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Mice Deficient in *N*-Acetylgalactosamine 4-Sulfate 6-*O*-Sulfotransferase Are Unable to Synthesize Chondroitin/Dermatan Sulfate containing *N*-Acetylgalactosamine 4,6-Bissulfate Residues and Exhibit Decreased Protease Activity in Bone Marrow-derived Mast Cells*[S]

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Chondroitin sulfate (CS) and dermatan sulfate (DS) containing *N*-acetylgalactosamine 4,6-bissulfate (GalNAc(4,6-SO_4)) show various physiological activities through interacting with numerous functional proteins. *N*-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) transfers sulfate from 3′-phosphoadenosine 5′-phosphosulfate to position 6 of *N*-acetylgalactosamine 4-sulfate in CS or DS to yield GalNAc(4,6-SO_4) residues. We here report generation of transgenic mice that lack GalNAc4S-6ST. GalNAc4S-6ST-null mice were born normally and fertile. In GalNAc4S-6ST-null mice, GalNAc(4,6-SO_4) residues in CS and DS disappeared completely, indicating that GalNAc4S-6ST should be a sole enzyme responsible for the synthesis of GalNAc(4,6-SO_4) residues in both CS and DS. IdoA-GalNAc(4,6-SO_4) units that account for ~40% of total disaccharide units of DS in the liver of the wild-type mice disappeared in the liver DS of GalNAc4S-6ST-null mice without reduction of IdoA content. Bone marrow-derived mast cells (BMMCs) derived from GalNAc4S-6ST-null mice contained CS without GlcA-GalNAc(4,6-SO_4) units. Tryptase and carboxypeptidase A activities of BMMCs derived from GalNAc4S-6ST-null mice were lower than those activities of BMMCs derived from wild-type mice, although mRNA expression of these mast cell proteases was not altered. Disaccharide compositions of heparan sulfate/heparin contained in the mast cells derived from BMMCs in the presence of stem cell factor were much different from those of heparan sulfate/heparin in BMMCs but did not differ significantly between wild-type mice and GalNAc4S-6ST-null mice. These observations suggest that CS containing GalNAc(4,6-SO_4) residues in BMMCs may contribute to retain the active proteases in the granules of BMMCs but not for the maturation of BMMCs into connective tissue-type mast cells.

Chondroitin sulfate (CS)[2] and DS chains are composed of GlcAβ 1(or IdoAα1)-3GalNAc repeating units bearing sulfate groups on various positions of each sugar residue. The sulfation pattern of CS appears to be important for the specific functions of CS. CS-E containing GlcAβ1–3GalNAc(4,6-SO_4) is implicated in immunological functions of mucosal mast cells (1–6), regulation of procoagulant activity (7), outgrowth of neurite (8–10), binding of chemokines (11, 12), midkine (13), and various heparin binding growth factors (14, 15), infection of herpes simplex virus (16, 17), metastasis of the Lewis lung carcinoma cells (18), migration of neuronal precursors during cortical development (19), and activation of matrilysin (20). CS of thrombomodulin, which is involved in the antithrombin-dependent anticoagulant activity, has been reported to bear GalNAc(di-SO_4)-GlcA-GalNAc(di-SO_4) at the nonreducing end (21). Rat glomeruli (22) and rat mesangial cells (23) were reported to synthesize glycosaminoglycans containing the IdoAα1–3GalNAc(4,6-SO_4) unit, which had been found in hag fish notochord (24). DS containing IdoA-GalNAc(4,6-SO_4)

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text, references, Figs. S1–S4, and Table S1.

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[2] The abbreviations used are: CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; GalNAc(4,6-SO_4), 4,6-bis-*O*-sulfo-*N*-acetylgalactosamine; GalNAc(4SO_4), 4-*O*-sulfo-*N*-acetylgalactosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; HexA, hexuronic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; GalNAc4S-6ST, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase; ΔDi-0S, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-6S, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-4S, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-diS_D, 2-acetamide-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS_E, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose; ΔDiHS-0S, 2-acetamide-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose; ΔDiHS-6S, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔDiHS-NS, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ΔDiHS-(N,6)diS, 2-deoxy-2-sulfamino-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔDiHS-(N,2)diS, 2-deoxy-2-sulfamino-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; and ΔDiHS-(N,6,2)triS, 2-deoxy-2-sulfamino-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-Sulfo-D-glucose; BMMC, bone marrow-derived mast cell; CTMC, connective tissue-type mast cell; SCF, stem cell factor; MMC, mucosal-type mast cell; PN-1, protease nexin-1; TM, thrombomodulin; RT, reverse transcription; HPLC, high performance liquid chromatography; ES, embryonic stem cell.

units from porcine intestinal mucosa was reported to activate heparin cofactor II (25).

GalNAc4S-6ST transfers sulfate to position 6 of GalNAc($4SO_4$) residues included in CS and DS (26, 27). GalNAc4S-6ST also sulfates (GalNAc($4SO_4$)) located at the nonreducing terminal of CS and DS. GalNAc4S-6ST is thus a key enzyme for the synthesis of CS and DS containing GalNAc(4,6-SO_4) residues. GalNAc4S-6ST was purified to the homogeneity from the squid cartilage (26). On the basis of the amino acid sequence of the squid GalNAc4S-6ST, cDNAs of human (27) and squid GalNAc4S-6ST (28) were cloned. Human GalNAc4S-6ST exhibited activity toward both the nonreducing terminal and internal GalNAc ($4SO_4$) residues of CS-A (27, 29). Squid GalNAc4S-6ST could produce oversulfated CS containing the E-D hybrid tetrasaccharide structure (28). Expression of GalNAc4S-6ST was reported to be regulated during development of the brain (30, 31), bone marrow-derived mast cells (32), and early mouse embryos (33).

Loss-of-function studies on glycosaminoglycan sulfotransferases have revealed the biological significance of the sulfation pattern of HS and CS (34, 35). In this report, we generated transgenic mice that cannot express GalNAc4S-6ST using a homologous recombination gene-targeting approach. We observed that GalNAc4S-6ST-null mice could not synthesize CS/DS containing GalNAc(4,6-SO_4) residues systemically. We also showed that bone marrow-derived mast cells obtained from GalNAc4S-6ST-null mice had decreased level of mast cell proteases.

EXPERIMENTAL PROCEDURES

Materials—The following commercial materials were used: $H_2{}^{35}SO_4$ was from PerkinElmer Life Sciences; chondroitinase ACII, chondroitinase ABC, heparitinase I, heparitinase II, heparinase, unsaturated disaccharides kit for HS, unsaturated disaccharides kit for CS, CS-A (whale cartilage), and DS (pig skin) were from Seikagaku Corp., Tokyo; a Partisil-10 SAX column (4.6 mm × 25 cm) was from Whatman; *N*-acetylgalactosamine 4-sulfate, *N*-acetylgalactosamine 6-sulfate, tetra-*n*-butylammonium hydrogen sulfate (Fluka), and ganciclovir were from Sigma; Hi Load 16/60 Superdex 30 pg, Hi Load 16/60 Superdex 200 pg, and Fast Desalting Column HR 10/10 were from Amersham Biosciences. DOCOSIL (4.6 × 150 mm) was from Senshu Scientific Co. Ltd., Tokyo; Geneticin was from Invitrogen; Nanosep Centrifugal Devices 3K was from Nihon Pall Ltd, Tokyo; WEHI-3 cells were from American Type Culture Collection; NIH-3T3 cells were from RIKEN Cell Bank, Tsukuba, Japan; stem cell factor (SCF) was from PeproTech, Inc., Rocky Hill, NJ; antibody against mMCP-6 (sc-32473) was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; anti-actin antibody was from Sigma; S-2586 for chymotrypsin-like proteases and S-2288 for trypsin-like proteases were from Chromogenix, Milano, Italy; M-2245 for carboxypeptidase A was from Bachem AG, Bubendorf, Switzerland. Protease Inhibitor Mixture Complete Mini was from Roche Diagnostics K.K., Tokyo. [^{35}S]PAPS was prepared as described (36). Unlabeled PAPS was a gift from YAMASA Corp. (Choshi, Japan). *N*-Acetylgalactosamine 4,6-bissulfate was prepared from ΔDi-diS_E, as described previously (37).

Construction of a Targeting Vector and Generation of GalNAc4S-6ST-deficient Mice—The 3′-phosphoadenosine 5′-phosphosulfate binding site of GalNAc4S-6ST, which is critical for the sulfotransferase activity, is located in exon 2; therefore, we planned to delete exon 2. A cDNA fragment that codes for exon 2 of GalNAc4S-6ST was amplified by PCR. The first PCR reaction was carried out using oligonucleotides GCGAAGCTTGTAATCCCCAGCAAATTCC and GCGC-TCGAGGCGGACAATTCCAAAGC, which were synthesized according to the sequence of mouse GalNAc4S-6ST cDNA clone (accession number AB187269), as primers, and the mouse GalNAc4S-6ST cDNA as a template. The amplified cDNA was sent to the DNA Microarray Facility, Roswell Park Cancer Institute (Buffalo, NY) to screen the Mouse 129 Female RPCI-22 BAC Library. From the positive clones, genomic DNA containing GalNAc4S-6ST (11 kb) was excised by digestion with EcoRV and subcloned into this site of Bluescript KS-plasmid (Stratagene) and sequenced. This genomic DNA contained exon 1 (1914–2912), exon 2 (3659–3998), exon 3 (6114–6260), and exon 4 (10078–10234). The targeting construct is depicted in Fig. 2*A*. The generated construct contained a deletion of the 2.1-kb SphI fragment, which contains full exon 2. Thus, no functional GalNAc4S-6ST protein should be present in the transgenic animals if homologous recombination occurs in the mouse genome. The short arm in the targeting construct contained a 2.4-kb PstI-SphI fragment that resided upstream of the deleted portion of the GalNAc4S-6ST gene. This genomic fragment was inserted downstream of the phosphoglycerate kinase neo-cassette. The long arm in the targeting construct contained a 3.7-kb SphI-HindIII fragment that resided downstream of the deleted portion of the GalNAc4S-6ST gene. It was inserted upstream of the neo-cassette. This targeting vector was linearized by digestion with ScaI and electroplated into E14 embryonic stem (ES) cells. The clones containing the *neo* gene but not the thymidine kinase gene (*tk*) were selected by incubation in a medium containing 200 μg/ml Geneticin (G-418) and 2 μM ganciclovir. Positive clones were subsequently screened by Southern blot analysis or PCR using the primers shown in Fig. 2*A*. The ES clones that had been successfully targeted were injected into C57BL/6 blastocysts, and the chimeric male mice were crossed with C57BL/6 female mice. The genotypes of the offspring were determined by PCR using the DNA isolated from the tail, as described below. Heterozygous mice were intercrossed to generate GalNAc4S-6ST-null mice. The use of animals for research complied with guidelines for care and use of animals, and permission was given by the Animal Research Committee of Aichi University of Education.

Genotype Analysis—The genotyping of the mutant ES clones or chimeric mice was performed by Southern blot analysis and/or PCR. Genomic DNA from the ES cell clones or mouse tails was isolated using a DNA purification kit (DNeasy tissue kit, Qiagen), digested with BamHI, and hybridized to the probe shown in Fig. 2*B*. The probe that hybridizes to the sequence within exon 1 was adopted because any 5′-external probe showed high homology with the mouse repetitive sequences and gave a smear band in the Southern blot. PCR amplification was performed by using the following two pairs of primers: forward primer 1 (5′-CCATCAGATTGGCCTGTAG-

GTCTTTAGAG-3′) and reverse primer 2 (5′-GCTTGGAGTA-GAGCACGTAGGAATTG-3′) for the analysis of the wild-type allele; forward primer 1 and reverse primer 3 (5′-CATCGCCTTC-TATCGCCTTCTTGAC-3′) for the mutated allele.

Measurements of GalNAc4S-6ST mRNA in Various Mouse Tissues by Real-time PCR—Total RNA was extracted by TRIzol reagent (Invitrogen) and was reverse-transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems). mRNAs for GalNAc4S-6ST and β-actin expression were quantified by real-time RT-PCR using a TaqMan gene expression assay in a 7300 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol.

Assay of Sulfotransferase Activity—The spleens from wild-type and homozygous mutant mice were homogenized by using a Polytron homogenized in 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.15 M NaCl, 20% glycerol, and protease inhibitors. To the homogenates, Triton X-100 was added to a final concentration of 0.5%, and the mixtures were further homogenized in a glass homogenizer. The homogenate was centrifuged at 10,000 × *g* for 10 min, and the supernatant solution was used as the enzyme. Sulfotransferase activity was assayed by the method described previously (29). The standard reaction mixture contained, in a final volume of 50 μl, 50 mM imidazole-HCl, pH 6.8, 10 mM $CaCl_2$, 20 mM reduced glutathione, 25 nmol (as galactosamine) of CS-A, 50 pmol of [^{35}S]PAPS (~5.0 × 10^5 cpm), and enzyme. The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, the reaction mixtures were treated by Actinase (0.2 mg/ml) at 37 °C for 16 h, and the ^{35}S-labeled glycosaminoglycans were isolated by the precipitation with ethanol followed by gel chromatography with a Fast Desalting Column as described previously (38). Radioactivity of the isolated glycosaminoglycans was determined. For determining the position of sulfate transferred to the acceptor, the ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ACII as described below. The radioactive products formed after the enzymatic digestion were separated with HPLC using a Whatman Partisil-10 SAX column as described below, and ^{35}S radioactivity was determined.

Disaccharide Composition Analysis of CS/DS and HS/Heparin—Disaccharide composition analysis of CS/DS extracted from various mouse tissues was carried out as follows. Various mouse tissues were homogenized in acetone, and the acetone-insoluble materials were dried. The dried materials (up to 30 mg) were suspended in 1 ml of 0.2 M NaOH and stirred for 16 h at room temperature. After neutralization with 3 M acetic acid, the samples were digested with DNase I and RNase A (0.1 mg/ml each) in 20 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$ for 2 h at 37 °C. After heating at 100 °C for 2 min, Actinase (0.4 mg/ml) was added, and the mixture was incubated at 50 °C for 24 h. To the digests, trichloroacetic acid (final 5%) was added, and the precipitates formed were removed by centrifugation at 10,000 × *g* for 15 min. To the resulting supernatant fractions were added 3 volumes of ethanol containing 1.3% potassium acetate, and glycosaminoglycans were precipitated by centrifugation at 10,000 × *g* for 10 min. The precipitates were dissolved in 300 μl of 4 M guanidine-HCl solution and filtered by a Nanosep Centrifugal Devices 3K. Glycosaminoglycans remaining on the filter were recovered by 100 μl of 1 M guanidine-HCl solution twice and mixed with 3 volumes of ethanol containing 1.3% potassium acetate. After the mixtures were placed at −80 °C for 30 min, glycosaminoglycans were precipitated by centrifugation at 10,000 × *g* for 30 min at 4 °C. The purified glycosaminoglycans were dissolved in 100 μl of water, and 25-μl aliquots were digested with 0.5 turbidity-reducing unit of *Streptomyces* hyaluronidase in 50 mM sodium acetate buffer, pH 5.0. After digestion, 10 μg of glycogen as a carrier and 2 volumes of ethanol containing 1.3% potassium acetate were added, and the mixtures were left at −80 °C for 30 min. Glycosaminoglycans were precipitated by centrifugation at 10,000 × *g* for 30 min at 4 °C. The precipitates were digested with chondroitinase ABC and chondroitinase ACII, or chondroitinase ACII alone as described below. The disaccharide products were analyzed according to the method of Toyoda *et al.* (39), with a slight modification of the elution conditions. For analysis of CS/DS and HS/heparin obtained from BMMCs, peritoneal cells and CTMC-like cells derived from BMMCs in the presence of SCF, MMC-like cells derived from bone marrow cells, and whole newborn embryos were carried out as follow. Cells or whole newborn embryos that had been homogenized in acetone and dried were treated with 0.2 M NaOH at 4 °C and digested with DNase I, RNase A, and Actinase as described above. The reaction was stopped by heating at 100 °C for 2 min, and samples were centrifuged at 10,000 rpm for 10 min to remove insoluble material. The supernatants were diluted with an equal volume of 20 mM Tris-HCl, pH 7.2, and loaded onto a DEAE-Sephacel column equilibrated with the same buffer. The column was washed with 10 column volumes of 20 mM Tris-HCl, pH 7.2, containing 0.2 M NaCl and then eluted with 3 column volumes of Tris-HCl, pH 7.2, containing 2 M NaCl. To the eluates, 20 μg of glycogen and 3 volumes of cold ethanol containing 1.3% potassium acetate were added, and the glycosaminoglycans were recovered by centrifugation. For disaccharide composition analysis of CS/DS, the purified glycosaminoglycans were treated with hyaluronidase and digested with chondroitinase ABC and chondroitinase ACII as described above. The disaccharide products were analyzed as above.

Expression Levels of mRNA Encoding Glycosaminoglycan Sulfotransferases, Mast Cell Proteases, and Serglycin Analyzed by RT-PCR—Total RNA samples were prepared from the BMMCs generated from 7-week-old female mice (wild-type, heterozygote, or homozygote) using TRIzol (Invitrogen). Reverse transcription was carried out using a High Capacity RNA-to-cDNA kit (Applied Biosystems) and 1 μg of total RNA as the template. The expression levels of glycosaminoglycan sulfotransferases were semiquantitatively determined by RT-PCR using the following eight primers pairs: *Galnac4s-6st* forward primer (CCCACTTCTACATCATTGGG) and reverse primer (TGGAACTTGCGAAGTAGAGA); *C6st-1* forward primer (CGCCACGTGCTTCTCATG) and reverse primer (CCGCAAAGGCCACTATGC); *C4st-1* forward primer (AGGTGACAGACACCTGCCG) and reverse primer (ACTCCTCGAACTTGACATCGT); *Hs6st-1* forward primer (AGGACCATGGTTGAGCG) and reverse primer (GCGGCGATTGGGCCGATA); *Hs6st-2* forward primer (CCGGTGCCGGATCC-

GTA) and reverse primer (GCGTCGCGCTTGCCATC); *Hs6st-3* forward primer (CAACTTCGGGGAGCAGC) and reverse primer (TCCACGAAGCGGGTCAG); *Hs2st* forward primer (TTCATGGGGCTCCTCAGG) and reverse primer (AAGCGCACCTGATCTTGC); and *Ndst-2* forward primer (GAGAGGCCAGGCCACAG) and reverse primer (TGCAACATTCCAGTGTG). The expression levels of mRNAs encoding mast cell proteases and serglycin were semiquantitatively determined by RT-PCR using the following seven primers pairs: *Mmcp-1* forward primer (GAGGATCCATGCAGGCCCTACTATTCCTGATG) and reverse primer (ATCTCGAGCTTGCCATTTATGACTGTTTTAATCC); *Mmcp-2* forward primer (GTGATGACTGCTGCACACTG) and reverse primer (CTTGAAGAGTCTGACTCAGG); *Mmcp-4* forward primer (GTAATTCCTCTGCCTCGTCCT) and reverse primer (CCCAAGGGTTATTAGAAGAGCTC); *Mmcp-5* forward primer (GAGGATCCATGAAGCCTGCAGCAGCCCTGAGG) and reverse primer (GACTCGAGATTCTCCCTCAAGATCTTATTGATCC); *Mmcp-6* forward primer (GAGCGGCCGCATGCTGAAGCGGCGGCTGCTGCTG) and reverse primer (GACTCGAGGGAATGCTCAGGGACATAGCGGTG); *Mmcp-7* forward primer (TGCCAATGACACCTACTGGATGC) and reverse primer (CAGGAGTCATGTCCTTCATTCC); *Cpa3* forward primer (ACACAGGATCGAATGTGGAG) and reverse primer (TAATGCAGGACTTCATGAGC); and *Srgn* forward primer (GAGGATCCATGCAGGTTCCCGTCGGCAGCA) and reverse primer (ATCTCGAGTATAATAAAATCGTCTTCTGGTTGGTCTTGG).

Culture of Mouse Bone Marrow Cells—Bone marrow cells were obtained from the femurs of 7-week-old BALB/c mice, and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 50% conditioned medium of WEHI-3 cells (40). To obtain further differentiated mast cells, BMMCs cultured for ~4 weeks as above were cultured in the same medium containing 10 ng/ml SCF on a NIH-3T3 cell layer, which was treated with 10 μg/ml mitomycin C, for 5 weeks (41). To obtain mucosal-type mast cell (MMC)-like cells, bone marrow cells obtained from the femurs of 9-week-old BALB/c mice were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 ng/ml recombinant human transforming growth factor-β (Peprotech), 1 ng/ml mouse interleukin (IL)-3 (Peprotech), 5 ng/ml mouse IL-9 (Peprotech), and 10 ng/ml mouse SCF (Peprotech) (42). The medium was replaced with the fresh medium every 3–4 days. After ~30 days, *in vitro* derived MMC-like cells were used for the following experiments.

Staining Protocols of Cells—BMMCs were suspended in phosphate-buffered saline and collected onto object glasses by cytospin (500 rpm, 5 min). A standard procedure for May Grünwald/Giemsa staining was used. Briefly, cells were air-dried and then stained in May Grünwald solution (10 min) followed by Giemsa (10 min), with washing steps between and after staining.

Metabolic Labeling and Enzymatic Digestion of Glycosaminoglycans Synthesized by Liver—Livers of wild-type and homozygous mutant mice were taken out aseptically and cut into cubic slices (2 mm thick) in phosphate-buffered saline. The slices were placed in 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 150 μCi/ml $^{35}SO_4$ and incubated for 24 h in a CO_2 incubator. The ^{35}S-labeled glycosaminoglycans were isolated by the methods described above for the determination of disaccharide composition of HS/heparin. Glycosaminoglycans thus obtained were treated with nitrous acid at pH 1.5 (43). After nitrous acid treatment, 3 volumes of ethanol containing 1.3% potassium acetate was added. The glycosaminoglycans were recovered by centrifugation at 10,000 $\times$ *g* for 10 min and dissolved in water. The glycosaminoglycan solution was applied to a Fast desalting column (Sephadex G-25 column) equilibrate with 0.1 M NH_4HCO_3, and the void volume fractions were collected. The purified glycosaminoglycans thus obtained were digested with chondroitinase ACII or chondroitinase ABC, and the degradation products were separated by HPLC using a Whatman Partisil-10 SAX column or by Superdex 30 chromatography as described below.

Metabolic Labeling of Chondroitin Sulfate Synthesized by BMMCs—BMMCs were plated in 6-cm dishes at a density of 1×10^6 cells/dish. $^{35}SO_4$ (30 μCi/ml) was added to the culture medium of BMMCs 2 days after plating. The labeling with $^{35}SO_4$ was continued for 24 h. The ^{35}S-labeled glycosaminoglycans were extracted from the combined cell and medium fractions by treatment with 0.5 M NaOH for 24 h at 4 °C, and digestion with Actinase (0.3 mg/ml) after neutralization. To the digested solution, trichloroacetic acid was added to the final concentration of 5%, and the precipitates formed were removed by centrifugation at 10,000 $\times$ *g* for 10 min. The resulting supernatant fractions were mixed with 3 volumes of ethanol containing 1.3% potassium acetate, and ^{35}S-labeled glycosaminoglycans were precipitated by centrifugation at 10,000 $\times$ *g* for 10 min. The precipitates were dissolved in water, and precipitation with 3 volumes of ethanol was repeated twice. The final precipitates were dissolved in water, and the radioactivity was determined. To determine the disaccharide composition, the ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ACII, and the radioactive products were separated by HPLC using a Whatman Partisil-10 SAX column.

Analysis of Protease Activities in BMMCs—BMMCs were solubilized in lysis buffer (phosphate-buffered saline containing 2 M NaCl and 0.5% Triton X-100 (44); 100 μl of lysis buffer/1×10^6 cells). The reaction mixtures contained, in a final volume of 120 μl, 10-μl aliquots of the lysed solution of BMMCs, 20 μl (S-2586 and S-2288) or 40 μl (M-2245) of 1.8 mM aqueous solutions of chromogenic substrates. S-2586, S-2288, and M-2245 were used for determination of chymotrypsin-like proteases, trypsin-like proteases, and carboxypeptidase A, respectively. The reaction mixtures were incubated at 37 °C, and the absorbance at 405 nm was monitored with a microplate reader. The enzyme activity was determined by using a freshly extracted enzyme solution.

Superdex 30 Chromatography, Superdex 200 Chromatography, and SAX-HPLC—A Superdex 30 16/60 column was equilibrated with 0.2 M NH_4HCO_3 and run at a flow rate of 2 ml/min. One-ml fractions were collected. A Superdex 200 16/60 column was equilibrated with 0.2 M NaCl/20 mM Tris-HCl and run at a flow rate of 1 ml/min. One-ml fractions were collected. Separation of the degradation products formed from ^{35}S-labeled glycosaminoglycans were carried out by HPLC using a What-

man Partisil-10 SAX column (4.6 mm × 25 cm). The column was equilibrated with 5 mM KH_2PO_4. The column was developed with 5 mM KH_2PO_4 for 10 min followed by a linear gradient from 5 to 500 mM KH_2PO_4. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min and a column temperature of 40 °C.

Western Blot Analysis—BMMCs were extracted with phosphate-buffered saline containing 2 M NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors for 30 min on a rotary shaker. The extract was separated by SDS-PAGE as described by Laemmli (45). The separated proteins were electrophoretically transferred to a Hybond ECL membrane (Amersham Biosciences) and stained with anti-mMCP-6 antibody. The blot was developed with polyclonal anti-goat IgG antibody coupled to horseradish peroxidase using an ECL detection kit and a Hyperfilm ECL (Amersham Biosciences).

Digestion with Chondroitinase ACII, Chondroitinase ABC, and Heparitinase I/Heparitinase II/Heparinase—Digestion of glycosaminoglycans with chondroitinase ACII or chondroitinase ABC was carried out for 4 h at 37 °C in the reaction mixture containing, in a final volume of 25 μl, 50 mM Tris acetate buffer, pH 7.5, 0.1 mg/ml bovine serum albumin, and 30 milliunits of chondroitinase ABC and/or 30 milliunits of chondroitinase ACII. For disaccharide composition analysis of HS/heparin, the purified glycosaminoglycans were digested with 0.5 milliunit of heparinase I, 0.25 milliunit of heparitinase II, and 0.5 milliunit of heparinase in 50 mM Tris acetate, pH 7.5, containing 1 mM $CaCl_2$ and 0.1 mg/ml bovine serum albumin at 37 °C for 2 h.

Transmission Electron Microscope—BMMCs were collected by centrifugation, and the cell pellets were fixed with modified Karnovsky fixative for ~6 h at 4 °C. After being washed with 0.1 M phosphate buffer (pH 7.2), they were postfixed with 1% OsO_4-0.1 M phosphate buffer for ~1.5 h at 4 °C. The samples were dehydrated in an alcohol-acetone series and were embedded in Quetol 812 (Nisshin EM Co., Tokyo). Ultrathin sections cut with a diamond knife were stained with uranyl acetate and lead citrate, then observed with a JEOL, JEM-2000FX electron microscope.

RESULTS

Expression Pattern of Mouse GalNAc4S-6ST mRNA in Various Tissues of Wild-type Mice—The expression patter of mouse GalNAc4S-6ST mRNA was determined by real-time PCR (Fig. 1). Various tissues were obtained from 7-week-old BALB/c mice. Relatively high expression was observed in the brain, heart, lung, spleen kidney, and liver.

Targeted Disruption of the GalNAc4S-6ST Gene—The construction of the targeting vector and the strategy for screening ES cells for homologous recombination are shown in Fig. 2*A*. In the recombinant allele, one of PAPS binding sites (5′-phosphosulfate binding site) was deleted. We generated chimeric mice from an ES clone that had undergone homologous recombination at the GalNAc4S-6ST locus. The chimeric mice were subsequently crossed with C57BL/6 mice to facilitate the germ line transmission. We confirmed the complete destruction of the GalNAc4S-6ST gene by Southern blot analyses and PCR (Fig. 2, *B* and *C*). We first analyzed a BamHI digest of the genomic

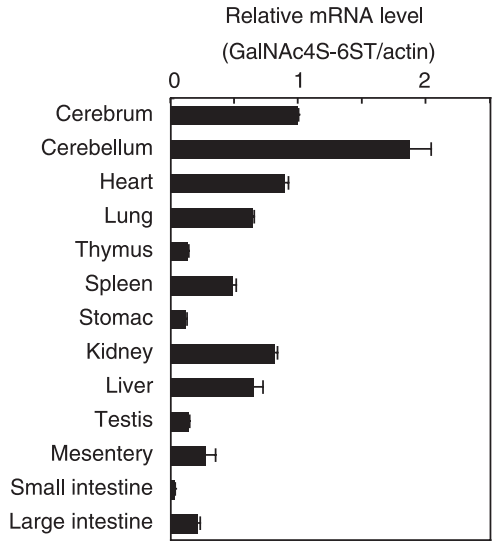

FIGURE 1. **Expression GalNAc4S-6ST mRNA in the various tissues in the wild-type mice.** Real-time RT-PCR was carried out as described under "Experimental Procedures." Values indicate averages of duplicate determination, and ranges of data were indicated above each *bar*.

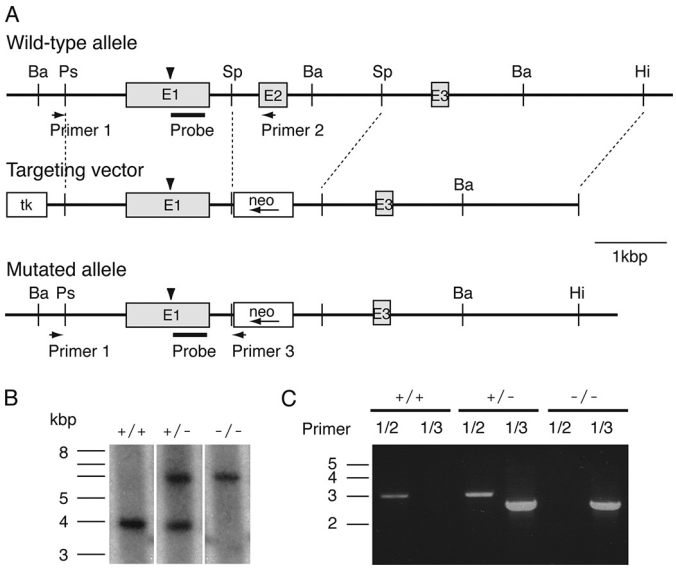

FIGURE 2. **Targeted disruption of *Galnac4s-6st* and the generation of GalNAc4S-6ST-deficient mice.** *A*, depiction of the targeting vector for *Galnac4s-6st* disruption. The restriction maps of the *Galnac4s-6st* gene (*top*), the targeting vector (*middle*), and the mutant allele (*bottom*) are shown. A 2.1-kb SphI fragment containing 5′- phosphosulfate binding sites was deleted and replaced with a *neo* gene cassette. The translation start site is indicated by *arrowheads*. The probe used for the Southern blot analysis is shown at the *bottom*. *Ba*, BamHI; *Ps*, PstI; *Sp*, SphI; *Hi*, HindIII. *B*, Southern blot analysis of tail genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice. The tail genomic DNA was digested with BamHI and hybridized to the probe shown in *A*. *C*, PCR analysis of tail genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice. The primers used for PCR are shown in *A*.

DNA from the wild-type mice and mutant mice using a probe indicated in Fig. 2*A*. Digests from the wild-type mice contained a single fragment of 3.8 kb that hybridized to the probe, whereas digests from the homozygous mutant mice contained a hybridizing fragment of 6.0 kb, and digests from the heterozygous mutant mice contained two fragments corresponding in size to the fragments detected individually in the digests from the wild-type and homozygous mutant mice (Fig. 2*B*). RT-PCR of

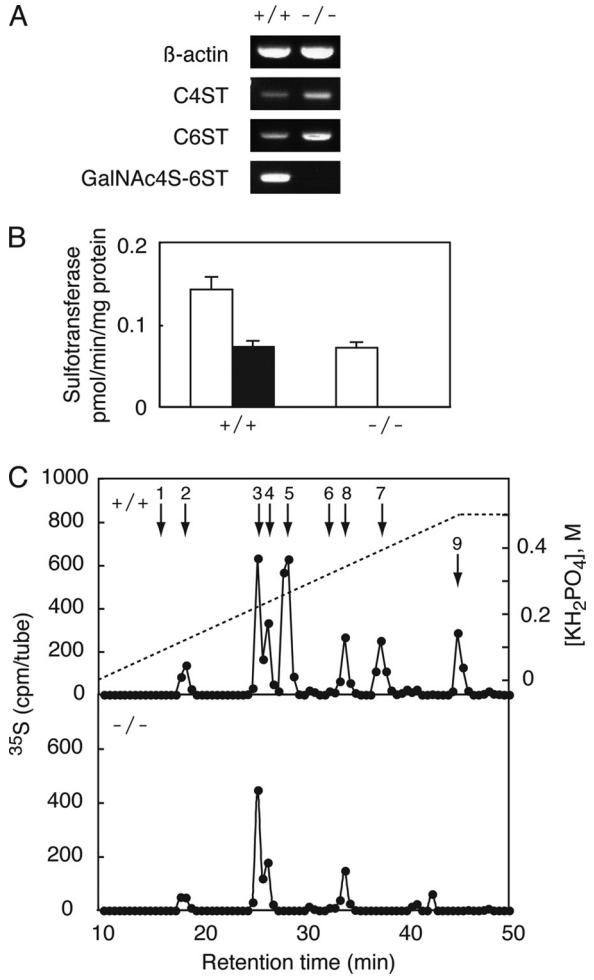

FIGURE 3. **Disappearance of GalNAc4S-6ST mRNA and GalNAc4S-6ST activity in the spleen of *Galnac4s-6st*$^{-/-}$ mice.** *A*, RT-PCR analysis of total spleen RNA from wild-type (+/+) and homozygous (−/−) mutant mice. *B*, sulfotransferase activity in the extracts from the spleen of wild-type (+/+) and homozygous (−/−) mutant mice. Sulfotransferase activity was assayed using CS-A as the acceptor. Total activity (*open bar*) and GalNAc4S-6ST activity calculated from *C* (*filled bar*) are shown. Each *bar* represents the mean ± S.D. from three determinations. *C*, SAX-HPLC separation of disaccharides and oligosaccharides formed after digestion with chondroitinase ACII. *Arrows* indicate the elution position of: *1*, GalNAc(6SO_4); *2*, GalNAc(4SO_4); *3*, ΔDi-6S; *4*, ΔDi-4S; *5*, GalNAc(4, 6-SO_4); *6*, ΔDi-diS_D; *7*, ΔDi-diS_E; *8*, oligosaccharide I (GalNAc(4SO_4)-GlcA(2SO_4)-GalNAc(6SO_4)); and *9*, oligosaccharide II (GalNAc(4,6-SO_4)-GlcA(2SO_4)-GalNAc(6SO_4)). The *dotted line* represents concentration of KH_2PO_4.

spleen RNA showed that the GalNAc4S-6ST gene is not expressed in the homozygous mutant mice. In contrast, levels of the C4ST-1 and C6ST-1 transcripts in the homozygous mutant mice were higher than those in the wild-type mice (Fig. 3*A*). Sulfotransferase activity contained in the extracts from the spleen was determined. The total sulfotransferase activity was decreased in the spleen of the homozygous mutant mice (Fig. 3*B*, *open bar*). To determine whether GalNAc4S-6ST activity is present in the spleen extracts, we digested the ^{35}S-glycosaminoglycans with chondroitinase ACII and separated the degradation products by SAX-HPLC (Fig. 3*C*). When the extract from the spleen of the wild-type mice was used, seven radioactive peaks were observed. Among these peaks, peaks corresponding to GalNAc(4,6-SO_4), ΔDi-diS_E, and oligosaccharide II could not be detected under the assay conditions when the extracts from the spleen of the homozygous mutant mice were used. We have previously demonstrated that GalNAc(4,6-SO_4), ΔDi-diS_E, and oligosaccharide II were released from the ^{35}S-labeled glycosaminoglycan formed from CS-A after incubation with GalNAc4S-6ST and [^{35}S]PAPS (29). The observation, that peaks corresponding to GalNAc(4,6-SO_4), ΔDi-diS_E, and oligosaccharide II disappeared when the extract from the spleen of the homozygous mutant mice was used, strongly supports the idea that the spleen extract was devoid of GalNAc4S-6ST activity. From the elution pattern of Fig. 3*C*, the GalNAc4S-6ST activity was calculated and is shown in Fig. 3*B* (*filled bar*). These results strongly suggest that expressions of the GalNAc4S-6ST mRNA and GalNAc4S-6ST activity were both abolished in the homozygous mutant mice. We analyzed genotypes of 222 pups (BALB/c background) obtained from intercrosses between the heterozygous mice. The genotypes agreed well with the Mendelian rule (26.1% wild-type, 46.4% heterozygous, and 27.5% homozygous), indicating that GalNAc4S-6ST is not crucial for embryonic development. The *Galnac4s-6st*$^{-/-}$ mice were born normally and fertile, showed no obvious developmental abnormality, although an average litter size appeared to be reduced in crossing between the homozygous mutant mice; 2.7 ± 1.2 pups (n = 3) for crossing between homozygous mutant mice *versus* 6.8 ± 2.5 pups (n = 35) for crossing between heterozygous mice.

Disappearance of HexA-GalNAc(4,6-SO_4) Disaccharide Units in CS/DS Isolated from Galnac4s-6st$^{-/-}$ *Mice*—GalNAc4S-6ST showed the activity to synthesize the GalNAc(4,6-SO_4) residue in CS/DS *in vitro*, but it has not been confirmed whether GalNAc4S-6ST is the sole enzyme responsible for the synthesis of this residue. If an enzyme other than GalNAc4S-6ST is present, GalNAc(4,6-SO_4) might still be synthesized in the *Galnac4s-6st*$^{-/-}$ mice. To rule out this possibility, we analyzed the structure of CS/DS obtained from various tissues of the *Galnac4s-6st*$^{-/-}$ mice. After removal of hyaluronic acid by digestion with *Streptomyces* hyaluronidase, glycosaminoglycans were digested with chondroitinase ABC plus chondroitinase ACII, and the disaccharide compositions were determined as described under "Experimental Procedures" (Fig. 4). ΔDi-diS_E, which was derived from disaccharide units of GlcA-GalNAc(4,6-SO_4) and IdoA-GalNAc(4,6-SO_4), was detected in various proportions in the tissues of the wild-type mice; relatively high proportions of ΔDi-diS_E (>10% of the total disaccharides) were observed in the liver, kidney, and spleen. Unexpectedly, the proportion of ΔDi-diS_E in the cerebrum and cerebellum was the lowest among these tissues, although GalNAc4S-6ST mRNA was highly expressed in these tissues as shown in Fig. 1. To determine the proportion of IdoA-GalNAc(4,6-SO_4) units in CS/DSs obtained from the liver, kidney, and spleen, we digested the CS/DSs by chondroitinase ACII alone, and disaccharide composition was analyzed. From the difference in ΔDi-diS_E between chondroitinase ABC digestion and chondroitinase ACII digestion, the ratios of IdoA-GalNAc(4,6-SO_4)/(GlcA-GalNAc(4,6-SO_4) plus IdoA-GalNAc(4,6-SO_4)) were calculated as 0.98, 0.83, and 0.60 for liver, spleen, and kidney, respectively. These results indicate that a major part of GalNAc(4,6-SO_4) residues of the CS/DS obtained from these tissues is included in IdoA-GalNAc(4,6-

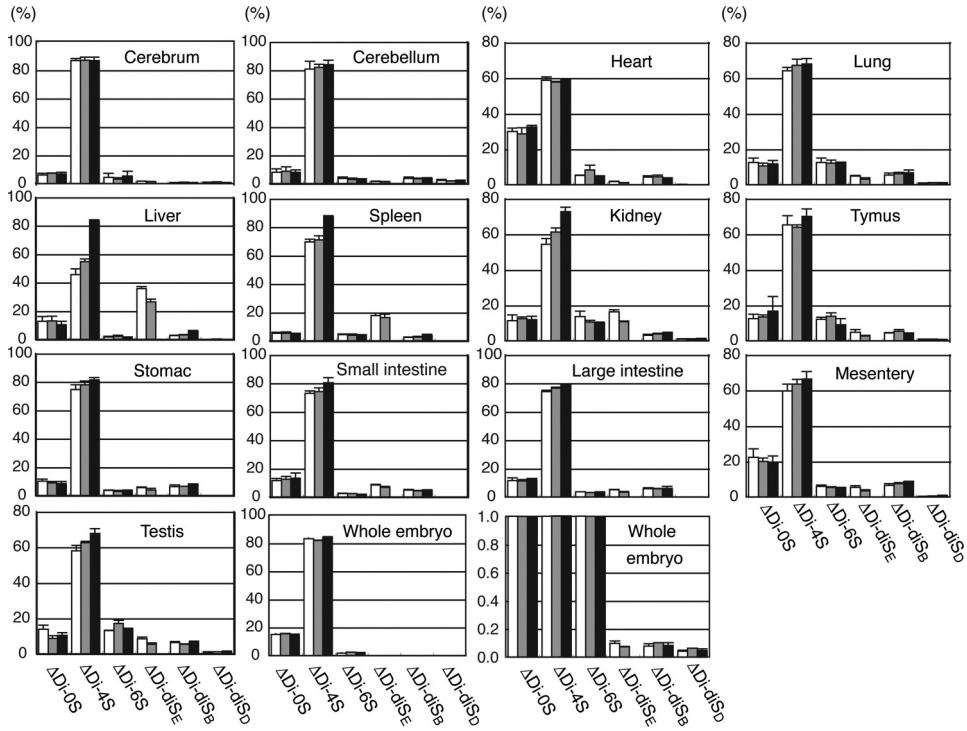

FIGURE 4. **Disappearance of HexA-GalNAc(4,6-SO$_4$) unit in CS/DS of various tissues taken from mutant mice.** CS/DS was isolated from various tissues of 7-week-old mice or whole newborn embryos as described under "Experimental Procedures." CS/DS was digested with a mixture of chondroitinase ABC and chondroitinase ACII and subjected to reversed-phase ion pair chromatography with postcolumn fluorescence labeling as described under "Experimental Procedures." The *histograms* show the percentage compositions of unsaturated disaccharide in the CS/DS isolated from various organs of wild-type (*open bar*), heterozygous (*gray bar*), and homozygous mutant mice (*filled bar*). The values were obtained from three independent experiments, and each *bar* represents the mean ± S.D. from three independent experiments. The *histograms* of whole embryos were enlarged in the *separate panel*. In all tissues as well as whole embryos, ΔDi-diS$_E$ disappeared completely in homozygous mutant mice.

SO$_4$). As shown in Fig. 4, when CS/DS prepared from various tissues of the homozygous mutant mice was analyzed, no ΔDi-diS$_E$ was detected in all tissues examined here. We also determined the disaccharide composition of CS/DS obtained from the whole newborn embryos. ΔDi-diS$_E$ was detected in the wild-type whole embryos albeit at a lower proportion (<0.1% of the total disaccharides) but could not be detected at all in the homozygous mutant embryos (Fig. 4 and supplemental Fig. S1). These observations support the idea that GalNAc4S-6ST should be a sole enzyme for the synthesis of GalNAc(4,6-SO$_4$) residues in CS and DS. In the heterozygous mutant mice, the proportion of ΔDi-diS$_E$ was lower than for the wild-type mice. These results clearly indicate that GalNAc4S-6ST is indispensable for the synthesis of not only the GlcA-GalNAc(4,6-SO$_4$) disaccharide unit but also the IdoA-GalNAc(4,6-SO$_4$) disaccharide unit. In most tissues, the decrease in ΔDi-diS$_E$ was compensated by the increase in ΔDi-4S, ΔHexA-GalNAc(4SO$_4$), but not by other oversulfated disaccharide units.

Analysis of Glycosaminoglycans Synthesized in the Liver—As described above, it became obvious that CS/DS containing a high proportion of IdoA-GalNAc(4,6-SO$_4$) is present in the liver. To examine whether IdoA content is relevant to the formation of GalNAc(4,6-SO$_4$) residues, we prepared ^{35}S-labeled CS/DS from the liver as described under "Experimental Procedures" and digested it with chondroitinase ABC or chondroitinase ACII. When the ^{35}S-labeled CS/DS obtained from the wild-type mice was digested with chondroitinase ABC and applied to SAX-HPLC, the radioactivity was recovered in ΔDi-4S and ΔDi-diS$_E$ (Fig. 5*A*). Considering that ΔDi-diS$_E$ has two radioactive sulfate groups, the molar ratio of ΔDi-diS$_E$ to the total disaccharides was calculated as 0.39, which was nearly the same as the ratio of CS/DS accumulated in the liver (Fig. 4). In contrast, when the ^{35}S-labeled CS/DS obtained from the homozygous mutant mice was digested with chondroitinase ABC, the peak of ΔDi-diS$_E$ disappeared, and most radioactivity was recovered in ΔDi-4S with a small peak eluted just before ΔDi-diS$_E$ (Fig. 5*A*). This small peak was assumed to be ΔHexA(2SO$_4$)-GalNAc(4SO$_4$) from the retention time. The ^{35}S-labeled CS/DSs obtained from wild-type mice and the homozygous mutant mice were both hardly degraded by chondroitinase ACII (Fig. 5*B*), and nearly completely degraded by chondroitinase ABC to disaccharides (Fig. 5*C*). These results indicate that uronic acid residues contained in CS/DSs obtained from both the wild-type mice and homozygous mutant mice are mostly IdoA, and therefore the IdoA content of the liver CS/DS appears to be independent of the formation of GalNAc(4,6-SO$_4$) residues.

We determined the core proteins of the proteoglycans bearing GalNAc(4,6-SO$_4$)-rich CS/DS (see supplemental "Experimental Procedures," Fig. S2, and Table SI) and found that the core proteins were mainly composed of decorin and biglycan.

Comparison of BMMCs Derived from Wild-type Mice and Galnac4s-6st$^{-/-}$ Mice—BMMCs were reported to contain CS-E as the granular components. To clarify the role of GalNAc4S-6ST in the synthesis and function of CS-E in the BMMCs, we compared properties of BMMCs obtained from wild-type, heterozygous mutant, and homozygous mutant mice.

Morphological Appearance—We compared morphological appearance of BMMCs. The cytospin slides were stained by May Grünwald-Giemsa. As shown in Fig. 6*A*, the wild-type BMMCs were mainly composed of cells showing metachromatic granular staining. In contrast, BMMCs derived from the *Galnac4s-6st$^{-/-}$* mice stained weakly. Instead, many cells containing empty vesicles were observed (Fig. 6*B*). Transmission electron micrographs of BMMCs indicated that various types of granules (granules filled with small vesicles or electron dense materials and granules with empty appearance) are present in

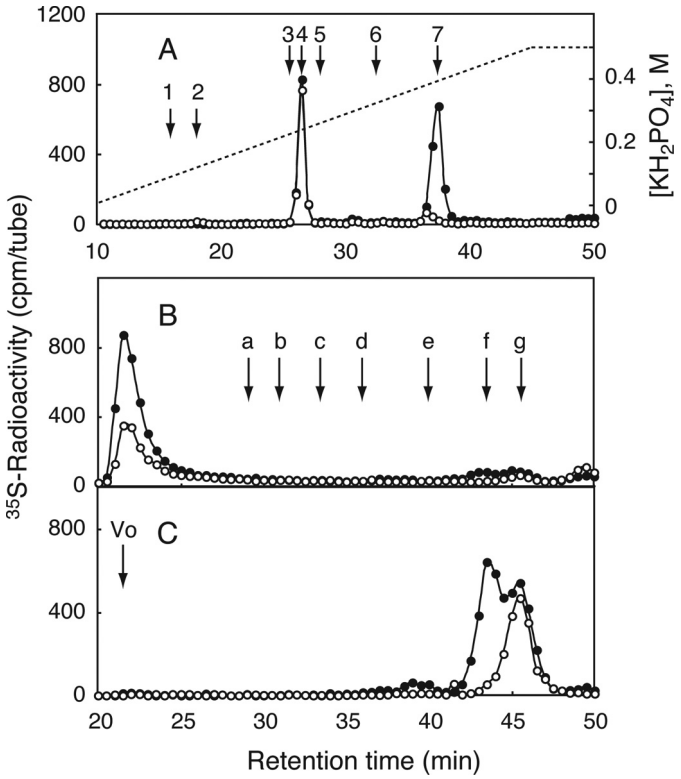

FIGURE 5. **Digestion of the ^{35}S-labeled liver CS/DS with chondroitinase ACII and chondroitinase ABC.** The ^{35}S-labeled liver CS/DS was prepared as described under "Experimental Procedures" from wild-type (*filled circle*) and homozygous mutant (*open circle*) mice and digested with chondroitinase ABC (*A* and *C*) or chondroitinase ACII (*B*). The digests were applied to SAX-HPLC (*A*) or the Superdex 30 column (*B* and *C*), and radioactivity of each fraction was determined. The standards used for SAX-HPLC were the same as those described under the legend for Fig. 3*B*. The *dotted line* in *A* represents concentration of KH_2PO_4. *Arrows* in *B* and *C* indicate the elution position of blue dextran (*Vo*): CS dodecasaccharide (*a*); CS decasaccharide (*b*); CS octasaccharide (*c*); CS hexasaccharide (*d*); CS tetrasaccharide (*e*), ΔDi-diS_E (*f*); and ΔDi-4S (*g*).

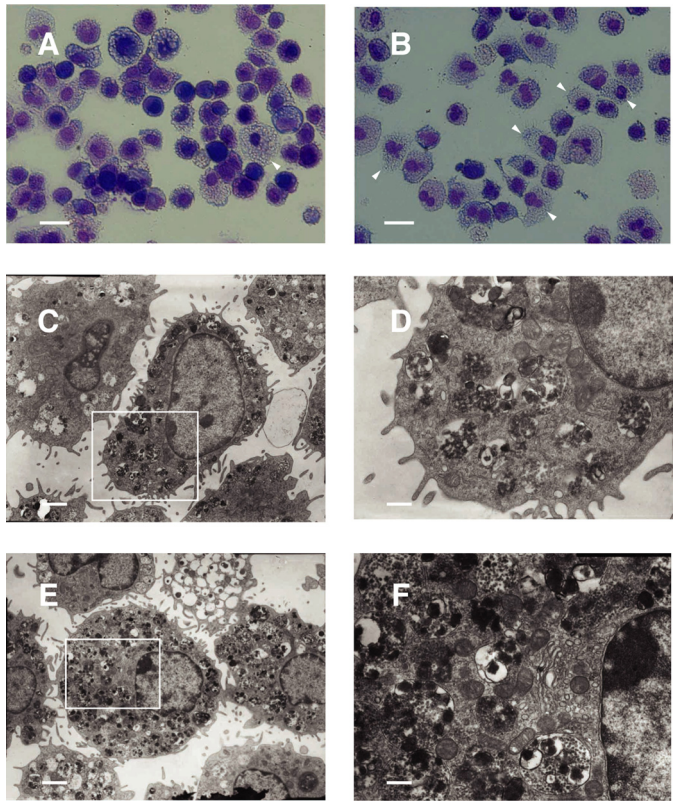

FIGURE 6. **Morphology of bone marrow-derived mast cells.** BMMCs were derived from wild-type mice (*A*, *C*, and *D*) and homozygous mutant mice (*B*, *E*, and *F*) as described under "Experimental Procedures" for 6 weeks. *A* and *B*, cytospin slides were prepared from BMMCs and stained with May Grünwald-Giemsa. Examples of cells containing empty vesicles are shown by *arrowheads*. *C–F*, transmission electron micrographs of BMMCs were taken as described under "Experimental Procedures." *D* and *F*, *boxed areas* of *C* and *E* are enlarged. *Bars*: *A* and *B*, 20 μm; *C* and *E*, 2 μm; and *D* and *F*, 500 nm.

the wild-type BMMCs (Fig. 6, *C* and *D*) and the *Galnac4s-6st*$^{-/-}$ BMMCs (Fig. 6, *E* and *F*). Unlike *Serglycin*$^{-/-}$ BMMCs, in which granules were filled with amorphous structure (46, 47), structural differences in these granules were not observed between the wild-type BMMCs and the *Galnac4s-6st*$^{-/-}$ BMMCs. These observation suggest that GalNAc4S-6ST and its product CS-E may not be indispensable for the formation of these granules, although the proportion of the cells with the empty granules was increased in the *Galnac4s-6st*$^{-/-}$ BMMCs.

Expression of mRNA of Glycosaminoglycan Sulfotransferases—Expression of sulfotransferases involved in the sulfation of CS/DS and HS/heparin were determined by using RT-PCR (supplemental Fig. S3). GalNAc4S-6ST mRNA was not expressed in the BMMCs derived from the homozygous mutant mice. Expression of other sulfotransferases was not altered significantly.

Structural Analysis of Glycosaminoglycans—Glycosaminoglycans were extracted from BMMCs derived from *Galnac4s-6st*$^{+/+}$ or *Galnac4s-6st*$^{-/-}$ mice, and disaccharide composition was analyzed (Fig. 7). The HexA-GalNAc(4,6-SO_4) unit disappeared completely and the HexA-GalNAc(4SO_4) unit was increased in CS/DSs from the homozygous mutant BMMCs (Fig. 7*A*). Disaccharide composition of HS/heparin obtained from the *Galnac4s-6st*$^{-/-}$ BMMCs was nearly the same as that of HS/heparin obtained from the wild-type BMMCs (Fig. 7*B*). BMMCs are thought to be immature type mast cells, because BMMCs infused into mast cell-deficient mice developed to both MMCs and connective tissue-type mast cells (CTMCs) (48). To determine if the deficiency in CS-E might affect the structure of HS/heparin in the mature CTMCs, we analyzed disaccharide composition of CS/DS and HS/heparin of the CTMC-like cells derived from BMMCs in the presence of SCF and a feeder layer of fibroblast. During the differentiation from BMMCs to the CTMC-like cells, the proportion of HS/heparin to the total glycosaminoglycans was increased as judged from the yield of disaccharides: 0.18 ± 0.09 to 0.75 ± 0.16 for the wild-type cells and 0.25 ± 0.04 to 0.76 ± 0.03 for the *Galnac4s-6st*$^{-/-}$ cells. Disaccharide compositions of CS/DS of the CTMC-like cells obtained from the wild-type and *Galnac4s-6st*$^{-/-}$ BMMCs were nearly the same as those of respective BMMCs (Fig. 7*C*). In contrast, disaccharide compositions of HS/heparin of the CTMC-like cells were much different from those of BMMCs; the proportion of ΔDiHS-(*N*,6,2)triS was increased in the CTMC-like cells (Fig. 7*D*). However, no significant differences in the disaccharide composition of HS/heparin obtained from the CTMC-like cells were observed between the wild-type cells and the *Galnac4s-6st*$^{-/-}$ cells. These observations suggest that deficiency in CS-E may not affect the dif-

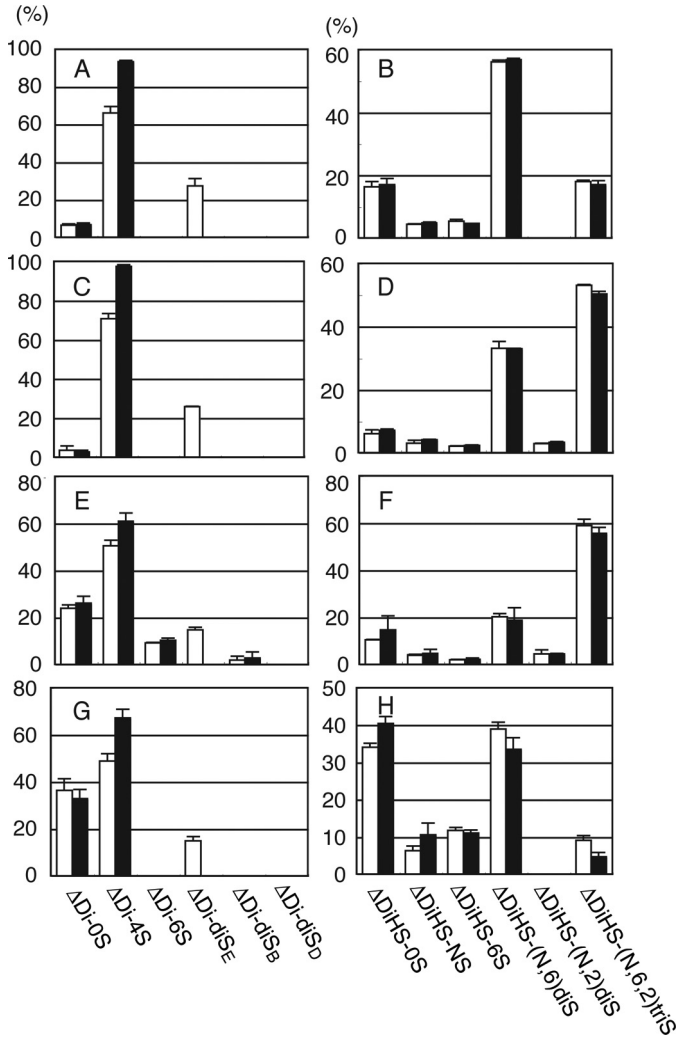

FIGURE 7. **Disaccharide compositions of CS/DS and HS/heparin obtained from BMMCs, CTMC-like cells, peritoneal cells, and MMC-like cells.** BMMCs were derived from wild-type and homozygous mutant mice as described under "Experimental Procedures" for 6 weeks. The CTMC-like cells were obtained from BMMCs as described in the presence of SCF/fibroblasts under "Experimental Procedures." The MMC-like cells were obtained from bone marrow cells in the presence of SCF, transforming growth factor-β, IL-3, and IL-9 as described under "Experimental Procedures." CS/DS and HS/heparin were isolated from the BMMCs (*A* and *B*), CTMC-like cells (*C* and *D*), peritoneal cells of 7-week-old mice (*E* and *F*), or MMC-like cells (*G* and *H*). Disaccharide compositions of CS/DS (*A*, *C*, *E*, and *G*) and HS/heparin (*B*, *D*, *F*, and *H*) were determined as described under "Experimental Procedures." The *histograms* show the percentage compositions of unsaturated disaccharide in the CS/DS or HS/heparin of wild-type (*open bar*) and homozygous mutant mice (*filled bar*). The values were obtained from two independent experiments in *A*, *B*, *C*, *D*, *G*, and *H* and three independent experiments in *E* and *F*. Each *bar* represents the mean ± range for *A*, *B*, *C*, *D*, *G*, and *H* and mean ± S.D. for *E* and *F*.

ferentiation into CTMCs. This idea seems to be supported by the observation that no significant differences in the disaccharide compositions of HS/heparin obtained from the peritoneal cells, in which mature CTMCs are abundant, were detected between the wild-type mice and the homozygous mutant mice (Fig. 7*F*). We also analyzed a disaccharide composition of CS/DS and HS/heparin of MMC-like cells derived from bone marrow cells in the presence of transforming growth factor-β, IL-3, IL-9, and SCF as described under "Experimental Procedures." The MMC-like cells derived from both the wild-type and the *Galnac4s-6st*$^{-/-}$ bone marrow cells contained mainly CS/DS; the proportion of HS/heparin to the total glycosaminoglycans was 0.017 ± 0.002 for the wild-type cells and 0.008 ± 0.001 for the *Galnac4s-6st*$^{-/-}$ cells. No significant difference in disaccharide compositions of HS/heparin was observed between the MMC-like cells derived from the wild-type bone marrow cells and the MMC-like cells derived from the *Galnac4s-6st*$^{-/-}$ bone marrow cells, and typical heparin structures seemed to be absent in HS/heparin of these cells, because the proportion of ΔDiHS-(N,6,2)triS was extremely low and the proportion of ΔDiHS-0S was high (Fig. 7*H*). These observations suggest that deficiency in CS-E may also not affect the differentiation into CS-rich MMC-like cells.

Synthesis of Glycosaminoglycan—Glycosaminoglycans synthesized by BMMCs were metabolically labeled with radioactive sulfate as described under "Experimental Procedures." Total incorporation into glycosaminoglycans was much lower in BMMCs derived from the heterozygous and homozygous mutant mice than in BMMCs derived from the wild-type mice (Fig. 8*A*). When the labeled glycosaminoglycans were applied to a Sephadex G-25 column after digestion with chondroitinase ACII, most radioactivity was recovered in the retarded fractions in all types of BMMCs (supplemental Fig. S4*A*), indicating that the major glycosaminoglycan synthesized by BMMCs was CS. In Mono Q chromatography, CS obtained from the wild-type BMMCs showed two peaks corresponding to the elution position of CS-A and heparin, respectively. In contrast, CS obtained from BMMCs derived from the homozygous mutant mice showed a single peak at the elution position of CS-A (Fig. 8*B*). When the labeled CS obtained from the wild-type BMMCs was digested with chondroitinase ACII and the degradation products were separated with SAX-HPLC, ΔDi-4S derived from GlcA-GalNAc(4SO$_4$) unit and ΔDi-diS$_E$ derived from GlcA-GalNAc(4,6-SO$_4$) unit were detected as major peaks with a small peak at the elution position of GalNAc(4,6-SO$_4$). On the other hand, only ΔDi-4S was detected when CS obtained from the homozygous mutant BMMCs was digested. In the heterozygous mutant BMMCs, the proportion of ΔDi-diS$_E$ was lower than the wild-type BMMCs (supplemental Fig. S4*B*). We compared molecular size of the labeled CS synthesized in the wild-type BMMCs and the homozygous mutant BMMCs by Superdex 200 chromatography (Fig. 8*C*). The homozygous mutant BMMCs synthesized larger CS than the wild-type BMMCs; from the elution time of standard dextran, molecular mass corresponding to the peak position was estimated to be 32 kDa for the wild-type BMMCs and 44 kDa for the homozygous mutant BMMCs. Introduction of the GalNAc(4,6-SO$_4$) residue may attenuate the elongation rate of CS chains.

Expression of Mast Cell Protease—Mast cells contain various mast cell proteases. These proteases are classified into chymase, tryptose, and carboxypeptidase A on the basis of the specificity. Storage of these mast cell proteases were reported to be dependent on heparin, because most proteases were lost in the peritoneal cells of heparin-deficient NDST-2 null mice (49). On the other hand, tryptases mMCP-7 and mMCP-6 were still present in skeletal muscle and BMMCs, respectively, obtained from NDST-2 null mice (50). To determine whether the expres-

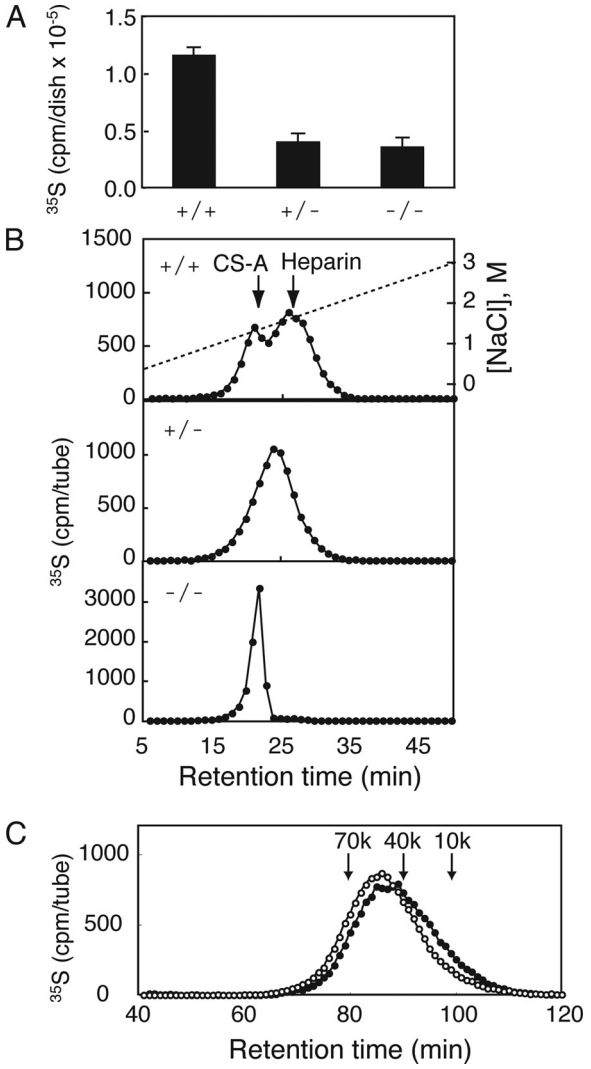

FIGURE 8. **Biosynthesis of CS in BMMCs.** BMMCs were derived from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice for 6 weeks and then labeled with $^{35}SO_4^{2-}$ for 24 h as described under "Experimental Procedures." *A*, total incorporation of ^{35}S-radioactivity into glycosaminoglycans synthesized in BMMCs. Each *bar* represents the mean ± S.D. from three culture dishes prepared simultaneously. *B*, the ^{35}S-labeled glycosaminoglycans synthesized by BMMCs were separated with a Mono Q column. Elution positions of CS-A and heparin are indicated by the *arrows*. The *dotted line* represents concentration of sodium chloride. *C*, the ^{35}S-labeled glycosaminoglycans synthesized by BMMCs derived from wild-type (*filled circle*), and homozygous (*open circle*) mutant mice were separated with a Superdex 200 column. The *arrows* indicate elution position of the standard dextran.

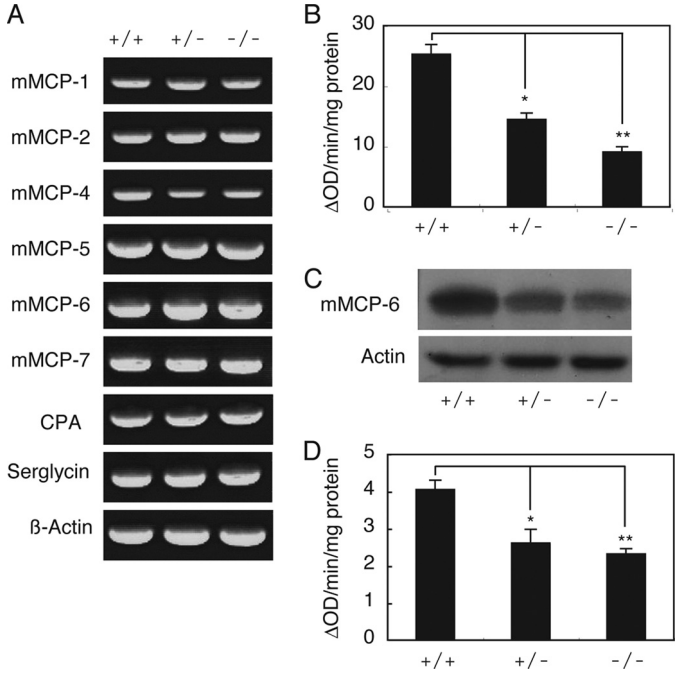

FIGURE 9. **Analysis of mRNA of mast cell proteases and activity of tryptase and carboxypeptidase A in BMMCs.** BMMCs were derived from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice for 6 weeks. *A*, total RNA was prepared from BMMCs and used for analysis of the expression of mouse mast cell protease-1 (mMCP-1), mMCP-2, mMCP-4, mMCP-5, mMCP-6, mMCP-7, carboxypeptidase A, serglycin, and β-actin. *B*, tryptase activity of the extracts from BMMCs was determined as described under "Experimental Procedures." Each *bar* represents the mean ± S.D. from three culture dishes prepared simultaneously. Similar results were obtained from the three independent experiments. *, $p < 0.001$; **, $p < 0.0001$. *C*, protein extracts from the BMMCs were subjected to 10% SDS-PAGE. Western blots were probed using an antibody against mMCP-6 or actin followed by ECL as described under "Experimental Procedures." *D*, carboxypeptidase activity of the extracts from BMMCs was determined as described under "Experimental Procedures." Each *bar* represents the mean ± S.D. from three culture dishes prepared simultaneously. Similar results were obtained from another independent experiment. *, $p < 0.01$; **, $p < 0.001$.

sion and activities of these mast cell proteases in BMMCs are affected by deficiency in CS-E, we compared mRNA expression and activities of proteases in BMMCs. As shown in Fig. 9*A*, expression of mast cell protease mRNAs as well as serglycin mRNA was not altered in the *Galnac4s-6st*$^{-/-}$ BMMCs. On the other hand, both tryptase activity (Fig. 9*B*) and carboxypeptidase A activity (Fig. 9*D*) of the *Galnac4s-6st*$^{-/-}$ BMMCs were much lower than the activity of the wild-type BMMCs. Chymase activity could not be detected in both the wild-type and the *Galnac4s-6st*$^{-/-}$ BMMCs under the assay conditions used. Decreased tryptase activity in the *Galnac4s-6st*$^{-/-}$ BMMCs was supported by decreased protein level of one of tryptase, mMCP-6 (Fig. 9*C*).

DISCUSSION

We generated GalNAc4S-6ST null mice by homologous recombination. Although GalNAc4S-6ST null mice showed no abnormality in gross appearance, a possibility has been raised that the average litter size may be reduced in crossing between the homozygous mutant mice. Deficiency in a serine protease inhibitor, protease nexin-1 (PN-1) resulted in male infertility (51). PN-1 was reported to form complexes with thrombomodulin (TM) and to modulate its anticoagulant activity. TM is a CS proteoglycan, and CS moiety of TM appeared to be involved in PN-1 binding to TM (52). Structural analysis of CS chain attached to rabbit TM showed that CS contained GalNAc(4,6-SO_4) residues at the nonreducing terminal region (21). Because GalNAc(4,6-SO_4) is present in CS/DS obtained from the testis (Fig. 4), it remains to be studied whether CS/DS containing GalNAc(4,6-SO_4) may modulate the activity of PN-1 in the testis, thereby affecting the male fertility.

BMMCs have been induced from bone marrow cells when bone marrow cells were cultured in the presence of IL-3 (54). BMMCs contain serglycin bearing CS-E instead of heparin (1, 55). We confirmed that BMMCs derived from *Galnac4s-6st*$^{-/-}$

mice contained CS-A without the GlcA-GalNAc(4,6-SO_4) unit. BMMCs derived from *Galnac4s-6st*$^{-/-}$ mice stained weakly by May Grünwald-Giemsa and showed many empty granules, whereas wild-type BMMCs showed metachromatic granular staining (Fig. 4). These observations are similar to those of seglycin$^{-/-}$ BMMCs (44). In transmission electron micrographs, the cytoplasm of BMMCs derived from both the wild-type and *Galnac4s-6st*$^{-/-}$ mice shows three types of morphologically distinct granules; granules displaying internal vesicles (exosomes) (type I), granules with an electron-dense core surrounded by membrane vesicles (type II), and electron-dense granules (type III) (56). In addition, empty granules are also observed. No obvious differences were observed in the intragranular structure of the individual type of granule between the wild-type mice and the *Galnac4s-6st*$^{-/-}$ mice. These observations are quite different from those of BMMCs derived from serglycin$^{-/-}$ mice, in which the granules were filled with amorphous materials (46, 47). CS-E may not be involved in the formation of intragranular structures such as exosomes.

BMMCs are thought to be immature-type mast cells (55, 48). To examine whether absence of CS-E could affect the structure of HS/heparin in the CTMCs and MMCs, we analyzed disaccharide composition of HS/heparin contained in the CTMC-like cells and MMC-like cells, which were developed *in vitro* from bone marrow cells. Disaccharide compositions of HS/heparin contained in the CTMC-like cells and MMC-like cells did not differ significantly between the wild-type mice and GalNAc4S-6ST-null mice, suggesting that differentiation into CTMCs or MMCs might be independent of CS-E.

It is poorly understood how elongation of the CS chain is regulated. βGalNAc transferase activity was shown to different activity toward oligosaccharides with different sulfation patter. When a hexasaccharide or a tetrasaccharide with the GalNAc(4,6-SO_4) residue at the penultimate position from the nonreducing end was used as the acceptor, βGalNAc transferase activity was not detected, suggesting that introduction of the GalNAc(4,6-SO_4) residue might suppress the elongation of the CS chain (57). We compared the disaccharide composition as well as chain length of CS chain synthesized by the wild-type BMMCs and the *Galnac4s-6st*$^{-/-}$ BMMCs. The ^{35}S-labeled CS synthesized by the *Galnac4s-6st*$^{-/-}$ BMMCs was found to be devoid of not only GlcA-GalNAc(4,6-SO_4) unit but also the nonreducing terminal GalNAc(4,6-SO_4) residue. The molecular size of CS synthesized by the *Galnac4s-6st*$^{-/-}$ BMMCs was larger than that of CS synthesized by the wild-type BMMCs. These observation suggest that formation of the GlcA-GalNAc(4,6-SO_4) unit or the nonreducing terminal GalNAc(4,6-SO_4) residue may regulate the elongation of CS chain. CS chain synthesized by the wild-type BMMCs exhibited two peaks with different negative charges in Mono Q chromatography, whereas CS chain synthesized by the *Galnac4s-6st*$^{-/-}$ BMMCs showed only one peak with lower negative charge. These observation suggest that 6-*O*-sulfation of GalNAc($4SO_4$) residues by GalNAc4S-6ST may preferentially occur at the regions where GalNAc(4,6-SO_4) residues are already present. We have also obtained two peaks with different negative charges in Mono Q chromatography when CS-A was sulfated by squid GalNAc4S-6ST *in vitro* (58).

We observed decreased activities of tryptase and carboxypeptidase and decreased mMCP-6 protein in the *Galnac4s-6st*$^{-/-}$ BMMCs, although mRNA levels of these proteins were not altered. These observations appear to support the idea that CS-E contributes to the storage of mMCP-6 in the granules of BMMCs. However, because BALB/c mice express mMCP-7 in addition to mMCP-6 as tryptase, the possibility could not be excluded that the decrease in mMCP-7 might contribute to the decrease in the tryptase activity. Formation of a tetramer of mMCP-6 through binding to heparin is required for the enzyme activity (59). Deficiency in CS-E in the *Galnac4s-6st*$^{-/-}$ BMMCs may prevent the tetramer formation, thereby hampering the formation of active tryptase.

Disaccharide analysis of CS/DS obtained from wild-type mice showed that GalNAc(4,6-SO_4) residues are present not only in CS chain but also in DS chain. Especially, CS/DS extracted from the liver was hardly degraded by chondroitinase ACII, indicating that GalNAc(4,6-SO_4) residues in the liver are mostly included in DS chain. In *Galnac4s-6st*$^{-/-}$ mice, GalNAc(4,6-SO_4) residues in DS chain also disappeared completely, indicating that GalNAc4S-6ST is required for the formation of GalNAc(4,6-SO_4) residues in both CS and DS chain. DS containing GalNAc(4,6-SO_4) residues isolated from porcine intestinal mucosa was reported to interact with heparin cofactor II (25). It remains to be studied whether the liver GalNAc(4,6-SO_4)-rich DS shows such function. The proportion of IdoA-GalNAc(4,6-SO_4) units in CS/DSs in the fibrous lesions of rat liver with cirrhosis was reported to be much lower than in the nonfibrous lesion (60). We found that GalNAc(4,6-SO_4)-rich DS proteoglycans were composed of mainly decorin and biglycan. Decorin was shown to have a fundamental role in regulating collagen fiber formation *in vivo* (61), and expression of decorin was regulated during liver fibrosis (62–64). It remains to be studied whether the liver CS/DSs with high IdoA-GalNAc(4,6-SO_4) units could regulate the function of decorin in liver fibrosis.

Recently it was reported that reduced IdoA blocks in CS/DS obtained from DS epimerase I knock-out mice were accompanied with a decrease in HexA($2SO_4$)-GalNAc($4SO_4$) unit but not HexA-GalNAc(4,6-SO_4) (65). These results seem to be compatible with our present results that deletion of the GalNAc(4,6-SO_4) residues in the liver CS/DS did not affect the IdoA content of the liver CS/DS.

Amino acid sequence of GalNAc4S-6ST shares with that of the human B-cell RAG-associated gene product (27, 66). The product of human B-cell RAG-associated gene was shown to be potentially involved in B-cell-specific regulation of the expression of RAG1; however, in our preliminary experiments, we could not detect any differences in serum antibody levels against ovalbumin or bacterial glutathione *S*-transferase between wild-type mice and *Galnac4s-6st*$^{-/-}$ mice.

Matrilysin (MMP7) activates pro-α-defensins (procryptdins), a family of structurally similar 3- to 4-kDa antimicrobial peptides found in the granules of Paneth cells at the base of the crypts of Lieberkühn. Highly sulfated glycosaminoglycans, such as heparin and CS-E, were shown to enhance not only the intermolecular autolytic activation of promatrilysin but also the activity of fully active matrilysin to cleave specific physiological

substrates, procryptdins (20). Alcian Blue, which is used for detection of sulfated glycosaminoglycans, was reported to stain an outer rim around each granule of Paneth cells (53, 67). It remains to be determined whether or not CS-E is present in the Paneth cells and involved in the regulation of procryptdins.

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