The Activation of Fibroblast Growth Factors (FGFs) by Glycosaminoglycans: Influence of the Sulfation Pattern on the Biological Activity of FGF-1

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Six synthetic heparin-like oligosaccharides have been used to investigate the effect of the oligosaccharide sulfation pattern on the stimulation of acidic fibroblast growth factor (FGF-1) induced mitogenesis signaling and the biological significance of FGF-1 trans *dimerization in the FGF-1 activation process. It has been found that some molecules with a sulfation pattern that does not contain the internal trisaccharide motif, which has been proposed for high affinity for FGF-1, stimulate FGF-1 more efficiently than those with the structure of the regular region of heparin. In contrast to regular region oligosaccharides, in which the sulfate groups are distributed on both sides of their helical three-dimensional structures, the molecules containing this particular sulfation pattern display the sulfate groups only on one side of the helix. These results and the fact that these oligosaccharides do not promote FGF-1 dimerization according to sedimentation-equilibrium analysis, confirm the importance of negative-charge distribution in the activation process and strongly suggest that FGF dimerization is not a general and absolute requirement for biological activity.*

Introduction

The fibroblast growth factors (FGFs) constitute a family of more than twenty signaling polypeptides that are involved in a variety of biological processes including cell proliferation, differentiation, and angiogenesis.[1] FGF-1 (acidic FGF) and FGFB2 (basic FGF) are the prototypical members of this family. FGF biological functions are triggered by binding of the polypeptide to specific transmembrane tyrosine kinase receptors (FGFRs) at the cell surface.[2] Both FGFs and FGFRs belong to the class of heparin binding proteins.[3, 4] In vitro assays have shown that FGF-2-induced mitogenesis is highly dependent on cell-surface heparan sulfate glycosaminoglycans (HS-GAGs), which can be replaced by soluble heparin when the cells are stripped of their GAG coating.[5–7] Soluble heparin is also a near absolute requirement for FGF-1-driven mitogenesis. However, in this case, heparin can be replaced by some nonphysiological compounds of relatively low molecular mass.[8, 9] The minimal structural requirements for GAGs to activate the different members of the FGF family have not been unequivocally established. On the other hand, it has been proposed that FGF dimerization is a key process in the formation of the FGF:FGFR signaling complex mediated by GAGs either free or bound to the cell surface[5, 10] There is convincing evidence that, in the presence of GAGs, FGF-1 and FGF-2 assembly occurs with different degrees of oligomerization.[11, 12] Moreover, crystal structure data of binary GAG – FGF-1[13] and GAG – FGF-2[14] complexes and of ternary FGF-1 – GAG – FGFR[15] and FGF-2 – GAG – FGFR[16] complexes reveal different assemblages of FGF molecules, which may take place in *cis* or *trans* about the GAG chain. These and other biochemical and biophysical results[17–19] make it difficult to ascertain the importance of FGF oligomerization in signaling.

The understanding of FGF activation in structural terms is seriously hindered by the heterogeneity of the HS-GAG component. HS-GAGs are a family of closely related linear polysaccharides consisting of alternating units of D-glucosamine and an uronic acid, D-glucuronic, or L-iduronic. The complex biosynthesis of these GAGs results in sequences of unsulfated and variously sulfated units distributed in different domains along the polysaccharide chain.[20] These chains present well-defined

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three-dimensional helical structures in terms of overall conformation.[21] The sequence, the charge distribution, and the conformational flexibility of the internal L-iduronate residues in these chains are believed to play a key role in the wide range of specific interactions exhibited by these molecules[22, 23] It is widely considered that these interactions are primarily electrostatic in nature with variable contributions of nonionic, hydrogen bonding, and hydrophobic interactions[4] A recent report has established that optimal van der Waals contact is also important in influencing the specificity.[24] The selectivity of the process seems to essentially depend on the spatially defined sulfate and carboxylate patterns, the shape, and the internal flexibility of the polysaccharide chains[21–23] Therefore, it should be expected that specifically designed synthetic HS-GAG-type oligosaccharides, with a defined three-dimensional orientation of sulfate and carboxylate groups, may provide valuable information on the mechanism of FGFR activation.

In this context, we now report new data on the influence of the sulfation pattern along the GAG oligosaccharide chain on the biological activity of the FGF system using synthetic oligosaccharide sequences. We also have addressed the problem of FGF dimerization using these synthetic molecules. The results provide additional conclusive evidence of the importance of the distribution of electrostatic potential along the oligosaccharide backbone and strongly suggest that GAG-induced FGF-1 dimerization is not an absolute requirement for biological activity.

Results and Discussion

We have previously reported the chemical synthesis of HS-GAG oligosaccharides containing the monosaccharide sequence of the major region of heparin with different size and sulfation pattern.[25–27] We have now investigated the influence of the different distribution of electronegative potential arising from these different sulfation patterns on the capacity of these oligosaccharides to regulate FGF-1-induced mitogenesis. Also, using these synthetic molecules, we have addressed the question of the significance of FGF-1 dimerization in the biological process.

Four different types of previously synthesized[25–27] HS-GAG-like oligosaccharides have been used in this investigation (Figure 1). Hexasaccharide **1** and octasaccharide **2** contain the structural motif of the major region of heparin.[20] Hexasaccharides **3**, **5** and **6** and octasaccharide **4** contain the same sequence of monosaccharide units with different charge distribution. The D-glucosamine $\alpha 1 \rightarrow 4$ L-iduronate repeating unit in the major heparin sequence (GlcN – IdoA) was maintained in the four series in order to conserve the main structural features of the glycan chain in all compounds studied. Only the size and the sulfation pattern have been varied in the different oligosaccharide constructs for comparison purposes. The size of these molecules was selected as the minimal chain length to be expected to stimulate FGF-1-induced mitogenic activity that could be obtained with a reasonable synthetic effort. It has been reported that octasaccharides and longer GAG fragments bind and activate FGFs, while controversial results have been published on the stimulatory activity of hexasaccharides.[2, 28] The sulfation pattern was designed in an attempt to obtain different distributions of electrostatic potential, assuming that **1** – **6** would adopt a helix-like solution conformation as found for HS-GAGs.[21] This was confirmed by NMR spectroscopy and molecular dynamics simulations.[25–27] Compounds **1** and **2** display the negatively charged groups regularly distributed on both sides of the helix. The sulfate groups in compounds **3** and **4** are oriented on only one side of the helical structure. The negative charge on the other side of the helix in **3** and **4** is greatly decreased with regard to **1** and **2**, since only the carboxylate groups on the A and E iduronate units in **3** and **4** would adopt this orientation. We reasoned that the ability of **3** and **4** to interact with FGF-1 to form a *trans* dimer similar to that observed by X-ray crystallography[13] would be greatly decreased as compared with **1** and **2**. The total negative charge in hexasaccharides **5** and **6** is similar to that in **3**, but it appears differently distributed on both sides of the helix. On the other hand, compounds **1** and **2** contain internal IdoUA(2-OSO_3) – $GlcNSO_3$(6-OSO_3) – IdoUA(2-OSO_3) trisaccharide motifs, which have been reported for GAG fragments with high affinity for FGF-1,[29] while this motif is lacking in **3**, **4**, **5** and **6**. Furthermore, compounds **1** – **4** contain the 6-OSO_3 and the 2-OSO_3 groups reported to be necessary for interaction with FGFRs to induce mitogenicity,[30] while the 6-OSO_3 groups are lacking in compound **5**, and the 2-OSO_3 groups are lacking in compound **6**.

We have analyzed the influence of these sulfation patterns on the electrostatic properties of hexasaccharides **1**, **3**, **5**, and **6** by mapping their electrostatic potential over their solvent accessible surfaces. Preliminary data indicated that the calculated electrostatic potential was strongly dependent on the values of the torsional angles that control the orientation of the negatively charged side groups. This strong dependence could obscure the results to the point of making a reasonable interpretation impossible. In order to overcome this difficulty, a careful selection of the structures to be used in the calculations had to be made. This was attempted by using several approaches starting from data either for the free oligosaccharides or for the FGF-bound counterparts, as reported in published X-ray studies. A first set of structures was constructed from those reported for natural heparin (PDB ID:1 hpn),[31] manually modifying the position of the sulfate groups for structures **3**, **5** and **6**. This set of structures afforded interpretable results but presented significant drawbacks. Having been constructed from a virtual structure, this set of structures, although it may represent a dynamic ensemble, did not necessarily constitute an energetic minimum. A second set of structures was therefore created from our previous molecular dynamics data in explicit water for **1**, **3**, **5**, and **6**.[25, 32] An ensemble of representative structures was created for each hexasaccharide by relaxing structures resulting from the average along several molecular dynamics simulation intervals. The energetically most favorable structure for each molecule was used to generate the electrostatic surface. However, this second set of structures was extremely sensitive to the orientation of the sulfate groups, as the side chain torsional angles showed a great dispersion along the representative structures and among the sulfation patterns. Then we

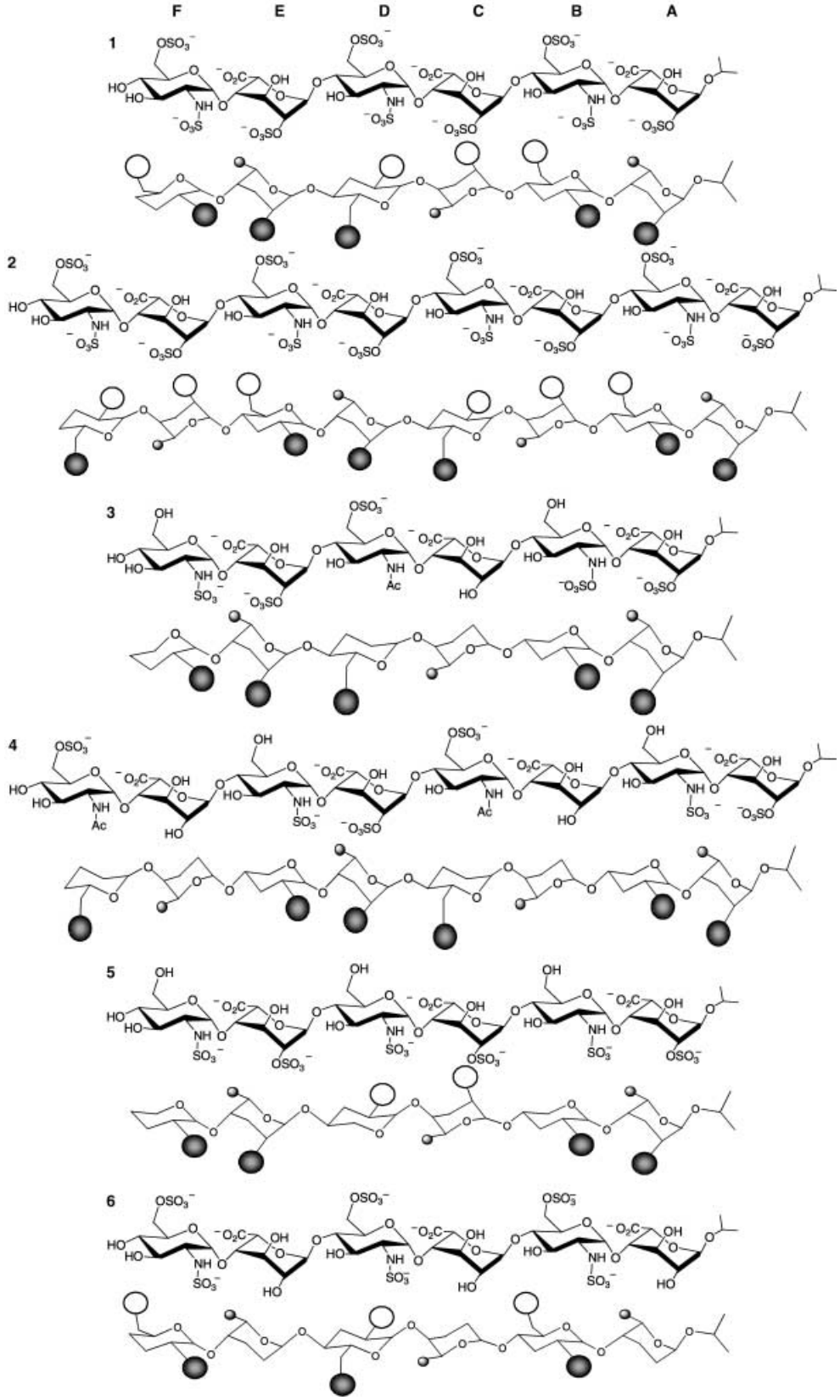

Figure 1. *Glycosaminoglycan-like synthetic oligosaccharides* ***1*** *–* ***6****. Formulae and schematic representation, small and large circles indicate carboxylate and sulfate groups, respectively, and filled and open circles indicate substitution on opposite sides.*

turned to the structure of the oligosaccharides in FGF – GAG fragment complexes as a criterion for structure selection. A heparin oligosaccharide fragment in a complex with FGF-1 (PDB ID:1 amx)[13] was chosen. This approach might be more realistic than the above as it considered the structure in the bound state as the prototype for selection. Only the $^1C_4$ conformation of the L-iduronate units was taken into account, as this is the only L-iduronate ring form that appears in the crystal structure of the complex. Structures that were incompatible with the helical geometry shown by the oligosaccharide fragment in the complex[13] and with those determined for **1** – **6** by NMR and modeling data[25–27, 32] were discarded. The positions of the center of the sulfate groups in the structures with a helical geometry were compared with those in the crystal, and the structure that was closer with regard to rmsd value was finally selected.

The results are summarized in Figure 2. It has been assumed that the region that primarily interacts with FGF-1 involves a cluster of sulfate groups contained in GlcN – IdoA – GlcN trisaccharide motifs. This is a widely accepted feature that has been commonly used in most of the interaction models based on X-ray crystallographic data.[33] These interacting regions are contained in trisaccharide substructures D, E, F and B, C, D, but substructures D, E, F conserve most of the sulfate groups in the different sulfation patterns of **1**, **3**, **5**, and **6**. Substructures D, E, F have therefore been taken as the interacting region. For the four different molecules, the electrostatic potential was quite similar on these faces closer to the protein (Figure 2 column B). However, the potentials on the opposite faces show clear differences between the structures with symmetrically (**1**, **5** and **6**) and asymmetrically (**3**) distributed charge (Figure 2, column C). On these opposite faces, compounds **5** and **6**, with a

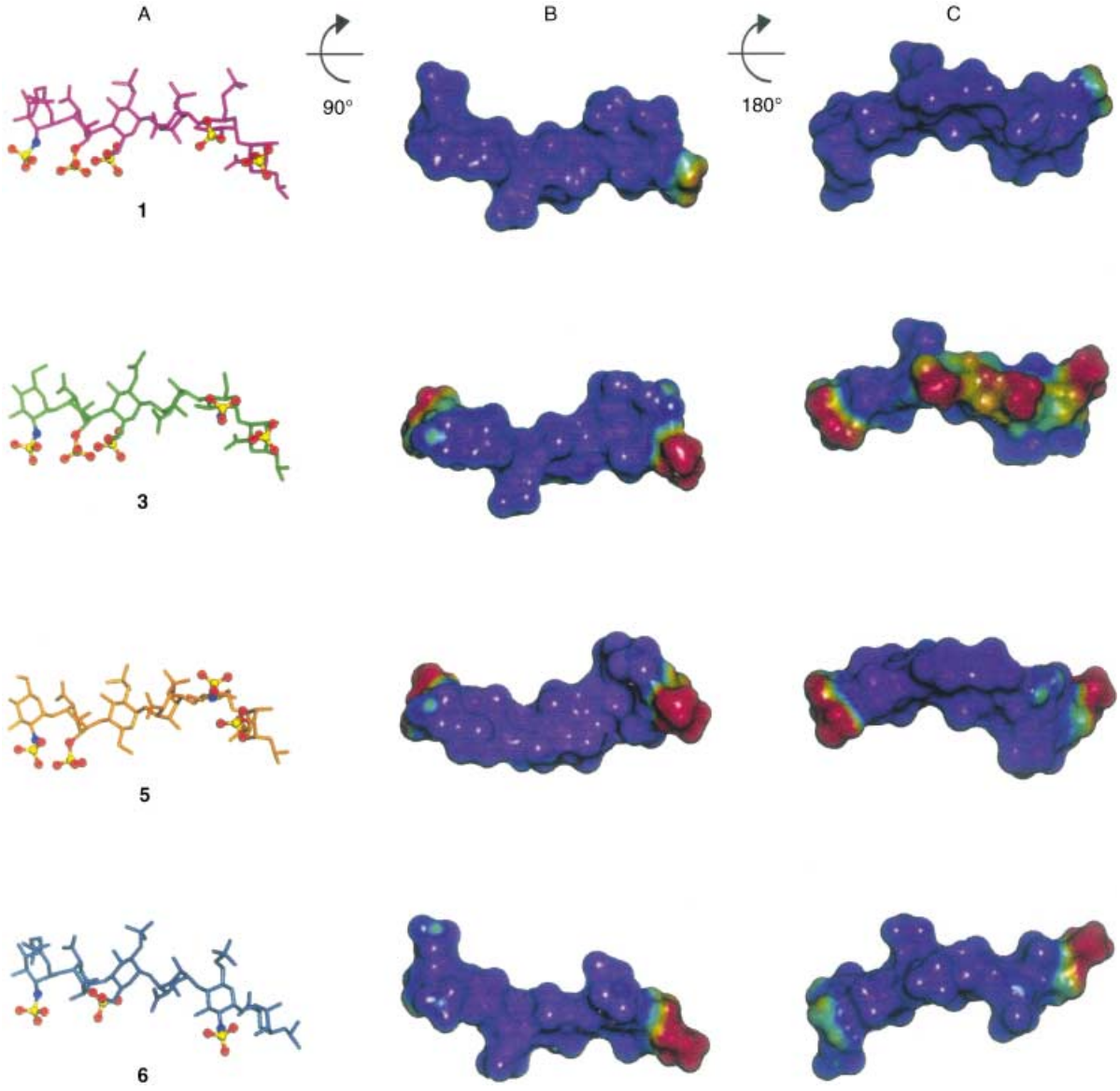

Figure 2. *Representative structures for the hexasaccharides **1**, **3**, **5**, and **6** compatible with the FGF1-heparin complex (column A); sulfate groups supposed to be in contact with the protein are displayed as balls and sticks. Columns B and C: Connoly surfaces mapping the electrostatic potential for synthetic hexasaccharides. The color scale is relative: blue and red represent the more and less negative potential regions, respectively.*

global charge closer to **3**, present a distribution of electrostatic potential similar to that of **1**.

We have investigated the induction of the mitogenic activity of FGF-1 by these synthetic molecules and the results are summarized in Figures 3 and 4. We have previously reported some results for compounds **1** and **2**[25] As shown in Figure 3, the

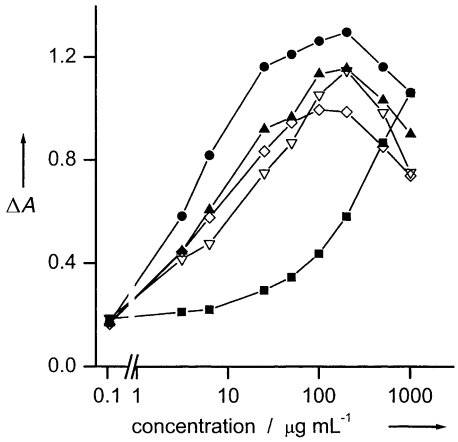

Figure 3. *Effect of increasing concentrations of heparin (●), hexasaccharides **1** (■) and **3** (▲), and octasaccharides **2** (◇) and **4** (▽) on the mitogenic activity of FGF1.*

maximum activating activity of octasaccharide **2** was reached at a concentration around 100 μg mL^{-1}, at which the maximum activating effect of heparin was also observed. The half-maximum activating concentration of **2** and heparin were also equivalent (6 – 10 μg mL^{-1}), and only the maximal activation level of **2** was somehow lower than that of heparin. For hexasaccharide **1** much higher concentrations were needed to reach a maximal activation level equivalent to those of **2** and heparin. Figure 3 also shows the induction of the mitogenic activity of FGF-1 by increasing concentrations of hexasaccharide **3** and octasaccharide **4** in comparison with the results obtained for **1** and **2**. The activating effect of hexasaccharide **3** and octasaccharide **4** reached a maximum at approximately the same concentration as heparin and octasaccharide **2**, and the half-maximum activating concentration of **3**, **4**, **2**, and heparin were equivalent. The maximal activation level for **3** and **4** was similar to that of heparin and somehow higher that in the case of octasaccharide **2**. Figure 4 summarizes the results obtained for the four hexasaccharides **1**, **3**, **5**, and **6**, here **5** and **6** did not show appreciable activating effect.

These results show that the size of the GAG chain, which has a dramatic influence at the hexasaccharide and octasaccharide level when the sulfation pattern corresponds to that of the regular region of heparin (compounds **1** and **2**),[25] does not have any remarkable effect when the sulfate groups are distributed as in **3** and **4**. These oligosaccharides activate FGF-1 almost as effectively as low-molecular-weight heparin. The lower total negative charge in **3** as compared with **1**, differently distributed and oriented mainly on one side of the helical structure, results in completely different behavior. The importance of the sulfation pattern is further demonstrated by comparison with the results

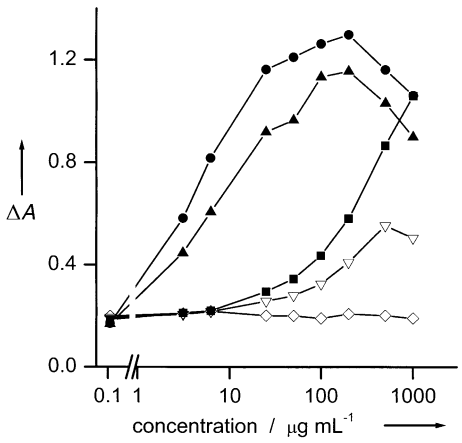

Figure 4. *Effect of increasing concentrations of heparin (●), hexasaccharides **1** (■), **3** (▲), **5** (◇), and **6** (▽) on the mitogenic activity of FGF1.*

obtained for **5** and **6**. Compounds **5** and **6** have the same size as **1** and **3**, a symmetric charge distribution regularly displayed on both sides of the helical structure, as in **1**, and a global negative charge that is lower than **1** and quite close to **3**. The remarkable differences observed for **1**, **3**, **5**, and **6** clearly show that subtle changes in the sulfation pattern may result in dramatic changes in biological activity. As has already been mentioned, none of **3**, **4**, **5**, or **6** contains the sulfation pattern that has been proposed for high affinity for FGF-1, which is only present in **1** and **2**.

These results constitute an experimental confirmation of the influence of the size and the sulfation pattern of the HS-oligosaccharide on the stimulation of FGF-1-induced mitogenesis signaling. The sulfation pattern determines the distribution of electronegative potential along the oligosaccharide chain. It could be speculated that, if the productive GAG – FGF interaction is primarily electrostatic in nature, the behavior of hexasaccharide **3** and the remarkable difference from that of **1**, **5**, and **6** indicate that a precise arrangement of the negative charges in this oligosaccharide structure may be needed to induce FGF-1 activity. This precise arrangement, which may be present in natural GAGs, would display an asymmetric distribution of electronegative potential on both faces of the oligosaccharide helical structure, as in hexasaccharide **3** and octasaccharide **4**. It could well be, however, that in the molecular recognition events between naturally occurring GAGs and FGFs, interactions other than electrostatic play a key role.[24] Further investigation in this regard is presently in progress.

Molecules (**3** and **4**) with a distribution of electronegative potential mostly on one face of the helix and a relatively small chain length as compared to naturally occurring GAGs do not seem well suited to effectively participate in the formation of either *trans* or *cis* FGF dimers. Effectively, equilibrium sedimentation analysis of FGF-1 preparations in the presence of activating concentrations of **3**, **4**, and heparin (Figure 5) shows that, except in the last case, FGF-1 sedimentation profiles correspond to that expected for a FGF-1 monomer. As shown in the same figure, the profile adopted the characteristics of a FGF-1 dimer in the case of heparin. Therefore, it seems obvious that, in the assayed experimental conditions (concentrations at which

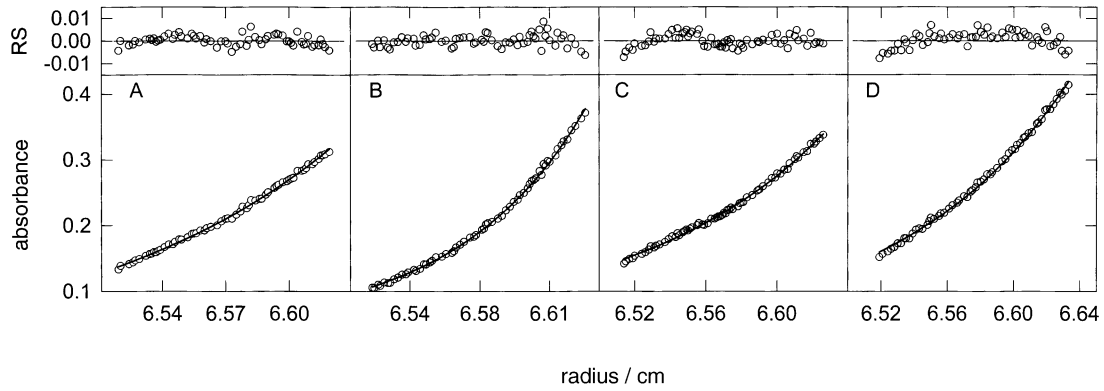

Figure 5. *Effect of heparin, hexasaccharide **3** and octasaccharide **4** on the sedimentation equilibrium of FGF-1. A) FGF1 without activators; B) FGF-1 in the presence of 100 μg mL^{-1} of heparin ; C) FGF-1 in the presence of 100 μg mL^{-1} hexasaccharide **3** ; D) FGF-1 in the presence of 100 μg mL^{-1} of octasaccharide **4**. Absorbance was normalized to the minimum and maximum values of the plotted gradient.*

the tested compounds elicit a nearly full FGF-1 mitogenic activity), only natural heparin fragments posses the structural requirements to cause FGF-1 to dimerize. In addition, results summarized in Figures 3, 4, and 5, strongly suggest that FGF-1 dimerization previous to FGF-1 – FGFR binding is not an absolute requirement for full FGF-1 mitogenic activity.

In conclusion, by using synthetic oligosaccharides with different size and sulfation patterns (compounds **1** – **6**), the elucidation of the molecular basis of the FGF activation process has been approached, avoiding the problems that arise from the inherent heterogeneity of the natural GAG fragments currently used in these studies. It has been confirmed that the previous finding that octasaccharide is the minimum saccharide size to stimulate FGF-1-induced mitogenesis signaling holds true for oligosaccharides with the structure of the major sequence of the regular region of heparin (compounds **1** and **2**). However, it has been found that for molecules with a specific arrangement of sulfate groups (**3** and **4**) a hexasaccharide (**3**) can activate FGF-1 as effectively as the regular region octasaccharide **2**. This specific sequence, which involves alternate *N*-sulfo-D-glucosamine, L-iduronate-2-sulfate, *N*-acetyl-D-glucosamine-6-sulfate, and L-iduronate units, does not contain the internal trisaccharide motif that has been proposed for high affinity for FGF-1. It was designed and synthesized in the search for structures with a hindered capacity to participate in the formation of FGF-1 *trans* dimers. Sedimentation-equilibrium analysis with FGF-1 and these synthetic molecules strongly suggest that FGF-1 dimerization is not an absolute requirement for biological activity.

Experimental Section

Molecular modeling: The solvent-accessible surfaces of compounds **1**, **3**, **5**, and **6** were calculated with Sybyl[34] by using a probe radius of 1.4 Å. The coulombic electrostatic potential at the Connolly surface was calculated by using the atomic partial charges taken from PIM carbohydrate-specific parameters for TRIPOS force field at http://www.cermav.cnrs.fr/databank/pim/index.html.[35, 36] The structures used in the calculations of the electrostatic potential were obtained as follows. The first set of structures was constructed with the reported heparin structure (PDB ID:1 hpn)[31, 37] by manually modifying the sulfation pattern. The second and the third sets were taken from the ensemble of structures constructed from 2.0 ns molecular-dynamics simulations in explicit solvent (TIP3P water) by using periodic boundary conditions and the Particle Mesh Ewald (PME) methodology for calculation of long-range electrostatic interactions.[32] The positions of the atoms were averaged every 100 ps (the first 100 ps were discarded) and the structures obtained were relaxed by using GLYCAM parameters and 1000 cycles of conjugate gradient minimization. Further processing of these structures was made with Sybyl[34] data base functionality. For each compound, the most stable structure among the ensemble was used to construct the second set of structures. The third set was also taken from the same ensemble by using the sulfate root mean square deviation with the crystallographic complex (PDB ID:1 amx)[12, 37] criteria.

Biological assays: Heparin-sepharose was obtained from Pharmacia, nitrocellulose filters were from Millipore, culture plates were from Costar, ITS + culture supplement came from Collaborative Research Inc., Na-heparin (average molecular weight 3 kDa) was obtained from Sigma, L-glutamine, Ham = s F-12 medium, and Dulbecco = s modified Eagle = s medium (DMEM) were from Flow. Distilled water filtered through a Milli-Q (Millipore) water purified fitted with an Organex column (Millipore) was used in all solutions.

Mitogenic activity was measured as previously described.[38] Cells were counted by measuring the total amount of crystal violet retained by cell nuclei by differential absorption (620 – 690 nm).[38, 39] For assaying the activation of FGF-1 by heparin and the synthetic oligosaccharides **1** – **6**, a mitogenic unit of the protein (320 pg mL^{-1}) was added to each well of the assay plate.[8] A 139 residue form of FGF-1 was used.[9] The protein was synthesized and purified as previously reported with expression vector pMG47.[39]

Short column (70 µL) sedimentation equilibrium experiments were performed at 293 K and at 25 000 rpm in a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics, by using an An-60Ti rotor and standard (12 mm optical path) six-channel centerpieces of Epon charcoal. Absorbance scans were carried out at 236 nm until equilibrium was reached. High-speed sedimentation was afterwards carried out for baseline determination. Analyzed solutions were always FGF-1 (6 µM) in phosphate solution (10 mM, pH 7.2) with NaCl (80 mM). Whole-cell weight-average bouyant molar masses (bM_w) were obtained by fitting the experimental data to the equation for radial concentration distribution of an ideal solute at sedimentation equilibrium with the program EQASSOC (BA). The

bouyant mass values allowed a first-hand analysis of the sedimentation data of the different mixtures to be performed. Analyses to determine whole-cell apparent weight-average molecular weights ($\tilde{M}_{w,a}$) are not straightforward in our case because the proteins and ligand have different partial specific volumes: vbars for a FGF (0.735 $cm^3 g^{-1}$)[39] and heparin (0.51 $cm^3 g^{-1}$).[40] Analysis of the sedimentation equilibrium data of the macromolecular mixtures was done by assuming the linear approximation for the bouyant masses (BK): $bM_{w,ij} = ibM_{w,A} + jbM_{w,B}$, here ij refers to the complex A_iB_j and $bM_{w,A}$ and $bM_{w,B}$ are the bouyant masses of pure A and pure B, respectively.

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