Cooperative Dimerization of Fibroblast Growth Factor 1 (FGF1) upon a Single Heparin Saccharide May Drive the Formation of 2:2:1 FGF1-FGFR2c-Heparin Ternary Complexes*

The related glycosaminoglycans heparin and heparan sulfate are essential for the activity of the fibroblast growth factor (FGF) family as they form an integral part of the signaling complex at the cell surface. Using size-exclusion chromatography we have studied the capacities of a variety of heparin oligosaccharides to bind FGF1 and FGFR2c both separately and together in ternary complexes. In the absence of heparin, FGF1 had no detectable affinity for FGFR2c. However, 2:2:1 complexes formed spontaneously in solution between FGF1, FGFR2c, and heparin octasaccharide (dp8). The dp8 sample was the shortest chain length that bound FGFR2c, that dimerized FGF1, and that promoted a strong mitogenic response to FGF1 through FGFR2c. Heparin hexasaccharide and various selectively desulfated heparin dp12s failed to bind FGFR2c and could only interact with FGF1 monomERICally. These saccharides formed 1:1:1 complexes with FGF1 and FGFR2c, which had no tendency to self-associate, suggesting that binding of two FGF1 molecules to the same saccharide chain is a prerequisite for subsequent FGFR2c dimerization. We found that FGF1 dimerization upon heparin was favored over monomeric interactions even when a large excess of saccharide was present. A cooperative mechanism of FGF1 dimerization could explain how 2:2:1 signaling complexes form at the cell surface, an environment rich in heparan sulfate.

The fibroblast growth factor (FGF) family of proteins control the proliferation, migration, differentiation, and survival of a wide range of cell types (1). The FGFs are of fundamental importance during embryogenesis where they orchestrate the complex processes of organ formation and limb development (2). Dysregulated FGF expression is often associated with malignant transformation, because it facilitates the growth, survival, and metastatic spread of cancer cells (3). In humans 22 FGFs have been described along with 4 FGF receptor (FGFR) subtypes (11), which can interact with several different FGFs. The FGFs themselves are also not limited to a single FGFR type, for example FGF1 (acidic FGF) is a universal ligand, recognized by all FGF receptors (10). On the other hand, FGF7 (keratinocyte growth factor) is only capable of binding FGFR2b (11). The D2 domain of the FGFRs contains a short stretch of basic amino acids recognized by all FGF receptors (10). On the other hand, FGF7 (keratinocyte growth factor) is only capable of binding FGFR2b (11). The D2 domain of the FGFRs contains a short stretch of basic amino acids referred to as the K18K loop (12). This region mediates interaction with heparin and HS (13) and is essential for receptor function. The FGFRs are also heparin-binding proteins, and it is now appreciated that signal transduction requires the formation of a ternary complex at the cell surface formed between FGF, FGFR, and heparan sulfate (HS).

Heparin and HS are two structurally and functionally related glycosaminoglycans (GAGs (14–16)). HS is expressed on core proteins in the extracellular matrix and on the surface of almost all mammalian cell types. In contrast, heparin is produced by mast cells where it is stored in granules for release as free chains. Biosynthesis occurs in the Golgi apparatus with the same enzymes involved for both GAGs (17). The in HS synthesis an unknown mechanism targets the modification steps to resulting in a significant degree of structural heterogeneity (17). During HS synthesis an unknown mechanism targets the modification steps to discrete regions of the polysaccharide chain. This creates a domain organization where sulfated and unsulfated regions of roughly equal length (14–18 disaccharides) alternate in a relatively uniform pattern along the HS chain (19). Within the sulfated regions are stretches of IdoA(2S)-GlcNS with varying patterns of 6-O-sulfation (S-domains, 2–7 disaccharides long (20)), which are flanked by stretches of alternat-
ing N-sulfated and N-acetylated disaccharides with highly variable patterns of O-sulfation (19). In contrast to HS, heparin chains are shorter and highly sulfated throughout. Heparin saccharides can be likened to the S-domains regions of HS, and the relative ease with which they can be prepared means that heparin is often used experimentally as a substitute for HS. The heterogeneity of sulfation present in HS, and to a lesser extent heparin, is key to their functions, because distinct patterns of sulfation define binding sites for specific protein ligands (14).

Variations in the distribution of basic amino acids upon the surface of the FGFs lead to markedly different heparin-binding properties (21). FGF2 (basic FGF) will bind to heparin/HS pentasaccharides (dp5) that are devoid of 6-O-sulfate groups as long as they contain one or more critically positioned N- and 2-O-sulfates (22). In contrast, FGF1 binding to heparin/HS oligosaccharides is thought to require N-, 2-0-, and 6-O-sulfation (23). Heparin adopts a helical secondary structure in solution, leading to the sulfate groups of contiguous disaccharide units alternating between opposite sides of the helical axis (24). This conformation allows protein ligands to dimerize through interactions with opposite faces of the same short saccharide sequence. Crystal structures of 2:1 complexes formed between FGF1 and heparin dp10 show this trans binding arrangement (25). Whether FGF dimerization can occur in vivo on HS chains has important implications for how ternary complexes form and what stoichiometry these complexes adopt.

Two crystal structures of FGF-FGFR-heparin ternary complexes have been published. Schlessinger, Mohammadi, and colleagues (26) describe a putative 2:2:2 complex formed between FGF2, FGFR1c, and heparin dp10 [excitation max., 425 nm; emission max., 520 nm]. All elution profiles (7.2). The same column and HPLC system were used for all runs. Proteins and/or heparin fragments were preincubated for 30 min at room temperature in 240 µl of column running buffer prior to column loading. These samples were then eluted isocratically at 0.5 ml/min, and the absorbance at 280 nm (aromatic amino acid side chains) or 232 nm (unsaturated bonds of heparin fragments) was recorded. The elution of AMAC-labeled heparin dp12 was monitored by fluorescence detection (excitation max., 425 nm; emission max., 520 nm). All elution profiles shown are representative of at least two repeats.

The Superdex 200 column was calibrated using gel filtration HMW and LMW protein calibration kits (Amersham Biosciences). The column void (V0) and total (VT) volumes were determined using blue dextran 2000 and sodium dichromate, respectively. The elution volumes (Ve) of protein standards were converted into a calibration chart of Kd against molecular mass [Kd = (V0 - Ve)/((Ve - Vt)]. A line of best fit was fitted to the calibration data using Microsoft Excel, and the equation of this line was used to estimate mass according to observed Kd. We also plotted the square root of −log10(Kd) against the Stokes radius for the protein standards. The equation for the line of best fit on this chart was used to calculate the Stokes radius of eluted species.

**Disaccharide Composition Analysis of Heparin Oligosaccharides**—Heparin saccharides (200 µg) were suspended in heparin buffer (50 mM NaAc, 0.5 mM CaAc2, 0.1 mg/ml bovine serum albumin, pH 7.0). Aliquots of heparinase I, II, and III were added to a concentration of 100 µU/ml, and digests were incubated at 37 °C for 16 h. Disaccharides were purified from the heparin digests using a Superdex peptide column (10 × 300 mm, Amersham Biosciences) running at 0.5 ml/min in 0.1 M NH4HCO3, while monitoring the absorbance of the eluate at 232 nm. Heparin disaccharides were then repeatedly lyophilized to remove NH4HCO3, until their conductivity in 1 ml of dH2O, pH 3.5, was <200 µS.

A single ProPac PA-1 strong anion-exchange HPLC column (4.6 × 250 mm, Dionex, Camberley, UK) was linked to a Hewlett Packard 1100 series high-performance chromatography system. The column was run at a flow rate of 1 ml/min in MilliQ ultrapure water, pH 3.5. Lyophilized disaccharides were resuspended in 1 ml of this buffer and loaded onto the column prior to the application of the gradient (45 min, 0–1.0 M NaCl). The eluate was monitored for absorbance at 232 nm, and peaks were identified by comparison to heparin disaccharide standards.

**Mitogenesis Assay**—BaF-32 cells are a lymphoblastoid cell line deficient for HS synthesis (33). These cells have no endogenous expression of the FGFs or FGFRs, but were transfected with FGFR2c. FGF1-stimulated proliferation of the transfected cells required the addition of HS or heparin saccharides to the culture medium. BaF-32 cells were routinely maintained in interleukin-3-conditioned medium as described above (34). For the mitogenesis assay, cells were serum-starved for 2 days, washed 3 times in phosphate-buffered saline then seeded at a low density (2.5 × 103 cells/ml) onto a 96-well plate in RPMI 1640 supplemented with 10% (v/v) horse serum. Cells were incubated for 4 days at 37 °C in medium containing FGF1 (100 ng/ml) and sized heparin oligo-
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Saccharides (0.1–2.0 μg/ml) or porcine mucosal HS (10 μg/ml). The CytoLite™ kit (Packard BioScience) was used as a chemiluminometric assay of viable cell number.

RESULTS

Size-exclusion Chromatography of FGF1, FGFR2c, and Heparin dp12—In this study we applied the technique to investigate the formation in solution of ternary complexes between FGF1, FGFR2c, and heparin. A Superdex 200 HR 10/30 column was connected to an HPLC system and equilibrated in a buffer of physiological ionic strength. A

FIGURE 1. Size-exclusion chromatography of FGF1, FGFR2c, and heparin dp12. Superdex 200 HR 10/30 size-exclusion chromatography (see “Experimental Procedures”). 5 nmol of FGF1 (thick black line), 5 nmol of FGFR2c (thick gray line), and 30 nmol of heparin dp12 (thin black line) were applied separately to the column. Elution of protein and saccharide was followed by absorbance at 280 and 232 nm, respectively.

TABLE ONE

Elution times, Stokes radii, and mass calculations for the major observed peaks

| Fig. | Incubation | Elution time (min) | Stokes radius (Å) | Apparent mass (kDa) | Predicted complex | Expected mass (kDa) | Actual mass (kDa) |
|------|------------|--------------------|-------------------|---------------------|-------------------|---------------------|-------------------|
| 1    | FGF1 (F1)  | 36.1               | 17.3              | 16                  | 2:1               | 55                  | 35                |
| 1    | FGFR2 (R2) | 33.0               | 26.2              | 33                  | 2:1               | 31                  | 18                |
| 2A   | F1 + dp12  | 30.6               | 32.6              | 57                  | 1:1               | 50                  | 34                |
| 2B   | F1 + R2 + dp12 | 27.6           | 41.4              | 113                 | 2:2:1             | 122                 | 84                |
| 3A   | F1 + dp6   | 33.8               | 23.9              | 28                  | 1:1               | 31                  | 18                |
| 3A   | F1 + dp8   | 32.0               | 29.2              | 42                  | 2:1               | 50                  | 34                |
| 3B   | R2 + dp8   | 31.4               | 31.1              | 49                  | 1:1               | 50                  | 27                |
| 3B   | R2 + dp10  | 30.5               | 33.8              | 60                  | 1:1               | 53                  | 27                |
| 4    | F1 + R2 + dp6 | 31.5           | 30.6              | 47                  | 1:1:1             | 64                  | 42                |
| 4    | F1 + R2 + dp8 | 29.1           | 38.4              | 84                  | 2:2:1             | 117                 | 83                |
| 4    | F1 + R2 + dp8 | 30.7           | 33.3              | 58                  | 1:1:1             | 67                  | 43                |
| 4    | F1 + R2 + dp10 | 28.4          | 40.6              | 99                  | 2:2:1             | 119                 | 84                |
| 6A   | F1 + DNS5-dp12 | 33.3         | 25.3              | 31                  | 1:1               | 38                  | 19                |
| 6A   | F1 + D5S-dp12 | 33.5           | 24.8              | 30                  | 1:1               | 36                  | 19                |
| 6A   | F1 + D6S-dp12 | 32.6           | 27.6              | 37                  | 1:1               | 36                  | 19                |
| 6B   | F1 + R2 + DNS-dp12 | 30.3         | 34.7              | 64                  | 1:1:1             | 71                  | 44                |
| 7    | F1 + dp12 (1:4 ratio) | 31.4       | 31.2              | 49                  | 2:1               | 55                  | 35                |
usly the elution of protein (absorbance at 280 nm) and saccharide (fluorescence) from the Superdex 200 column. The presence of the AMAC-tag had no significant effect on the elution position of the FGF1-dp12 and FGF1-FGFR2c-dp12 complexes observed. The sensitivity of this approach also allowed us to detect the same complexes at concentrations below that which protein absorbance could be monitored (<200 pmol, data not shown). SEC with fluorescent end labeling of saccharides could be used in future studies to investigate protein–heparin complex formation at physiologically relevant concentration levels.

A 2:1 incubation of FGF1 and AMAC-dp12 led to the formation of a single complex (\( R_s = 32.6 \) Å) in which all of the FGF1 and saccharide was present (Fig. 2A). The crystal structure of a 2:1 FGF1-dp10 complex has been previously published (25) and has a predicted hydrodynamic radius of 29.1 Å (using the program Crysol (39)). These data indicate that the observed FGF1-dp12 complex has a 2:1 stoichiometry. Such a complex would have a mass of 35 kDa, however, the apparent mass of the peak calculated from the calibration data was 57 kDa. This could be explained by the large apparent mass of the heparin dp12. The sum of the apparent masses of the components in the 2:1 complex was 55 kDa (16 + 16 + 22 kDa).

Using SEC we failed to detect any interaction between FGF1 and FGFR2c in the absence of heparin (Fig. 2B). However, when these two proteins were incubated in a 2:2:1 ratio with heparin dp12, a single major peak was observed with a much earlier elution position. All of the AMAC-tagged dp12 was present within this complex indicating that a 2:2:1 complex was formed. The Stokes radius of this ternary complex (41.4 Å, using heparin dp12) was also closer to that predicted from the crystal structure of the 2:2:1 Pellegrini complex (40.9 Å, dp10), than that of the 2:2:2 Schlessinger/Mohammadi complex (37.7 Å, dp10, calculated using Crysol (39)). An apparent mass of 113 kDa was calculated for the ternary complex. The discrepancy between this mass and the predicted 122-kDa apparent mass of a 2:2:1 FGF1-FGFR2c-dp12 complex is likely to be a consequence of the reduced hydrodynamic volume of the components when they are brought closely together into a multicomponent complex. In particular, the small dp12 saccharide is surrounded by protein within the ternary complex and so probably contributes less to the overall hydrodynamic volume (and therefore apparent mass) than would be predicted from its relatively large Stokes radius. Ternary complexes prepared under exactly the same conditions have been prepared by Harmer et al. (29) (using heparin dp10), with a 2:2:1 stoichiometry confirmed by mass spectrometry and analytical ultracentrifugation.

### TABLE TWO

| Heparin saccharide | Elution time | Stokes radius | Apparent mass | Actual mass |
|--------------------|-------------|---------------|---------------|------------|
| dp6                | 36.6        | 15.7          | 14.6          | 1.72       |
| dp8                | 35.9        | 17.7          | 17.0          | 2.29       |
| dp10               | 35.3        | 19.4          | 19.5          | 2.97       |
| dp12               | 34.8        | 21.0          | 22.2          | 3.44       |
| DNS-dp12           | 34.9        | 20.7          | 21.6          | 3.15       |
| D2S-dp12           | 35.2        | 19.7          | 20.0          | 2.97       |
| D65-dp12           | 35.4        | 19.1          | 19.1          | 2.97       |

#### FIGURE 2. Heparin dp12 binds FGF1 dimerically and forms 2:2:1 FGF1-FGFR2c-saccharide complexes.

Superdex 200 HR 10/30 size-exclusion chromatography. A, 2 nmol of FGF1 were incubated with (thick black line) or without (thin gray line) 1 nmol of AMAC-tagged heparin dp12 with the protein absorbance at 280 nm monitored. Additionally, elution of AMAC-dp12 in the FGF1 incubation was followed by fluorescence detection (thin black line). B, 2 nmol of FGF1 and FGFR2c were incubated with (thick black line) or without (thin gray line) 1 nmol of AMAC-tagged heparin dp12 with the protein absorbance at 280 nm monitored. Additionally, elution of AMAC-dp12 in the FGF1 incubation was followed by fluorescence detection (thin black line).

#### Octasaccharides Are the Shortest Heparin Fragments Capable of Dimerizing FGF1 and Forming 2:2:1 FGF1-FGFR2c-Heparin Complexes—

We investigated the ability of a range of sized heparin saccharides to bind FGF1 and FGFR2c. Incubation with heparin dp4 had little effect on the elution position of FGF1 (Fig. 3A). In contrast FGF1 was split equally by dp6 into heparin-bound and -unbound peaks (Fig. 3A). This would be expected for a 1:1 FGF1-dp6 complex, because incubations were carried out in a 2:1 ratio of FGF1 to saccharide. When FGF1 was incubated with heparin dp8, a major peak was observed with a significantly larger hydrodynamic volume (\( R_s = 29.2 \) Å) than the FGF1-dp6 complex (23.9 Å, Fig. 3A). This peak is likely to represent a 1:1 association of FGF1-dp8 as the apparent mass (42 kDa) was 16 kDa larger than that observed for the 1:1 FGF1-dp6 complex (28 kDa). In contrast to FGF1, the elution position of FGFR2c was unaffected by heparin dp6, indicating that no stable complex formed (Fig. 3B). However, when incubated with heparin dp8 a peak consistent with a 1:1 FGFR2c-dp8 complex was observed. An octasaccharide has previously been reported as the minimal heparin length for binding to FGFR1c (40) and FGFR4 (41). The FGFR2c-dp12 complex also appeared to have a 1:1 stoichiometry (\( R_s = 33.1 \) Å, apparent mass 57 kDa, data not shown).
The same sized heparin oligosaccharides were tested for their ability to promote ligand-receptor binding (Fig. 4). Heparin dp6, which bound FGF1 in a 1:1 ratio (23.9 Å, Fig. 3A), formed a complex with a larger hydrodynamic volume when incubated with both FGF1 and FGFR2c (30.6 Å). This indicates that, although FGFR2c cannot bind to FGF1 or dp6 alone, it can bind to FGF1:dp6 to form a 1:1:1 complex. We observed no tendency for these 1:1:1 ternary complexes to associate with one another to form structures with a 2:2:2 stoichiometry, like that reported by Schlessinger et al. (26). The elution profile for the FGF1:FGFR2c:dp8 incubation was clearly resolved into two peaks with different hydrodynamic properties (Fig. 4). The apparent mass estimates (84 kDa) of the early eluting peak suggest that it represents a 2:2:1 FGF1:FGFR2c:dp8 ternary complex (TABLE ONE). A small subpopulation of our octasaccharides may have lacked one or more critical sulfate groups. The later eluting peak could represent a FGF1:FGFR2c:dp6 complex to which one or more of the protein components was unable to bind. The heparin dp10 sample formed one major peak with a Stokes Radius of 40.6 Å (Fig. 4), almost identical to the 40.9 Å predicted using Crysol (39). This structure has been extensively characterized previously as 2:2:1 FGF1:FGFR2c:dp10 (27, 29).

Octasaccharides Are the Shortest Heparin Fragments Capable of Stimulating FGF1-mediated Mitogenesis through FGFR2c—Heparin dp6 was the shortest oligosaccharide capable of forming 2:2:1 FGF1:FGFR2c:heparin complexes (Fig. 4). In contrast, heparin dp6 formed ternary complexes with a 1:1:1 stoichiometry. To investigate the relevance of these complexes to FGF1 signaling we used a viable cell assay to quantify the effects of various sized heparin oligosaccharides on FGFR2c-mediated cell proliferation (Fig. 5). BaF-32 cells were transfected to express FGFR2c as their sole FGF receptor. These cells were deficient for HS synthesis, and therefore mitogenesis in response to FGF1 only occurred when exogenous HS or heparin was added to the media. At the heparin concentrations used in this assay both dp4 and dp6 had little effect on FGF1-mediated cell proliferation. In contrast, FGF1 with either heparin dp8 or dp10 raised cell numbers significantly in comparison to FGF1 alone. At a 2 µg/ml concentration both dp8 and dp10 were almost as effective as the HS control (10 µg/ml) at promoting FGF1 activity. These data suggest that if 1:1:1 FGF1:FGFR2c:heparin complexes form at the cell surface, they are incapable of mediating FGF1 signal transduction.

Selectively Desulfated Heparin 12-mers Bind FGF1 Monomerically and Form 1:1:1 FGF1:FGFR2c:Heparin Ternary Complexes—To investigate how the sulfation pattern of heparin contributed to its binding of FGF1 and FGFR2c, we prepared a range of heparin dodecasaccharides that were deficient for sulfation at either the N-, 6-O-, or 2-O-positions. The Stokes radii of these saccharides on the Superdex 200 column (19.1–20.7 Å, TABLE TWO) were close to that of the untreated dp12 sample (21.0 Å), showing that the hydrodynamic properties of these saccharides were largely unaffected by selective desulfation. Their disaccharide composition was assessed to determine the selectivity and efficiency of the desulfation procedures (data not shown). The N-de-
sulfated/N-reacetylated (DNS) dp12 had its N-sulfated disaccharide content reduced from 96% to just 2% with negligible loss of O-sulfation. Similarly, the 2-O-desulfated (D2S) sample had its 2-O-sulfate content reduced from 90% of disaccharides to just 5%. However, whereas N-sulfation was essentially unaffected in the D2S dp12, there was some loss of 6-O-sulfates (from 88% to 49%). The partially 6-O-desulfated (D6S) dp12 saccharide had its 6-O-sulfate content reduced from 88% to 21% (~24% of the level of the parent heparin) with negligible loss of N- and 2-O-sulfation.

All three selectively desulfated dp12 saccharides were found to form complexes with FGF1 (Fig. 6A). However, the Stokes radii of these complexes (24.8 – 27.6 Å, Table ONE) were all significantly less than that of the 2:1 complex formed between FGF1 and the native dp12 (32.6 Å, Fig. 2A). It is likely that these saccharides are binding FGF1 in 1:1 complexes and that, unlike with the parent heparin dp12 saccharide, this interaction precludes the binding of a second FGF1 molecule. The affinity of FGFR2c for the desulfated dp12 saccharides was also assessed, but in each case, unlike with the parent heparin dp12, there was no evidence for a stable interaction (data not shown).

Having determined that the selectively desulfated heparin dp12s failed to dimerize FGF1 and were unable to bind FGFR2c, we investigated whether they could still mediate binding between FGF1 and FGFR2c. The DNS-dp12 sample formed 1:1 complexes with FGF1 of which the Stokes radius was 25.3 Å (Fig. 6B and Table ONE). When incubated with both FGF1 and FGFR2c, a larger complex was observed (34.7 Å). This complex eluted much later than the 2:2:1 complex formed by the unmodified parent dp12, and had an apparent mass consistent with a 1:1:1 ternary complex (64 kDa, Fig. 6B and Table ONE). Equivalent results were also obtained with the D2S and D6S heparin dp12 samples (data not shown).

The Effect of Oligosaccharide Concentration on FGF1 Dimerization and Ternary Complex Formation—Our data suggest that the formation of a 2:1 FGF1-saccharide complex is necessary for subsequent receptor dimerization. However, saccharide (HS) at the cell surface will be much more abundant than the FGF1 ligand. Therefore we investigated the effect that heparin dp12 concentration had on the stoichiometry of FGF1-dp12 complexes (Fig. 7). As observed previously (Fig. 2A), the incubation of FGF1 and dp12 at a 2:1 molar ratio resulted in full recruitment of the FGF1 into 2:1 complexes (Rg = 32.6 Å). At a 1:1 ratio of incubation there should have been sufficient saccharide present to bind all of the FGF1 in 1:1 complexes; however, the Stokes radius of the observed peak (32.4 Å) suggests that 2:1 FGF1-dp12 complexes still predominated. Although for this incubation no 1:1 peak was present, we observed that the major peak was broader and eluted slightly later from

FIGURE 5. A heparin octasaccharide is necessary to stimulate mitogenic signaling of FGF1 through FGFR2c. BaF-32 cells transfected with FGFR2c were serum-starved for 2 days, washed three times with phosphate-buffered saline, then seeded at a low density (2.5 x 10⁴ cells/ml) in 96-well plates (see “Experimental Procedures”). Cells were then incubated for 4 days with 100 ng/ml FGF1 in the presence of various concentrations of heparin dp4 (white columns), dp6 (gray columns), dp8 (striped columns), or dp10 (black columns). Viable cell number was assayed using the CytoLite™ kit (100% = CytoLite™ incorporation for FGF1 with porcine mucosal HS [10 μg/ml], 0% = CytoLite™ incorporation for FGF1 alone). This chart represents one of three experiments with each data point performed in triplicate.

FIGURE 6. Selectively desulfated heparin dp12 saccharides bind FGF1 monomerically and form 1:1 complexes with FGF1 and FGFR2c. Superdex 200 HR 10/30 SEC. A, 3 nmol of FGF1 were incubated with 1.5 nmol of N-desulfated/N-reacetylated (NDS, thick black line), 2-O-desulfated (D2S, thick gray line), or preferentially 6-O-desulfated (D6S, thin black line) heparin dp12. The predetermined elution positions of FGF1 and the 2:1 FGF1-dp12 complex are indicated. B, 3 nmol of FGF1 were incubated alone (thick black line), with 1.5 nmol of NDS heparin dp12 (thick gray line), or with 3 nmol of FGFR2c and 1.5 nmol of NDS heparin dp12 (thin black line). The predetermined elution position of the 2:2:1 FGF1-FGFR2c-dp12 complex and predicted stoichiometries of the protein-heparin complexes are indicated.

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The FGF signaling species at the cell surface is a ternary complex formed between the FGF ligand, its receptor, and heparin/HS (42). Two separate models of FGF ternary complexes have been published. Schlessinger, Mohammadi, and colleagues presented the crystal structure of a symmetrical dimer formed by two 1:1 FGF2:FGFR1c decasaccharide complexes (26). In contrast, Pellegrini and colleagues (27) described an asymmetrical structure in which two FGFR2c subunits were bridged by an FGF1 dimer formed upon a single heparin decasaccharide. This 2:2 complex was present in crystals as part of a higher oligomeric structure defined by intermolecular crystal packing interactions (28). The higher oligomeric structure also allowed a further 2:2:1 complex to be tentatively identified as a candidate for a structure existing in solution; this involved direct interactions between the two FGF-FGFR half-complexes, resembling those of the 2:2:2 complex described by Schlessinger et al. (26, 28). Further analysis suggested that, by varying the conditions of preparation, both architectures can be formed from the same proteins (29). However, in solution the complex following the Schlessinger preparation contains just one heparin chain (29). Therefore, there appears to be two potential architectures for the 2:2 FGF-FGFR signaling complex, both of which can be formed upon a single HS chain in vivo. We have expanded on these studies by using a variety of defined heparin fragments in a SEC approach to investigate the role that heparin plays in the assembly of 2:2 FGF-FGFR complexes.

We observed that FGF1:FGFR2c-heparin ternary complexes formed spontaneously in solution. The Stokes radius and apparent mass calculations from SEC both suggest that these complexes had a 2:2:1 stoichiometry. In addition, by simultaneously measuring protein absorbance (280 nm) and saccharide fluorescence (AMAC tagging), we demonstrated that at a 2:2:1 FGF1:FGFR2c:dp12 incubation ratio all of the protein and saccharide was colocalized in the ternary complex (Fig. 2B). Ternary complexes pooled from an identical column were recently characterized by both analytical ultracentrifugation and mass spectrometry as having a 2:2:1 stoichiometry (29). These data offer compelling evidence that, when formed in solution, 2:2 FGF1:FGFR2c-heparin oligosaccharide complexes predominate. FGF1 has previously been crystallized in a 2:1 complex with heparin decasaccharide (25). Again, using a combination of Stokes radius, apparent mass calculation, and ratio of incubation (with AMAC-tagged saccharide, Fig. 2A) we demonstrated that a 2:1 FGF1:dp12 complex predominated in solution.

A range of size-defined heparin oligosaccharides were tested both for their ability to dimerize FGF1 and their ability to bind FGFR2c in ternary complexes with FGF1. In all cases, the heparin fragments that dimerized FGF1 formed 2:2 complexes between FGF1 and FGFR2c. The shortest saccharide capable of inducing the formation of FGF1 dimers was found to be a heparin dp6 (Fig. 3A), as observed previously (25). This saccharide was also the shortest capable of interacting with FGFR2c alone (1:1 complex, Fig. 3B) and the shortest that formed 2:2:1 complexes (Fig. 4) (29). Notably, octasaccharides were also the shortest heparin fragments that efficiently induced FGF1 mitogenesis in a HS-deficient FGFR2c-transfected cell line (Fig. 5). These data suggest that the ability of a saccharide to dimerize FGF1 may be a critical factor in determining whether it can promote FGF1 signaling by bringing together two FGFR2c subunits.

The heparin dp6 sample bound quantitatively to FGF1 in a 1:1 stoichiometry (Fig. 3A). A study of the heparin-protein interactions in the 2:2:1 Pellegrini-type complex correctly predicted that the hexasaccharide would be the shortest heparin fragment capable of forming 1:1:1 FGF1:FGFR2c-heparin complexes (Fig. 4) (28). FGFR2c was recruited into 1:1:1 complexes despite showing no stable binding to either FGF1 or heparin dp6 alone (Figs. 2B and 3B). In common with the heparin hexasaccharide, various selectively desulfated heparin dp12 samples also bound FGF1 monomerically (Fig. 6C) and showed no affinity for FGFR2c. In each case these saccharides bound FGF1 and FGFR2c in an apparent 1:1:1 stoichiometry (Fig. 6B). Therefore, saccharides that are incapable of binding FGFR2c alone will bind this receptor in ternary complexes provided they have an affinity for FGF1. This observation suggests that FGF1 binding precedes that of FGFR2c, as has been suggested by other studies of this system using different techniques (43). In the 2:2:1 Pellegrini complex, the FGFR2c subunit interacted with the three sulfate groups on the disaccharide at the non-reducing end of the heparin dp10 (28). Our data show that there could be some flexibility in these interactions as all three selectively desulfated heparin dp12s formed 1:1:1 complexes with FGF1 and FGFR2c (Fig. 6B). Alternatively, the FGF1:FGFR2c interaction may not require a direct heparin interaction with the receptor. One of the FGFR2c subunits in the ternary complex described by Pellegrini et al. (27) was bound to an FGF1 molecule without additional heparin contacts. Heparin-HS interactions with FGF have previously been reported to reduce the conformational flexibility of the protein ligand (44, 45). It is possible that binding to heparin saccharides might induce a subtle conformational change within FGF1 that stabilizes a favorable topology of the FGFR binding site.

The 1:1:1 FGF1:FGFR2c-heparin complexes that we observed showed no inherent affinity for one another. The formation of complexes in which FGFR2c is dimerized therefore appears to be dependent on the ability of a single heparin saccharide to act as a surface upon which FGF1, and subsequently FGFR2c, can be dimerized. This function of

![Figure 7. The effects of heparin dp12 concentration on FGF1 dimerization. Superdex 200 HR 10/30 SEC. 2 nmol of FGF1 were incubated with 1 nmol (2:1 ratio of FGF1 and dp12, thick black line), 2 nmol (1:1 ratio, thick gray line), and 8 nmol (1:4 ratio, thin black line) of heparin dp12. The predetermined elution positions of FGF1, the 1:1 FGF1:DNS-dp12 complex, and the 2:1 FGF1:dp12 are indicated.](image-url)
Heparin-mediated FGF1 Dimerization and Signal Complex Formation

A signaling mechanism that requires two binary FGF-FGFR complexes to form on adjacent sides of the same stretch of sequence within an HS chain would not appear to be particularly efficient. However, this does not take into account factors that may drive the process of dimerization. We investigated how changing the concentration of heparin far outweighs that of FGF1 (Fig. 7). A cooperative mode of FGF dimerization could drive the formation of 2:2 FGF-FGFR-HS complexes at the cell surface where HS chains are abundant (Fig. 8).

In this study we describe the use of the SEC technique to study the heparin-mediated interaction of FGF1 with FGFR2c. This approach has several advantages over more established techniques (48). Heparin-protein complexes were both formed and resolved in a physiologically relevant buffer solution. Direct visualization of protein by absorbance at UV wavelengths allowed us to keep our complexes free in solution without having to immobilize components or bind them with antibodies or other detecting agents. Alternatively, fluorescent tagging of saccharides can be used to detect complexes formed between components incubated at physiologically relevant concentrations. The SEC column proved fast and simple to run with good reproducibility. Calibration using protein standards gave mass estimates and Stokes radii for our heparin structure is the ligand upon which FGFR dimerization occurs. The receptor subunits within this 2:2:1 FGF-FGFR-heparin Pellegrini-like complex (27) are then activated by transphosphorylation to initiate FGF signal transduction.

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