INFLUENCE OF HEPARIN MIMETICS ON THE ASSEMBLY OF THE FGF-FGFR4 SIGNALING COMPLEX

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Fibroblast growth factor (FGF) signaling regulates mammalian development and metabolism, and its dysregulation is implicated in many inherited and acquired diseases including cancer. Heparan sulfate glycosaminoglycans (HSGAGs) are essential for FGF signaling as they promote FGF-FGFR binding and dimerization. Using novel organic synthesis protocols to prepare homogeneously sulfated heparin mimetics (HM) including hexasaccharide (HM₆), octasaccharide (HM₈) and decasaccharide (HM₁₀), we tested the ability of these HM to support FGF1 and FGF2 signaling through FGFR4.

Biological assays show that both HM₈ and HM₁₀ are significantly more potent than HM₆ in promoting FGF2-mediated FGFR4 signaling. In contrast, all three HM have comparable activity in promoting FGF1-FGFR4 signaling. To understand the molecular basis for these differential activities in FGF1/2-FGFR4 signaling, we used nuclear magnetic resonance (NMR) spectroscopy, isothermal titration calorimetry (ITC) and size exclusion chromatography (SEC) to characterize binding interactions of FGF1/2 with the isolated Ig-domain 2 (D2) of FGFR4 in the presence of HM, and binary interactions of FGFs and D2 with HM. Our data confirm the existence of both a secondary FGF1-FGFR4 interaction site and a direct FGFR4-FGFR4 interaction site thus supporting the formation of the symmetric mode of FGF-FGFR dimerization in solution. Moreover, our results show that the observed higher activity of HM₈ relative to HM₆ in stimulating FGF2-FGFR4 signaling correlates with the higher affinity of HM₈ to bind and dimerize FGF2. Notably FGF2-HM₈ exhibits pronounced positive binding cooperativity. Based on our findings we propose a refined symmetric FGF-FGFR dimerization model which incorporates the differential ability of HM to dimerize FGFs.

The fibroblast growth factors (FGF) (1,2) comprise a family of secreted polypeptides that are encoded by 18 distinct genes (FGF1-FGF10 and FGF16-FGF23) in mammals. FGFs play pleiotropic roles in development as well as metabolism (3-7). FGFs transmit their effects by binding and activating FGF receptor tyrosine kinases (FGFRs) a subfamily within receptor tyrosine kinase superfamily (3,8,9).

The core homology domain of FGFs is about 120 amino acid long and adopts a β-trefoil fold consisting of 12 β strands (10-19). All FGFs interact with heparan sulfate glycosaminoglycans (HSGAGs) (20). The HSGAG-binding site of FGFs is composed of the β1-β2 loop and the region encompassing β10 till β12 (10,21-25). Based on their high sequence and structural homology to FGFs, four additional genes (FGF11-FGF14) are also regarded FGF family members.
However, recent structural and biochemical data show that FGF11-FGF14 are functionally unrelated to FGFs (26-28).

Four genes in mammalian organisms (FGFR1-4) code for FGFRs (9). The ectodomain of a prototype FGFR comprises three Ig domains (D1-D3). An unique property of FGFR is the presence of a stretch of acidic residues in the linker connecting D1 to D2, referred to as “acid box”. A wealth of structural and biochemical studies have demonstrated that D2, D3 and D2-D3 linker are necessary and sufficient for FGF binding (8,29). Like FGFs, FGFRs are also HSGAG-binding proteins (30). HSGAG-binding site of FGFR resides in D2 and is comprised of residues from g-helix A, β strands B and D, and gA-βA’ and βA’-βB loops (23,24). D1 and the D1-D2 linker are dispensable for FGF binding and in fact negatively regulate FGFR signaling (31-33).

Two major alternative splicing events take place in the ectodomains of FGFR1-3: one involving D1 and the D1-D2 linker, and the other D3 (9,34). Alternative splicing of D1 and the D1-D2 linker serves as a mechanism to modulate receptor autoinhibition whereas alternative splicing in D3 determines ligand binding specificity (35,36). In FGFR1-FGFR3, the second half of D3 is encoded by two mutually exclusive exons (“b” and “c”) that are used in a tissue-specific fashion (37-41). The “b” exon is used in epithelial tissues, whereas the “c” exon is preferentially used in mesenchymal tissues. As a result of this splicing event, the number of principal FGFRs is increased to seven isoforms namely FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, and FGFR4. Importantly, alternative splicing of D3 sets a specificity barrier such that epithelially expressed FGFs activate FGFRc isoforms while mesenchymally expressed FGFs activate FGFRb isoforms (42). Most FGFs bind and activate more than one of the seven principal FGFRs, although they do not cross the specificity barrier set by the D3 alternative splicing (43). For example, FGF2 exhibit high affinity to both FGFR1c and FGFR2c but does not bind to FGFR1b and FGFR2b. The affinity of FGF2 to FGFR3c and FGFR4 is also negligible (44). FGF1 is an exception as it is capable of indiscriminately binding both “b” and “c” isoforms of FGFR1-3 and FGFR4 (42). Crystallographic studies of several FGF-FGFR complexes have shown that the D3 alternative splicing regulates FGF-FGFR binding specificity and promiscuity by modifying the primary sequences of the key ligand binding sites in D3 (14,16).

Genetic analysis of mice and flies deficient in enzymes involved in heparan sulfate (HS) biosynthesis, and cell-based assays using cell lines devoid of HSGAG have established that FGF signaling requires HSGAGs (45-47). HSGAGs impinges on FGF signaling through multiple mechanism including promotion of FGF-FGFR binding and dimerization (8), control of FGF diffusion and gradient formation in the extracellular matrix (18,48,49), providing thermal stability and protection from proteolytic degradation (50,51).

Crystallographic analyses of FGF-FGFR-heparin ternary complexes have provided two conceptually different models by which HSGAG promote FGF-FGFR dimerization. According to a symmetric model [(Mohammadi-model, FGFR1-FGF2-heparin(decasaccharide), PDB entry 1FQ9)] (23) HS promote formation of a symmetric 2:2:2 FGF-FGFR signaling complex. The dimer interface is mediated by protein-protein contacts between the two adjacent FGF-FGFR halves and is strengthened by interaction of heparin with FGF and FGFR. The dimer interface comprises direct receptor-receptor contacts mediated by D2, and interactions of FGF from one FGF-FGFR half with D2 of FGFR in the second FGF-FGFR half (referred to as secondary FGF-FGFR interface). At the membrane distal end of the dimer, the individual HS binding sites of two FGFs and FGFRs unite into one HS binding canyon into which two HS chains bind. By engaging the HS binding sites of FGF and FGFRs, HS augments FGF-FGFR affinity within each FGF-FGFR half as well as promotes the dimer interface (8,52).

In this structure, only the first six sugar units at the non-reducing end of the decasaccharide are in contact with the protein (FGFR or FGF). The remaining four sugar units of one of two decasaccharides are disordered. In the other decasaccharide, two additional sugar units are visible due to the favorable crystal contacts they make with an adjacent FGF molecule. Thus, based on the symmetric model, a hexasaccharide should be sufficient for dimerization and hence biological activity (8,52).

A different model has been proposed by Blundell and coworkers [(Blundell-model, FGFR2c-FGF1-HS, PDB entry 1E0O)] (24). This
model displays a 2:2:1 FGF-FGFR-HS stoichiometry in which a single HS chain bridges two FGFs in trans and each FGF binds to one FGFR only. In contrast to the symmetric model, there are no direct protein-protein contacts between the two FGF-FGFR halves in this model. In other words, the asymmetric dimer is held together solely by the ability of HS to dimerize FGFs and consequently this mode of dimerization is strictly HS-dependent.

The entire cocrystallized decasaccharide is visible in this structure. Two sugar units at the non-reducing end of oligosaccharide engage the D2 domain of one of the two FGFR chains while the HSGAG-binding site of the other FGFR chain remains unoccupied. Seven sugar rings bridge the two FGFs in trans. Based on this model, the shortest biologically active HS would be an octasaccharide, although maximal activity would require a dodecasaccharide as it will be long enough such that it could engage D2 of both FGFRs (24). It should be noted, however, that an alternative interpretation of this crystal structure also leads to a model similar to the symmetric one (52,53).

Numerous in vitro and cell-based studies have been attempted to test the salient features of each model. Most data support the physiological relevance of the symmetric dimerization model. For example, mutations that ablate interactions of FGF from one FGF-FGFR half to D2 of FGFR in the second FGF-FGFR half diminish the ability of the mutated FGF to signal while these mutated FGFs retain the ability to form a 1:1 FGF-FGFR complex (54). A further unbiased piece of evidence in support of a symmetric model comes from analysis of the naturally occurring A172F mutation in FGFR2. This gain-of-function mutation, responsible for the Pfeiffer syndrome, maps to the direct FGFR-FGFR interface and confers gain-of-function by promoting direct D2-D2 contacts and hence receptor dimerization (54).

Biological studies with size-fractioned heparin oligosaccharides have also been used to test the validity of each model. However, no consensus has been reached with regard the minimal oligosaccharide length needed for FGF signaling. Some studies show that a hexasaccharide and even smaller sugars are capable of promoting FGF signaling (55-57). In contrast, other data show that a hexasaccharide has either poor or no activity at all, and that an octasaccharide is the shortest biologically active heparin (53). In all these prior studies, the heparin oligosaccharides were prepared by either enzymatic hydrolysis or chemical cleavage of heparin isolated from natural sources. Therefore, a potential reason for the disparity between these data could be differences in the homogeneity of oligosaccharide preparations used. Our ability to de novo synthesize homogeneously sulfated heparin oligosaccharides of various degrees of polymerization including hexasaccharide (HM₆), octasaccharide (HM₈) and decasaccharide (HM₁₀) (Fig. 1A) provided us with the unique opportunity to revisit the minimum oligosaccharide length requirement for FGF signaling. Using a BaF3 cell line overexpressing a chimeric FGFR4, we show differences in the abilities of these HM to promote FGF1 and FGF2 signaling through FGFR4. All three HM have comparable capacity to stimulate FGF1-FGFR4 signaling. In contrast, in the case of FGF2, a major activity difference in signaling is seen for the transition from HM₆ to HM₁₀/₁₀.

To understand the molecular basis for the observed prominent activity differences between HMs in promoting FGF1- and FGF2-FGFR4 signaling, we used NMR spectroscopy, ITC and SEC to study the interactions of FGF1 and FGF2 with the isolated D2 of FGFR4 (the HSGAG-binding domain of FGFR4) in the presence and absence of HM₆ and HM₈. Since HM₈ and HM₁₀ displayed similar receptor activation level in biological assays, our biophysical studies were restricted to HM₈. NMR chemical shift mapping, signal attenuation, and T₂ relaxation studies show the existence of a secondary FGF-FGFR D2 interaction site as well as direct D2-D2 interaction site corresponding to those seen in 1FQ9 thus providing direct evidence that the symmetric mode of dimerization occurs in solution. Our data show that the two HM make identical sets of contacts with FGF and FGFR D2 in the 2:2 FGF-D2 dimers. Therefore, the higher activity of HM₈ relative to HM₆ is not due to the additional contacts of the two extra sugar rings of HM₈ with FGF or FGFR D2. Rather, our data show that differences in the binding affinity of HM for FGFs along with differences in the ability of HM to dimerize FGFs correlate with the differences in the biologic activities of the FGFs. Based on our findings we propose a refined symmetric model that takes into account the differential abilities of HM to dimerize FGF2 in a cooperative fashion.
Experimental Procedures

**Cloning, expression and purification of FGF and FGFR**—DNA encoding the human FGF1 (G21-D155), FGF2 (P10-S155), FGFR4 D2 (N138-L242) was amplified by PCR and cloned into E. coli vector pETTEV (N-terminal His-tag followed by a TEV protease cleavage site) or pET21b (C-terminal His-tag) (Novagen). The identity of the clones was verified by sequencing and the E. coli expression plasmids were transformed into BL21(DE3) CodonPlus-RIPL (Stratagene) cells. Expression and purification of FGF1 (58) and FGF2 (59) was performed as previously described. The successful expression, purification and refolding of the D2 domain is similar for FGFR1, 2, 4 with minor modifications (60,61).

**ECD of FGFR4 and its mutated transmembrane parts**—(LALAVLLAGLEELLLRWQ to LALAVLLLLRWQ amino acid x-y) were fused to the intracellular domain of hMpl and cloned into a modified pEF6/V5-His (C-terminal HA tag) (Invitrogen). The construct with chimeric FGFR4-hMpl (pEF6mut-HA FGF-R4αIIIcmut-hMpl2) was stably integrated by electroporation into murine BaF3 cells genome. The cell line was selected with 20 ng/ml FGF2 (R&D, 234-FSE-025) and 10 ng/ml heparin (Sigma, H3149).

**BaF/3 FGFR4-hMpl proliferation assay**—BaF3 expressing FGFR4-hMpl was grown in a supplemented RPMI1640 medium. Proliferation of cells was quantified by luminescence ATP measurement with a cell titer glo luminescent cell viability assay (Promega, G7571).

**NMR spectroscopy**—NMR spectra for all proteins and compounds were recorded at 298 K and were referenced to internal 3-Trimethylsilyl-2,2,3,3-tetradeuero-propionate-sodium salt (TSP). The experiments were carried out on a BRUKER three-channel DRX600 and on a BRUKER four-channel DRX800 spectrometer using standard pulse programs. Specific parameters including buffer conditions and concentrations are summarized in the Figure captions.

**Isothermal titration calorimetry**—ITC measurements were carried out on a VP-ITC ultrasensitive titration calorimeter (MicroCal LLC) and data analyses were performed as described previously (62).

**RESULTS**

**Synthesis of heparin mimetics**—For the synthesis of hexameric HM6, we have developed a strategy based on chain elongation from a common disaccharide building-block (63). The elongation may then be repeated multiple times until the fully protected hexasaccharide is obtained. As in previous heparin mimetics synthesis, acyl groups were employed to protect the hydroxyl groups to be sulfated, benzyl ethers were used for the free hydroxyl groups and azido groups masked the amino groups to be sulfated.

We have used a similar strategy for the synthesis of oligosaccharides HM8 and HM10 and their preparation will be published elsewhere.

**HM6 and HM10 are more effective than HM6 in promoting FGFR2-dependent FGFR4 dimerization and signaling**—The murine pre-B cell line BaF3 requires interleukin-3 (IL-3) to proliferate. This cell line does not naturally express FGFRs and HSGAG. However, when transfected with FGFRs, BaF3 cells acquire the ability to proliferate in response to exogenous FGF and soluble heparin. Thus, the FGFR-overexpressing BaF3 cell lines are ideal to compare the biological activity of heparin mimetics to promote FGF- FGFR signaling. To compare the efficacy of HM6, HM8 and HM10 (Fig. 1A) in promoting FGFR-dependent dimerization of FGFR we established a stable cell line expressing chimeric FGFR4 (Fig. 1B, C), termed FGFR4-hMpl, in which the ectodomain of FGFR4 was fused to transmembrane and intracellular domain of thrombopoietin receptor (hMpl). Addition of FGF and heparin causes the ectodomain of the FGFR4-hMpl to dimerize which in turn juxtaposes the intracellular hMpl regions, leading to subsequent Mpl activation and ultimately evoking a proliferation response. Therefore, cellular proliferation can be used as readout to compare the capacity of FGFs and/or HM to dimerize the ectodomain of FGFR4.

FGFR4-hMpl expressing BaF3 cells were deprived of IL-3 and treated with increasing concentrations of FGF1 or FGF2 in the presence or absence of 100 nM of HM6, HM8 or HM10. In the absence of HM, both FGF1 and FGF2 induced proliferation of the FGFR4-hMpl cells with a half maximal effective concentration (EC50) of 16.6 ng/ml and 11.8 ng/ml, respectively (Fig. 1C). HM6/8/10 modestly decreased the EC50 of FGF1. In contrast, HM8/10 led to 10-fold decrease in the EC50
values for FGF2 (Fig. 1C), suggesting that HM₈/₁₀ are more potent than HM₆ in assisting FGF2 to dimerize and activate FGFR4-hMpl.

The observation that FGF1 and FGF2 are able to dimerize FGFR4-hMpl in the absence of HM is intriguing and can only be reconciled by the symmetric FGF-FGFR-heparin dimerization assembly model (Mohammadi model). In contrast, the asymmetric mode of dimerization (Blundell model) lacks protein-protein contacts between the two FGF-FGFR halves, the ability of FGF1 and FGF2 to dimerize and activate FGFR4-hMpl in the absence of HM cannot be reconciled with this model. On the other hand, the higher efficacy of HM₈/₁₀ compared to HM₆ fits better with the asymmetric model because according to the symmetric model a hexasaccharide should be sufficient to promote FGF-FGFR binding and dimerization.

NMR studies indicate a symmetric FGF-FGFR dimerization model in solution

We decided to use NMR spectroscopy techniques to investigate the molecular basis for the higher potency of HM₈ over HM₆ in promoting FGF2-FGFR4 dimerization and signaling. Crystal structures of several FGF-FGFR complexes have established that both D2 and D3 are needed for FGF binding (8). In contrast to the well resolved Ig domains D1 (64) and D2 (60,65), the D3 domain of all investigated FGFR (FGFR1/2/4) constructs (D1D2D3, D2D3, D3) lead to line broadening beyond detection in solution. The structural integrity of the investigated D3 domain was confirmed by X-ray analysis (successful crystallization of FGFR2 D2D3/FGF1). Therefore, our NMR study focused on the Ig domain 2, the heparin binding domain of the receptor.

We used chemical shift perturbations (CSPs) and changes in the signal intensities of one ¹⁵N-labeled protein component upon addition of the second, unlabeled component to test if the isolated D2 domain of FGFR4 is capable of binding FGF. Backbone assignments of FGF1 and FGF2 have been reported previously (58,59) and were used in this study. Consistent with published FGF-FGFR X-ray structures, no chemical shift perturbations (CSPs) in 2D-[¹⁵N,¹³C]-correlation spectra of ¹⁵N-labeled FGF1 or FGF2 were observed upon titration with unlabeled FGFR4 D2. We also prepared uniformly ¹⁵N-labeled D2 and titrated it with unlabeled FGF1 and FGF2. No CSPs were observed in HSQC of ¹⁵N-labeled D2 at protein concentrations of 50 to 100 µM. These data are consistent with published crystal structures indicating that both D2 and D3 are needed for FGF binding (8).

The crystal structures of FGF-FGFR-heparin ternary complex show that D2 mediates binding of FGF to HM. In both these structures heparin interacts simultaneously with HM binding on D2 of FGFR1 (23) and FGFR2 (24) and the HSGAG binding site of FGFs. This observation suggests that, although D2 alone is not sufficient for high affinity FGF binding by itself, the presence of heparin increases the binding affinity of FGF to D2. Indeed a pervious ITC study has shown that the presence of sucrose octasulfate (SOS), a heparin analog, enhances binding of FGFR1 D2 to FGF1 (60).

To test if HM₆ and HM₈ can promote binding of FGF1 and FGF2 to D2 of FGFR4 we first confirmed that isolated D2 of FGFR4 is capable of binding heparin. Addition of HM₆ and HM₈ to ¹⁵N-labeled FGFR4 D2 led to CSPs in 2D-[¹⁵N,¹³C]-correlation spectra (Supplementary Fig. 1). To identify the HSGAG binding residues of D2 we then assigned the NMR backbone resonances of FGFR4 D2 using standard triple resonance NMR experiments on a [¹³C,¹⁵N]-labeled sample (unpublished results). D2 showing CSPs include Lys 158, Leu 159, Val 168, Lys 169. These D2 residues are homologous to the heparin-binding residues of FGFR1 and FGFR2 D2 seen in the X-ray structures of ternary complexes (PDB entries 1FQ9 (23) and 1E0O (24)). Interestingly, in addition to CSPs close to the HM binding site, strong CSPs and severe line broadening were observed for D2 residues Ser 141 and Tyr 142 located at the N-terminal tip of D2 in the presence of either HM.

Next, we tested whether HM can support binding of FGF1 and FGF2 to D2. Two complementary titration experiments were performed: In the first experiment, uniformly ¹⁵N-labeled FGFR4 D2 was titrated with a 1:1 mixture of unlabeled FGF with HM₆ or with HM₈. In the second experiment, 1:1 mixtures of ¹⁵N-labeled FGF and HM were titrated with unlabeled FGFR4 D2. In both these titration experiments, a major reduction in signal intensity of backbone resonances of the ¹⁵N labeled component (FGF1, FGF2 or D2) was observed suggesting that indeed, in the presence of HMs, D2 is capable of binding FGF. Analysis of the line width of individual
backbone amide resonances of D2 and FGF allowed us to identify residues in FGF and D2 that are involved in protein-protein and protein-HM interactions (Supplementary Table I).

No structure of FGFR4 D2 has been reported so far. However, the sequence identity between the ECDs of FGFR1, FGFR2 and FGFR4 is very high (over 50%). Therefore, NMR results on FGFR4 D2 can be analyzed using the available ternary complex structures of FGFR1 and FGFR2. The regions in FGF1 and D2 that are affected in the FGF1-D2-HM8 titration experiment are mapped onto both X-ray structures (the symmetric 2:2:2 complex 1FQ9 (Fig. 2) and the asymmetric 2:2:1 complex 1E0O (Fig. 3)). FGFR4 D2 residues that undergo signal attenuation upon titration with an unlabeled FGF1:HM8 mixture include the predicted HSGAG-binding site of FGFR4 D2 and the predicted primary ligand binding site of D2. These two regions are identical between the two crystallographic models. Interestingly, other affected D2 residues include Gly 165, Asn 166, Thr 167, Glu 212 and correspond to the D2 regions that are expected to mediate the direct D2-D2 interface (Figure 2 and 3, cyan circles) and the secondary FGF-FGFR D2 interface (Fig. 2 and 3, yellow circles) observed in the symmetric model.

Residues in FGF1 that undergo signal attenuation upon titration with unlabeled D2 correspond to the FGF1’s HSGAG-binding site, and primary receptor binding site (Supplementary Table I). Interestingly also CSPs and/or signal attenuation were observed for the residues mapping to the region on FGF1 that is predicted to mediate the secondary FGF-FGFR D2 interface (Fig. 2 and 3, yellow circles) based on the symmetric dimerization model. This secondary FGF-D2 interaction site was also described by Kochoyan and co-workers for a complex between FGFR1 D2 and FGF1 in the absence of heparin (65). Notably, the NMR data show that HM8 and HM9 generally induce perturbations and signal attenuation of the same residues in FGFR4 D2 and in FGF1 and FGFR2 (FGFR4 D2:FGF2-HM8:HM8 mapped on 1FQ9 X-ray structure: Supplementary Fig. 2 and 3). We therefore conclude that NMR data show the symmetric dimerization model in solution.

Strong line broadening of D2 and FGF residues indicates that the FGF-D2-HM complex is highly dynamic with exchange rates between different oligomerization states on the µs to ms timescale. Thus, we carried out T2 NMR relaxation measurements to gain more insights into the dynamics and stoichiometry of the FGF-D2-HM complexes. T2-derived apparent molecular weights of FGF1/2 and FGFR4 D2 in the absence and presence of either HM8 or HM9 are summarized in Table I. Consistent with the results of NMR titration experiments described above in the absence of HM, the predicted molecular weights for monomeric FGF1, FGF2 and FGFR4 D2 were observed. In the presence of HM, however, higher molecular weight species were detected (Table II). The highest molecular weights were observed for FGF1 and FGFR4 D2 in the presence of HM8. The measured apparent molecular weight of FGF1 increased from 21 kDa to 44 kDa for 15N-FGF1 in complex with unlabeled FGFR4 D2 and HM8. At the same time, the apparent molecular weight of FGFR4 D2 increased from 21 kDa to 37 kDa for 15N-FGFR4 D2 in the complex. For a stable 2:2 dimeric FGF-D2 complex, values around 60 kDa would have been expected for both complexes. Therefore, smaller apparent molecular weights together with different apparent molecular weights for FGF and FGFR4 D2 suggest that both proteins are in a dynamic equilibrium between the free form and higher molecular weight complexes with a stoichiometry greater than 1:1:1 FGF-D2-HM (theoretical molecular weight of 32 kDa) but smaller than 2:2:2 in solution at concentrations of 50 µM. The increase in T2-derived apparent molecular weights was greater for FGF1 than for FGF2, and greater with HM8 than with HM9 indicating that the equilibrium between ternary complex and lower molecular weight species in solution is shifted prominently towards the ternary complex for FGF1 vs. FGF2 and for HM8 vs. HM9.

We also studied the interaction of FGFR4 D2 with FGF1 and FGF2 in the absence and presence of HM using ITC. In the absence of HM, titrations of FGFR4 D2 into FGF1 or FGF2 resembled control titrations of FGFR4 D2 into buffer (Supplementary Fig. 4). Titrations of D2 into a 1:1 mixture of FGF-HM8 also generated no heat signal. In contrast, titration of FGFR4 D2 into a 1:1 mixture of FGF1-HM8 and FGFR2-HM8 showed a binding event with a KD ~ 1 µM and a stoichiometry near unity (Table I, Supplementary Fig. 4). These data indicate that HM8 forms a ternary complex with FGF and D2, whereas complexes mediated by HM9 are not stable at these protein concentrations (10 µM). The prominent
dilution heat signal that was observed in the titrations of FGFR4 D2 into buffer possibly indicates dissociation of protein oligomers; a tendency for self-association of FGFR4 D2 was also observed by NMR spectroscopy (discussed below).

Taken together, NMR and ITC experiments indicate differences between HM₆ and HM₈ in their ability to promote FGF-D2 complex formation and dimerization. However, NMR CSP and signal attenuation studies show HM₆ and HM₈ generally induce effects on the same residues in FGFR4 D2 and in FGF1 and FGF2 suggesting that the greater efficacy of HM₈ relative to HM₆ to promote dimerization cannot be explained by additional contacts of two extra sugar units of HM₈ with FGFR D2 or FGF. These findings imply that differences in the binary interactions of HM with FGF and/or FGFR D2 may underlie the observed differences in the biological potency of these two HM.

**HM display major differences in their ability to dimerize FGFs —**

It has been reported previously that heparin can induce dimerization/higher-order oligomerization of FGF1 or FGF2 (66,67). Therefore, we compared the binding affinities of HM for FGF1 and FGF2 and the abilities of HM to induce oligomerization of FGF1/2 using NMR spectroscopy. As expected, titration of HM₆ and HM₈ to both ¹⁵N-labeled FGF1 and FGF2 (NMR backbone assignments: (58), (59)) caused strong CSPs and line broadening of the amino acid residues comprising the HSGAG-binding site of the ligands (Supplementary Fig. 5, Supplementary Table II). Compared to HM₆, HM₈ induced stronger CSPs and signal attenuation of ¹⁵N-labeled FGF1 and FGF2 than HM₆ in agreement with higher affinity of HM₈ over HM₆ to FGF1 and FGF2.

Next we used NMR ¹H T₂ relaxation measurements to study HM-induced FGF dimerization. The ¹H T₂ derived apparent molecular weights of the FGF-HM complexes are given in Supplementary Table III. The data show that both HM₆ and HM₈ are capable of dimerizing FGF. However, the smaller apparent molecular weight of the FGF dimer in the presence of HM₈ pointed to dynamic monomer-dimer equilibrium on the NMR time scale. The dynamic equilibrium in the case of HM₆ was also confirmed by size exclusion chromatography (SEC) (Supplementary Fig. 6, Supplementary Table IV). In the presence of HM₈, FGF eluted at a retention time midway between the expected retention times for monomeric and dimeric FGF. In contrast upon addition of HM₈, the FGF2 peak shifted to a retention volume which correspond to the FGF2 dimer (Supplementary Table IV).

Stepwise titration of HM to either FGF followed by NMR ¹H T₂ relaxation measurements allowed us to further elucidate the dimerization mechanism. The T₂ derived apparent molecular weight as a function of the HM:FGF ratio was measured (Fig. 4E). Maximum dimerization was achieved at a HM:FGF2 ratio of 0.5. This finding indicates a HM:FGF2 stoichiometry of 1:2, suggesting a two-step binding model (Fig. 4F). At ratios higher than 0.5, the apparent molecular weight of FGF2 decreased again, in line with a partial dissociation of the HM-FGF dimer in the presence of an excess of HM. While dimer association at low HM:FGF ratios was similar for both HM, dimer dissociation was more pronounced for HM₈ than for HM₆.

Theoretical fitting of the HM₆ titration into FGF2 resulted in a two step binding model with similar K_D values for the two binding events (Fig. 4E, open triangles, black line; K_D₁=160 nM, K_D₂=120 nM). In contrast, the HM₈-FGF2 interaction showed a pronounced positive cooperativity (Fig. 4E, filled triangles, black line: K_D₁=100 nM and K_D₂=5.8 nM). Taken together, the NMR data suggest that for the HM₈-FGF2 interaction, the recruitment of the second FGF2 to form the dimeric complex HM₈-(FGF2)₂ occurs with higher affinity than the first step indicating positive cooperativity of binding, in agreement with previous reports (68).

The ability of HM to dimerize FGFs was also compared using ITC at tenfold lower protein concentration than in the NMR ¹H T₂ relaxation measurements (Fig. 4C, D). Both HM bound to FGF with sub-micromolar affinities (Table I). Within experimental error, HM₆ exhibited similar affinity for FGF1 and FGF2. Compared to HM₆, HM₈ bound two-fold tighter to FGF1. In case of FGF2 the difference in binding affinities between the HM was substantially larger: HM₈ bound with 15-fold higher affinity than HM₆ (54 vs. 843 nM) to FGF2.

We also used ITC to compare the efficacy of HM to dimerize FGF1 and FGF2. ITC experiments showed that HM₆ bound to FGF with a 1:1 binding
FGF interacts with all HM units in a degenerate, dynamic fashion

Based on the published FGF-heparin crystal structures FGF binds to an internal trisaccharide binding motif (21,22). Thus we decided to study binding of HM to both FGF1 and FGF2 by monitoring the anomic proton resonances of HM in $^1$H 1D NMR spectra (Fig. 4A, B). Conveniently, the anomic protons of the oligosaccharides fall in the region between 5.0 and 5.5 ppm where no protein signals occur. At a HM:FGF ratio of 1:1, the anomic resonances of all sugar units of HM$_6$ and HM$_8$ become broadened beyond detection indicating the presence of multiple bound conformations, which interchange on the $\mu$s to ms time scale. No difference could be detected in the line broadening between the terminal and non-terminal sugar units. Based on the FGF-heparin crystal structures we would have expected to observe differences in line broadening between the internal trisaccharide binding motif and the terminal units. The lack of difference in the extent of line broadening between the terminal and internal sugar units indicate that all sugar units participate in ligand binding. Thus, based on our NMR data, FGF interacts in a degenerate fashion with all sugar units of HM with exchange of the units between the free and different protein-bound states on a time scale of $\mu$s to ms.

Mathematically formulation of the degeneracy in the FGF2-HM interaction show that an increased degeneracy of FGF binding sites for HM$_8$ relative to HM$_6$ alone is not sufficient to explain the differences in the affinity of HM$_6$ and HM$_8$ for FGF2. Based on a trisaccharide binding motif (see below), HM$_8$ offers three degenerate binding sites compared to only two binding sites on HM$_6$. Supplementary derivation I demonstrates that the increased number of FGF binding sites results for HM$_8$ in a higher affinity for both binding events (1.5-fold for binding of the first FGF; 1.3-fold for the second FGF). This theoretical formulation agrees well with the experimental data for the first HM-FGF interaction. However, for the second binding event, a 20-fold difference in affinity is observed experimentally for HM$_6$ compared to HM$_8$. In addition, binding site degeneracy alone results in a lower affinity for the second FGF-HM interaction compared to the first one (HM$_6$: 4-fold higher affinity; HM$_8$: 4.5-fold) and therefore cannot explain the observed positive cooperativity.

Interaction of heparin mimetics with FGFR4 D2—We also studied the ability of HMs to induce dimerization/oligomerization of FGFR4 D2. T$_2$ experiments indicate an increase in apparent molecular weight from 21±3 kDa in the absence of HM$_8$ to 28±7 kDa in the presence of HM$_8$ (expected monomeric molecular weight for FGFR4 D2: 15 kDa). These findings provide structural evidence for a FGF-independent, HM-induced dimerization of FGFR4, which was previously indicated by biological data (69).
Binding of HM to FGFR4 D2 was also measured by ITC (Supplementary Fig. 8). For both HM, similar affinities in the low micromolar range (HM<sub>6</sub>: 2.4 μM, HM<sub>8</sub>: 3.4 μM) and stoichiometries near unity were obtained (Table I). In a previous study, similar affinities of 0.3-0.4 μM have been reported for the HM-FGFR4 interaction (70,71). Lower stoichiometries for HM<sub>8</sub> than for HM<sub>6</sub> (Table I) possibly indicate a partial HM<sub>8</sub>-induced dimerization as observed by SEC (Supplementary Table IV) and NMR. Binding thermodynamics differed strongly for the two HM. HM<sub>8</sub> had a much larger binding enthalpy compared to HM<sub>6</sub> (-9.3 vs -1.2 kcal/mol). In contrast binding of D2 to HM<sub>6</sub> was primarily entropy-driven, indicating a different binding mode for HM<sub>6</sub> as compared to HM<sub>8</sub>. It has been reported previously that an heparin octamer constitutes the minimal chain length for stable HM binding to FGFR4 (70). The deviating binding thermodynamics observed with HM<sub>6</sub> could therefore point to an alternative, possibly nonspecific binding mode. Taken together, our data show that HM<sub>6</sub> and HM<sub>8</sub> differ greatly in their affinity FGF1/2 and have different ability to dimerize FGFs whereas both HMs exhibit comparable affinities for D2 and have poor ability to dimerize FGFR4 D2.

**DISCUSSION**

Using organic chemistry methods we were able to de novo synthesize chemically pure sulfated heparin hexasaccharide (HM<sub>6</sub>), octasaccharide (HM<sub>8</sub>) and decasaccharide (HM<sub>10</sub>). We showed that HM<sub>8</sub> is significantly more potent than HM<sub>6</sub> while HM<sub>10</sub> displays similar potency as HM<sub>8</sub> in promoting FGF2-mediated FGFR4 signaling. Interestingly, the prominent effect in biological activity of the octasaccharide relies on its interaction with FGF2, since FGF1-HM<sub>6/8/10</sub> activation only resulted in relatively small changes in FGFR4-signaling.

To delineate the underlying molecular basis for higher efficacy of HM<sub>8</sub> relative to HM<sub>6</sub> we used NMR, ITC and SEC to characterize binding interactions of FGF1 and FGF2 with isolated Ig-domain 2 (D2) of FGFR4 in the presence of heparin mimetics, and binary interactions of FGFs and D2 with HM. Our NMR data in solution support the symmetric dimerization model proposed by Mohammadi and colleagues (23) for HM<sub>6</sub> and HM<sub>8</sub> in contrast to the conclusion of Goodger and coworkers (53) who proposed an asymmetric ternary complex formation (24) for octasaccharides and larger heparin fragments. Our observed symmetric complex in solution is transient, and HM<sub>8</sub> (compared to HM<sub>6</sub>) shifts the equilibrium more strongly towards the ternary complex for both FGF1 and FGF2. Importantly, the NMR data show that the higher efficacy of HM<sub>8</sub> relative to HM<sub>6</sub> is not due to the ability of HM<sub>8</sub> to make additional contacts with FGF or D2.

Analysis of the binary interaction of HM with the D2 domain of FGFR4, which harbors the heparin binding site, show that HM<sub>6</sub> and HM<sub>8</sub> bind D2 with ~1 µM Kd, in agreement with previous reports (70,71). Therefore, the differences in affinity appear too small to account for the observed differences in potency between HM<sub>6</sub> and HM<sub>8</sub>. A tendency of HM<sub>8</sub> to dimerize the receptor already in the absence of FGF could be observed, an interesting finding in light of the reported heparin sensitivity of FGFR4 (69).

In contrast to the findings of Goodger and coworkers (53) that an octasaccharide is the shortest heparin fragment to form a 2:1 FGF-heparin complex, in our study both HM<sub>6</sub> and HM<sub>8</sub> were able to dimerize FGF1 and FGF2, above a protein concentration of 50 μM. NMR-based epitope mapping revealed closely overlapping binding sites of HM<sub>6</sub> and HM<sub>8</sub> on FGF1 and FGF2. The observed binding epitopes correspond to the heparin binding sites observed in the published crystallographic studies (21,22).

Importantly, FGF2 dimerization upon HM<sub>8</sub> addition reveals strong positive cooperativity, in agreement with previous studies on both FGF1 (68) and FGF2 (53). Furthermore, HM<sub>8</sub> has a stronger propensity to dimerize FGF than HM<sub>6</sub> and binds with 15-fold higher affinity to FGF2. By contrast, for FGF1 the affinity for HM<sub>8</sub> is only two-fold higher than for HM<sub>6</sub>.

There are two potential mechanisms that could account for the differences in affinities of HM<sub>6</sub> and HM<sub>8</sub> towards FGFs. Firstly, the affinity can be increased due to a degeneracy effect observed in multivalent interactions. Secondly, the interactions of HM with both FGF and FGFR are highly dynamic, as observed by NMR line broadening experiments. Our findings are in line with recent surface plasmon resonance data (72), where rapid association and dissociation kinetics for the FGF-HM complex and rapid dissociation for the receptor-HM complex were demonstrated.
As the oligosaccharides offer multiple binding motifs for FGF, the fast dynamics result in multivalent binding. As a consequence, for both HM₆ and HM₈ all sugar units are involved in the interaction with at least one FGF molecule in agreement with a previous NMR study (73). The presence of multiple, overlapping binding sites in the longer oligosaccharide could result in apparent higher FGF binding affinities (57). However, a mathematical formulation of the influence of multivalency on the affinities of the hexameric vs. octamer oligosaccharide (Supplementary Derivation I) shows that the degeneracy effect can neither explain the observed cooperativity nor the difference between FGF1 and FGF2. Rather, we detect strong cooperativity for the second binding event only in the case of FGF2 with HM₈.

In order to gain insights into the molecular basis of the observed cooperativity in FGF2-HM₈ binding on the basis of the reported structures, we analyzed the interactions of FGF1 and FGF2 with heparin oligosaccharides in the published crystal structures (PDB-entries 2AXM, 1E00, 1FQ9, 1BFB, 1BFC). Striking differences between the binding modes of FGF1 and FGF2 to heparin oligosaccharides are observed. Based on the structures of FGF1 in complex with a decasaccharide (22) (PDB entry 2AXM) or FGF-R2 D2D3-decasaccharide (24) (PDB entry 1E00), FGF1 interacts with the HM sulfate groups of the sequence GlcN-IdoA-GlcN (GIG) (Supplementary Fig. 9, left). In contrast to FGF1, the available crystal structures of FGF2 in complex with heparin differ in their sugar interaction pattern: in the FGF2-FGFR1c-decasaccharide structure (1FQ9) FGF2 binds to a IdoA-GlcN-IdoA (IGI) motif whereas in the FGF2-FGFR1c-decasaccharide structure (1FQ9) FGF2 binds to a IdoA-GlcN-IdoA (IGI) motif (Supplementary Table I) we suggest that FGF2 binds to IGI motif for FGF2 in solution as observed in 1FQ9 (23) (Supplementary Fig. 9, right).

Different symmetries are observed for the interactions of FGF1 and FGF2 with HM in the ternary complexes. The conformations of the sugars bound to FGF1 and FGF2 are therefore also different (Supplementary Fig 9). The affinity for the first binding event of HM₈ is similar for FGF1 and FGF2. Since the structure of free HM (PDB entry 1HPN) differs from the conformation of bound HM, the first binding event of both FGF1 and FGF2 must change the conformation of the HM. In structural terms, positive cooperativity is consistent with the reported data that binding of the first FGF induces a kink (74) in the structure of HM which then, together with entropic factors, facilitates binding of a second FGF. However, the affinity of the second binding event for FGF1 is in the same K_D range as the first step. Therefore, there is no indication for a difference in the binding mode for the first and second step for FGF1. In stark contrast, for FGF2 the second step affinity is 17-fold stronger than the first association of FGF2 to HM₈. Thus according to our data, the strong ability of HM₈ to bind and dimerize FGF2 molecules in a cooperative fashion correlates with the higher efficacy of HM₈ relative to HM₆ to promote FGF2 signaling. Recent studies of heparin dendrimers (75) induced FGF-oligomers, synthetically polymerized FGFs (76) and covalently cross-linked FGF-dimers (77) suggest that oligomerization of FGF is required for an agonistic effect of HM on FGFR signaling. Therefore, the oligomerization of FGF in the presence of longer HM such as HM₆ and HM₈ needs to be considered in the formation of FGF-FGFR cell surface signaling assembly.

Based on our findings we propose a refined symmetric FGF-FGFR dimerization model (Fig. 5) which incorporates the differential ability of HM to dimerize FGFs and the presence of a symmetric 2:2:2 ternary complex in solution (Fig. 5A, B). The (FGF2)_2-HM dimer is structurally not part of the symmetric ternary crystal complex, which therefore implies a dissociation of the (FGF2)_2-HM for the assembly of the ternary crystal complex. Due to the cooperativity of the FGF2-HM₆ dimerization, it is unlikely that a FGF2-HM₆ (1:1) monomer interacts with the receptor. This discrepancy is addressed in Fig. 5 by postulating a 2:1:1 intermediate state (Fig. 5B, IVb). This model could explain the transition from the (FGF2)_2-HM dimer to the symmetric 2:2:2 complex. Assuming that FGF2 binds to an IGI motif (Supplementary Fig. 9), the initial 1:1 FGF2-HM₆ complex can form through two alternative ways for HM₆ (Fig. 5A, IIA & b). Binding of a second FGF molecule leads to one unique dimeric species (Fig. 5A, III). This dimeric species cannot directly bind to the receptor because of potential steric clashes between FGF2 and the receptor (Fig. 5A, IVa, Fig. 6A). Therefore, the 1:1:1 FGF2-FGFR-
HM₆ complex (Fig. 5A, IVb) can only be formed when the binding of (FGF2)₂-HM₆ to the receptor and dissociation of the second FGF2 are synchronous or from the FGF2-HM₆ complex with a single FGF2 bound to HM₆ directly. In contrast to HM₆, HM₈ can form three different 1:1 FGF2-HM₈ complexes (Fig. 5B, IIA-c). Presuming the conformation of the oligosaccharide in the symmetric ternary complex, the dimerization step can either lead to an FGF dimer in analogy to the HM₆ complex (Fig. 5B, IIIa), or the FGF molecules bind one to the middle binding site and one to the reducing end site of the saccharide (Fig. 5B, IIIb). The cis-binding mode where both FGF2s are at the outer binding sites can be excluded because it would lead to direct FGF2-FGF2 contacts which are not observed in 2D-NMR experiments. The dimerization step has a significantly higher affinity compared to the first FGF2 association step for HM₈. Since HM₈ is chemically contained within HM₈, it can be assumed that the 2:1 FGF2-HM₈ (Fig. 5B, IIIa) has a similar structure and is equally stable as the corresponding complex with HM₆ (Fig 5A, III). Therefore, the high cooperativity upon HM₈-induced dimerization is likely a result of complex formation involving the additional sugar units of HM₈ at the reducing end (Fig. 5B, IIIb). We speculate that cooperativity is mediated by a different binding mode of the second FGF2 binding to HM₈ leading to a GIG binding motif shown in IIIb to be the favored dimer. The crystal structure of FGF2-FGFR1-decasaccharide (1FQ9) shows a GIG binding motif for the second FGF2 at the reducing end of decasaccharide (23). The fact that eight sugars are necessary for the biological effect further supports that the binding mode of the formation of IIIb is according to a GIG motif. Additionally, the contacts of HM to the second FGF2 are formed by three sulfate groups (GIG) instead of two sulfates and a carboxy group for the IGI motif, which could also explain higher affinity for the second FGF2 binding. The direct binding of the (FGF2)₂-HM₈ (Fig. 5B, IIIa) complex in analogy to the (FGF2)₂-HM₆ (Fig. 5A, III) complex to the receptor would also lead to the same steric clashes as for HM₆ (Fig. 5A, IVa, Fig. 6A). The favored (FGF2)₂-HM₈ complex, however, can form a 2:1:1 FGF2:FGFR:HM₈ complex (Fig. 5B, IVb) as an intermediate state. The ability of the (FGF2)₂-HM dimer to interact directly with FGFR constitutes the key difference between the octameric as compared to the hexameric HM. The formation of a symmetric ternary assembly of stoichiometry 2:2:2 in analogy to PDB entry 1FQ9 would require the dissociation of one FGF2 and subsequent dimerization of two 1:1:1 complexes. Alternatively, we propose that the resulting complex could dimerize directly to a 4:2:2 FGF2:FGFR:HM₈ complex. As depicted in Fig. 6B and 6C, the proposed complex is sterically possible. This mechanism could explain the increased potency of HM₈ vs. HM₆ based on a symmetric complex, because HM induced dimerization is one of its steps and therefore this step can also be rate limiting. On the basis of our model, it remains unclear if the 4:2:2 complex induces signaling directly or if it constitutes an intermediate state.

Our biophysical studies provide a detailed view on the interaction of FGF, FGF receptors and heparin. The striking biological observation that oligosaccharide length and specificity towards FGF2 translates into differences in signaling is based on unique dimerization properties of the FGF2-HM₈ complex due to both multivalent, dynamic interaction of FGF with HM and the positive cooperativity only observed for FGF2-HM₈. Given the fact that there is significant interest in pharmaceutical development of mimetics of HSGAG as potential drugs to modulate FGF signaling, our data should facilitate the rational design of heparin mimetics as agonists of FGF2-FGFR signaling.

REFERENCES


**FOOTNOTES**

The abbreviations used are: DSF, differential scanning fluorimetry; ECD, extracellular domain; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HM, heparin mimetics; HS, heparan sulfate; HSGAGs, heparin sulphate glycosaminglycans; HSPG, heparan sulfate proteoglycan; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; SEC, size exclusion chromatography.

**FIGURE LEGENDS**

Fig. 1. Effect of HM$_6$, HM$_8$ and HM$_{10}$ addition on FGFR4 sensitivity to FGF1/2 in a BaF/3 FGFR4-hMpl proliferation assay. (A) Constitution of the heparin-mimetics decasaccharide HM$_{10}$, octasaccharide HM$_8$ and hexasaccharide HM$_6$. The residue nomenclature used in the text is indicated. The non-reducing end of the oligosaccharides is shown on the left side. IdoA: $\alpha$-L-iduronic acid, GlcN: $\alpha$-D-glucosamine. (B) Increasing doses of FGF1 or FGF2 have been used in absence and in presence of 100 nM HM of different lengths. (C) EC$_{50}$ values of FGF1 or FGF2 with or without HM have been calculated and are given in the table.

Fig. 2. 2D NMR results of the FGFR4 D2-FGF1-HM$_8$ complex mapped onto the X-ray structure of the ternary complex of FGFR1-FGF2-HM$_{10}$ (1FQ9) in two different views. FGFR1 and FGF2 are represented by blue and green surfaces, respectively; one heteromeric half (FGFR1-FGF2) is depicted in light colors, the second one (FGFR1’-FGF2’) in dark colors; HM are displayed as a stick model. Strong amide signal broadening and CSPs larger than 0.03 ppm are colored in red. (A) NMR mapping of $^{15}$N FGFR4 D2 amide signals with strong line broadening observed upon addition of unlabeled FGF1-HM$_8$ complexes. The affected residues are located at the interface between FGF, FGFR and HM$_8$. In addition to the FGF-FGFR interaction sites within the heteromeric half complexes (FGFR-FGF or FGFR’-FGF’), also interactions between the heteromeric half complexes (FGFR-FGF’ – cyan circles; FGFR-FGF’ and FGFR’-FGF’-yellow circles) are detected. Orange circles indicate the receptor-receptor-contacts only seen in the asymmetric X-ray structure (Fig. 3). (B) NMR mapping of $^{15}$N FGF1 amide signals with strong line broadening observed upon addition of unlabeled FGFR4 D2 in presence of HM$_8$. The interaction sites between FGF and HM$_8$ as well as the binding surface to FGFR and FGFR’ are affected. The unique FGF-heparin interaction of the asymmetric X-ray structure is depicted as a dark blue circle.

Fig. 3. Mapping of the CSP results explained in figure caption 2 (same representation and color coding) to the asymmetric complex of FGFR2 D2D3-FGF1-Heparin (1E0O). Surface areas where CSP data confirm the symmetric model (1FQ9, Fig. 2) but do not fit to the depicted complex are marked with circles: Yellow and cyan circles show contacts in 1FQ9 between an FGF and a receptor molecule and between two receptor molecules, respectively. Dark blue circles mark contacts between heparin and FGF and orange circles between the two receptor molecules present in 1E0O that could not be confirmed by CSP data.

Fig. 4. HM-FGF2 interaction (A, B). Anomeric region of the 1D $^1$H NMR spectra of HM$_6$ (A) and HM$_8$ (B) in absence (bottom) and in presence (top) of FGF2. Spectra were recorded with 50 $\mu$M HM and 50
μM FGF2 in 50 mM NaH₂PO₄/ Na₂HPO₄ buffer, pH 6.8, 100 mM NaCl. (C, D) ITC titrations of FGF with heparin mimetics. (C) Sample titration of FGF2 with HM₆. Top panel: raw heating power data; the first peak represents a small pre-injection (5 µl) that is omitted in the integrated data. Bottom panel: data after peak integration and concentration normalization. Curve fit of the data to a single site binding model. (D) Isotherms for binding of HM₆ (open squares) or HM₈ (filled squares) to FGF1, and for binding of HM₆ (open triangles) or HM₈ (filled triangles) to FGF2. Dotted vertical lines indicate the equivalence point of the titrations with HM₆ and HM₈ at a molar ratio (HM : FGF) of 1 and 0.5. The sample cell contained 5 μM FGF, and HM were titrated from a 65 μM stock solution. Measurement conditions: see Table II. (Bottom) (E) HM-induced FGF2 dimerization determined by NMR ¹H T₂-relaxation. Apparent molecular weight of FGF2 upon titration with HM₆ (open triangles) and HM₈ (filled triangles) determined from ¹H T₂ measurements of the bulk imino protons at 30 μM FGF2 concentration. Equilibrium concentrations and apparent molecular masses were fitted assuming the two-step mechanism depicted in (F) as a model. A least square fit to the experimental data was performed by variation of K_D1 and K_D2. The fitted theoretical masses are depicted as solid lines. For HM₆, K_D1=160 nM and K_D2=120 nM were found, for HM₈, K_D1=100 nM and K_D2=5.8 nM. (F) The assumed two-step interaction scheme of FGF2 (grey) with HM (black) with binding constants K_D1 and K_D2.

Fig. 5. FGF-FGFR-HM assembly model. Model for the interaction of HM₆ (A) and HM₈ (B) with FGF2 and FGFR. The oligosaccharides are symbolized by white balls for iduronic acid (IdoA) and black balls for glucosamine (GlcN), the sulfate groups are indicated by small gray balls. The carboxylate group of the iduronic acid is shown as small white ball. The sulfate group at the non-reducing end of HM, which required for interaction with the receptor, is depicted in red. (A) The first association of FGF2 to the HM₆ (I) is possible in two ways for HM₆ (IIa+b). The following dimerization step leads to one unique species (III). Direct binding of this complex to the receptor (IVa) is not possible for sterical reasons. The 1:1:1 FGF2-FGFR-HM complex (IVb) can either be formed by interaction of the FGF2-HM₆ complex IIb with FGFR or in a concerted step of binding of (FGF2)₂-HM₆ to the receptor and simultaneously dissociation of the second FGF. (B) HM₈ (I) can form three different 1:1 FGF:heparin complexes (IIa-c). The dimerization step can lead to two different FGF2 dimer forms (IIIa+b). The favored (FGF2)₂-HM₈ complex (IIb) can then form a 2:1:1 FGF2-FGFR-HM₈ complex (IVb) or dimerize to a 4:2:2 FGF2-FGFR-HM₈ complex. Alternatively, the ternary 1:1:1 complex (V) formed from the interaction of FGFR-FGF2-HM₈ complex (IIb) with FGFR can also result to a signaling competent assembly (2:2:2).

Fig. 6. Modeled structures of FGF2-FGFR-HM complexes based on the symmetric ternary complex (1FQ9). (A, B) FGF2 modeled to the 1:1:1 FGF2-FGFR1 D2D3-HM₆ heteromeric half-complex of the crystal structure. FGF2 is depicted as green cartoons, HM in sticks. FGFR1 D2D3 is symbolized by a blue surface. (A) Modeled structure according to the HM₆ like (FGF2)₂-HM complex (Fig. 5A and B, IVa). FGFR1 D2 and FGF2 overlap sterically. This complex cannot be formed. (B) Modeled structure with the second possible (FGF2)₂-HM₈ binding mode (Fig. 5B, IVb). No sterical clashes indicate that this complex is a possible intermediate of ternary complex formation. (C) Modeled ternary complex of a 4:2:2 FGF-FGFR-HM stoichiometry according to the heteromeric half-complexes depicted in (B). This proposed complex is sterically possible as an intermediate assembly state for FGF induced FGFR signaling.

TABLES
Table I. Thermodynamic parameters for the interactions measured by ITC. Binding parameters were obtained from a fit of the calorimetric data to a single site binding model. Where applicable, the standard error from 2-3 measurements is indicated. All titrations were performed in 50 mM NaH₂PO₄/ Na₂HPO₄, 100 mM NaCl, 25 mM arginine, pH 6.8, T = 25 °C. Protein concentrations in the sample cell, 5-10 µM; ligand concentrations in the syringe, 50-100 µM; final ratios of ligand over sample protein was 2-3.

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FGFR4 D2 - HM interaction

| FGFR4 D2      | HM<sub>6</sub>    | 0.9 ± 0.2    | 2400 ± 700      | -1.2 ± 0.3   | 6.5 ± 0.1      |
| FGFR4 D2      | HM<sub>8</sub>    | 0.7 ± 0.1    | 3400 ± 1000     | -9.3 ± 3.5   | -1.9 ± 3.7     |

FGF - FGFR4 D2 - HM interaction

| FGF1 + HM<sub>6</sub> | FGFR4 D2      | 1.2           | 893             | -1.5         | 6.8            |
| FGF2 + HM<sub>8</sub> | FGFR4 D2      | 1.2           | 1100            | -1.1         | 7              |

<sup>a</sup>Apparent stoichiometry (ligand: sample protein) of the complex

Table II. Overview of interaction sites visible in the symmetric ternary complex (1FQ9) identified by 2D NMR spectra upon addition of FGFR4 D2 to the complex of FGF with HM<sub>6</sub>/HM<sub>8</sub>. The last column gives the apparent molecular weight of the complex of FGF1/FGF2 with receptor FGFR4 D2 together with HM<sub>6</sub> or HM<sub>8</sub>. These values are determined from bulk amide 1H T<sub>2</sub> times from 1H 1D T<sub>2</sub> data<sup>a</sup> and 1H T<sub>2</sub> times obtained from line width fits to isolated resonances in 2D-[1H,15N]-correlation spectra<sup>b</sup> as described in the Supplementary data. The first number gives the value measured for 15N FGF in the presence of unlabeled FGFR4 D2 and HM and the second for 15N FGFR4 D2 in the presence of unlabeled FGF and HM. The FGF-FGFR-HM ratio was 1:1:1. Estimated error is ±5kDa. (+ indicates line broadening/CSPs detected)

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<td>FGF1 + HM8</td>
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<td>4.6-15.3</td>
</tr>
<tr>
<td>FGF1 + HM10</td>
<td>11.0</td>
<td>7.4-16.3</td>
</tr>
<tr>
<td>FGF2: w/o HM</td>
<td>11.8</td>
<td>/</td>
</tr>
<tr>
<td>FGF2 + HM6</td>
<td>11.2</td>
<td>9-13.8</td>
</tr>
<tr>
<td>FGF2 + HM8</td>
<td>1.6</td>
<td>0.9-3</td>
</tr>
<tr>
<td>FGF2 + HM10</td>
<td>0.68</td>
<td>0.47-0.91</td>
</tr>
</tbody>
</table>
Figure 2:
Figure 4:

A

B

C

D

E

F
Influence of heparin mimetics on the assembly of the FGF - FGFR4 signaling complex

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