Oligomerization reduces heparin affinity but enhances receptor binding of fibroblast growth factor 2

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The biological response of cells to fibroblast growth factors (FGFs) depends on heparan sulphate glycosaminoglycans sharing particular structural motifs. Heparin induced FGF dimerization has been suggested to mediate receptor dimerization and activation. Here we demonstrate that heparin-derived oligosaccharides that promote receptor binding and activation specifically induce the dimerization of basic FGF (FGF2). These heparin-induced dimers of FGF2 acquire high affinity for receptor binding and are biologically active. Using biotinylated FGF2 bound to immobilized streptavidin gradually saturated with biotin, enabled a quantitative analysis of heparin-dependent and heparin-independent FGF2 monomers and oligomers. Streptavidin induced FGF2 dimers bind and activate FGF receptors only in the presence of heparin. An excess of strepta-

vidin, forcing biotin-FGF2 into monomers, reduces receptor binding and blocks FGF-dependent cell proliferation. All these suggest predominant receptor binding and activation by heparin associated FGF2 oligomers. Unexpectedly, heparin induced dimers and higher order oligomers lose most of their affinity towards heparin. Direct binding of soluble FGF receptors (FGFRs) to either monomers or dimers of FGF2, immobilized on heparin, confirm the preferred association of FGFRs with dimers of FGF2. Computerized molecular docking predicts a *cis*-oriented FGF2 dimer, stabilized by heparin, which complies with all the experimental data.

Key words: heparan sulphate, molecular docking, proteoglycans, receptor tyrosine kinase.

INTRODUCTION

Fibroblast growth factors (FGFs) constitute a family of at least nineteen structurally related heparin-binding polypeptides that are expressed in a wide variety of cells and tissues. They are potent mitogens that stimulate proliferation, migration and differentiation of cells of mesenchymal and neuroectodermal origin [1,2]. The biological response of cells to FGF is mediated through specific, high-affinity (K_d 20–500 pM) cell-surface receptors that possess intrinsic tyrosine kinase activity and are phosphorylated upon the binding of FGF [3]. A lower affinity (K_d 2×10^{-9} M), large capacity (10^6 sites/cell) class of binding sites has also been identified [4]. These sites, composed primarily of heparan sulphate moieties of proteoglycans, are found on the cell surface and in the extracellular matrix of almost every cell and tissue [5].

Studies of the involvement of heparin-like structures in FGF binding and activation identified a novel role for these molecules in the formation of distinct FGF–heparan sulphate complexes that enhance high-affinity binding and are essential for the biological activity of FGF [6–8]. Heparin-dependent receptor binding and activation applies to all FGFs investigated [9,10] as well as to other heparin-binding growth factors like vascular endothelial growth factor [11,12], heparin-binding epidermal growth factor [13,14] and hepatocyte growth factor [15]. The minimal heparin-derived fragment, which is capable of binding to basic FGF (FGF2), is a tetra- or pentasaccharide [16,17]. However, the minimal heparin oligosaccharide, which is capable of enhancing FGF2 receptor binding and activation, is a sulphated decasaccharide [8,17–19].

Ligand induced receptor dimerization is a key event in transmembrane signalling of receptor tyrosine kinases. Receptor dimerization leads to an increase in kinase activity, resulting in autophosphorylation and the induction of diverse biological

responses [20]. Many growth factors that serve as ligands to receptor tyrosine kinases are dimeric and this property is crucial for their function in receptor binding and dimerization [20–22]. Acidic FGF (FGF1) and FGF2 have been suggested to dimerize upon their interaction with heparin [8,23,24], thereby leading to receptor dimerization and activation. In a recent study we have utilized NMR to demonstrate that *cis*-oriented FGF dimers, i.e. two FGF2 molecules bound to each other on the same side of a heparin-derived decasaccharide are capable of receptor binding, whereas *trans*-oriented dimers of FGF2, formed on a heparin tetrasaccharide, are biologically inactive [25].

Several models have been proposed for the interaction between FGF, heparin and the FGF receptor (FGFR) [7,23,24,26,27] sharing the idea of a biologically preferred ternary complex but different in considerations regarding the stoichiometry, relative orientation and specific intermolecular interactions between the components forming the active ternary complex. In an attempt to further resolve the nature of these interactions, we made use of defined heparin fragments, recombinant ligands and soluble FGFRs, all of which were differentially tagged to carefully control for their purification and quantification. Our studies demonstrate the importance of heparin for the correct dimerization of FGF2, which in turn greatly facilitates the binding of this ligand and oligomerization of the receptor. We also present a model structure of the FGF2 dimer complexed with heparin and the FGFR ligand-binding domain, which complies with the experimental data.

MATERIALS AND METHODS

Materials

Anti-FGF2 antibodies (FB-8) were from Sigma (Rehovot, Israel). Disuccinimidyl suberate (DSS) and bis(sulphosuccinimidyl)

Abbreviations used: FGF, fibroblast growth factor; FGFR, FGF receptor; FGF1 (aFGF), acidic FGF; FGF2 (bFGF), basic FGF; DSS, disuccinimidyl suberate; AP, alkaline phosphatase.

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suberate were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Na¹²⁵I was from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.) and heparin–Sephacrose was from Pharmacia Biotech AB (Uppsala, Sweden). Biotin hydrazide and streptavidin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human FGF2 and biotinylated FGF2 were generously given by Andrew Seddon (Pfizer Research Center, Groton, CT, U.S.A.). Heparin was from Hepar Industries (Franklin, OH, U.S.A.) and the deca- and tetrasaccharides were generously given by Carl Svahn (Pharmacia, Uppsala, Sweden). The XTT[®] cell proliferation kit was from Biological Industries Co. (Beit Haemek, Israel).

Radiolabelling of recombinant growth factors

Recombinant growth factors (1 µg each) were labelled with Na¹²⁵I (1 mCi), using the chloramine T method [28]. Iodinated growth factors were separated from free iodine on heparin–Sephacrose columns (200 µl), the eluted factors were divided into aliquots and kept at –20 °C until use.

Western immunoblotting

Proteins separated by SDS/PAGE were electrophoretically transferred to nitrocellulose paper, incubated with the indicated antibodies and identified using horseradish-peroxidase-conjugated Protein A and the ECL[®] kit (Amersham) according to the manufacturer's instructions.

Ligand dimerization

FGF2 (2 µg/ml) was incubated, for 1 h, in the absence or presence of different heparin oligosaccharides in binding buffer [14]. The cross linker DSS (15 mM) was added for an additional 20 min. Dimerization was analysed by Western blotting using monoclonal antibody FB-8.

Binding of FGFR1 to biotinylated FGF2 *in vitro*

A 96 well ELISA plate was coated with streptavidin (1 µg/well) for 18 h at 4 °C. Biotin, at the indicated concentration, was added for 2 h and the plate washed three times with PBS containing 0.05 % Tween-20. Biotinylated FGF2 was then added at either 100 ng/ml or 1 µg/ml for an additional 2 h. The wells were washed sequentially with 2 M NaCl then PBS and incubated for 2 h with soluble FGFR1 ectodomain coupled to alkaline phosphatase (AP) at the indicated concentration, with or without heparin (1 µg/ml). After washing, the plates were incubated with the AP substrate, *p*-nitrophenyl phosphate (Sigma) and the absorbance read at 410 nm using an ELISA plate reader.

Heparin-dependent, FGFR1-mediated cell proliferation induced by monomers or oligomers of FGF2

A mouse myeloid cell line, FDC-P1, expressing FGFR1 [53] was grown (2×10^4 cells/well) at 37 °C in 96 well ELISA plates in Iscove's modified Dulbecco's medium containing 10 % (v/v) fetal calf serum, 10 ng/ml FGF1 and 5 µg/ml heparin. The cells were washed three times with Iscove's modified Dulbecco's medium and incubated for 24 h with the indicated concentrations (0–30 ng/ml) of biotinylated FGF2 and streptavidin (0.01–10 µg/ml) in the absence and presence of 5 µg/ml heparin. An XTT assay was used to quantify the cell number by measuring absorbance at 490 nm using an ELISA plate reader.

Relative affinity of monomeric and dimeric forms of FGF2 to immobilized heparin

Human recombinant FGF2 (0.5 µg) was incubated with 250 µl of heparin–Sephacrose beads for 2 h at room temperature. After extensive washing with PBS the heparin–Sephacrose beads were washed twice with the indicated concentrations of NaCl. After each wash a sample of the beads was taken out and the rest washed with the next salt concentration. All the samples were then washed again with PBS and subjected to chemical cross linking with DSS (15 mM) for 20 min. The samples were analysed by SDS/PAGE and Western immunoblotting with monoclonal antibodies to FGF2 (FB-8).

Binding of soluble FGFR1 to FGF2 and to heparin

Soluble FGFR1–AP fusion protein in conditioned medium was incubated with heparin–Sephacrose beads pre-incubated with 0.5 µg of FGF2 for 2 h at room temperature. After extensive washing with PBS, the beads were washed twice with increasing concentrations of NaCl as indicated. All the samples were then washed once with PBS and the bound receptor level estimated according to the associated AP activity upon incubation with the phosphatase substrate *p*-nitrophenyl phosphate and reading the absorbance at 410 nm.

Computer modelling of the FGF2 dimer

To model the structure of the FGF2 dimer we used the program MolFit, based on the previously described docking algorithm [29,30]. This algorithm treats the two monomers as rigid bodies and scans stepwise all the possible relative positions (rotations and translations) searching for good geometric matches between their surfaces. Each match is given a score, which quantifies the degree of surface complementary offset by the amount of interpenetration. The X-ray structure of the FGF2 monomer [31–33], as deposited in the Protein Data Bank ([34], entry 4fgf), was used in the computations. Some conformational changes may occur upon dimerization and therefore the same 'surface softening' procedure was used as was used for the prediction of the structure of the β -lactamase-inhibitor complex [35,36], i.e. the surface layer was made 3–3.5 Å (1 Å = 10^{-10} m) thick and the penalty for interpenetration was reduced. The translations were scanned at an interval of 1.253 Å and the rotations at an interval of 12°. The two highest scoring matches for each of the 8760 rotations were saved for further analysis, resulting in 17 520 putative complexes with surface complementary scores that ranged between 277 and 513 (arbitrary score units).

Statistical analysis of the distribution of the scores was performed as follows: the number of matches with a given score was plotted as a function of the score producing a somewhat skewed distribution, due to the elimination of weak matches by our procedure. A Gaussian curve was therefore matched to the descending part of the distribution, providing an estimate for the mean score (332 units) and for the standard deviation (39.5 score units).

The available biochemical/biological information on the proposed receptor-binding site was used to screen the top ranking 1809 docking solutions whose scores exceed 513–3 σ . The receptor-binding site in either FGF2 monomer has to be exposed in the biologically active dimer. Therefore, docking solutions in which any atom in residues identified as a part of the receptor-binding site (E96, L98, S100, N101, N102 and L140; the numbering of the residues here and thereafter follows that of entry 4fgf in the Protein Data Bank) in one monomer comes within 5 Å of any

atom in the other monomer, were eliminated. Only 193 complexes passed this test and the 19 top ranking ones (scores 513–2 σ) were checked manually on a graphics screen. The two heparin hexasaccharide chains were positioned based on the experimental X-ray structure, while the putative decasaccharide was positioned after omitting the terminal sugars using a modified conformation of the sugar units preceding them. The possible binding of FGFRs IgD2 and IgD3 to the predicted FGF2 dimer was constructed based on the recently published co-structure of interleukin-1 with its receptor [54].

RESULTS

Effect of heparin oligosaccharides on FGF2 dimerization

Since heparin-mediated ligand dimerization may play a key role in FGF signal transduction, we investigated the effect of activating and inhibitory oligosaccharides on FGF dimerization. To this end, FGF2 was incubated with heparin-derived tetra- or decasaccharides and dimer formation was analysed by chemical cross-linking and SDS/PAGE. Dimerization was followed in the presence of 1 mM dithiothreitol to control for spontaneous, disulphide-linked dimers of FGF2, which may appear as FGF dimers with impaired receptor binding. Analysis of FGF2 dimers induced by either a tetra- or decasaccharide revealed that, similar to intact heparin, only the decasaccharide could efficiently induce formation of covalently linked dimers of FGF2 (Figure 1). The short tetrasaccharide not only failed to promote the formation of covalently linked FGF2 dimers but also efficiently inhibited, in a

dose-dependent manner, ligand dimerization induced by the active decasaccharide (Figure 1).

Effects of ligand dimerization and heparin on receptor binding of FGF2

In order to test the hypothesis that dimers of FGF2 may constitute a high-affinity binding and activating unit for FGFRs, we devised a cell free system that allowed us to regulate the formation of FGF2 oligomers, in a manner independent of heparin. For that purpose we made use of fully active, biotinylated FGF2 and immobilized tetrameric streptavidin. A 96-well plate coated with streptavidin was pre-incubated with increasing concentrations of free biotin in order to saturate to different degrees the streptavidin-binding sites present in each well. An identical amount of biotin–FGF2 was added to the wells. Taking into consideration the practically irreversible interaction of biotin and streptavidin, the level of dimers and higher-order oligomers of FGF2 on this presaturated streptavidin should decrease as the concentration of free biotin increases. As can be seen in Figure 2(A), there is a marked decrease in the specifically bound fraction of soluble FGFR1 as a function of free biotin, reaching a plateau level above 0.2 μ g/ml biotin, where the ratio of biotin to immobilized streptavidin is such that one or less FGF molecules are bound to each molecule of immobilized streptavidin. This suggests that the high-order oligomeric forms of FGF2 share a significantly higher capacity to bind receptor. Interestingly, binding to oligomeric FGF2 still requires heparin and immobilized FGF2 interacts with soluble FGF receptors mainly in the presence of heparin, irrespective of the biotin concentration used (Figure 2A). This suggests that heparin may have at least two roles in FGF signalling, by both stabilizing ligand oligomerization and promoting receptor binding of these oligomers.

The immobilization of biotin-FGF2 on to streptavidin provided us with a unique opportunity to monitor the interaction of FGF2 with the FGFR in the complete absence of heparin or heparin-like molecules (Figure 2B). Omitting the 2 M NaCl wash prior to receptor binding resulted in significant levels of bound receptors in an apparent heparin-independent manner (results not shown), suggesting that some residual heparin or heparin-like molecules may have either leaked or co-purified with FGF2 or with the soluble receptors that are produced in mammalian cells and are capable of shedding large quantities of heparan sulphate [37]. Direct binding of soluble FGFR1 was therefore determined after an extensive salt wash of the immobilized ligand. As can be seen in Figure 2(B), the capacity of soluble FGFR1 to bind the oligomeric form of FGF2, in the presence of heparin, is much higher than the capacity to bind to either the oligomeric form without heparin or the monomeric forms, still present in significant amounts even at 5 μ g/ml of free biotin, with or without heparin. While we have no evidence that multiple FGF2 molecules immobilized on streptavidin are in any way similar to heparin-induced oligomers, it is clearly demonstrated that under conditions where streptavidin-mediated oligomers are not formed, there is no significant receptor binding even at saturating heparin concentrations.

Figure 1 Dimerization of FGF2 in the presence of heparin-derived oligosaccharides

FGF2 (2 μ g/ml) was incubated with either tetra- or decasaccharides or with the indicated combinations for 1 h at room temperature. After chemical cross-linking the samples were resolved by electrophoresis and Western immunoblotting with anti-FGF2 antibodies (inset). The relative absorbance (O.D.) was quantified by densitometry.

Effect of FGF2 oligomerization and heparin on receptor-mediated cell proliferation

FDC-P1 cells expressing FGFR1, which depend for their survival on both FGF and heparin [53], were tested for their capacity to proliferate in response to biotin–FGF2 at increasing concen-

Figure 2 Binding and activation of FGFR1 by oligomers of FGF2

(A) Biotinylated FGF2 (100 ng/ml) was incubated in a plate coated with streptavidin with

trations of streptavidin and in the absence or presence of heparin (Figure 2C). No survival of cells was observed in the absence of heparin at any of the streptavidin concentrations tested. At low streptavidin levels and in the presence of heparin, where dimers and high-order oligomers of FGF2 are readily formed (Figure 2), there is full biological activity in response to biotin-FGF2, comparable to that obtained under streptavidin free conditions. At 1 µg/ml streptavidin, where biotin-FGF2 is present predominantly as a monomer, there is no cell proliferation, even in the presence of heparin, and this inhibition can be reversed by an excess of ligand (Figure 2C). Streptavidin by itself had no effect on cell survival at any of the concentrations used above (results not shown).

FGF2 dimers have a lower affinity for heparin

The formation of an FGF-heparin-receptor complex involves multiple interactions between each of the components. While dimeric FGF2 seems to be the preferred ligand for the receptor, its capacity to bind heparin may be altered upon dimerization. To gain insight into this interaction, FGF2 was incubated with immobilized heparin, under reducing conditions to avoid disulphide-bridged dimers, and the formation of monomers and dimers was analysed by chemical cross-linking and SDS/PAGE. The relative affinity of these forms to immobilized heparin was evaluated by sequential elution at increasing concentrations of NaCl (Figure 3A). Strikingly, while monomeric FGF2 was retained on the column at high salt and eluted, as expected, only at 1.5 M NaCl [52], the dimeric and higher-order oligomeric forms of FGF2 were either dissociated or displaced from heparin at 0.7–0.8 M NaCl.

Low-affinity heparin-bound dimers of FGF2 preferentially bind to the receptor

To further test the observed dramatic drop in the affinity for heparin of FGF2 upon dimerization and its implications for receptor binding, we analysed the capacity of soluble FGFR1 to interact with monomeric or mixed dimeric and monomeric forms of FGF2 immobilized on to heparin, at an increasing ionic strength (Figure 3B). There is a dramatic decrease in FGFR1 binding upon increasing the salt concentration of the wash solution above 0.7 M NaCl. This correlates with the elution profile for FGF2 dimers from heparin at the same ionic strength (Figure 3A). Notably, soluble FGFR1 in itself binds very weakly to heparin and is displaced from heparin at less than 0.5 M NaCl, a concentration at which FGF-receptor binding is not at all affected, indicating that the loss of receptor binding at 0.7 M salt is most likely due to the loss of oligomeric FGF2 bound to heparin, rather than due to its effects on direct heparin-receptor

increasing concentrations of free biotin. After washing the plate with 2 M NaCl to eliminate residual FGF-associated heparin the plate was incubated for a further 2 h with soluble FGFR1-AP (approx. 0.1 µg/well) in the presence or absence of heparin and the bound receptor was estimated by measuring AP. (B) Biotinylated FGF2 (1 µg/ml) was incubated with the streptavidin coated plate in the presence or absence of 5 µg/ml biotin. After washing with 2 M NaCl the plate was incubated with increasing concentrations of soluble FGFR1-AP in the absence or presence of (1 µg/ml) heparin. Receptor binding was quantified by a colorimetric reaction using *p*-nitrophenyl phosphate as a substrate and measuring absorbance (O.D.) at 410 nm. (C) FDC-P1 cells expressing FGFR1 were incubated with the indicated concentration of biotin-FGF2 and streptavidin in the absence or presence of heparin for 24 h. An XTT assay (measured at 490nm) was used to quantify the cell number. □, 0.1 µg/ml streptavidin; ◇, 5 µg/ml heparin; ■, 0.1 µg/ml streptavidin, 5 µg/ml heparin; ◆, 1 µg/ml streptavidin, 5 µg/ml heparin.

Figure 3 Heparin affinity chromatography (A) and receptor binding (B) of monomeric and dimeric forms of FGF2

(A) FGF2 was bound to heparin-Sepharose for 2 h and washed with a stepwise gradient of NaCl (0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2 M). After each wash a sample was subjected to chemical cross-linking, resolved by SDS/PAGE and Western immunoblotting and FGF2 was detected using anti-FGF2 antibodies. (B) Soluble FGFR-1-AP was incubated with the heparin-Sepharose beads with (filled bars) or without (hatched bars) FGF2 for 2 h at increasing concentrations of NaCl as indicated in (A). Specifically bound receptor-AP was quantitated by colorimetric reaction using *p*-nitrophenyl phosphate as a substrate, measuring the absorbance at 410 nm. Receptor binding could be fully reconstituted by reincubating the 0.8 M NaCl fraction with FGFR-1-AP in PBS for another 4 h (*).

interactions. Monomeric FGF2 retained on the column, albeit at significant levels, did not support FGF-receptor binding. These, heparin associated, apparently non-functional monomers of FGF2, were found to bind receptor after being further incubated in PBS for 4 h at room temperature (Figure 3B), suggesting that the retained monomers of FGF2 are functional once they are allowed to re-oligomerize on heparin.

A proposed model structure for active FGF2 dimers

Our experimental results indicate that FGF2 dimers are the most active moieties that bind to and activate FGFRs. It seems, however, that dimers can also be formed, although inefficiently, in the absence of heparin [38,39,51]. This suggests that while heparin may facilitate dimer formation, it does not necessarily mediate monomer-monomer interactions in the biologically active FGF2 dimer. Rather, it may stabilize the correct biologically active dimer, in which two FGF2 molecules in direct contact with one another bind a chain of about ten monosaccharide units of heparan sulphate.

The top ranking FGF2 dimer structure obtained by molecular docking, as described in the Materials and methods section, is a symmetrical structure (Figure 4). Water accessible Connolly surfaces are drawn for each monomer and coloured as follows: yellow and white for each monomer, blue for the heparin-binding residues and red and green for the putative primary and

Figure 4 Proposed model structure of the FGF2 dimer, heparin and the FGF receptor

(A) Model structure of FGF2 dimer. Solvent accessible Connolly surfaces of the two monomers in a dimer are shown in white and yellow. Residues which are known to bind heparin (HBS) are coloured blue and the major and minor receptor binding sites (RBS) are in red and green respectively. Two heparin hexamers (Protein Data Bank entry 1bfc), shown as black stick diagrams, are positioned as was determined in the experimental X-ray structure. (B) Illustration of the binding of a single chain of ten saccharides to the proposed FGF2 dimer. (C) Possible binding of two FGF-receptor molecules to a single FGF2 *cis*-oriented dimer, based on the structure of interleukin-1/interleukin-1 receptor (Protein Data Bank entry 1itb). The Figure was drawn using the program Insight II (MSI/Biosym).

secondary receptor-binding sites respectively. The primary receptor-binding site was used in the screening, as described in the Materials and methods section. Notably, the secondary receptor-binding site and the heparin-binding surface are also exposed in the proposed structure of the dimer. Moreover, the two heparin-binding sites are adjacent, forming a contiguous surface that can bind a single chain of heparin [40,41].

Two short heparin chains were placed in the appropriate binding sites, according to the experimental X-ray structure of FGF2 complexed with a heparin hexamer (Protein Data Bank

entry 1bfc) [41]. These are shown as black stick diagrams in Figure 4(A). Omission of the terminal saccharide residues and adjustment of the torsion angles of the preceding residues in each heparin hexamer (shown in Figure 4B) bring the two pentamers close together, suggesting that a heparin decamer can span the two adjacent heparin-binding sites. The ends of the proposed heparin decamer are very close to the receptor-binding sites (red patches on the surfaces of the FGF2 monomers), compatible with the suggested possibility that heparin may be directly involved in FGF–receptor binding [26,42].

The dimer interface includes residues H35, D37, R39, D41, D79, R81, L83, K119, T121, Q123, Y124, L126 and K129. It comprises mostly polar and charged residues, allowing the formation of hydrogen bonds and ion pairs. The few hydrophobic-aliphatic side-chains point toward hydrophobic or polar (but not charged) fragments in the other monomer. Notably, residues K119 and K129, whose mutation was shown to dramatically reduce heparin affinity and heparin-induced receptor binding [40], are found at the edge of the proposed dimer interface, where interactions with either the other monomer or heparin are possible.

The co-structure of interleukin-1, a close structural homologue of FGFs, and its receptor, was recently solved [54]. Modelling FGFR immunoglobulin-like domains 2 and 3, which contain all the necessary information for ligand binding according to the interleukin-1–receptor complex, yielded a molecular complex whereby each receptor molecule interacts with two FGF molecules comprising a single dimer, through the major receptor-binding site on one FGF and the minor receptor-binding site on the other. The model also predicts that a single heparin decasaccharide molecule can form interactions with both FGF heparin-binding sites as well as with the predicted heparin-binding site of FGFRs [42]. Notably, IgD3, comprising a major alternatively spliced ligand-binding site, and the linker between IgD2 and IgD3 interact with the major receptor-binding site of FGF, whereas elements from IgD2 interact with the minor receptor-binding site.

DISCUSSION

There seems to be a marked difference in structure between heparin oligosaccharides capable of binding FGF and the ones that can facilitate its high-affinity receptor binding and biological activity. We and others, have shown that the minimum size of heparin capable of binding and displacing heparin bound FGF2 is a tetrasaccharide, while only oligosaccharides containing ten units or more can efficiently enhance high-affinity receptor binding [16,17,19,26,43]. The results presented here demonstrate a strong correlation between the capacity of these decasaccharides to induce dimerization of FGF2 and their ability to enhance high-affinity receptor binding and activation. Tetrasaccharides, which are devoid of any biological or receptor-binding activity, can induce only *trans*-oriented dimers of FGF2, but decasaccharides can induce *trans*-oriented dimers, *cis*-oriented dimers or both, forming a complex tetramer [17,19,25,44]. It may well be that it is the highly, sometimes fully, sulphated nature of the oligosaccharides used that permits the formation of the *trans*-oriented dimers [25,44]. While such extensively sulphated structures are uncommon in heparan sulphate, the natural ligand for FGF–receptor interactions *in vivo*, one cannot exclude that some highly sulphated domains, on which the assembly of *cis*- and mixed *cis*- and *trans*-oriented dimers of FGF, may exist. A high content of sulphated disaccharides within a heparan sulphate oligosaccharide, in itself, however, does not ensure FGF2 activation [19]. Our proposed FGF2-dimer–heparin com-

plex, based on the X-ray structure of FGF2–heparin, predicts that each monomer binds two to three sulphate groups, in agreement with the experimentally determined composition of active heparan sulphate fragments. [43,45]. Specific sulphate moieties that are not involved in FGF binding and probably point away from the FGF molecule may directly interact with the receptor's heparin-binding domain [42]. These may involve glucosamine *O*⁶-sulphates, which favourably bind the FGF receptor ectodomain [26] and are required for its activation [19].

In analysing the structures produced by our molecular docking procedure, we expected that in the correct dimer (1) the secondary receptor-binding site and the heparin-binding site in each monomer would be exposed, in addition to the primary receptor-binding site, which was used for screening the raw docking results, (2) the relative position of the heparin-binding sites could explain the preference for a decamer heparin chain, and (3) the lower affinity of the dimer towards heparin compared with the monomer could be explained. The top ranking FGF2 dimer structure (Figure 4) complies with these requirements. As mentioned above, the receptor- and heparin-binding sites are exposed and the two heparin-binding sites are adjacent so that a single decamer heparin chain can span the combined binding site, while its ends are close to the receptor-binding site. Residues K119 and K129, which have been shown to affect heparin and heparin-induced receptor binding [40] and affect the spontaneous dimerization of FGF2 (M. Safran and A. Yayon, unpublished work), are found in our model at the edge of the proposed monomer–monomer interface, where they can form hydrogen bonds with polar residues from the other monomer (T121 and Q123). It therefore appears that K119 and K129, which contribute most of the binding energy to heparin in the FGF2 monomer [40], can form inter-monomer interactions that stabilize the FGF2 dimer, probably at the expense of the monomer–heparin interactions. This duality provides a plausible explanation for the observed lower affinity for heparin of the FGF2 dimer compared with that of the monomer.

FGF2 is associated predominantly with heparan sulphate on the cell surface and in the extracellular matrix. The relatively high affinity of FGF2 for heparin-like molecules and the large number of these sites which outnumber the signalling receptors, sometimes by two orders of magnitude, may raise the problem of the probability of an FGF molecule to bind and form an active signalling complex at any given moment. Our results demonstrate a sharp decrease in heparin affinity upon ligand dimerization, which may provide a logical basis for resolving this ambiguity. The markedly reduced heparin affinity upon dimerization, potentially through the diversion of heparin-binding residues, i.e. K119 and K129, towards the dimer interface, may also explain why multimeric forms of FGF2, although quite abundant, are rarely observed in FGF2 freshly purified on a heparin column. It is important to note that FGF2 dimers are still capable of binding heparin, although with a markedly reduced affinity, and this retained capacity may account for the stabilization of an active ternary signalling complex. The reduced affinity of FGF dimers for heparin therefore enables fast dissociation rates, increased diffusion capacity in the extracellular matrix and lateral mobility across the cell surface, all of which can markedly enhance receptor binding and activation.

A model of a heparin-oriented FGF dimer is also attractive with respect to the potential of specific heparan sulphate domains to support coupling of particular FGFs to specific FGFRs *in vitro* [43,46–48] and *in vivo* [49]. These heparan sulphate domains, which vary in size and in the density and distribution of charges, contributed by *O*- and *N*-linked sulphates, can provide an enormously large number of distinct binding motifs. Each

structurally distinct 'code' can, according to our model, bind two given FGF molecules in a specific relative orientation to create a distinct receptor-specific binding surface.

The exact way in which ligand dimerization may lead to receptor oligomerization and activation is not at all clear. One plausible mechanism is the one utilized by covalently linked dimeric growth factors that are believed to support receptor activation by properly orienting two monomeric receptors. As in the case of platelet derived growth factor [21], structural elements from both dimer subunits, which in the case of FGF could be stabilized and oriented by a heparan sulphate molecule, may contribute to the formation of a contiguous single high-affinity receptor-binding surface. Structural considerations of FGFR1 kinase, however, suggest that more than one receptor dimer may be required for efficient *trans*-phosphorylation to take place [55], similar in that respect to interleukin-6 ligand-receptor complexes, where activation involves the oligomerization of two receptor dimers through direct binding at two independent sites on interleukin-6 [56]. In such a case, heparin, in addition to its role in creating a high-affinity binding site on a dimeric FGF, could serve to create and properly orient higher-order receptor complexes for efficient *trans*-phosphorylation and signal transduction to take place as well. Alternatively, upon binding of a dimeric ligand, receptor dimers and oligomers may be formed and stabilized by direct receptor-receptor interactions, as shown for *c-kit*, the stem-cell factor receptor [50]. The exact mode of functional FGFR oligomerization, however, and its regulation by a heparan sulphate-FGF complex remains to be elucidated.

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