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Glycosaminoglycan affinity of the complete fibroblast growth factor family

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ABSTRACT

Background: Many fibroblast growth factor family proteins (FGFs) bind to the heparan sulfate/heparin (HP) subtypes of sulfated glycosaminoglycans (GAGs), and a few have recently been reported to also interact with chondroitin sulfate (CS), another sulfated GAG subtype.
Methods: To gain additional insight into this interaction, we prepared all currently known FGFs (i.e., FGF1–FGF23) and assessed their affinity for HP, CS-B, CS-D and CS-E. In addition, midkine, hepatocyte growth factor and pleiotrophin were studied as other known HP-binding proteins.
Results: We found that members of the FGF19 subfamily (i.e., FGF15, 19, 21 and 23) had little or no affinity for HP; all of the other secretable growth factors tested had strong affinities for HP, as was indicated by the finding that their elution from HP-Sepharose columns required 1.0–1.5 M NaCl. We also found that FGF3, 6, 8 and 22 had strong affinities for CS-E, while FGF5 had a moderate affinity for CS-D. The interactions between FGFs and GAGs thus appear to be more diverse than previously understood.
General significance: This is noteworthy, as the differential interactions of these growth factors with GAGs may be key determinants of their specific biological activities.

1. Introduction

Fibroblast growth factor (FGF) family proteins, including FGF homologous factors (FHFs), are encoded by 22 genes in both mice and humans. They are expressed by various cell types and are involved in regulating growth, differentiation and gene expression at the cellular level, thereby regulating development, homeostasis and metabolism at the organ level. Their activities are mainly exerted through their high-affinity binding to specific FGF receptor tyrosine kinases (FGFRs) on the cell surface. In addition, like several other polypeptide growth factors, most FGFs have an affinity for heparin, which modulates their receptor specificity; that is, in establishing an active FGF–FGFR signaling complex, heparin or its structural analog heparan sulfate (HS) plays an important role as a cofactor [1]. In addition, HS also serves as a low-affinity binding reservoir for FGFs on the cell surface, and as a binding molecule that stabilizes the three-dimensional structure of FGFs. Recent reports also suggest that HS tissue-specifically regulates the reactivity of the FGF–FGFR interaction [2,3]. Thus both the spatiotemporal expression and microstructure of HS appear to be as important for induction of specific biological processes via the FGF system as the spatiotemporal expression of FGFs and FGFRs.

The biological significance of chondroitin sulfate (CS) also has been addressed recently. Using the glycosyltransferases involved in CS biosynthesis as molecular tools, it was demonstrated that chondroitin or CS is indispensable for cell growth and differentiation [4]. In addition, Mizuguchi et al. showed that chondroitin is essential for cell division in *C. elegance* [5], and Thiele et al. described disruption of bone formation caused by abnormal sulfation of CS [6]. In both of these cases, the findings strongly suggest that dysregulation of polypeptide growth factor activity might be involved. Indeed, several growth factors have been shown to interact with CS, including midkine (MK) with CS-E [7], and hepatocyte growth factor (HGF) with CS-B [8]. Moreover, CS-E reportedly interacts with FGF16 and FGF18 [9], and CS-B enhances FGF7 activity [10].

With that as background, we assessed the affinities of all known FGFs for HS and CS to better understand the importance of GAG interactions in the biology of the FGF system.

2. Materials and methods

2.1. Cells and reagents

COS-1 cells were purchased from the American Type Culture Collection (ATCC) and cultivated in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, D1152) supplemented with

Abbreviations: FGF, fibroblast growth factor; GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; MK, midkine; HGF, hepatocyte growth factor; PTN, pleiotrophin; SM, skim milk; TBS, tris-buffered saline; KGF, keratinocyte growth factor

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Table 1
Recombinant proteins used in this study

	cDNA source	Number of amino acid residues[a]	Expected Mw[b]
FGF1	Human brain	167	19.2
FGF2	Human kidney	186	21.0
FGF3	Mouse brain	260	29.1
FGF4	Mouse ES cells, EB3/5	205	22.8
FGF5	Human tumor cell line	280	31.1
FGF6	Mouse brain	203	22.8
FGF7	Mouse skin	196	22.7
FGF8	Mouse mammary tumor	225	26.3
FGF9	Rat brain	239	27.2
FGF10	Mouse skin	203	23.2
FGF15	Mouse ES cells, EB3/5	225	26.4
FGF16	Rat heart	238	27.5
FGF17	Mouse brain	223	26.1
FGF18	Mouse skin	213	24.9
FGF19	Human adenocarcinoma, SW480	226	25.6
FGF20	Human adenocarcinoma, SW480	242	27.3
FGF21	Mouse liver	213	23.8
FGF22	Mouse skin	172	20.4
FGF23	Human leukemia, K-562	259	29.2
MK	HUVEC	154	17.3
HGF	Human placenta	728	83.4
PTN	Mouse brain	166	19.0

[a] With 3xFLAG–6xHis tag but without the signal sequence.
[b] Calculated using <http://paris.chem.yale.edu/extinct.html>.

10% fetal bovine serum (JRH Biosciences Inc., Lenexa,, KS) under 5% CO_2 at 37 °C. In addition, the cells used for recombinant protein production were incubated in Opti-MEM I serum-free medium (Invitrogen, Carlsbad, CA).

Heparin-Sepharose CL-6B was purchased from Amersham Bioscience AB (Uppsala, Sweden). HS, CS-B, CS-D and CS-E were all purchased from Seikagaku Kogyo (Tokyo, Japan).

Total RNA was isolated from tissues or cultured cells using TRIZOL (Invitrogen). Subsequent reverse transcription and polymerase chain reaction (RT-PCR) were carried out using Superscript II (Invitrogen) and KOD-Plus-polymerase (TOYOBO, Japan), respectively. Synthetic oligonucleotides for PCR were purchased from Tsukuba Oligo Service Co., Ltd. (Tsukuba, Japan). The sequences of the isolated cDNAs and expression vectors were verified using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.2. cDNA preparation and expression vector construction

The sources for the cDNAs encoding FGFs are summarized in Table 1. The cDNAs for FGF3 and 17, as well as PTN, were prepared from mouse brain RNA by RT-PCR; those for FGF7, 10, 18 and 22 were from mouse skin; FGF21 cDNA was from mouse liver; cDNAs for FGF4 and 15 were from mouse ES cells (EB3/5); those for FGF19 and FGF20 were from human colorectal adenocarcinoma cells (SW480); FGF23 cDNA was from human chronic myelogenous leukemia cells (K-562), and MK cDNA was from human umbilical vein endothelial cells. Full-length cDNAs were amplified by PCR, and the resultant DNA fragments were cloned into pCR-Blunt II-TOPO vector (Invitrogen). Isolation of cDNAs for human FGF1 and FGF2, mouse FGF6 and rat FGF16 was previously described [11–14]. cDNAs for human FGF5 [15], mouse FGF8 [16], rat FGF9 [17] and human HGF [18] were kindly provided by Drs. Xi Zhan (Holland Laboratory of American Red Cross, MD), Craig A. MacArthur (Univ. Southern California, CA), Tsutomu Kurokawa (Takeda Chemical Industries Company, Ltd., Tsukuba, Japan) and Keiji Miyazawa (Univ. of Tokyo, Tokyo, Japan), respectively.

2.3. Construction of expression vectors for mammalian cells

Oligonucleotides #466 (5′-ccg ctc gag act aca aag acc atg acg gtg att ata aag atc atg aca tcg act aca ag-3′) and #467 (5′-tgc ggg ccc tca atg gtg atg gtg atg atg acc ctt gtc atc gtc atc ctt gta gtc ga-3′) were subjected to intended dimer formation with KOD-Plus-, after which the product was digested with Apa I and Xho I. The resultant DNA fragment was cloned in pcDNA3.1(+) (Invitrogen) pre-digested with Apa I and Xho I. One clone, designated FLAG-His/pcDNA3.1(+)/cl. 9, was verified for the expected DNA sequence and used for subsequent experiments.

In almost all cases, the full open reading frame (ORF) for each growth factor was amplified by PCR using a sense primer containing an EcoR I adaptor and Kozak sequence, and an antisense primer containing a Xho I adaptor. The PCR product was digested with EcoR I and Xho I, and the resultant fragment was cloned into FLAG-His/pcDNA3.1(+) pre-digested with EcoR I and Xho I. When the ORF contained a Xho I recognition sequence, a Sal I adaptor was used for the antisense primer, as it generates compatible cohesive ends. When the ORF contained an EcoR I recognition sequence, an EcoR V or BamH I adaptor was used for the sense primer.

2.4. Expression of recombinant proteins by COS-1 cells and their purification

The day before transfection, 2×10^6 COS-1 cells were seeded into 10-cm cell culture dishes. Expression vectors comprised of MK, HGF or PTN cDNAs cloned into FLAG-His/pcDNA3.1(+) were transfected using FuGENE 6 (Roche Diagnostics Ltd., Basel, Switzerland) following the

Table 2
Amino acid sequences of N-terminus of the recombinant FGFs used in this study

FGFs	Amino acid sequences
FGF1	MANYKKPKLLYCSNGGHFLRILPDGTVDGTRDRSDQHIQLQLSAESVGEVYIKSTETGQY…
FGF2	MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHI…
FGF3	MTTGPGTRLRRDAGGRGGVYEHLGGAPRRRKLYCATKYHLQLHPSGRVNGSLENSAYSIL…
FGF4	MAPNGTRHAELGHGWDGLVARSLARLPVAAQPPQAAVRSGAGDYLLGLKRLRRLYCNVGI…
FGF5	MHGEKRLTPEGQPAPPRNPGDSSGSRGRSSATFSSSSASSPVAASPGSQGSGSEHSSFQW…
FGF6	MSPAGARANGTLLDSRGWGTLLSRSRAGLAGEISGVNWESGYLVGIKRQRRLYCNVGIGF…
FGF7	MACNDMSPEQTATSVNCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQ…
FGF8	MQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQVLANKRINAMAEDGD…
FGF9	MAPLGEVGSYFGVQDAVPFGNVPVLPVDSPVLLSDHLGQSEAGGLPRGPAVTDLDHLKGI…
FGF10	MLGQDMVSQEATNCSSSSSSFSSPSSAGRHVRSYNHLQGDVRWRRLFSFTKYFLTIEKNG…
FGF15	MRPLAQQSQSVSDEDPLFLYGWGKITRLQYLYSAGPYVSNCFLRIRSDGSVDCEEDQNER…
FGF16	MAEVGGVFASLDWDLQGFSSSLGNVPLADSPGFLNERLGQIEGKLQRGSPTDFAHLKGIL…
FGF17	MENHPSPNFNQYVRDQGAMTDQLSRRQIREYQLYSRTSGKHVQVTGRRISATAEDGNKFA…
FGF18	MAEENVDFRIHVENQTRARDDVSRKQLRLYQLYSRTSGKHIQVLGRRISARGEDGDKYAQ…
FGF19	MRPLAFSDAGPHVHYGWGDPIRLRHLYTSGPHGLSSCFLRIRADGVVDCARGQSAHSLLE…
FGF20	MAPLAEVGGFLGGLEGLGQQVGSHFLLPPAGERPPLLGERRSAAERSARGGPGAAQLAHL…
FGF21	MYPIPDSSPLLQFGGQVRQRYLYTDDDQDTEAHLEIREDGTVVGAAHRSPESLLELKALK…
FGF22	MGYPHLEGDVRWRRLFSSTHFFLRVDLGGRVQGTRWRHGQDSIVEIRSVRVGTVVIKAVY…
FGF23	MYPNASPLLGSSWGGLIHLYTATARNSYHLQIHKNGHVDGAPHQTIYSALMIRSEDAGFV…

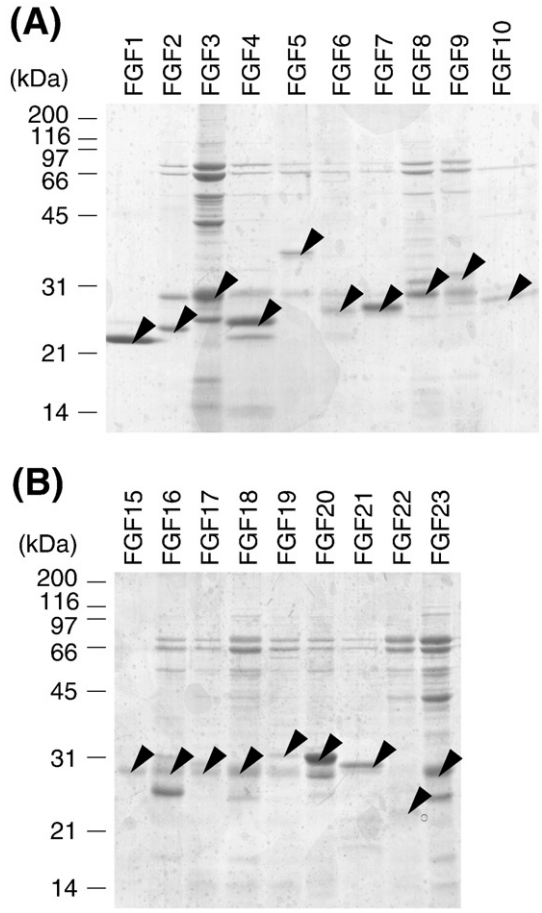

Fig. 1. Electrophoresis of recombinant FGFs prepared in this study. The cDNAs encoding FGF1–23 were cloned into pET-3c and expressed in BL21(DE3)/pLysS *E. coli* cells. The proteins were purified on Ni-NTA agarose columns. Each protein (approximately 1 μg) was resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Arrowheads indicate the FGF protein of interest. The calculated molecular weight of each protein is listed in Table 1. The mobilities of the molecular weight standards are indicated at the left in kDa.

manufacturer's instructions. The cells were then cultivated in Opti-MEM I serum-free medium for 72 h, after which the conditioned medium was collected. To semi-purify the secreted proteins, Ni-NTA agarose (QIAGEN GmbH, Hilden, Germany) was added to the medium and, after washing with wash buffer (50 mM Tris–HCl [pH 8.0], 300 mM NaCl, 20 mM imidazole), the bound proteins were eluted using elution buffer (50 mM Tris–HCl [pH 8.0], 300 mM NaCl, 250 mM imidazole). After the fractions were analyzed by immunoblot and ELISA, those containing the protein of interest were pooled and subjected to extensive dialysis against Tris-buffered saline (TBS; 10 mM Tris–HCl [pH 7.4], 0.15 M NaCl). The concentrations of the semi-purified proteins were then determined by ELISA, after which the proteins were subjected to further analyses. The yields of the semi-purified proteins ranged from 0.3 μg to 1 μg.

2.5. Construction of expression vectors for E. coli

When *E. coli* were used as the host cells to express mammalian secretable proteins, the cleavable secretion signal sequence at the N-terminus of the protein was not required and even diminished the yield. Therefore, all FGFs were expressed in soluble form by removing the cleavable N-terminal signal sequences. This was accomplished by deleting the cDNA encoding the signal sequence when the expression vector was constructed. SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) [19] was used to predict signal peptide cleavage sites within the amino acid sequences. To facilitate better understanding, the amino acid sequences of the N-terminal regions of the FGFs used in this study are summarized in Table 2. For all the FGFs, the natural C-terminal sequences were maintained and linked with the 3xFLAG-$(His)_6$ tag. Thereafter, cDNAs encoding each of the growth factors, without their signal sequence but with a 3xFLAG-$(His)_6$ tag, were amplified by PCR using the mammalian expression vector described above as a template, a sense primer containing an Nde I adaptor and an antisense primer containing a BamH I adaptor. The PCR product was digested with Nde I and BamH I, and the resultant fragment was cloned into pET-3c vector (Novagen, Merck KGaA, Germany) pre-digested with Nde I and BamH I. When the ORF contained a BamH I recognition sequence, a Bgl II adaptor was used for the antisense primer, as it generates compatible cohesive ends.

2.6. Expression of recombinant proteins by E. coli and their purification

Complementary DNAs encoding FGFs were cloned into pET-3c expression vector and used to transform BL21(DE3)/pLysS *E. coli* cells, after which positive clones surviving in the presence of ampicillin and chloramphenicol were selected. Protein expression was then induced using an Overnight Express Autoinduction System (Novagen), and the soluble proteins were liberated by disrupting the cell wall using BugBuster Reagent (Novagen) following the manufacturer's instructions. Briefly, confluent *E. coli* suspension was diluted 1:1000 (routinely 0.1 ml to 100 ml) in Overnight Express System medium supplemented with ampicillin and chloramphenicol and then incubated for 18 h at 37 °C with constant shaking at 300 rpm. After centrifugation, the cell pellet was suspended in BugBuster Protein Extraction reagent (routinely 5 ml) containing Benzonase Nuclease and incubated at room temperature on a rotating mixer for 30 min. The insoluble debris was then removed by centrifugation, and the supernatant was filtered through a 0.45 μm filter. The resultant clarified extract was added to Ni-NTA agarose (QIAGEN), and

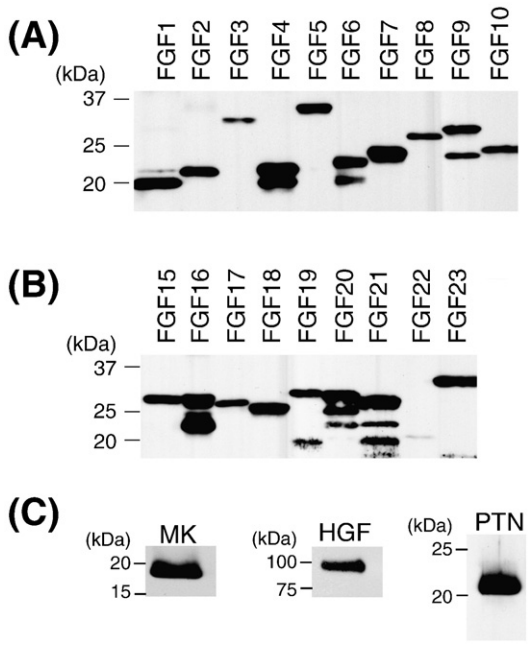

Fig. 2. Immunoblot analysis of recombinant growth factor proteins prepared in this study. FGF1–23 was prepared as described in the legend of Fig. 1, and the cDNAs encoding MK, HGF and PTN were cloned into pcDNA3.1(+) and expressed in COS-1 cells. The proteins were purified on Ni-NTA agarose columns. Each protein (approximately 50 ng) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized using anti-FLAG monoclonal antibody. The calculated molecular weight of each protein is listed in Table 1. The mobilities of the molecular weight standards are indicated at the left in kDa.

recombinant proteins were semi-purified as described above. The yields of the semi-purified FGFs were approximately 50–200 μg, depending on the construct.

2.7. ELISA based on FLAG epitope to determine the concentrations of recombinant proteins

Protein samples were loaded into microtiter plates (NUNC Immuno plate, C96 maxisorp) along with standard samples. The loaded proteins were fixed on the plate by incubation overnight at 4 °C. After washing with TBS containing 0.05% Tween-20 (TBS-T), 5% skim milk (SM; Wako Pure Chemical Industries, Ltd., Japan) dissolved in TBS was added and incubated for 1 h at 37 °C. Thereafter, monoclonal anti-FLAG antibody M2 (100 μg/ml in 1% SM/TBS; Sigma-Aldrich) was added and incubated for 1 h at 37 °C. After washing the plates 3 times with TBS-T, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (1/1000 dilution in 1% SM/TBS; Zymed Laboratories Inc., CA) was added and incubated for 1 h at 37 °C. Final washing was repeated 4 times, after which TMB(+) reagent (DAKO, Denmark) was added to visualize the HRP activity. After terminating the enzyme reaction by adding 1 N H_2SO_4, the absorbance at 450 nm was measured. 3xFLAG-$(His)_6$-tagged FGF1 prepared previously was used as the standard sample in this ELISA system.

2.8. Electrophoresis and immunoblot analysis of semi-purified growth factors

Semi-purified recombinant proteins (approximately 1 μg) were separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining. For immunoblot analysis, semi-purified recombinant proteins (approximately 50 ng) were separated in the same way and electronically transferred to nitrocellulose membranes (Schleicher & Schuell BioScience Inc., Keene, NH). The membranes were soaked in 5% SM/TBS for 18 h at 4 °C, after which the proteins on the membrane were probed with anti-FLAG monoclonal antibody M2 (10 μg/ml in 5% SM/TBS; Sigma-Aldrich Co.). HRP-conjugated goat anti-mouse IgG (H+L) (1/10,000 dilution in 5% SM/TBS; Zymed Laboratories Inc.) was used as the secondary antibody and visualized with ECL (Amersham Bioscience) according to the manufacturer's instructions.

2.9. Immobilization of glycosaminoglycans

GAGs were immobilized on Sepharose using the carbodiimide method, basically following the protocol described in the reference [20]. The carboxyl groups of GAGs were activated using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; Wako Pure

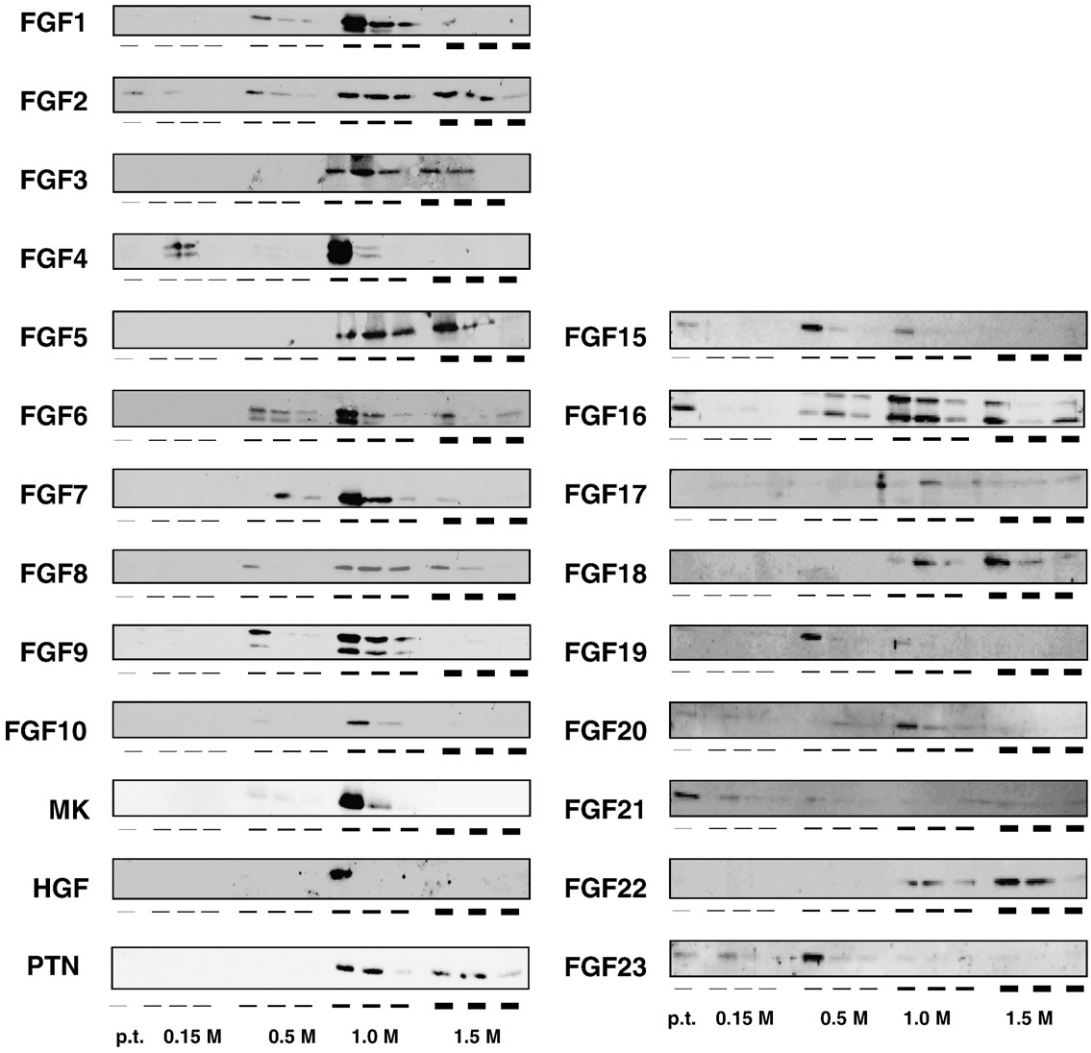

Fig. 3. Affinity of growth factors for immobilized heparin. All of the FGF proteins used in this set of experiments were expressed in *E. coli*. Each growth factor was applied to an open heparin-Sepharose column, which was then sequentially run with three column volumes each of 10 mM Tris–HCl containing 0.15 M, 0.5 M, 1.0 M and 2.0 M NaCl. Aliquots from each fraction (one-column volume) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized using anti-FLAG monoclonal antibody. The NaCl concentration at which a protein was eluted is indicative of the protein's affinity for heparin. The results are summarized in Table 3.

Table 3
Summary of glycosaminoglycan affinity of the growth factors

	HP	CS-E	CS-B	CS-D
FGF1	+	+/−	+/−	−
FGF2	+~++	+/−	+/−	+/−
FGF3	+	+	−	−
FGF4	+	+/−	−	+/−
FGF5	+~++	+/−~+	+/−~+	+
FGF6	+	+	−	−
FGF7	+	+/−	+/−	+/−
FGF8	+~++	+	+/−	+/−
FGF9	+	+/−	+/−	+/−
FGF10	+	+/−	+/−	+/−
FGF15	+/−	−	Nd	Nd
FGF16	+	++	Nd	Nd
FGF17	+	−	Nd	Nd
FGF18	++	+~++	Nd	Nd
FGF19	+/−	−	Nd	Nd
FGF20	+	+/−~+	Nd	Nd
FGF21	−	−	Nd	Nd
FGF22	++	+	Nd	Nd
FGF23	+/−	−	Nd	Nd
MK	+	+	+/−	+/−
HGF	+	+	+	+/−
PTN	+	++	Nd	Nd

Abbreviations: HP, heparin; CS-E, chondroitin sulfate-E; CS-B, chondroitin sulfate-B; CS-D, chondroitin sulfate-D.
"–, +/–, + and ++" indicate no binding (flow through at 0.15 M NaCl), weak binding (eluted at 0.5 M NaCl), moderate binding (eluted at 1.0 M NaCl), and strong binding (eluted at 1.5 M NaCl), respectively.
"Nd" indicates "not determined".

Chemical Industries, Ltd., Osaka, Japan) to generate carboxylic anhydride, which was then coupled to amino groups provided by EAH-Sepharose 4B (Amersham Bioscience AB). The excess amino groups were blocked using acetic anhydride. In these experiments, approximately 4 mg of GAG were immobilized on 1.0 ml of Sepharose (bed volume). The GAG-immobilized Sepharose beads were extensively washed with TBS and saved in TBS with 0.02% NaN_3 at 4 °C until use.

The coupling efficiency was evaluated by using the carbazole–sulfuric acid method to quantify uronic acid before and after the coupling. As no uronic acid was detected in the flow-through fraction after coupling (data not shown), the protocol employed to immobilize the GAG sugars on Sepharose was deemed effective.

2.10. Analysis of the affinity between the growth factors and immobilized glycosaminoglycan

Minicolumns were prepared using the GAG-immobilized Sepharose. Disposable pipette tips were plugged with glass wool and packed with a slurry of GAG-Sepharose (bed volume, ~100 μl). After extensive washing with 10 mM Tris–HCl (pH 7.4) containing 2.0 M NaCl, the column was equilibrated with TBS. Aliquots of recombinant growth factors, ranging between 40 and 200 ng, were dissolved in 300 μl TBS and loaded onto the minicolumns. The columns were then washed three times with 300 μl TBS containing 0.15 M NaCl, after which they were washed three times with 10 mM Tris–HCl (pH 7.4) containing either 0.5 M NaCl, 1.0 M NaCl or 2.0 M NaCl. Aliquots that pass through and each eluted fraction were then subjected to SDS-PAGE and immunoblot analysis, as described above.

2.11. Analysis of the affinity of mammalian cell-expressed FGF19 subfamily members for immobilized heparin

Complimentary DNAs for human FGF19, 21 and 23 were cloned into pcDNA3.1/His, and the expression vector was constructed as described previously. [21] COS-1 cells were transfected with the plasmid in serum-free medium (Opti-MEM I), after which the conditioned medium were collected as described above. An aliquot of the conditioned medium was then applied to an AKTA-FPLC chromatographic system equipped with a HiTrap heparin column (Amersham Bioscience). The NaCl concentration of the buffer was gradually increased from 0.15 M to 2.5 M, and the eluate was fractionated. Aliquots of each fraction were separated by SDS-PAGE, and the FGFs were detected by immunoblotting using anti-penta-His monoclonal antibody (QIAGEN).

3. Results

3.1. Expression and purification of recombinant growth factors

Complementary DNAs encoding FGFs were cloned into pET-3c expression vector together with the 3xFLAG-$(His)_6$ tag and were used to transform *E. coli* cells (BL21(DE3)/pLysS). The expressed proteins were purified on Ni-NTA columns and dialyzed against TBS. The results of SDS-PAGE (Fig. 1) indicated that, in addition to the FGF protein of interest (indicated by arrowheads), partially degraded FGF proteins and/or *E. coli*-derived proteins were also present in some of the samples, despite the inclusion of protease inhibitors during the purification procedure. Nonetheless, the intact FGF of interest was clearly detected in each preparation, and immunoblot analysis revealed that the molecular weights of the FGF proteins were consistent with the calculated molecular weights listed in Table 1 (Fig. 2A and B). The main bands were examined further in later experiments, and the impurities do not appear to have affected our conclusions about the interactions of FGFs with GAGs determined by western blotting.

To assess the biological activities of the recombinant 3xFLAG-$(His)_6$ tagged FGFs, we initially compared the activity of 3xFLAG-$(His)_6$-tagged FGF1 with that of untagged FGF1 and confirmed that their effects are indistinguishable [22]. Because FGF family members have highly conserved three-dimensional structures, as well as conserved primary structures in a core region of approximately 120 amino acids, it is expected that the C-termini do not form solid structures; instead, they are "sticking out from a trefoil barrel structure", as was seen in the crystal structures of FGF1, FGF2, FGF4, FGF7, FGF8, FGF9, FGF10, FGF19 and FGF23 [23–30]. We therefore expect that tagging their C-termini have little or no effect on the biological activities of the FGF proteins used in this study.

Complementary DNA encoding MK, HGF and PTN were cloned into pcDNA3.1(+) expression vector with 3xFLAG-$(His)_6$ tag and transiently

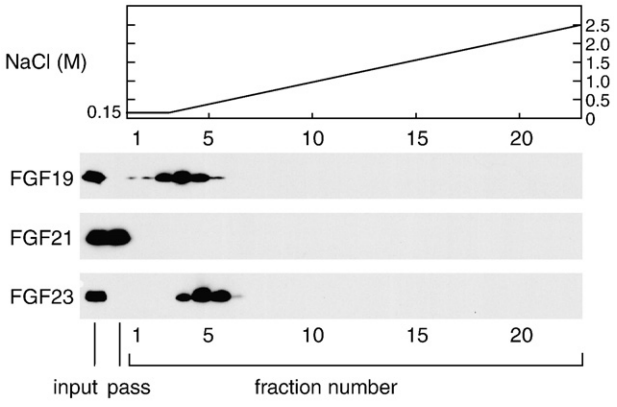

Fig. 4. FGF19 subfamily members show little or no affinity for immobilized heparin. In this set of experiments FGF19, 21 and 23 were expressed in COS-1 cells. The respective COS-1 conditioned media were applied to a HiTrap heparin column on an AKTA-FPLC system. In 10 mM Tris–HCl (pH 7.4), the NaCl concentration was gradually increased from 0.15 M to 2.5 M (top panel), and the eluate was fractionated. Each fraction was then resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized using an anti-His antibody. "Input" indicates the equivalent amounts of sample loaded onto the column.

transfected into COS-1 cells. The proteins secreted into a serum-free medium during the subsequent 72 h were collected, purified on Ni-NTA columns and dialyzed against TBS. As shown in Fig. 2C, each purified protein had the expected molecular weight listed in Table 1.

3.2. Evaluation of the growth factor affinities for immobilized heparin

Although FGFs were once designated heparin-binding proteins, not all have been examined with respect to their affinity for heparin. To address this issue, we subjected all known FGFs to heparin-affinity chromatography (Fig. 3, Table 3). FGF11, FGF12, FGF13 and FGF14 are also known as FHF3, FHF1, FHF2 and FHF4, respectively, and are sometimes not thought of as true FGFs because they do not bind to and activate FGFRs. We therefore excluded the description of FHFs (FGF11–14) and the results obtained with them from this manuscript.

We found that FGF1–10 in physiological buffer were retained on heparin-Sepharose columns and were eluted with buffer containing 1.0 M or 1.5 M NaCl, which is in good agreement with earlier studies [31]. Likewise, FGF16–18, 20 and 22 showed strong affinity to heparin, and were eluted with 1.0 M or 1.5 M NaCl. By contrast, FGF21 did not bind to heparin-Sepharose at all, while human FGF19 and its paralogue, mouse FGF15, and FGF 23 exhibited similar low affinities for heparin-Sepharose and were eluted with 0.5 M NaCl. The other heparin-binding growth factors examined (i.e., MK, HGF and PTN) exhibited high affinity for heparin-Sepharose and were eluted with 1.0 M NaCl (Fig. 3), which is also in good agreement with the earlier reports [32,33]. We also assessed the affinity of FGF1–10 for immobilized HS prepared from bovine kidney, and found that their elution profiles were similar to those for heparin-Sepharose (data not shown).

3.3. FGF19 subfamily members have little or no affinity for immobilized heparin

Because step-wise elution showed that members of the FGF19 subfamily (i.e., FGF15, FGF19, FGF21 and FGF23) have little or no affinity for immobilized heparin (Fig. 3), we assessed their heparin affinity more precisely using AKTA-FPLC. In addition, to eliminate the possibility that the low affinity of these proteins for heparin reflects inappropriate folding resulting from their expression in *E. coli*, we expressed them in a mammalian expression system for these experiments. As shown in Fig. 4, FGF21 in conditioned medium did not interact with heparin at all. FGF19 and 23 weakly bound to heparin and were eluted with 0.33 M and 0.50 M NaCl, respectively. Even after dialysis against phosphate-buffered saline or 25 mM phosphate buffer containing 50 mM NaCl, FGF21 passed through the heparin column

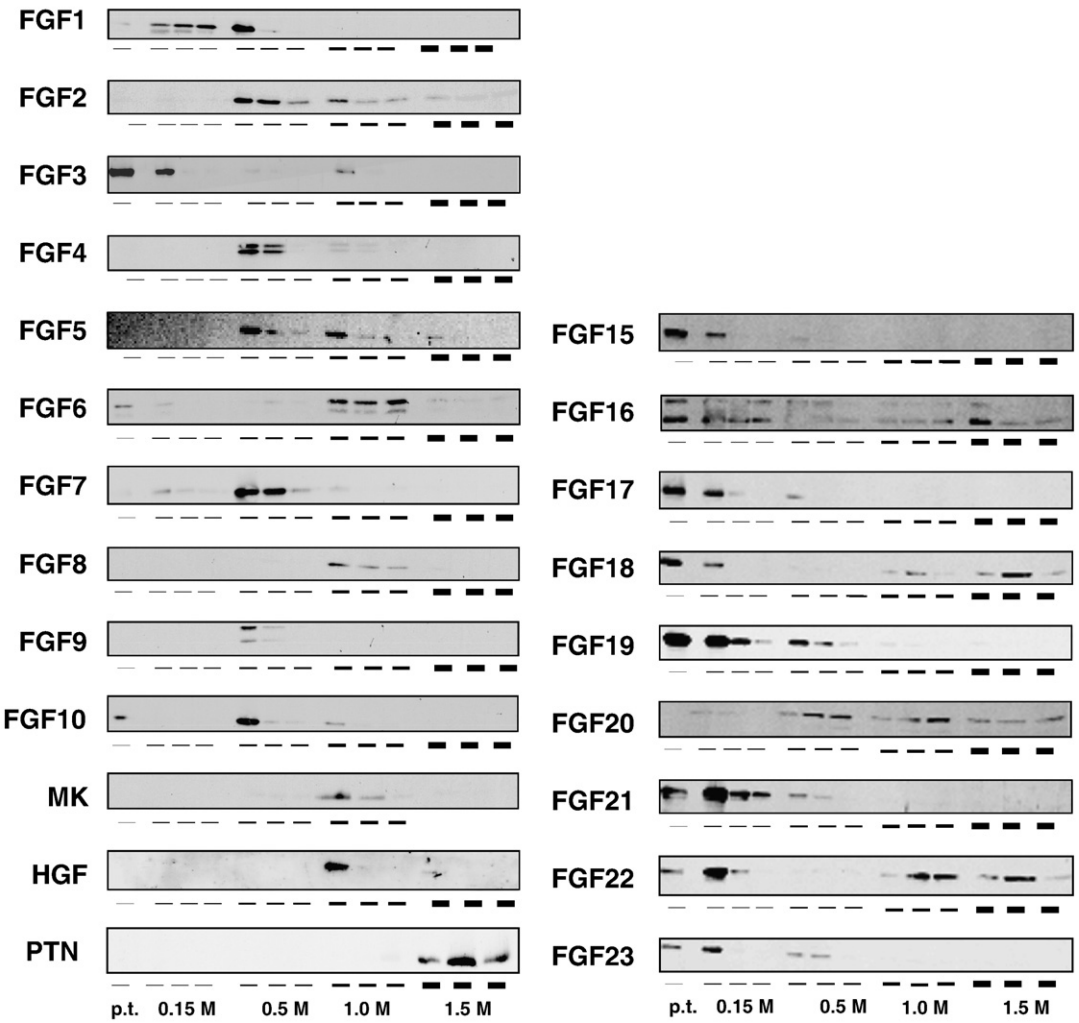

Fig. 5. Affinity of growth factors for immobilized CS-E. Each growth factor was applied to a CS-E-Sepharose column, which was then sequentially run with three column volumes each of 10 mM Tris–HCl containing 0.15 M, 0.5 M, 1.0 M and 2.0 M NaCl. Aliquots from each fraction (one-column volume) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized using an anti-FLAG monoclonal antibody. The NaCl concentration at which a protein was eluted is indicative of the protein's affinity for CS-E. The results are summarized in Table 3.

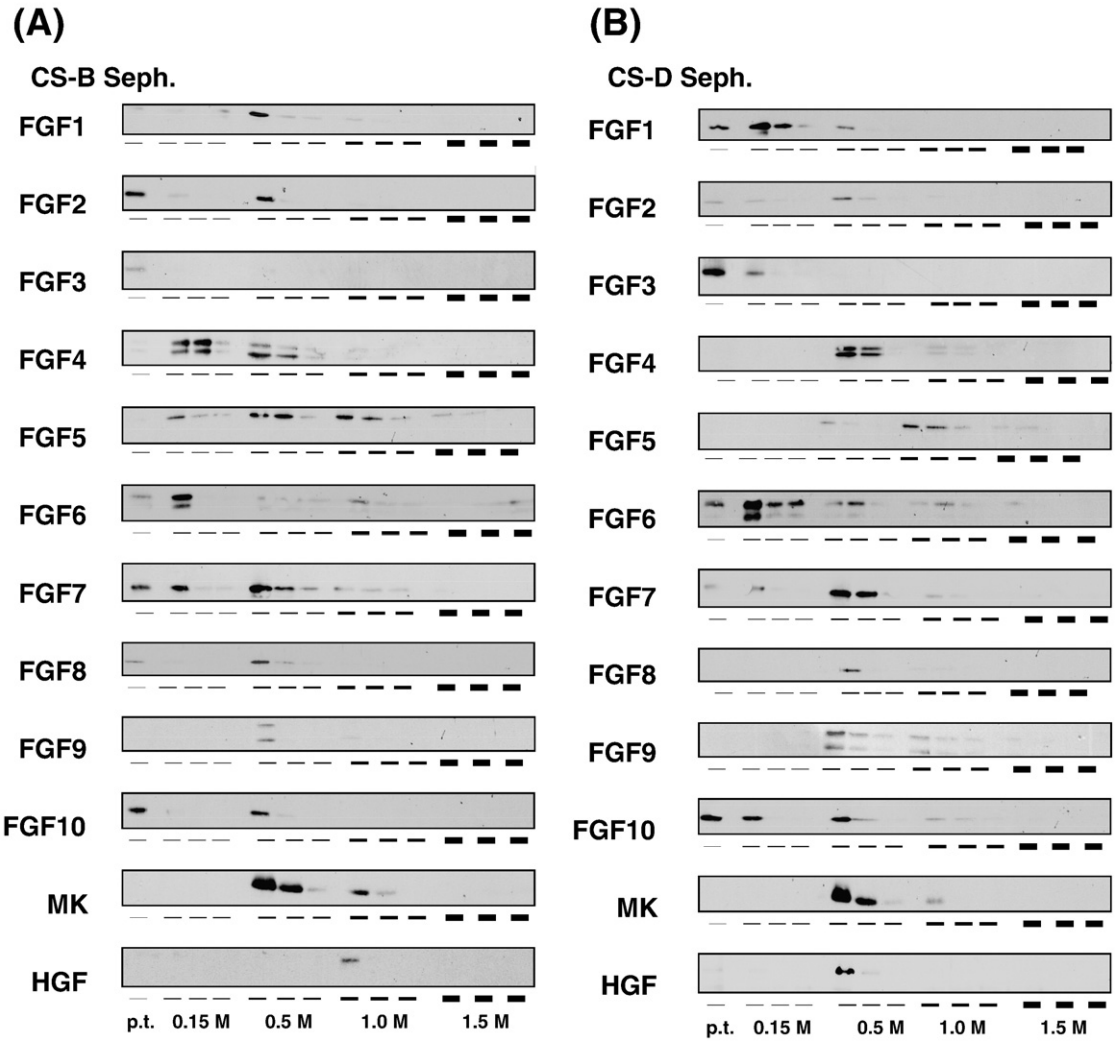

Fig. 6. Affinity of growth factors for immobilized CS-B (A) and CS-D (B). The experimental design was identical to that used for CS-E.

(data not shown), which is consistent with an earlier study carried out using surface plasmon resonance spectroscopy [30].

3.4. Affinity of growth factors for immobilized chondroitin sulfate-E, -B and -D

Recent filter binding assays and surface plasmon resonance analyses revealed an interaction between some FGFs and CS; in particular, FGF16 and 18 bind to CS-E [9]. We therefore conducted an affinity chromatography study using immobilized CS with the same experimental design used with immobilized heparin. Because immobilized CS beads are not commercially available, we immobilized various classes of CS on EAH-Sepharose beads using the coupling reagent EEDQ. When we subjected MK, HGF and PTN to CS-E column chromatography, we found that all three bound to the immobilized CS-E and were eluted with 1.0 M, 1.0 M or 1.5 M NaCl, respectively (Fig. 5), which is in good agreement with earlier results [7]. Moreover, we found that HGF also bound to immobilized CS-B and was eluted with 1.0 M NaCl (Fig. 6A), which is also consistent with earlier findings [8].

While most FGFs had little or no affinity for CS-E, FGF3, 6, 8 (all eluted at 1.0 M NaCl), 16, 18 and 22 (all eluted at 1.5 M NaCl) bound to CS-E (Fig. 5). In addition FGF5 showed moderate affinity for CS-D, eluting at 1.0 M NaCl (Fig. 6B), but no FGF exhibited any affinity for CS-B (Fig. 6A). Many growth factors also showed weak affinity (eluted at 0.5 M) for CS-E, CS-D and CS-B (Figs. 5 and 6). These results are summarized in Table 3.

4. Discussion

Most FGF family members have been characterized as having an affinity for heparin; indeed, this feature was considered essential because heparin, or the cell surface component HS, is actively involved in forming FGF–FGFR complexes. However, the affinity for heparin of a number of recently identified FGF proteins had not been characterized. Moreover, some FGFs reportedly bind CS [9], raising the possibility that this feature is shared by other FGF proteins. We therefore comprehensively analyzed the affinity of FGFs for heparin, HS and CS.

The finding that FGF15, 19, 21 and 23, which are all the members of the FGF19 subfamily, have little or no affinity for heparin is noteworthy, and while this manuscript was in preparation, similar observations for FGF19, 21 and 23 were reported elsewhere [30]. These FGF19 subfamily members reportedly regulate various aspects of metabolism. Consistent with those activities, it appears that the ligand-expressing cells and the target organs are remote from one another, which is in contrast to many other FGFs that utilize paracrine/autocrine modes of action to exert their effects. We suggest this lack of affinity for heparin enables these FGFs to be freely mobile in the extracellular and intravenous environment, which is rich in HS. In fact,

HS likely mediates the rapid clearance of intravenously administered FGFs from plasma [34]. Nonetheless, FGF21, which is primarily expressed in liver, is able to reach its major target in adipose tissue [21,35–37]. Thus FGF19 subfamily members appear to have an endocrine mode of action, and the molecular mechanism enabling this distinct mode is a subject of interest. In the present study, we clearly demonstrate that FGF19 subfamily members, FGF21 in particular, have a very low affinity to heparin. We suggest that they are not (or only weakly) trapped within heparin-bearing tissues such as the lumen of blood vessels and the extracellular matrix, making it possible for them to be recruited from the circulation to remote targets.

We and others have reported that FGF19 subfamily members require *Klotho* family protein to serve as a co-receptor [21,35–37]. Highly expressed in adipose tissue, beta-*Klotho* acts to mediate FGFR activation and the biological activity of FGF21. It is therefore reasonable to suggest that freely floating FGF19 subfamily members are able to act at target organs expressing *Klotho* family protein, and that the lack of heparin affinity enables them to exert their biological effects in an endocrine fashion.

Our analyses of the affinity of FGFs for CS yielded several new findings. In addition to FGF16 and 18, which were previously reported to bind to CS-E, we found that FGF22 strongly bound to CS-E (eluted at 1.5 M NaCl), while FGF3, 6, and 8 bound to CS-E with moderate affinity (eluted at 1.0 M NaCl), and FGF5 bound to CS-D with moderate affinity (eluted at 1.0 M NaCl). In addition, a number of FGFs showed weak affinity (eluted at 0.5 M NaCl) for CS-E, CS-D and CS-B. Whether this binding affects their ability to form complexes with FGFRs and exert their biological effects will be the subject of our future studies.

Among the FGF family members expressed in mammals, the structures of FGF1, 2, 4, 7, 8, 9, 10, 19 and 23 have been determined [23–30]. Among these, only FGF1 and FGF2 have been co-crystallized with heparin. The information obtained with those two crystal structures confirmed that the interaction with heparin can be fully explained in the context of their three-dimensional structure, although it is based on their primary structures. Furthermore, to our knowledge there is no information currently available on the structural basis for the interaction between any FGF and CS. The absence of three-dimensional, structural information on the binding of any FGF to CS makes it difficult to compare the interactions of FGFs and HS with those of FGFs and CS. Nonetheless, we considered this subject at the level of the primary structure. According to Goetz et al. [30], the three-dimensional topology of the heparin-binding site of FGF19 subfamily members differs from that of the paracrine-acting FGFs and this structural divergence can be attributed to the lack of GxxxxGxxT/S motif found only in paracrine-acting FGFs. Their report is consistent with our finding that these FGFs have a low affinity for heparin.

FGF7, also known as KGF (keratinocyte growth factor), is expressed in dermal tissue [38] and is thought to be involved in wound healing [39]. CS-B, otherwise known as dermatan sulfate, is also abundant in dermal tissues [40]. Although it was previously reported that dermal CS-B enhances the activity of FGF7 [10], we found that there is only weak interaction between FGF7 and CS-B (eluted at 0.5 M NaCl). This discrepancy likely reflects the fact that those earlier investigators did not examine the direct interactions among the proteins within the signaling complex (i.e., CS-B, FGF-7 and FGFR). Consequently, it is unclear whether or not the reported enhancement of KGF activity by CS-B resulted from some other mechanism, perhaps a structural alteration of FGFR through interaction with CS-B. Further structural analysis will be needed to shed additional light on this issue. As already mentioned, the primary structure of a given FGF, including FGF7, is not sufficient to fully explain its degree of affinity for HS or CS. Therefore the mechanism by which CS-B enhances FGF7 activity needs to be studied in the context of three-member complexes involving FGF7, its receptor (FGFR2IIIb) and CS-B. The interaction of FGF7 with CS-D or CS-E also was weak, as it was eluted at less than 0.5 M NaCl.

Like FGF, HGF binds to HS, especially to its highly sulfated domain. HGF also reportedly interacts with CS-B, perhaps via the 4-O-sulfate group in *N*-acetylgalactosamine [8]. Consistent with that idea, we found that HGF interacts with CS-E, which also contains an *N*-acetylgalactosamine 4-O-sulfate group. It is not yet known whether these HGF-interactive CSs are functionally involved in HGF signaling.

Although CS proteoglycans are expressed by many cell types and are abundant in the extracellular matrix of connective tissues, until recently their biological importance remained unclear. However, molecular cloning of the enzymes involved in CS biosynthesis has enabled discovery of some of their biological functions. For example, Kitagawa et al. reported variable, developmental stage-dependent changes in the sulfation patterns of CS [41], while Hikino et al. and Ichijo reported that CS participated in neuronal elongation and axon guidance, respectively [42,43]. In the present study, we found that a number of FGFs bound to CS, and elsewhere such FGF–CS binding has been studied in the context of the regulation of the development and function of central nervous system. For example, earlier studies utilizing *in situ* hybridization have shown that FGF3 is expressed in Purkinje cells early during development, that FGF5 is expressed in the central nervous system at later stages of development, and that FGF6 is expressed in both the developing central nervous system and skeletal muscle [44]. It is thus possible that variations in the structure of CS moieties and their spatiotemporal expression may modulate the biological activity of these FGFs.

To our knowledge, the structural basis for the affinity of proteins for CS has not been reported. Although FGF3, 6, 8, 16, 18 and 22 interact with CS-E with high affinity, the primary structures of these FGFs are not very similar, not even the positioning of their basic amino acids. Perhaps future resolution of the three-dimensional structures of CS–FGF complexes will shed light on this question.

The results of several studies suggest CS may modulate the biological activity of the polypeptide growth factors that bind to it. One of the MK receptors, a receptor-type tyrosine phosphatase ζ (PTP ζ), is a CS-modified proteoglycan [45]. Moreover, that MK is reported to strongly interact with CS-E [46] suggests that the binding of MK to the CS sugar chain on PTP ζ may significantly contribute to the overall affinity of MK for its receptor. Indeed, it has been suggested that the binding of MK to CS-E contributes importantly to MK-mediated PTP ζ signaling that contributes to the regulation of osteoblast migration and neuron survival [47,48]. How CS is involved in the active signaling complex is not yet known, however.

Regulation of FGF signaling by HS is complex and dependent on a variety of intermolecular interactions. In addition to the direct interaction of HS with FGFs, interactions between HS and FGFRs also appear to be important for active complex formation and the regulation of signal transduction [49]. The currently accepted model of the active molecular complex for FGF signaling involves dimer formation by a three-member complex comprised of FGF ligand, FGF receptor tyrosine kinase (FGFR), and heparin (HP). For many, if not all, FGF ligands there are direct interactions among these three components – i.e., FGF–FGFR, FGF–HP and HP–FGFR interactions. Consequently, interactions between FGF and FGFR, and between HP and FGFR, are also important for understanding and/or predicting the overall signaling of the given FGF ligand through a given receptor. These issues in the context of receptor signaling will be the subjects of future studies.

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