Crystal Structure of a Ternary FGF-FGFR-Heparin Complex Reveals a Dual Role for Heparin in FGF Binding and Dimerization

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Summary

The crystal structure of a dimeric 2:2:2 FGF:FGFR:heparin ternary complex at 3 Å resolution has been determined. Within each 1:1 FGF:FGFR complex, heparin makes numerous contacts with both FGF and FGFR, thereby augmenting FGF-FGFR binding. Heparin also interacts with FGFR in the adjoining 1:1 FGF:FGFR complex to promote FGFR dimerization. The 6-O-sulfate group of heparin plays a pivotal role in mediating both interactions. The unexpected stoichiometry of heparin binding in the structure led us to propose a revised model for FGFR dimerization. Biochemical data in support of this model are also presented. This model provides a structural basis for FGFR activation by small molecule heparin analogs and may facilitate the design of heparin mimetics capable of modulating FGF signaling.

Introduction

The fibroblast growth factor (FGF) family, composed of 21 members to date, is involved in a variety of critical biological processes such as cell proliferation, differentiation, cell migration, morphogenesis, and angiogenesis reviewed by Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Galzio et al., 1997; Naski and Ornitz, 1998; Nishimura et al., 2000). Four receptor tyrosine kinases, FGFR1–4, mediate the biological responses of FGFs. FGFRs are comprised of an extracellular ligand binding portion consisting of three immunoglobulin (Ig)-like domains (D1–D3), a single transmembrane helix, and a cytoplasmic portion with protein tyrosine kinase activity reviewed by Jaye et al., 1992; Johnson and Williams, 1993). The overall intricacy of the FGF-FGFR system is further enhanced by alternative splicing of FGFR mRNA, such as the splicing event in exon III of D3 that plays an important role in determining ligand-receptor specificity reviewed by Jaye et al., 1992; Johnson and Williams, 1993). Each of the FGFRs binds to a unique subset of FGFs accounting for the tight regulation and diversity in response to FGF stimulation (Ornitz et al., 1996; Plotnikov et al., 2000).

Receptor dimerization is an essential step in FGF signaling and requires heparan sulfate proteoglycans (HSPG) (Rapraeger et al., 1991; Yayon et al., 1991; Ornitz et al., 1992; Spivak-Kroizman et al., 1994). A dimerization model has emerged, based upon the crystal structure of FGF2 bound to the ligand binding domain (D2–D3) of FGFR1, that explains how FGFs act in concert with heparin to induce FGFR dimerization and activation (Plotnikov et al., 1999). According to the model, two 1:1 FGF:FGFR complexes form a symmetric dimer. Direct receptor–receptor interactions and secondary ligand–receptor interactions between an FGF of one 1:1 complex and the FGFR of the adjoining 1:1 complex stabilize this dimer. No direct ligand–ligand interactions are observed. The D2 domains of the two receptors form a canyon of positive potential that continues onto the top side of both ligands. It was postulated that heparin traverses this canyon and bridges the two 1:1 FGF:FGFR complexes. A recently reported crystal structure of another dimeric assemblage of two FGFR2 complexes lends additional support to this model (Stauber et al., 2000).

Based on the dimeric structure, manual docking experiments have shown that a maximally active dodecasaccharide perfectly traverses the canyon and engages both the high- and low-affinity heparin binding sites of the ligands. In contrast, an octasaccharide placed centrally into the canyon can only interact with the low-affinity heparin binding sites of the ligands. A cytoplastically docked hexasaccharide is unable to interact with any heparin binding sites of the ligands, implying that oligosaccharides smaller than an octasaccharide do not possess biological activity. However, there has been some controversy in determining the minimal length of heparin necessary for FGF signaling. It has been proposed that the shortest biologically active heparin oligosaccharide is an octasaccharide and that an increase in heparin length parallels an increase in biological activity up to a dodecasaccharide (Ornitz et al., 1992). However, other studies report that hexasaccharides are biologically active (Gambarini et al., 1993; Zhou et al., 1997) and that even disaccharides possess biological activity (Ornitz et al., 1995).

The inability of this model to fully reconcile all of the previous literature led us to further characterize the role of heparin in FGF signaling. In this report, we describe the crystal structure of a ternary FGF2-FGFR1-heparin complex. Interactions between heparin, FGF, and FGFR provide a molecular basis for the dual role of heparin in augmenting 1:1 FGF:FGFR affinity and promoting dimerization of two FGF-FGFR complexes. Moreover, the unexpected stoichiometry of FGF:FGFR:heparin observed in the structure led us to propose a model that...
Five percent of the reflections were used for calculation of R free.

Assembly is maintained. Traversing of the canyon by 2-O-sulfate groups of heparin. This provides an explanation between FGF and heparin involve N-sulfate and sulfate-mediated interactions. The ligands. Consequently, the symmetry of the dimeric FGF2±hexasaccharide structure (Faham et al., 1996).

Heparin Structure

At the FGF±heparin interface, a total of 16 hydrogen bonds are made, of which 10 are predicted to be mediated by the helical conformation of the decasaccharide (data not shown). The GlcN rings are all found in a chair conformation. The IdoA rings are in either a chair or a skewed boat conformation as previously observed in the solution structure of a dodecasaccharide (Mulloy et al., 1993), suggesting that IdoA can adopt multiple conformations depending on the contacts it makes with FGF or FGFR. It is likely that the conformational flexibility of IdoA plays a role in specific recognition of various FGFs or FGFRs.

Results and Discussion

Structure Determination

Since the heparin binding canyon is present in the FGF2-FGFR1 crystals, we reasoned that incubation of these crystals with decasaccharide would facilitate obtaining a ternary FGF-FGFR-heparin complex. The structural basis of the ternary FGF2-FGFR1-decasaccharide was solved using the phases obtained from the FGF2-FGFR1 structure (Plotnikov et al., 1999). Data collection and refinement statistics are given in Table 1. We anticipated finding a single decasaccharide molecule traversing the canyon and bridging the ligands. However, the difference Fourier electron density map clearly shows two decasaccharide molecules in the canyon (Figure 1). Only five of the six sugar rings (A±F) are observed to interact with protein. Consequently, the electron density is well defined for these rings. In addition, due to favorable lattice contacts, two additional sugars (rings G and H) could be modeled for one of the decasaccharides.

Heparin Structure

The decasaccharide can be approximated as a helix generated by repeating disaccharide units of D-glucosamine (GlcN) and L-iduronic acid (IdoA) joined by α-1→4 linkages. Each disaccharide unit is sulfated at three positions, one at the 2-hydroxyl group of IdoA and two at the 2-amino and 6-hydroxyl groups of GlcN. Sulfate and carboxylate groups form the negatively charged edges of the heparin helix and appear on a given side of the helix every 17±19 Å on average. These helical parameters are in agreement with the X-ray fiber diffraction values of 8.7 Å and 180° for a heparin polymer (Nieduszynski et al., 1977). Heparin polysaccharides are polar entities with a nonreducing end (O4) and a reducing end (O1). In the crystal structure, the decasaccharides bind with their nonreducing ends in the center of canyon and run out onto the high-affinity heparin binding sites of the ligands. Consequently, the symmetry of the dimeric assembly is maintained. Traversing of the canyon by one polar heparin molecule disrupts the 2-fold symmetry of the system.

Several intramolecular hydrogen bonds stabilize the helical conformation of the decasaccharide (data not shown). The GlcN rings are all found in a chair conformation. The IdoA rings are either in a chair or a skewed boat conformation as previously observed in the solution structure of a dodecasaccharide (Mulloy et al., 1993), suggesting that IdoA can adopt multiple conformations depending on the contacts it makes with FGF or FGFR. It is likely that the conformational flexibility of IdoA plays a role in specific recognition of various FGFs or FGFRs.

Heparin–FGF and Heparin–FGFR Interactions

Each decasaccharide makes a total of 30 hydrogen bonds with FGF and both FGFRs (Figure 2). Within one 1:1 FGF:FGFR complex, 25 hydrogen bonds are made with heparin. The remaining five hydrogen bonds with heparin originate from the FGFR of the adjoining 1:1 FGF:FGFR complex. Lysines 160, 163, 172, 175, and 177, located on the heparin binding surface of D2 make nine hydrogen bonds with heparin in the context of a 1:1 FGF:FGFR complex. The bulk of these hydrogen bonds are sulfate mediated with all three types of heparin sulfate groups (N-sulfate, 2-O-sulfate, and 6-O-sulfate) being employed (Figure 2).

At the FGF–heparin interface, a total of 16 hydrogen bonds are made, of which 10 are predicted to be mediated (Figure 2). The remaining six hydrogen bonds involve the carboxylate, linker, or ring oxygens of heparin. Surface residues Asn-27 (located in the β1-β2 loop), Arg-120, and Thr-121 (located in the β9-β10 loop), Lys-125, Lys-129, Gin-134, Lys-135, and Ala-136 (located in β11-β12 loop) form the heparin binding site on FGF. These residues are the same ones that interact with heparin in the FGF2–hexasaccharide structure (Faham et al., 1996). However, since the orientation of the heparin helix with respect to FGF is flipped between these two structures, the hydrogen bonding pairs are not identical.

Except for two hydrogen bonds (one between Lys-135 of FGF2 and the 6-O-sulfate of ring B and the other between Thr-121 of FGF2 and the 6-O-sulfate group of ring E), the remainder of the sulfate-mediated interactions between FGF and heparin involve N-sulfate and 2-O-sulfate groups of heparin. This provides an explanation for why FGF2 retains binding ability to 6-O-desulfated heparin (Guimond et al., 1993; Rusnati et al., 1994;
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Figure 1. Electron Density Map of Decasaccharides Soaked into Preformed Crystals of an FGF2-FGFR1 Complex

(A) Location of decasaccharides in the dimeric assemblage. Only the Cα traces of D2s (cyan) and FGFs (orange) are shown. The decasaccharides are rendered in white sticks.

(B) Stereo view of F1 – F6, electron density map computed after simulated annealing with decasaccharide omitted from the atomic model. The map is computed at 3.0 Å resolution and contoured at 1.8 σ. Sugar rings are labeled A through H starting at the nonreducing end of the decasaccharide. Atom coloring is as follows: oxygens in red, sulfurs in yellow, nitrogens in blue, and carbons in gray. This figure was made using Bobscript (Esnouf, 1997).

Nevertheless, 6-O-desulfated heparin oligosaccharides are still ineffective in promoting FGF2-FGFR interaction (Guimond et al., 1993; Rusnati et al., 1994; Pye et al., 1998). In the present crystal structure, the 6-O-sulfate of ring B (Figure 2) makes hydrogen bonds with heparin binding residues of both FGF and FGFR. Concurrent binding of both FGF and FGFR to the same sulfate group of heparin clearly serves to increase the apparent affinity of FGF for FGFR. Hence, the present structure also provides a molecular basis for the well-documented heparin-dependent 1:1 FGF:FGFR interaction (Ornitz et al., 1992).
Figure 2. Detailed Interactions between Decasaccharide, FGF, and FGFR

(A) Stereo view of the interactions between decasaccharide, FGF, and FGFRs. Only the side chains of interacting residues are shown. The two D2s of the adjoining FGFRs are colored cyan and green, respectively. Atom coloring is the same as in Figure 1. Carbon atoms in FGFRs have the same coloring as the D2 to which they belong. Dotted lines represent hydrogen bonds. This figure was made using Molscript (Kraulis, 1991) and Raster3D (Merrit and Bacon, 1997).

(B) Schematic diagram of interactions between decasaccharide, FGF, and FGFRs. Dashed lines represent hydrogen bonds. Hashed lines represent hydrophobic interactions. The sugar rings of heparin are labeled starting at the nonreducing end from A through F. The backbone carbon atoms of heparin are numbered according to IUPAC nomenclature. The type and the number of interacting residues are colored based on the molecule to which they belong. Coloring is as follows: FGF in red, D2 of the primary FGFR in blue, and D2 of the adjoining FGFR in green. Only the relevant functional groups and backbone atoms of the interacting amino acids are shown. This figure was made using ChemDraw Pro 4.5 (Cambridge Software).
In addition to promoting FGF-FGFR interaction within the 1:1 FGF:FGFR complex, heparin also interacts with the adjoining receptor across the 2-fold dimer. A total of five hydrogen bonds are made at this interface between FGFR residues Lys-207 and Arg-209 and sugar rings A–D of heparin (Figure 2). Hydrophobic contacts between Ile-216 and the nonreduced ring A of heparin further fortify this interface. The hydrogen bonds between Lys-207 and heparin are mediated via carboxylate, linker, and ring oxygens of heparin. In contrast, Arg-209 makes hydrogen bonds with the 2-O-sulfate group of ring C and the G-O-sulfate group of ring D, thereby emphasizing the critical dual role of 6-O-sulfate in promoting 1:1 FGF2:FGFR interaction and inducing 2:2 FGF:FGFR dimer formation. The crystal structure provides a plausible explanation for the well-documented inability of 6-O-desulfated heparin oligosaccharides to promote mitogenic activities by failing to induce receptor dimerization (Guimond et al., 1993; Rusnati et al., 1994; Pye et al., 1998).

While the heparin binding residues in D2 of FGFRs are highly conserved, the heparin binding residues of the FGF family display considerable diversity (Faham et al., 1998; Venkataraman et al., 1999). Moreover, the β1–β2 heparin binding loop is of variable length in different FGFs. As a result of this heterogeneity, it is likely that different FGFs require heparan sulfates of distinct sulfation and/or length to exert their optimal biological activity. In fact, it has been demonstrated that FGF2 requires 2-O-sulfate for heparin binding but not 6-O-sulfate. In contrast, FGF1 requires both sulfate groups to bind to heparin (Ishihara, 1994). Pericellular HSPGs from different cells exhibit significant heterogeneity in sulfation patterns, carbohydrate content, and length. These variations could have a profound effect on FGF-FGFR interactions. Moreover, remodeling of the extracellular matrix during development may be a means to regulate the biological activities of FGFs.

The heparin binding mode in the present structure disputes the previous findings regarding the minimal length requirement for heparin to promote FGF-FGFR dimerization as well as the stoichiometry of FGF:FGFR:heparin interactions. The tripartite interactions between FGF, FGFR, and heparin observed in the crystal structure suggest that heparin hexasaccharides are sufficient to promote receptor dimerization. Therefore, we decided to test the ability of a hexasaccharide to promote dimerization of FGF-FGFR complexes in vitro. Homogeneously sulfated hexasaccharide was mixed at various molar ratios with a purified 1:1 FGF1:FGFR2 complex, and the reaction mixtures were analyzed by size exclusion chromatography to quantitate dimerization (Figure 3). Addition of hexasaccharide at a molar ratio of 0.5:1 hexasaccharide:FGF1:FGFR2 complex, and the reaction mixtures were analyzed by size exclusion chromatography to quantitate dimerization (Figure 3). Addition of hexasaccharide at a molar ratio of 0.5:1 hexasaccharide:complex dimerized half of the FGF1:FGFR2 complex (Figure 3B). Hexasaccharide at a molar ratio of 1:1 hexasaccharide:complex led to the quantitative dimerization of all the FGF1:FGFR2 complexes (Figure 3C). Excess hexasaccharide reduced dimerization and resulted in the appearance of a peak that elutes slightly earlier than the control (Figure 3D). This peak corresponds to the ternary 1:1:1 hexasaccharide:FGF1:FGFR2 complex and is expected since excess heparin would compete with the secondary heparin interaction site of the adjoining FGFR. Thus, dimer formation would be reduced and accompanied by a concurrent increase in the amount of the monomeric, tight 1:1:1 FGF:FGFR:hexasaccharide ternary complex. It is noteworthy that in the absence of heparin, FGF-FGFR complexes tend to dissociate under size exclusion chromatography conditions, indicating that heparin increases

Figure 3. Hexasaccharide Can Induce FGFR Dimerization Homogeneously sulfated hexasaccharide was mixed in various ratios with purified 1:1 FGF1:FGFR2 complex, and the reaction mixtures were analyzed on a Superdex 200 (Pharmacia) for dimer formation. (A) Control (no hexasaccharide added); (B) hexasaccharide was added at a molar ratio of 0.5:1 hexasaccharide:complex; (C) hexasaccharide was added at a molar ratio of 1:1 hexasaccharide:complex; (D) hexasaccharide was added at a molar ratio of 2.85:1 hexasaccharide:complex. The positions of monomers and dimers are indicated by the letters M and D, respectively. The letter T shows the position of the tight monomeric ternary 1:1:1 hexasaccharide:FGF1:FGFR2 complex. The letter L shows the position of free FGF1.
the affinity of FGF toward FGFR and stabilizes dimer formation.

We repeated the dimerization assays with longer heparin oligosaccharides to further confirm the 2:2:2 stoichiometry of FGF:FGFR:heparin for optimal dimerization. A similar stoichiometry of dimer formation was observed with both octasaccharide and decasaccharide (data not shown). Thus, the binding stoichiometry in solution strongly confirms the observed mode of heparin binding in the crystal structure. Moreover, though a decasaccharide can traverse the canyon to bridge the ligands, it still requires a 2:2:2 molar ratio of FGF:FGFR:heparin to induce dimerization. Based on these results, we propose that this structure most likely represents the major, biologically relevant ternary FGF:FGFR-heparin structure. To obtain additional independent verification of the heparin binding mode observed in the soaked crystals, we have recently cocrystallized the FGF2-FGFR1 complex with hexa-, octa-, and decasaccharides (M. M., unpublished results). The electron density map of the FGF2-FGFR1-hexasaccharide cocrystal structure, albeit at low resolution, clearly shows the same mode of heparin binding as the one seen in the soaked crystal, demonstrating that the model presented is not biased by the soaking procedure.

It is noteworthy that the purified unliganded extracellular domains of FGFRs are monomeric in solution as judged by size exclusion chromatography. Nevertheless, the existence of preformed FGFR dimers on the cell surface cannot be precluded since the high local concentration on the plasma membrane could lead to dimerization that cannot be detected in solution. In this case, an alternative mechanism, similar to the one proposed for the erythropoietin receptor (Livnah et al., 1999), may account for heparin-dependent FGF-FGFR dimerization, whereby FGF, aided by heparin, alters the conformation of a preformed inactive FGFR dimer.

Conclusions
Based upon the crystal structure and supporting biochemical experiments, we propose a “two-end” model by which heparin induces FGF-dependent FGFR dimerization (Figure 4). According to this model, heparin interacts via its nonreducing ends with both FGF and FGFR and promotes the formation of a stable 1:1:1 FGF:FGFR:heparin ternary complex. A second 1:1:1 FGF:FGFR:heparin ternary complex is then recruited to the first complex via direct FGFR:FGFR contacts, secondary interactions between FGF in one ternary complex and FGFR in the other ternary complex, and indirect heparin-mediated FGFR-FGFR contacts. In the absence of heparin, the direct receptor–receptor contacts and secondary ligand–receptor interactions are not sufficient for appreciable dimerization. Clearly, heparin augments direct FGFR-FGFR and secondary FGF-FGFR interactions.

The proposed two-end model presented in this report is entirely consistent with the chemical architecture of heparan sulfate chains of HSPGs. Based on chemical and enzymatic hydrolysis analyses, heparan sulfate chains of HSPGs can be roughly divided into low and high sulfate regions (Gambarini et al., 1993). The low sulfate region, at the reducing end, is linked to the protein core and is mainly N-sulfated. This region is highly susceptible to hydrolysis with heparitinase I. Since 6-O-desulfated heparins are ineffective in promoting FGF-dependent FGFR dimerization, we believe that these low sulfate regions at the reducing end are not likely to play a major role in FGF signaling. The high sulfate region, at the nonreducing end, is highly susceptible to treatment with heparitinase II. Moreover, the chemical nature of the highly sulfated nonreducing end resembles heparin and is made up of trisulfated disaccharide units, [IdoA,2S-GlcNS,6S], considered to be the building block of heparin (Gambarini et al., 1993). These highly sulfated regions of heparan sulfate have been
shown to be the major determinants of the potentiating effect of heparan sulfate on FGF1 mitogenic activity (Gambarini et al., 1993). Based on the crystal structure, these highly sulfated nonreducing ends are expected to interact with both FGF and FGFRs to induce dimerization.

A survey of the nature of the tripartite interactions between FGF, FGFR, and heparin shows that about half of these interactions are mediated through carbohydrate, linker, and ring oxygens of heparin. Therefore, the results presented here afford a structural basis for the ability of certain synthetic nonsulfated heparan-derived di- and triasaccharides to promote FGF-dependent FGF activation in vivo (Ornitz et al., 1995). Large scale synthesis of heparin molecules with an homogeneous sulfation pattern remains an obstacle. On the basis of the structure presented here, it is possible to design small molecule heparin analogs in which the sulfate groups are replaced with similar functional groups. Thus, our structural studies establish a framework for the rational design of heparin mimetics capable of modulating FGF activity. Given the important roles FGF plays in angiogenesis and other biological processes, synthetic heparin agonists and antagonists may have potential therapeutic value.

Experimental Procedures

Expression, purification, and crystallization of FGF2-FGFR1 complex were carried out as described previously (Plotnikov et al., 1999). Crystals of the native FGF2-FGFR1 complex were incubated in 10 µl of stabilizing solution [40% PEG 8000, 0.25 M ammonium sulfate, 0.1 M Tris–HCl (pH 8.3)] containing 1 mM decasaccharide for 1 week at 20°C. Data were collected on a flash-frozen crystal (in a dry nitrogen stream using mother liquor containing 10% glycerol as cryo-protectant) on a CCD detector at beamline X4A at the National Synchrotron Light Source, Brookhaven National Laboratory. Data processing were performed using DENZO and SCALEPACK (Otwinowski, 1993).

Difference Fourier electron density maps were computed using the FGF2-FGFR1 structure (Plotnikov et al., 1999). The initial model for the oligosaccharide was taken from the crystal structure of FGF2 in complex with hexasaccharide (1BF) (Faham et al., 1998). The parameters for the oligosaccharide were generated using the HIC-Up server (Kleywegt and Jones, 1998). Simulated annealing and positional/B factor refinement were performed using CNS (Brunger et al., 1998). Model building into 2Fo − Fc and Fc − F electron density maps was performed with the program O (Jones et al., 1991). The average B factor is 36.9 Å² for all atoms, 35.0 Å² for FGF2, 35.3 Å² for FGFR1, and 72.4 Å² for decasaccharide molecules.

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References


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