BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 8379–8386 8379
DOI: 10.1021/bi9006379

Compositional Analysis of Heparin/Heparan Sulfate Interacting with Fibroblast Growth Factor·Fibroblast Growth Factor Receptor Complexes†

Fuming Zhang,*,‡ Zhenqing Zhang,‡ Xinfu Lin,‡ Andrew Beenken,§ Anna V. Eliseenkova,§ Moosa Mohammadi,§ and Robert J. Linhardt‡

‡*Departments of Chemistry and Chemical Biology, Biology, and Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, and* §*Department of Pharmacology, New York University School of Medicine, New York, New York 10016*

Received April 14, 2009; Revised Manuscript Received July 3, 2009

ABSTRACT: Heparan sulfate (HS) proteoglycans (PGs) interact with a number of extracellular signaling proteins, thereby playing an essential role in the regulation of many physiological processes. One major function of HS is to interact with fibroblast growth factors (FGFs) and their receptors (FGFRs) and form FGF·HS·FGFR signaling complexes. Past studies primarily examined the selectivity of HS for FGF or FGFR. In this report, we used a new strategy to study the structural specificity of HS binding to 10 different FGF·FGFR complexes. Oligosaccharide libraries prepared from heparin, 6-desufated heparin, and HS were used for the interaction studies by solution competition surface plasmon resonance (SPR) and filter trapping assays. Specific oligosaccharides binding to FGF·FGFR complexes were subjected to polyacrylamide gel electrophoresis (PAGE) analysis and disaccharide compositional analysis using liquid chromatography and mass spectrometry. The competition SPR studies using sized oligosaccharide mixtures showed that binding of each of the tested FGFs or FGF·FGFR complexes to heparin immobilized to an SPR chip was size-dependent. The 6-desulfated heparin oligosaccharides exhibited a reduced level of inhibition of FGF and FGF·FGFR complex binding to heparin in the competition experiments. Heparin and the 6-desulfated heparin exhibited higher levels of inhibition of the FGF·FGFR complex binding to heparin than of FGF binding to heparin. In the filter trapping experiments, PAGE analysis showed different affinities between the FGF·FGFR complexes and oligosaccharides. Disaccharide analysis showed that HS disaccharides with a degree of polymerization of 10 (dp10) had high binding selectivity, while dp10 heparin and dp10 6-desulfated heparin showed reduced or no selectivity for the different FGF·FGFR complexes tested.

Heparan sulfate (HS)[1] proteoglycans (PGs) are essential components of both the extracellular matrix (ECM) and the cell surface membrane. Heparan sulfate (HS) is a linear sulfated glycosaminoglycan (GAG), consisting predominantly of a repeating disaccharide motif comprised of β-D-glucuronic acid and *N*-acetyl-α-D-glucosamine residues connected through 1→4 glycosidic linkages. Each disaccharide unit can be differentially substituted with 2-*O*-sulfo groups in the uronic acid residue and 6-*O*-, 3-*O*-, and *N*-sulfo groups in the glucosamine residue (*1*, *2*). Each biosynthetic modification is incomplete, thus resulting in sequence heterogeneity thought to serve as an important mechanism in the regulation of HS interaction specificity with cellular proteins, including various growth and differentiation factors and morphogens, extracellular matrix components, protease inhibitors, protease, lipoprotein lipase, and various pathogens (*2*, *3*). These interactions have been shown to play a pivotal role in various patho-physiological phenomena as well as in tissue morphogenesis. For example, genetic studies in flies and more recently in mice demonstrate that HSs are indispensable for proper development (*4*−*6*).

The fibroblast growth factor (FGF) family consists of 18 structurally related proteins with a core region of homology of 100−120 residues known as a β-trefoil core, in addition to variable N- and C-terminal regions (*7*, *8*). In development, FGFs are regulators of mesenchymal-epithelial communication and are required for organogenesis and pattern formation (*8*). FGFs continue to regulate tissue homeostasis in the adult and play important roles in wound healing, tissue repair, cholesterol metabolism and serum phosphate regulation (*7*). FGFs perform their diverse functions by binding and activating cell surface FGF receptors (FGFRs) that form a subfamily within the receptor tyrosine kinase (RTK) superfamily (*9*). FGF·FGFR binding specificity is essential for the regulation of FGF signaling and is

†This work is supported by National Institutes of Health Grants GM 38060 and HL 62244 (to R.J.L.), DE13686 to M.M., and T32-GM066704-05 (to NYU Dept. of Pharmacology for A.B.).

*To whom correspondence should be addressed. Phone: (518) 276-6839. Fax: (518) 276-3404. E-mail: zhangf2@rpi.edu.

[1]Abbreviations: HS, heparan sulfate; PGs, proteoglycans; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; dp, degree of polymerization; PG, proteoglycan; ECM, extracellular matrix; GAG, glycosaminoglycan; RTK, receptor tyrosine kinase; IdoA, iduronic acid; S, sulfo; GlcN, glucosamine; IPTG, isopropyl β-D-thiogalactoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; MW, molecular weight; CO, cutoff; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*-[(dimethylamino)propyl]carbodiimide; RU, response unit; Fc, flow cell; LC, liquid chromatography; MS, mass spectrometry; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; Ac, acetyl; 0S, ΔUA-GlcNAc; NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; triS, ΔUA2S-GlcNS6S; HPLC, high-performance liquid chromatography; ESI, electrospray ionization.

Published on Web 07/10/2009

determined by primary sequence differences among the 18 FGFs and 7 FGFRs (*10*−*13*). Receptor dimerization in FGF signaling requires the presence of the highly sulfated heparin/HS polysaccharide chains of HSPGs. Aberrant FGF signaling can cause a wide spectrum of human pathological conditions including skeletal syndromes, olfactory syndromes, phosphate-wasting disorders, reproductive disorders, and cancer (*13*).

FGF signaling begins with the formation of a ternary complex of FGF, FGFR, and heparan sulfate. Early models suggested that heparin/HS serves primarily as a template for FGF dimerization with two molecules of FGF bound to the heparin helix in either a cis or trans orientation (*14*). Heparin/HS binds tightly to FGFs having dissociation constants ranging from 100 nM to 10 μM (*15*). Cellular studies with selectively desulfated heparins show that different types of sulfo groups may be acquired for promotion of FGF signaling (*16*−*19*). FGF1 and FGF2, the most studied members of the family, bind to specific sulfo groups in heparin oligosaccharides (*15*, *20*, *21*). FGF2 recognizes a heparin/HS pentasaccharide containing an iduronic acid (IdoA) 2-*O*-sulfo residue (*22*) with no requirement for 6-*O*-sulfo groups in its glucosamine (GlcN) residue (*20*, *23*) but requiring larger 6-*O*-sulfo group containing sequences for signaling (*24*, *25*). FGF1 recognizes a specific octasaccharide (*26*) containing an internal IdoA2SGlcNS6SIdoA2S (where S is sulfo) trisaccharide motif (*22*) and also requires 6-*O*-sulfo groups for signaling (*22*, *25*, *27*). Early studies were focused exclusively on the interaction of FGF with heparin/HS. However, structural data clearly established heparin/HS interacts with both growth factor and receptor, thus requiring the study of binding of heparin/HS to the FGF·FGFR complex, the subject of this study. In this report, an oligosaccharide library prepared from heparin, 6-desufated heparin, and HS was used to analyze heparin/heparan sulfate sequences that interacted with FGF·FGFR complexes by solution competition using surface plasmon resonance (SPR) and filter trapping. Specific oligosaccharides binding to FGF·FGFR complexes were subjected to polyacrylamide gel electrophoresis (PAGE) analysis and disaccharide analysis.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. All FGFRs were refolded and purified from inclusion bodies as previously described (*28*). The purification procedures for FGF1 (*29*), FGF8 and FGF17 (*30*), FGF9 (*31*), and FGF10 (*12*) have all been published previously. Full-length FGF3 was expressed in pET30a, refolded, and purified by heparin affinity, nickel affinity, and size exclusion chromatography. Full-length FGF4 was expressed in pET28a, and the ligand was obtained from inclusion bodies via salt extraction with 2 M NaCl, 25 mM Hepes (pH 7.5), and 10% glycerol. FGF4 was then purified by heparin affinity and size exclusion chromatography. Full-length FGF5 and FGF6 were both expressed in pET28a, refolded, and then purified by heparin affinity and size exclusion chromatography. All proteins are of human origin except FGF3, which is from mouse; all proteins are expressed in BL21 DE3 cells, and refolding protocols for all ligands follow that previously described (*28*). The FGFRs and some of the FGFs were refolded using slow dialysis as follows. Bacterial cells transformed with expression vectors for the D2−-D3 fragments of FGFR1c, FGFR2c, and FGFR2b were induced with isopropyl β-D-thiogalactoside (IPTG) for 5 h and centrifuged, and the bacterial pellet was lysed in 25 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 10% glycerol using a French press. Following centrifugation, the pellets containing ectodomains were dissolved in 6 M guanidinium hydrochloride and 10 mM dithiothreitol (DTT) in 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0). The solubilized ectodomains were refolded by dialysis against 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) or Tris buffer (pH 7.5) containing 150 mM NaCl, 10% glycerol, and 1 mM L-cysteine. The refolded FGFR1 and FGFR2 proteins were purified by heparin Sepharose affinity chromatography followed by size exclusion chromatography on a Superdex 200 (Pharmacia) column equilibrated with 25 mM Tris-HCl buffer (pH 7.5) containing 1.0 M NaCl. To generate the desired complexes, purified ectodomains were mixed with different FGFs in a 1:1 ratio and concentrated using Centricon 30 (Amicon) and then the FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, and FGF17·FGFR1c were run over a Sephadex 200 size exclusion column in 1 M NaCl and 25 mM Hepes (pH 7.5) to prepare the FGF·FGFR complexes.

Preparation of Oligosaccharide Libraries. The porcine intestinal heparin and porcine intestinal heparan sulfate were from Celsus (Celsus Laboratories, Cincinnati, OH). 6-Desulfated heparin was prepared by the method with a silylating reagent, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTSTFA) (*19*). The oligosaccharide libraries from heparin, 6-desulfated heparin, and HS were prepared using enzymatic depolymerization by using the combination heparin lyase I, II, and III digestion. Undigested saccharides and enzymes were removed by ultrafiltration with a membrane molecular mass cutoff (MMCO) of 5 kDa. The low-molecular mass oligosaccharides ($<$ 5000 Da) obtained were fractionated on a Bio-Gel P-6 column. Individual fractions consisting of hexasaccharides, octasaccharides, and decasaccharides were collected, desalted, and used as oligosaccharide libraries in this study.

Preparation of the Heparin Biochip. Albumin-heparin (Sigma) was covalently immobilized to the sensor surface (Fc2) through its primary amino groups (*32*). Briefly, the carboxymethyl groups on the C1 chip (GE Healthcare, Uppsala, Sweden) surface were first activated using an injection pulse with a duration of 10 min (50 μL, with a flow rate of 5 μL/min) of an equimolar mix of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*-[(dimethylamino)propyl]carbodiimide (EDC) (final concentration of 0.05 M, mixed immediately prior to injection). A solution of albumin-heparin [200 μg/mL in sodium acetate buffer with the addition of 2 M guanidine (pH 4.0)] was then applied (20 μL) by manual injection. Excess unreacted sites on the sensor surface were blocked with a 50 μL injection of 1 M ethanolamine. The successful immobilization of albumin-heparin was confirmed by the observation of an ~300 response unit (RU) increase in the sensor chip. To prepare the control flow cell (Fc1), bovine serum albumin was immobilized on the surface with a similar amine coupling procedure. After the surface had been activated with NHS/EDS, 5 μL of an albumin [20 μg/mL in sodium acetate buffer (pH 4.0)] solution was then injected by manual injection, yielding ~300 RU immobilized.

Solution Competition SPR Study. We performed solution/surface competition experiments by SPR (BIAcore 3000, GE Healthcare) to examine the effect of saccharide chain size and structure of different heparin/HS on the heparin−FGF·FGFR interaction. Proteins (FGFs, or the FGF·FGFR complex,

1000 nM) premixed with a certain concentration (2000 nM) of hexasaccharides (dp6), octasaccharides (dp8), and decasaccharides (dp10) were injected over the heparin chip at a flow rate of 30 μL/min. For each set of competition experiments on the SPR, a control experiment (only protein without added oligosaccharides) was performed to make certain the surface was completely regenerated and that the results obtained between runs were comparable. The response was monitored as a function of time (sensorgram) at 25 °C.

"Fishing" for Specific Oligosaccharides Binding to the FGF·FGFR Complex from Oligosaccharide Libraries. To characterize the compositions of heparin or HS required to bind the FGF·FGFR complex, initially three different FGF·FGFR complexes (FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c) were used to bind decasaccharide libraries from heparin, 6-desulfated heparin, and HS. FGF·FGFR complexes [225 μg in 75 μL of buffer (25 mM Hepes buffer), with 1 M NaCl (pH 7.5)] were mixed with 30 μg of different decasaccharide libraries in 100 μL of buffer [25 mM Hepes, with 150 mM NaCl (pH 7.5)] and incubated at room temperature for 1 h. The nonbinding oligosaccharides were removed from the mixture using ultracentrifugation with nanosep tubes (MMCO of 30 kDa), and remaining complexes were washed three times with buffer. The FGF·FGFR−oligosaccharide ternary complexes obtained were heated to 100 °C to break the complex, and then protein was removed from each sample using a centrifugal membrane filter (MMCO of 10 kDa). The high-affinity oligosaccharides were subjected to structural analysis by gradient PAGE and disaccharide compositional analysis. Next, seven additional FGF·FGFR complexes (FGF3-FGFR2b, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, FGF8b·FGFR2c, FGF10·FGFR2b, and FGF17·FGFR1c) were similarly used to fish for specific HS structures from dp10 HS using the same approach.

Structural Analysis of the Specific Oligosaccharides Binding to the FGF·FGFR Complex. (*i*) *PAGE Analysis*. Polyacrylamide gel electrophoresis (PAGE) was applied in analyzing the molecular weight and polydispersity of the oligosaccharides. In each lane, ~5 μg of oligosaccharide was subjected to electrophoresis against a standard composed of heparin oligosaccharides prepared enzymatically from bovine lung heparin, and the gel was visualized with Alcian blue.

(*ii*) *Disaccharide Compositional Analysis Using Liquid Chromatography and Mass Spectrometry* (*LC−MS*). A mixture of recombinant heparinase I, II, and III (a generous gift from J. Liu of the University of North Carolina, Chapel Hill, NC) was added to the FGF·FGFR−oligosaccharide ternary complex and the mixture incubated at 37 °C overnight. The products were filtered by the centrifugal filter devices (3 kDa MMCO, Millipore), through which the heparin/HS disaccharides were obtained. A set of unsaturated disaccharide standards of heparin/HS (Seikagaku) [0S, ΔUA-GlcNAc (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid and Ac is acetyl); NS, ΔUA-GlcNS (where S is sulfo); 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; and triS, ΔUA2S-GlcNS6S] were used in the analysis. Solutions A and B for high-performance liquid chromatography (HPLC) were 15 and 70% acetonitrile, respectively, containing the same concentration of 37.5 mM NH_4HCO_3 and 11.25 mM tributylamine. The pH values of the solutions were adjusted to 6.5 with acetic acid. The flow rate was 10 μL/min. The separation was performed on a C-18 column (Agilent) using solution A for 20 min, followed by a linear gradient from 20 to 45 min from 0 to 50% solution B. The column effluent entered the source of the electrospray ionization (ESI) mass spectrometer for continuous detection by MS (Agilent) (*33*).

RESULTS

Solution Competition SPR Study. Competitive binding studies with heparin (immobilized on the SPR chip) and soluble sized oligosaccharides, derived from heparin and 6-desulfated heparin, were performed using SPR. FGF or FGF·FGFR complexes (1 μM), with or without bound oligosaccharide, were passed over the surface of a SPR biochip on which heparin was immobilized (Figures 1 and 2 and Table 1). Different oligosaccharides of defined length [from hexasaccharide (dp6) to decasaccharide (dp10)] were used in the competition study. The results showed that (1) the dissociation rates of the FGF·FGFR complex injections were much slower than those observed when FGF alone was injected, based on the overall shapes of SPR sensorgrams, demonstrating that the ternary FGF·FGFR·HS complexes are considerably more stable than the FGF·HS binary complexes; (2) heparin-derived, sized oligosaccharide mixtures inhibit the binding of FGF1, FGF2, and their complexes (FGF1·FGFR1c and FGF2·FGFR1c) to immobilized heparin and the level of inhibition decreased with decreasing oligosaccharide size, demonstrating a chain length dependence; (3) 6-desulfated heparin oligosaccharides showed a reduced level of inhibition in the competition experiments, demonstrating the importance of either the 6-*O*-sulfo groups or overall sulfation level for binding to FGF1 and FGF2 and their FGF·FGFR complexes; and (4) the 6-desulfated heparin was a better inhibitor of binding of the FGF·FGFR complex to heparin than of binding of FGF to heparin, indicating that the 6-*O*-sulfo group or overall sulfation level was less critical for high-affinity binding to the FGF·FGFR complex than to FGF, and that the FGF·FGFR·heparin ternary complex is more stable.

Compositional Analysis of the Specific Oligosaccharides Binding to FGF·FGFR Complexes. In the first set of fishing experiments, three FGF·FGFR complexes (FGF1·FGFR2c FGF2·FGFR1c, and FGF2·FGFR1c, in 1 M NaCl) were examined for their binding to heparin or HS-derived oligosaccharide mixtures. FGF·FGFR complexes have poor solubility in low-salt buffers, and therefore, they need to be stored in high-salt (1 M) buffers. High salt can weaken interactions of the HS/heparin with the FGF·FGFR complex. Thus, in the binding experiments, the salt was diluted when the oligosaccharide mixture was added. After it had been mixed with the oligosaccharide (in 150 mM NaCl), the salt concentration of the FGF·FGFR complex was reduced to ~500 mM, which kept the complex soluble while allowing protein−oligosaccharide binding. A molar excess of each of the three sized decasaccharide mixtures (dp10 from heparin, 6-desulfated heparin, and HS) was incubated in HBS buffer with each of the three FGF·FGFR complexes (FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c). The complexes each had a molecular mass of ~45 kDa, while the individual oligosaccharides had molecular masses of < 3.3 kDa (calculated for the fully sulfated heparin decasaccharide). The nonbinding oligosaccharides were removed from the mixtures using ultracentrifugation (MMCO of 30 kDa). PAGE analysis (Figure 3) was used to examine

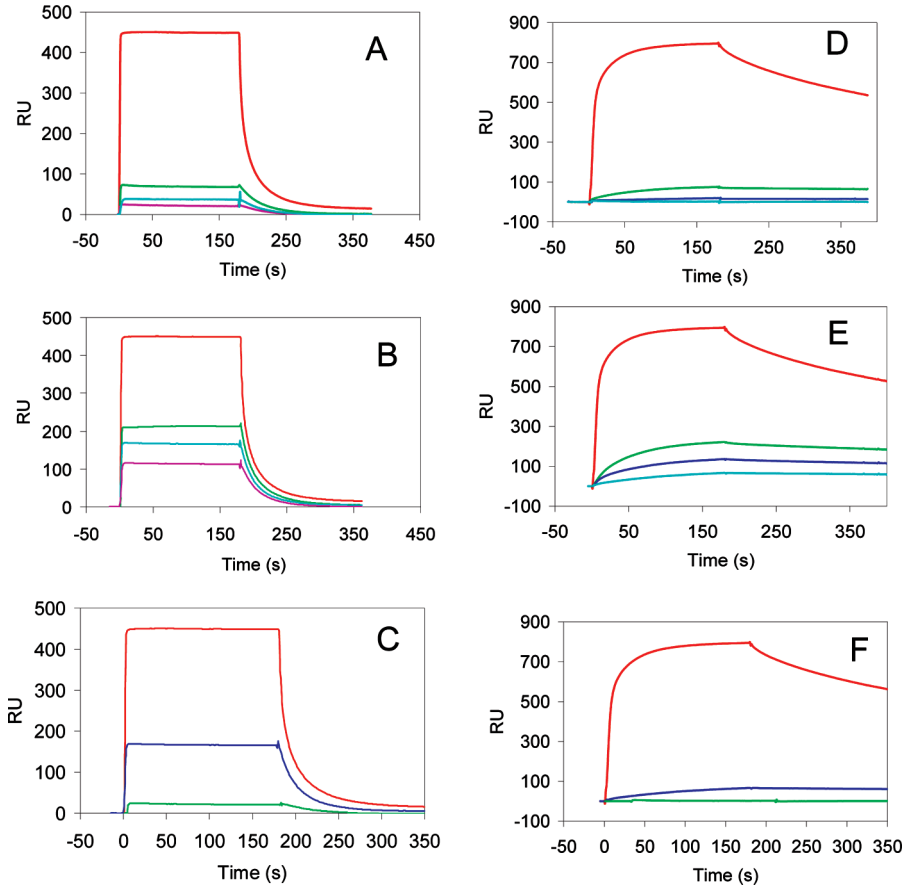

FIGURE 1: (A) Inhibition of FGF1 binding to immobilized heparin by a sized heparin oligosaccharide mixture: control (no oligosaccharides) in red, dp6 in green, dp8 in turquoise, and dp10 in purple. (B) Inhibition of FGF1 binding to immobilized heparin by sized 6-desulfated heparin oligosaccharide mixtures: control (no oligosaccharides) in red, dp6 in green, dp8 in turquoise, and dp10 in purple. (C) Comparison of the inhibition of FGF1 binding to immobilized heparin by dp8 oligosaccharide mixtures: red, control; blue, heparin 6-desulfated heparin dp8; green, heparin dp8. (D) Inhibition of binding of the FGF1·FGFR1c complex to immobilized heparin by sized heparin oligosaccharide mixtures: control (no oligosaccharides) in red, dp6 in green, dp8 in blue, and dp10 in turquoise. (E) Inhibition of binding of the FGF1·FGFR1c complex to immobilized heparin by sized 6-desulfated heparin oligosaccharide mixtures: control (no oligosaccharides) in red, dp6 in green, dp8 in blue, and dp10 in turquoise. (F) Comparison of the inhibition of FGF1·FGFR1c binding to immobilized heparin by dp8 oligosaccharide mixtures, red, control; blue, 6-desulfated heparin dp8; green: heparin dp8. The concentrations of FGF1, FGF1·FGFR1c complex, and the oligosaccharide were 1000, 500, and 2000 nM, respectively.

affinity differences between the complex and decasaccharides. All three complexes exhibited similar band intensities for the high-affinity decasaccharides, suggesting that there was little selectivity or that PAGE was not able to detect subtle differences in oligosaccharide selectivity. The overall band intensity (total staining in each lane) of the interacting HS and 6-desulfated heparin decasaccharides showed a similar pattern indicating the order of affinity (FGF·FGFR complex to HS or 6-desulfated heparin) is as follows: FGF1·FGFR1c > FGF2·FGFR1c > FGF2·FGFR2c. In contrast, the overall band intensity for heparin decasaccharides was similar for all three complexes.

Next, the disaccharide compositions of decasaccharides (dp10) with high affinity for FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c complexes were determined. The results showed little FGF·FGFR complex binding selectivity for heparin and 6-desulfated heparin decasaccharides (Figure 4 A,B). This is undoubtedly due to the highly uniform repeating structures in both heparin, the tri-S disaccharide, and the 6-desulfated heparin, the 2SNS disaccharide. Therefore, the more highly variable HS decasaccharide mixture was examined. The results (Figure 4 C) showed major composition differences in the dp10 HS oligosaccharides binding to the different complexes, suggesting a very high level of selectivity (diversity of the disaccharide compositional structures). The dp10 HS that bound to the FGF2·FGFR1c complex, for example, contained substantially more 2SNS disaccharide than did the dp10 HS that bound to the FGF1·FGFR1c and FGF2·FGFR2c complexes. In addition, the dp10 HS that bound to the FGF2·FGFR2c complex contained substantially more tri-S disaccharide than did the dp10 HS that bound to the FGF1·FGFR1c and FGF2·FGFR1c complexes. Binding studies using FGF1 and FGF2, in the absence of FGFR2c, were next conducted as a control experiment to ensure that the FGF·FGFR complexes remained intact in the oligosaccharide binding studies. The results (Table 1 of the Supporting Information) show clear differences between growth factor and complex binding to HS decasaccharides. In particular, HS decasaccharides binding FGF1 and the FGF1·FGFR1c complex had remarkably different compositions. Moreover, the affinity of FGF1 for decasaccharides rich in triS and FGF2 for decasaccharides rich in NS2S is consistent with literature reports (*20*−*22*, *34*).

Since the dp10 HS showed highest binding selectivity for the first three FGF·FGFR complexes, we examined seven additional FGF·FGFR complexes (FGF3·FGFR2b, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, FGF8b·FGFR2c, FGF10·

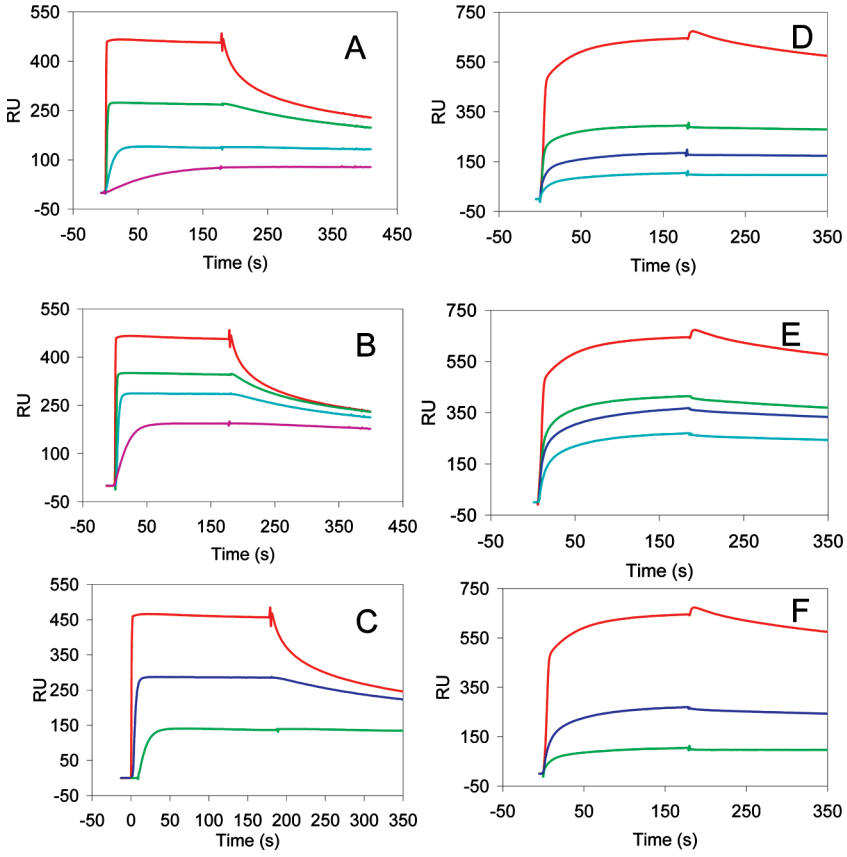

FIGURE 2: (A) Inhibition of binding of FGF2 to immobilized heparin by sized heparin oligosaccharide mixtures: control (no oligosaccharides) in red, dp6 in green, dp8 in turquoise, and dp10 in purple. (B) Inhibition of binding of FGF2 to an immobilized heparin chip by sized 6-desulfated heparin oligosaccharide mixtures: control (no oligosaccharides) in red, dp6 in green, dp8 in turquoise, and dp10 in purple. (C) Comparison of the inhibition of binding of FGF2 to immobilized heparin by dp8 oligosaccharide mixtures: red, control; blue, heparin 6-desulfated heparin dp8; green: heparin dp8. (D) Inhibition of binding of the FGF2·FGFR1c complex to immobilized heparin by a sized heparin oligosaccharide mixture: control (no oligosaccharides) in red, dp6 in green, dp8 in blue, and dp10 in turquoise. (E) Inhibition of binding of the FGF2·FGFR1c complex to immobilized heparin by a sized 6-desulfated heparin oligosaccharide mixture: control (no oligosaccharides) in red, dp6 in green, dp8 in blue, and dp10 in turquoise. (F) Comparison of the inhibition of FGF2·FGFR1c binding to immobilized heparin by dp8 oligosaccharide mixtures, red, control; blue, 6-desulfated heparin dp8; green: heparin dp8. The concentrations of FGF2, FGF2·FGFR1c complex, and the oligosaccharide were 1000, 500, and 2000 nM, respectively.

Table 1: Summary of the Inhibition Percentage of dp10 Oligosaccharides for Binding of FGF or the FGF·FGFR Complex to Heparin Based on the Solution Competition SPR

	FGF1	FGF2	FGF1·FGFR1c complex	FGF2·FGFR1c complex
dp10 heparin	95%	70%	99.7%	84%
6-desulfated dp10 heparin	63%	38%	92%	58%

FGFR2b, and FGF17·FGFR1c) were similarly studied using the same approach. Again, the binding decasaccharides obtained were determined by disaccharide compositional analysis. The disaccharide compositional analysis (Figure 5) showed that triS disaccharide was the only interacting structure in the FGF8b·FGFR2c and FGF10·FGFR2b complexes. This triS disaccharide was the dominant interacting structure with the FGF3·FGFR2b complex, but a small fraction of 0S, NS, and 6S disaccharide were also observed. The four remaining complexes (FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, and FGF17·FGFR1c) also selected for HS decasaccharide that were diverse in their disaccharide composition.

DISCUSSION

Information regarding the structural specificity of protein−HS interactions has been afforded by technical improvement in the methods for the structural analysis of HS oligosaccharides, mutational analysis of protein HS binding sites, molecular modeling, and, recently, crystal or NMR structures of protein−HS complexes (*35*). Interactions between heparin/HS and proteins have been characterized quantitatively using a number of techniques, including trapping and quantifying HS−protein complexes on surfaces, affinity co-electrophoresis, optical biosensors, and isothermal titration calorimetry. The sequences in HS that interact with FGF1 or FGF2 have been studied by biochemical and X-ray crystallographic analysis (*20*, *36*). It was concluded from initial studies that heparin/HS needs to interact with both FGF and FGFR for the signaling (*37*). In addition to the studies on FGF1 and FGF2, the HS sequences that mediate binding and/or activation of some HBGFs have been reported in the systems including FGF4 (*38*, *39*), FGF8b (*18*), hepatocyte growth factor (*4*, *5*, *19*), and platelet-derived growth factor (*6*). These studies on the binding structures

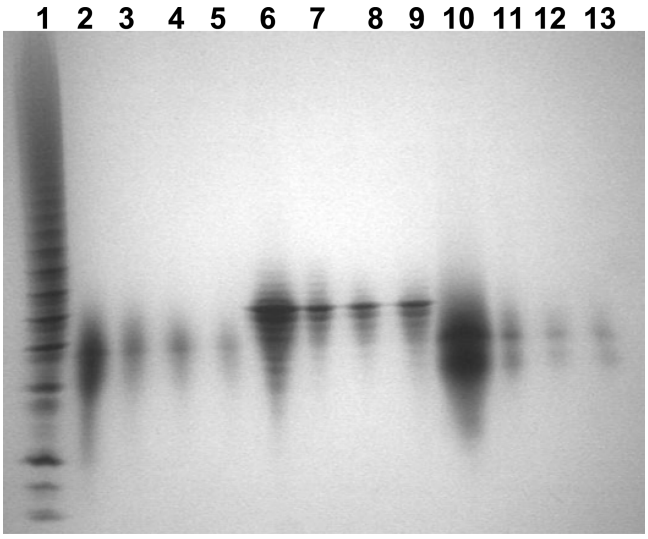

FIGURE 3: PAGE analysis on oligosaccharide released from FGF·FGFR specific binding: Lane 1, bovine lung heparin oligosaccharide standards; Lane 2: HS dp10; Lane 3 to 5, HS dp10 binding to FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c, respectively; Lane 6, heparin dp10; Lane 7 to 9, heparin dp10 binding to FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c, respectively; Lane 10, 6-desulfated heparin dp10; Lane 11 to 13, 6- desulfated heparin dp10 binding to FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c, respectively.

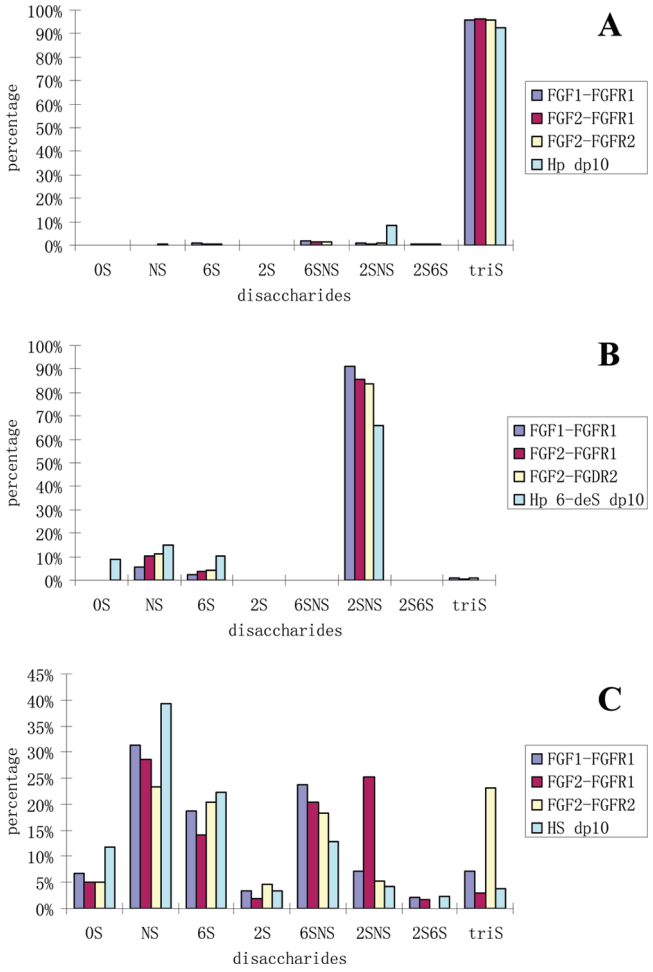

FIGURE 4: Disaccharide compositional determination of decasaccharides binding to FGF1·FGFR1c, FGF2·FGFR1c, and FGF2·FGFR2c complexes using LC−MS: (A) heparin (dp10) to different FGF·FGFR complexes, (B) dp10 6-desulfated heparin to different FGF·FGFR complexes, and (C) dp10 HS to different FGF·FGFR complexes. The light blue bars in panels A, B and C correspond to the composition of unfractionated dp 10 oligosaccharides.

in HS appear to support the idea that each heparin binding growth factor may specifically recognize unique structures in HS. A recent systematic study (*18*) using sequences modified with specific sulfotransferases shows that there were at least five classes of HS octasaccharide recognition sites for FGFs: (1) requiring a 2-*O*-sulfo group, FGF2; (2) requiring a 6-*O*-sulfo group, FGF10; (3) requiring a 2-*O*-sulfo, with a partial requirement for a 6-*O*-sulfo group, FGF18; (4) requiring both 2-*O*-sulfo and 6-*O*-sulfo groups, FGF4 and FGF7; and (5) no binding to an octasaccharide sequence, FGF8. Although the importance of HS in FGF signaling has been well documented over the past decade, the heparin/HS structure involved in the interaction with most FGFs is still largely undetermined. Most importantly, HS binds to both FGF and FGFR to form a signal transduction complex, and structural analysis of HS with binding activity to FGF·FGFR complexes has not been studied (*13*). In this study, we provide a new strategy for the study of the structural specificity of HS binding to FGF·FGFR complexes rather than FGFs or FGFRs alone.

The competition SPR studies between heparin and sized oligosaccharides (derived from heparin and 6-desulfated heparin) using FGF or the FGF·FGFR complex showed that all binding events were size-dependent (Figures 1 and 2), consistent with previous reports (*14*). It is generally held that binding to FGFs required oligosaccharides of tetrasaccharide to hexasaccharide length, whereas activation required larger oligosaccharides, octasaccharide to decasaccharide length. In comparison to heparin oligosaccharide, the 6-desulfated heparin oligosaccharide showed weaker inhibition in the competition, suggesting 6-desulfated heparin has a lower affinity for FGF1 and FGF2 and for FGF1·FGFR1c, FGF2·FGFR1c complexes. In comparison to FGF alone (Table 1), heparin decasaccharide and the 6-desulfated heparin decasaccharide more strongly inhibited binding of the FGF·FGFR complex to heparin, demonstrating that the affinity for the complex is higher than that for the single FGF and the ternary FGF·FGFR−heparin complex is more stable. These studies also clearly show that a 6-*O*-sulfo group was more important in the interaction of either oligosaccharide or GAG with FGF1 than with FGF2, consistent with literature reports (*20*, *22*, *23*, *34*), and similarly, a 6-*O*-sulfo group was more important in the interaction of either oligosaccharide or GAG with the FGF1·FGFR1c than with the FGF2·FGFR1c complex. These results also demonstrate that SPR can be utilized in competitive binding studies of FGF·FGFR complexes between GAG and GAG oligosaccharides.

During the fishing for specific oligosaccharides, 10 FGF·FGFR complexes FGF1·FGFR1c, FGF2·FGFR1c FGF2·FGFR2c, FGF3·FGFR2b, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, FGF8b·FGFR2c, FGF10·FGFR2b, and FGF17·FGFR1c) were used with a filter trapping method. Three sized oligosaccharides (dp10 from heparin, 6-desulfated heparin, and HS) were bound to each of three FGF·FGFR complexes in the first set of filter trapping experiments. PAGE analysis showed affinity differences between the complexes, suggesting the presence of unique high-affinity decasaccharides that could be used for the sequencing studies. All three complexes exhibited similar overall banding intensities for the tightly interacting heparin decasaccharides, suggesting that

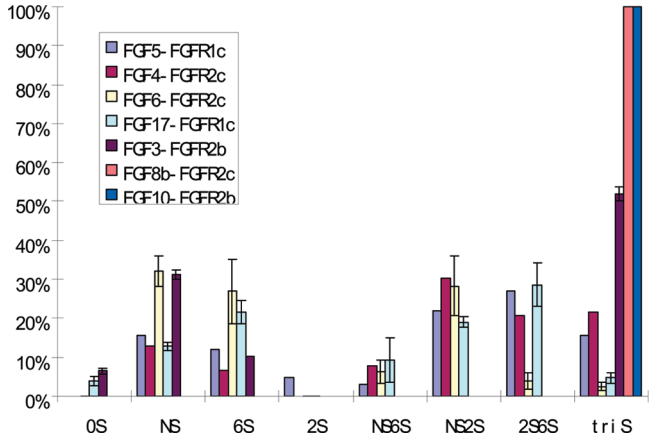

FIGURE 5: Disaccharide compositional analysis of dp10 HS binding to seven different complexes (FGF3·FGFR2b, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, FGF8b·FGFR2c, FGF10·FGFR2b, and FGF17·FGFR1c).

they have comparable high affinity for heparin. The overall banding intensity of interacting HS and 6-desulfated heparin decasaccharides suggests HS and 6-desulfated heparin exhibit decreasing relative affinities: FGF1·FGFR1c > FGF2·FGFR1c > FGF2·FGFR2c. These studies demonstrate that FGF·FGFR complexes can be used for affinity capture of specific oligosaccharides but suggest that it is necessary to increase the structural diversity of the oligosaccharide mixture being examined to optimize the molar ratio of diverse components and to identify structure for high-affinity binding. Since the binding of dp10 HS to different complexes displays very high selectivity (diversity of the disaccharide compositional structures) by disaccharide compositional analysis (Figure 4C), the remaining filter trapping experiments examined binding of dp10 HS binding to seven additional FGF·FGFR complexes (FGF3·FGFR2b, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, FGF8b·FGFR2c, FGF10·FGFR2b, and FGF17·FGFR1c). Disaccharide compositional analysis of interacting HS decasaccharides showed some complexes (FGF3·FGFR2b, FGF8b·FGFR2c, and FGF10·FGFR2b) binding a similar disaccharide compositional pattern with dominant triS structure and the rest of the tested complexes (FGF1·FGFR1c, FGF2·FGFR1c, FGF2·FGFR2c, FGF4·FGFR2c, FGF5·FGFR1c, FGF6·FGFR2c, and FGF17·FGFR1c) binding the decasaccharide having diverse composition of HS disaccharides (Figures 4 and 5). These results suggest that the FGF·FGFR complex binds with diverse structures of HSs, which depends on the abundance of different HSs available at the cell surface. These data are in agreement with a recent report (*37*) demonstrating the ability of HS chains to promote formation of a ternary complex among FGF and their receptors. These receptors may depend primarily on the abundance, length, and overall sulfation domains and possibly to a lesser degree on the selective saccharide sequence/precise location of sulfo groups. Moreover, FGF·FGFR complexes often select HS decasaccharide binding partners different from the FGF component alone (Table 1 of the Supporting Information).

In conclusion, SPR and filter trapping techniques were used to investigate FGF·FGFR−heparin/HS interactions and provide important structural information, in particular from HS decasaccharide libraries. The use of such libraries should facilitate the identification of critical structural features required for particular interactions and can greatly simplify qualitative and quantitative analysis. The methodology described may be useful in the discovery of novel glycotherapeutics that target disease-related protein−HS interactions.

SUPPORTING INFORMATION AVAILABLE

A table comparing the disaccharide composition of dp10 HS binding to FGF and dp10 HS binding to the related FGF·FGFR complex. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Linhardt, R. J., and Loganathan, D. (1990) Heparin, heparinoids and heparin oligosaccharides: Structure and biological activities. In Biomimetic Polymers (Gebelein, G., Ed.) pp 135−173, Plenum Press, New York.
2. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem. 68*, 729–777.
3. Iozzo, R. V. (1998) Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem. 67*, 609–652.
4. Bullock, S. L., Fletcher, J. M., Beddington, R. S., and Wilson, V. A. (1998) Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev. 12*, 1894–1906.
5. Merry, C. L., Bullock, S. L., Swan, D. C., Backen, A. C., Lyon, M., Beddington, R. S., Wilson, V. A., and Gallagher, J. T. (2001) The molecular phenotype of heparan sulfate in the Hs2st−/− mutant mouse. *J. Biol. Chem. 276*, 35429–35434.
6. Kamimura, K., Fujise, M., Villa, F., Izumi, S., Habuchi, H., Kimata, K., and Nakato, H. (2001) *Drosophila* heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. Structure, expression, and function in the formation of the tracheal system. *J. Biol. Chem. 276*, 17014–17021.
7. Itoh, N. (2001) Fibroblast growth factors. *Genome Biol. 2*, 1–12.
8. Ornitz, D. M. (2000) FGFs, heparan sulfate and FGFRs: Complex interactions essential for development. *BioEssays 22*, 108–112.
9. Bennasroune, A., Gardin, A., Aunis, D., Cremel, G., and Hubert, P. (2004) Tyrosine kinase receptors as attractive targets of cancer therapy. *Crit. Rev. Oncol. Hematol. 50*, 23–38.
10. Olsen, S. K., Garbi, M., Zampieri, N., Eliseenkova, A. V., Ornitz, D. M., Goldfarb, M., and Mohammadi, M. (2003) Fibroblast growth factor (FGF) homologous factors share structural but not functional homology with FGFs. *J. Biol. Chem. 278*, 34226–34236.
11. Jaye, M., Schlessinger, J., and Dionne, C. A. (1992) Fibroblast growth factor receptor tyrosine kinases: Molecular analysis and signal transductiom. *Biochim. Biophys. Acta 1135*, 185–199.
12. Yeh, B. K., Igarashi, M., Eliseenkova, A. V., Plotnikov, A. N., Sher, I., Ron, D., Aaronson, S. A., and Mohammadi, M. (2003) Structural basis by which alternative splicing confers specificity in fibroblast growth factor receptors. *Proc. Natl. Acad. Sci. U.S.A. 100*, 2266–2271.
13. Mohammadi, M., Olsen, S. K., and Ibrahimi, O. A. (2005) Structural basis for fibroblast growth factor activation. *Cytokine Growth Factor Rev. 16*, 107–137.
14. Wu, Z. L., Zhang, L., Yabe, T., Kuberan, B., Beeler, D. L., Love, A., and Rosenberg, R. D. (2003) The involvement of heparan sulfate (HS) in FGF1/HS/FGFR1 signaling complex. *J. Biol. Chem. 278*, 17121–17129.
15. Tumova, S., Woods, A., and Couchman, J. R. (2000) Heparan sulfate proteoglycans on the cell surface: Versatile coordinators of cellular functions. *Int. J. Biochem. Cell Biol. 32*, 269–288.
16. Ostrovsky, O., Berman, B., Gallagher, J., Mulloy, B., Fernig, D. G., Delehedde, M., and Ron, D. (2002) Differential effects of heparin saccharides on the formation of specific fibroblast growth factor (FGF) and FGF receptor complexes. *J. Biol. Chem. 277*, 2444–2453.
17. Lundin, L., Larsson, H., Kreuger, J., Kanda, S., Lindahl, U., Salmivirta, M., and Claesson-Welsh, L. (2000) Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis. *J. Biol. Chem. 275*, 24653–24660.
18. Ashikara-Hada, S., Habuchi, H., Kariya, Y., Itoh, N., Reddi, A. H., and Kimata, K. (2004) Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. *J. Biol. Chem. 279*, 12346–12354.
19. Kariya, Y., Kyogashima, M., Suzuki, K., Isomura, T., Sakamoto, T., Horie, K., Ishihara, M., Takano, R., Kamei, K., and Hara, S. (2000) Preparation of completely 6-*O*-desulfated heparin and its ability to enhance activity of basic fibroblast growth factor. *J. Biol. Chem. 275*, 25949–25958.
20. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science 271*, 1116–1120.

21. DiGabriele, A. D., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J., and Hendrickson, W. A. (1998) Structure of a heparin-linked biologically active dimer of fibroblast growth factor. *Nature 393*, 812–817.
22. Kreuger, J., Salmivirta, M., Sturiale, L., Giménez-Gallego, G., and Lindahl, U. (2001) Sequence analysis of heparan sulfate epitopes with graded affinities for fibroblast growth factors 1 and 2. *J. Biol. Chem. 276*, 30744–30752.
23. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. *J. Biol. Chem. 269*, 931–935.
24. Lyon, M., and Gallagher, J. T. (1998) Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion. *Matrix Biol. 17*, 485–493.
25. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) Activating and inhibitory heparin sequences for FGF2 (basic FGF). Distinct requirements for FGF1, FGF2, and FGF4. *J. Biol. Chem. 268*, 23906–23914.
26. Fromm, J. R., Hileman, R. E., Weiler, J. M., and Linhardt, R. J. (1997) Interaction of fibroblast growth factor-1 and related peptides with heparan sulfate and its oligosaccharides. *Arch. Biochem. Biophys. 346*, 252–262.
27. Ishihara, M. (1994) Structural requirements in heparin for binding and activation of FGF1 and FGF4 are different from that for FGF2. *Glycobiology 4*, 817–824.
28. Plotnikov, A. N., Hubbard, S. R., Schlessinger, J., and Mohammadi, M. (2000) Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity. *Cell 101*, 413–424.
29. Olsen, S. K., Ibrahimi, O. A., Raucci, A., Zhang, F., Eliseenkova, A. V., Yayon, A., Basilico, C., Linhardt, R. J., Schlessinger, J., and Mohammadi, M. (2004) Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity. *Proc. Natl. Acad. Sci. U.S.A. 101*, 935–940.
30. Olsen, S. K., Li, J. Y., Bromleigh, C., Eliseenkova, A. V., Ibrahimi, O. A., Lao, Z., Zhang, F., Linhardt, R. J., Joyner, A. L., and Mohammadi, M. (2006) Structural basis by which alternative splicing modulates the organizer activity of FGF8 in the brain. *Genes Dev. 20*, 185–198.
31. Plotnikov, A. N., Eliseenkova, A. V., Ibrahimi, O. A., Shriver, Z., Sasisekharan, R., Lemmon, M. A., and Mohammadi, M. (2001) Crystal structure of fibroblast growth factor 9 reveals regions implicated in dimerization and autoinhibition. *J. Biol. Chem. 276*, 4322–4329.
32. Zhang, F., Fath, M., Marks, R., and Linhardt, R. J. (2004) A highly stable covalent conjugated heparin biochip for heparin-protein interactions studies. *Anal. Biochem. 304*, 271–273.
33. Thanawiroon, C., Rice, K. G., Toida, T., and Linhardt, R. J. (2004) LC/MS sequencing of highly sulfated heparin-derived oligosaccharides. *J. Biol. Chem. 279*, 2608–2615.
34. Guglier, S., Hricovíni, M., Raman, R., Polito, L., Torri, G., Casu, B., Sasisekharan, R., and Guerrini, M. (2008) Minimum FGF2 binding structural requirements of heparin and heparan sulfate oligosaccharides as determined by NMR spectroscopy. *Biochemistry 47*, 13862–13869.
35. Powell, A. K., Yates, E. A., Fernig, D. G., and Turnbull, J. E. (2004) Interactions of heparin/heparan sulfate with proteins: Appraisal of structural factors and experimental approaches. *Glycobiology 14*, 17R–30R.
36. Jastrebova, N., Vanwildemeersch, M., Rapraeger, A. C., Giménez-Gallego, G., Lindahl, U., and Spillmann, D. (2006) Heparan Sulfate-related Oligosaccharides in Ternary Complex Formation with Fibroblast Growth Factors 1 and 2 and Their Receptors. *J. Biol. Chem. 281*, 26884–26892.
37. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science 252*, 1705–1708.
38. Powell, A. K., Fernig, D. G., and Turnbull, J. E. (2002) Fibroblast growth factor receptors 1 and 2 interact differently with heparin/heparan sulfate. *J. Biol. Chem. 277*, 28554–28563.
39. Woods, A., and Couchman, J. R. (1992) Heparan sulfate proteoglycans and signaling in cell adhesion. *Adv. Exp. Med. Biol. 313*, 87–96.