Specific Heparan Sulfate Saccharides Mediate the Activity of Basic Fibroblast Growth Factor*

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In a previous study, we showed that heparitinase releases a 14-saccharide sequence (Oligo-H) from heparan sulfate (HS) with the structure \( \text{AGlGUAu}1,4\text{GlcNSO}_3\alpha_1,4\text{Idc}c\text{A}(2S)\alpha_1,4\text{GlcNSO}_3\beta_1,4\text{Idc}c\text{A}\alpha_1,4\text{GlcNAc} \) (where \( \text{Idc}c\text{A}(2S) \) represents iduronic acid 2-sulfate), which binds to basic fibroblast growth factor (bFGF) with high affinity (Turnbull, J. E., Fernig, D. K., Ke, Y., Wilkinson, M. C. & Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341). This paper describes further work on the binding properties of HS saccharides and their capacity to mediate bFGF activity in a mitogenesis assay, in which responsiveness is dependent on the addition of HS or heparin. Saccharides prepared by heparinase or nitrous acid digestion and heparinase-resistant fragments five saccharide units (degree of polymerization \( dp = 10 \)) or less in size were unable to activate bFGF. However, heparinase-resistant saccharides of \( dp = 12-16 \) were active in the assay; the \( dp = 14 \) and \( dp = 16 \) fractions were equivalent in activity to heparin and more active than the parent HS. Saccharides of the same size and basic structure as the active fractions \( (=dp = 12) \) bound to bFGF with high relative affinity. Active saccharides were composed mainly of N-sulfated disaccharides, the predominant unit being \( \text{Idc}c\text{A}(2S)\text{-GlcNSO}_3 \). This was enriched at least 5-fold in the active saccharides by comparison with the original HS. In addition, the \( dp = 12 \) and \( dp = 14 \) active fractions had a notably low content of trisulfated disaccharides \( \text{Idc}c\text{A}(2S)\text{-GlcNSO}_6 \) (where GlcNSO\(_6\)) represents N-sulfated glucosamine 6-sulfate), which are the major repeat units of heparin. The data show that sequences similar in size and basic structure to Oligo-H can mediate the mitogenic activity of bFGF. Overall, the results provide further evidence that specific HS sequences are generated biosynthetically in order to fulfill particular biological functions such as activation of bFGF.

A number of growth factors, often structurally unrelated and acting on a variety of target cells, display a substantial affinity for heparin and heparan sulfate (HS), and in some cases, the interaction is essential for the induction of mitogenesis (1–5). HS is found almost ubiquitously on the cell surface and in the pericellular matrix of mammalian cells, whereas heparin has a much more restricted distribution, being produced exclusively by connective tissue-type mast cells and stored within secretion granules (6). HS is therefore the principal cell-associated polysaccharide involved in the interaction of this general class of growth factors with their signal-transducing receptors.

The most widely studied of the HS-dependent growth factors is basic fibroblast growth factor (bFGF), which elicits a cellular response through a dual receptor mechanism involving both cell-surface HS and a family of membrane proteins (fibroblast growth factor receptors (FGFRs)) with tyrosine kinase activity (3–5, 7–10). The precise role of HS in signal transduction is unclear, but one possibility is that HS induces a conformational change in the growth factor that is essential for recognition by the FGFRs (3–5, 7–10). An alternative view suggests that a conformational change is unnecessary and that HS is a component of a ternary complex in which strong cooperative binding occurs through the simultaneous interaction of bFGF with both HS and the FGFR (5, 11). A complex of this type could be further stabilized by the direct binding of HS to the FGFR, which has recently been reported to occur (5, 7, 12). These concepts, which are not mutually exclusive, suggest a central role for HS in molecular recognition and signal transduction and prompt the question of the structural requirements in the polysaccharide that are essential for mediating bFGF activity.

The heparan sulfates have a unique molecular design in which N- and O-sulfate groups are mainly clustered in iduronic-rich domains separated by regions of low sulfation containing predominantly N-acetylated disaccharides (13–16). Structural analysis of HS has shown that heparitinase-resistant oligosaccharides enriched in 2-O-sulfated iduronic acid (IdcA) residues bind strongly to bFGF (17, 18). In addition, we previously identified a major high affinity bFGF binding sequence in fibroblast HS to be a \( dp = 14 \) saccharide (Oligo-H) with the structure \( \Delta\text{GlcGUAu}1,4\text{GlcNSO}_6\alpha_1,4\text{Idc}c\text{A}(2S)\alpha_1,4\text{GlcNSO}_6\beta_1,4\text{Idc}c\text{A}\alpha_1,4\text{GlcNAc} \) (17).

A useful property of Oligo-H that facilitated its isolation from the HS chain was its resistance to the enzyme heparitinase, which acts only in the regions of low sulfation, at linkages between glucosamine (GlcNAc) or GlcNSO\(_3\) and glucuronic acid (GlcUA) residues (13, 14, 17). However, the Oligo-H sequence is degraded by heparinase (17), which specifically cleaves at the linkage between GlcNSO\(_6\) and IdcA(2S) (14, 17, 19). Here, we report studies aimed at defining the sequences within HS that mediate the biological activation of bFGF, using an assay of the mitogenic activity of bFGF on chlorate-treated 3T3 fibroblasts developed by Rapraeger et al. (9) in which responsiveness is dependent upon the addition of heparin or HS.

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‡The abbreviations used are: HS, heparan sulfate; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; \( dp \), degree of polymerization (i.e. for disaccharide, \( dp = 2 \), etc.); \( \text{Idc}c\text{A}(2S) \), iduronic acid 2-sulfate; \( \text{GlcNSO}_6 \), N-sulfated glucosamine 6-sulfate; \( \text{GlcUA} \), unsaturated hexuronic acid residue formed at nonreducing end of disaccharides and oligosaccharides by lyase scission; \( \text{Idc}c\text{A} \), masturated hexuronic residue defined as in original polymer on the basis of the known specificity of heparitinase (14, 19); DCS, donor calf serum; MEM, minimum Eagle's medium; DMEM, Dulbecco's minimum Eagle's medium; HFIC, high pressure liquid chromatography.

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Using this bioassay, we have studied the structure-activity relationships of HS saccharides released by heparitinase or heparinase in terms of their ability to activate bFGF.

**EXPERIMENTAL PROCEDURES**

**Materials**—Donor calf serum (DCS), MEM, and sulfate-free DMEM (lacking MgSO₄ and containing 4.5 mg/liter glucose and reduced cysteine (0.065%, w/v)) were obtained from Life Technologies, Inc. (Gibco Biocult, Paisley, Scotland). Bovine brain-derived bFGF for use in cell growth assays was obtained from British Biotechnology (Oxford, Great Britain). Recombinant human bFGF coupled to Affi-Gel 10 (Bio-Rad) was prepared as described previously (17) and provided by Dr. D. Fernig (Department of Biochemistry, University of Liverpool, Liverpool, Great Britain). Bovine kidney HS (0.77 sulfates/disaccharide), heparin, and chondroitin sulfate were obtained from Sigma. Porcine mucosal HS (0.81 sulfates/disaccharide) was obtained from Organon (Oss, The Netherlands). Heparitinases I and II, heparinase, and unsaturated HS disaccharide standards were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Heparitinase and heparinase were also obtained from Sigma or Grampian Enzymes (Aberdeen, Scotland).

**Cell Culture**—Donor calf serum was heat-inactivated at 66°C for 20 min prior to use. To remove inorganic sulfate for use in FGF assays, heparin-dialysed DCS was dialyzed against 4 x 5 liters of Dulbecco’s A phosphate-buffered saline, pH 7.2. 3T3 fibroblasts from Swiss mouse embryos (ICN Biomedicals Ltd.) were maintained in MEM containing 10% DCS and 5% fetal bovine serum. Cells were used when passages 10 and 20 for experiments.

**HS-dependent Assay for bFGF Activation**—Stock cultures that were 70–90% confluent were transferred into sulfate-free DMEM containing 4% dialyzed DCS and 20 mM chloride (Fluka Chemie AG). After 4 h, the cells were removed from the culture dishes by treatment with trypsin and EDTA (0.05% and 2 mM, respectively). The trypsin activity was inhibited by the addition of an equal volume of sulfate-free DMEM containing 4% dialyzed DCS and 20 mM chloride. The cells were pelleted by centrifugation (500 x g for 5 min) and resuspended in the same medium. Cells were then seeded into 24-well plates at a density of 5 x 10³/well in 0.5 ml of medium and left at 37°C for 16 h to adhere and spread. The monolayers were then transferred into sulfate-free DMEM containing 0.5% dialyzed DCS and 20 mM chloride and incubated for a further 24 h. The growth-arrested, HS-deficient cells were treated with bFGF (between 0.625 and 10 ng/ml) in the presence or absence of exogenous glycosaminoglycans or glycosaminoglycan fragments. Glycosaminoglycan and bFGF were added in fresh sulfate-free medium containing 0.5% dialyzed DCS and 20 mM chloride. Each experimental variable was performed in triplicate. Each plate contained a positive control (cells treated with 10% nondialyzed DCS in normal MEM) and a negative control (cells treated with fresh sulfate-free DMEM containing 0.5% dialyzed DCS and 20 mM chloride). Replicative DNA synthesis was assessed 24 h after the addition of the growth factor and glycosaminoglycan by the inclusion of 1.0 pCi/ml [α-³²P]thymidine (ICN Biomedicals Ltd.) for 3 h. Incorporation was determined by liquid scintillation counting following fixation of the cell layers (methanol, 4°C) and washing with 5% trichloroacetic acid.

**Preparation of HS Oligosaccharides**—Porcine mucosal HS, bovine kidney HS, and metabolically radiolabeled HS (isolated from human skin fibroblast cultures (13, 14); 0.76 sulfates/disaccharide) were depolymerized with heparitinase, heparinase, or fresh low pH nitrous acid as described before (17). Gel filtration on Bio-Gel P-6 and Sepharose CL6B columns was carried out as described previously (14). Peaks were detected by measuring UV absorbance (232 nm), a molar extinction coefficient of 9320 M⁻¹ cm⁻¹ for a molecular mass of ~500 Da/disaccharide.

**Strong Anion-exchange HPLC of Disaccharides**—Disaccharide composition was analyzed by complete depolymerization of HS or HS oligosaccharides to disaccharide products with a mixture of heparitinases, followed by Bio-Gel P-2 chromatography (yields ~90%) and separation by HPLC on a ProPac PA1 analytical column (4 x 250 mm; Dionex) as described previously (17). The elution positions of specific disaccharides, detected by UV absorbance (232 nm), were established by comparison with authentic standards.

**RESULTS**

**Response of Chlorate-treated 3T3 Cells to bFGF is Dependent on HS**—Quiescent 3T3 cells maintained in sulfate-free medium and 20 mM sodium chloride produced HS characterized by reduced sulfation (<5% of control) and an inability to bind bFGF (data not shown). These cells were essentially unresponsive to bFGF in a concentration range of 0.625–10 ng/ml (Fig. 1A). However, in the presence of 10 ng/ml heparin, a clear dose response to bFGF was observed, with [³H]TdR incorporation reaching 60% of the positive control (response with 10% nondialyzed serum in normal MEM) (Fig. 1A). When the heparin concentration was varied, activation of bFGF reached a maximum value at 50 ng/ml and then declined (Fig. 1B); significant responses were obtained at doses as low as 1 ng/ml (data not shown). HS also activated bFGF, but was less potent than heparin at low concentrations (up to 50 ng/ml). Maximum activity with HS was seen at 500 ng/ml (40% of positive control) (Fig. 1B). Chondroitin sulfate was inactive even at concentrations as high as 500 ng/ml (Fig. 1B).

**Structural Characteristics of HS Saccharides That Mediate bFGF Activity**—To investigate the ability of different sequences from HS to activate bFGF, the polysaccharide was degraded with heparitinase, heparinase, or low pH nitrous acid, and the degradation products were tested in the mitogenesis assay. Saccharides produced by treatment with low pH nitrous acid or heparinase were inactive (Fig. 2A), indicating the importance of N-sulfated disaccharides and IdceA(2S) residues, respectively. In contrast, heparitinase treatment did not alter the ability of HS to activate bFGF (Fig. 2A), demonstrating that the active sequences were located in heparitinase-resistant domains.

The ability of the heparitinase-resistant saccharide fractions to activate bFGF was then studied in more detail. Heparitinase-digested HS was fractionated on Bio-Gel P-6, and a series of saccharides ranging in size from dp2 (main product) to dp16...
were resolved (data not shown). Individual peaks of resistant saccharide components (dp6-16) were prepared and tested for activity in the bio assay at a fixed concentration of 50 ng/ml. The results showed that activity was clearly dependent on fragment size (Fig. 2B); saccharides of five or less disaccharide units (≤dp10) were completely inactive, whereas dp12 fragments gave a moderate response (30% of positive control). The dp14 and dp16 fractions were the most active, inducing 60 and 65% responses, respectively (Fig. 2B), and, interestingly, were equal in activity to heparin at this concentration (Fig. 2B).

The activation of bFGF by the heparitinase-resistant saccharides was studied over a wide concentration range. The dose-response curve for the dp10 fraction demonstrated that these saccharides were inactive over the whole concentration range tested (1–200 ng/ml) (Fig. 2C). Similar results were obtained for the dp6 and dp8 fractions (data not shown). In contrast, dose-response curves obtained for the dp12 and dp14 fractions showed that activity increased sharply in the range of 1–50 ng/ml and thereafter plateaued (Fig. 2C). The dp14 fraction was consistently more active than the dp12 fraction over the concentration range tested (1–200 ng/ml) (Fig. 2C). Results for the dp16 fraction (not shown) were essentially identical to those for the dp14 saccharides.

Comparison of Molecular Sizes of Heparinase- and Heparitinase-resistant Fragments—Size-exclusion chromatography on Bio-Gel P-6 showed that heparinase cleaved a limited number of linkages in metabolically labeled HS (10–12%; see Refs. 13 and 14), producing only small amounts of di- and tetrasaccharide products (Fig. 3A). The most abundant fragments ran in the void volume (saccharides larger than approximately dp20 (5 kDa) are excluded on this gel), and there were very low levels of intermediate-size fragments. In contrast, heparitinase treatment extensively degraded the HS (~63% of linkages cleaved), yielding a range of resistant saccharide species (dp4–16) in addition to a major disaccharide peak (Fig. 3B). It was apparent from size-exclusion chromatography on Sepharose CL-6B that the majority of the fragments produced by heparitinase scission were ~9–10 kDa in size (Kav = 0.66) (Fig. 3C), compared to the intact chains, which were ~45 kDa (Kav = 0.34). By comparison, the resistant fragments generated by heparitinase were much smaller (Kav = 0.8–0.9; ~1500–4000 Da), in agreement with the Bio-Gel P-6 data (Fig. 3B).

Relationship between HS Saccharide Structure and Affinity for bFGF—To investigate further the relationships among saccharide structure, size, and affinity, we studied the binding of the full range of metabolically labeled saccharides produced by heparitinase action to immobilized bFGF (Fig. 4). There was a positive correlation between saccharide size and binding strength, and only the larger saccharides (~dp12) bound to bFGF with an apparent affinity similar to that of the parent polysaccharide. The proportion of high affinity material (defined as elution with 1–1.5 M NaCl) was considerably greater in the intact chains and dp14 and dp12 fractions (60, 68, and 49%, respectively) compared to the smaller saccharides (18 and 6%
fractionated by gel filtration on a Bio-Gel. These oligosaccharides for bFGF was then assessed by bFGF affinity separately and freeze-dried as described previously indicated. The data show the percentage of total saccharides one to seven disaccharides in size (dp2-14) were pooled as described for Fig. 3. Fractions corresponding to peaks containing oligosaccharides one to seven disaccharides in size (dp2-14) were pooled separately and freeze-dried as described previously (15). The affinity of these oligosaccharides for bFGF was then assessed by bFGF affinity chromatography as described previously (16). Bound material was eluted with a step gradient of sodium chloride at the concentrations indicated. The data show the percentage of total $^3$H label recovered that eluted at each concentration. None of the disaccharide fractions bound to the column (data not shown).

![Graph](image)

**Fig. 4. Fractionation of heparitinase-resistant oligosaccharides on bFGF affinity column.** $^3$H-Labeled fibroblast HS chains were treated with heparitinase, and the resulting oligosaccharides were fractionated by gel filtration on a Bio-Gel P-6 column (1 x 120 cm) as described for Fig. 3. Fractions corresponding to peaks containing oligosaccharides one to seven disaccharides in size (dp2-14) were pooled separately and freeze-dried as described previously (15). The affinity of these oligosaccharides for bFGF was then assessed by bFGF affinity chromatography as described previously (16). Bound material was eluted with a step gradient of sodium chloride at the concentrations indicated. The data show the percentage of total $^3$H label recovered that eluted at each concentration. None of the disaccharide fractions bound to the column (data not shown).

for dp10 and dp8, respectively, and 0 for dp6 and smaller fractions. These findings demonstrate a direct relationship between the size of heparitinase-resistant saccharides and their affinity for bFGF. Since the same type and size of saccharides have the capacity to support bFGF activity (Fig. 2, B and C), these results indicate that heparitinase-resistant sequences six disaccharide units or more in size have the appropriate structural characteristics both to bind bFGF with high relative affinity and to mediate its biological activity.

**Disaccharide Composition of HS Saccharides That Activate bFGF**—Analysis of the composition of the heparitinase-resistant fractions (dp10-16) indicated that saccharides that activate bFGF are significantly enriched in the disulfated disaccharides with the structure $\Delta$HexUA(2S)-GlcNS03 (in which HexUA would be IdecA in the HS chains (14, 17)). The proportion of these disaccharides was high (52-59%) in the active fractions (dp12-16), compared to 11% in the parent HS and only 7% in the inactive dp10 fraction (Table 1). Interestingly, the triulfated disaccharide $\Delta$HexUA(2S)-GlcNS03(6S), which is the main structural unit in heparin (20), was present in only trace quantities in the dp12 and dp14 saccharide fractions (Table 1). The content of N-acetylated disaccharides was calculated to be approximately one/oligosaccharide, and these are probably located at the nonreducing ends of the saccharides (17). These analytical data strongly suggest a major role for clusters of IdecA(2S)-GlcNS03 units in the activation of bFGF.

**DISCUSSION**

In this study, we have demonstrated that the ability of HS to activate bFGF resides in saccharides that can be released by heparitinase. Both activation of bFGF (Fig. 2, B and C) and high apparent affinity for bFGF (Fig. 4) correlated strongly with size of heparitinase-resistant sequences. Activation in particular demonstrated a sharp cutoff in its dependence on saccharide size, with the most active fragments containing seven to eight disaccharide units (dp14-16). In fragments composed of six disaccharide units, activity was lower by ~50% (Fig. 2B), and smaller saccharides were completely inactive even though dp10 and dp8 fractions bound relatively strongly to bFGF (Fig. 4). Similarly, despite exhibiting an appreciable affinity for bFGF (18), heparitinase-released saccharides were also inactive (Fig. 2B), indicating that relatively strong binding of saccharides to bFGF is not in itself sufficient for activation of the growth factor and suggesting that specific structural features are required. The functional relevance of sequences with moderate to low relative affinity for bFGF, which are obviously abundant in HS and rare in heparin, has yet to be established. The notion that they are responsible for functions other than activation of bFGF (5, 21) clearly requires further investigation.

In view of their resistance to heparitinase, the basic structure of the saccharides that activate bFGF can be described by the general formula $\Delta$GlcUA1,4GlcNS03(6S)-IdecA(2S)-GlcNS03(6S)-IdecA(2S)-GlcNAc. Based on the analysis of disaccharide composition (Table I), the majority of the internal IdecA residues will be substituted with O-sulfate groups at C-2. Inactivation of HS by heparitinase treatment (Fig. 2) confirms the presence of IdecA(2S) residues in the active sequences. Since heparinase acts on HS to produce predominantly large fragments (Fig. 3), cleavage at these key IdecA(2S) residues within the active domains is probably responsible for the loss of activity, rather than an unspecific effect of extensive depolymerization to small inactive saccharides. The virtual absence of the triulfated disaccharides of the type IdecA(2S)-GlcNS03(6S), which are characteristic of heparin structure, in the dp12 and dp14 fractions suggests strongly that this structural unit is not required for bFGF activation.

About 8% of saccharides within the active dp12 and dp14 fractions contained GlcNS03 residues O-sulfated at C-6 (Table I). This amount is substoichiometric, so that on average, a single GlcNS03(6S) residue will be present in about half of the oligosaccharides in these fractions. It is not known whether this moiety is influential in bFGF activation (21). In an earlier study, we showed that a high affinity bFGF binding sequence (Oligo-H) contained an internal repeat of five disaccharides of the type IdecA(2S)-GlcNS03 and no 6-O-sulfated amino sugars (17). Relative affinity for bFGF was closely linked to the IdecA(2S) content, and in oligosaccharides with lower contents of IdecA(2S), a compensatory presence of C-6 O-sulfation of GlcNS03 and GlcNAc was not sufficient to support strong...
The disaccharide composition of heparitinase-resistant oligosaccharides (dp10-16) from porcine mucosal HS was assessed by strong anion-exchange (SAX) HPLC as described under "Experimental Procedures." The data shown here have some bearing on a recent study of the HS glycan structure.

| Sulfation                  | Disaccharide structure            | dp10 | dp12 | dp14 | dp16 | Intact |
|---------------------------|-----------------------------------|------|------|------|------|--------|
| Non-                      | ΔHexUA-GlcNAc                     | 18.6 | 8.0  | 7.2  | 8.1  | 46.0   |
| Mono-                     | ΔHexUA-GlcNAc(6S)                 |      | 2.3  | 2.4  | 1.7  | 9.2    |
| Mono-                     | ΔHexUA(2S)-GlcNAc                 | 7.3  | 4.7  | 3.3  | 3.4  | 1.3    |
| Mono-                     | ΔHexUA-GlcNSO3                    | 66.7 | 23.6 | 19.3 | 14.7 | 22.4   |
| Di-                       | ΔHexUA-GlcNSO3(6S)                |      | 8.5  | 7.4  | 4.4  | 4.2    |
| Di-                       | ΔHexUA(2S)-GlcNSO3                | 7.4  | 52.0 | 59.2 | 55.5 | 10.9   |
| Tri-                      | ΔHexUA(2S)-GlcNSO3(6S)            |      | 0.9  | 7.2  | 14.2 | 6.0    |
| N-SO₄/disaccharide        |                                   | 0.74 | 0.85 | 0.87 | 0.87 | 0.44   |
| 2-O-SO₄/disaccharide      |                                   | 0.15 | 0.68 | 0.64 | 0.71 | 0.18   |
| 6-O-SO₄/disaccharide      |                                   |      | 0.12 | 0.11 | 0.22 | 0.19   |

* ND, not detected.

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