Diversity does make a difference: fibroblast growth factor–heparin interactions

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Fibroblast growth factors (FGFs) are members of a protein family with a broad range of biological activities. The best characterized FGFs interact with two distinct extracellular receptors – a transmembrane tyrosine kinase FGF receptor (FGFR) and a heparan sulfate-related proteoglycan of the extracellular matrix. These components form a FGF–FGFR–proteoglycan complex that activates the FGF-mediated signal transduction process through FGFR dimerization. Recent crystal structure determinations of FGF–heparin complexes have provided insights into both the interactions between these components and the role of heparin-like proteoglycans in FGF function. Future advances in this field will benefit enormously from an ability to specifically prepare homogenous heparin-based oligosaccharides of defined sequence for use in biochemical and structural studies of FGF and many other systems.

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Abbreviations

FGF	fibroblast growth factor
FGFR	FGF receptor
GlcN	glucosamine
IdoA	iduronic acid
KGF	keratinocyte growth factor
KGFR	KGF receptor
PDB	Protein Data Bank

Introduction

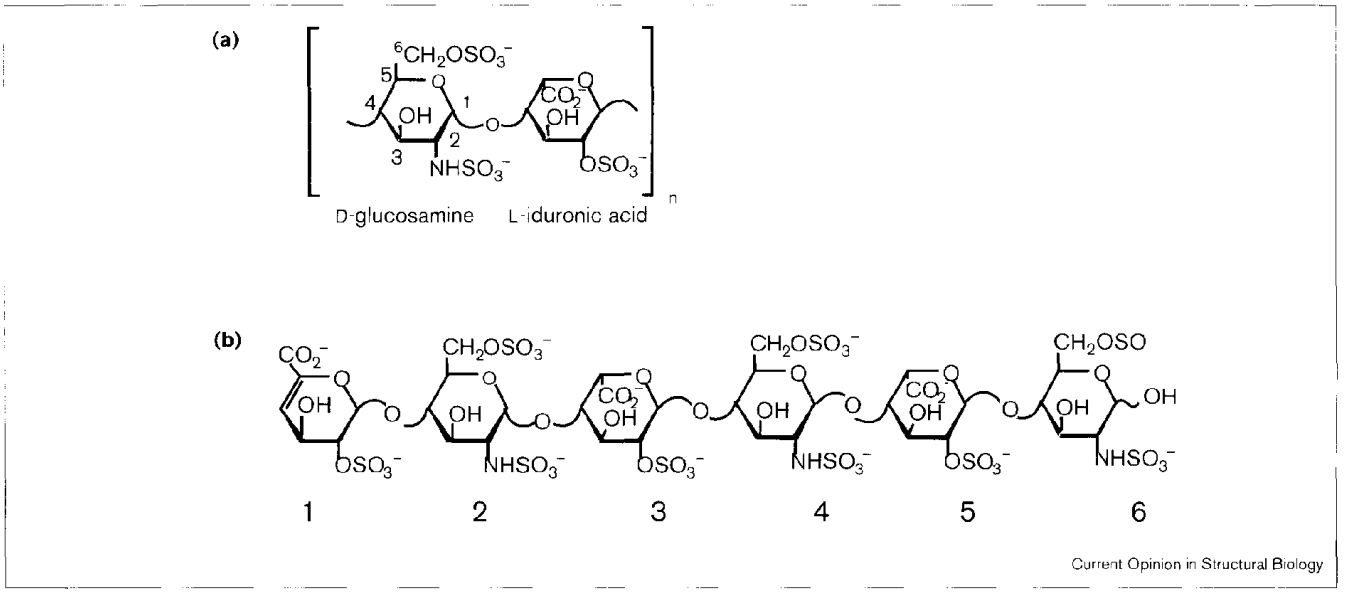
Many growth factors, including the fibroblast growth factors (FGFs), bind to the extracellular matrix of target tissues by interacting with heparan sulfate and related proteoglycans [1,2]. A variety of roles for proteoglycans in this process have been proposed, including protection from proteolysis, localization, storage and internalization of growth factors. Interactions with heparan sulfate-like proteoglycans are required for the activation of the FGF-mediated signal transduction process. Members of the FGF family participate in a wide variety of biological activities involving cell growth and differentiation, including angiogenesis, morphogenesis and wound healing (reviewed in [3–7]). Presently, 18 (and counting) members of the FGF family have been identified [8], as well as four transmembrane tyrosine kinase receptors for FGF (FGFRs), which exist as multiple splice variants with different affinities for different FGFs (see [9]). The diverse activities exhibited by the FGF family reflect an intricate combinatorial process involving potential interactions between multiple FGFs, FGFRs and the naturally heterogeneous glycosaminoglycan sidechains of heparan sulfate proteoglycans. In common with other receptor-mediated pathways, FGF-stimulated signal transduction involves the dimerization of FGFR and it is probable that heparan sulfate proteoglycans participate in this process through a direct, divalent cation-dependent interaction with FGFR [10,11] and/or through the oligomerization of FGFs [12–15].

High resolution structural studies into the role of proteoglycans in the FGF system have so far focused exclusively on the characterization of interactions between FGFs and heparan sulfate-related proteoglycans, in particular heparin (see [16••] for an excellent recent review of this area). Although anecdotal reports surface periodically of efforts to extend structural analyses to include the extracellular FGF-binding domains of the FGFR, to date, the structure of the intracellular tyrosine kinase domain is the only FGFR-related component to have been solved [17]. As a starting point for this discussion of heparin–FGF interactions, the basic features of the heparin and FGF structures, and the energetics and stoichiometry of their interaction will be briefly summarized.

Heparin structure

The heparin polymer (reviewed in [18,19]) is generated from disaccharide repeat units consisting of D-glucosamine (GlcN) and L-iduronic acid (IdoA) joined by α1-4 linkages (Figure 1). Heparin and, to a greater degree, heparan sulfate can also contain D-glucuronic acid, *N*-acetylated D-glucosamine and, rarely, unsubstituted D-glucosamine. Successive disaccharides within heparin are related by a twofold screw operation, generated by a rotation of approximately 180°, coupled with a translation of 8.0–8.7 Å along the helix axis. A typical heparin disaccharide contains three sulfate groups — one attached to the 2-hydroxyl group of IdoA and two linked to the 2-amino and 6-hydroxyl groups of GlcN. The negatively charged sulfate and carboxyl groups are positioned along the edges of the ribbon-like heparin molecule. Heparan sulfate, found in cell surface proteoglycans, has a similar structure to heparin, although it is typically less extensively sulfated than heparin. Extensive heterogeneity exists in heparan sulfate proteoglycans — including the length of the saccharide chain, the extent of sulfation and the core carbohydrate sequence — and it is quite likely that this

Figure 1



Chemical structure of heparin. **(a)** Structure of the repeating disaccharide unit of heparin. **(b)** Structure of the heparin-derived hexasaccharide used in the crystallographic studies described in [36], with the numbering scheme of the sugars indicated.

'heterogeneity' is utilized for the carbohydrate sequence-specific binding of particular molecules, including FGFs.

FGF structure

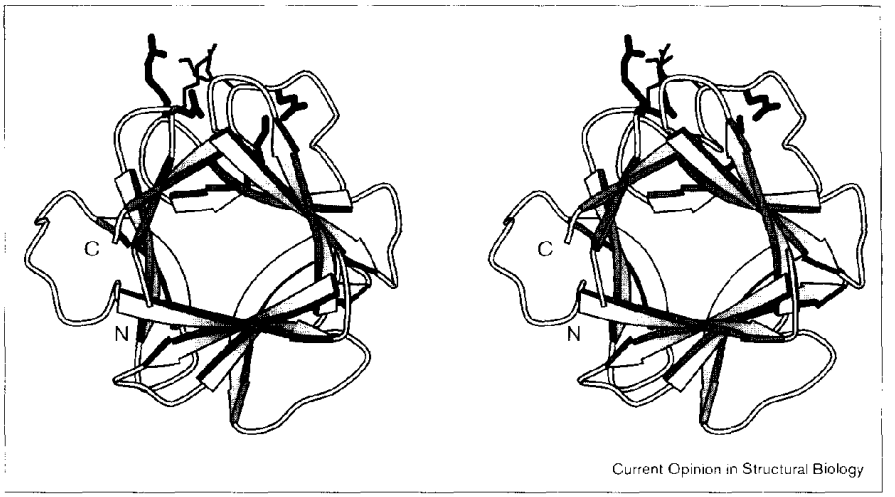
Crystallographic analyses of FGF-1 (acidic FGF, [20,21]) and FGF-2 (basic FGF, [20,22–25]) structures have demonstrated that these proteins adopt a 'β trefoil' [26] fold, which consists of three copies of a basic four-stranded antiparallel β sheet (Figure 2). A variety of studies, performed primarily on FGF-2, have identified a stretch of basic residues in the polypeptide chain, encompassing β strands 10 and 11, as participating in the heparin-binding site (see [15,27] and the general reviews).

Binding stoichiometry and energetics

Dissociation constants in the 0.1–10 μM range have been reported for the binding of FGF-2 to various heparin-derived oligosaccharides [13], with longer oligosaccharides generally exhibiting tighter binding. Longer oligosaccharides can also bind to more than one FGF molecule. The relationship between heparin length, biological activity and FGF binding has been extensively studied [28] and there is general agreement for the fact that longer heparin oligosaccharides tend to be more biologically active. A key issue, and one that remains controversial, concerns whether a minimum heparin length is required for activity and, if so, what that length is. Even though the heparin

Figure 2

Stereo view of FGF-2 (Protein Data Bank [PDB] coordinates 1BFC, [36]), illustrating the polypeptide fold adopted by members of the FGF protein family. The sidechains of residues forming the high and low affinity heparin-binding sites are represented by thick and thin black lines, respectively. The molecular representations in this paper were prepared with the program MOLSCRIPT [47].



tetrasaccharide can bind two FGF molecules at high concentrations [29•], it has not been found to support FGF activity (for recent reports, see [30,31]). Interestingly, however, nonsulfated disaccharides and trisaccharides based on the heparin pattern have been reported to activate the FGF signaling pathway [32]. Most studies indicate that heparin octasaccharides and longer species can support FGF activity [12,28,30,33,34]. The most controversial results concern the ability of heparin hexasaccharides in stimulating FGF activity; hexasaccharides have been reported to be inactive in some studies [12,28,33,34], whereas other studies have observed stimulatory activity for this species [31,35]. It is likely that different assays are sensitive to different aspects of the FGF–heparin interaction, so different results can be obtained when the reagents used are nominally the same. Perhaps the safest conclusions at present are that neither the length of the heparin fragment nor the stoichiometry of FGF binding to heparin are the sole determinants of activity, but rather specific types of complexes are required.

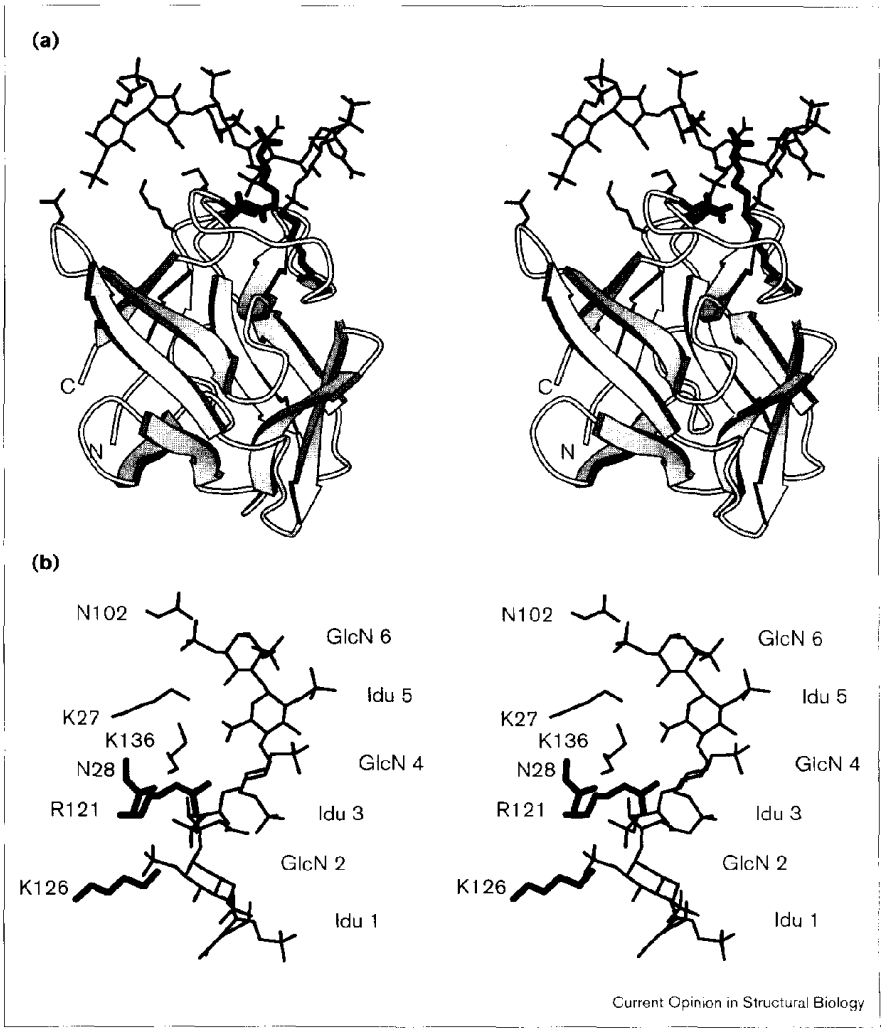
Structures of heparin–FGF complexes

Crystal structures were initially determined for FGF-2 complexed with a heparin tetrasaccharide and a heparin hexasaccharide [36]. The two oligosaccharides bind to FGF-2 in similar fashion, with the nonreducing ends closely superimposed. Both oligosaccharides interact with residues Asn28, Arg121, Lys126 and Gln135, and the longer hexasaccharide also contacts residues Lys27, Asn102 and Lys136 (Figure 3). Since the shorter tetrasaccharide and the longer hexasaccharide overlap in the tetrasaccharide-binding region, this must represent a high affinity site for the binding of FGF to heparin oligosaccharides that are based on the disaccharide sequence repeat of heparin. The high affinity site was previously observed to be occupied by a sulfate group in the binding of sucrose octasulfate to FGF-1 [37] and by a sulfate group from the crystallization buffer for FGF-2 [23,24].

Many of the specific interactions between FGF-2 and the heparin hexasaccharide utilize negatively charged groups on heparin (Table 1). In particular, the 2-*N*-sulfate groups

Figure 3

Protein–heparin interactions in FGF-2. **(a)** Stereo view of the binding of a heparin-derived hexasaccharide to FGF-2 (PDB coordinates 1BFC, [36]). The sidechains of the FGF-2 residues involved in the high and low affinity heparin-binding sites are represented by the thick and thin black lines, respectively. Relative to Figure 2, this figure is rotated approximately 90° about a vertical axis in the plane of the page. **(b)** Stereo view detailing the sidechain-mediated interactions between FGF-2 and a heparin-derived hexasaccharide. The sidechains of residues forming the high and low affinity heparin-binding sites are represented by thick and thin black lines, respectively. The sidechain of Gln135 in the high affinity binding site was removed for clarity.



of GlcN residues 2 and 6, the 2-*O*-sulfate group of IdoA residue 3, and the carboxylate group of IdoA residue 5 are involved in salt bridges and hydrogen-bonding interactions with polar groups on FGF-2. The surface areas lost by the burial of FGF residues upon binding of the tetrasaccharide and hexasaccharide have been calculated to be ~180 Å^2 and ~290 Å^2, respectively. Only a few residues contribute the bulk of the surface area buried in these complexes; in the tetrasaccharide complex, over half of the buried surface area is contributed by three residues, Arg121, Lys126 and Lys136, whereas Lys27 and Asn102 each provide an additional ~30 Å^2 of buried surface area in the hexasaccharide complex. Significantly, the binding of these oligosaccharides to FGF-2 was not associated with any significant conformational changes of the polypeptide backbone, indicating that it is unlikely that heparin-induced conformational changes in FGFs are required for the activation of the signal transduction pathway.

The structure of FGF-1 crystallized in the presence of a heparin decasaccharide has recently appeared [38••]. Dimers of FGF-1 exist in these crystals with the monomers bridged exclusively by heparin (Figure 4); that is, no protein–protein interactions occur. The interface consists of five to six heparin monosaccharide units, with the remaining sugar residues of the decasaccharide disordered and not visible in the electron density maps. There are strong parallels between the heparin complexes of FGF-1 and FGF-2 — the orientation of the helix axis of heparin with respect to the FGF-1 polypeptide fold is virtually identical to that previously observed in FGF-2–heparin complexes and many of the contacts formed between FGF-1 and heparin correspond closely to the high affinity binding site identified on FGF-2 (Figure 4, Table 1). An important aspect of this most recent structure is that the FGF-1 molecules bind to adjacent disaccharides on opposite sides of the heparin helix, forming a heparin-bridged dimer; these interactions are mediated in part through contacts between FGF-1 and the 6-*O*-sulfate groups that are not utilized in the binding of FGF-2 to heparin. Since the two FGF-1 monomers are approximately related by a twofold rotation, while the heparin helix is polar, with consecutive units related by a 2_1 screw axis, the two FGF-1 molecules form distinct contacts with heparin. One FGF-1 molecule

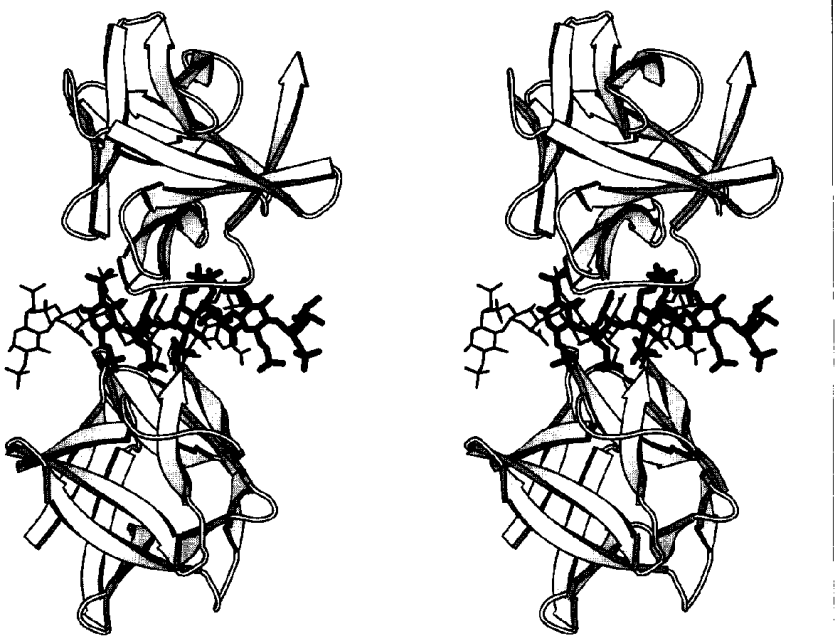
Table 1

Polar contacts between FGF-2 or FGF-1 residues and heparin-derived oligosaccharides, as observed crystallographically [36, 38••] in the 1BFC and 2AXM PDB entries.

Heparin		FGF-2	FGF-1†	
Residue number*	Atom (or group)	Residue number	Residue number (A)	Residue number (B)
IdoA −1	6-O-SO_3^-		R122 (130)	
GlcN 0	6-O-SO_3^-		R122 (130)	
GlcN 0	2-SO_3^-			K118 (126)
GlcN 0	2-SO_3^-			K128‡ (136)
GlcN 0	2-SO_3^-			A129‡ (137)
IdoA 1	2-O-SO_3^-			K113‡ (121)
IdoA 1	2-O-SO_3^-			N18 (28)
IdoA 1	2-O-SO_3^-			K118 (126)
IdoA 1	6-CO_2^-			K113 (121)
GlcN 2	2-SO_3^-	N28	N18 (28)	
GlcN 2	2-SO_3^-	R121‡		
GlcN 2	2-SO_3^-	K126	K118 (126)	
GlcN 2	6-O-SO_3^-			K113‡ (121)
GlcN 2	6-O-SO_3^-			N114‡ (122)
IdoA 3	3-OH	N28	N18 (28)	
IdoA 3	3-OH	R121		
IdoA 3	2-O-SO_3^-	K126	K118 (126)	
IdoA 3	2-O-SO_3^-	Q135	Q127 (135)	
IdoA 3	2-O-SO_3	K136‡	K128‡ (136)	
IdoA 3	2-O-SO_3^-	A137‡	A129‡ (137)	
IdoA 3	6-CO_2^-			K112 (120)
IdoA 3	6-CO_2^-			R122 (130)
GlcN 4	2-SO_3^-			R122 (130)
GlcN 4	6-O-SO_3^-			K128‡(136)
IdoA 5	6-CO_2^-	K136		
GlcN 6	2-SO_3^-	K27		
GlcN 6	2-SO_3^-	N102		
GlcN 6	3-OH	K27		

*The heparin sugar residues are numbered according to the FGF-2–hexasaccharide complex. The hexasaccharide observed in the FGF-1 structure is shifted by a disaccharide related to the FGF-2 complex, so that the four sugar residues in common are numbered 1–4, while the two remaining residues at the reducing end of heparin in the FGF-1 complex are numbered 0 and −1, respectively. †For heparin contacts to FGF-1, the FGF-1 residue numbers are indicated, with the corresponding positions in FGF-2 provided in parentheses. A and B designate the two FGF-1 molecules of the heparin-bridged dimer. ‡These residues form a backbone contact with a heparin group.

Figure 4



Stereo view of the heparin-mediated FGF-1 dimer (PDB coordinates 2AXM, [38••]), with the hexasaccharide observed complexed to FGF-2 superimposed (PDB coordinates 1BFC, [36]). FGF-2 and the A subunit of FGF-1 were superimposed for this illustration. The heparin molecules of the FGF-1 and FGF-2 structures are indicated by thick and thin black lines, respectively.

interacts with heparin in a similar fashion to that observed in the FGF-2–hexasaccharide complex, while the contacts made by the other FGF molecule require swapping the positions of the GlcN-2-SO_3^- and the IdoA-2-*O*-SO_3^- groups. The exact pattern of interactions is not precisely defined, however, as variations are observed for the interactions between FGF-1 and heparin in different molecules related by noncrystallographic symmetry and in different crystal forms. A similar variability in the interactions between FGF-1 and sucrose octasulfate has also been noted [37].

The conformations of the heparin oligosaccharides in the crystal structures determined to date are generally similar. From these structures, the helical parameters of an idealized heparin helix can be estimated; average helical parameters of a 174° rotation and an 8.6 Å translation per disaccharide were calculated for the FGF-2–hexasaccharide complex [36], whereas average helical parameters of an approximately 160° rotation and an 8.0 Å translation per disaccharide can be calculated from the coordinates of the FGF-1–heparin complex [38••]. These values are in reasonable agreement with the X-ray fiber diffraction values of approximately 180° and 8.0–8.7 Å, for the rotation and translation, respectively, of the heparin polymer [18]. The observed torsion angles of the heparin oligosaccharides are within the calculated allowed region for the heparin polymer and are close to those previously derived from NMR data [39,40••]. Both NMR and X-ray crystallographic studies indicate that the GlcN rings adopt the chair conformation, whereas the IdoA residues exist in either the chair or skew boat forms, with the equilibrium position being sensitive to environmental conditions, including binding to proteins.

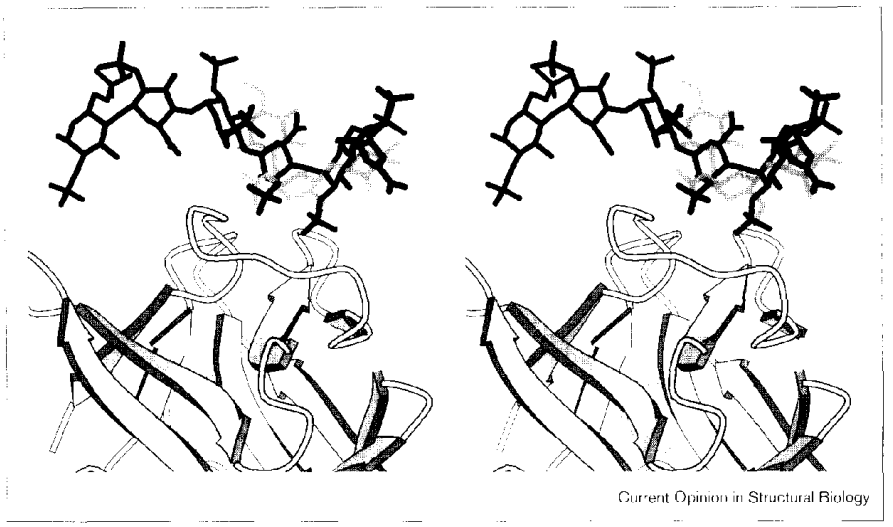
Somewhat surprisingly, although FGF–heparin interactions are dominated by interactions involving the sulfate groups, nonsulfated oligosaccharides can also bind to the same site, as revealed by the structure of a complex between FGF-2 and a heparin-derived desulfated trisaccharide [32]. Two binding regions for this ligand were identified on FGF-2; one corresponds to the high affinity binding site seen in the heparin complexes (Figure 5), whereas the second region occupies a site located at a lattice contact between two molecules. This latter region has not been observed as being occupied in the complexes with heparin and its location at a protein–protein interface is consistent with the observation that the ligand promotes FGF dimerization.

Sequence variability in the heparin-binding region of the FGF family

A sequence alignment of the FGF family reveals that none of the residues in the heparin-binding region are completely conserved throughout the FGF family. Significantly, although ion-pair interactions are critical for the binding interactions between heparin and FGFs, even the charge of the residue sidechain fails to be strictly maintained (Table 2). As a result of these sequence variations, it is clear that different members of the FGF family utilize

Figure 5

Superposition of a nonsulfated heparin-derived trisaccharide–FGF-2 complex ([32], coordinates courtesy of G Waksman) and the FGF–2-heparin hexasaccharide [36] structures, illustrating the overlap in the high affinity heparin-binding site, despite the lack of sulfate groups in the trisaccharide. The hexasaccharide and trisaccharide are represented by the black and gray lines, respectively.



different contacts for binding to heparin. Furthermore, different members of the FGF family will undoubtedly exhibit different affinities for heparin and some may not bind heparin or related proteoglycans at all. The differential requirements and responses to heparan sulfate of the FGF family members have been documented [41]; for example, heparin inhibits the binding of FGF-7 (keratinocyte growth factor, KGF) to the KGF receptor (KGFR) member of FGFRs, while enhancing the binding of FGF-1 to KGFR [42]. The differential effects of FGF-1 and FGF-2 upon 3T3 cells have been reported to be due to the effects of heparan sulfate [43]. Other experiments have shown that FGF-2 requires the *2-O*-sulfate for heparin binding, but not the *6-O*-sulfate, whereas FGF-1 requires both the *2-O*-sulfate and the *6-O*-sulfate for heparin binding, and FGF-4 can bind heparin in the absence of either the *6-O*-sulfate or the *2-O*-sulfate [41,44]. Significantly, these observations are consistent with the current crystal structures of FGF–heparin complexes [36,38••]; FGF-1 utilizes the *6-O*-sulfate to bind heparin,

Table 2

Sequence alignment in the heparin-binding region of members of the FGF family, generated with the Wisconsin Sequence Analysis Package.

Member	FGF-2 residue number*						
	27	28	102	121	126	135	136
FGF-1	S	N	N	K	K	Q	K
FGF-2	K	N	N	R	K	Q	K
FGF-3	A	T	L	G	R	Q	K
FGF-4	N	V	N	K	K	M	K
FGF-5	R	V	N	K	K	H	I
FGF-6	N	V	N	K	K	M	T
FGF-7	R	T	N	Q	V	Q	K
FGF-8	R	T	N	R	R	Q	R
FGF-9	R	T	N	K	R	Q	K
FGF-10	F	T	N	G	R	N	T
FGF-11	R	Q	N	K	M	K	A
FGF-12	Q	Q	N	K	M	K	P
FGF-13	R	Q	N	K	M	K	P
FGF-14	R	Q	N	K	M	K	P
FGF-15	A	G	L	H	E	S	L
FGF-16	R	T	N	K	R	Q	K
FGF-17	R	T	N	R	R	Q	R
FGF-18	R	T	N	K	R	Q	Q
Heparin contact	GlcN2-SO_3^- GlcN3-OH	GlcN2-SO_3^- IdoA3-OH	GlcN2-SO_3^-	IdoA3-CO_2^-	GlcN2-SO_3^- IdoA2-OSO_3^-	IdoA2-OSO_3^-	IdoA6-CO_2^-
†Affinity	Low	High	Low	High	High	High	Low

*The residues are numbered according to the FGF-2 sequence. †'High' and 'low' affinity refer to the heparin-binding sites described in the text.

whereas FGF-2 does not; furthermore, FGF-2 makes a specific contact to the 2-*O*-sulfate group of IdoA through the sidechain of Gln135, so that the substitution of this residue by methionine in FGF-4 would prevent this interaction, consistent with the observation that FGF-4 can bind heparin in the absence of 2-*O*-sulfate groups [41].

Models for the role of heparin in the FGF signal transduction pathway

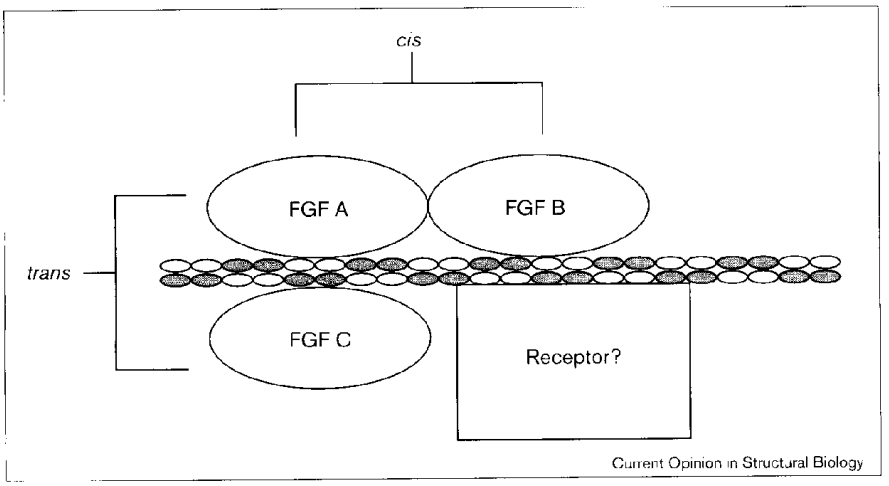
Two general roles for heparin have been proposed that could lead to the activation of the FGF signal transduction pathway by facilitating receptor dimerization — an indirect mechanism, in which heparin binds multiple FGF molecules that in turn bind multiple FGFRs, and a direct mechanism, in which heparin can bind FGFR and directly participate in receptor dimerization. These roles need not be exclusive; indeed, biochemical studies demonstrate that heparin can bind both FGFs and FGFRs [10]. In the absence of any direct structural information concerning the interaction of heparin with FGFR, the molecular details of the latter interaction remain speculative at this time. Consequently, the most detailed models of receptor dimerization have focused on the ability of heparin to bind multiple FGF molecules. As noted above, heparin may be approximated by a ribbon, in which the same residues appear on a given side every approximately 17 Å. Consequently, two classes of binding modes between heparin and FGFs (or other ligands) can be identified (Figure 6) — those in which FGF molecules bind to the same side of the ribbon (*cis* binding model) and those in which FGF molecules bind to opposite sides of the heparin ribbon (*trans* binding model).

Structure-based arguments have been presented supporting both binding modes as being physiologically relevant (keeping in mind that different binding modes may be required for different activities). *Cis* binding models are based on the close correspondence between the length of a heparin octasaccharide (two turns of the heparin helix) and the length of an FGF molecule (~34 Å). Since most studies indicate that octasaccharides are active, this correspondence suggests that heparin must be of a sufficient length to span two FGF molecules on the same side of the heparin helix [39,45,46••]. At present, no structures have been reported of FGFs bound to heparin in a *cis* fashion. *Trans* binding models, in contrast, utilize the potential availability of two binding surfaces on the heparin helix to complex two FGF molecules per approximately five saccharide residues (or about one turn of the heparin helix), as observed in the recent FGF-1–heparin complex [38••]. As beautifully illustrated by the present structural results on FGF-1 and FGF-2, the differences between the *cis* and *trans* models may reflect effects as subtle as the presence or absence of a particular sulfate group on heparin or the ability of a protein to interact with a particular sulfate group. Higher order models are also possible; for example, an FGF tetramer complexed to heparin through both *cis* and *trans* interactions has been proposed to represent the minimal biologically active unit of FGF-2 [29•].

Conclusions

The challenge posed by the FGF system is to understand how multiple FGFs, heparin and FGFRs are integrated in order to create the distinctive functions of the FGF family. Structural studies at present have concentrated on the interactions of FGF-1 and FGF-2 with homogeneous heparin oligosaccharides. Undoubtedly, an important development will be the structure determination of an FGF–heparin–FGFR complex, which will directly address the role of heparin in receptor dimerization. Equally important developments will involve the extensive characterization of the association thermodynamics between FGFs, heparin and FGFR in order to more quantitatively evaluate the stoichiometry and binding energetics of the system.

Figure 6



Schematic representation of the *cis* and *trans* modes of FGF binding to heparin. The filled and open circles serve to distinguish the two saccharide components of heparin with two disaccharides present per full helix turn. The potential availability of binding sites on heparin for interacting with non-FGF components, such as FGFR, is schematically indicated.

Given the diversity and complexity of the FGF system, however, it would seem prudent not to overgeneralize a few specific observations and apply them to the entire FGF family. This consideration is especially relevant to the role of heparin and related proteoglycans, since significant heterogeneity, in terms of sulfation patterns, carbohydrate sequence and length, is present in these components and this has been difficult to study experimentally. Variations in the carbohydrate sequence or sulfation pattern might be used to specify the presence or absence of binding sites for FGF and other molecules in heparin, thereby permitting the assembly of the appropriate complex of molecules for the initiation of signal transduction. A key advance in deciphering the structural and biological consequences of this diversity will undoubtedly be the development of synthetic methods for specifically preparing homogeneous oligosaccharides of defined sequence that can be used to address many of these outstanding issues using both biochemical and structural approaches.

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