Heparan Sulfate-related Oligosaccharides in Ternary Complex Formation with Fibroblast Growth Factors 1 and 2 and Their Receptors*

Received for publication, January 26, 2006, and in revised form, June 27, 2006 Published, JBC Papers in Press, June 28, 2006, DOI 10.1074/jbc.M600806200

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Biosynthesis of heparan sulfate (HS) is strictly regulated to yield products with cell/tissue-specific composition. Interactions between HS and a variety of proteins, including growth factors and morphogens, are essential for embryonic development and for homeostasis in the adult. Fibroblast growth factors (FGFs) and their various receptors (FRs) form ternary complexes with HS, as required for receptor signaling. Libraries of HS-related, radiolabeled oligosaccharides were generated by chemo-enzymatic modification of heparin and tested for affinity to immobilized FR ectodomains and for affinity to immobilized FR ectodomains, and this interaction was further promoted by FGF1 but not by FGF2. In all systems studied, the stability of FGF-oligosaccharide-FR complexes correlated with a multitude of processes in development and homeostasis, due to their ability to interact with a variety of proteins (1–3). Such interactions involve basic amino acid residues and are mediated by negatively charged carboxyl and sulfate groups along the HS chains. Biosynthesis of HS is initiated by the formation of a [4GlcAβ1-4GlcNAca1]₃p precursor polysaccharide that is synthesized on a proteoglycan core protein. Subsequent enzymatic modification reactions involve partial replacement of N-acetyl by N-sulfate groups, C5-epimerization of GlcA to L-iduronic acid (IdoUA), and finally O-sulfation at various positions (reviewed in Ref. 4). N- and O-sulfate groups typically occur clustered in domains of high negative charge that provide primary sites of interactions with proteins. The biosynthetic polymer-modification reactions are regulated to yield a vast number of different sequences that appear to be cell and tissue specific (5–8) and to change during development (9, 10) and aging (11). HS has also been implicated in a variety of disease conditions (12, 13).

It is generally held that the structural diversity of HS confers selectivity to interactions with distinct proteins (14, 15). This notion applies to the family of fibroblast growth factors (FGFs) that induce a variety of signaling events essential to development and homeostasis (for review, see Ref. 16). FGFs are polypeptides with a conserved core of ~120 amino acid residues and more variable N- and C-terminal domains. The human FGF family consists of 22 species that are grouped in seven different subfamilies based on phylogenetic analysis (for review, see Ref. 17). The growth factors bind with variable selectivity to four different FGF receptors (FRs); in addition, FR1–3 present isoforms (b and c forms) due to alternative splicing of the IgIII domain in the extracellular ligand-binding part of the receptor. HS is required for binding of FGFs to their receptors (18, 19), and several lines of evidence indicate that the polysaccharide interacts with both proteins, in at least partly selective manner (10, 20–24). Attempts at defining HS structures required for binding of different FGFs revealed some distinctive features but also considerable sharing of binding sites (25, 26).

In the present study, we have sought to identify HS-related saccharide structures needed to form ternary complexes with two well characterized FGFs, FGF1 and FGF2, and their receptors. These growth factors, among others, regulate a number of events during embryogenesis and homeostasis (27–29) (for review, see Ref. 16). FGF1 is the only FGF able to activate both b and c splice variants of FR1–3 as well as FR4, whereas FGF2 activates all receptors except FR2b and FR3b (30, 31). We have used affinity chromatography based on immobilized FR ectodomains, soluble FGF1 or FGF2, and radiolabeled oligosaccharide libraries. The libraries were generated by chemo-enzymatic modification of size-defined, heparin-derived oligosac-
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charides and covered an extended sequence space. The results point to non-selective involvement of oligosaccharides in complexes between both growth factors and FR1c, FR2c, and FR3c, whereas FGF1 and FGF2 differ in their saccharide requirement for complex formation with FR4.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FGF1 and FGF2 were produced in a bacterial expression system as described (32). Soluble FGF receptor-alkaline phosphatase (FR/AP) fusion proteins consisted of the three extracellular Ig-like loop domains of FR1, FR2, and FR3 (all IIIc splice variants) and the extracellular part of FR4 fused to the N-terminus of human placental-AP (33). FR/AP cDNAs were kindly provided by D. Ornitz (Washington University, St. Louis, MO). The receptor proteins were expressed in COS-7 cells and purified as described (22). Anti-human placenta AP-agarose beads were obtained from Sigma. Heparin from bovine lung was purified as described (34). HS from porcine intestinal mucosa was a kind gift by G. van Dedem (Diosynth, Oss, The Netherlands). HS from porcine intestinal mucosa was a kind gift by G. van Dedem (Diosynth, Oss, The Netherlands). BioGel P-10 and empty 10-ml columns were purchased from Bio-Rad (Sundbyberg, Sweden), and a ProPac PA-1 column was from Dionex (Surrey, UK). All other materials was of best quality available.

Preparation of Radiolabeled Oligosaccharides—Heparin and HS radiolabeled oligosaccharides were obtained as described (36). Briefly, heparin was subjected to partial deaminative cleavage with HNO2 (pH 1.5), and the products were reduced with NaB3H4, separated according to size, and recovered 10- and 12-mer fractions were further fractionated with regard to 6-O-sulfation by anion-exchange HPLC on ProPac PA-1. Subfractions were pooled and desalted for subsequent affinity chromatography.

Affinity Chromatography—FR/AP proteins were immobilized by incubating 50 pmol of each protein for 2 h at 4 °C with 0.2 ml of anti-human placental AP-agarose beads equilibrated with 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl. The FR/AP suspension was transferred to 10-ml Bio-Rad columns (0.1 ml/column), washed with 100 volumes of 50 mM Tris/HCl, pH 7.4, 2 M NaCl, and finally re-equilibrated with the initial buffer. 3H-radiolabeled oligosaccharides (2–20 × 103 cpm corresponding to 0.3–3 pmol), FGF (3–30 pmol of FGF1 or FGF2), or a combination of both (mixed just prior to application to the column), in 50 μl of equilibration buffer, were applied to the columns that were maintained for 30 min at 4 °C and subsequently eluted with a stepwise gradient ranging from 0.15 to 2 M NaCl in 50 mM Tris/HCl, pH 7.4. Effluent fractions (0.2 ml) were collected and analyzed for radioactivity by scintillation counting. Control runs with labeled heparin oligosaccharides with and without FGFs on beads not substituted with FR/AP showed no binding under the conditions of the experiments. Heparin oligomers bound the FR1c–3c at very low ionic strength (i.e. 50 mM Tris/HCl, pH 7.4), as predicted by interaction data reported by others (40, 41) but were completely eluted at 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl. Moreover, FGFs were applied to FR/AP columns in the absence of saccharides. Resultant effluent fractions were precipitated with trichloroacetic acid and analyzed by SDS-PAGE (42) followed by silver staining (43). In all affinity separations, the number of receptor molecules exceeded those of oligosaccharides by a factor of 10–100 to avoid saturation of the system. The molar ratio of oligosaccharides to growth factors was ~1:10, as optimized in pilot runs.

Effluent fractions from affinity chromatographies of oligosaccharide libraries were pooled as indicated, desalted on PD-10 columns, and further analyzed by anion-exchange HPLC on ProPac PA-1. Reaplication experiments were performed with a number of combinations of FGF and FR, in which bound and unbound pools were separately reapplied to the FR/AP columns and eluted as described above.

RESULTS

Formation of FGF-Heparin-FR Ternary Complex—The ability of HS-related oligosaccharides to support formation of ternary complexes with FGFs and FRs was assessed by affinity chromatography of radiolabeled oligosaccharides on immobilized FR/AP in the presence of the growth factors. The FR/AP fusion proteins were immobilized through their AP domains, presumably in dimerized form due to AP dimer formation in solution (44). In pilot experiments aimed at defining the experimental conditions conducive to FGF-saccharide–FR ternary
complex formation, large $^3$H-heparin oligomers (>18-mers) (Fig. 1A) were applied to the receptor columns in the presence or absence of FGFs. In the absence of FGF, the oligosaccharides emerged quantitatively in the unbound pool (unretarded in or absence of FGFs. In the absence of FGF, the oligosaccharides (Fig. 1B) were observed in fractions (12-mer in size) applied to an affinity column with immobilized FR2c in the absence of FGF. When added instead together with FGFs and FRs, size-defined, radiolabeled heparin oligomers (>6-mer) were applied to the receptor columns in the presence of FGF1 or FGF2. Results are presented as the proportion of total amounts applied, eluted at moderate (>0.2-0.4 M NaCl, open bars) or high ionic strength (>0.4-2.0 M NaCl, closed bars). Similar results were obtained in repeat experiments.

**FIGURE 1.** FGF-heparin-FR complex formation on immobilized FR2c. A, structure of a heparin oligosaccharide with n repeats of the typical heparin disaccharide, consisting of 2-O-sulfated idoUA and N-/6-O-sulfated GlcN residues. Dark ellipsoid, 2-O-sulfate; light ellipsoid, 6-O-sulfate; empty rectangle, N-sulfate. B, $^3$H-radiolabeled heparin fragments (>18-mer in size) were applied to an affinity column with immobilized FR2c in the absence of FGF (——), $^3$H-radiolabeled heparin 10-mer (—■—) and 12-mer (—▲—) oligomers were applied to the same affinity column together with FGF1. The column was eluted using a stepwise salt gradient (NaCl concentration, ——), and effluent fractions were analyzed for radioactivity by scintillation counting. Highly similar results were obtained for FR1c–3c. Inset, results are presented as the proportion of the total amounts of each oligosaccharide applied that was eluted at moderate (>0.2-0.4 M NaCl, open bars) and high (>0.4-2.0 M NaCl, closed bars) ionic strength (10, 10-mer oligosaccharides; 12, 12-mer oligosaccharides).

**FIGURE 2.** Binding of heparin oligomers to FR2c and FR4 in the presence of FGF1 or FGF2. A, binding of size-defined $^3$H-heparin oligosaccharides (6-12-mer) to FR2c in the presence of FGF1 or FGF2 as indicated. B, binding of 6-12-mers to FR4 alone (——) and in the presence of FGF1 or FGF2 as indicated. Binding of heparin oligomers to FR2c is representative for FR1c and FR3c. Results are presented as the proportion of total amounts applied, eluted at moderate (>0.2-0.4 M NaCl, open bars) or high ionic strength (>0.4-2.0 M NaCl, closed bars). Similar results were obtained in repeat experiments.

Dependence on Oligosaccharide Size—A variety of techniques, including crystallography as well as several binding and signaling assays, have been used to demonstrate that heparin oligosaccharides of different sizes mediate interactions between various FGFs and FRs. We therefore tested our assembly assay for oligosaccharide size requirements, in different combinations with FGFs and FRs. Size-defined, radiolabeled heparin oligomers (>6-mer) were applied together with FGF1 or FGF2 to FR affinity columns. Two typical experiments are shown in Fig. 1B, in which heparin 10- and 12-mer fractions showed a broad distribution of retarded components that emerged from 0.3 to 0.8 M NaCl and from 0.4 to 1.4 M NaCl for 10- and 12-mer oligomers, respectively. Based on these pilot experiments, oligomers eluted with >0.2 M salt were all considered capable of complex formation, whereas complex stability was reflected by the actual elution position in the salt gradient. In the following, an arbitrary distinction between “moderate affinity binding” (>0.2-0.4 M NaCl) and “high affinity binding” (>0.4 M NaCl) will be applied.

Heparin 8-mers were the shortest fragments capable of (largely moderate affinity) complex formation of FGF1 with FR1c, FR2c, or FR3c (Fig. 2A). Decasaccharides revealed a larger proportion of complex-bound species, as well as an increase in the proportion of high affinity binders. With 12-mers, no further increase in complex generation was observed, although a slight increase in affinity was noted. FGF2, on the other hand, required smaller heparin oligomers than FGF1 for complex formation with each of the three receptors (Fig. 2A). An 8-mer induced maximal complex formation, only a slight increase in apparent affinity being observed with larger oligosaccharides (Fig. 2A and data not shown). Repeat analyses
gave patterns very similar to those shown in Fig. 2A, with no significant deviations between different FRs (data not shown). Contrary to FR1c–3c, FR4 retained >18-mer heparin oligosaccharides even in the absence of FGF ligands, with an affinity reflected by elution at 0.4 – 0.6 M NaCl (data not shown). This property also applied to shorter oligosaccharides, essentially down to 10-mer size (Fig. 2B). Co-application with FGF1 promoted complex formation of even smaller heparin fragments (6- and 8-mers) and also led to increased complex stability. Only minor changes were observed upon co-application of FGF2 when compared with control runs without FGF (Fig. 2B). When compared with FGF2, FGF1 thus was more prone to complex formation with heparin oligosaccharides and FR4, contrary to the effects seen with FR1c–3c (Fig. 2, A and B, and data not shown).

Dependence on O-Sulfation—The oligosaccharides obtained by deaminative cleavage of unmodified heparin were predominantly N-sulfated and contained mostly 2-O-sulfated IdoUA and 6-O-sulfated GlcN residues. To assess the importance of different O-sulfate substituents, we generated endgroup 3H-labeled, size-defined oligosaccharide libraries that were designed to provide extensive sequence diversity with regard to O-sulfation. Oligosaccharides devoid of 6-O-sulfate groups were fractionated with regard to the extent of 2-O-sulfation, and each subclass was subjected to enzymatic 6-O-sulfation (see “Experimental Procedures”). Each resulting library thus contained species with a fixed number of 2-O-sulfate but a variable number of 6-O-sulfate groups. Both the 2-O-sulfate and the 6-O-sulfate groups were variably distributed within the oligomers (37). The libraries were separately applied to the FR-columns, in different combinations with FGF1 and FGF2.

2-O-Sulfation—Oligosaccharides with two 2-O-sulfate groups (and variable 6-O-sulfation) generated small proportions of moderate affinity complexes with FGF1 and FR1c or FR2c. Three and four 2-O-sulfate groups lead to increased amounts of ternary complex, still of largely moderate affinity type, and also to some complex formation with FR3c (Fig. 3). Affinity separations with FGF2 showed generally higher yields of complexes and larger proportions of high affinity species. These binding patterns were underpinned in corresponding experiments with 10-mer libraries, with a tendency toward promotion of high affinity complexes. The relative but consistent preference for FGF2 over FGF1 in complex formation of oligosaccharide libraries with receptors FR1c–3c agrees well with the results obtained with fully sulfated heparin oligomers (Fig. 2A).

Similar to the fully sulfated 10-mers derived from unmodified heparin (Fig. 2B), 10-mer libraries containing at least three 2-O-sulfate groups showed appreciable binding to FR4 even in the absence of FGFs (Fig. 3D). Again, the addition of FGF2 had only marginal effect. FGF1, on the other hand, greatly promoted complex formation of both 8- and 10-mer libraries with FR4 (Fig. 3D). Although significant binding was observed already with mono-2-O-sulfated fragments, increased 2-O-sulfation led to augmented complex formation and to higher affinity interaction with FGF1 and FR4.

6-O-Sulfation—The impact of 6-O-sulfation on ternary complex formation was evaluated in two types of experiments. First, library oligosaccharides separated on FR columns in the presence of FGF1 or FGF2. Library pools containing the indicated numbers of 2-O-sulfate (and variable numbers of 6-O-sulfate) groups were separated on immobilized FR1c (A), FR2c (B), FR3c (C), and FR4 (D). Oligosaccharides were also analyzed on FR4 in the absence of FGFs (–) (D). Results are presented as the proportion of total amounts applied, eluted at moderate (>0.2 – 0.4 M NaCl, open bars) or high ionic strength (>0.4 – 2.0 M NaCl, closed bars). Similar results were obtained in repeat experiments.

![FIGURE 3. Binding of octa- and decasaccharide libraries to FRs in the presence of FGF1 or FGF2. Library pools containing the indicated numbers of 2-O-sulfate (and variable numbers of 6-O-sulfate) groups were separated on immobilized FR1c (A), FR2c (B), FR3c (C), and FR4 (D). Oligosaccharides were also analyzed on FR4 in the absence of FGFs (–) (D). Results are presented as the proportion of total amounts applied, eluted at moderate (>0.2 – 0.4 M NaCl, open bars) or high ionic strength (>0.4 – 2.0 M NaCl, closed bars). Similar results were obtained in repeat experiments.](image-url)
Anion-exchange HPLC of affinity-fractionated oligosaccharides by anion-exchange HPLC. A, anion-exchange HPLC separation of a 10-mer oligosaccharide library before affinity fractionation. The library was prepared from a 10-mer oligosaccharide fraction containing three 2-O-sulfate groups by microsomal modification as described under "Experimental Procedures." The numbers above the peaks denote the number of 2-O- and 6-O-sulfate groups of the fragments represented by the respective peaks. The 10-mer oligosaccharide library (A) was separated on an FR1c column in the presence of FGF1 (Fig. 3A). The unbound pool (eluted at 0.15–0.2 M NaCl) (B) and the bound pool (eluted at >0.2–2.0 M NaCl) (C) were analyzed by anion-exchange HPLC, as described under "Experimental Procedures."

sulfate groups. The unfractonated 10-mers were found to range from zero to four 6-O-sulfate groups (Fig. 4A, (3 + x), indicating numbers of 2-O- and 6-O-sulfate groups, respectively). Separation of this library on an FR1c column in the presence of FGF1 yielded ~60% unbound fraction (Fig. 3A). Analysis of the unbound material by anion-exchange HPLC showed (3 + 0) components but no significant amounts of 6-O-sulfated oligosaccharides (Fig. 4B). The bound fraction (~40%), eluted within the >0.2–2.0 M range of the gradient (thus including both moderate affinity and high affinity species) contained virtually all 6-O-sulfated components ((3 + 2), (3 + 3), (3 + 4); (3 + 1) was difficult to evaluate due to low abundance in starting material) of the unfractonated library (Fig. 4C). A small proportion of (3 + 0) components found in the bound pool (Fig. 4C), was due to incomplete resolution during affinity chromatography (see the second approach below). Taken together, these results suggested that N-sulfated 10-mers with three 2-O-sulfate groups could mediate binding of FGF1 to immobilized FR1c, only if they carried in addition a minimum of two (possiby only one) 6-O-sulfate residues.

In a second approach, library oligosaccharides were first fractionated by anion-exchange HPLC followed by affinity chromatography of the separated fractions. The results obtained, illustrated in Fig. 5 for the 10-mer library carrying three 2-O-sulfate groups per molecule, confirm and extend the conclusions of separations performed in the reverse order (Fig. 4). Isolated (3 + 0) 10-mers, thus lacking 6-O-sulfate groups, were not retained by the FR1c column in the presence of FGF1, at 0.2 M NaCl concentration, whereas a small proportion of the (3 + 2) fraction bound with moderate affinity. Fragments with three or four 6-O-sulfate groups yielded larger proportions of ternary complex and increased stability of complexes, as predicted (Fig. 5). In this particular 10-mer library, penta O-sulfated components within the (3 + 2) subfraction represented the least sulfated species compatible with FGF1-FR1c complex formation. To investigate whether both 2-O- and 6-O-sulfate groups were required, fragments lacking either type of substituents were prepared (see "Experimental Procedures") and tested for FGF1-FR1c binding. An N-sulfated decamer containing four 2-O- or 6-O-sulfate groups thus showed minor binding, which was slightly increased by the addition of a fifth 2-O- or 6-O-sulfate group (Fig. 5). Taken together, these findings suggest that N-sulfated 10-mers containing four or five O-sulfate residues, irrespective of type, are capable of interacting with FGF1 and FR1c at higher than physiologic ionic strength.

The experimental strategies illustrated in Figs. 4 and 5 were applied to various combinations of oligosaccharides, FGFs and FRs. In Fig. 6 are oligosaccharides classified as non-binding, moderately binding, and strongly binding, according to the criteria defined. FGF1 required 8- or 10-mers with at least 5 O-sulfate groups for moderate binding to FR1c–3c and 10-mers with

FIGURE 4. Anion-exchange HPLC of affinity-fractionated oligosaccharides by anion-exchange HPLC. A, anion-exchange HPLC separation of a 10-mer oligosaccharide library before affinity fractionation. The library was prepared from a 10-mer oligosaccharide fraction containing three 2-O-sulfate groups by microsomal modification as described under "Experimental Procedures." The numbers above the peaks denote the number of 2-O- and 6-O-sulfate groups of the fragments represented by the respective peaks. The 10-mer oligosaccharide library (A) was separated on an FR1c column in the presence of FGF1 (Fig. 3A). The unbound pool (eluted at 0.15–0.2 M NaCl) (B) and the bound pool (eluted at >0.2–2.0 M NaCl) (C) were analyzed by anion-exchange HPLC, as described under "Experimental Procedures."

FIGURE 5. Complex formation of FGF1 and FR1c with fractions isolated from oligosaccharide libraries and from depolymerized, 2-O-desulfated heparin. Library 10-mers containing three 2-O-sulfate groups were fractionated by anion-exchange HPLC (Fig. 4A), and fractions differing with regard to number of 6-O-sulfate groups (3 + 0; 3 + 2; 3 + 3; 3 + 4) were isolated and individually tested for binding to FR1c in the presence of FGF1. Fragments with four or five 2-O-sulfate groups (4 + 0; 5 + 0) isolated from two other 10-mer libraries were also tested for binding. In addition, 10-mers with three or four 6-O-sulfate groups, lacking 2-O-sulfate (0 + 3:0 + 4), and a 12-mer with five 6-O-sulfate groups (0 + 5)*, derived from 2-O-desulfated heparin, were assessed in a similar fashion. Results are presented as the proportion of total amounts applied, eluted at moderate (>0.2–0.4 M NaCl, open bars) and high ionic strength (>0.4–2.0 M NaCl, closed bars). Similar results were obtained in repeat experiments.
7 0-sulfate groups for strong binding (Fig. 6, A and E). Corresponding requirements for FGF2 were similar but generally satisfied at somewhat lower degrees of O-sulfation (Fig. 6, B and F; see also Fig. 3). The difference between FGF1 and FGF2 was reversed, and more pronounced, in interactions with FR4. Octasaccharides carrying only 3 O-sulfate groups thus managed complex formation with FGF1 and FR4, whereas 6 O-sulfate groups were required with FGF2 (Fig. 6, C, D, and G). Notably, 2-O- and 6-O-sulfate groups could generally compensate for each other in complex formation involving all combinations of FGFs and FRs tested. To assess the element of reversibility in complex formation, bound and unbound fractions derived from a charge-homogeneous (3 + 4) library 10-mer were reapplied to the same receptor matrix along with new FGF. Each sample yielded new bound and unbound fractions in the same proportions initially observed (data not shown). In all, these results implicate overall charge/sulfate density as the major selection parameter in recruitment of saccharides to complex formation with the two growth factors and their receptors but provide no evidence for more selective structural preference.

Oligosaccharides from Authentic Heparan Sulfate—To assess the relevance of the binding data obtained using library oligosaccharides, complex formation was also studied using N-sulfated 10-mers derived from two species of authentic HS. The two oligosaccharide preparations, from liver and intestine HS, generated ternary complexes with FGF1, FGF2, and FR1c, FR2c, and FR4 (Fig. 7). The extent of complex formation varied between different FGF and FR combinations, essentially in accord with data obtained with library oligosaccharides (Fig. 3). In combination with FR2c, FGF2 thus tended to form more stable complexes with both HS oligosaccharides when compared with FGF1. With FR4, these relations were clearly reversed since the HS oligosaccharides promoted amplex complex formation with FGF1 but not with FGF2 (Fig. 7; cf. Fig. 3D).

FIGURE 6. FGF-FR binding properties of library oligosaccharides. Octasaccharide libraries were tested as described with FGF1 and FR1c–3c (A); with FGF2 and FR1c–3c (B); with FGF1 and FR4 (C); and with FGF2 and FR4 (D). Decasaccharide libraries were analyzed with FGF1 and FR1c–3c (E); with FGF2 and FR1c–3c (F); and with FGF1 and FR4 (G). Decasaccharides binding to FR4 was only marginally augmented by FGF2, and these combinations were therefore not tested in detail. Open boxes, no oligosaccharide binding above 0.2 M NaCl; gray boxes, 10–50% of oligosaccharides eluted at >0.2–0.4 M NaCl concentration (e.g. Fig. 5, 3 + 2 + 3); black boxes, >50% of oligosaccharides eluted at >0.2–2.0 M NaCl concentration (Fig. 5, 3 + 4). a), number of 2-O-sulfate groups in left column, b), number of 6-O-sulfate groups in top row. *, 12-mer oligosaccharides were used. †, binding with moderate affinity to FR1c and FR2c but not to FR3c.

FIGURE 7. Complex formation of FGFs and FRs with decasaccharides from authentic HS. N-Sulfated 10-mers isolated from liver (Li) or intestine (In) HS were tested for binding to FR1c, FR2c, or FR4 in the presence of FGF1 or FGF2. Results are presented as the proportion of total amounts applied, eluted at moderate and high ionic strength, as in Fig. 1. Similar results were obtained in repeat experiments.

Only minor differences in complex formation were noted between oligosaccharides from liver and intestine.

DISCUSSION

The requirement for heparin- or HS-related saccharides in FGF-FR signaling has been recognized for over a decade, following the pioneering discoveries by different groups (18, 19, 47). Attempts to define the functional role of the carbohydrate have been largely based on mitogenic assays with HS-deficient cells, stimulated with FGF in the presence of exogenous saccharide. Conclusions of such early studies, that the saccharide needs to interact with both the FGF and the FR moieties (20, 48), were later verified through crystallization of ternary complexes (45, 46). Use of fully sulfated heparin or partially desulfated heparin as exogenous saccharide reagent in signaling systems precluded any detailed analysis of structural requirements. Binding studies with isolated FGFs pointed to some differences regarding preferred saccharide ligand structure but also to extensive sharing of saccharide epitopes (25, 26, 49). Information regarding the more complex problem of identifying minimal saccharide structures required for simultaneous interaction with FGF and FR in ternary complexes has remained inconclusive and partly contradictory (21, 23, 24, 50).

In the present study, we have used oligosaccharide libraries to define structures required to support formation of ternary complexes with the growth factors, FGF1 and FGF2, and the receptors, FR1c, FR2c, FR3c, and FR4. Receptor constructs (FR-AP) were immobilized, and radiolabeled 8- and 10-mer N-sulfated oligosaccharides with systematically varied proportions of O-sulfate groups were separated on these receptor columns in the presence of FGF under conditions precluding FR1c–3c binding of oligosaccharides on their own. Salt elution profiles were interpreted in terms of amount and stability of ternary complexes formed. Although such profiles may overemphasize the ionic component of interaction (51), they presumably reflect the relative affinities of different oligosaccharides toward the same receptor-ligand complex. Oligosaccharide fractions capable of supporting complex formation were identified and distinguished from those that were relatively inactive. By and large, closely similar oligosaccharide
fractions promoted complex formation between either FGF1 or FGF2 and all four receptors tested, i.e. FR1c–3c and FR4. Some quantitative difference was noted in that FR1c–3c was more prone to oligosaccharide-mediated complex formation with FGF2 than with FGF1, whereas FR4 showed the reverse preference (Figs. 3 and 6). FR4 was unique in binding sulfated oligosaccharides, under the chosen conditions, even in the absence of FGF, in accord with previous findings (52, 53). With all FGF-FR combinations tested, increased levels of oligosaccharide O-sulfation correlated with amounts and stability of complexes formed. No striking preference for 2-O- or 6-O-sulfate groups was noted in our assay system (Figs. 3, 5, and 6). Oligosaccharides isolated from authentic HS substituted as predicted for library oligosaccharides in FGF-FR complex formation (Fig. 7). The ability of HS chains to promote ternary complex formation between FGF1 or FGF2 and their receptors thus would seem to depend primarily on the abundance, length, and overall O-sulfation of their N-sulfated domains and not on the selective saccharide sequence/precise location of sulfate groups. Whether similar conditions also apply to other FGFs remains an open question that is currently under investigation. It is moreover recognized that ternary complex formation, under the conditions of this study, does not necessarily reflect cell signaling potential, and it will therefore be important to test the oligosaccharides in cell-based signaling assays.

A number of studies have actually correlated HS structure with cell activation potential (21, 23, 24, 50, 54). Authentic HS decamers with an average of 2.5 2-O- and 1.5 6-O-sulfate groups represented minimal structures, in terms of size and charge required to activate FR1c-transfected BaF3 cells challenged with FGF2 in a proliferation assay (50). These results agree with our findings of ternary complex formation supported by a minimum of 4 O-sulfate groups (Fig. 6F). Further, cell stimulation capacity was linearly related to increasing 6-O-sulfation over a constant background of N- and 2-O-sulfation. More pronounced activation, however, was seen with 12-mer oligosaccharides upon substoichiometric increase in 6-O-sulfation, conceivably indicative of position-specific effects (50). Similar heparin lyase-generated authentic HS-oligosaccharides were further subfractionated by anion-exchange chromatography and used for stimulation of FR-transfected BaF3 cells in the presence of FGF2 (21). The cell stimulatory effects of the resultant species did not correlate with their degree of sulfation. Further contradictory results were obtained in studies on the effects of variously O-desulfated heparins on signaling (20, 23, 24, 54). A critical issue and potential explanation for different results in all of these studies could be the different types of cells and test conditions used, with a downstream readout in the form of cell proliferation over 2–3 days.

The relative lack of specificity regarding carbohydrate structure in FGF1- or FGF2-HS-FR interactions is also inferred from observations of quite disparate experimental systems. Compelling indications of structurally lax interactions of HS with FGF-FR derive from observations in vivo of phenotype traits in mice genetically deficient in enzymes required for HS biosynthesis. Several FGF-dependent patterning events in embryonic development, involved in the appropriate formation of the gut/intestine and heart (FGF9 and 10 (29, 55)), brain (FGF2 and 8 (56) and other FGFs, for review, see Ref. 31), and liver (FGF1 and 2 (28)), appear normal in mice deficient in GlcA C5-epimerase or 2-O-sulfotransferase (57, 58). These enzymes are required for the formation of IdoUA and the subsequent 2-O-sulfation of these units, respectively, and their absence results in severely deranged HS structures that either lack IdoUA altogether or contain IdoUA residues that are non-sulfated. On the other hand, both N-sulfation and GlcN 6-O-sulfation are augmented in the mutant when compared with wild-type HSs such that the overall charge density of the polysaccharide remains essentially unchanged. Apparently, such “mutated” HSs can substitute for normal HS in several physiologically essential interactions with FGF-FR ligands. These findings argue against specificity at sequence level with regard to HS structure and rather suggest preference based on the occurrence of N-sulfated domains of the appropriate length and overall degree of O-sulfation.

Yet another type of experimental data, to be reconciled with our present findings, was obtained using AP-tagged FR-ectodomains together with FGFs to target endogenous HS in mouse embryo tissues at different developmental stages (10, 22). The major conclusions from those studies were that there exist (i) developmental stage-specific, and in some cases, organ-specific differences in HS and (ii) that some of these differences could be traced to variations in HS sulfation pattern (e.g. differences in 2-O-sulfation). However, the major differences were observed with FGF4 and FGF8b, whereas FGF2 (and possibly FGF1) appeared more promiscuous in their HS-binding requirements (10, 22). Notably, FGF1 and FGF2 belong to the same FGF subfamily with common properties distinctive from those of other members of the FGF superfamily (17). FGFs from other subfamilies, especially those with more restricted expression patterns regarding time and cell subsets (e.g. FGF4 and FGF8b), could conceivably depend on more restricted HS structures. Preliminary findings with FGF8b suggest that structural requirements other than sulfation degree are important for complex formation.

A second contrast between the current study and studies in which the FGF and FR-AP probes are provided in soluble form to a limited number of HS chains in a tissue section (10, 22) is that receptor complex detected in the ligand and carbohydrate engagement assay may involve oligomerization of multiple FGFs and FR-APs, even on different HS chains. Although such oligomerization could highlight binding requirements different from those measured here, we assume that both systems may contribute information relevant to the in vivo signaling systems.

Interestingly, our results indicate that the stability of FGF-HS-FR complexes can be modulated through graded O-sulfation of N-sulfated-binding sites. Such modulation may be of pathophysiological significance. Mutations in the FGF-binding domains of FR1–3, leading to increased affinity between FGFs and FRs, and thus to augmented receptor signaling, are associated with a number of pathological conditions (59). Such mutated receptors may bind FGF even without the help of

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HS. Moreover, FGF concentration is clearly of importance. Changes in FGF2 concentration thus were found to profoundly influence cellular response to FR signaling such that cells would proliferate, differentiate, or die (60). Together, these observations suggest that the effects of FGF concentration may be “edited” through the sulfation degree of participating HS chains, signaling induced by FGF at low concentration being upgraded through involvement of highly sulfated HS domains, and vice versa. Conversely, effects of increasing or decreasing FGF concentration could be accentuated by high affinity and low affinity HS, respectively.

Questions regarding structure-function relations of HS and their dependence on regulated HS biosynthesis remain major challenges in proteoglycan research. The biosynthetic process appears strictly regulated, as assessed through compositional or immunohistochemical analysis of HS species from various sources (7, 8). What is the purpose of this regulation? The limited number of sequence-specific HS-protein interactions described depend on the occurrence of “rare” constituents, such as 3-O-sulfated and N-unsubstituted GlcN units (61–64). Incompletely characterized but functionally important interactions involved in skeletal and renal development apparently require the common constituents, IdoUA and IdoUA-2-O-sulfate (57, 58). Yet other events, involving FGF-induced FR signaling, may depend on HS domains of adequate length and sulfate density, but without any need for specific residues nor for selective sequences. Conceivably, similar requirements may apply in the generation of morphogen gradients stabilized by cell surface HS-proteoglycans during embryonic development (65, 66). Whether these requirements fully explain the need for regulation in HS biosynthesis remains to be established.

Acknowledgments—We thank Gunilla Pettersson for excellent technical assistance, Sindhulakshmi Kurup for size fractionated heparin immunohistochemical analysis of HS species from various sources, and acknowledge for providing FR reagents.

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