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6-O-Sulfation of Heparan Sulfate Differentially Regulates Various Fibroblast Growth Factor-dependent Signalings in Culture^{*[5]}

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Heparan sulfate (HS) interacts with diverse heparin-binding growth factors and thereby regulates their bioactivities. These interactions depend on the structures characterized by the sulfation pattern and isomer of uronic acid residues. One of the biosynthetic modifications of HS, namely 6-O-sulfation, is catalyzed by three isoforms of HS6-O-sulfotransferase. We generated HS6ST-1- and/or HS6ST-2-deficient mice (6ST1-KO, 6ST2-KO, and double knock-out (dKO)) that exhibited different phenotypes. We examined the effects of HS 6-O-sulfation in heparin-binding growth factor signaling using fibroblasts derived from these mutant mice. Mouse embryonic fibroblasts (MEF) prepared from E14.5 dKO mice produced HS with little 6-O-sulfate, whereas 2-O-sulfation in HS from dKO-MEF (dKO-HS) was increased by 1.9-fold. HS6-O-sulfotransferase activity in the dKO-MEF was hardly detected, and HS2-O-sulfotransferase activity was 1.5-fold higher than that in wild type (WT)-MEFs. The response of dKO-MEFs to fibroblast growth factors (FGFs) was distinct from that of WT-MEFs; in dKO-MEFs, FGF-4- and FGF-2-dependent signalings were reduced to ~30 and 60% of WT-MEFs, respectively, and FGF-1-dependent signaling was moderately reduced compared with that of WT-MEFs but only at the lower FGF-1 concentrations. Analysis with a surface plasmon resonance biosensor demonstrated that the apparent affinity of dKO-HS for FGF-4 was markedly reduced and was also reduced for FGF-1. In contrast, the affinity of dKO-HS for FGF-2 was 2.5-fold higher than that of HS from WT-MEFs. Thus, 6-O-sulfate in HS may regulate the signalings of some of HB-GFs, including FGFs, by inducing different interactions between ligands and their receptors.

Heparan sulfate (HS)² proteoglycans interact with numerous proteins such as growth factors and morphogens, their recep-

tors, extracellular matrix molecules, and enzymes. These interactions regulate the activity, gradient formation, and stability of the corresponding ligands. Thus, HS has important functions in various developmental, morphogenic, physiological, and pathogenic processes (1–4). The interaction of HS with heparin-binding growth factors (HB-GFs) is thought to be dependent not only upon the patterns of sulfate and hexuronic acid isomer residues but also on their negative charge (5–10). The polysaccharide of HS is synthesized by the alternative transfer of a glucuronic acid residue and an *N*-acetylglucosamine residue. It is then partially modified through the coordinate action of various enzymes in the Golgi apparatus, namely, *N*-deacetylase/*N*-sulfotransferases, C5-epimerase, and 2-O-, 6-O-, and 3-O-sulfotransferases to yield HS with a variety of structures (11, 12). HS is further modified by 6-O-endosulfatase at the cell surface (13–15). During these processes, the fine and divergent structure of HS is generated.

The destruction of the *Hs6st* gene has been reported to cause the abnormal phenotypes by the perturbation of HB-GF-dependent signaling. For example, *Drosophila* *Hs6st* functions in tracheal development that is mediated by FGF signalings (16, 17). Zebrafish *Hs6st* appears to function in Wnt-dependent muscle differentiation (18) and vascular endothelial growth factor-dependent angiogenesis in caudal vein (19). *Caenorhabditis elegans* lacking the *Hs6st* gene exhibits abnormal axon branching, which is mediated by Kal-1/Anosmin-1 (20). HS 6-O-endosulfatase, exhibiting substrate specificity for the trisulfated IdoUA2S-GlcNS6S disaccharide units of HS/heparin, promotes Wnt activity (14, 21), whereas it inhibits FGF signaling during mesoderm induction (22). Recently, Pratt *et al.* (23) reported that HS6ST-1-deficient mice exhibit erroneous axon navigation at the optic chiasm. We observed that HS6ST-1-deficient mice exhibit aberrant angiogenesis in the placental labyrinthine microvessels (24). Thus, a line of evidence suggests that the 6-O-sulfate in HS plays a crucial role in development and regulates the functions of HB proteins, although the defects in HS6ST appear to vary in a species-dependent manner.

Biochemical and crystal structure studies of FGF binding to HS/heparin and the formation of the ternary FGF·FGF receptor

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3.

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² The abbreviations used are: HS, heparan sulfate; CS, chondroitin sulfate; GAG, glycosaminoglycan; FGF, fibroblast growth factor; FGFR, FGF receptor; HS6ST, HS 6-O-sulfotransferase; HS2ST, HS 2-O-sulfotransferase; Glc-

NSO₃, *N*-sulfoglucosamine; WT, wild type; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; FBS, fetal bovine serum; BSA, bovine serum albumin; BrdUrd, bromodeoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dig-FGF, digoxigenin-conjugated FGF; MEF, mouse embryonic fibroblast; dKO, double knock-out; CDSNS, completely desulfated *N*-sulfated.

(FGFR)-HS complex have demonstrated that some of these interactions require the 6-O-sulfate residues of heparin/HS (25–28). Furthermore, immunohistochemical studies have revealed that various FGF-FGFR pairs bind differentially to tissues; this phenomenon might reflect the preference structure of HS (7, 29). However, *in vitro* studies using oligosaccharides generated by the chemoenzymatic modification of heparin have indicated that the stability of FGF-oligosaccharides-FGFR complexes is correlated with the negative charge of the oligosaccharides rather than with the precise distribution of sulfate groups (30).

We have previously demonstrated that mammals possess three HS6ST genes (known as *HS6ST-1*, *-2*, and *-3*) (31). The second member of this sulfotransferase family undergoes alternative splicing to yield *HS6ST-2S* (32). Although the *in vitro* substrate specificities of three isoforms partially overlap, each isoform exhibits a characteristic substrate preference. The expression patterns of three HS6ST isoforms also exhibit tissue- and development stage-specific variations (33, 34). We have generated HS6ST-1- and/or HS6ST-2-null mice. The HS6ST-1-null mice die at E15.5, whereas the HS6ST-2-null mice survive and are fertile. However, dKO mice die at a slightly earlier stage than HS6ST-1-null mice, suggesting important roles of the preference structure of HS in the development of various tissues, which might result in the different survival rate. Although several HB proteins are involved in development, it remains poorly understood how HB-GF-inducible signaling is regulated in the response to the *in vivo* altered sulfation pattern of HS.

In this study we isolated mouse embryonic fibroblasts (MEFs) derived from the skin of dKO mice (dKO-MEFs) and investigated the function of 6-O-sulfation of the HS chain in the FGF response. We demonstrated that HS from the dKO-MEFs has undetectable 6-O-sulfate residues and that the dKO-MEFs exhibit a differential response to FGF-1, FGF-2, and FGF-4, respectively. The results suggest important roles of 6-O-sulfation patterns at least in FGF signaling.

EXPERIMENTAL PROCEDURES

Materials—Heparitinase I (*Flavobacterium heparinum*, EC 4.2.2.8.), heparitinase II (*F. heparinum*, no number assigned), and heparinase (*F. heparinum*, EC 4.2.2.7) completely desulfated *N*-sulfated (CDSNS)-heparin and an unsaturated glycosaminoglycan (GAG) disaccharides kit were obtained from Seikagaku Corp. Sensu Pak Dicosil was purchased from Sensu Scientific. A silica-based polyamine (PAMN) column was from YMC. DEAE-Sephacel and Superose 6 columns were from Amersham Biosciences. $\text{H}_2^{35}\text{SO}_4$ was from PerkinElmer Life Sciences. Hyaluronan (104, 27, and 10.4 kDa) as the standard for molecular mass analysis of the polysaccharides was from Seikagaku Corp. Recombinant human FGF-1 and FGF-4 were purchased from PeproTech and FGF-2 from PROGEN Biotechnik GmbH. The sensor chip SA was from BIAcore. Anti-phospho-MAPK42/44 was from Cell Signaling; anti-phospho-FGFR1 (Y766)(p-Flg) was from Santa Cruz Biotechnology; anti-actin was from Sigma; anti-digoxigenin was from Roche

Applied Science. HS6ST-1 (24) and/or HS6ST-2 knockout mice was generated as described.³

Isolation of Embryonic Fibroblasts—MEFs were isolated from 14.5-day normal, HS6ST-1^{-/-}, HS6ST-2^{-/-}, and dKO embryos. After the internal organs were removed from the trunks, the trunks were cut to small pieces and digested with 0.25% trypsin, 0.53 mM EDTA in PBS(–) for 10 min at 37 °C. The residues were repeatedly digested, and the supernatants were passed through a 100- μm mesh. The cells contained in the filtrate were collected by centrifugation at 1,000 rpm for 10 min, and were subsequently grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MEFs from the third passage were used.

Preparation and Structural Analysis of HS—Subconfluent MEF layers in 100-mm dishes were washed with PBS(–) twice; the cells were collected with 2 ml of 0.2 M NaOH using a cell scraper. The cell suspending solution was incubated for 16 h at room temperature, neutralized with 4 M acetic acid, and then incubated at 37 °C for 2 h with DNase I, RNase A, and 10 mM MgCl_2 in 50 mM Tris-HCl (pH 8.0). Subsequently, 0.5 mg of Pronase (actinase E; Kaken-Seiyaku Corp., Tokyo) was added to the cell, and incubation was continued at 37 °C for 16 h. The reaction was stopped by heating at 100 °C for 5 min, and the samples were centrifuged at 13,000 rpm for 10 min to remove any insoluble material. The supernatants were diluted with an equal volume of 20 mM Tris-HCl buffer (pH 7.2) and loaded on a DEAE-Sephacel column equilibrated with the same buffer. The columns were washed with 10 column volumes of buffer containing 0.2 M NaCl and then eluted with 4 column volumes of 2 M NaCl in Tris-HCl buffer. The eluates were precipitated with 2.5 volumes of cold 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate and 1 mM EDTA, and then the GAGs were recovered by centrifugation. An aliquot of the GAG was digested at 37 °C for 2 h with a mixture of 0.2 milliunits of heparitinase I, 0.1 milliunits of heparitinase II, and 0.2 milliunits of heparinase in 50 μl of 50 mM Tris-HCl buffer (pH 7.2), 1 mM CaCl_2 , and 5 μg of bovine serum albumin. After filtration of the digests with Ultrafree-MC (5,000 molecular weight limit; Millipore Corp.), the unsaturated disaccharide products in the filtrates were analyzed by reverse phase ion-pair chromatography using Sensu Pak column Dicosil with a fluorescence detector according to Toyoda's method (33) with slightly modified elution conditions. To examine the molecular size of HS, the MEFs were incubated with 100 $\mu\text{Ci}/\text{ml}$ of ^{35}S -inorganic sulfate for 24 h, and ^{35}S -labeled GAGs were prepared as described above. An aliquot of the GAGs was digested with a heparitinase mixture and/or chondroitinase ABC and then subjected to gel chromatography on a Superose 6 column equilibrated with 0.2 M NH_4OAc at a flow rate of 0.5 ml/min. The disaccharide compositions of ^{35}S -labeled chondroitin sulfate (CS) in the MEFs were analyzed by digesting them with chondroitinase ABC and then subjecting to polyamine-bound silica (PAMN) column chromatography as described previously.

Assay for Sulfotransferase Activity—For the extraction of sulfotransferase from the MEFs, the cell layer was washed with

³ N. Nagai, H. Habuchi, and K. Kimata, manuscript in preparation.

Effect of 6-O-Sulfated HS on FGF-1, -2, and -4 Signaling

PBS(–) and then scraped off the dish in 2 ml of buffer containing 0.15 M NaCl, 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.2), 20% glycerol, 10 mM MgCl₂, 2 mM CaCl₂, and protease inhibitors (35). After 1 h of gentle stirring, the homogenate was centrifuged at 4 °C for 10 min at 10,000 × *g*. The supernatant was used as the enzyme. Sulfotransferase activity was determined as described previously (31). Briefly, the standard reaction mixture (50 μl) contained 2.5 μmol of imidazole HCl (pH 6.8), 3.75 μg of protamine chloride, 25 nmol (as hexosamine) of CDSNS-heparin, 50 pmol of [³⁵S]3'-phosphoadenosine 5'-phosphosulfate (~5 × 10⁵ cpm), and cell extract containing 10 μg of protein. After incubation at 37 °C for 20 min, the reaction was terminated by heating at 100 °C for 1 min. Next, ³⁵S-labeled CDSNS-heparin was isolated by precipitation with 95% ethanol containing 1.3% potassium acetate and 1 mM EDTA, followed by gel chromatography on a Fast desalting column. The isolated product was digested with a heparitinase mixture. The digests were subjected to PAMN column chromatography, and the amount of ³⁵S-labeled disaccharides was measured using a scintillation counter. One unit of enzyme activity was defined as the amount required to transfer 1 pmol of sulfate per min.

Expression Levels of mRNA Encoding FGFRs, HS6STs, and HS2ST Analyzed by RT-PCR—Total RNA from WT- and dKO-MEFs was isolated using an RNeasy kit (Qiagen, Tokyo, Japan). A reverse transcription reaction was performed using the high capacity cDNA archive kit (ABI) and 1.0 μg of total RNA as the template. The expression levels of *Hs6st* isoforms and *Hs2st* were semiquantitatively determined by RT-PCR using the following four primers pairs: *Hs6st-1* forward primer (AGGACCATGGTTGAGCG) and reverse primer (GCGGCGATTGGCCGATA); *Hs6st-2* forward primer (CCGGTGCCGGATCCGTA) and reverse primer (GCGTCGCGCTTGCCATC); *Hs6st-3* forward primer (CAACTTCGGGGAGCAGC) and reverse primer (TCCACGAAGCGGGTCAG); and *Hs2st* forward primer (TTCATGGGGCTCCTCAGG) and reverse primer (AAGCGCACCTGATCTTGC). The expression levels of mRNA encoding FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3, and FGFR4 were semiquantitatively determined by RT-PCR using the following six primers pairs: *fgfr1-F* forward primer (GTGGAACGATCTCCGCAC) and *fgfr1b-R* reverse primer (GCCACGCAGACTGGTTAG) for *fgfr1b*; *fgfr1-F* forward primer and *fgfr1c-R* reverse primer (CAGAGTGATGGAGAGTC) for *fgfr1c*; *fgfr2b-F* forward primer (GATGTGGAGTTTGTCTGCAAGGTTT) and *fgfr2b-R* reverse primer (GACTGGTTGGCTGCCCTATATAAT) for *fgfr2b*; *fgfr2c-F* forward primer (CAGCATCGGAGCCTTATTATGG) and *fgfr2c-R* reverse primer (GTTACATTCCGAATATAGAGACC) for *fgfr2c*; *fgfr3-F* forward primer (GGGCTCCTTATTGACTCG) and *fgfr3-R* forward primer (GCCGGATGCTGCCAAACT) for *fgfr3*; *fgfr4-F* forward primer (CACTGGCTCAAGGATGGA) and *fgfr4-R* reverse primer (CTACCCAGAGATTCTCC) for *fgfr4*; and GAPDH-F forward primer (GGTGAAGGTCGGTGTGAACG) and GAPDH-R reverse primer (GCATGGACTGTGGTCATGAG) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The PCR was carried out in a final volume of 25 μl containing 12.5 pmol of each primer and 2-μl aliquots of reverse transcriptase reaction mixture under the condition of 37 cycles for *Hs6sts* and *Hs2st* or 35

cycles for *fgfrs*, denaturation at 95 °C for 1 min, annealing at 56.5 °C for 30 s for *Hs6sts* and *Hs2st* or 56 °C for *fgfrs*, and extension at 72 °C for 30 s. The PCR products were subjected to 2% agarose gel electrophoresis. The data were obtained from three independent experiments.

Surface Plasmon Resonance Analysis—The interaction of growth factors with HS was analyzed using a BIAcore 1000 surface plasmon resonance biosensor. A streptavidin-conjugated sensor Chip SA was used to immobilize the HS prepared from the WT- and dKO-MEFs. Next, 5 μg of HS or heparin was incubated with 5.6 μg of NHS-LS-Biotin (Pierce) in 100 μl of 50 mM sodium bicarbonate buffer (pH 8.5) for 30 min at room temperature. An excess of NHS-LS-Biotin was reacted with 0.1 M ethanolamine for 30 min at room temperature. The biotinylated GAGs were precipitated with 3 volumes of cold 95% (v/v) ethanol containing 1.3% potassium acetate and 1 mM EDTA, and the process was then repeated to remove any unreacted biotin. The precipitates from HS preparation were digested with 5 milliunits of chondroitinase ABC for 10 min at 37 °C to degrade CS. After the reaction was stopped by heating at 95 °C for 2 min, biotinylated HS was recovered by precipitation with 3 volumes of cold 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate and 1 mM EDTA. 0.5–2 μg/ml biotinylated GAGs in PBST were injected at the constant flow rate of 5 μl/min. The biotinylated heparin and WT- or dKO-HS, of ~10 and 700 resonance units, respectively, were immobilized onto a streptavidin-conjugated sensor chip SA. Various concentrations of growth factors in PBST were perfused at a flow rate of 30 μl/min. The sensor chip was regenerated with injection of 2 M NaCl/PBST. All measurements were carried out at room temperature. At least two independent experiments were performed. Resonance data from sensorgrams were evaluated using the BIAevaluation software (version 2) according to the manufacturer's instructions. Dissociation constant (*K_D*) for binding was calculated by Scatchard plot analysis. The response at equilibrium, *Req* (*y* axis) was plotted against *Req/C* (*x* axis), where *C* was the molar concentration of growth factor. The slope of this plot equals $-K_D$.

Preparation of Digoxigenin-conjugated FGFs—Digoxigenin-conjugated FGF was prepared as described previously (36) with a slight modification. Briefly, 10 μg of FGF-2 or FGF-1 was added in 500 μl of 0.15 M NaCl, 20 mM phosphate buffer (pH 8.5), containing 50 μg of *N*-acetylated heparin and then mixed with 8.75 nmol of digoxigenin-3-*O*-methylcarbonyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester (Roche Applied Science) in ethanol, followed by 2 h at room temperature. The 1 ml of heparin-Sepharose gel equilibrated with PBS containing 0.1% (w/v) BSA (solution A) was added into FGF mixture and incubated at 4 °C for 1 h. The column was washed with 5 ml of solution A and then eluted with 1 ml of solution A containing 2 M NaCl and 0.02% (v/v) Tween 20 to recover the digoxigenin-conjugated FGFs (Dig-FGFs).

Pulldown Assay—To examine the interaction between FGF and FGFR1c with or without various glycosaminoglycans, 20 ng of Dig-FGF-1 or Dig-FGF-2 and 50 ng of FGFR1c-Fc (R & D Systems, Minneapolis, MN) were mixed in 500 μl of pulldown buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Nonidet P-40, and 1 tablet/10 ml of Complete Mini protease inhibitor

mixture tablets (Roche Applied Science)) containing various glycosaminoglycans. After the incubation at 4 °C for 1 h, 20 μ l of protein G-Sepharose beads were added into the mixtures and incubated at 4 °C for 2 h. To remove Dig-FGFs from the ternary complex (Dig-FGFs-FGFR1c-Fc-HS), the beads were washed with 2 M NaCl in 20 mM HEPES buffer (pH 7.5). Then they were washed with PBS two times and suspended in SDS sample buffer and subjected to 15% SDS-PAGE. Western blots were probed with an anti-digoxigenin antibody.

Analysis of FGFs Signaling—MEFs (1×10^5) were seeded in 24-well plates. After incubation in DMEM containing 0.4% FBS for 18 h, the cells were washed with fresh serum-free DMEM containing 0.1% BSA (medium A) and incubated for an additional 24 h. Different concentrations of FGF-1, FGF-2, and FGF-4 were added into the wells in duplicate. After incubation at 37 °C for exactly 5 min, the medium was immediately discarded and 0.5 ml of LIPA buffer (10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1% Triton X-100, 1.5 mM EDTA, 1 mM Na_3PO_4 , 25 mM NaF, 1 mM Na_3VO_4 , and 1 tablet/10 ml of Complete Mini protease inhibitor mixture tablets (Roche Applied Science)) was added to the cell layers. After the cells were frozen in a refrigerator at -80 °C, cells lysates were prepared at 4 °C for 60 min with rotation and clarified by centrifugation at $10,000 \times g$ for 30 min. Then 30 μ g of protein was subjected to 8% SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). Western blots were probed with antibodies against phospho-ERK1/2 and phospho-FGFR1, followed by a horseradish peroxidase-conjugated secondary antibody, and ECL detection was performed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and x-ray film (GE Healthcare) according to the manufacturer's instructions. The band density of pERK1/2 or pFGFR1 was measured using ImageGauge software and normalized by actin bands. The data were obtained from three independent experiments performed in triplicate.

Digestion of Cell Surface/Extracellular Matrix HS or CS—The WT-MEFs were digested with 10 milliunits of heparitinase I, 5 milliunits of heparitinase II, and 10 milliunits of heparinase and/or 10 milliunits of chondroitinase ABC at 37 °C before the addition of FGFs. After 15 min, the enzyme and the digested HS or CS were removed by washing twice with DMEM containing 0.1% BSA, and the cells were then stimulated as described above.

Assay of DNA Synthesis—MEFs (1×10^4) were seeded in 96-well plates, and incubated in serum-free DMEM containing 0.1% BSA for 48 h. Different concentrations of FGF-1, FGF-2, FGF-4, and BrdUrd labeling solution were added into the wells in duplicate. After further incubation for 24 h, BrdUrd incorporated to DNA was measured according to the manufacturer's instructions (Roche Applied Science).

RESULTS

Characterization of HS Isolated from MEF—We isolated fibroblasts from WT, HS6ST-1^{-/-}, HS6ST-2^{-/-}, and HS6ST-1^{-/-}/HS6ST-2^{-/-} mice at E14.5 (designed WT-MEFs, 6ST1KO-MEFs, 6ST2KO-MEFs, and dKO-MEFs, respectively). We first measured the expression levels of *Hs6st-1*, *Hs6st-2*, and *Hs6st-3* mRNA in WT-MEFs using semiquantita-

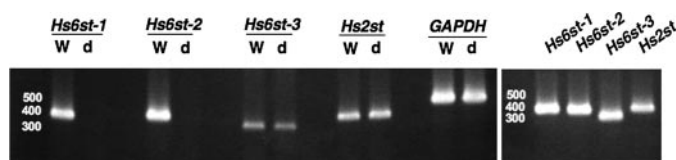


FIGURE 1. Expression levels of the *Hs6st-1*, *Hs6st-2*, *Hs6st-3*, and *Hs2st* mRNAs in WT-MEFs and dKO-MEFs. The expression levels of *Hs6st-1*, *Hs6st-2*, *Hs6st-3*, and *Hs2st* mRNAs in the WT- and dKO-MEFs were semi-quantitatively measured using RT-PCR with each primer pair, as described under "Experimental Procedures." GAPDH was used as the control. To compare the relative expression levels of these transcripts, PCR was carried out using equal molar concentration of each plasmid containing their cDNA as the templates (right-hand side, 4 lanes). W, WT-MEFs; d, dKO-MEFs.

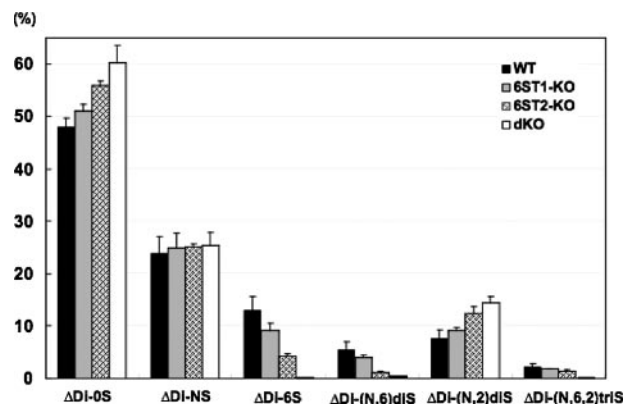


FIGURE 2. Comparison among disaccharide compositions of HS from the MEFs of WT, HS6ST-1^{-/-}, HS6ST-2^{-/-}, and dKO mice. HS was prepared from the cell layers of MEFs as described under "Experimental Procedures." HS was digested with a heparitinase-I, -II, and heparinase mixture, and these digests were then subjected to reverse phase ion-pair chromatography with postcolumn fluorescence labeling as described under "Experimental Procedures." The histogram shows the percentage of unsaturated disaccharide compositions (Δ Di-OS, Δ Di-6S, Δ Di-NS, Δ Di-(N,6)diS, Δ Di-(N,2)diS, and Δ Di-(N,6,2)triS are the disaccharide units derived from -HexA-GlcNAc-, -HexA-GlcNAc(6SO₄)-, -HexA-GlcNSO₃(6SO₄)-, -HexA(2SO₄)-GlcNSO₃-, and -HexA(2SO₄)-GlcNSO₃(6SO₄)-, respectively) in the HS isolated from the MEFs of WT, HS6ST1-KO, HS6ST2-KO, and dKO mice. Values (heights of the histograms) are the means of triplicate experiments and error bars represent the standard deviation. Δ Di, 2-acetamide-2-deoxy-4-O-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose.

tive RT-PCR with equal concentration of the plasmids containing each cDNA as a standard template (Fig. 1). The transcription level of *Hs6st-2* RNA was the highest, and the expression level of *Hs6st-3* mRNA (~40% of *Hs6st-2* mRNA) was the lowest. We then examined the structure of HS prepared from the four MEFs (Fig. 2). The analysis of the disaccharide compositions in HS revealed that compared with the WT-HS, 6-O-sulfation in the HS isolated from 6ST1KO-MEF (6ST1KO-HS) was decreased in GlcNAc6SO₄ residue (70% of WT-HS), HexA-GlcNSO₃(6SO₄) unit (74%), and HexA(2SO₄)-GlcNSO₃(6SO₄) unit (82%). In contrast, 6-O-sulfation in the HS isolated from 6ST2KO-MEF (6ST2KO-HS) was markedly decreased in GlcNAc6SO₄ residue (33%) and HexA-GlcNSO₃(6SO₄) unit (21%) but was moderately decreased in HexA(2SO₄)-GlcNSO₃(6SO₄) unit (64%). In the dKO-HS, GlcNAc6SO₄ residue, HexA-GlcNSO₃(6SO₄) unit and HexA(2SO₄)-GlcNSO₃(6SO₄) unit were hardly detectable. The content of 2-O-sulfate in the HS isolated from all the mutant cells was increased by 1.2- to 1.9-fold that in WT-HS (Fig. 2). Then we examined the expression levels of *Hs2st* mRNA. The expression level of *Hs2st* mRNA in the dKO-MEFs was 1.2-fold

TABLE 1**The activities of HS6ST and HS2ST in WT- and dKO-MEF cell layers**

The preparation of crude extracts from WT- and dKO-MEFs and the measurement of enzyme activities were described under "Experimental Procedures." The activities of HS2ST and HS6ST were determined from the ^{35}S radioactivity incorporated to position 2 of HexA residues and position 6 of GlcNSO₃ residues in the substrate (CDSNS-heparin), respectively.

MEF	Activity of HS2ST	Activity of HS6ST	Total activity
	units/ μg protein	units/ μg protein	units/ μg protein
WT	4.55	1.89	6.44
dKO	7.18	0.08	7.26

higher than that in WT-MEFs (Fig. 1). The level of *Hs6st-3* mRNA in dKO-MEFs was slightly lower than that in the WT-MEFs. We then examined the activities of HS6ST and HS2ST in the cells (Table 1). The activity of HS6ST in the dKO-MEFs was less than 4% that in the WT-MEFs, which is probably because of the action of HS6ST3. These data are also consistent with almost complete loss of 6-O-sulfate residues observed in the dKO-HS. The activity of HS2ST in the dKO-MEF was 1.6-fold higher than that in the WT-MEFs. These results are considerably consistent with the increase in 2-O-sulfation observed in the dKO-HS.

To assess the effects of the absence of 6-O-sulfation in HS on the biosynthesis of CS and HS, we characterized the contents and the sizes of HS and CS in the cell layers of the four MEFs. After incubation in the medium containing ^{35}S -inorganic sulfate for 24 h, GAGs were isolated from the cell layer and subjected to chromatography on a Superose 6 column after the digestion with heparitinases and/or chondroitinase ABC (supplemental Fig. 1). WT-HS and dKO-HS (the chondroitinase ABC digests of GAGs from WT-MEFs and dKO-MEFs) were both mainly eluted as two peaks, a larger broad peak and a smaller peak (supplemental Fig. 1B). The larger WT-HS peak was eluted around 63 kDa and the larger dKO-MEF peak at ~57 kDa. This slight size difference might be caused by a decrease in the number of 6-O-sulfate residues. In contrast, the smaller dKO-HS (~8 kDa) was almost the same size as the smaller WT-HS. WT-CS and dKO-CS (the heparitinases digests of GAGs from WT-MEFs and dKO-MEFs) were both eluted as a single peak around 27 kDa (supplemental Fig. 1D). The HS and CS contents in the dKO-MEFs were almost identical to those in WT-MEF, and the CS contents in both dKO- and WT-MEFs were ~1.5 times greater than the HS content (Table 2). The disaccharide compositions of the dKO-CS were very similar to that of the WT-CS and the content of oversulfated disaccharides in both CSs was ~3% of the total disaccharides (Table 3). These results indicated that neither the structure nor the amount of CS was altered by the defects of 6-O-sulfation in HS. Thus, we used WT- and dKO-MEF to examine the function of 6-O-sulfation of HS in FGF signaling.

Expression Level of mRNA Encoding FGFRs—To address the effect of 6-O-sulfation on the response of FGFs, we examined the species and the expression levels of *fgfrs* mRNA by RT-PCR. Both the WT- and dKO-MEF strongly expressed *fgfr1b*, *fgfr1c*, *fgfr2c*, and *fgfr3* and moderately expressed *fgfr2b*, whereas they weakly expressed *fgfr4* mRNA. The expression of *fgfrs* mRNA was at very similar levels in the WT- and dKO-MEF (supplemental Fig. 2), suggesting that the 6-O-sulfation defect in HS had a negligible

TABLE 2**Contents of HS and CS in WT- and dKO-MEFs**

The total radioactivity incorporated into HS was calculated from disaccharide fractions produced by digestion with a mixture of heparitinase, and the total radioactivity incorporated into CS was calculated from disaccharide fractions produced by the digestion with chondroitinase ABC, respectively.

MEFs	HS content	CS content	Ratio of [^{35}S]CS/[^{35}S]HS
	% ^a	% ^a	
WT	100	100	1.6
dKO	97	107	1.5

^a The values represent the relative ^{35}S radioactivity in the HS or CS from WT-MEFs.

TABLE 3**Disaccharide compositions of CS prepared from WT- and dKO-MEFs**

GAG prepared from the cell layers of WT- and dKO-MEFs was digested with chondroitinase ABC. The digests were analyzed by high pressure liquid chromatography as described under "Experimental Procedures." The values are % of the total disaccharide and the means $\pm$ S.D. in three independent experiments. ND means not detectable. $\Delta\text{Di-OS}$, $\Delta\text{Di-4S}$, $\Delta\text{Di-6S}$, $\Delta\text{Di-diSE}$, and $\Delta\text{Di-diSD}$ are the disaccharide units derived from -HexA-GalNAc-, -HexA-GalNAc(4S)-, -HexA-GalNAc(6S)-, -HexA-GalNAc(4,6-SO₃)-, and -HexA(2SO₃)-GalNAc(6SO₃)-, respectively, in the CS isolated from MEFs.

MEF	Unsaturated disaccharide				
	$\Delta\text{Di-OS}$	$\Delta\text{Di-4S}$	$\Delta\text{Di-6S}$	$\Delta\text{Di-diSE}$	$\Delta\text{Di-diSD}$
	%	%	%	%	%
WT	5.1 $\pm$ 1.5	92.1 $\pm$ 2.2	ND	2.2 $\pm$ 0.4	0.7 $\pm$ 0.4
DKO	5.1 $\pm$ 1.6	91.4 $\pm$ 1.8	ND	2.5 $\pm$ 0.3	1.0 $\pm$ 0.4

effect on the expression of *fgfr* mRNA. Because it has been reported that the signaling of FGF-1, FGF-2, and FGF-4 is mainly mediated through all FGFRs, FGFR1c and FGFR3c, and FGFR1c and FGFR2c, respectively (36, 37), we analyzed the effects of 6-O-sulfation on the signaling of these FGFs by the phosphorylations of FGFR1 and ERK.

Control of 6-O-Sulfated HS on FGF-dependent Signaling—To measure FGF-dependent phosphorylation of ERK1/2 and FGFR1, FGF-1, FGF-2, or FGF-4 was added into serum-starved WT-MEF and dKO-MEF. In both WT-MEFs and dKO-MEFs, FGF-dependent phosphorylation of ERK1/2 and FGFR1 was evident since the first 5 min (Fig. 3). The phosphorylation of ERK1/2 was increased until 10 min and then decreased at 15 min. The phosphorylation of FGFR1 was increased until 15 min and then decreased at 30 min. The time-dependent response showed the similarity between WT-MEF and dKO-MEF. We next ascertained whether or not the cell surface HS in the MEFs contributed to the regulation of FGF signaling (Fig. 4). To remove HS and/or CS/dermatan sulfate from the cell surface, WT-MEFs were digested with a heparitinase mixture and/or chondroitinase ABC. FGF-1, FGF-2, or FGF-4 was then added to these treated cells, and the phosphorylation of ERK1/2 and FGFR1 was analyzed. As shown in Fig. 4, digestion with a heparitinase mixture but not chondroitinase ABC resulted in a marked reduction in FGF-dependent phosphorylation of FGFR1 with regard to all three FGFs. These results demonstrated that cell surface HS but not CS/dermatan sulfate in the MEFs stimulated the activities of FGF-1, FGF-2, and FGF-4. The phosphorylation of ERK induced by FGF-2 and FGF-4 was also remarkably inhibited when HS was removed, indicating that the signal transduction of both FGF-2 and FGF-4 requires the HS chain at least in the MEFs. In contrast, FGF-1-dependent phosphorylation of ERK was moderately inhibited by the removal of HS. Furthermore, the double digestion with hep-

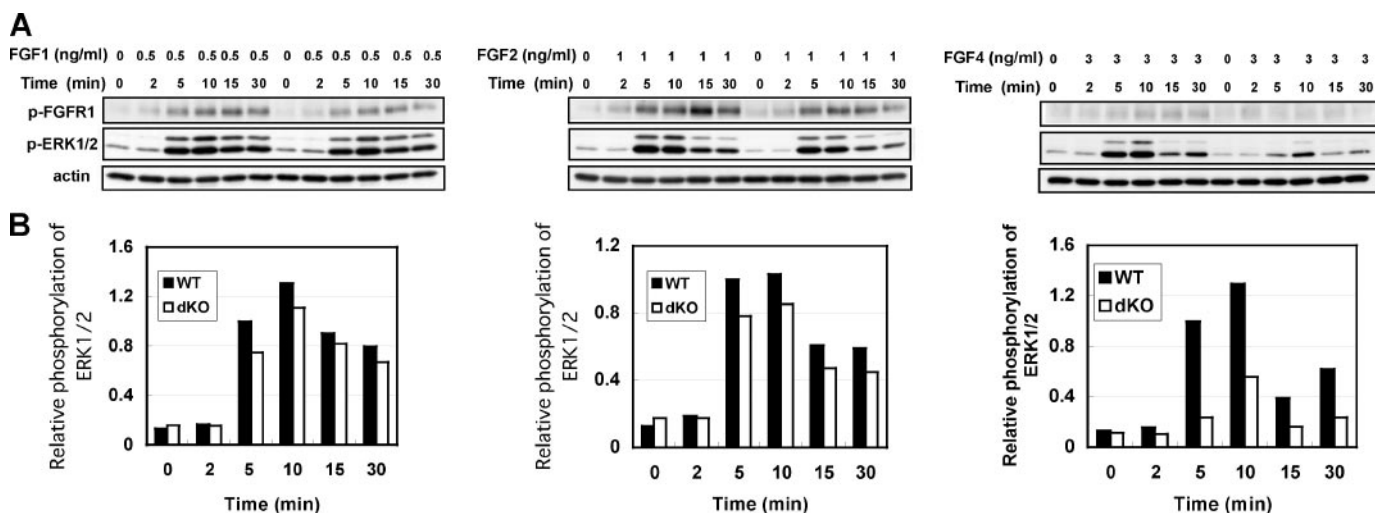


FIGURE 3. Kinetics of FGF-1- (left panel), FGF-2- (middle panel), and FGF-4-dependent (right panel) signaling. WT-MEFs and dKO-MEFs (1×10^5) were seeded in 24-well plates. After incubation in DMEM containing 0.4% FBS for 18 h, the cells were washed with fresh serum-free DMEM containing 0.1% BSA (medium A) and incubated for an additional 24 h. The WT-MEFs and dKO-MEFs were incubated with 1 ng/ml of FGF-2, 3 ng/ml of FGF-4, or 0.5 ng/ml of FGF-1 for the indicated time (min). The phosphorylation of FGFR1 and ERK1/2 in the cells was analyzed by Western blotting as described under "Experimental Procedures." The Western blots are shown in the upper panels and representative photograph (A). The amount of pERK1/2 was quantified from each Western blot in Fig. 3 by using BAS5000 software and was normalized by actin bands (lower panel, B). The data (the mean values) were obtained from two independent experiments with duplicate wells.

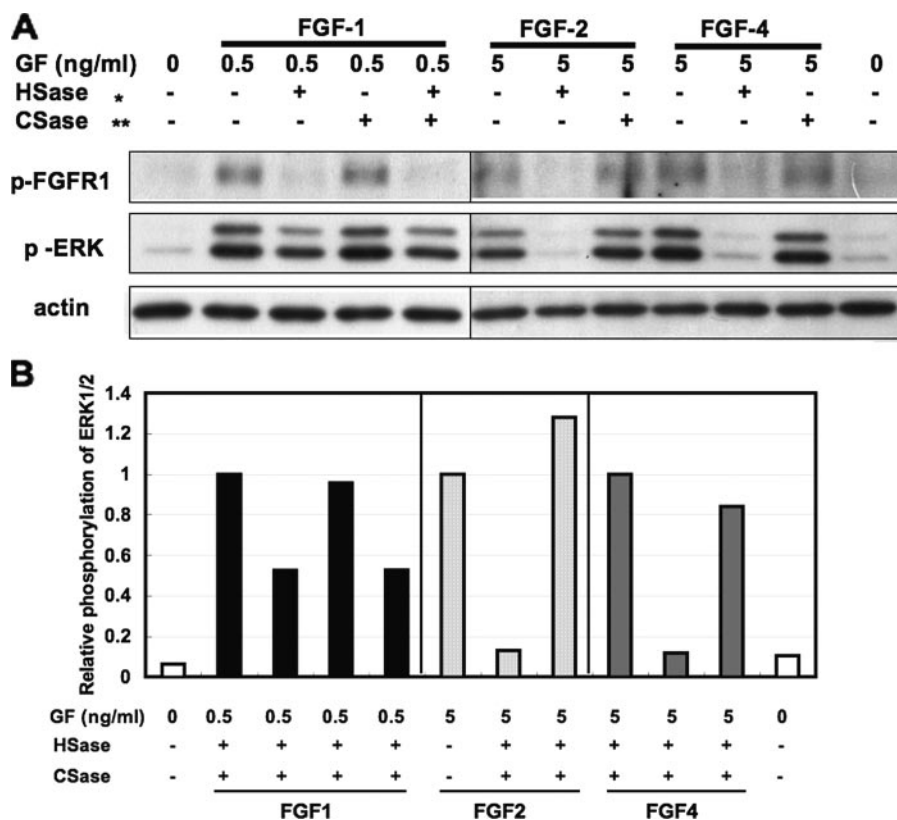


FIGURE 4. FGFs signaling depends on cell surface HS but not CS. WT-MEFs (1×10^5) were seeded in 24-well plates. After incubation in DMEM containing 0.4% FBS for 18 h, the cells were washed with fresh serum-free DMEM containing 0.1% BSA (medium A) and incubated for an additional 24 h. The WT-MEFs were digested with a heparitinase mixture and/or chondroitinase ABC prior to the addition of FGFs. After 15 min, the enzyme and the digested HS or CS were removed by washing the wells with medium A, and 5 ng/ml FGF-2 and FGF-4 or 0.5 ng/ml FGF-1 was then added into the wells. The phosphorylation of FGFR1 and ERK1/2 in the cells was analyzed by Western blotting as described under "Experimental Procedures." The Western blots are shown in the upper panels and representative photograph (A). The amount of pERK1/2 was quantified from each Western blot in Fig. 4 by using BAS5000 software and was normalized by actin bands (lower panel, B). The data were obtained from two independent experiments with duplicate wells.

aritinases and chondroitinase ABC did not alter the extent of inhibition. These results suggest that the signaling of FGF-1 mediated through FGFR1 depends on HS, but that mediated through other FGFRs, such as FGFR2 and/or FGFR3, appears to depend only partially on HS. However, we cannot rule out the possibility that HS might be protected in part from heparitinase attack by the proteins bound to it and that such fragmented HS preferentially stimulates FGF-1 activity. We next examined the effect of dKO-MEF response to FGF (Fig. 5). The FGF-1-dependent phosphorylation of FGFR1 was clearly reduced in the dKO-MEFs (Fig. 5A), indicating that 6-O-sulfate residues in HS stimulate FGF-1 signaling mediated through FGFR1. At a lower concentration of FGF-1 (0.2–1 ng/ml), FGF-1-inducible phosphorylation of ERK1/2 in the dKO-MEF was decreased to ~70% that in the WT-MEFs. However, at a higher concentration of FGF-1 (3 ng/ml), the signaling in both MEFs exhibited similar activation (Fig. 5B).

At the FGF-2 concentration examined, ERK phosphorylation in the dKO-MEFs decreased to ~60% that in the WT-MEFs. The level of

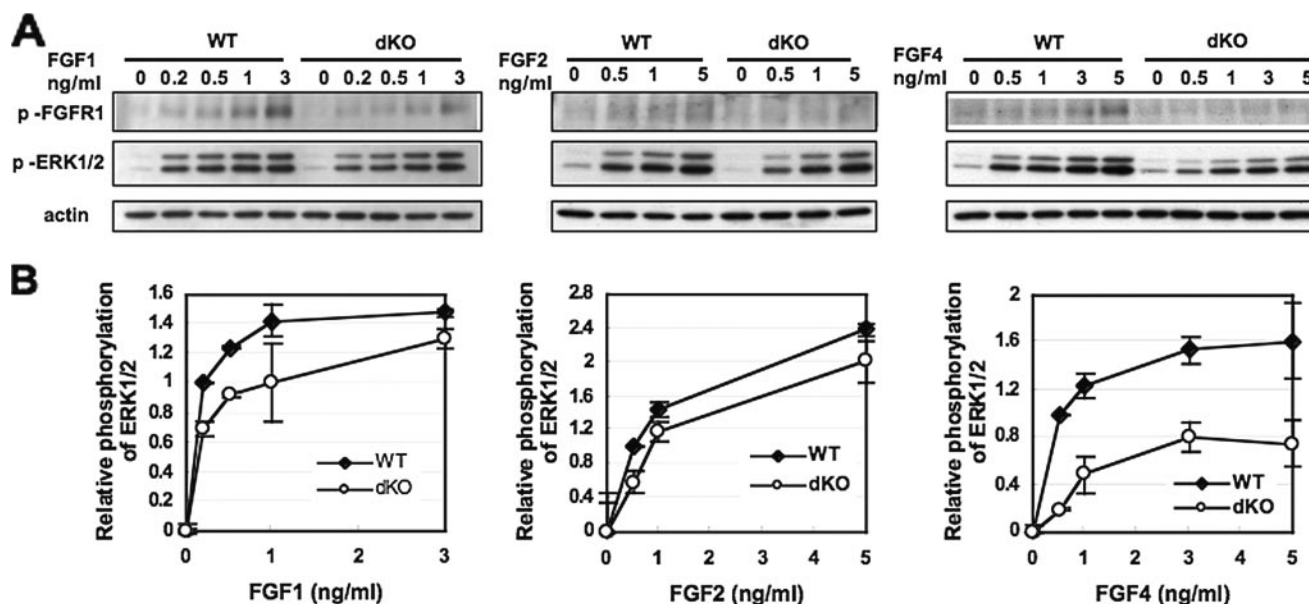


FIGURE 5. **FGF-1, FGF-2, and FGF-4-induced phosphorylation of ERK1/2 and FGFR1 in WT- and dKO-MEFs.** MEFs (1×10^5) were seeded in 24-well plates. After incubation in DMEM containing 0.4% FBS for 18 h, the cells were washed with fresh medium A and incubated for an additional 24 h. The WT- and dKO-MEFs were incubated with different concentrations of FGF-1, FGF-2, and FGF-4 for 5 min at 37 °C. **A**, phosphorylation of FGFR1 and ERK1/2 in the cells was analyzed by Western blotting as described under "Experimental Procedures." **B**, amount of pERK1/2 was quantified from **A** by using BAS5000 software and was normalized by actin bands. The data were obtained from three independent experiments with duplicate wells used in each experiment.

TABLE 4

Dissociation constants for bindings of FGF-1, FGF-2, and FGF-4 to WT-, dKO-HS, and heparin

Ligand GAG	FGF-1, K_D^a	FGF-2			FGF-4		
		k_a	k_d	K_D^b	k_a	k_d	K_D^b
	nM	$M^{-1} s^{-1}$	s^{-1}	nM	$M^{-1} s^{-1}$	s^{-1}	nM
WT-HS	194	8.53×10^5	1.25×10^{-2}	14.7	1.76×10^5	1.5	
dKO-HS	553	33.2×10^5	1.86×10^{-2}	5.57	0.472×10^5	2.11×10^{-2}	449
Heparin	98	7.63×10^5	0.29×10^{-2}	3.82	1.82×10^5	0.179×10^{-2}	9.80

^a Dissociation constants for FGF-1 were determined from Fig. 7, A–C, which were analyzed by the response at equilibrium.

^b Dissociation constants for FGF-2 and FGF-4 were determined from Fig. 7 (D–F, G and H) based on the responses in the kinetics state model using BIAevaluation software (version 2). K_D values were calculated from the ratio of k_d to k_a .

FGFR1 phosphorylation in the dKO-MEFs was apparently decreased compared with that in WT-MEFs, although the level of FGFR1 phosphorylation was very low in both MEFs. FGF-2 is known to preferentially bind to 2-O-sulfated heparan sulfate (38–41). Because the content of the 2-O-sulfate residues in HS was increased by 1.9-fold in the dKO-MEFs (Fig. 1), FGF-2 might interact rather strongly with dKO-HS. Indeed, as shown in Table 4, the affinity between FGF-2 and dKO-HS was higher than that between FGF-2 and WT-HS. Therefore, the reduction in the FGF-2-dependent signaling might be due to the reduced formation of the FGF-2-FGFR-HS ternary complex.

Among three FGFs, FGF-4 signaling was most distinctly affected by the decreased 6-O-sulfation in HS. ERK phosphorylation in the dKO-MEF was reduced to 15–40% that in the WT-MEFs at FGF-4 concentrations ranging from 0.5 to 5 ng/ml. Furthermore, the phosphorylation of FGFR1 in the dKO-MEF was apparently decreased compared with that in the WT-MEF. FGF-4 signaling uses mainly the c splice forms of FGFR, *i.e.* FGFR1c and FGFR2c. Taken together, the results suggest that the FGF-4 signaling mediated through FGFR2c and FGFR1c appears to be stimulated by 6-O-sulfated HS.

Reduction of FGF-dependent DNA Synthesis in dKO-MEFs—As described above, the decreased 6-O-sulfation in HS caused the reduction of FGF-induced phosphorylation of ERK1/2.

Therefore, we asked whether the phosphorylation of ERK1/2 was correlated with the cell proliferation. WT-MEF and dKO-MEF incorporated approximately equally BrdUrd into DNA in the medium containing FBS (Fig. 6A). However, three FGF-dependent S-phase DNA biosyntheses in dKO-MEF were decreased compared with that in WT-MEF within the concentration examined. The ratios of dKO-MEF to WT-MEF on the BrdUrd incorporation were reduced to 0.43, 0.81, and 0.38 at the concentration of 1 ng/ml FGF-1, FGF-2, and FGF-4, respectively (Fig. 6, B–D). On cell proliferation, FGF-4 activity was affected most strongly among three FGFs by the 6-O-sulfation in HS. The ratio of dKO-MEF to WT-MEF became a lower value, 0.29 at the concentration of 5 ng/ml. These results indicate that 6-O-sulfation in HS plays the critical role on the cell proliferation as well as the phosphorylation of ERK1/2.

Affinity of FGFs for WT-HS and dKO-HS Analyzed Using Surface Plasmon Resonance—As described above, FGF activation, particularly that of FGF-4, was decreased in the dKO-MEFs. It is likely that this inhibition is caused by the reduced affinity of FGFs for HS lacking 6-O-sulfation. Thus, we analyzed the interaction of FGF-1, FGF-2, and FGF-4 with WT-HS and dKO-HS using the BIAcore system. The sensorgrams and their dissociation constants are shown in Fig. 7 and Table 4, respectively. The K_D values of FGF-1/HS and FGF-1/heparin were calculated

by Scatchard analysis, because the detachment of FGF-1 from WT-HS and dKO-HS was too fast to determine the dissociation rate (k_d). Our K_D value of FGF-1/heparin was nearly comparable with the reported values, 91 nM (43) and 149 nM (47). It has been reported previously that the binding of FGF-1 to heparin requires the presence of *N*-, 2-*O*-, and 6-*O*-sulfate groups in heparin (41, 42). The K_D value of FGF-1/dKO-HS was 2.8-fold higher than that of FGF-1/WT-HS. These data indicated that

the interaction of HS with FGF-1 was also stimulated by 6-*O*-sulfate in HS.

The K_D value of FGF-2/dKO-HS was 2.5-fold lower than that of FGF-2/WT-HS. FGF-2 exhibits stronger binding to heparin with 2-*O*-sulfate groups than to heparin with 6-*O*-sulfate groups (8, 39–42). Therefore, this higher affinity between FGF-2 and dKO-HS is probably because of the higher 2-*O*-sulfate content in dKO-HS compared with that in WT-HS. Nevertheless, FGF-2-dependent phosphorylation of ERK1/2 in the dKO-MEF was less than 70% that in the WT-MEFs, suggesting that 6-*O*-sulfate residues in HS regulate FGF-2-dependent signaling.

The affinity of FGF-4 for dKO-HS was markedly decreased to approximately one-fifth that of FGF-4 to WT-HS. These data are consistent with our previous finding that FGF-4 binding to HS/heparin requires both the 6-*O*-sulfate and 2-*O*-sulfate groups in HS/heparin (8). This remarkable difference between the affinity of FGF-4 for WT-HS and dKO-HS would probably cause the markedly reduced signaling in dKO-MEFs of FGF-4 compared with that of FGF-1 or FGF-2.

Effects of 6-*O*-Sulfation on the Ternary Complex Formation of FGF-FGFR1c-HS—As FGF-2 indicated the higher affinity for dKO-HS rather than for WT-HS, we examined the effect of 6-*O*-sulfation on the ternary complex formation. The FGF-2-FGFR1c complexes were slightly formed in the absence of glycosaminoglycan (2nd lane from left in Fig. 8). However, the addition of glycosaminoglycans extremely promoted the complex formation, and the stimulation was detected at 100 ng/ml WT-HS and dKO-HS. However, the ability of WT-HS on the ternary complex formation was apparently higher than that of dKO-HS at 1 μ g/ml and was about 10-fold lower than that of heparin. These results indicated that 6-*O*-sulfate residues in HS

promote the ternary complex formation of FGF-2-FGFR1c-HS and support the reduction of FGF-2-dependent signaling in dKO-MEF. Likewise, FGF-1-FGFR1c-HS ternary complex with dKO-HS was apparently decreased at 1 μ g/ml in comparison with that of WT-HS.

Recovery of FGF Signaling in dKO-MEFs by Heparin—We then examined whether the reduced signaling in the mutant cells could be rescued by the addition of heparin or HS. We first examined the effects of exogenous heparin on FGF-4-dependent signaling. FGF-4-dependent signaling in dKO-MEF increased when 10 ng/ml heparin was added, and it recovered to the same level as that in the WT-MEFs when 100 ng/ml heparin was added. In contrast, the addition of 1–1000 ng/ml heparin to WT-MEF had no effect on FGF-4 signaling (Fig. 9). These results suggest that exogenous heparin

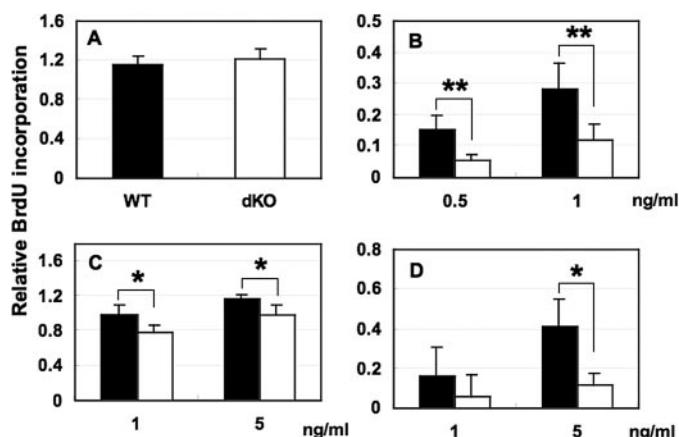


FIGURE 6. FGF-dependent dKO-MEF proliferation was apparently decreased in comparison with that of WT-MEF. MEFs (1×10^4) were seeded in 96-well plates and incubated in serum-free DMEM containing 0.1% BSA for 48 h. A, 10% of FBS; B, different concentrations of FGF-1 (0.5, 1 ng/ml); C, FGF-2 (1, 5 ng/ml); D, FGF-4 (1, 5 ng/ml) and BrdUrd labeling solution were added into the wells in the duplicate. After further incubation for 24 h, BrdUrd incorporated to DNA was measured as described under "Experimental Procedures." The values were obtained from three independent experiments with duplicate wells, and each bar (filled bars, WT-MEF; open bars, dKO-MEF) represents the mean $\pm$ S.D. from the three independent experiments. * $p < 0.01$; ** $p < 0.005$ comparing WT-MEF with dKO-MEF by Student's *t* test.

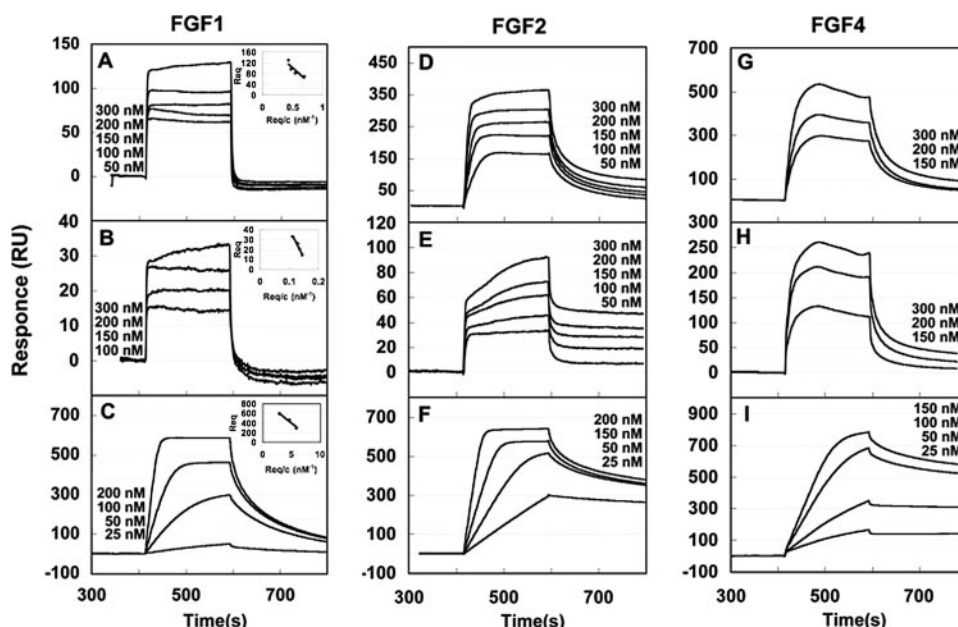


FIGURE 7. Distinct kinetics of the binding of FGF-1, FGF-2, and FGF-4 to WT- and dKO-HS. Various concentrations of FGF-1 (A–C), FGF-2 (D–F), and FGF-4 (G–I) were injected onto the surface of WT-HS- (A, D and G), dKO-HS- (B, E and H), or heparin-immobilized (C, F and I) sensor chips. Association phase began at 420 s and dissociation phase began at 600 s. For determination of K_D values to FGF-1, Scatchard plot analysis was used as described under "Experimental Procedures" (inset in A–C). Binding kinetics for FGF-2 and FGF-4 were determined based on the responses in the kinetics state model using BIAevaluation software (version 2).

Effect of 6-O-Sulfated HS on FGF-1, -2, and -4 Signaling

acts as a substitute for very little 6-O-sulfated HS on the cell surface of the dKO-MEFs, and that the exogenous heparin could not inhibit the formation of FGF-4·FGFR·WT-HS complex in the WT-MEF. As another possibility, exogenous heparin may form the functional ternary complex comparable to the cell surface HSPG in the WT-MEF. Next, we examined whether the addition of WT-HS rescued FGF-4-dependent signaling in dKO-MEFs. Unlike heparin, 1000 ng/ml WT-HS or dKO-HS did not recover the activity of FGF-4 in the dKO-MEFs (Fig. 9). Even at the higher concentration of WT-HS, 10 μ g/ml, FGF-4-dependent signaling could not be recovered and rather was inhibited (Fig. 9).

Porcine aorta HS having the similar disaccharide composition to WT-HS also did not rescue the signaling (data not shown). These results suggest that HSPG might be effective for the activation of FGFs.

DISCUSSION

HS6ST-deficient mutants in various animals exhibit aberrant morphology (17–20, 23, 24). The analysis of these mutants suggests a disturbance of the signaling of various HB-GFs. In this study, we demonstrated that the MEFs derived from HS6ST-1/HS6ST-2 dKO mice synthesize HS with few 6-O-sulfate residues and that the degree of 6-O-sulfation in HS differentially regulates the signalings of various FGFs.

The expression levels of *Hs6st* mRNA in the WT-MEFs increase in the following order: *Hs6st-3* < *Hs6st-1* < *Hs6st-2*. These data support the results of an *in situ* hybridization study conducted by Sedita *et al.* (34), which demonstrated that *Hs6st-2* mRNA has a predominantly mesenchymal distribution, whereas *Hs6st-1* mRNA is mainly expressed in epithelium- and neuron-derived tissues during organogenesis. The levels of *Hs6st-1* and *Hs6st-3* mRNA expression in the WT-MEFs were ~83 and 40% that of the *Hs6st-2* transcript, respectively. The expression of *Hs6st-3* mRNA was similar in the dKO- and WT-MEFs. WT-HS contains 20.6 mol of 6-O-sulfate residues per 100 mol of disaccharide, whereas dKO-HS contains very few 6-O-sulfate residues. Consistent with the lack of 6-O-sulfation in dKO-HS, the HS6ST activity in the dKO-MEFs was less than 4% that in the WT-MEFs. This indicates that the *Hs6st-3* mRNA in the MEFs hardly contributes to the 6-O-sulfation of

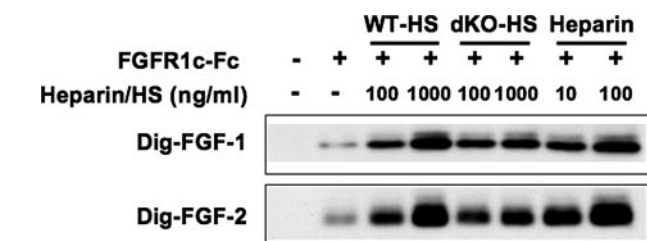


FIGURE 8. Effects of 6-O-sulfation on the ternary complex formation of FGF-FGFR1-HS. Twenty ng of Dig-labeled FGF-1 or FGF-2 and 50 ng of FGFR1c-Fc were added into 500 μ l of pull-down buffer containing WT-HS, dKO-HS, or heparin of the indicated concentration. After the incubation at 4 $^{\circ}$ C for 1 h, 20 μ l of protein G-Sepharose bead was added into the mixtures and incubated at 4 $^{\circ}$ C for 2 h. The beads were washed with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) and then with PBS two times and suspended in SDS sample buffer. The coprecipitated Dig-FGF was determined by an anti-digoxigenin antibody as described under "Experimental Procedures." The Western blots are shown in the representative photograph. Two different experiments showed similar results.

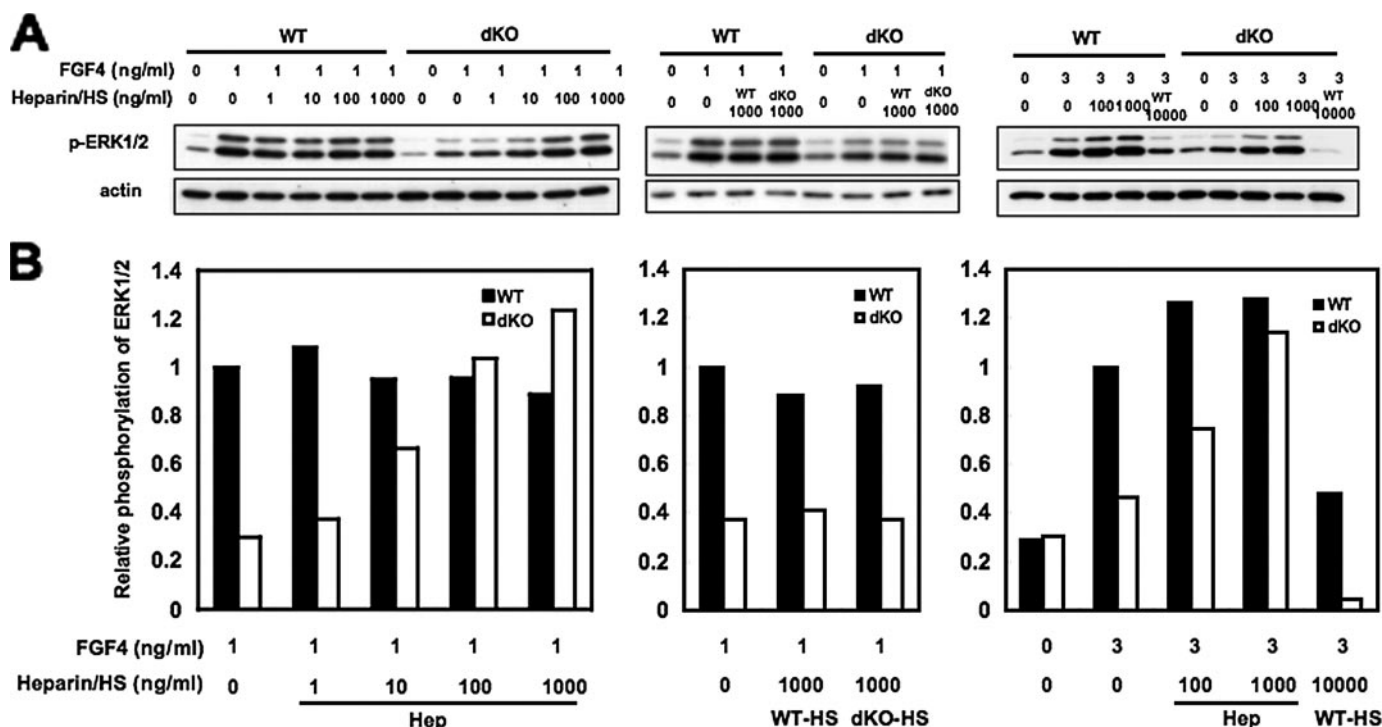


FIGURE 9. FGF signaling in dKO-MEFs was recovered by exogenous heparin. MEFs (1×10^5) were seeded and cultured as described under "Experimental Procedures." After incubation in DMEM containing 0.4% FBS for 18 h, the cells were washed with fresh medium A and incubated for an additional 24 h. The cells were incubated for 5 min at 37 $^{\circ}$ C with 1 or 3 ng/ml FGF-4 in the absence or presence of heparin (1–1000 ng/ml), WT-HS (1, 10 μ g/ml), and dKO-HS (1, 10 μ g/ml). The phosphorylation of ERK1/2 in these cell lysates was analyzed by Western blotting (A) as described under "Experimental Procedures" and quantified by BAS5000 software (B). The relative phosphorylation was normalized by actin band. The data were obtained from three independent experiments with duplicate wells used in each experiment.

HS. WT-HS contains 9.8 mol of 2-O-sulfate residues per 100 mol of disaccharide, and the amount of these residues in dKO-HS increased to 14.3 mol per 100 mol of disaccharide. This increase in the 2-O-sulfate content was consistent with the increase in the HS2ST activity in the dKO-MEFs. However, the increased level of 2-O-sulfation was substantially lower than the reduced level of 6-O-sulfate residues. Thus, the lack of 6-O-sulfation in dKO-HS was partially compensated for by 2-O-sulfotransferase. In contrast, the HS produced by the MEFs from HS2ST^{-/-} mice was enriched mainly in 6-O-sulfate, almost maintaining the overall negative charge (44). HS6ST-1 and HS6ST-2 preferentially transfer sulfates to glucosamine residues neighboring IdoUA and GlcUA, respectively (31). However, HS2ST preferentially transfers sulfate to IdoUA and rarely transfers sulfate to GlcUA (45, 46). The difference in the substrate preferences of these HS-modified enzymes might be one of the causes of the incomplete compensation for HS biosynthesis by the dKO-MEFs. In contrast, in the case of *Drosophila* that has only one *Hs6st* gene (16), the HS from Hs6st-defective mutants exhibited remarkably high 2-O- and N-sulfation, resulting in the same degree of sulfation as in the wild type (17). These results suggest that compensatory changes appear to vary in a species-specific manner when one sulfation is disturbed. The different compensation among animals and/or organs may be due to differences in the substrate specificity and expression levels of modification enzymes.

It is known that CS chains containing the E unit [GlcUA-GalNAc(4,6)diS] are bound to members of the FGF family (45). CS chains produced by MEFs contain the E unit. Thus, we examined whether CS was correlated with FGF signal transduction. Although the MEFs were digested with chondroitinase ABC to remove the CS chains, FGF-dependent signaling remained unaltered (Fig. 5). In contrast, FGF-dependent signaling was almost completely lost when MEFs were digested with a heparitinase mixture. Moreover, double digestion with heparitinases and chondroitinase ABC did not lead to an additional reduction in FGF-dependent signaling. These results suggest that CS merely has the ability to bind to FGF and may not contribute to the formation of the FGF·FGFR·CS ternary complex and/or that the structure of the CS produced by the MEFs may be inappropriate for activation of FGF signaling.

It should be noted that the affinity of dKO-HS for each FGF exhibited different characteristics. FGF-1-, FGF-2-, and FGF-4-dependent signaling through FGFR1 requires strong binding to the HS chain and is correlated to the 6-O-sulfate content. These results support the previous x-ray crystal structure analysis that the Arg-209 amino acid residue in FGFR1 forms a hydrogen bond with 6-O-sulfate residues in heparin and this induces FGF/FGFR1 dimer formation (28). The K_D value of FGF-1/HS exhibited a correlation with the 6-O-sulfation in HS as FGF-1 binding to heparin was reported to require both 6-O- and 2-O-sulfate residues (42, 48). Lower phosphorylation and BrdUrd incorporation in dKO-MEF are likely to be caused by the lower affinity to FGF-1 and lower ternary complex formation compared with WT-MEF.

With respect to heparin/HS binding, FGF-2 exhibits a stronger preference for heparin/HS containing 2-O-sulfate residues

than for heparin/HS containing 6-O-sulfate residues (8, 39–42, 48). The affinity of FGF-2 for dKO-HS was three times higher than that for WT-HS; this could be because the dKO-HS contains considerably more 2-O-sulfate residues than the WT-HS. FGF-2-dependent signaling was strongly regulated by HS. However, the FGF-2-induced phosphorylation of ERK was moderately reduced in the dKO-MEFs, suggesting that 6-O-sulfate residues would be important for the formation of the FGF-2·FGFR·HS ternary complex to the signaling. In fact, the ability of dKO-HS on the ternary complex formation with FGF-2/FGFR1c was lower than that of WT-HS (Fig. 8).

Biochemical (8, 42) and x-ray crystallography (49) studies have demonstrated that when using oligosaccharides derived from heparin, both 2-O- and 6-O-sulfate groups in heparin are crucial for the interaction of FGF-4 with heparin. In contrast, it has been demonstrated that both 2-O-desulfated heparin and 6-O-desulfated heparin polymer stimulated the mitogenic activity of FGF-4, *i.e.* the presence of either the 6-O-sulfate or 2-O-sulfate group is sufficient to activate FGF-4 (41). This study demonstrated that FGF-4-dependent phosphorylation of FGFR1 was almost completely abolished in the dKO-MEF, whereas the phosphorylation of ERK was reduced to a lesser extent. It has been suggested previously that under restricted HS conditions, FGF-4 preferentially interacts with FGFR2 compared with FGFR1 (50). Together with the present data, it is most likely that the 6-O-sulfation of HS regulates differentially the signaling of FGF-4 mediated by each FGFR.

FGF-4 signaling was recovered by the exogenous addition of heparin, suggesting that the lower responses of the dKO-MEFs to FGFs were principally because of the altered structure of HS. Because the K_D value of WT-HS for FGF-4 was 9-fold higher than that of heparin for FGF-4, HS was added to the medium at 10 and 100 times the heparin concentration. However, FGF-4 signaling was not recovered at 1 μ g/ml of WT-HS, and at 10 μ g/ml the signaling was rather inhibited. These results suggest that heparin having multivalent binding sites may be anchored into extracellular matrix through heparin-binding protein such as fibronectin, and forms the ternary complex. On the other hand, the free HS chain prepared from WT-MEFs probably has only a few binding sites. Therefore, these HS chains were less effective in forming the ternary complex than HS-proteoglycan bearing some HS chains. Furthermore, HS associated with the cell surface via the proteoglycan might be more effective on the functional ternary complex formation than soluble HS chain. As a model of 2:2:2 FGF-1/FGFR1/nonreducing site in HS was shown previously (54), one FGF-4 and one FGFR1 might form a half-complex at the nonreducing end of the HS chain, and two sets of such a half-complex might dimerize and form the active ternary complex between each nonreducing end of HS. However, the free HS chain might only form a half-ternary complex (55).

In the WT-MEFs, the FGF-1-dependent phosphorylation of FGFR1 was almost completely inhibited by the removal of HS; a similar trend was observed for FGF-2 and FGF-4. Interestingly, the FGF-1-dependent phosphorylation of ERK was only partially decreased by the removal of HS, whereas FGF-2- and FGF-4-dependent phosphorylation of ERK have a marked requirement for HS. From these data, it might be possible that the HS frag-

ments that remain undigested, even after treatment with a heparitinase mixture, persist on the cell surface and that these fragments could stimulate FGF-1-dependent signaling but not FGF-2- or FGF-4-dependent signaling. Alternatively, compared with FGFR1-mediated signaling, FGF-1-inducible signaling through receptors other than FGFR1 might have a weak requirement for HS. In any case, 6-O-sulfation appears to distinctly regulate the formation of the FGF-FGFR-HS ternary complex. Future studies will investigate the 6-O-sulfate requirement of various FGF/FGFR combinations of the ~23 members in the FGF family. HS also interacts with many HB proteins other than growth factors and regulates cellular behaviors such as adhesion and invasion (1, 51–53). The function and mechanism of the regulation of HS 6-O-sulfation during such phenomena could be clarified by using the cells isolated from various organs of 6ST1-KO, 6ST2-KO, and dKO mice.

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