozano, R. M., Ortega, S., Varela, J., D az-Orejas, R., Ram rez, J. M., and Gim nez-Gallego, G. (1992) *Gene (Amst.)* **113**, 231–238
- Juhasz, P., and Biemann, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 4333–4337
- Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R. (1999) *Science* **286**, 537–542

24. Beavis, R. C., and Chait, B. T. (1990) *Anal. Chem.* **62**, 1836–1840
25. Casu, B., and Lindahl, U. (2001) *Adv. Carbohydr. Chem. Biochem.* **57**, 159–206
26. Johnston, W. K., Unrau, P. J., Lawrence, M. S., Glasner, M. E., and Bartel, D. P. (2001) *Science* **292**, 1319–1325
27. Gesteland, R. F., Check, T. R., and Atkins, J. F. (eds) (1999) *The RNA World*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Stemmer, W. P. C. (1994) *Nature* **370**, 389–391
29. Cramer, A., Raillard, S.-A., Bermudez, E., and Stemmer, W. P. C. (1998) *Nature* **391**, 288–291
30. Joo, H., Lin, Z., and Arnold, F. H. (1999) *Nature* **399**, 670–673
31. Flitsch, S. L. (2000) *Curr. Opin. Chem. Biol.* **4**, 619–625
32. Bartolozzi, A., and Seeberger, P. H. (2001) *Curr. Opin. Struct. Biol.* **11**, 587–592
33. Kobayashi, M., Habuchi, H., Habuchi, O., Saito, M., and Kimata, K. (1996) *J. Biol. Chem.* **271**, 7645–7653
34. Wu, Z. L., Zhang, L., Beeler, D. L., Kuberan, B., and Rosenberg, R. D. (2002) *FASEB J.* **16**, 539–545
35. Hagner-McWhirter, Å., Lindahl, U., and Li, J.-P. (2000) *Biochem. J.* **347**, 69–75
36. Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) *Biochem. J.* **285**, 805–813
37. Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem.* **268**, 23898–23905
38. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) *J. Biol. Chem.* **267**, 10337–10341
39. Ishihara, M., Shaklee, P. N., Yang, Z., Liang, W., Wei, Z., Stack, R. J., and Holme, K. (1994) *Glycobiology* **4**, 451–458
40. Kreuger, J., Prydz, K., Pettersson, R. F., Lindahl, U., and Salmivirta, M. (1999) *Glycobiology* **9**, 723–729
41. Merry, C. L. R., Bullock, S. L., Swan, D. C., Backen A. C., Lyon, M., Beddington, R. S. P., Wilson, V. A., and Gallagher, J. T. (2001) *J. Biol. Chem.* **276**, 35429–35434
42. Merry, C. L. R., Lyon, M., Deakin, J. A., Hopwood, J. J., and Gallagher, J. T. (1999) *J. Biol. Chem.* **274**, 18455–18462
43. Lander, A. D. (1998) *Matrix Biol.* **17**, 465–472

Biosynthetic Oligosaccharide Libraries for Identification of Protein-binding Heparan Sulfate Motifs: EXPLORING THE STRUCTURAL DIVERSITY BY SCREENING FOR FIBROBLAST GROWTH FACTOR (FGF) 1 AND FGF2 BINDING

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