Control of Fibroblast Growth Factor (FGF) 7- and FGF1-induced Mitogenesis and Downstream Signaling by Distinct Heparin Octasaccharide Motifs*

Received for publication, February 17, 2006, and in revised form, May 5, 2006. Published, JBC Papers in Press, May 25, 2006, DOI 10.1074/jbc.M601559200

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Variation in length, disaccharide composition, and sulfation of heparan sulfate (HS) affects fibroblast growth factor (FGF) signaling. However, it is unclear whether the specific distribution of groups within oligosaccharides or random variations in charge density underlies the effects. Recently we showed that a mixture of undersulfated octasaccharides exhibiting 7 and 8 sulfates (7,8-S-OctaF7) generated from heparin had the highest affinity for FGF7 monitored by salt resistance (>0.60 m salt) of octasaccharide-FGF7 complexes. 7,8-S-OctaF7 also had the highest specific activity for formation of a complex with dimeric FGFR2IIIb competent to bind FGF7. Here we show that when endogenous HS was inhibited by chlorate treatment, 7,8-S-OctaF7 specifically supported FGF7-stimulated DNA synthesis and downstream signaling in FGFR2IIIb-expressing mouse keratinocytes. It failed to support FGF1 signaling in both HS-deficient mouse keratinocytes and 3T3 fibroblasts. In contrast, abundant, more highly sulfated and heterogeneous mixtures of octasaccharides with lower affinity (0.30–0.60 m salt) for FGF7 supported FGF1-induced signaling in both cell types. In contrast to the two-component 7,8-S-OctaF7 mixture from FGF7, the high affinity octasaccharide fraction from FGF1 was a heterogeneous mixture with components ranging from 8 to 12 sulfates with 11-S-octasaccharides the most abundant. The high affinity fraction exhibited similar properties to the lower affinity fractions from both FGF1 and FGF7. Octasaccharide mixtures eluting from FGF1 between 0.30 and 0.60 m and above 0.60 m salt were nearly equal in support of FGF1 signaling in fibroblasts and keratinocytes. Both were deficient in support of FGF7-induced signaling in keratinocytes. The results show that both variations in overall charge density and specific distribution of charged groups within HS motifs exhibit FGF-specific control over formation of FGF-HS-FGFR complexes and downstream signaling.

‡ This work was supported by United States Public Health Service National Institutes of Health, NIDDK Grant DK35310 and NCI Grant CA59971 and by GS PlatZ, Ltd. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviations used are: FGF, fibroblast growth factor; 7,8-S-OctaF7, FGF7 affinity-purified mixture of 7- and 8-sulfated octasaccharides; FGFR, fibroblast growth factor receptor tyrosine kinase; GlC6A, glucuronic acid; GlCN, glucosamine; GST, glutathione S-transferase; HexA2SGlcNS6S, 2-O-sulfate; H or PIMH, porcine intestinal mucosa heparin; HS, heparan sulfate; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SAX-HPLC, strong anion exchange high performance liquid chromatography; MAP kinase, mitogen-activated protein kinase; MK, mouse skin keratinocytes; ERK, extracellular signal-regulated kinase.

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in FGF signaling similar to what has been demonstrated for anticoagulation (23).

Recently we exploited the scalability, stability, and selectivity in regard to heparin oligosaccharide motifs of bacterial-derived FGF7 fused at the N terminus with GST to fractionate and characterize heparin oligosaccharides that interact with its heparin-binding domain (18, 24–26). Only 0.7–3.7% of oligosaccharide mixtures from 8 to 14 monosaccharide units, respectively, bound FGF7 with high affinity based on salt resistance of oligosaccharide-FGF7 complexes. The same minority fractions bound independently to dimers of FGFR2IIIb to form HS-FGFR2IIIb complexes that then bind FGF7. The shortest high affinity oligosaccharide capable of supporting binding of FGF7 to FGFR2IIIb was an undersulfated octasaccharide exhibiting 7 and 8 sulfates (7,8-S-OctaF7). Based on disaccharide analysis and its anticoagulant activity, it was deduced that 7,8-S-OctaF7 contains the rare N-unsubstituted ΔHexA2SGlcN6S, a trisulfated disaccharide most likely exhibiting a 3-O-sulfate, and a variably unsulfated/menosulfated disaccharide. This was in contrast to more abundant and heterogeneous octasaccharides with lower affinity. They were more highly sulfated and exhibited predominantly 11 and 12 sulfates and the most common trisulfated disaccharide in heparin, ΔHexA2SGlcNS6S. Surprisingly, these results indicated that affinity for FGF7 based on salt resistance of binding does not increase proportionally to anionic density of heparin octasaccharides. Moreover the undersulfated 7,8-S-OctaF7 mixture with highest affinity for FGF7 exhibited the highest specific activity for binding to FGFR2IIIb. (7,8-S-OctaF7)-FGFR2IIIb complexes supported the binding of FGF7, whereas the more highly sulfated octasaccharides with lower affinity failed to support an FGF7-HS-FGFR2IIIb complex (24, 26).

The above results demonstrated that specific distribution of sulfate groups within an undersulfated octasaccharide might play a role in formation of oligomeric FGF7-HS-FGFR2IIIb complexes involved in transmembrane signaling. To determine whether 7,8-S-OctaF7 also exhibits specificity for FGF7 signaling, we compared its ability to support FGF7 and FGF1 mitogenesis and downstream signaling in mouse keratinocytes that express FGFR2IIIb. Because native HS in keratinocytes is at levels sufficient to mask a response to external heparin, cells were made responsive by depression of HS sulfation by treatment with chlorate. 7,8-S-OctaF7 specifically supported FGF7-induced signaling, but failed to support signaling induced by FGF1. Moreover, 7,8-S-OctaF7 failed to support FGF1-induced signaling in mouse fibroblasts expressing predominantly FGFR1. In contrast, more highly sulfated octasaccharide mixtures with lower affinity for FGF7 supported FGF1 signaling in both cell types. Similar to octasaccharide mixtures with lower affinity for both FGF7 and FGF1, those with high affinity for FGF1 were also more highly sulfated than 7,8-S-OctaF7. They supported FGF1 signaling, but were deficient in support of FGF7-induced signaling in keratinocytes. The results show that both the degree of sulfation and distribution of sulfate groups in HS motifs control FGF-induced mitogenesis and downstream signaling in an FGF isotype-specific manner.

**Experimental Procedures**

**Purification and Analysis of Heparin Oligosaccharide Mixtures**—Size-defined heparin oligosaccharides were generated by controlled cleavage (10–30%) of crude porcine intestinal mucosal heparin (6,000–30,000 Da, 170 USP units/mg, Sigma) with a low concentration of GST-heparinase 1 (about 20 ng/ml) in the presence of 1 mM dithiothreitol and 1 mM Ca$^{2+}$ (26). Heparinase 1 with about 50 times specific activity of commercial preparations was cloned from *Bacteroides thetaiotaomicron* and expressed as a GST fusion in *Escherichia coli* and purified by GSH chromatography. Upon reaching the desired degree of degradation, GST-heparinase 1 was removed by GSH-Sepharose. After a screen of common chemical and enzymatic depolymerization methods, heparinase 1 treatment was shown the best compromise between the yield of short oligosaccharides and preservation of activity of motifs for support of FGF7 binding to FGFR2IIIb and anticoagulant activity. The size-defined oligosaccharides were purified from the partially cleaved crude heparin mixture by gel filtration chromatography that was repeated until purity in respect to size was achieved. Purity of oligosaccharides was assessed by gradient PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics Inc., Billerica, MA) (26).

Oligosaccharide mixtures were affinity fractionated by graded salt elution from columns of GST-FGF7 (26) or GST-FGF1 immobilized on GSH-Sepharose that retain high affinity for heparin. Specific activity of FGF7 and FGF1 prior to and after use in affinity columns was assessed in heparin-dependent FGF binding assays and mitogenic activity in mammalian cells to ensure use of the highest quality material in affinity separations (26). The purity of each oligosaccharide fraction in respect to size was again monitored prior to further structural and functional analysis.

Affinity purification, activity assessment, and primary structural analysis including the disaccharide compositional analysis of active 7,8-S-OctaF7 has been described in detail (26). Octasaccharide fractions from FGF1 bioaffinity were also analyzed by strong anion exchange high performance liquid chromatography (SAX-HPLC), MALDI-TOF-MS, and disaccharide composition as described (26). Anticoagulant activity of heparin oligosaccharide fractions was monitored by the antithrombin-mediated inhibition of Factor Xα activity as described (24).

**Activity of Heparin Oligosaccharides in Support of FGF-induced Signaling in Mouse Keratinocytes and Fibroblasts**—The activity of 7,8-S-OctaF7 for support of the FGF-HS-FGFR ternary complex formation was analyzed as described (26). Here we further examine the activity of isolated oligosaccharide fractions for support of FGF-induced cellular proliferation. Endogenous cellular HS was depressed by chlorate treatment to induce maximum dependence of FGF-stimulated mitogenesis and signaling on external HS (27, 28). Stock cultures of mouse skin keratinocytes (MK) (29) were maintained in serum-free RITC 80-7 medium (Masaki Corp. Tokyo, Japan) supplemented with 1 ng/ml FGF1, 1 μg/ml insulin, 10 μg/ml holo-
transferrin, 10^{-9} M dexamethasone, 1 mg/ml bovine serum albumin/4 μg/ml oleic acid complex, 5 μg/ml ethanolamine, 0.4 ng/ml FeSO_{4}, 0.1 μM L-ascorbic acid, 1 mM pyruvic acid, 60 μg/ml hypoxanthine, 30 mM sodium selenite, 1 mM glutamine, 15 μg/ml L-methionine, 30 μg/ml L-proline, 0.05 mM dithiothreitol, and 50 μg/ml kanamycin sulfate. Culture flasks were coated with Vitrogen 100 (Cohesion, Palo Alto, CA) prior to introduction of medium and cells. To maintain a maximum proliferative response with minimal terminal differentiation, MK cultures were maintained below 85% confluence. Stock cultures of mouse NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of RPMI 1640 and Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal bovine serum according to the standard 3T3 protocol (1:3 dilution after subculture every 3 days). MK and 3T3 cells were synchronized by withdrawal of FGF1 and fetal bovine serum, respectively, and followed by treatment with 30 and 1 mM sodium chloride, respectively, for 24 h prior to harvest for use in assays. MK (2 × 10^{4}) and 3T3 (3 × 10^{5}) cells were placed in each well (about 60 mm^2 surface) of 48-well tissue culture plates (BD Biosciences) containing 0.3 ml of medium for 1 h. After cell attachment for 1 h at 37°C, the medium was replaced with fresh FGF1- or fetal bovine serum-free medium containing 30 and 1 mM sodium chloride for MK and 3T3 cells, respectively. Test oligosaccharide fractions and then FGF were sequentially added. After 16 h, 0.5 μCi/ml [3H]thymidine was added and incorporation of [3H]thymidine into DNA was determined after 4 h as described (30). The activity of 300 nM oligosaccharide fractions was expressed as a percent of DNA synthesis supported by 0.4 and 4 μg/ml crude PIMH standard for MK and 3T3 cells, respectively. These were the minimal amounts of crude PIMH that promoted maximum mitogenic activity as determined in separate dose-response curves and the activity was designated as 100% output for the particular assay conditions. A concentration of single oligosaccharide addition at 300 nM was chosen for comparative studies because this concentration of 7,8-S-OctaF7 elicited peak mitogenic stimulation of FGF7 in mouse keratinocytes. Background DNA synthesis in the absence of oligosaccharide was subtracted. All mitogenic stimulation experiments were repeated at least three times with each sample from three independent preparations.

**HS-dependent FGF Induction of MAP Kinase Phosphorylation and Cyclin D1 Expression**—MK (1 × 10^{5}) and 3T3 cells (3 × 10^{5}) in each well of 48-well plates prepared as described above for analysis of DNA synthesis were exposed to 5 ng/ml FGF in the presence or absence of 300 nM of the indicated FGF-affinity fractionated octasaccharides. Cells were harvested after 15 min and 16 h, respectively, for analysis of MAP kinase phosphorylation and cyclin D1 expression. Cells were washed with cold phosphate-buffered saline and lysed in 60 μl of cold buffer comprised of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.5 mM Na3VO4, and 1 mM freshly prepared phenylmethylsulfonyl fluoride. lysates were clarified by centrifugation at 14,000 × g at 4°C for 5 min, then 30 μl of supernatant from each of the test samples was mixed with SDS sample buffer and analyzed on 10% SDS-PAGE and immunobotted. Phosphorylation of ERK1/2 was assessed with mouse monoclonal antibody 12D4 (Upstate, Lake Placid, NY) and expression of cyclin D1 was analyzed with rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences) after treatment with secondary antibodies conjugated with horseradish peroxidase. Sample loading level was standardized by analysis with β-actin antibody.

**RESULTS**

7,8-S-OctaF7 Specifically Supports FGF7-induced Mitogenic Activity in FGFR2IIIb-expressing MK—In previous studies (26), we isolated a unique and rare undersulfated 7,8-S-OctaF7 by FGF7 bioaffinity. By a combination of quantitative anticoagulant activity analysis and strong anionic exchange, ion-pair reverse phase HPLC and MALDI-TOF-MS applied to both the purified octasaccharide and its reduction to disaccharides, we deduced a composition of two rare AHexa2SGalcN6S units, one trisulfated disaccharide, and a third type of disaccharide that could be either unsulfated or monosulfated. The enrichment of anticoagulant activity indicated that the trisulfated disaccharide may bear 3-O-sulfate that is essential for the activity. In contrast, the much more highly sulfated octasaccharides with moderate affinity for FGF7 were comprised of predominantly trisulfated disaccharides (26). No acetyl groups were detected in either the high or moderate affinity fractions for FGF7 (26) despite the fact that heparin fractions that failed to bind or bind with low affinity to either factor were enriched in N-acetyl groups. 4 FGF7 mediates directionally specific signaling from stroma to epithelium in numerous parenchymal tissues comprised of both compartments (31–34). FGF7 is expressed only in stromal cells that express no FGFR2IIIb and acts on FGFR2IIIb whose expression is restricted to epithelial cells that express no FGF7. 7,8-S-OctaF7 forms complexes with FGFR2IIIb that bind FGF7 to form FGF7-(7,8-S-OctaF7)-FGFR2IIIb complexes with high specificity and specific activity relative to octasaccharides with lower affinity for FGF7 (26). We sought to determine whether the FGF7-(7,8-S-OctaF7)-FGFR2IIIb complex represents a specific active signaling complex in epithelial cells expressing physiological levels of FGFR2IIIb. Both FGF1 and FGF7 bind to FGFR2IIIb in vitro in the presence of crude heparin (35). MK cells exhibit FGFR2IIIb, and both FGF1 and FGF7 stimulate its DNA synthesis and downstream signaling (28, 30). The stimulation by FGF1 is enhanced by exogenous heparin, whereas stimulation by FGF7 is unaffected and inhibited by increasing levels of external heparin that enhance FGF1 stimulation (28, 36). This indicated that the endogenous repertoire of HS in MK cells was optimal for support of FGF7 signaling, but deficient for support of FGF1 signaling through FGFR2IIIb. To induce dependence of signaling on external heparin, MK cells were treated with the sulfation inhibitor sodium chloride for 24 h prior to addition of FGF and heparin. This was followed by comparative assessment of DNA synthesis and activation of

4 Y. Luo and W. L. McKeelhan, unpublished results.
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FIGURE 1. Effect of FGF7 affinity-fractionated oligosaccharides on FGF7-stimulated DNA synthesis in mouse keratinocytes. A, dose-dependent activity of octasaccharide fractions for support of FGF7-induced mitogenic activity. The indicated concentrations of heparin octasaccharides were added with 5 ng/ml of FGF7 to chlorate-treated MK DNA synthesis was assessed by incorporation of radiolabeled thymidine as described under "Experimental Procedures." Activity was expressed in cpm after subtraction of background in the absence of heparin. The octasaccharide mixture with the highest specific activity eluting at 1.0 M NaCl exhibiting two species in respect to degree of sulfation is referred to as 7,8-S-OctaF7 (26). B, comparative activity of octasaccharide, decasaccharide, and dodecasaccharide fractions from FGF7. Fractions eluted from FGF7 at 300 nM were added to MK cells. Effect on DNA synthesis was expressed as a percent of 0.4 μg/ml crude PIMH (H), the maximum output of the assay. One hundred percent activity represents a stimulation of 2300 cpm due to addition of heparin after subtraction of a background output of the assay. One hundred percent activity elicited by 4 μg/ml crude PIMH represented 4500 cpm after subtraction of 5500 cpm elicited by FGF1 alone. Data are the mean ± S.E. (n = 3) from three experiments performed with three independent oligosaccharide preparations. Crude, unreacted oligosaccharide.

downstream signaling pathways induced by both FGF1 and FGF7.

Fig. 1A indicates that in contrast to normal MK cells, 7,8-S-OctaF7 is strongly supportive for mitogenic activity induced by FGF7 in HS-deficient cells. Its specific activity is increased many times over crude octasaccharide and octasaccharide mixtures with lower affinity for FGF7. Stimulation was detectable at about 3 nM with half-maximum and maximum activity at 120 and 300 nM, respectively. At 300 nM, 7,8-S-OctaF7 elicited 90% of the maximal activity elicited by unfractonated PIMH at 0.4 μg/ml that was used as a 100% control. The same amount of crude octasaccharide elicited only 27% of maximum (Fig. 1B).

The activity of 7,8-S-OctaF7 was 90% or greater than that of the high affinity fractions eluting at 1.0 M NaCl of longer oligosaccharide fractions from FGF7 (data not shown). Oligosaccharides of all lengths with lower affinity for FGF7 were deficient in support of FGF1-induced DNA synthesis in both keratinocytes and fibroblasts. The mouse fibroblasts are thought to express predominantly FGFR1 and fail to respond to FGF7 under any conditions (35). Similar to the lack of stimulation of FGF1-induced DNA synthesis in MK cells, 300 nM 7,8-S-OctaF7 also failed to support FGF1-stimulated DNA synthesis in 3T3 cells (Fig. 2B).

In contrast, 7,8-S-OctaF7 at 300 nM failed to support the mitogenic activity of FGF1 in the MK cells (Fig. 2A). Separate experiments indicated that no stimulation could be demonstrated at concentrations up to 10 μM (not shown). However, at 300 nM the octasaccharide fraction with lower affinity for FGF7 supported FGF1-induced mitogenesis to 70% of the 0.4 μg/ml PIMH standard (Fig. 2A). As expected, activity of longer oligosaccharides mixtures containing motifs with high affinity for FGF7 increased with increasing length of oligosaccharide (Fig. 2A). This indicates that motifs within the two-component 7,8-S-OctaF7 are deficient for support of FGF1-induced DNA synthesis in keratinocytes.

7,8-S-OctaF7 Fails to Support FGF1-induced Mitogenesis in Mouse Fibroblasts—Because 7,8-S-OctaF7 was specific for support of FGF7 relative to FGF1 signaling in keratinocytes that bear only FGFR2IIIb, we examined the effect of FGF7-purified oligosaccharides on FGF7-induced DNA synthesis in NIH 3T3 fibroblasts. The mouse fibroblasts are thought to express predominantly FGFR1 and fail to respond to FGF7 under any conditions (35). Similar to the lack of stimulation of FGF1-induced DNA synthesis in MK cells, 300 nM 7,8-S-OctaF7 also failed to support FGF1-stimulated DNA synthesis in 3T3 cells (Fig. 2B).

No stimulation by 7,8-S-OctaF7 could be detected up to 10 μM (data not shown). Similar to FGF1 activity in MK cells, the more highly sulfated octasaccharide fraction with lower affinity for FGF7 was equal to 90% of the PIMH standard (Fig. 2B). Longer oligosaccharides with both moderate and high affinity for FGF7 were equal to or exceeded activity of the 4 μg/ml PIMH standard. Thus motifs within the two-component 7,8-S-OctaF7 are deficient for support of FGF1-induced DNA synthesis in both keratinocytes through FGFR2IIIb and 3T3 cells through FGFR1 or other FGFR isotypes.

Specificity of 7,8-S-OctaF7 for FGF7-induced Activation of ERK1/2 and Cyclin D1—Activation of the FGFR signaling complex recruits FRS2 and Grb2/Sos complexes resulting in activa-
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FIGURE 3. Downstream signaling supported by FGF7 affinity-fractionated octasaccharides. The indicated heparin fragments were added into culture medium containing 5 ng/ml of FGF7 or FGF1 for MK (A) or 3T3 (B) cells prepared as described under "Experimental Procedures." PIMH (H) was added at 0.4 and 4 μg/ml for MK and 3T3 cells, respectively, and octasaccharides at 300 nM. Cell extracts were analyzed by immunoblot and relative band intensities were quantified by densitometry. Density of each band was divided by the density of the β-actin control. Band intensity in the absence of heparin (no H) was assigned a value of 1. Numbers indicate relative band intensities. Experiments were repeated three times with three independent oligosaccharide preparations. Data are representative of at least three independent assays with samples from three independent preparations.

Affinity of oligosaccharides for FGF1 and FGF7

Oligosaccharides eluting at 0.14, 0.3, 0.6, and 1.0 M NaCl were designated as the unretained, low, moderate, and high affinity fractions, respectively. F1 = FGF1; F7 = FGF7.

| Oligosaccharide (units) | 0.14 M NaCl | 0.3 M NaCl | 0.6 M NaCl | 1.3 M NaCl | 1.0 M NaCl |
|------------------------|-------------|-------------|-------------|-------------|------------|
| F1                     |             |             |             |             |            |
| F7                     |             |             |             |             |            |

Percent of applied oligosaccharide

- 6
- 8
- 12

FGF7 at 0.60 M NaCl exhibited stimulation by 16-fold (Fig. 3). FGF7-induced phosphorylation of ERK1/2 by phosphorylation and an increase in cyclin D1. To determine the activity of FGF7 affinity-fractionated octasaccharides on these indicators, extracts of chlorate-treated MK cells were analyzed after stimulation with FGF7 or FGF1 and the indicated oligosaccharide fractions (Fig. 3). 300 nM 7,8-S-OctaF7 stimulated FGF7-induced phosphorylation of ERK1/2 by 22-fold and cyclin D1 expression by 17-fold over base levels in the presence of FGF7 alone. This equals or exceeds activity of the 0.60 and 1.0 M NaCl eluates from FGF1 were subjected to MALDI-TOF mass spectrometric analysis as described for octasaccharide fractions from FGF7 (26). Heparinase I cleavage leaves the reducing end intact and non-reducing end with a double bond without randomly affecting other groups. The number of sulfate and acetyl groups can be calculated by the mass of the resultant size-defined oligosaccharides, which can be stated as m/z = 4225.2 + 337.29N + 420.37N\textsubscript{Ac} + 80.058N\textsubscript{SO3} in which N\textsubscript{Ac}, N\textsubscript{SO3}, and N\textsubscript{Ac} indicate the number of disaccharides, acetyl groups, and sulfate groups, respectively. Mass of the peptide carrier (RG\textsubscript{19}R) is 4225.2. Addition of one sulfate (SO\textsubscript{3}) or acetyl group will show a new peak with an 80- or 42-Da mass interval. Both 0.60 and 1.3 M eluates from FGF1 exhibited a similar range of sulfated octasaccharides bearing from 8 to 12 sulfates (Fig. 4) even though the high affinity fraction is less than 10% of the lower affinity octasaccharide for FGF1 (Table 1). There was no indication of acetyl groups in either fraction. Notably the yield of 11-sulfated octasaccharides exceeded that of 12-sulfated octasaccharides. This was in marked contrast to the 0.60 and 1.0 M salt eluates from FGF7 in which only the former was highly sulfated and the latter much less sulfated (26). The highly sulfated profile exhibited by both moderate and high affinity octasaccharides for FGF1 was similar to the 0.60 M eluate from FGF7 with the exception that a 12-sulfated octasaccharide was most abundant in the latter case (26). A separate analysis of the disaccharide composition of high affinity octasaccharides from FGF1 by complete reduction to disaccharides and subsequent SAX analysis revealed predominantly ΔHexA2SGLcN5S6S (data not shown). This was similar to the moderate affinity octasaccharides from FGF7 (26).

Analysis by SAX chromatography indicated that all three fractions retained by FGF1 were much more heterogeneous support FGF-induced mitogenesis was compared with those from FGF7 affinity chromatography. Heparin hexa-a, octa-, and dodecasaccharides bound to FGF1 at 0.14 M NaCl were eluted stepwise at 0.3, 0.6, and 1.3 M NaCl. The 1.3 M NaCl eluate above which no additional material was recovered was designated as the high affinity fraction. Six times the FGF1-bound octasaccharide and dodecasaccharide was resistant to salt above 0.60 M salt than that bound to FGF7 (Table 1). The difference in amounts bound at lower affinity eluting at 0.60 M or below from both factors was much less. Notably, there is significantly more hexasaccharide bound to FGF1 with moderate and high affinity than bound to FGF7.
than those retained by FGF7 with a similar salt resistance (26) (Fig. 5). Low affinity octasaccharide fractions eluting at 0.3–0.6 M salt from FGF1 exhibited a spectrum spanning 0.7 to 1.7 M NaCl on SAX relative to the low affinity octasaccharide fraction from FGF7 that eluted much more narrowly between 1.3 and 1.6 M NaCl (26). Material eluting at 0.6 and 1.3 M salt concentrations from FGF1 was generally distributed among the same peaks that spanned 1.45 to 1.8 M NaCl eluates on SAX. The SAX column is incapable of retention or fractionation of material above 1.8 M NaCl. This spread was in marked contrast to comparable fractions with moderate and high affinity for FGF7 whose predominant peaks both eluted together at the 1.8 M NaCl resolution limit of SAX (26).

Overlapping subfractions eluting at similar salt concentrations from SAX chromatography of the 0.6 and 1.3 M eluates from FGF1 were desalted and analyzed by MALDI-TOF-MS. The first peak eluting at 1.45 M NaCl on SAX for the 0.60 M eluate from FGF1 exhibited predominantly 8-, 9-, and 10-sulfated octasaccharides (not shown). The peak A doublet at about 1.65 M NaCl on SAX for the 0.60 M eluate from FGF1 exhibited peaks of 6–11-sulfated octasaccharides increasing in respective order (Fig. 6A). Peak B eluting at the same salt concentration on SAX for the 1.3 M eluate from FGF1 (Fig. 5) was a mixture of 8–11-sulfated octasaccharides (Fig. 6B). Peaks C and D eluting at 1.75 M salt on SAX (Fig. 5) for the 0.60 and 1.3 M salt eluates from FGF1, respectively, exhibited the same 7–11-sulfated species in similar relative quantities (Figs. 6C and 6D). Peaks E and F for the 0.60 and 1.3 M salt eluates from FGF1, respectively, exhibited the same 7–11-sulfated species in similar relative quantities (Figs. 6C and 7D). Peaks E and F for the 0.60 and 1.3 M salt eluates from FGF1, respectively, both eluted at the resolution limit of SAX (1.8 M salt) and exhibited 9-, 10-, 11-, and 12-sulfated octasaccharides (Fig. 6, E and F). It is especially noteworthy that both fractions exhibited significant amounts of less than fully sulfated octasaccharides despite the fact that they run at the highest salt elution point possible on SAX. Moreover, it is notable that fraction F for the high affinity eluate from FGF1 exhibited more 11-sulfated octasaccharides than fraction E as well as significant amounts of 8-sulfated species (Fig. 6F). These results confirm...
that FGF1 binds to a broader and more heterogeneous spectrum of octasaccharides with overall higher charge density than FGF7. However, similar to what was observed for FGF7 (26), high affinity to FGF1 based on resistance to high salt is not always a strict correlate of high charge density and high degree of sulfation.

Anticoagulant and Mitogenic Activity of FGF1 Affinity-fractionated Oligosaccharides—Independent of length heparin oligosaccharides as well as crude heparin mixture with increasing affinity for FGF7 based on salt resistance exhibited a proportional increase in anticoagulant activity (24, 26). Fig. 7 indicates that in contrast to FGF7, the high affinity octasaccharide fraction from FGF1 exhibited no increase in activity over fractions with lower affinity and the crude octasaccharide. Although some activity could be detected at higher levels, anticoagulant activity at 100 nM was barely detectable in the FGF1 affinity-fractionated material. Longer heparin oligosaccharides exhibited increased anticoagulant activity. However, in contrast to fractions from FGF7, no enrichment of anticoagulant activity was observed for fractions from FGF1. In fact, anticoagulant activity of longer oligosaccharides appeared to decrease with increasing affinity for FGF1 (24).

The ability to support both FGF1- and FGF7-induced DNA syntheses in MK cells of FGF1-fractionated octasaccharide and dodecasaccharide fractions was then compared. Fig. 8A shows that the octasaccharide fractions with moderate (0.60 M salt) and high affinity (1.3 M salt) for FGF1 exhibited 63 and 91% of the activity, respectively, of the 0.4 μg/ml PIMH standard for support of FGF1-induced DNA synthesis. Notably the moderate affinity fraction was 70% as effective as the high affinity fraction. Both fractions exhibited a similar activity in support of FGF1-stimulated DNA synthesis in 3T3 cells (not shown). In contrast both octasaccharide fractions from FGF1 were severely deficient in support of FGF7 activity in MK cells (Fig. 8A). Activity of the high affinity fraction eluting at 1.3 M salt was about equal to the crude unfractionated octasaccharide (Fig. 8A). A dose-response curve similar to that shown in Fig. 1 for the high affinity octasaccharide from FGF1 indicated that the maximum level of FGF7-induced DNA synthesis observed at any concentration was about 25% of the level supported by 300 nM 7,8-S-OctaF7 before inhibitory elements dominated (data not shown). As expected the activity of all dodecasaccharide fractions was increased over that of octasaccharides (Fig. 8B). However, fractions from FGF1 affinity columns were markedly superior for FGF1-stimulated DNA synthesis relative to FGF7-stimulated synthesis in MK cells. Activity of dodecasaccharide fractions with moderate and high affinity from FGF1 for FGF7-stimulated DNA synthesis again was about equal to that of crude dodecasaccharide. This indicates that FGF1 selectively binds oligosaccharide motifs that support FGF1-induced signaling, despite the fact that it binds a much wider range of oligosaccharides with overall higher charge density than does FGF7. In contrast to FGF7-bound oligosaccharides, there is little difference in activity between moderate and high affinity fractions from FGF1 for FGF1-induced DNA synthesis.

DISCUSSION

Here we show that a structurally specific undersulfated heparin octasaccharide mixture (7,8-S-OctaF7) purified by high affinity to FGF7 based on salt resistance (26) specifically supports the induction of FGF7-mediated mitogenic activity and downstream signaling in mouse keratinocytes expressing FGFR2IIb. This is in parallel to its high specific activity and specificity in formation of the oligomeric FGF7-HS-FGFR2IIb complex (26). In contrast, 7,8-S-OctaF7 fails to support FGF1 stimulated activity in the same keratinocytes through FGFR2IIb and in mouse fibroblasts that express other FGF1-activated FGFR. We show that FGF1 binds with high affinity to a much wider spectrum of heparin oligosaccharides with overall higher average charge density than does FGF7. In contrast to FGF7, octasaccharides with moderate affinity for FGF1 based on salt resistance cannot be clearly distinguished from those with high affinity in respect to degree of sulfation and average charge density. They also cannot be distinguished from the heterogeneous mixture of octasaccharides with lower affinity than 7,8-S-Octa7 for FGF7 (26). This is consistent with predictions based on differences in the overall density and topography of...
Our results show that the heparin oligosaccharide fractions with the highest affinity for FGF1 and FGF7, respectively, exhibit respective specificity for FGF1 and FGF7 in support of signaling. Undersulfated 7,8-S-OctaF7 fails to support FGF1 signaling. Similar to fractions with lower affinity for FGF7 and FGF1, the heterogeneous and more highly sulfated octasaccharide mixture with high affinity for FGF1 was deficient for support of FGF7-induced signaling. The failure of undersulfated 7,8-S-OctaF7 to support FGF1 signaling suggests that formation of the active FGF1-HS-FGFR signaling complex requires a different oligosaccharide motif than the active complex of FGF7-HS-FGFR2IIb. The low activity of both moderate and high affinity fractions from FGF1 in FGF7 signaling is consistent with the lack of enrichment of 7,8-S-OctaF7 and related motifs from crude oligosaccharide by FGF1 bioaffinity. The heterogeneity and similar high charge density of moderate and high affinity factions from FGF1 precludes a prediction on the nature of possibly specific motifs required for FGF1 signaling beyond restrictions required for interaction with FGFR (37). It is noteworthy that the octasaccharide fraction with high affinity for FGF1 exhibited a consistently higher activity than the fraction with moderate affinity. This was despite the fact that both fractions appear to be of similar overall charge density and contain minor undersulfated octasaccharide species. This may indicate some selectivity of FGF1 for oligosaccharide motifs that work best for FGF1 signaling that are hidden within the overall heterogeneity.

Based on an affinity analysis of heparin oligosaccharides for FGF7 related to function, we previously proposed a three affinity class model for control of FGF function in the tissue environment (26). The model provides a conceptual basis for differential control of FGF1 and FGF7 signaling by HS in parenchymal tissues comprised of both epithelial cells expressing FGFR2IIb and stromal cells expressing FGFR other than FGFR2IIb. FGFs encounter an abundant low affinity class of matrix and cell surface HS sites with large overlap among FGFs. In heparin, which is used here as a model compound for HS (38, 39), this class of sites are estimated to comprise about half of the total sites. They are characterized by the lowest degree of sulfation and are represented by oligosaccharides that elute from FGF at 0.30 M salt. Their number appears generally similar for both FGF1 and FGF7. These sites may affect movement and gradients of FGFs in the tissue environment with quantitative differences among FGFs dependent on overall basicity of the FGF.

The second class of sites comprises the majority of remaining binding sites. They are represented by fractions that are eluted from FGF between 0.30 and 0.60 M salt. These sites are characterized by higher average charge density rather than specific distribution of charged groups that fit the particular FGF HS-binding domain. These sites may exhibit more selectivity than the first class of sites among FGFs. Selectivity is still largely dominated by overall basicity of the FGF and particularly its HS-binding domain. These sites are expected to more actively control stability, storage, intercellular trafficking, and access of FGF to membrane FGFR. Our comparative studies of FGF1 and FGF7 indicate that both factors also share a significant fraction of this class of HS-binding sites. However, the sites appear much more abundant and in shorter oligosaccharide sequences for FGF1 than for FGF7. Although the results here were deduced from heparin as the experimental prototype, we predict that the relative abundance and location of the two classes of sites and the third one discussed below may vary among diverse tissues.

The third class of sites revealed by an analysis of affinity based on salt resistance of binding to FGF7 related to function was rare and surprisingly pure in respect to degree of sulfation. More surprisingly, the charge density of this class of sites exhibiting highest resistance to salt when complexed with FGF7 was less than sites that eluted at lower salt concentration. We have proposed that this inverse relationship between salt resistance of HS-FGF complexes and charge density of the oligosaccharide reflects composite forces affecting overall affinity beyond simple interactions of negatively- and positively-charged groups (26). The composite interaction affecting salt elution is likely affected by charge clashes and topographical compatibilities and incompatibilities between elements of the HS oligosaccharide motif and the FGF HS-binding domain. This rare third class of sites represented by relatively homogenous 7,8-S-OctaF7 in respect to sulfation exhibits rare disaccharide composition and disposition of sulfate groups that presumably best fit the specific HS-binding domain of FGF7. Moreover, part or all motifs within 7,8-S-OctaF7 also exhibit structural elements sufficient for high affinity binding to FGFR2IIb independent of FGF7 (26, 37, 40). Based on our previous results, we argued that because 7,8-S-OctaF7 is rare and specific for FGF7 and has high affinity for both FGF7 and FGFR, 7,8-S-OctaF7 or similar cellular tissue HS motifs most likely exist in cell surface complexes of (7,8-S-OctaF7)-FGFR2IIb (16, 26, 37, 40). This is instead of a random dispersion in the tissue matrix. Such cell membrane-localized complexes constitute the single most competitive and specific sites for FGF7 in the microenvironment. Our current results predict that FGF1 cannot activate complexes of (7,8-S-OctaF7)-FGFR2IIb. In contrast, FGF1 activation requires different HS motifs within the wider range of high affinity binding motifs for FGF1 that also possess the structural elements sufficient for high affinity binding to FGFR2IIb and FGFR1. In contrast to 7,8-S-OctaF7, we propose that the more abundant high affinity FGF1 sites, only part of which may be capable of forming the FGF signaling complex, are likely distributed broadly in the tissue matrix.

The differences in abundance, distribution, and signaling requirements of the three classes of sites for FGF1 and FGF7 are consistent with their different roles in parenchymal tissues. The primary role of FGF7 is to mediate stromal instruction to parenchymal cells for maintenance of tissue homeostasis (31–34). Low levels of FGF7 of stromal cell origin must constantly traverse the abundant low affinity sites in HS-rich basement membranes and the tissue matrix to reach high affinity HS-FGFR2IIb complexes on epithelial cells. In contrast, FGF1 and probably its homologue FGF2 that originates from multiple cell types are likely sequestered in the matrix among the abundant HS sites that include those with high affinity. Such depots constitute a store of FGF1 and FGF2 for an acute repair
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response available for release upon acute perturbation of matrix HS sites (41).

Our results may explain some of the novel and sometimes conflicting observations concerning the role of HS in FGF7 signaling compared with FGF1 and other FGFs (20, 28, 36, 42). Originally it was proposed that unlike other FGFs, HS was not essential for FGF7 signaling and exerted a net negative control (20, 36, 43, 44). This was due to the observed inhibition of FGF7-induced signaling in keratinocytes displaying FGFR2IIb by external heparin concentrations that stimulated FGF1 signaling. Subsequent studies under conditions where HS was deficient revealed that similar to other FGFs the binding of FGF7 to membrane-anchored FGFR2IIb and FGF7 signaling require HS (20, 36, 42, 45). These observations are consistent with the rare nature and specificity of high affinity 7,8-S-OctaF7 for FGF7 and the notion that such motifs are primarily in complex with cell membrane FGFR2IIb rather than distributed randomly throughout the tissue matrix (26). Endogenous (7,8-S-OctaF7)-FGFR2IIb complexes in keratinocytes that cannot recognize FGF1 are apparently sufficient for activation of keratinocytes by FGF7 in the absence of external heparin. Only by depression of endogenous HS synthesis or extraction of FGFR2IIb away from cellular HS (28, 36, 45) can an external effect of heparin be generated. Endogenous keratinocyte HS-FGFR2IIb complexes are apparently not sufficient for support of FGF1 signaling. Therefore there is a requirement for external heparin for maximal FGF1 stimulation. Motifs within crude heparin capable of forming HS-FGFR2IIb complexes competent to bind FGF1 are likely to be far more abundant than FGF7-specific 7,8-S-OctaF7. Such abundant motifs likely compete, exchange with, and dominate endogenous (7,8-S-OctaF7)-FGFR2IIb complexes resulting in an apparent inhibition of FGF7-induced signaling while supporting signaling by FGF1. The large number of low affinity FGF7-binding motifs in crude heparin that cannot interact with FGFR2IIb may also contribute to the apparent inhibition. Consistent with this, we have shown that unlike crude heparin, FGF7 affinity purified 7,8-S-OctaF7 fails to inhibit FGF7-induced signaling in native keratinocytes except at very high concentrations. Lower affinity fractions for FGF7 and both moderate to high affinity fractions for FGF1 exhibit an inhibitory effect similar to crude heparin on FGF7 signaling while at the same concentrations stimulate FGF1 signaling.3

In conclusion, our results provide a structural basis for the large number of reports that predict that structurally specific HS motifs are involved in control of FGF signaling (13–22, 46). Our results are also in agreement that FGFs extensively share HS epitopes with low to moderate affinity that are likely dominated by random variations in charge density of HS and the basicity of the FGF heparin-binding interface (23, 46). Specific position of charged groups may contribute to quantitative differences, but is unlikely to play strict FGF-specific roles in these interactions. These abundant epitopes are restricted to FGFR-independent functions. In contrast, rare less than fully sulfated HS motifs with specifically distributed sulfate groups that are capable of concurrent interaction with both FGFR and FGF play FGF-specific roles in assembly and activation of the oligomeric FGF-HS-FGFR signaling complex. In the case of FGF1 and FGF7 coupled to FGFR2IIb, these rare motifs appear highly specific. It is conceivable that FGF receptor complexes formed with these motifs may be shared with other untested FGFs, however, this remains to be tested. Isolation and structural analyses are needed to determine the degree of overlap among different members of the FGF family in this class of HS motifs.

7,8-S-OctaF7 provides the first unique undersulfated FGF-specific prototype. Its identification and extraction was dependent on an FGF7 affinity reagent of highest structural integrity and bioactivity to eliminate nonspecific protein-HS polyelectrolyte interactions (18, 25, 26). Preliminary structural characterizations indicate it may contain within a single octasaccharide motif two rare side groups (unmodified N-acetyl- and O-sulfate) (26). This justifies scale-up and dedication of the rare material to determination of the complete sequence, distribution of sulfate groups, and proof of functional importance of position of each residue by modification during enzymatic and chemical synthesis. Identification of specific FGF-binding motifs for FGF1 will require more alternative purification strategies than affinity purification based on FGF1. We predict that similar to FGF1, FGF affinity approaches alone will also be insufficient to identify active signaling HS motifs for FGF2, -3, -5, -8, -10, and -18 because of clusters of high basic charge density and topography of charged groups in the HS-binding domains.3 In contrast we predict on the same basis that FGF4, -6, -9, -11, -16, -17, -19, -20, -21, -22, and -23 could be amenable to the direct affinity approach described here for FGF7. The elucidation of FGF- and FGFR-specific motifs should aid in design of agonists and antagonists for pharmacological intervention in FGF signaling.

Acknowledgment—We thank Courtney Bowles for technical assistance.

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