In the clutches of proteoglycans: how does heparan sulfate regulate FGF binding?

Fibroblast growth factors and their receptors bind to heparan sulfate glycosaminoglycans. This is thought to promote ligand–receptor binding and enhance signaling by promoting receptor multimerization. Synthetic mimetics designed to occupy these binding sites may provide the means to understand and to regulate FGF signaling.

Chemistry & Biology October 1995, **2**:645–649

Members of the fibroblast growth factor (FGF) family, which now includes nine related polypeptides, are unified by an affinity for heparan sulfate, with estimated K_d values of 1–100 nM [1,2]. The affinity for heparan sulfate was initially discovered due to tight binding of FGF-2 to heparin, a mast cell product that is a close cousin of the heparan sulfate glycosaminoglycans that are found attached to core proteins at the periphery of all adherent cells. Once referred to as the 'heparin-binding' growth factor family, the FGFs now share this distinction with a number of other growth factors

A critical feature of the FGF binding to heparan sulfate is that it is central to FGF signaling. As is known for most other growth factors, FGFs signal by activating a receptor tyrosine kinase (RTK), which initiates a phosphorylation cascade within the cell that culminates in multiple cellular outcomes [1]. Unlike most other growth factors, however, the signaling complex that assembles at the cell surface includes heparan sulfate. Heparan sulfate-binding sites have been identified on the FGF and on the RTK and the participation of heparan sulfate in the interaction between FGF and its receptor has repercussions on effective receptor activation [3,4]. The participation of heparan sulfate in this signaling has attracted the attention of scientists interested in throwing a monkey wrench into the works. Their reasoning is that the correct assembly of this complex may be disrupted by heparan sulfates that almost, but do not quite, fit. This may occur naturally in biological systems, where the sulfation of heparan sulfate and thus its ability to appropriately recognize domains on the FGF and the receptor may vary. Researchers using chemically modified heparins hope to identify potent mimetics that can regulate abnormal FGF signaling in diseases such as atherosclerosis or in neoplasia by, for example, inhibiting the angiogenesis that supports the growth of solid tumors.

The FGFs (Table 1) are characterized by their prototypes, FGFs 1 and 2, which have roles in numerous tissues and in early development [1]. The FGF family is an enigma in many ways, not the least of which is the issue of how a signal is generated. The idea that they may signal by receptor transphosphorylation is attractive; in this model, RTKs are caused to form dimers or multimers by ligand binding and, once brought into proximity, actively phosphorylate one another. The phosphorylated tyrosines then act as a scaffold for the assembly of an aggregate of cytosolic signaling molecules. As FGFs and their receptors exist as monomers, however, it is unclear how they form multimers and as yet unproved that the receptors must dimerize in order to signal.

FGF receptor tyrosine kinases (RTKs)

The FGFs initiate a cell surface signal by binding to a family of RTKs (FGFR1–4), represented by four family members and splicing variants [5]. The receptors activate multiple signaling pathways and the stimulation of specific pathways can be uncoupled by mutating specific tyrosines in the cytoplasmic domain [6]. Thus, regulation of transphosphorylation by manipulating the dimerization process may be a potential means of fine-tuning the response to FGF.

In their extracellular domains, these receptors contain immunoglobulin (Ig)-like loop domains that place them in the Ig superfamily of receptors. All four receptor types contain three Ig domains, whereas FGFRs 1–3 also display splice variants lacking the distal Ig loop (loop I). In addition, Ig loop III, proximal to the plasma membrane, displays splicing variation that dramatically affects affinity for specific FGF family members. This is perhaps most apparent for FGFR-2, where splicing variation in the latter half of the third Ig loop converts an affinity of FGFR-2 for either FGF-1 or FGF-2 into a

Table 1. Numerical designation for members of the FGF family.

Number	Alternate name(s)
FGF-1	Acidic FGF
FGF-2	Basic FGF
FGF-3	int-2
FGF-4	hst, k-FGF
FGF-5	–
FGF-6	–
FGF-7	KGF
FGF-8	AIGF
FGF-9	GAF

AIGF, Androgen-induced growth factor; GAF, glia-activating factor; KGF, keratinocyte growth factor.

keratinocyte growth factor (KGF) receptor, with much lowered affinity for FGFs 1 and 2 [7].

Another feature of the extracellular domain of the FGF RTKs is a heparan sulfate binding region (Fig. 1). This is located just distal to the second Ig loop and is contained in all spliced forms of the RTKs [8]. Modeling of the receptor conformation suggests that this domain participates in anchoring heparan sulfate and FGF within a receptor dimer [9]. A region that contains the heparan sulfate domain, Ig-loop II, the interloop region between loops II and III and the distal portion of loop III is protected from tryptic digestion when bound by heparan sulfate [10]. This region constitutes the minimal portion of the receptor that will bind FGF. The remaining section of loop III undergoes splicing variation and has a major role in determining specificity of FGFRs for specific FGFs [5,10].

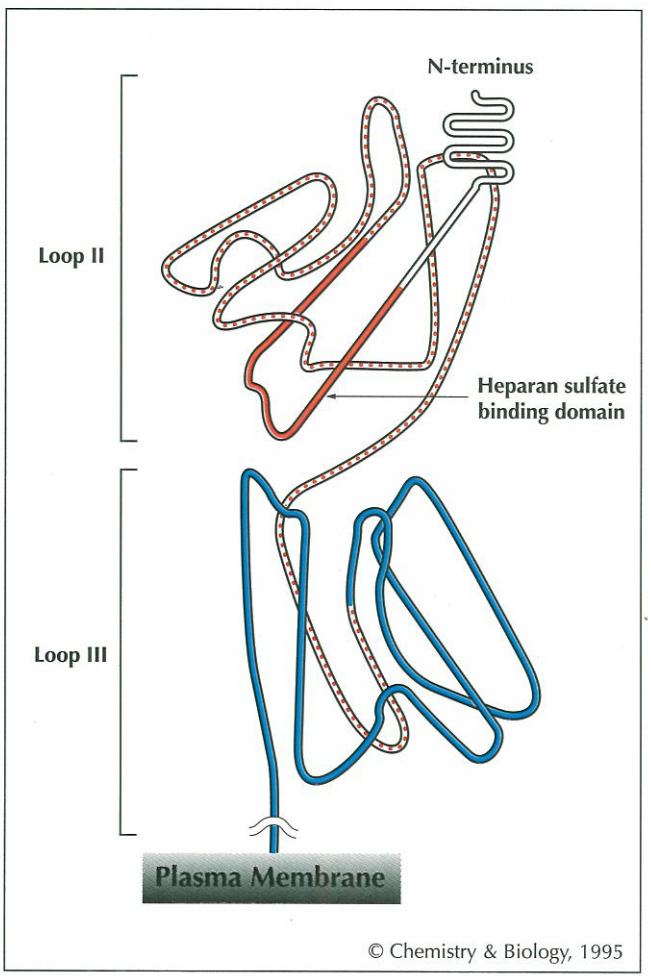

Fig. 1. Model of the extracellular, FGF-binding region of the FGF receptor tyrosine kinase. Shown are the second and third Ig-loops of the extracellular domain, with the heparin-binding region shown in solid red. The dotted red region, including the heparin-binding domain, is protected by heparan sulfate from tryptic digestion; this fragment has been shown by Wang *et al.* [10] to represent the minimal binding region for FGF. The blue region, which includes the variably spliced IIIc domain of loop III influences receptor specificity. Adapted from Wang *et al.* [10].

Heparan sulfate proteoglycans: regulators of receptor function

Heparan sulfate proteoglycans are found in the extracellular matrix adjacent to cell surfaces and as integral components of the plasma membrane [11,12]. Perlecan is a ubiquitous component of basement membranes and is also secreted into the matrix surrounding stromal cells. On cell membranes, the syndecans and betaglycan are present as transmembrane proteins, together with a group of phosphatidylinositol-linked heparan sulfate proteoglycans. These proteoglycans present a dense array of heparan sulfate chains that bind a variety of matrix components and growth factors. The binding to matrix is believed to be important in matrix organization and in the formation of focal contacts (sites of adhesion between the cell and its substratum).

Heparan sulfate glycosaminoglycans: specificity within the polymer?

The binding of heparan sulfate to its ligands is influenced by the construction of the glycosaminoglycan chain [11,12]. Synthesis begins with the synthesis of a tetrasaccharide linkage region on the core protein to which is added a repeating 1→4 glycosidically-linked copolymer of D-glucuronic acid and N-acetyl-D-glucosamine. The chain is then subjected to enzymatic modifications as the proteoglycan passes through the Golgi apparatus, culminating in a highly modified polyanion; at its greatest extent, the modified polymer displays disaccharides where glucuronate has been epimerized to iduronate, and sulfate residues have been added at several sites. The repercussions of these modifications are still not fully understood, but recent findings suggest that binding sites for specific ligands are present within the chain. The chains can be digested with heparinases that cleave at discrete sites, not unlike the use of restriction enzymes to examine the frequency of specific sites within a DNA strand; unfortunately this technique is limited to two or possibly three useful enzymes. Heparan sulfate or heparin fragments bearing different patterns of modifications can be derived in this manner, however, and some of these fragments display affinity for defined ligands [13,14]. Additionally, these fragments can be desulfated by chemical means to demonstrate the requirement for specific sulfate groups in the binding [15–17]. A further intriguing finding is that the modifications are likely to be cell type specific; isolation of a single heparan sulfate proteoglycan from different cell types and analysis of its chain structure by heparinase cleavage has shown differences in the modification pattern [18]. And it is quite possible that these modifications are also proteoglycan-specific.

Is FGF's affinity for heparan sulfate its Achilles' heel?

From the time of their discovery, the FGFs have been known to have an affinity for heparan sulfate. The classical method for purifying FGFs is on heparin affinity columns where elution requires up to 1.5 M NaCl [2]. At cell surfaces, more than 10^6 binding sites for FGF exist within the meshwork of heparan sulfate. This serves to limit the diffusion of FGF but is also argued to directly augment FGF binding to its RTK. The binding of FGFs to heparan

sulfates shows a considerable degree of specificity. FGF-2 binding depends on specific modifications ; the important modifications appear to differ for FGF-1 and FGF-4 [15]. Fragmentation of heparan sulfate with heparinases allows the isolation of a highly active fragment for FGF-2 that displays high amounts of iduronosyl-2-O-sulfate; calculations suggest that roughly one such site may occur per heparan sulfate chain [13]. But these modifications may not occur on all proteoglycans or in all tissues. It has been argued, for example, that the heparan sulfate present on perlecan of human lung fibroblasts rather than that of cell surface forms of heparan sulfate proteoglycans is responsible for FGF binding and activation [19]. Indeed, the heparan sulfate chains present on some proteoglycans appear to bind FGF-2, but actually inhibit its activity by depriving it from heparan sulfates that would augment its activity. Presumably, these inhibitory heparan sulfates fail to participate in formation of a signaling complex at the cell surface. A similar example of proteoglycan specificity is derived from the embryonic neuroepithelium, where the specificity of a heparan sulfate proteoglycan changes during development in unison with a change in FGF expression [20].

Formation of the receptor/FGF/heparan sulfate complex

Modeling of the binding of FGF to its receptor has taken many forms, from activity analyses using truncated or modified forms of the participants to crosslinking studies and physical chemical imaging. The central issues are an understanding of how the receptor assimilates FGF into a high affinity complex and how subsequent signaling is initiated. These two issues share a common uncertainty, namely, does the FGF and/or the receptor need to dimerize and how might this be accomplished? Physical mapping of FGF on a heparin glycosaminoglycan demonstrates that a single FGF may occupy as few as five saccharides within a chain of over twenty [16,17]. Thus, multiple FGFs binding to a single chain would achieve 'dimerization' of the growth factor. Use of high concentrations of FGF together with heparan sulfates show that the FGF can be cross-linked into dimers [21]. Similarly, a fraction of the FGF receptors appear to be dimerized at the cell surface by cross-linking methods [22]. It remains to be formally shown how these dimers are assembled and that they are responsible for all signaling, however.

Using FGF-2 and FGFR1 as models, important domains in FGF-2 have been identified that mediate its binding and activity [9,23] (Fig. 2). One is a hydrophobic region that binds the receptor and is composed of Y24, R44, N101, Y103, L140 and M142, with Y24, Y103 and L140 being especially critical. These latter three amino acids are highly conserved in the FGF family and are likely to represent an important receptor-binding domain. A second domain that binds the receptor is a loop comprising amino acids 106–115; mutations in amino acids K110, Y111 and W114 or removal of this loop dramatically influence FGF activity [24]. This loop has a lower affinity for the receptor and is postulated in one model to play a role in receptor dimerization. Mutations within

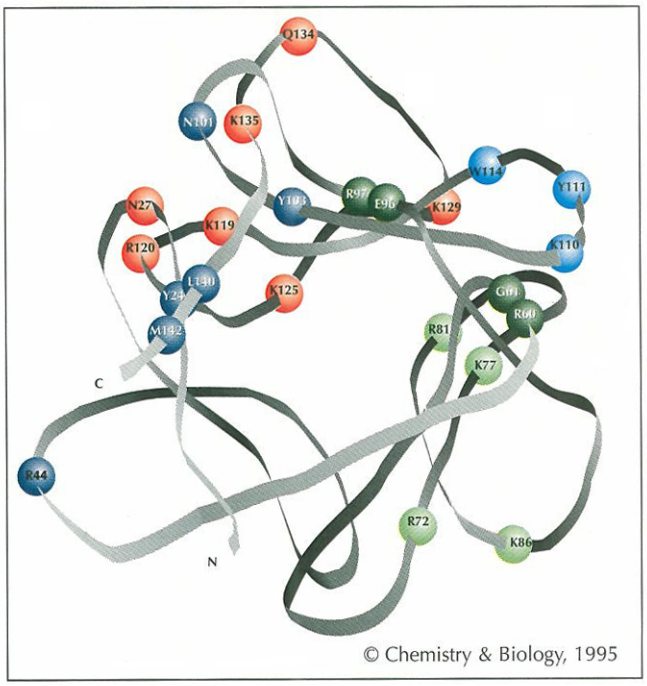

Fig. 2. Model of FGF-2 showing important binding domains. Two important classes of binding domains have been proposed within the growth factor. A primary receptor-binding domain is comprised of amino acids Y24, R44, N101, Y103, L140 and M142 (shown in dark blue). A secondary receptor binding domain (K110, Y111 and W114) is in light blue. The primary heparan-sulfate-binding domain is shown in red (N27, K119, R120, K125, K129, Q134 and K135). Two secondary sites (shown in dark and light green) have been identified by binding di- or trisaccharides and are proposed by Ornitz *et al.* [21] to take part in bFGF dimerization when occupied by the oligosaccharides. Thus, the region shown in dark green (R60, G61, E96 and R97) is proposed to interact with a second bFGF via the region shown in light green (R72, K77, R81 and K86). Adapted from Ericksson *et al.* [27].

this domain do not interfere with receptor binding *per se*, but do reduce activity [24]. The third domain is the heparin (heparan sulfate) binding domain that encompasses amino acids N27, K119, R120, K125, K129, K135 and Q134 (8,23); these amino acids comprise a cationic site that binds heparin fragments a short as five sugars, but enhancement of activity is seen as the length of these fragments approaches 12 sugars [14,15].

The identification of these domains provides insight into important binding events when FGF-2 binds to FGFR1: receptor binding to FGF, receptor binding to heparan sulfate, FGF binding to heparan sulfate, the complex of FGF/heparan sulfate binding to receptor and, finally, a second receptor with or without another FGF binding to this assembly. This latter event does not appear to occur in the absence of heparin or heparan sulfate and failure of this binding to occur dramatically reduces FGF signaling [3]. The role of the heparan sulfate is likely to be two-fold: first, to mask positive charges on the FGF and its receptor, allowing them to approach each other, and, second, to provide anchorage between molecules in the complex. Indeed, models of FGF:receptor interaction suggest that a heparan sulfate

consisting of 10–12 saccharides would anchor them together [9]. Heparin fragments of less than this size are usually found to be inhibitory, presumably because they occupy FGF-2 but cannot participate effectively in facilitating receptor binding.

It is interesting to speculate how heparan sulfates longer than 12 saccharides, or proteoglycans bearing multiple chains, would help to generate multimers of these FGF:receptor complexes. Such complexes may be necessary for efficient transphosphorylation and their formation may stabilize FGF binding. That is, the strength of these interactions may depend on the degree to which a 'complete' complex is formed. This speculation is intriguing due to the current interest in the 'strength of signal' hypothesis extended for other growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF) [25]. This hypothesis suggests that the strength of the growth factor signal, which is a function of the degree to which receptor signaling is sustained, determines which intracellular pathways can be activated. One model for this is that the signal may need to be strong enough to overcome the intracellular phosphatases necessary to keep the signal turned off. Thus, the amount of time that FGF is resident on its receptor and the retention of the receptor as a signaling entity would determine the strength of the signal. Heparan sulfate proteoglycans may have a very interesting role in such signaling.

A final variable to be considered is multiple configurations of the FGF receptor. The heparin-binding domain appears to be variably exposed in splice variants that display either two or three Ig loops. When the variably expressed Ig loop I is present, it can interact with loop II, masking the heparin-binding site on the receptor and influencing the participation of heparan sulfate in the binding of FGF-1 [26].

Oligosaccharides as mediators of FGF action

Pinpointing several important heparan sulfate binding sites within the FGF signaling complex suggests appealing notions for the design of signaling competitors. These sites may either be *bona fide* sites on the FGF or receptor which normally participate in complex formation, or be hidden sites that prevent complex formation when bound by small synthetic oligosaccharides. Maccarana *et al.* [16] described a minimal pentasaccharide of heparin that appears capable of binding FGF-2, although longer fragments appear likely to participate in signaling. However, Ornitz *et al.* [21] have now described smaller synthetic di- and trisaccharides that either stimulate or inhibit FGF activity on lymphoid cells transfected with the cDNA for FGFR1. These cells are devoid of endogenous heparan sulfate proteoglycans and respond to the FGF only when presented with exogenous heparan sulfate. Two new sites are described on FGF-2 (Fig. 2); sites which when occupied by small synthetic heparin fragments may promote the dimerization of two FGFs. This is of interest for several reasons. First, it identifies sites that may be part of an important mechanism for FGF signaling, although it remains to be shown that native heparan sulfates would bind to these regions. Second, it demonstrates that synthetic oligosaccharides or their homologs can bind to specific sites within FGF, potentially affecting activity. It is intriguing to note that the ability of the oligos to promote FGF signaling does not necessarily correlate with their ability to promote binding of FGF to a soluble form of receptor. Comparison of a di- and trisaccharide that promote equivalent binding of FGF to receptor shows that the trisaccharide is 10-fold more effective at promoting mitogenic stimulation. The explanation for this may lie in the several potential heparin binding sites on the FGF and receptor. Thus, small fragments may be envisioned to facilitate receptor signaling either by enhancing the affinity of FGF binding to receptor, or by enhancing the ability of two FGFs to dimerize. One might speculate that heparan sulfate proteoglycans at the cell surface participate in both events, leading to the formation of a stable, multimeric complex that carries out signaling. Equally plausible is that oligosaccharides that bind but fail to 'fit' would succeed in preventing one or more of these interactions. This leads to the speculation that small heparinoids may successfully be used to either completely inhibit the formation of such a complex, or even manipulate the stability of the complex formed, and thus the activity of the signaling pathway.

References

1. Basillico, C. & Moscatelli, D. (1992). The FGF family of growth factors and oncogenes. *Adv. Cancer Res.* **59**, 115–165.
2. Rapraeger, A.C., Guimond, S., Krufka, A. & Olwin, B.B. (1994). Regulation by heparan sulfate in FGF signaling. *Methods Enzymol.* **245**, 219–140.
3. Rapraeger, A.C., Krufka, A. & Olwin, B.B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705–1708.
4. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., & Ornitz, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841–848.
5. Johnson, D.E., Lee, P.L., Lu, J. & Williams, L.T. (1990). Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Mol. Cell. Biol.* **10**, 4728–4736.
6. Peters, K.G., *et al.*, & Williams, L.T. (1992). Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca^{2+} flux but not mitogenesis. *Nature* **358**, 678–681.
7. Miki, T., *et al.*, & Aaronson, S. (1992). Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc. Natl. Acad. Sci. USA* **89**, 246–250.
8. Kan, M., Wang, F., Xu, J., Crabb, J.W., Hou, J. & McKeehan, W.L. (1993). An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* **259**, 1918–1921.
9. Pantoliano, M.W., *et al.*, & Sisk, W.P. (1994). Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* **33**, 10229–10248.
10. Wang, F., Kan, M., Xu, J., Yan, G. & McKeehan, W.L. (1995). Ligand-specific structural domains in the fibroblast growth factor receptor. *J. Biol. Chem.* **270**, 10222–10230.
11. Kjellén, L. & Lindahl, U. (1991). Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* **60**, 443–475.
12. Rapraeger, A.C. (1993). The coordinated regulation of heparan sulfate, syndecans and cell behavior. *Curr. Opin. Cell Biol.* **5**, 844–853.
13. Turnbull, J.E., Fernig, D.G., Ke, Y., Wilkinson, M.C. & Gallagher, J.T. (1992). Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* **267**, 10337–10341.
14. Ishihara, M., Tyrrell, D.J., Stauber, G.B., Brown, S., Cousens, L.S. &

Stack, R.J. (1993). Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological activities mediated by basic fibroblast growth factor. *J. Biol. Chem.* **268**, 4675–4683.
15. Guimond, S., Maccarana, M., Olwin, B., Lindahl, U. & Rapraeger, A. (1993). Activating and inhibitory heparin sequences for FGF-2 (basic FGF): distinct requirements for FGF-1, FGF-2 and FGF-4. *J. Biol. Chem.* **268**, 23906–23914.
16. Maccarana, M., Casu, B. & Lindahl, U. (1993). Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* **268**, 23898–23905.
17. Habuchi, H., *et al.*, & Kimata, K (1992). Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. *Biochem. J.* **285**, 805–813.
18. Bernfield, M., *et al.*, & Lose, E.J. (1992). Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **8**, 365–393.
19. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G. & Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. *Cell* **79**, 1005–1013.
20. Nurcombe, V., Ford, M.D., Wildschut, J.A. & Bartlett, P.F. (1993). Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science* **260**, 103–106.
21. Ornitz, D.M., Herr, A.B., Nilsson, M., Westman, J., Svahn, C.-M. & Waksman, G. (1995). FGF binding and FGF receptor activation by synthetic heparan-derived di and trisaccharides. *Science* **268**, 432–436.
22. Spivak-Kroizman, T., *et al.*, & Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation and cell proliferation. *Cell* **79**, 1015–1024.
23. Thompson, L.D., Pantoliano, M.W. & Springer, B.A. (1994). Energetic characterization of the basic fibroblast growth factor-heparin interaction: identification of the heparin binding domain. *Biochemistry* **33**, 3831–3840.
24. Springer, B.A., *et al.*, & Book, G.W. (1994). Identification and concerted function of two receptor binding surfaces on basic fibroblast growth factor required for mitogenesis. *J. Biol. Chem.* **269**, 26879–26884.
25. Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185.
26. Wang, F., Kan, M., Yan, G., Xu, J. & McKeehan, W.L. (1995). Alternatively-spliced NH2-terminal immunoglobulin-like loop I in the ectodomain of the fibroblast growth factor (FGF) receptor 1 lowers affinity for both heparin and FGF-1. *J. Biol. Chem.* **270**, 10231–10235.
27. Ericksson, A.E., Cousens, L.S. & Matthews, B.W. (1993). Refinement of the structure of human basic fibroblast growth factor at 1.6 Å resolution and analysis of presumed heparin binding sites by selenate substitution. *Protein Struct.* **2**, 1274–1284.

Alan C Rapraeger, Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53706, USA.