Evidence That Heparin Saccharides Promote FGF2 Mitogenesis through Two Distinct Mechanisms*

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Heparin-like saccharides play an essential role in binding to both fibroblast growth factors (FGF) and their receptors at the cell surface. In this study we prepared a series of heparin oligosaccharides according to their size and sulfation level. We then investigated their affinity for FGF2 and their ability to support FGF2 mitogenesis of heparan sulfate-deficient cells expressing FGFR1c. Tetra- and hexasaccharides bound FGF2, but failed to dimerize the growth factor. Nevertheless, these saccharides promoted FGF2-mediated cell growth. Furthermore, whereas enzymatic removal of the non-reducing end 2-O-sulfate group had little effect on the 1:1 interaction with FGF2, it eliminated the mitogenic activity of these saccharides. This evidence supports the symmetric two-end model of ternary complex formation. In contrast, even at very low concentrations, octasaccharide and the symmetric two-end model of ternary complex formation. In this study we prepared a series of heparin oligosaccharides according to their size and sulfation level. We then investigated their affinity for FGF2 and their ability to support FGF2 mitogenesis of heparan sulfate-deficient cells expressing FGFR1c. Tetra- and hexasaccharides bound FGF2, but failed to dimerize the growth factor. Nevertheless, these saccharides promoted FGF2-mediated cell growth. Furthermore, whereas enzymatic removal of the non-reducing end 2-O-sulfate group had little effect on the 1:1 interaction with FGF2, it eliminated the mitogenic activity of these saccharides. This evidence supports the symmetric two-end model of ternary complex formation. In contrast, even at very low concentrations, octasaccharide and larger heparin fragments conferred a potent mitogenic activity that was independent of terminal 2-O-sulfation. This correlated with the ability to dimerize FGF2 in an apparently cooperative manner. This data suggests that potent mitogenic signaling results from heparin-mediated trans-dimerization of FGF2, consistent with the asymmetric model of ternary complex formation. We propose that, depending on saccharide structure, there are different architectures and modes of ternary complex assembly that differ in stability and/or efficiency of transmembrane signaling.

FGF1 and FGF2 are the prototypic members of the fibroblast growth factor (FGF) family. They can induce proliferation, differentiation, motility, adhesion, survival, and apoptosis of cells at both embryonic and adult stages (1, 2). A wide variety of cells express FGF1 and FGF2, for example, vascular smooth muscle cells, neurons, epithelial cells, and fibroblasts (3). FGF2 can also be released by macrophages in response to inflammation (4) or by platelets in response to vessel injury (5). FGF1 and FGF2 are potent angiogenic factors (6). Angiogenesis, the formation of new blood vessels, is important in human disease; it is induced by malignant tumors, and is a cause of arthritis and visual impairment in diabetic retinopathies. Tumors secrete pro-angiogenic molecules, such as the FGFs, in an autocrine or paracrine manner, and increased FGF levels are associated with poor prognosis for many cancers (2, 7).

Heparan sulfate (HS) proteoglycans are membrane-associated co-receptors that coordinate the interaction of FGFs with their high-affinity tyrosine kinase receptors, the FGFRs (8). These high-affinity receptors are the products of distinct mammalian genes: FGFR1 (flt), FGFR2 (bek), FGFR3, and FGFR4. FGFR1–3 can be alternatively spliced to produce a, b, and c isoforms. The a isoform is secreted and may function as a soluble receptor antagonist (9). The b and c receptor variants are particularly important as they display different ligand binding specificities and affinities (10). For example, FGFR2c was reported to bind FGF1 and FGF2 with equal affinity, whereas FGFR2b bound FGF2 with a thousand-fold lower affinity than FGF1 (11). It has also been suggested that the expression of b and c FGFR isoforms can be "reversibly switched" by the influence of FGF1 or FGF2 (12). Aberrant receptor switching has been reported to be involved in the progression of certain tumors (13).

The interaction of HS proteoglycans with FGFs provides a physiological mechanism for regulation of FGF signaling. The ability of HS- or heparin-derived saccharides to recognize different FGFs depends on their size and sulfation pattern. Crystal structures show that FGF1 and FGF2 interact with 4–5 sugar residues within short heparin oligosaccharides (14, 15), although saccharides of this length are generally inactive or weak activators in mitogenesis assays. FGF2 is known to interact with N-sulfoglucosamine (GlcNCS) and 2-O-sulfated idurionate residues (IdoUA(2S)) in heparin and HS (15, 16), but the additional presence of 6-O-sulfation is required for biological activity (17, 18); whereas FGF1 requires N-, 2-O-, and 6-O-sulfate groups for both high-affinity binding and activation (14, 19, 20). Variations in the FGF receptor subtypes expressed by different cells also influence the saccharide requirements for FGF-mediated cell growth (19, 21). However, for each FGF-FGFR combination the optimum location of sulfate groups upon activatory saccharides is unclear. Indeed, there is...
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FIGURE 1. Schematic of the crystallographic models for the FGF-FGFR-heparin ternary complex. FGF extracellular domains II and III are shown as dark-gray ovals, FGF as shaded/white circles, and heparin as a dashed black line (with an arrow indicating the reducing end). A, symmetrical model. Two FGF-FGFR pairs interact through FGF-FGFR interactions, FGF-FGF interactions across the pairs, and through contacts with heparin. The right-hand view, looking down on the apex of the structure, shows the two non-reducing heparin ends meeting. B, asymmetric model. Two FGF-FGFR pairs are linked through interactions of both FGFs and one FGFR with the heparin chain. There are no significant protein-protein contacts between the two heterodimers. C, a 1:1:1 FGF-FGFR-heparin complex (shown in the middle) is common to both models (shown at either side). D, a crystallographic symmetry operation (faded) in the asymmetric model generates a complex that is reminiscent of the symmetric model (highlighted within the dashed box). The resulting complex is common to both crystal systems, but contains only one molecule of heparin for two FGFs and FGFRs, and so remains asymmetric.

some dispute in the literature on whether the overriding influence is the sulfate density of the saccharides or the sulfate patterning (22, 23).

Two crystal structures of FGF-FGFR-heparin ternary complexes have been published, which are predicted to form at different sites upon HS chains (24). The symmetrical 2:2:2 FGF-FGFR-saccharide complex (25) involves two saccharide chains that terminate at the center of the structure, and so this species could form only on the non-reducing ends of two separate but proximal HS chains (Fig. 1A). In contrast, the asymmetrical 2:2:1 complex (26) is formed around a single saccharide and could therefore assemble internally upon HS chains (Fig. 1B). However, the two FGF-FGFR half-complexes in the asymmetrical structure make no significant protein-protein interactions and so are reliant on the probability that they become aligned on opposite faces of the same short stretch of HS. This might be achieved through induction of favorable local conformational changes in the saccharide; indeed, there is strong evidence for the existence of this in solution (27). A comparison of the two ternary complex structures demonstrates that a similar substructure exists in both crystals (Fig. 1, C and D), comprising two 1:1 FGF-FGFR units interacting as a dimer through both FGF-FGFR and FGFR-FGFR contacts (24).

Studies of ternary complex formation using gel-filtration, ultracentrifugation, and mass spectrometry suggest that this substructure exists in solution but contains just one heparin saccharide (27). It is possible that the proposed symmetrical 2:2:2 structure (25) is in fact a 2:2:1 complex disordered around the local dyad axis in the crystals, giving apparent occupancy at both sites.

In a recent study using a size exclusion chromatography approach to examine ligand-saccharide interactions, we reported that one of the key biochemical properties of heparin saccharides that correlated with induction of FGF1 mitogenesis was a capacity to dimerize the growth factor. FGF1 dimerization appeared to be a highly cooperative process because the formation of 2:1 FGF1-saccharide complexes was favored even in the presence of excess saccharide. In the present investigation we have examined the interaction of heparin saccharides of variable length and sulfation with FGF2 and equated their binding properties with bioactivity in an FGF2-FGFR1c-mediated mitogenesis assay. The results indicate some similarities with FGF1, but also reveal an underlying level of complexity in structure-activity relationships that may reflect the existence of different mechanisms of co-receptor function in native HS chains.

EXPERIMENTAL PROCEDURES

Materials—The FGFR-transfected BaF3 cell lines, originally created by Dr. D. Ornitz (Washington University Medical School, St. Louis, MO), were obtained from Dr. J. Whitelock (University of New South Wales, Sydney, Australia). Human recombinant FGF2 was purchased from R & D Systems (Abingdon, United Kingdom). Grampian Enzymes (Orkney, UK) supplied heparinase (EC 4.2.2.7), heparinase II (no EC number assigned), and heparinase III (EC 4.2.2.8), whereas Glyko Inc. (Bicester, UK) supplied recombinant α-L-iduronate 2-O-sulfatase (12Sase; EC 3.1.6.13) exoenzyme. The ProPac PA1 analytical column was from Dionex (Camberley, UK). The Superdex 75 HR, Superose 12 HR, and PD-10 columns were purchased from GE Healthcare. Bio-Gel P-10 resin was purchased from Bio-Rad. Invitrogen supplied cell culture media and horse serum, whereas the Cytolite™ cell proliferation assay kit was from PerkinElmer Life Science. All other reagents were from BDH-Merck Ltd (Lutterworth, UK).

Preparation of Heparin Oligosaccharides—Partial heparinase digests of heparin were performed as described previously (28). The resultant fragments were size separated on a Bio-Gel P-10 column (1.5 × 170 cm) at 4 ml/h in 0.5 M ammonium hydrogen carbonate. A range of peaks, corresponding to heparin fragments of different length, were detected by UV absorbance at 232 nm (unsaturated bonds of heparin fragments). Fractions (1 ml) were collected and each peak was pooled separately, then freeze-dried several times to remove residual ammonium hydrogen carbonate. Removal of the non-reducing end 2-O-
sulfate group from these saccharides was achieved by treatment with 40 milliunits/ml I2Sase exoenzyme overnight at 37 °C in 50 mM sodium acetate buffer (pH 5.0) containing 100 μg/ml bovine serum albumin.

Subfractions of the sized heparin oligosaccharide pools were prepared by resolution on an analytical ProPac PA1 (4 × 250 mm) strong anion exchange-high performance liquid chromatography (SAX-HPLC) column. The column was run at 1 ml/min in Milli-Q H2O (pH 4.0) with oligosaccharides eluted over the NaCl gradients indicated (Fig. 2). Elution was monitored at 232 nm and the identified peaks were pooled, then desalted using a PD-10 column. Each pooled sample was reapplied to the ProPac PA1 column to confirm purity.

Disaccharide Analysis—Purified oligosaccharides (15–30 μg) were incubated with 20 milliunits/ml heparinase for 24 h at room temperature, then 20 milliunits/ml each of heparinase, heparinase II, and heparinase III for a further 24 h. To verify that the oligosaccharides had been fully digested down to disaccharides, the sample was applied to the Superose 12 HR SEC column using 0.25 mM ammonium hydrogen carbonate at a flow rate of 0.75 ml/min. The disaccharides were pooled and freeze dried to remove ammonium hydrogen carbonate. Each disaccharide sample was then resolved on a ProPac PA1 column with elution monitored at 232 nm. For this a two-step linear NaCl gradient was applied at a flow rate of 1 ml/min (0 – 0.12 M NaCl, pH 4.0, over 90 min, followed by 0.12 – 1 M NaCl, pH 4.0, over 45 min) and peaks were identified by comparison with known disaccharide standards.

Mitogenic Activity Screening—BaF3 lymphoblastoid cells (transfected with FGFR1c) were routinely maintained in RPMI 1640 supplemented with 10% (v/v) horse serum and 5% (v/v) interleukin-3 (IL-3)-conditioned medium at 37 °C. The IL-3-conditioned medium was prepared from Wehi-3b cells (mouse myelomonocytes) and filtered prior to use with the BaF3 cells. For the mitogenesis assay, BaF3 cells were serum starved for 2 days, then washed three times with phosphate-buffered saline at 37 °C to remove residual IL-3. The cells were then seeded onto a 96-well plate at low density (2.5 × 10^5 cells/ml) in RPMI 1640 supplemented with 10% (v/v) horse serum, 10 ng/ml FGF2, and heparin saccharides. Porcine mucosal HS at a concentration of 1 μg/ml, with the appropriate FGF, was used as a positive control in all assays. After a 4-day incubation period, the cells were assessed for mitogenic activity using the chemiluminometric CytoLite™ assay kit on a Top Count system.

Size-exclusion Chromatography (SEC) of FGF2-Saccharide Complexes—An Agilent 1100 series HPLC system was used to equilibrate a pre-packed Superdex 200 GL column (10 × 300 mm) in 150 mM NaCl, 50 mM phosphate buffer (pH 7.2). FGF2 protein (2 nmol) was added to heparin samples (1 nmol) suspended in column buffer (final volume of 250 μl) and incubated at room temperature for 15 min prior to column loading. Samples were eluted isocratically at a flow rate of 0.5 ml/min and the absorbance at 214 nm (peptide bond) recorded. The column was equilibrated using protein molecular weight standards as described previously (29). The void and total volumes were determined using blue dextran and acetone, respectively.

RESULTS

Preparation of Size-fractionated Heparin Oligosaccharides—SEC on a Bio-Gel P-10 column was used to separate a partial heparinase digest of heparin into a series of well resolved peaks ranging in size from 2 to 26 monosaccharide units (dp2 to dp26; degree of polymerization). The tetra-, hexa-, octa-, and decasaccharide fractions were pooled and subfractionated using SAX-HPLC. Three tetrasaccharide peaks (Fig. 2A) and five hexasaccharide peaks (Fig. 2B) were identified and pooled for use in subsequent experiments. The most anionic species (latest eluting peaks) from the heparin octasaccharide (dp8-fs) and decasaccharide peaks (dp10-fs) profiles were also pooled (Table 1).

The Effect of Heparin Oligosaccharides on FGF2-FGFR1c Activity—To clarify the shortest fragment of heparin that activated FGF2, activity screening was carried out using BaF3 cells transfected with the FGFR1c receptor (30). BaF3 cells are a
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TABLE 1
Disaccharide analysis of SAX-HPLC-purified heparin oligosaccharides

Heparin oligosaccharide species prepared by SAX-HPLC (Fig. 2) were treated with a combination of heparinase, heparinase II, and heparinase III. The resultant disaccharides were resolved on the ProPac PA1 column, quantified by absorbance at 232 nm, and identified by comparison to heparin disaccharide standards. Data are shown as percentage of total disaccharides and represent the mean of three repeats.

| Disaccharide    | UA-GlcNS | UA-GlcNS(6S) | UA(2S)-GlcNS | UA(2S)-GlcNS(6S) |
|-----------------|----------|--------------|--------------|------------------|
| dp4-1           | 6        | 35           | 59           |                  |
| dp4-2           | 44       | 56           |              |                  |
| dp4-3           |          |              |              |                  |
| dp6-1           | 2        | 23           | 62           |                  |
| dp6-2           | 28       | 29           | 43           |                  |
| dp6-3           | 6        | 24           | 70           |                  |
| dp6-4           | 44       |              | 56           |                  |
| dp6-5           |          |              | 100          |                  |
| dp8-8s          |          |              | 100          |                  |
| dp10-fs         |          |              | 100          |                  |

FIGURE 3. FGF2-FGFR1c mitogenic activity with size-defined heparin oligosaccharides. BaF3 cells transfected with the receptor subtype FGFR1c were plated out at a density of 2.5 × 10⁴ cells/ml with increasing concentrations of heparin and 10 ng/ml FGF2. Viable cell numbers were measured using the CytoLite™ kit. 100% = CytoLite incorporation for FGF and porcine mucosal HS (1 μg/ml) and 0% = CytoLite incorporation for FGF alone. The ♦ represents unfractionated heparin tetrasaccharides; ▲, unfractionated heparin hexasaccharides; △, fully sulfated heparin octasaccharide (dp8-fs); and ×, fully sulfated heparin decasaccharide (dp10-fs). Data points for all mitogenesis experiments were performed in triplicate and each graph is representative of three repeat experiments.

The Effect of Subfractionated Heparin Oligosaccharides on FGF2-FGFR1c Activity—Disaccharide analyses of the HPLC-prepared oligosaccharide subfractions (Fig. 2) revealed that they were all fully N-sulfated with varying degrees of 2-O- and 6-O-sulfation (Table 1). The last tetra- and hexasaccharide peaks to elute from the SAX-HPLC column were found to be relatively homogeneous fully sulfated species. In contrast, earlier eluting peaks appeared to contain more than one major species as the disaccharide ratios obtained were substoichiometric. The hexasaccharide pools dp6-1 and dp6-3 were notable as they eluted from the SAX-HPLC column at different positions (Fig. 2B), yet contained IdoUA(2S)-GlcNS and IdoUA(2S)-GlcNS(6S) in an approximately equal ratio (Table 1). These two fractions possibly contained major species that were isomers in respect to the position of 6-O-sulfate groups.

For comparison we also tested the dp8-fs and dp10-fs oligosaccharides (Fig. 2, C and D). Both were very strong activators of FGF2 with FGFR1c, with dp10-fs activity even exceeding that of the HS positive control (Fig. 3). Moreover, the activation curves for these two oligosaccharides showed a sharp dose-response profile with near-maximal activity reached at just 0.1 μg/ml. Although this was not a strictly like-for-like comparison, the striking difference in mitogenic activity between the dp4/6 and dp8/10 samples prompted us to look more closely at the composition of the tetra- and hexasaccharides and to test whether individual subfractions (Fig. 2, A and B) varied in their capacity to stimulate mitogenesis.

The fully sulfated dp4-3 (Table 1) was able to promote proliferation much more effectively than the unfractionated tetrasaccharide sample (Figs. 3 and 4A). At 2 μg/ml this response was equivalent to the positive control. In contrast, the dp4-1 and dp4-2 fractions, which appeared to lack one 2-O- or one 6-O-sulfate, respectively (Table 1), were unable to activate FGF2 (Fig. 4A). The lower activity of the un fractionated tetrasaccharide sample, compared with purified dp4-3, can be explained by the fact that these inactive species constitute a significant proportion of the tetrasaccharides.

The fully sulfated dp6-5 fraction (Table 1) was markedly more effective at stimulating cell growth than the other hexasaccharides tested (Fig. 4B). The dp6-2, dp6-3, and dp6-4 pools had an intermediate activity, whereas dp6-1, the earliest eluting...
SAX-HPLC peak, stimulated only a weak mitogenic response. The disaccharide analyses suggest that the differences in activity were due to the O-sulfation pattern (Table 1). The oligosaccharide peaks, dp6-1 and dp6-3, had very similar disaccharide compositions but differed in their degree of growth promoting activity. Conversely, dp6-2 and dp6-3 displayed comparable mitogenic activity, but distinct disaccharide compositions. These oligosaccharide subfractions illustrate the potential importance of sulfation pattern in the activation of the FGF2-FGFR1c signaling complex.

The dp4-3 and dp6-5 fractions showed more mitogenic activity than the unfractionated tetra- and hexasaccharide samples from which they were derived. However, the activity curves for these fully sulfated fractions were notably different from those obtained with the equivalent fully sulfated octa- and decasaccharides. The dp8-fs and dp10-fs gave very sharp dose-response curves that reached a plateau at saccharide concentrations as low as 0.1 μg/ml (Fig. 3). In contrast, the fully sulfated dp4 and dp6 fractions had only a weak activity at these low levels of saccharide and their dose-response curves rose more gradually without appearing to reach a plateau (Fig. 4). This was even despite the fact that for equivalent microgram/ml quantities, the shorter heparin samples contained a greater number of oligosaccharide chains.

**Removal of the Terminal 2-O-Sulfate Group and Its Effect on FGF2-FGFR1c Activity**—Although dp4-3 stimulates FGF2 activation of the FGFR1c receptor subtype in our assay (Fig. 4A), HS fragments of 10–12 monosaccharide units in length appear to be necessary to stimulate the same ligand-receptor pairing (17, 18). This could be a consequence of their different preparation methods. Heparin fragments are produced by partial heparinase digestion that leaves a non-reducing end 2-O-sulfate group, whereas HS fragments are more commonly produced using heparinase III and so do not possess this terminal sulfation (31, 32). To investigate this matter, we treated our heparin oligosaccharides with I2Sase, an exoenzyme that specifically removes the non-reducing end 2-O-sulfate (33). The I2Sase-treated samples were resolved by SAX-HPLC to confirm the enzymatic reaction had gone to completion (Fig. 2).

I2Sase-treated oligosaccharide samples were compared with untreated samples for their ability to stimulate FGF2-FGFR1c mitogenesis. The tetra- and hexasaccharides were rendered inactive by I2Sase treatment (Fig. 5A), whereas dp8-fs and dp10-fs showed no significant loss of biological activity (Fig. 5, A and B). In addition, we also tested the hexasaccharide dp6-5 fraction before and after treatment with I2Sase (Fig. 5B). The enzyme effectively eliminated the mitogenic activity of dp6-5. This was despite the fact that the resultant hexasaccharide still contained a fully sulfated tetrasaccharide at its reducing end, identical in sequence to the fully sulfated heparin tetrasaccharide dp4-3 that was active in the same assay. In common with the I2Sase-treated dp6-5, untreated dp6-2 and dp6-4 also lacked a single 2-O-sulfate group (Table 1), but from internal positions (because these oligosaccharides are the products of heparinase cleavage). Both of these saccharides were modest activators of FGF2-FGFR1c mitogenesis (Fig. 4B). These findings indicate that for short saccharides the FGF signaling complex is sensitive to the sequence of 2-O-sulfation. The non-reducing end 2-O-sulfate group may facilitate receptor-receptor interactions in the symmetrical “two ends” model of ternary complex formation (25). However, the retention of full

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**FIGURE 5.** FGF2-FGFR1c mitogenic activity with I2Sase-treated tetra- and hexasaccharides. The mitogenesis assay was performed as described in the legend to Fig. 3. BaF3 cells transfected with FGFR1c were incubated with 10 ng/ml FGF2 and increasing concentrations of: A, unfractionated tetrasaccharides before () and after ( ) I2Sase; B, unfractionated hexasaccharides before () and after ( ) I2Sase.

**FIGURE 6.** FGF2-FGFR1c mitogenic activity with I2Sase-treated octa- and decasaccharides. The mitogenesis assay was performed as described in the legend to Fig. 3. BaF3 cells transfected with FGFR1c were incubated with 10 ng/ml FGF2 and increasing concentrations of: A, dp8-fs () and dp8-fs (); B, dp10-fs () and dp10-fs () after I2Sase.
activity by the I2Sase-treated octa- and decasaccharides suggests that they may form ternary complexes in a different manner.

Comparing the FGF2 Affinity of Heparin Oligosaccharides by SEC—Previously we determined the FGF1 binding ability of various heparin oligosaccharides using an SEC-based approach (29). We applied this technique here to determine whether the removal of the non-reducing end 2-O-sulfate group from our heparin oligosaccharides affected FGF2 affinity. Using the SEC method we could detect only a very weak interaction between FGF2 and the heparin tetrasaccharide sample. In contrast, hexasaccharide bound FGF2 in stable 1:1 complexes, whereas octasaccharide and larger fractions formed 2:1 FGF2-heparin complexes (Fig. 7A). This pattern of binding was identical to that which we reported for FGF1 previously (29). In all cases, pre-treatment of the sized heparin saccharides with I2Sase had little discernable effect on their interaction with FGF2 (Fig. 7B). This was despite the fact that SAX-HPLC confirmed the complete removal of the non-reducing end 2-O-sulfate groups (Fig. 2). Therefore, the failure of I2Sase-treated hexasaccharides to promote FGF2 signaling through FGFR1c (Fig. 5) was not a consequence of a failure to form 1:1 complexes with FGF2.

Our previous studies revealed that 2:1 FGF1-heparin complexes formed even in the presence of a large excess of heparin saccharide, indicating a cooperative mechanism of FGF1 dimerization (29). We repeated this experiment with FGF2 and found that 2:1 FGF2-heparin complexes were also highly favored over 1:1 complexes (Fig. 7C). Thus at the biochemical level the potent FGF2 activators (dp8 and dp10) can be distinguished from the weak activator (dp6) by their propensity to stably dimerize FGF2, and do so in a strongly cooperative manner.

DISCUSSION

It has been appreciated for some time that FGF signaling requires the formation of a ternary complex between the FGF ligand, FGFR, and HS (35); however, the stoichiometry and molecular arrangement of these components has remained controversial. Two conflicting ternary complex models have been suggested on the basis of x-ray crystallography. The asymmetrical model (26) described a 2:1 FGF1-FGFR2-heparin complex, with the two FGF-FGFR units dimerized in a trans configuration upon a heparin decasaccharide (Fig. 1B). In contrast, a symmetrical 2:2:2 FGF2-FGFR1c-heparin ternary complex was also proposed (25). In this two ends model a cationic canyon was formed at the interface of the two FGF-FGFR pairs, in which two heparin saccharides lie with their non-reducing ends at the center (Fig. 1A). These saccharides serve to stabilize each FGF-FGFR pair and, through interactions with the adjacent receptor, facilitate FGFR dimerization. The differences in the two published structures have come to dominate the discussion of ternary complex architecture; however, a 2:2 FGF-FGFR-heparin substructure in the Pellegrini crystal shares a lot of similarities with the symmetrical complex (Fig. 1, C and D), and it is possible that both might co-exist in higher order assemblies (36). Additional models should also not be discounted; for example, mass spectrometric data suggests that a 2:2 arrangement of FGF-FGFR, similar to that proposed by Schlessinger and Mohammadi (25), could form with just one heparin saccharide occupying the cationic cleft (27). Alternatively, a putative secondary FGFR binding site identified on FGF2 suggests that 1:2:1 FGF-FGFR-HS ternary complexes
might be possible (37). The different architectures of the various models lead to significant differences in the interactions predicted between the protein components and heparin. This could result in heparin/HS saccharides of different lengths and sulfation patterns favoring certain ternary complex architectures over others.

Our mitogenic studies using FGF2 with FGFR1c (the same pairing crystallized in the symmetrical complex (25)) revealed that a fully sulfated heparin tetrascaricride was capable of supporting mitogenic signaling (Fig. 4A). SEC analysis revealed that an octascaricride was the shortest heparin fragment that formed stable 2:1 FGF2-heparin complexes (Fig. 7A), whereas hexa- and tetrascaricrides formed only 1:1 complexes (although short-lived 2:1 complexes might not be detectable using this method). Given that the two FGF-FGFR pairs in the asymmetrical ternary complex are held together primarily through extensive interactions with the heparin saccharide, it seems unlikely that a tetrascaricride could afford anything greater than a transient stabilization of this type of complex. In contrast, the symmetrical 2:2 FGF-FGFR architecture observed in both the Schlessinger/Mohammadi and Pellegrini crystals (24) has extensive protein-protein contacts. It is perhaps this type of complex that is responsible for mitogenic signaling in response to the heparin dp4 (Fig. 4A). A heparin tetrascaricride, or a pair of them, could occupy the heparin-binding canyon and cooperate with the FGF-FGFR and FGFR-FGFR interactions to sufficiently stabilize the ternary complex.

The crystal structure of the symmetrical 2:2:2 complex suggested a potentially specific interaction between the non-reducing end 2-O-sulfate group of heparin, and the receptor (25). Significantly, this interaction was not predicted to be important in the “asymmetrical” 2:2:1 crystal structure of FGF1-FGFR2c-heparin (26). The data presented here highlight the significance of the non-reducing end 2-O-sulfate group when short heparin fragments were used to stimulate FGF2-FGFR1c signaling. Removal of this terminal sulfate group abolished the activating properties of tetra- and hexascaricrides (Fig. 5). This was true even for dp6-5, which was otherwise fully sulfated at all other positions (8 sulfate groups). Therefore, these findings appear to be consistent with the two end symmetrical model of ternary complex formation (25). In contrast, loss of terminal 2-O-sulfation had very little effect on the bioactivity of octa- and decascaricrides (Fig. 6, A and B). In the two end model, the saccharide non-reducing ends meet in the center at the receptor-receptor interface. So whereas an octascaricride might further stabilize ligand-receptor interactions through additional contacts at its reducing end, the interactions of its non-reducing end with the receptor-receptor interface should be identical to those observed of tetra- and hexascaricrides. The fact that the non-reducing end 2-O-sulfate group is essential for tetra- and hexascaricrides but not for longer heparin fragments suggests that two different mechanisms of ternary complex formation may be in action.

We have shown previously that a heparin octascaricride is the minimal size requirement for detection of 2:2:1 ternary complexes. Using SEC, this is presumably because an octascaricride is the shortest heparin fragment that can stably dock two FGFs in a trans dimer orientation to form a 2:1 FGF1-heparin complex (29). Moreover, solution binding studies suggest that a cooperative mechanism may favor such FGF1 dimerization over monomeric FGF1-heparin complexes (29). SEC data presented here shows that an octascaricride was also the shortest heparin fragment that dimerized FGF2 (Fig. 7A). This could explain why the removal of the terminal 2-O-sulfate group from octascaricride and longer heparin fragments had little effect on their ability to stimulate FGF2-FGFR1c mitogenesis. Very short heparin fragments (dp4 and dp6) cannot dimerize FGF2, but support mitogenic signaling through the 2:2:2 symmetrical ternary complex for which non-reducing end 2-O-sulfation of the saccharide is essential. In contrast, longer heparin fragments dimerize FGF2 in a cooperative manner (Fig. 7C) and therefore induce a potent mitogenic signal through the 2:2:1 asymmetrical complex even in the absence of terminal 2-O-sulfation.

We consider the cooperative trans-dimerization of FGF2 by saccharides dp8 and larger as the critical event in signaling that accelerates the dimerization of FGFRs and enhances signal transduction across the plasma membrane. This proposition is supported by the observation that the dimerization of FGF2 by heparin saccharides (Fig. 7A) is associated with a potent mitogenic activity at very low saccharide concentrations (0.1 µg/ml; Fig. 3). The clear differences in dose-response profiles for heparin dp6 that bound just one FGF2, and heparin dp8 that efficiently dimerized FGF2, are suggestive of different mechanisms of receptor activation (see Fig. 8). This correlation between cooperative dimerization of FGF2 on heparin saccharides and strong mitogenic activity is compatible with the 2:2:1 molar ratio for the putative asymmetrical FGF-FGFR-heparin assembly predicted by the Pellegrini/Blundell model (Fig. 8B). When monomer complexes are formed between FGF2 and short heparin saccharides the symmetrical 2:2:2 Schlessinger/
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Mohammadi complex might prevail (Fig. 8A). However, FGF2 binding to the heparin saccharides is not in itself sufficient to elicit a mitogenic signal. FGF2 will bind hexasaccharides that lack the terminal 2-O-sulfation (Fig. 7B) even though these structures are inactive in mitogenesis assays (Fig. 5). From this we can conclude that end-chain 2-O-sulfation is essential for the mitogenic action of short heparin saccharides (dp4 and dp6) and that end chain 2-O-sulfation might be important for interactions at the receptor-receptor interface.

Acknowledgment—We are grateful to Dr. Malcolm Lyon for critically reading this manuscript.

REFERENCES

1. Ornitz, D. M., and Itoh, N. (2001) Genome Biol. 2, REVIEWS3005
2. Powers, C. J., McLeskey, S. W., and Wellstein, A. (2000) Endocr.-Relat. Cancer 7, 165–197
3. Botta, M., Manetti, F., and Corelli, F. (2000) Curr. Pharm. Des. 6, 1897–1924
4. Carmeliet, P., and Jain, R. K. (2000) Nature 407, 249–257
5. Browder, T., Folkman, J., and Pirie-Shepherd, S. (2000) J. Biol. Chem. 275, 1521–1524
6. Presta, M., Dell’Era, P., Mitola, S., Moroni, E., Ronca, R., and Rusnati, M. (2005) Cytokine Growth Factor Rev. 16, 159–178
7. Grose, R., and Dickson, C. (2005) Cytokine Growth Factor Rev. 16, 179–186
8. Eswarakumar, V. P., Lax, I., and Schlessinger, J. (2005) Cytokine Growth Factor Rev. 16, 139–149
9. Duan, D. S., Werner, S., and Williams, L. T. (1992) J. Biol. Chem. 267, 16076–16080
10. Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., and Ornitz, D. M. (2006) J. Biol. Chem. 281, 15694–15700
11. Dell, K. R., and Williams, L. T. (1992) J. Biol. Chem. 267, 21225–21229
12. Scotet, E., and Houssaint, E. (1998) Oncogene 17, 67–76
13. Yan, G., Fukabori, Y., McBride, G., Nikolaropolous, S., and McKeehan, W. L. (1993) Mol. Cell. Biol. 13, 4513–4522
14. DiGabriele, A. D., Lax, I., Chen, D. I., Svanh, C. M., Jaye, M., Schlessinger, J., and Hendrickson, W. A. (1998) Nature 393, 812–817
15. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Science 271, 1116–1120
16. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341
17. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) J. Biol. Chem. 268, 23906–23914
18. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) J. Biol. Chem. 273, 22936–22942
19. Ostrovsky, O., Berman, B., Gallagher, I., Mulloy, B., Fernig, D. G., Delehedde, M., and Ron, D. (2002) J. Biol. Chem. 277, 2444–2453
20. Pye, D. A., Vives, R. R., Hyde, P., and Gallagher, J. T. (2000) Glycobiology 10, 1183–1192
21. Guimond, S. E., and Turnbull, J. E. (1999) Curr. Biol. 9, 1343–1346
22. Gallagher, J. T. (2006) Biochem. Soc. Trans. 34, 438–441
23. Kreuger, J., Spillmann, D., Li, J. P., and Lindahl, U. (2006) J. Cell Biol. 174, 323–327
24. Pellegrini, L. (2001) Curr. Opin. Struct. Biol. 11, 629–634
25. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J., and Mohammadi, M. (2000) Mol. Cell 6, 743–750
26. Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B., and Blundell, T. L. (2000) Nature 407, 1029–1034
27. Harmer, N. J., Ilag, L. L., Mulloy, B., Pellegrini, L., Robinson, C. V., and Blundell, T. L. (2004) J. Mol. Biol. 339, 821–834
28. Goger, B., Halden, Y., Rek, A., Mosl, R., Pye, D., Gallagher, J., and Kungl, A. J. (2002) Biochemistry 41, 1640–1646
29. Robinson, C. J., Harmer, N. J., Goodger, S. J., Blundell, T. L., and Gallagher, J. T. (2005) J. Biol. Chem. 280, 42274–42282
30. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. (1992) Mol. Cell. Biol. 12, 240–247
31. Desai, U. R., Wang, H. M., and Linhardt, R. J. (1993) Biochemistry 32, 8140–8145
32. Desai, U. R., Wang, H. M., and Linhardt, R. J. (1993) Arch. Biochem. Biophys. 306, 461–468
33. Bielicki, J., Hopwood, J. J., Wilson, P. J., and Anson, D. S. (1993) Biochem. J. 289, 241–246
34. Deleted in proof
35. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) Science 259, 1918–1921
36. Harmer, N. J., Robinson, C. J., Adam, L. E., Ilag, L. L., Robinson, C. V., Gallagher, J. T., and Blundell, T. L. (2006) Biochem. J. 393, 741–748
37. Springer, B. A., Pantoliano, M. W., Barbera, F. A., Gunzyulu, P. L., Thompson, L. D., Herblin, W. F., Rosenfeld, S. A., and Book, G. W. (1994) J. Biol. Chem. 269, 26879–26884