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Minimal Heparin/Heparan Sulfate Sequences for Binding to Fibroblast Growth Factor-1

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The glycosaminoglycans heparin and heparan sulfate (HS) bind to fibroblast growth factor FGF1 and promote its dimerization, a proposed prerequisite for binding to a cellular receptor and triggering mitogenic signals. The problem of minimal structural requirements for heparin/HS sequences to bind FGF1 was approached by surface plasmon resonance (SPR), NMR spectroscopy, and MALDI mass spectrometry studies using the three synthetic tetrasaccharides $GlcNSO_36OR$-$IdoA2SO_3$-$GlcNSO_36OR'$-$IdoA2SO_3OPr$ (AA, R = R′ = SO_3; BA, R = H, R′ = SO_3; BB, R = R′ = H; Pr, propyl). AA and BA significantly interact with the protein, whereas BB is practically inactive. The NMR spectra show that, whereas the interaction of AA primarily involves the $GlcNSO_36SO_3IdoA2SO_3$ disaccharide moiety at its nonreducing end, residues at both the nonreducing (NR) and reducing side (R) appear to be involved in the weaker complex of BA. Furthermore, MALDI experiments show that, in addition to 1:1 protein:tetrasaccharide complexes, AA and BA are able to form 2:1 complexes, indicating that heparin/HS-induced dimerization of FGF1 requires only one 6-OSO_3 group per tetrasaccharide.

Key Words: **heparin/heparan sulfate; FGF1; tetrasaccharides; binding; SPR; MALDI; NMR.**

The mammalian fibroblast growth factors (FGFs) are structurally related polypeptides involved in a number of biological functions such as cell proliferation, differentiation, and angiogenesis. The most extensively studied members of the FGF family are FGF1 and FGF2, formerly designated as "acidic" and "basic" FGF, respectively (1–3). The glycosaminoglycan heparan sulfate (HS), as part of a proteoglycan, plays a key role as mediator of FGF activity, by facilitating and stabilizing the formation of properly oriented FGF oligomers, an essential step to promote the oligomerization and activation of tyrosine kinase cellular receptors (FGFRs) (4). Exogenous heparin displaces FGFs from their ternary complexes with HS and FGFRs (5). Both HS and heparin are glycosaminoglycans constituted by alternating disaccharide sequences of a uronic acid (β-D-glucuronic acid, GlcA, or α-L-iduronic acid, IdoA) and α-D-glucosamine (GlcN) residues. The uronic acid residues (especially IdoA) can be 2-O-sulfated, and GlcN can be either N-acetylated or N-sulfated, and variously O-sulfated. The structures of HS and heparin differ from each other in the relative proportion and arrangement of individual residues. Whereas heparin chains are largely made up of -$IdoA2SO_3$-$GlcNSO_36SO_3$-sequences, HS is much more heterogeneous, and contains significant proportions of GlcA and N-acetylated GlcN residues. In both glycosaminoglycans, the low-sulfated and the high sulfated sequences are usually arranged in blocks, and regions containing $IdoA2SO_3$ and $GlcNSO_3$ residues have been recognized as those containing the binding sites for FGFs (6).

Considerable efforts have been made to elucidate the growth factor activation processes at the molecular level and to characterize the minimum heparin binding sequence for FGF1 and FGF2 necessary to promote assembly of active structures (7–13). It has been shown that even short oligomers (tri- and pentasaccharides) are able to bind FGF2 (8, 14–16). However, longer oligosaccharides are usually necessary for promoting dimerization and activation of FGFs (7, 12). It has also been shown that, whereas 6-O-sulfate groups are needed both for binding and activation of FGF1, these groups are unnecessary for binding to FGF2 (15, 17), but are essential for FGF2-mediated signaling (7, 12, 18). The heparin-derived tetrasaccharide **1** (Fig. 1) is the shortest oligosaccharide yet described to bind FGF1 with significant affinity (16). **1** also binds to FGF2, and the X-ray structure of its 1:1 complex with

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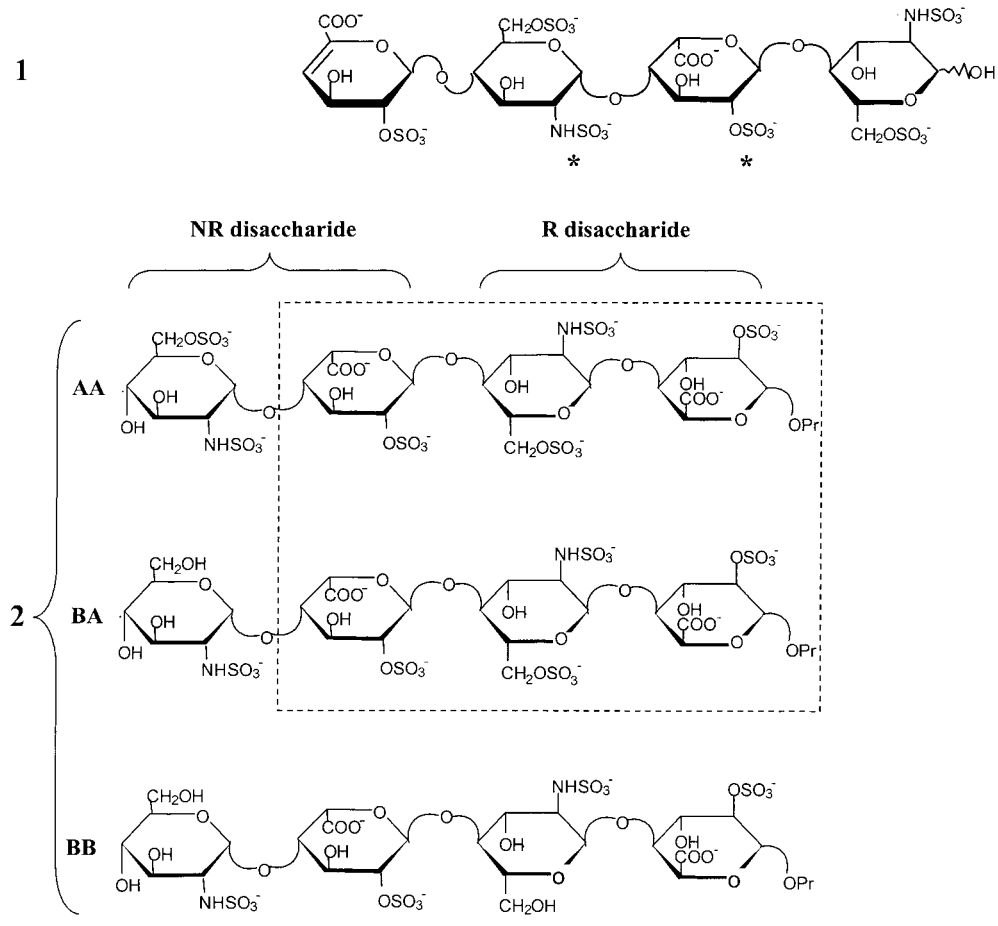

FIG. 1. Structure of a FGF1-binding heparin-derived tetrasaccharide (**1,** Ref. 16) and of three synthetic tetrasaccharides (**2**); NR and R are the nonreducing and reducing disaccharide moieties, respectively. The dashed frame shows minimal FGF1-binding motifs (18) in **AA** and **BA.**

the growth factor clearly shows that complexation primarily involves the sulfate groups labeled with asterisks in Fig. 1, i.e., one glucosamine NSO_3 group and one 2-OSO_3 group of the iduronate residue (14, 19). No X-ray structure is yet available for corresponding 1:1 complexes of FGF1. However, in a crystalline "dimeric" complex where one heparin decasaccharide chain is sandwiched between two FGF1 molecules, two different sets of contacts are apparent for the tetrasaccharide core of the decasaccharide (20).

In the present work, assessing the minimal heparin/HS sequence binding to FGF1 was approached using synthetic tetrasaccharides **2** (**AA, BA,** and **BB**) (Fig. 1). **AA** was obtained by coupling two disaccharide blocks **A** ($GlcNSO_36SO_3$-$IdoA2SO_3$), **BA** by coupling the 6-O-nonsulfated disaccharide **B** ($GlcNSO_3$-$IdoA2SO_3$) with **A,** and **BB** by coupling two **B** disaccharides (21, 22). It should be noted that two tetrasaccharides (**AA** and **BA**) contain the $IdoA2SO_3$-$GlcNSO_36SO_3$-$IdoA2SO_3$ trisaccharide sequence recently identified as a common motif of FGF1-binding HS octasaccharide fragments (18). Differing from each other in sulfation patterns at C-6 of $GlcNSO_3$ residues, the three tetrasaccharides permit evaluation of the contribution to FGF1 binding of each of two component disaccharide moieties (i.e., the nonreducing [NR] and the "reducing" [R] sides), as well as contribution of the 6-O-sulfate groups. Interaction with the protein was studied using surface plasmon resonance (SPR) to determine the relative strength of complexes, ^1H NMR spectroscopy to identify the sulfate groups involved in binding, and MALDI mass spectrometry to assess formation of both 1:1 and 2:1 protein oligosaccharide complexes.

MATERIALS AND METHODS

Materials. Recombinant human FGF-1 was provided by Professor G. Giménez-Galliego, Madrid, through the courtesy of Professor M. Martin-Lomas, Seville. Peptide $(Arg\text{-}Gly)_{19}Arg$ analysis was a gift from Drs. R. Sasisekharan and G. Venkataraman, MIT (Cambridge, MA). Sinapinic and caffeic acid were purchased from Aldrich (St. Louis, MO). Heparin was a pig intestinal preparation from LDO (Trino Vercellese, Italy). Low-molecular-weight heparin (LMWH) was a preparation from Sandoz, Nürnberg, Germany (provided by Professor J. Fareed (Loyola Univ., Maywood, U.S.A.). Tetrasaccharides **AA, BA,** and **BB** were synthesized as previously described (21).

Surface plasmon resonance (SPR). Measurements were performed with a Biacore 1000 surface plasmon resonance-based biosensor (Pharmacia Biosensor AB) and a CM5 sensor chip, at 25°C. The LMWH was immobilized on the sensor surface through biotinylation of the aldehyde group of its terminal 2,5-anhydro-D-mannose residue. LMWH (10 mg/ml) was incubated with a 6-fold molar excess of biotinamidocaproylhydrazide (BACH) in 1 mM acetate buffer, 0.15 M NaCl, pH 5.5, for 24 h at 20°C. Excess of biotin was removed by ultrafiltration on 500D Diaflo membranes (Amicon, U.S.A.). Activation of the sensor chip and immobilization of streptavidin were performed as described previously (16). Injection of biotinylated LMWH (1 mg/ml in 10 mM Hepes, pH 7.4, 0.3 M NaCl, 3.4 mM EDTA, 0.05% Tween 20) onto the biosensor surface, at a flow rate of 5 μl/min, produced 170 RU. Non specifically bound heparin was eliminated by three 20 μl injections of 3M KSCN. For binding experiments, stock FGF1 solutions (1 mg/ml) were diluted in running buffer (PBS, pH 7.2, 5 mM Na_2SO_4, 1 mM EDTA, 0.05% Tween 20) containing 1 mg/ml carboxymethyldextran, and 1 mM β-mercaptoethanol at a concentration of 500 nM, were injected onto the heparin surface. Different amounts of tetrasaccharides (1–800 nM) were mixed with FGF1 prior to the injection. The injected volume was 30 μl at a constant eluent flow rate of 100 μl/min. This relatively high flow rate was chosen to minimize binding rate limitations due to mass transport (23). The heparinized biosensor surface was regenerated with 2 M NaCl in PBS (phosphate-buffered saline). The specific binding of FGF1 was calculated by subtracting the binding to a totally desulfated heparin prepared as described (24) and linked to the sensor chip as for LMWH. Quantification of the binding data was performed using the Bia Evaluation software (Biacore).

NMR experiments. The tetrasaccharide samples (metal ions removed by EDTA) were dissolved in 10 mM sodium phosphate buffer, pH 6.0, 50 mM Na_2SO_4, 0.1 M NaCl, 1 mM β-mercaptoethanol d_6, prepared using D_2O (99.995% D). Recombinant FGF-1 was dissolved in the same buffer and tetrasaccharides **AA** and **BA** were added to achieve molar ratios of 10:1 (**AA:**FGF-1) and 20:1 (**BA:**FGF-1), respectively. ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AMX spectrometer, equipped with a 5 mm inverse probe and a shielded z-axis gradient, at 9°C. ^{1}H spin–spin relaxation times were measured by Carr-Purcell-Meiboom-Gill spin-echo experiments using 12 points in the variable delay list. Two-dimensional COSY, TOCSY, and HSQC experiments were performed using z-gradients for the coherence selection. Two-dimensional phase-sensitive NOESY experiments were recorded with 32 scans and with 4 different mixing time (100, 200, 300, and 500 ms). Transferred NOESY spectra were collected in a similar way except for a (25-ms) spin-lock pulse inserted after the 90° excitation pulse.

Computational procedures. The computed structure of tetrasaccharide **AA** in the complex FGF1 was obtained using the MacroModel program version 7.0 (25). The geometry, based on the crystal structure, was optimized with the AMBER force field. The effect of solvent was estimated using continuum model with extended cutoffs.

MALDI mass spectrometry. MALDI analyses were carried out in the positive ion linear mode on a Bruker Biflex III time-of-flight mass spectrometer equipped with a pulsed nitrogen laser (λ = 337 nm). A matrix solution (sinapinic acid 10 g/L in 30% AcCN/H_2O) containing equimolar concentrations (2.5 pmol/μl) of both FGF1 and basic peptide $(Arg\text{-}Gly)_{19}$-Arg (as free base, after ionic exchange on a Dowex 1X2 resin) (26) was mixed with an aqueous solution of the tetrasaccharide at the desired molar ratios. In each case, 1 μl of the final mixture was deposited on the stainless steel target of the spectrometer favoring sample crystallization at room temperature and atmospheric pressure. Mass spectra were calibrated externally with BSA and Cytochrome c. MALDI acquisitions were performed by collecting and summing "batches" of a few laser shots at the threshold laser irradiance (27). The final spectra are the result of average sampling at different crystal surface position.

RESULTS

FGF1-Binding Affinities

The affinity of **AA, AB,** and **BB** for FGF-1 was evaluated by SPR by their ability to displace the protein from a Biacore surface to which a LMWH was covalently bound through biotinylation. Displacement curves are shown in Fig. 2 and compared with that of heparin. Although with values consistently less than heparin, **AA** and **BA** significantly inhibited binding of the protein to the heparinized surface in a dose-dependent manner. The fully sulfated compound **AA** had an affinity ($IC_{50} = 2.4 \times 10^{-5}$ mol/L) about one order of magnitude higher than that of compound **BA** ($IC_{50} = 2.9 \times 10^{-4}$ mol/L) which lacks the 6-OSO_3 group on the nonreducing end glucosamine. The effect of tetrasaccharide **BB** (without 6-O-sulfate) was negligible ($IC_{50} \gg 10^{-3}$ mol/L).

FGF1-Induced Perturbation of NMR Signals

FGF1 induced variations of chemical shifts and line widths of **AA** and **BA,** and generated transferred NOE effects for both tetrasaccharides. Induced selective shifts and broadening are illustrated by the partial ^{1}H-NMR spectrum of **AA** (Fig. 3). In the corresponding spectrum for **BA** (not shown), induced broadening is consistently smaller, and induced shifts larger, than for **AA.** Values of ^{1}H and ^{13}C chemical shifts are reported in Table 1.

The ^{1}H- and ^{13}C-induced chemical shifts and line broadening indicate that kinetics of the interaction with FGF1 involves a fast-to-intermediate exchange rate between free and bound states. An indirect estimate of k_{off} values could be made considering that the proton and carbon chemical shift changes and the linewidths observed for **BA** in the presence of FGF1 are comparable with those measured for a heparin tetrasaccharide in the presence of antithrombin (28). A lower k_{off} is accordingly expected in the bound state for **AA** with respect to **BA,** thus confirming the lower affinity for FGF1 of the latter tetrasaccharide.

Line widths were measured for selected signals of **AA** and **BA** in the absence and presence of FGF1. In the presence of FGF1, line widths of the anomeric signals of the R moiety of **AA** consistently increased for the ^{1}H anomeric signals of both the amino sugar (A) and iduronate (I) residues (18 Hz for A_R-1 and 9 Hz for I_R-1). Stronger effects (more than 50 Hz) were observed for the anomeric signals of the NR disaccharide moiety (both A_{NR}-1 and I_{NR}-1). This effect is paralleled by the measured spin-spin relaxation times of anomeric protons, which considerably differ in these two moieties in the presence of FGF1: (A_R-1, 86 ms; I_R-1, 120 ms) compared to 48 ms and 41 ms of A_{NR}-1 and I_{NR}-1, respectively.

Similarly, several proton chemical shifts changed for both I_{NR}-1 and A_{NR}-1 in **AA.** However, the changes for A

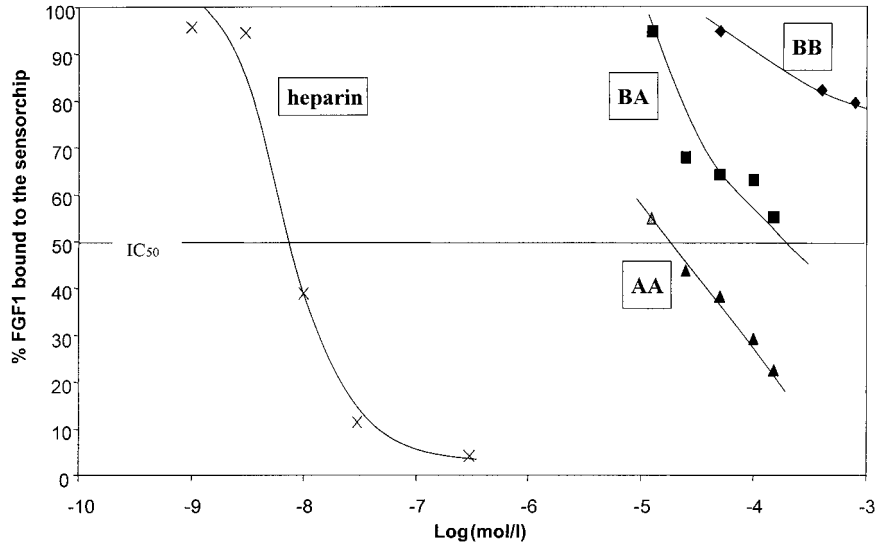

FIG. 2. Inhibition of FGF1 binding to heparinized sensor chip surfaces by tetrasaccharides **AA, BA,** and **BB** in comparison with heparin. Tetrasaccharides were mixed with 500 nM FGF-1 at different concentrations before injection: 12.5, 25, 50, 100, and 150 μM for both **AA** and **BA** and 50, 400, and 800 μM for **BB.** The heparin concentrations were 1, 3, 10, 30, and 300 nM. The percentage inhibition of FGF1 binding to surface-immobilized heparin was taken as] $(R_{FGF} - R_{TETRA})/R_{FGF}]$. 100, where R_{FGF} is the FGF1 binding capacity (in RU units) of the heparinized surface, R_{TETRA} is the FGF1 binding capacity in the presence of tetrasaccharide. IC_{50} is defined as the concentration of heparin/tetrasaccharide inducing 50% of inhibition of FGF1 binding.

and I residues were in opposite directions, with I_{NR}-1 being shifted downfield and A_{NR}-1 upfield. Data in Table 1 illustrate the shifts for various signals, where significant changes were seen for A_{NR}-1, A_{NR}-3, A_{NR}-5, I_{NR}-4, A_R-5, and I_R-5; a slightly smaller variation was also observed for I_{NR}-1, I_R-1, I_R-2, and I_R-4. The protein induced ^{13}C shielding variations as well. The largest differences were detected for C-6, both in the A_R unit (0.32 ppm) and in the A_{NR} unit (0.28 ppm) in **AA** and suggest that both 6-O-sulfates strongly interact with FGF1, especially the 6-OSO_3 group of the R moiety.

Though at higher ligand:protein ratios than for **AA,** FGF1-induced perturbations were also observed for tetrasaccharide **BA,** the largest effects being observed in HSQC spectra for A_{NR}-5, I_{NR}-5, I_R-2, I_R-3, and I_R-5. Interestingly, nearly a constant values of both proton and carbon resonances for A_{NR}-6 were observed and contrast with the observation for the fully sulfated tetrasaccharide **AA.** Since the electrostatic interaction among sulfate groups and the positively charged amino acid residues in the FGF1 binding site have the major effect upon changes in chemical shift values of both protons and carbons (28, 29), A_{NR}-6 shifts are well within expectations as the nonsulfated hydroxymethyl group seems not to be involved in FGF1 binding.

FGF1–tetrasaccharide interactions were further studied by evaluation of transferred NOE data. Here we discuss the results only qualitatively as the full quantitative interpretation is still in progress. The cross-peak intensities in 2D NOESY spectra of tetrasaccharides **AA** and **BA** in the free state were found to be small (~1%) indicating that $\omega\tau \approx 1$. In the presence of FGF1, transferred NOEs showed remarkably strong intensities as a consequence of the influence of the protein (28) upon cross-relaxation rates in both **AA** and **BA** (data not shown). Differences in the transferred NOE cross-peak intensities (those in **AA** being

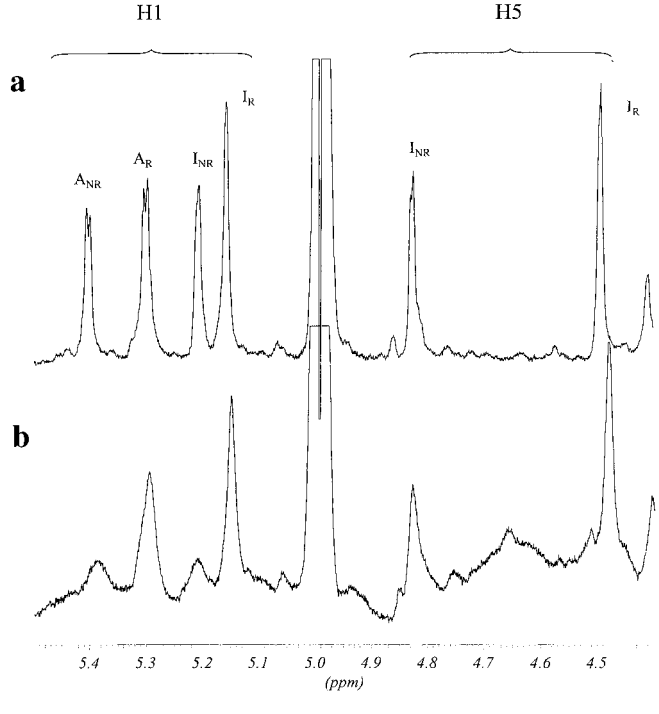

FIG. 3. Partial 500-MHz ^1H NMR spectra of tetrasaccharide **AA** at 9°C in buffer (a) and in the presence of FGF1 (b).

TABLE 1

^{1}H and ^{13}C Chemical Shifts of Tetrasaccharides **AA** and **BA** Alone (Free) and in the Presence of FGF1 (Bound)

	AA				**BA**			
	^{1}H		^{13}C		^{1}H		^{13}C	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound
A_{NR}-1	5.409	5.384	99.69	[b]	5.414	5.398	100.03	99.82
A_{NR}-2	3.254	[a]	[a]	[a]	3.201	3.186	60.93	60.98
A_{NR}-3	3.619	3.599	73.96	73.90	3.613	3.610	73.90	73.92
A_{NR}-4	3.544	3.540	72.08	71.85	3.461	3.448	72.60	72.69
A_{NR}-5	3.975	3.963	72.18	72.08	3.760	3.795	74.80	74.69
A_{NR}-6	4.335	[b]	69.43	69.15	3.800	3.808	62.97	63.10
A_{NR}-6′	4.178	[b]						
I_{NR}-1	5.214	5.222	102.06	101.95	5.223	5.212	102.09	102.21
I_{NR}-2	4.315	4.319	78.57	78.41	4.328	4.320	78.43	78.60
I_{NR}-3	4.190	4.196	71.90	71.86	4.238	4.218	71.63	71.86
I_{NR}-4	4.076	4.058	78.72	78.53	4.093	4.075	78.70	78.58
I_{NR}-5	4.832	4.831	72.15	72.16	4.945	4.863	71.69	72.00
A_{R}-1	5.308	5.308	99.99	99.96	5.317	5.317	100.62	100.25
A_{R}-2	3.217	[a]	[a]	[a]	3.268	3.254	60.59	60.87
A_{R}-3	3.677	3.666	72.45	72.39	3.686	3.677	72.23	72.32
A_{R}-4	3.765	3.756	78.63	[b]	3.765	3.771	78.54	78.52
A_{R}-5	3.961	3.936	72.85	72.70	3.947	3.974	72.27	72.25
A_{R}-6	4.408	[b]	69.36	69.04	4.400	4.399	69.07	69.29
A_{R}-6′	4.242	4.231			4.243	4.233		
I_{R}-1	5.163	5.154	101.21	101.21	5.197	5.163	101.23	101.36
I_{R}-2	4.210	4.201	77.89	77.93	4.245	4.230	77.37	77.87
I_{R}-3	4.208	4.204	70.71	70.75	4.272	4.234	70.06	70.65
I_{R}-4	4.014	4.006	78.72	78.84	4.045	4.025	78.90	78.80
I_{R}-5	4.500	4.485	70.64	70.44	4.651	4.543	69.85	70.37

Note. NR, nonreducing disaccharide moiety; R, reducing disaccharide moiety; A, glucosamine residue; I, 2-O-sulfated iduronic acid residue.
[a] Could not be determined because of signal overlap.
[b] Could not be determined because of large signal linewidth.

larger than in **BA**) were caused by different binding affinities of these tetrasaccharides to FGF1 as well as by different molar ratios of **AA** and **BA** in the complex. Furthermore, transferred NOEs were used to determine the conformation of iduronic acid residues since the magnitudes of intraresidue proton-proton three-bond coupling constants were not measurable due to the increased linewidth in the presence of protein. Small magnitudes of NOEs between protons I5 and I2 likely indicate that I residues adopt the $^{1}C_{4}$ chair form as in the free state (28, 29). However, full quantitative analysis of intra and interresidue NOEs is being used for future determination of the 3D structure at the glycosidic linkages and for conformation of monosaccharide residues in the bound state (work in progress).

Tetrasaccharide-Induced FGF1 Dimerization

MALDI mass spectra provided further evidence supporting the above conclusions on interaction of **AA** and **BA** with FGF1. MALDI spectra of tetrasaccharides **AA, BA,** and **BB** in the presence of FGF1 at the same molar ratio were compared with a spectrum of FGF1 "alone" (in the absence of the oligosaccharides) (Fig. 4). The *m*/*z* values for the FGF1 monomer and dimer were found to be, respectively, 15,062 and 30,119 (both peaks are split because of the lack of an amino acidic terminal residue). Molecular ions corresponding to trimers and tetramers, at *m*/*z* 45,095 and 60,349, were also observed at higher mass range (not shown). This experimental evidence, in spite of the elevated sample dispersion into matrix crystals (the tetrasaccharide-to-matrix molar ratio is about 1:10,000) led to exclusion of the presence of artifacts associated with nonspecific aggregation (30). MALDI spectra of FGF1 in the presence of **AA** and **BA** (Figs. 4b and 4c) show extra peaks (clarified in mass scale expansions) related to the corresponding complex 2:1 FGF-1/**AA** (*m*/*z* 31332) and FGF-1/**BA** (*m*/*z* 31218). The intensity of the complex peaks increases with increasing molar ratio between tetrasaccharides **AA** (31) and **BA.** Such a dimeric signal is absent in the corresponding spectrum obtained for the FGF1/**BB** (Fig. 4d).

DISCUSSION

Several studies have been made to establish the minimal length of heparin/HS oligosaccharides required

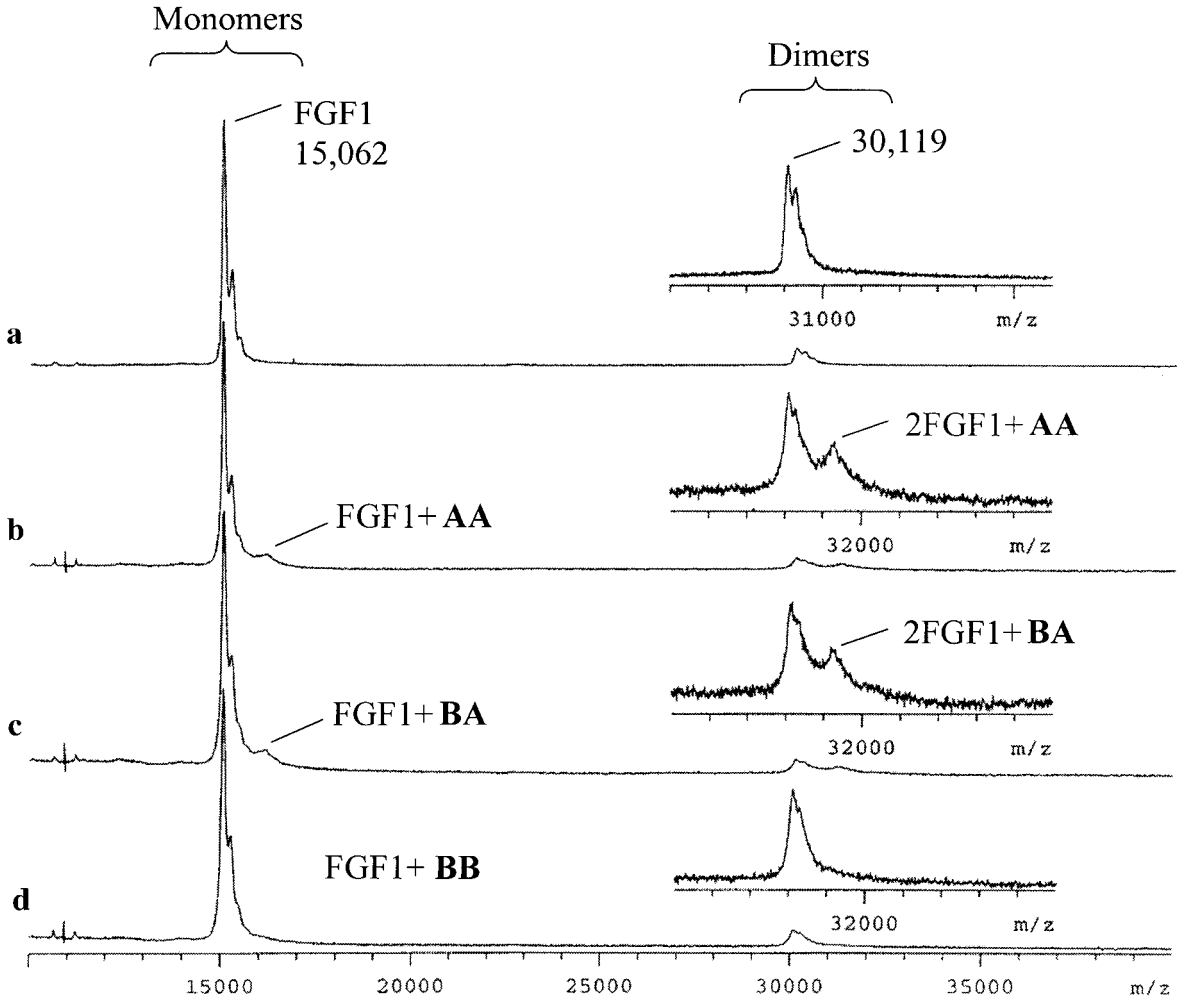

FIG. 4. MALDI mass spectra of FGF1 without (a) and in presence of tetrasaccharide **AA** (b), **BA** (c), and **BB** (d). Each measurement was performed by loading 2 pmol of FGF1 and 2 pmol of tetrasaccharide. The expansions of the dimer region (b) and (c) clearly show mass peaks corresponding to 2:1 complexes between FGF-1 and tetrasaccharide **AA** and **BA.** No 2:1 complex is observed for tetrasaccharide **BB** (d).

for binding to FGF1 and for inducing its dimerization (11, 16, 20). Whereas binding to FGF1 was reported to require at least a tetrasaccharide such as **1** (16), oligosaccharides smaller than octasaccharide could not facilitate dimerization of the growth factor and contribute to eliciting mitogenic signaling (16, 32–36). From the crystal data of the 2:1 FGF1: heparin decasaccharide complex, the electron densities of the ligand could be determined only for 5–6 saccharide residues (20). It has been proposed that shorter saccharide chains may not suffice to neutralize the positively charged heparin-binding regions of FGF1 and thus avoid strong electrostatic repulsion between FGF1 monomers, or that the low-affinity binding sites of the proteins are not sufficiently occupied or displaced by crystal packing forces (20). The optimized structure of tetrasaccharide **AA** in the 2:1 complex with FGF1 is presented in Fig. 5a. The structure of **AA** contains minimal structural requirements (with arrays of sulfate groups on both sides of the saccharide chain) necessary for binding two FGF1 molecules in the *trans* arrangement found in the crystalline complex. Such an oligosaccharide core contributes 8 of 9 interactions with protein monomer A and 11 of 12 with protein monomer B (19). Both the contacts observed for monomer A and for monomer B may be envisaged for 1:1 complexes, for which crystal data are not yet available. Computed structures of such complexes of tetrasaccharide **AA** involving only monomers A or B are depicted in Figs. 5b and 5c, respectively. Inspection of these structures reveals that all major interactions between amino acids in the binding site and sulfates are retained in both 1:1 complexes as found in the dimer form. However, because the solution structures of both tetrasaccharides and the protein (mainly the terminal residues) are flexible and their 3D structures may not entirely correspond to those in the crystal state, other nonbonded interactions were observed in addition to those described in the crystal (20).

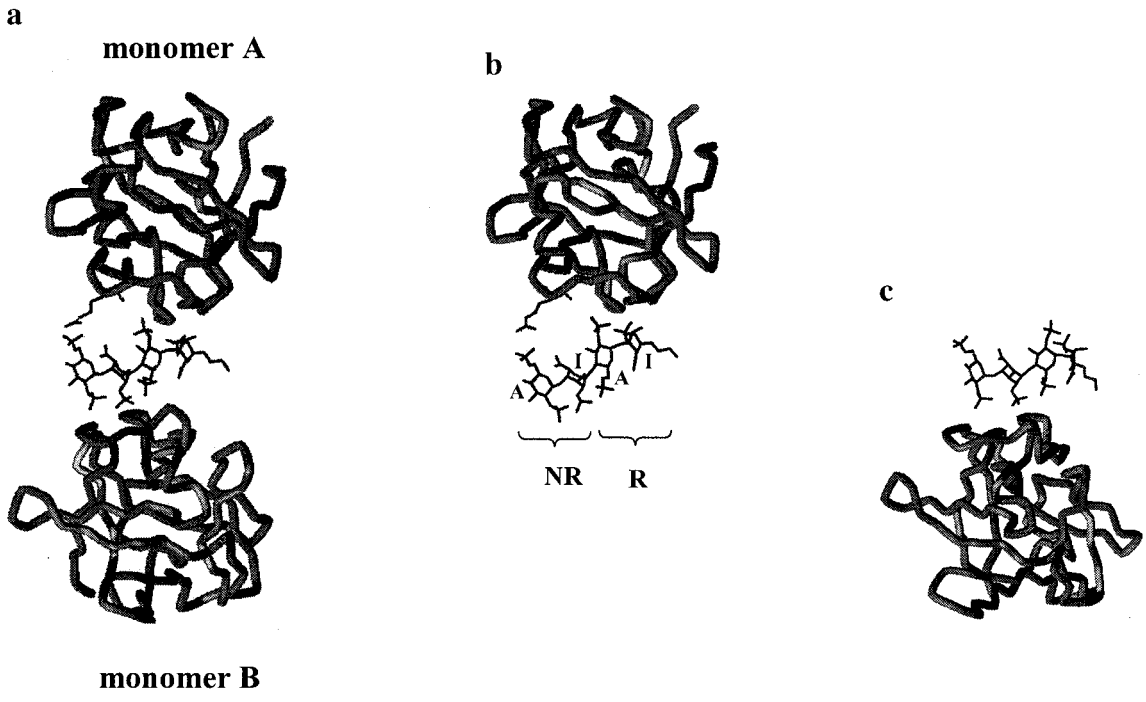

FIG. 5. Structure of synthetic tetrasaccharide **AA** in the complex with FGF1 obtained from protein crystal coordinates (Ref. 20; PDB, 1AXM and 2AXM) by molecular modeling. 2:1 complex (FGF1:**AA**) in *trans* arrangement (a). 1:1 complexes of monomer A with **AA** (b) and monomer B with **AA** (c), respectively. NR and R designates nonreducing and reducing disaccharide subunits in **AA,** respectively. A and I are glucosamine and iduronic acid residues, respectively.

In 1:1 complexes, for example, interaction between Lys113 monomer A and the NSO_3 group in A_R and the carboxylate group in I_R. On the other hand, a nonbonded interaction is seen between Lys112 not only involving the carboxylate in I_R (similarly as in crystal state in a structurally related hexasaccharide) but also the 6-OSO_3 group in A_R. The latter interaction, not described in the solid state, is in agreement with the observation of the considerable variations of chemical shifts of C-6 in A_R and the key role of this group during the binding with FGF1.

The present data confirm that tetrasaccharides with appropriate structures bind to FGF1 under conditions of the SPR and MALDI experiments, as well as of the solution NMR experiments. They also indicate that tetrasaccharides are long enough to favor formation of dimers of the growth factor, at least under conditions of the MALDI experiments. In addition, this study also complement previous information on the role of the 6-O-sulfate groups in formation of both 1:1 and 2:1 FGF1:tetrasaccharide complexes. In SPR experiments, **AA,** the structure of which corresponds to the regular sequences of heparin, has shown the highest relative affinity for FGF1. Though with lower affinity, **BA,** which is non-6-O-sulfated in the NR disaccharide moiety, also binds to the growth factor. On the other hand, lack of 6-O-sulfate groups in both the NR and R moieties (as in **BB**) leads to complete loss of affinity. Interestingly, both **AA** and **BA** contain the trisaccharide motif (Fig. 1) recently identified as minimal heparin/HS binding sequence to FGF1, involving a $GlcNSO_36SO_3$ residue flanked by two $IdoA2SO_3$ residues (18). Also the observed perturbation of NMR signals [typically associated with intermolecular associations (28, 29)] is significantly stronger for **AA** than for **BA.** In addition, selective broadening of ^{1}H-NMR signals of **AA** (Fig. 3) indicates a preferential involvement of the disaccharide moiety NR in complexation with FGF1. FGF1 also induces shifts of several ^{1}H and ^{13}C resonances (Table 1). It is important to note that the chemical shifts of signals associated with 6-OSO_3 groups of both the NR and R moieties of **AA** and **BA** are significantly perturbed by FGF1, suggesting that both are involved in binding to the protein and play a key role in formation of the complex with FGF1. In contrast, chemical shifts associated with the nonsulfated CH_2OH group of **BA** are unaffected. Combining the foregoing observations suggests that, whereas the NR disaccharide moiety of both **AA** and **BA** is primarily involved in interaction with FGF1 also involving the 6-OSO_3 group of the contiguous glucosamine residue as depicted for monomer B (Fig. 5c), the alternative interaction as shown for monomer A (Fig. 5b) also takes place to some extent. The present results are thus compatible with an equilibrium mainly involving two 1:1 complexes, with prevailing contribution of the interaction as depicted in Fig. 5b. Since the MALDI experiments (Fig. 4) show that **AA** and **BA** also form 2:1 FGF1:tetrasaccharide

complexes, the possibility that these dimeric complexes also contribute to the equilibria in solution cannot be disregarded, and will be further investigated in more detailed NMR studies.

Further support to the importance of at least one 6-O sulfate was provided by MALDI spectra. Whereas peaks corresponding to the complexes of FGF1 with **AA** and **BA** were observed, they were not generated by the non-6-O-sulfated **BB.** This evidence demonstrates that even relatively short chains of heparin-derived oligosaccharides may induce dimerization of FGF1, provided that they meet structural requirements necessary to promote this type of molecular assembly.

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