The role of heparin and heparan sulfate in the binding and signaling of fibroblast growth factors (FGFs) has been subject to intense investigation, but the studies have largely been confined to two species (FGF1 and FGF2) of the family with ~20 members. We have investigated the structural requirements for heparin/heparan sulfate binding in activation of FGF8 (splice variant b). We present evidence that the minimal FGF8-binding saccharide domain encompasses 5–7 monosaccharide units. The N-, 2-O-, and 6-O-sulfate substituents of heparin/heparan sulfate (HS) are all involved in the interaction, preferentially in the form of trisulfated IdoUA(2-OSO3)GlcNSO3(6-OSO3) disaccharide constituents. These structural characteristics resemble those described earlier for FGF1. By contrast, the saccharide structures required for the biological activity of FGF8b differed significantly from those characteristic for FGF1 and FGF2. Experiments with cells lacking active HS indicated that extended ≥14-mer heparin domains were needed to enhance cell proliferation and Erk phosphorylation by FGF8b, whereas in cells stimulated with FGF1 or FGF2 the corresponding responses were achieved by much shorter, 6–8-mer, oligosaccharides. Furthermore, still longer domains were needed to activate FGF8b in cells with “non-optimal” FGF receptor expression. Collectively, our data suggest that the heparin/HS structures enhancing the biological activity of FGFs were influenced by the FGF species involved as well as by the cellular composition of FGF receptors.

Fibroblast growth factor (FGF) 8 belongs to the family of ~20 related polypeptides that bind and activate FGF receptors (FGFR). FGF8 was originally identified as the gene encoding an androgen-induced growth factor that stimulated the proliferation of mouse mammary carcinoma cells in an autocrinic fashion androgen-induced growth factor that stimulated the proliferation of mouse mammary carcinoma cells in an autocrinic fashion androgen-induced growth factor that stimulated the proliferation of mouse mammary carcinoma cells in an autocrinic fashion androgen-induced growth factor that stimulated the proliferation of mouse mammary carcinoma cells in an autocrinic fashion.


Published, JBC Papers in Press, June 19, 2002, DOI 10.1074/jbc.M204961200

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This paper is available online at http://www.jbc.org

* This work was supported by the European Commission ("Biologically Active Novel Glycosaminoglycans") Grant QLK-CT-1999.00538, the Swedish Medical Research Council Grant K99–03X, the Swedish Cancer Society Grant 3919-B97, Polysackaridforskning AB (Uppsala, Sweden), the Finnish Cancer Union, the Academy of Finland, the Sigrid Juselius Foundation, and BioTie Therapies Corp. 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; FGF8, FGF receptor; HS, heparan sulfate; Erk, extracellular signal-regulated protein kinase; CHO, Chinese hamster ovary.

its expression has been demonstrated in human malignancies, including prostate and breast cancers (4–6). On the other hand, FGF8 appears to play important physiological roles in embryonic development and tissue patterning (7, 8).

FGF8 shares 30–40% sequence homology with other FGFs (FGF1–10 (1)). However, the structure of the Fgf8 gene is more complex than that of other related genes, comprising at least four exons, which can be alternatively spliced to yield different FGF8 protein isoforms (9, 10). The functional differences between the isoforms are not fully understood, but they seem to influence the transforming potential and receptor-binding properties of FGF8 (3, 8). Generally, FGF8 binds preferentially to the IIc splice variant of FGFR1–3 and to FGFR4 (8, 11, 12). Upon ligand binding, the receptors are activated through dimerization and phosphorylation by the intracellular tyrosine kinase domains (13). This process activates the Ras signal transduction pathway, the key components of which are the MAP kinases Erk1 (p44mapk) and Erk2 (p42mapk) (for reviews seeRefs. 14 and 15).

Heparan sulfate (HS) proteoglycans, abundant components of cell surfaces and the extracellular matrix, appear critical for FGF signaling. Cells lacking endogenous HS respond poorly to FGFs, whereas the response can be readily restored by addition of exogenous heparin (16, 17). Heparin/HS chains are initially synthesized as polymers of alternating glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) units (for reviews see Refs. 18–20). In HS biosynthesis, the polymer is first modified by partial N-deacetylation/N-sulfation of the GlcNAc residues. The further modification reactions, C-5 epimerization of GlcA to iduronic acid (IdoUA) units and O-sulfation at various positions (C-2 of IdoUA and GlcA units and C-3 and C-6 of GlcN units), all occur in the vicinity of previously incorporated N-sulfate groups. The modifications occur in a regioselective fashion, such that contiguous N-sulfated domains are interspersed with essentially unmodified N-acetylated sequences and with domains containing alternating N-acetylated and N-sulfated disaccharide units (21, 22). Heparin, a highly specialized product of connective tissue-type mast cells, is more extensively modified than HS, and the modifications are more evenly distributed along the polymer (18).

The interactions of HS with FGFs are believed to be mediated by the N-sulfated domains of the polysaccharide. The minimal HS domains binding to FGF1 and FGF2 are both contained in sequences encompassing 5–7 monosaccharide units (23–26) or perhaps in still smaller saccharide sequences (27). Apparently, the two factors require different O-sulfation for their binding to HS, such that the binding of FGF2 is critically dependent on a single 2-O-sulfated IdoUA residue, whereas FGF1 binding requires a 6-O-sulfated GlcNSO3 residue and 2-O-sulfated IdoUA units (26). The minimal FGF-binding HS domains do not, however, appear sufficient to stimulate FGF signaling which instead requires longer ~8–12-mer
domains containing both 2-O- and 6-O-sulfate substituents (28–31). Such activating domains are thought to mediate the formation of “ternary complexes” in which the polysaccharide interacts with both the FGF and FGFR components of the complex.

Whereas the HS sequences that bind to FGF1 and FGF2 have been characterized in detail (26) and the HS structures mediating their biological activities are becoming increasingly understood (32), much less is known of the HS structures involved in the binding and activation of other species of the FGF family. Here we have investigated the binding of heparin/HS to the splice variant b of FGFR8. FGF8b seems to have higher receptor-binding properties and higher transfomatory potential than the other FGF8 isoforms, and it has been implicated in various forms of cancer (3, 4, 8). We further investigated the role of heparin/HS in the signaling of FGF8b, and we performed comparative experiments with FGF1 and FGF2. These results indicate that the minimal FGF8b-binding HS domain encompasses ~5 monosaccharide units and contains both 2-O- and 6-O-sulfate groups and may thus resemble the sequence interacting with FGFR1. By contrast, FGF8b differs markedly from FGFR1 and FGFR2 with regard to the length of the heparin domains that are required for biological activity. Furthermore, we provide evidence that the requirement for the activating heparin domain is influenced by the cellular FGFR composition.

MATERIALS AND METHODS

Protein Production—The FGF8b expression clone was a kind gift from Dr. Craig MacArthur (Washington University). FGF8b was produced in Escherichia coli and purified through its C-terminal His6 tag under denaturing conditions as described elsewhere (9). After refolding by dialysis, FGF8b was passed through a column of heparin-Sepharose CL-6B (Amersham Biosciences AB), followed by elution of bound protein with NaCl. Recombinant FGFR1 and FGFR2 as well as untagged FGF8b were purchased from PeproTech EC Ltd. (London, UK).

Glycosaminoglycan Preparations—Heparin from pig intestinal mucosa (stage 14, Inotex Pharmaceutical Division), was purified as described previously (33). It was used either unlabeled or radiolabeled by [3H]-acetylation of free amino groups as described earlier (34) (specific activity ~75,000 dpm/nmol). The selectively desulfated heparin and heparin oligosaccharide preparations (35, 36), all derived from bovine lung heparin, were kindly provided by Dr. Dorothe Spillmann (Uppsala University). Heparin from bovine lung and HS from bovine kidney were from Sigma. HS from bovine aorta, lung, and intestine were generously provided by Dr. Keiichi Yoshida (Seikagaku Corp.). Heparin from bovine intestinal HS were applied to the column in phosphate buffer, followed by washing with phosphate buffer and elution of the bound material with a step gradient of NaPO4. The disaccharide peaks were identified by comparing their elution positions to those of standard heparin disaccharides. To analyze the proportions of non-O-sulfated disaccharides, the disaccharides were separated by high voltage paper electrophoresis on Whatman 3MM paper in pyridine/ acetic acid buffer, pH 5.3, for 80 min at 2000 V (38). The paper was dried, and the paper strips cut into 1-cm pieces and analyzed for radioactivity in a β-counter.

Cell Culture and Transfections—The heparan sulfate-deficient CHO cell line, pBsd 677 (40), was a kind gift from Dr. Jeffrey D. Esko (University of California). The cells were cultured in α-minimum essential medium containing glutamax-1 (Invitrogen) supplemented with 100 µg/ml streptomycin, 100 IU/ml penicillin G (Sigma), and 5% fetal bovine serum (Autogen Bioclear, UK). S115 mouse mammary carcinoma cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 5% fetal bovine serum (Autogen Bioclear), 1 mM sodium pyruvate, 1 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10 mM testosterone (all from Sigma).

FGFR4 cDNA in the pLTR2HX vector (41, 42) was a kind gift from Dr. Carlo Melino (University of Helsinki). The EcoRI fragment of FGFR4 cDNA was subcloned into the pcDNA3.1-plasmid (Invitrogen). The FGFR1 cDNA, encoding a receptor with three Ig loops, in the pcDNAIneo vector, was kindly provided by Dr. Lena Claesson-Welsh (Uppsala University). The BamHI-XbaI fragment of FGFR1 was subcloned into the pcDNA3.1-plasmid (Invitrogen). Transfections of CHO677 cells were made with the calcium phosphate precipitation method (43), followed by selection of transfected cells in medium containing 750 µg/ml gentamicin (G418; Sigma). The expression of the FGFR1 and FGFR4 proteins was detected by Western blotting with polyclonal anti-FGFR4 antibodies (C-16) and polyclonal anti-FGFR1 antibodies (C-15; Santa Cruz Biotechnology, Santa Cruz, CA). After the selection was completed, the transfected cells were maintained in a medium containing 300 µg/ml gentamicin.

Cell Proliferation Assays—CHO677 cells were plated onto 24-well plates, at a density of 20,000 cells/well. The cells were serum-starved for 48–72 h, after which fresh serum-free medium containing FGFs and glycosaminoglycans were added, and the cells were grown for an additional 24 h. To assess the cellular DNA synthesis, [methyl-3H]thymidine (1 µCi/well; PerkinElmer Life Sciences) was added for the last 2–4 h of the incubation. The cells were trypsinized, washed with cold PBS (calcium- and magnesium-free; Reagen Ltd., Kuopio, Finland), and incubated in 5% trichloracetic acid on ice. Following centrifugation, the pellet containing acid-insoluble material was dissolved in 0.5 ml NaOH, 0.5% SDS, and the incorporated radioactivity was measured by liquid scintillation counting.

S115 cells were plated onto culture dishes in low sulfate Dulbecco’s modified Eagle’s medium (16) containing 5% dextran-coated charcoal-treated fetal calf serum (44) and 30 mM sodium chloride (Merck). For 48 h, the cells were trypsinized and plated on 24-well plates at a density of 50,000 cells/well in low sulfate Dulbecco’s modified Eagle’s medium supplemented with 0.1% dextran-coated charcoal-treated fetal calf serum and 30 mM sodium chloride. After 24 h the medium was replaced, and FGFs and glycosaminoglycans were added. Incorporation of [3H]thymidine was measured as described above.

Erk Phosphorylation Assay—Cells were plated onto 24-well plates at a density of 20,000 cells/well. Following 48–72 h of incubation in serum-free medium, fresh medium containing FGFs and glycosaminoglycans was added to the cells. At various time points, the cells were lysed in 2× SDS sample buffer. The samples were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes by semi-dry blotting. The phosphorylated forms of Erk1 and Erk2 were detected by monoclonal anti-activated protein kinase antibodies (Sigma) according to the instructions of the manufacturer. To verify that the same amount of protein was loaded in each lane, the membrane was reprobed with polyclonal anti-Erk2 antibodies (C-14; Santa Cruz Biotechnology).

RESULTS

Minimal FGF8b-binding Saccharide Domain—To study the binding of FGF8b to heparin, increasing amounts of [3H]heparin were incubated with FGF8b in solution at physiological ionic strength. The formed protein-saccharide complexes were trapped on nitrocellulose filters, followed by quantification of
the bound saccharide. Heparin appeared to bind to FGF8b in a
dose-dependent and saturable manner (Fig. 1), and the binding
was abolished by addition of excess cold heparin (data not
shown). To identify the minimal FGF8b-binding heparin/HS
domain, similar experiments were performed with even num-
bered,3H-end-labeled heparin/HS oligosaccharides. Heparin
and HS hexasaccharides were the smallest oligosaccharides
with appreciable binding to FGF8b (Fig. 2), suggesting that the
minimal FGF8b-binding HS domain contains at least five in-
tact monosaccharide units (the reducing terminal unit in the
oligosaccharides being an3H-labeled 2,5-anhydromannitol res-
idue). The binding increased with increasing saccharide length,
possibly reflecting the presence of multiple, overlapping bind-
ing sites in the longer oligosaccharides. The apparent decrease
in the binding seen with ≥12-mer heparin oligosaccharides
probably reflected the binding of several FGF8b molecules to
the same fragment. In fact, when excess protein was added no
such decline in the binding was observed (data not shown).

Sulfation of FGF8b-binding Saccharide Domains—Distinct
patterns of sulfation appear essential for the binding of HS to
FGF1 and FGF2 (26). To elucidate the importance of
N-, 2-O-, and 6-O-sulfate groups of heparin for its binding to FGF8b,
selectively desulfated heparin species were tested for their
ability to inhibit binding of [3H]heparin to FGF8b in solution
using the filter-trapping assay. Although low concentrations (1
µg/ml) of unlabeled native heparin blocked the binding almost
completely, the desulfated heparin preparations showed lim-
ited inhibitory capacity (Fig. 3). Thus at a concentration of 50
µg/ml the binding was inhibited only by 20–30%. Each of the
preparations exhibited approximately similar inhibitory capac-
ity, suggesting a role for all three sulfate substituents in the
interaction.

To assess further the O-sulfation of FGF8b-binding saccha-
ride domains, compositional disaccharide analyses of FGF8b-
ning and non-binding HS domains were performed. Deca-
meric3H-labeled N-sulfated domains, from bovine intestinal
HS, were fractionated according to their activity toward FGF8b
on an affinity column (decasaccharides were used because it
was not possible to recover sufficient amounts of the smaller
FGF8b-binding oligosaccharides for analysis). Upon chroma-
tography on the FGF8b affinity matrix, 15–20% of the starting
decasaccharide material bound to FGF8b at a physiological
ionic strength. Almost all of the bound decasaccharides were
evolved from the column at 0.2 or 0.3 M NaCl (~45% at each ionic
strength). The separated decasaccharide pools were cleaved
with nitrous acid and3H-end-labeled by reduction with
NaB3H4. The resulting3H-labeled disaccharides were recov-
ered by gel filtration and further separated with strong anion-
exchange chromatography and/or high voltage paper electro-
phoresis. The anion-exchange chromatography analysis
indicated that the FGF8b-bound saccharides were enriched in
disulfated IdoUA(2-OSO3)-GlcNSO3 units (~1.4-fold) and
particularly in trisulfated IdoUA(2-OSO3)-GlcNSO3(6-OSO3)
units, the proportions of which were increased ~5-fold in the
fraction with the highest apparent affinity as compared with

FIG. 1. Binding of heparin to FGF8b. [3H]Heparin was incubated
in solution with FGF8b (6 µg/ml) for 2 h. The formed FGF8b-heparin
complexes were trapped on nitrocellulose filters, and the filter-bound
radioactivity was quantified by liquid scintillation counting (see “Ma-
terials and Methods”).

FIG. 2. Binding of heparin/HS oligosaccharides to FGF8b. To
assess the minimal length of FGF8b-binding heparin/HS domains,3H-
labeled heparin oligosaccharides (10,000 cpm) (A) or N-sulfated do-
 mains from bovine intestinal HS (20,000 cpm) (B) were incubated with
FGF8b (15 and 12.5 µg/ml, respectively) followed by the filter-trapping
procedure and quantification of the bound saccharide.

FIG. 3. Binding of selectively desulfated heparin preparations
to FGF8b. Unlabeled native heparin (H), selectively N- (N-DS), 2-O-
(2-O-DS), or preferentially 6-O-desulfated (6-O-DS) heparin prepara-
tions were incubated at the indicated concentrations with [3H]heparin
(10,000 cpm) and FGF8b (12.5 µg/ml). FGF8b-bound [3H]heparin was
trapped on nitrocellulose and quantified as described under “Materials
and Methods.” Heparin binding to FGF8b in the absence of any com-
petitor was set to 100%.
Disaccharide composition of FGF8b-binding HS domains. Decameric N-sulfated domains from bovine intestinal HS were fractionated on FGF8b-affinity column. Saccharides that did not bind to the affinity matrix at physiological ionic strength (Unbound) and saccharides that required 0.2 or 0.3 M NaCl for their elution from the matrix were subjected to cleavage by nitrous acid, pH 1.5. The resultant disaccharides were radiolabeled by reduction with NaB3H4 and recovered by gel chromatography on a column of Sephadex G-15. The proportions of the non-O-sulfated disaccharides were analyzed by high voltage paper electrophoresis, and the O-sulfated disaccharides were analyzed by anion-exchange high performance liquid chromatography. A, compositional disaccharide analysis of the unbound and bound decasaccharides. The peaks correspond to the following disaccharide structures in the native saccharide: peak 1, GlcA(2-OSO3)-GlcNSO3; peak 2, GlcA-GlcNSO3(6-OSO3); peak 3, IdoUA-GlcNSO3(6-OSO3); peak 4, IdoUA(2-OSO3)-GlcNSO3; and peak 5, IdoUA(2-OSO3)-GlcNSO3(6-OSO3). The asterisk indicates tetrasaccharides, in part due to "anomalous" ring contraction (39), that were not included in the quantification of disaccharides shown in B. B, schematic representation of the total disaccharide composition.

The Erk1 and Erk2 kinases are important signal transducers downstream of FGF8s and critical for the mitogenic FGF response (46). To further study the importance of heparin domain length for the biological activity of FGF8b, we assessed the effect of heparin oligosaccharides on FGF-induced Erk phosphorylation. FGF8b alone induced a transient phosphorylation analogously to heparin. By contrast, upon stimulation with FGF1, decameric heparin domains were sufficient to promote a strong and sustained Erk1/2 phosphorylation, whereas FGF1 alone did not induce detectable phospho-

deficient CHO677 cells were used to assess the ability of various exogenously added saccharides to restore the cell proliferation induced by FGF8b. FGF8b and/or heparin were added to serum-depleted cells, and the cell proliferation was assessed after 24 h by measuring the incorporation of [3H]thymidine into DNA by liquid scintillation counting. For additional information see "Materials and Methods."

To identify the minimal heparin domain capable of supporting the biological activity of FGF8b, similar experiments were conducted with size-defined heparin oligosaccharides (4–24 monosaccharide units in length). The results indicated that long oligosaccharides, ≥18-mers, were required to support proliferation induced by FGF8b (Fig. 6). The results were thus very different from the experiments employing FGF1 or FGF2, in which octasaccharides were sufficient to enhance growth factor activity (Fig. 6), in agreement with previous data (28, 45). We note that the recombinant FGF8b used in this experiment contained a His6 tag at the C terminus. To exclude the possibility that this would influence the results we also studied commercially available FGF8b without any tag; consistently with the data pertaining to the His-tagged FGF8b, the untagged FGF8b alone did not induce proliferation of the CHO677 cells, whereas concomitant treatment with ≥18-mer oligosaccharides stimulated proliferation (data not shown).

FIG. 4. Disaccharide composition of FGF8b-binding HS domains. The peak 2, GlcA-GlcNSO3; peak 3, IdoUA-GlcNSO3(6-OSO3); and peak 4, IdoUA(2-OSO3)-GlcNSO3 are important for the FGF8b-HS interaction.
rylation. These data are in good agreement with the results from the cell proliferation studies and further support the idea that extended heparin domains are required for the biological activity of FGF8b.

The major FGF-receptor expressed on CHO677 cells is FGFR1 (45), but it is not known whether the cells express the IIIc isoform of FGFRs that would be the preferred isoform for FGF8b (8, 11, 12). To study whether the cellular FGFR expression influenced the minimal heparin domain length required for the biological activity of FGF8b, CHO677 cells were transfected with FGFR4, which is considered as an efficient receptor for FGF8b (8, 11). Furthermore we made use of S115 mammary carcinoma cells, which are known to be highly responsive toward FGF8b and express at least FGFR1–3 (2, 47–49). For the experiments, the S115 cells were treated with sodium chlorate to inhibit the sulfation of endogenous HS and thus to exclude its contribution to the FGF8b response. In both cell systems, 14-mer instead of 18-mer heparin domains were sufficient to enhance the proliferative response to FGF8b, as studied by [3H]thymidine incorporation experiments (Fig. 8A). In addition, 14-mer heparin domains efficiently promoted the phosphorylation of Erk1/2 in the FGFR4-transfected CHO677 cells (Fig. 8B). We note that in FGFR4-transfected cells, lower concentrations of FGF8b (10 ng/ml) than those required for efficient stimulation of untransfected CHO677 cells (100 ng/ml) were sufficient to enhance the thymidine incorporation remarkably (data not shown), and FGF8b alone (i.e., in the absence of heparin) had a weak stimulatory effect. The above observations likely relate specifically to the expression of appropriate FGFR isoforms, because FGFR1-transfected CHO677 cells displayed similar requirements with regard to the length of the stimulatory fragment as untransfected CHO677 cells (Fig. 8A, inset).

These results indicate that the cellular pattern of FGFR expression influences the length of the heparin domain needed to promote the biological activity of FGF8b. However, despite the FGFR expression pattern, a distinct requirement of extended heparin domains for the FGF8b activity was seen in each of the systems studied (14-mer versus 6–8-mer required for FGF1 and FGF2 activity).

To characterize further the heparin/HS structures required for the biological activity of FGF8b, we studied the ability of selectively desulfated heparin preparations to promote FGF8b induced cell proliferation. In untransfected CHO677 cells the N-, 2-O-, and 6-O-desulfated heparin preparations did not stimulate the cell proliferation in the presence of FGF8b, whereas they had some stimulatory effect on the proliferation by FGF1 and FGF2 (Fig. 9A). In FGFR4-transfected CHO677 cells, the FGF8b-induced proliferation was not significantly affected by any of the desulfated heparin preparations (Fig. 9B). These data suggest that the N-, 2-O-, and 6-O-sulfate groups of heparin are all involved in the formation of productive FGF8b-FGFR complexes.

In the body, heparin is confined to connective tissue-type mast cells, whereas HS is present rather ubiquitously and thus likely the major physiological polysaccharide ligand to FGF8b. To investigate whether HS would serve that purpose, HS prep-
arations isolated from different tissues were tested for their ability to support FGF8b-induced proliferation. Cell proliferation experiments with untransfected and FGFR1- or FGFR4-transfected CHO677 cells indicated that HS from bovine aorta, kidney, or lung did not support the proliferation in any of the cell systems (Fig. 10). Similar results were obtained when the cells were stimulated with FGF2 (Fig. 10). By contrast, intestinal HS seemed to enhance the activity of both FGF8b and FGF2 in FGFR4-transfected cells, whereas in untransfected or FGFR1-transfected cells such an enhancing effect was seen only upon stimulation with FGF2 (Fig. 10). These results point to distinct differences in the ability of the various HS preparations to enhance the biological activity of FGF8b. Whereas intestinal HS clearly increased the cell proliferation induced by FGF8b, other HS species such as aorta HS appeared to have an inhibitory rather than stimulatory effect.

**DISCUSSION**

Following the discovery that heparin-like polysaccharides are required for the biological activity of FGF2 (16, 17), a number of studies have addressed the role of specific heparin/HS structures in the binding and signaling of FGF1 and FGF2.
CHO677 cells were serum-starved for 48 h and stimulated with FGF8b. Untransfected, FGFR1-transfected, and FGFR4-transfected CHO677 cells were serum-starved for 48 h and stimulated with FGF8b.

N-acetyl monosaccharide units and suggest that the minimal FGF8b-binding heparin domain encompasses a subdomain of the tumor vascularization (9, 50, 51). The results indicate that the FGF species, which has been implicated particularly in cancer and tumor vascularization (34, 35, 36), limited data have been available of other FGF species.

Sequence analyses of FGF8b-binding domains are in progress to address this issue in detail. By contrast, in terms of the structural requirements for heparin/HS in the interactions with both FGFs and FGFRs, our results suggest that the differential saccharide requirements do not originate from the properties of the heparin/HS structures required in the process. Wild-type CHO677 cells, which lack endogenous HS but express low levels of FGFRs (at least FGFR1) (45) or FGFR1-transfected CHO677 cells with higher receptor density, did not show any response to FGF8b in the absence of exogenous heparin, although they responded well to FGF2. Moreover, long ≥18-mer heparin oligosaccharides were required for the mitogenic effect of FGF8b. By contrast, in CHO677 cells transfected with FGFR4 as well as in chlorate-treated S115 breast carcinoma cells, some FGF8b response was seen without exogenous heparin, and 14-mer oligosaccharides were sufficient to enhance the activity remarkably. These findings indicate that the length of the heparin domain needed for FGF8b signaling depends on the pattern of cellular FGFR expression.

X-ray crystallography data support the idea that FGF signaling involves a ternary complex consisting of the FGF, FGFR, and HS components. Based on such studies, two somewhat different structural arrangements have been proposed, one involving FGF2 and FGFR1 and the other involving FGF1 and FGFR2. In the first one, two FGF2 molecules bind to the opposite sides of two FGFR1s (52–54). The complex is stabilized by direct receptor-receptor interactions and by contacts of the FGF component with the two receptor molecules (55). The central region of a heparin oligosaccharide is proposed to make contact with both FGFR components, whereas the ends of the heparin domain would interact with the two FGF2 molecules (56). This model was later revised to contain two heparin chains with their reducing end toward the center of the complex (55). In the FGF1-FGFR2 complex, a heparin decasaccharide interacts with the two FGF components and one receptor molecule (56). In this model the saccharide seems to have a more important stabilizing effect on the complex than what was proposed for the FGF2-FGFR1 complex. Whereas both models suggest that ≥10–12-mer heparin oligosaccharides would sufficiently contribute to the various interactions between the saccharide and the protein domains, our data pertaining to FGFR4 and FGF8b point to the requirement of longer oligosaccharide structures for productive interactions. In FGFR4-transfected myeloid 32D cells (2, 57), which entirely lack endogenous FGFRs, the biological activity of FGF8b is stimulated by ≥14-mer oligosaccharides, whereas a corresponding activation of FGFR1 and FGFR2 is seen with much shorter oligomers. It would therefore be reasonable to assume that the differential saccharide requirements do not originate from the properties of FGF8b but rather from those of FGF8b. It is possible that the distance of the heparin-binding domains of FGF8b from those of the receptor component is larger than in other FGF species. Alternatively, the FGFR4-binding affinity of FGF8b may be lower than that of FGFR1 and FGFR2, such that stronger interactions via heparin would be needed to stabilize the complex. In FGF2, mutation of Asn-104 to alanine decreases its affinity toward FGFR1 400-fold (57), presumably by impaired hydrogen bonding with the receptor (58). Although the FGFR-binding affinity of FGF8 has not been studied, we note that FGF8 differs from FGF2 in having a threonine residue in the corresponding position. The side chain of threonine is shorter than that of asparagine and might thus be less likely to be involved in FGFR binding.

The finding that extended ≥14-mer heparin domains were needed to stimulate FGF8b signaling raises intriguing questions with regard to the structure of physiological polysaccharides.

\[ \text{CHO677 cells} \]

**FIG. 10. Effect of heparan sulfate on FGF-induced proliferation.** Untransfected, FGFR1-transfected, and FGFR4-transfected CHO677 cells were serum-starved for 48 h and stimulated with FGF8b (100 ng/ml) or FGF2 (1 ng/ml) and HS (1 μg/ml). [3H]Thymidine incorporation into DNA was measured. The heparan sulfate preparations tested were isolated from aorta, kidney, lung and intestine. GF, FGF alone; H, heparin; −, no treatment.

studies all indicate that while short (4–5-mer) saccharide sequences are sufficient to interact with FGFs (23–25, 27), longer 8–12-mer sequences are needed for the formation of FGF-FGFR complexes and thus FGF signaling (28–31). Although the structural requirements for heparin/HS in the above processes are now understood in some detail with regard to FGFR1 and FGFR2, limited data have been available of other FGF species.

In this paper we have studied the role of heparin/HS in the binding and biological activity of FGF8b, a splice variant of FGF8, which has been implicated particularly in cancer and tumor vascularization (9, 50, 51). The results indicate that the minimal FGF8b-binding heparin domain encompasses ≥5 intact monosaccharide units and suggest that the N2, 2-O-, and 6-O-sulfate substituents of heparin are all involved in the interaction. The size and sulfation of the FGF8b-binding heparin domains may thus resemble those interacting with FGFR1 (25, 26). Sequence analyses of FGF8b-binding domains are in progress to address this issue in detail. By contrast, in terms of biological activity, markedly longer heparin oligosaccharides were required to enhance the biological activity of FGF8b as compared with FGF1 or FGF2. In cells lacking endogenous HS, ≥14-mer heparin oligosaccharides were needed to stimulate the mitogenic effect of FGF8b, whereas the activity of FGF1 and FGF2 was enhanced by much shorter, 6–8-mer, oligosaccharides.

It is now generally recognized that heparin/HS play an important role in FGF signaling, presumably because of their interactions with both FGFs and FGFRs. Our results suggest that the cellular pattern of FGFR expression influences not only the responsiveness of the cells to various FGF species but also the heparin/HS structures required in the process. Wild-type CHO677 cells, which lack endogenous HS but express low levels of FGFRs (at least FGFR1) (45) or FGFR1-transfected CHO677 cells with higher receptor density, did not show any response to FGF8b in the absence of exogenous heparin, although they responded well to FGF2. Moreover, long ≥18-mer heparin oligosaccharides were required for the mitogenic effect of FGF8b. By contrast, in CHO677 cells transfected with FGFR4 as well as in chlorate-treated S115 breast carcinoma cells, some FGF8b response was seen without exogenous heparin, and 14-mer oligosaccharides were sufficient to enhance the activity remarkably. These findings indicate that the length of the heparin domain needed for FGF8b signaling depends on the pattern of cellular FGFR expression.

X-ray crystallography data support the idea that FGF signaling involves a ternary complex consisting of the FGF, FGFR, and HS components. Based on such studies, two somewhat different structural arrangements have been proposed, one involving FGF2 and FGFR1 and the other involving FGF1 and FGFR2. In the first one, two FGF2 molecules bind to the opposite sides of two FGFR1s (52–54). The complex is stabilized by direct receptor-receptor interactions and by contacts of the FGF component with the two receptor molecules (55). The central region of a heparin oligosaccharide is proposed to make contact with both FGFR components, whereas the ends of the heparin domain would interact with the two FGF2 molecules (56). This model was later revised to contain two heparin chains with their reducing end toward the center of the complex (55). In the FGF1-FGFR2 complex, a heparin decasaccharide interacts with the two FGF components and one receptor molecule (56). In this model the saccharide seems to have a more important stabilizing effect on the complex than what was proposed for the FGF2-FGFR1 complex. Whereas both models suggest that ≥10–12-mer heparin oligosaccharides would sufficiently contribute to the various interactions between the saccharide and the protein domains, our data pertaining to FGFR4 and FGF8b point to the requirement of longer oligosaccharide structures for productive interactions. In FGFR4-transfected myeloid 32D cells (2, 57), which entirely lack endogenous FGFRs, the biological activity of FGF8b is stimulated by ≥14-mer oligosaccharides, whereas a corresponding activation of FGFR1 and FGFR2 is seen with much shorter oligomers. It would therefore be reasonable to assume that the differential saccharide requirements do not originate from the properties of FGF8b but rather from those of FGF8b. It is possible that the distance of the heparin-binding domains of FGF8b from those of the receptor component is larger than in other FGF species. Alternatively, the FGFR4-binding affinity of FGF8b may be lower than that of FGFR1 and FGFR2, such that stronger interactions via heparin would be needed to stabilize the complex. In FGF2, mutation of Asn-104 to alanine decreases its affinity toward FGFR1 400-fold (57), presumably by impaired hydrogen bonding with the receptor (58). Although the FGFR-binding affinity of FGF8 has not been studied, we note that FGF8 differs from FGF2 in having a threonine residue in the corresponding position. The side chain of threonine is shorter than that of asparagine and might thus be less likely to be involved in FGFR binding.

The finding that extended ≥14-mer heparin domains were needed to stimulate FGF8b signaling raises intriguing questions with regard to the structure of physiological polysaccharides.

\[ \text{CHO677 cells} \]

**FIG. 10. Effect of heparan sulfate on FGF-induced proliferation.** Untransfected, FGFR1-transfected, and FGFR4-transfected CHO677 cells were serum-starved for 48 h and stimulated with FGF8b (100 ng/ml) or FGF2 (1 ng/ml) and HS (1 μg/ml). [3H]Thymidine incorporation into DNA was measured. The heparan sulfate preparations tested were isolated from aorta, kidney, lung and intestine. GF, FGF alone; H, heparin; −, no treatment.
ride ligands to FGF8-FGFR complexes. Heparin is found only in connective tissue-type mast cells, but no reports of FGF8b expression by mast cells are available. However, mast cell infiltration is frequently seen in various malignant tumors (59–61), raising the possibility that heparin released from tumor-associated mast cells might stimulate FGF8b activity in the tumor. The extended N-sulfated domains required for the activity of FGF8b occur rarely in HS (21, 22). However, heparin-like HS with a high degree of N-sulfation have been found in glial progenitor cells, whereas the differentiated astrocytes and oligodendrocytes originating from such progenitor cells express HS with a more typical N-sulfation pattern (62). Notably, FGF8b has been implicated in astroglial differentiation (63). Alternatively, the activating structural arrangement in HS might encompass two N-sulfated domains separated by an N-acetylated/N-sulfated domain. Our findings that HS from bovine aorta, kidney, and lung displayed inhibitory rather than stimulating effects on FGF8b activity suggest that many HS species lack the ability to stimulate FGF8b signaling. However, intestinal HS enhanced FGF8b activity in FGFR4-transfected CHO cells, suggesting that this HS species, or so far uncharacterized HS domains that mediate FGF8 signaling. Moreover, it is possible that other HS species contain domains that activate FGF8b but that a partial depolymerization of the intact polysaccharide is required for such activity (as has been described for FGF2 previously (64)).

Acknowledgments—We thank Susanna Pykäri and Susana Kalevo-Mattila for technical assistance. Prof. Ulf Lindahl and Dr. Johan Kreuger (Uppsala University) are acknowledged for fruitful discussions.

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