Chondroitin sulfate N-Acetylgalactosaminyltransferase 1 Is Necessary for Normal Endochondral Ossification and Aggrecan Metabolism*

Received for publication, June 30, 2010, and in revised form, December 3, 2010 Published, JBC Papers in Press, December 10, 2010, DOI 10.1074/jbc.M110.159244

Takashi Sato1, Takashi Kudo9, Yuzuru Ikehashi9, Hiroyasu Ogawa9, Tomoko Hirano1, Katsue Kiyohara1, Kozue Hagiwara1, Akira Togayachi1, Masatsugu Ema1, Satoru Takahashi1, Koji Kimata1, Hiideto Watanabe2, and Hisashi Narimatsu3

From the 1Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Open Space Laboratory Central-2, 1-1-1 Umemizo, Tsukuba, Ibaraki 305-8568, the 8Department of Anatomy and Embryology, Doctoral Program in Life System Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, the 8Institute for Molecular Science of Medicine, Aichi Medical University, Karimata 21, Yazakoz, Nagakute, Aichi-gun, Aichi 480-1195, and the 8Department of Orthopaedic Surgery, Gifu University, Graduate School of Medicine, Yanagido 1-1, Gifu 501-1194, Japan

Chondroitin sulfate (CS) is a glycosaminoglycan, consisting of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid residues, and plays important roles in development and homeostasis of organs and tissues. Here, we generated and analyzed mice lacking chondroitin sulfate N-acetylgalactosaminyltransferase 1 (CSGalNACT-1). Csgalnact1−/− mice were viable and fertile but exhibited slight dwarfism. Biochemically, the level of CS in Csgalnact1−/− cartilage was reduced to ~50% that of wild-type cartilage, whereas its chain length was similar to wild-type mice, indicating that CSGalNACT-1 participates in the CS chain initiation as suggested in the previous study (Sakai, K., Kimata, K., Sato, T., Gotoh, M., Narimatsu, H., Shinomiya, K., and Watanabe, H. (2007) J. Biol. Chem. 282, 4152–4161). Histologically, the growth plate of Csgalnact1−/− mice contained shorter and slightly disorganized chondrocyte columns with a reduced volume of the extracellular matrix principally in the proliferative layer. Immunohistochemical analysis revealed that the level of both aggrecan and link protein 1 were decreased in Csgalnact1−/− cartilage. Western blot analysis demonstrated an increase in processed forms of aggrecan core protein. These results suggest that CSGalNACT-1 is required for normal levels of CS biosynthesis in cartilage. Our observations suggest that CSGalNACT-1 is necessary for normal levels of endochondral ossification, and the decrease in CS amount in the growth plate by its absence causes a rapid catabolism of aggrecan.

Chondroitin sulfate (CS)2 is a glycosaminoglycan (GAG), consisting of repeating disaccharide units of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcUA) residues with sulfate residues at various positions. CS is usually attached to a core protein, thereby forming a proteoglycan (PG). Depending on the core protein, CS is localized either in the extracellular matrix (ECM) or on the cell surface and is particularly abundant in cartilage and brain. In cartilage, a large CS PG aggrecan, which is thought to contain approximately 100 CS chains, forms PG aggregates together with hyaluronan and link protein 1 (LP-1). The aggregates contribute to water retention and resistance to compression (1–3). In brain, CS PGs such as aggrecan, versican, and neurocan are present in perineuronal nets, which wrap around the neuronal cell bodies and control neuronal plasticity and interneuronal activity (4). Recently, oversulfated CS has been shown to play critical roles in the neuronal migration in cerebral cortex (5). In addition, biochemical analyses have revealed that CS binds to cytokines and growth factors including midline and pleiotrophin (6), implying that CS plays an important role in development and homeostasis of organs and tissues.

CS biosynthesis is initiated by the transfer of a GalNAc residue to the linkage region of a GlcUA-β1,3-galactose (Gal)-β1,3-Gal-β1,4-Xyl tetrasaccharide primer that is attached to a serine residue of the core protein (Fig. 1A). Chain elongation then occurs by the alternate addition of GalNAc and GlcUA residues. In mammals, six glycosyltransferases have been identified, which are classified into three pairs based on amino acids sequence similarity. The first pair of glycosyltransferases comprises chondroitin sulfate synthase 1 (CSS1)/chondroitin synthase 1 (ChSy1) and chondroitin sulfate synthase 3 (CSS3), which both possess β3GT and β4GT motifs and exhibits dual glycosyltransferase activities of β1,3-glucuronyltransferase (GlUCaT) and β1,4-N-acetylgalactosaminyltransferase (GalNAcT) for chondroitin chain elongation (7, 8). The second pair comprises CSS2/chondroitin-polymerizing factor (ChPF) and chondroitin sulfate glucuronyltransferase (CSGlUCaT). CSS2 exhibits dual glycosyltransferase activities of glucuronyltransferase; CSS, chondroitin sulfate synthase; ECM, extracellular matrix; GAG, glycosaminoglycan; GlUCaT, glucuronic acid; GlUCaT, glucuronyltransferase; β3GT or β4GT GT, β1,3- or β1,4-glycosyltransferase; LP, link protein; PG, proteoglycan.
syngalactosyltransferase activity, similar to CSS1 and CSS3 (9). It was also reported that CSS2 behaves as a ChPF that cooperatively contributes to chondroitin chain polymerization by interacting with CSS1/ChSy1 (10). CSGalNAcT has a catalytic activity of each glycosyltransferase. Half-filled diamonds, open squares, open circles, and star refer to GlcUA, GalNAc, Gal, and Xyl, respectively. B, targeting strategy for conditional deletion of Csgalnact1 gene. Exon containing initiation codon and transmembrane domain was put between loxP sites and deleted away by mating with Ayu1-Cre, ubiquitously expressing transgenic line to remove the center arm containing an exon encoding the translational start codon and transmembrane domain of Csgalnact1. The resultant heterozygous (+/−) mice were then interbred to yield wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Csgalnact1 mutant mice. Genotypes were confirmed by PCR using the following primers containing restriction enzyme recognition sites (shown in lowercase): left arm, 5’−gggatccATATTGACGTGGATGTGGT−3’ and 5’−gggatccATATTGACGTGGATGTGGT−3’T; center arm, 5’−ggtgcacACTAATATATATATGAATG−3’; right arm, 5’−ggtgcacCTCCTATATCTACAAATAGA−3’ and 5’−ggtgcacCTCCTATATCTACAAATAGA−3’T. The amplified products were inserted into the original vector using PmeI and KpnI sites for the left arm, Sall sites for the center arm, and Ascl sites for the right arm after sequence confirmation. The targeting vector was linearized by digestion with NotI and transfected into C57BL/6J mouse embryonic stem (ES) cells (16). The resulting cells were selected in medium containing 0.3 mg/ml G418 (Naquarai Tesque, Kyoto, Japan). Anticipated homologous recombination was subsequently confirmed by PCR and Southern hybridization. Targeted ES cells were injected into ICR mouse blastocysts to generate chimeric mice. Male mice chimeric for the targeted allele were mated with female Ayu1-Cre mice (17), which was a general deleter Cre recombinase-expressing mouse. Probe position of Southern hybridization is indicated by the bold line. C, genotyping by PCR using genomic DNA. D, Southern blot analysis of progeny from intercross of heterozygotes. The positions of the WT and targeted fragments are indicated by arrowheads. E, quantitative analysis of Csgalnact1 and Csgalnact2 transcripts in brain of WT (n = 3) and Csgalnact1−/− mice (n = 3) by real-time PCR. The expression levels of each transcript were normalized to that of the β-actin transcript. Data were obtained from triplicate experiments and are presented as the mean ± S.E. (error bars). N.D., not detected.

been shown to be a critical enzyme for CS biosynthesis in cartilage (15).

Although biochemical characterization of these glycosyltransferases involved in CS biosynthesis has been performed extensively, their individual functions in vivo are not yet understood. In this study, we generated and characterized Csgalnact1−/− mice as the first gene knock-out mouse model of CS glycosyltransferases. The knock-out mice were viable and fertile, but slightly dwarf-like. Analysis of their cartilage showed it to contain a lower amount of CS and a reduced volume of the ECM, where aggrecan is mainly processed. These observations suggest that CSGalNAcT-1 is necessary for normal levels of endochondral ossification, and the decrease in CS amount in the growth plate by its absence causes a rapid catabolism of aggrecan.

**EXPERIMENTAL PROCEDURES**

**Mice**—Mice were maintained in specific pathogen-free conditions in the Laboratory Animal Resource Center at the University of Tsukuba. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the National Institute of Advanced Industrial Science and Technology and the University of Tsukuba.

**Generation of Csgalnact1−/− Mice**—The targeting vector for Csgalnact1 gene disruption was constructed by ligation of three PCR fragments into a conditional targeting vector cassette. Three arms (left arm, center arm, and right arm) for homologous recombination were amplified by PCR using the following primers containing restriction enzyme recognition sites (shown in lowercase): left arm, 5’−ggtgatattgccgctcaccATATTGACGTGGATGTGGT−3’T; center arm, 5’−ggtgcacCTCCTATATCTACAAATAGA−3’T; right arm, 5’−ggtgcacCTCCTATATCTACAAATAGA−3’T and 5’−ggtgcacCTCCTATATCTACAAATAGA−3’T. The amplified products were inserted into the original vector using PmeI and KpnI sites for the left arm, Sall sites for the center arm, and Ascl sites for the right arm after sequence confirmation. The targeting vector was linearized by digestion with NotI and transfected into C57BL/6J mouse embryonic stem (ES) cells (16). The resulting cells were selected in medium containing 0.3 mg/ml G418 (Naquarai Tesque, Kyoto, Japan). Anticipated homologous recombination was subsequently confirmed by PCR and Southern hybridization. Targeted ES cells were injected into ICR mouse blastocysts to generate chimeric mice. Male mice chimeric for the targeted allele were mated with female Ayu1-Cre mice (17), which was a general deleter Cre recombinase-expressing transgenic line to remove the center arm containing an exon encoding the translational start codon and transmembrane domain of Csgalnact1. The resultant heterozygous bearing the null allele were subsequently interbred to yield wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Csgalnact1 mutant mice. Genotypes were confirmed by PCR using the following three primers: 5’−ATGTAAGATGAACTGTCCATCCTATCATACTGATT-3’ and 5’−AATGGCGACCTGCCTATCTGGA−3’T, and 5’−GACGGTCTCCTGCTCTCGGA−3’T. For Southern hybridization, 40 μg of BamHI-
digested genomic DNA was resolved on a 0.8% agarose gel and probed with an ~1-kbp DNA probe amplified by PCR with the following primers: 5’-GAAGTGAGAATTCTTGGCCTGCT-3’ and 5’-TTGGAAAAGGAAATTGGCTCA-3’. Probe labeling and signal detection were performed according to the manufacturer’s instructions of AlkPhos labeling and detection system (GE Healthcare).

Quantitative Analysis of Csgalnact1, Csgalnact2, Aggrecan (Acan), and Link Protein 1 (Haplin1) Transcripts by Real-time PCR—Total RNAs were isolated from fresh brain of adult mice and humerus, tibial, and rib cartilages of newborn mice using an RNaseasy Mini kit (Qiagen), and cDNA templates were synthesized from the total RNA with a QuantiTect Reverse Transcription kit (Qiagen). The primers and probes were synthesized from the total RNA with a QuantiTect Reverse Transcription kit (Qiagen). The primers and probes were selected from TaqMan Gene Expression Assays (Applied Biosystems). Mm01345503_m1, Mm01189028_m1, and 5’-AGCTCCCACTGACAGCAAG-3’ were used for the quantitative analysis of Csgalnact1, Csgalnact2, aggrecan (Acan), link protein 1 (Haplin1), Adamts1, and Adamts5 genes, respectively. PCR products were measured continuously with an ABI PRISM 7700 Sequence Detection System. The relative amounts of the transcripts were normalized to the amount of β-actin (Actb) (Mm00477355_m1) and β-actin (Hapln1) (Mm00607939_s1) transcript in the same cDNA samples.

Skeletal Histology and Immunohistochemistry—The whole skeletons of embryos (embryonic day (E) 18.5) were fixed in 95% ethanol and stained overnight in a solution containing Alcian blue 8GX (Sigma-Aldrich). Samples were placed in 1% KOH (v/v)/Alizarin red S (Sigma-Aldrich) for 2 h and then destained with 2–0.2% KOH (v/v)/20–80% glycerol for several days. Cleared specimens were stored in 100% glycerol. Anatomically removed mice tissues were fixed in Mildform 10N (Wako Pure Chemical Industries; Osaka, Japan) after two immediate washings in PBS and then embedded in paraffin. The hard tissues from 2-week-old mice were decalcified in Morse’s solution (10% sodium citrate and 22.5% formic acid) for 24 h after fixing in 4% formalin before embedding in paraffin. Four-micrometer-thick sections were prepared from humerus and tibia and subjected to staining with hematoxylin & eosin, Masson’s trichrome staining, Alcian blue staining (Muto Pure Chemicals Co., LTD, Tokyo, Japan), and immunohistochemical staining of link protein-1 mAb, 8A4 (Developed Studies Hybridoma Bank at the University of Iowa), and collagen II Ab (Research Diagnostics, Flanders, NJ). Staining of aggrecan using mAb, 1C6 (Developed Studies Hybridoma Bank) was performed on sections treated with chondroitinase ABC (Seikagaku Corp., Tokyo, Japan). Hyaluronan staining was performed using biotinylated hyaluronic acid-binding protein (Seikagaku Corp.) according to the manufacturer’s instructions. All sections were observed with a Keyence Biozero fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

Estimation of Bone Length—To measure bone length, isolated humerus and tibia from 2-week-old mice were stained with Alizarin red S and Alcian blue 8GX, observed with a digital microscope (VH-8000; Keyence), and then measured with ImageJ software. Statistical analyses were calculated by Student’s t test. Skeletal Histology and Immunohistochemistry—Total RNAs were isolated from fresh brain of adult mice and humerus, tibial, and rib cartilages of newborn mice using an RNaseasy Mini kit (Qiagen), and cDNA templates were synthesized from the total RNA with a QuantiTect Reverse Transcription kit (Qiagen). The primers and probes were selected from TaqMan Gene Expression Assays (Applied Biosystems). Mm01345503_m1, Mm01189028_m1, and 5’-AGCTCCCACTGACAGCAAG-3’ were used for the quantitative analysis of Csgalnact1, Csgalnact2, aggrecan (Acan), link protein 1 (Haplin1), Adamts1, and Adamts5 genes, respectively. PCR products were measured continuously with an ABI PRISM 7700 Sequence Detection System. The relative amounts of the transcripts were normalized to the amount of β-actin (Actb) (Mm00477355_m1) and β-actin (Hapln1) (Mm00607939_s1) transcript in the same cDNA samples.

Skeletal Histology and Immunohistochemistry—The whole skeletons of embryos (embryonic day (E) 18.5) were fixed in 95% ethanol and stained overnight in a solution containing Alcian blue 8GX (Sigma-Aldrich). Samples were placed in 1% KOH (v/v)/Alizarin red S (Sigma-Aldrich) for 2 h and then destained with 2–0.2% KOH (v/v)/20–80% glycerol for several days. Cleared specimens were stored in 100% glycerol. Anatomically removed mice tissues were fixed in Mildform 10N (Wako Pure Chemical Industries; Osaka, Japan) after two immediate washings in PBS and then embedded in paraffin. The hard tissues from 2-week-old mice were decalcified in Morse’s solution (10% sodium citrate and 22.5% formic acid) for 24 h after fixing in 4% formalin before embedding in paraffin. Four-micrometer-thick sections were prepared from humerus and tibia and subjected to staining with hematoxylin & eosin, Masson’s trichrome staining, Alcian blue staining (Muto Pure Chemicals Co., LTD, Tokyo, Japan), and immunohistochemical staining of link protein-1 mAb, 8A4 (Developed Studies Hybridoma Bank at the University of Iowa), and collagen II Ab (Research Diagnostics, Flanders, NJ). Staining of aggrecan using mAb, 1C6 (Developed Studies Hybridoma Bank) was performed on sections treated with chondroitinase ABC (Seikagaku Corp., Tokyo, Japan). Hyaluronan staining was performed using biotinylated hyaluronic acid-binding protein (Seikagaku Corp.) according to the manufacturer’s instructions. All sections were observed with a Keyence Biozero fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

Estimation of Bone Length—To measure bone length, isolated humerus and tibia from 2-week-old mice were stained with Alizarin red S and Alcian blue 8GX, observed with a digital microscope (VH-8000; Keyence), and then measured with ImageJ software. Statistical analyses were calculated by Student’s t test.

Csgalnact1-deficient Mice Display Dwarf Phenotype

**Extraction of GAG**—Humerus, tibial, and rib cartilages were obtained from newborn mice after general anesthesia. GAGs were released from the core protein of 20 cartilage samples with 0.2 M NaOH for 16 h at room temperature. The samples were neutralized by an addition of 4 M acetate, treated with DNase and RNase for 2 h at 37 °C, and digested with 1 mg/ml proteinase K in 50 mM Tris-HCl, pH 8.0, for 2 h at 37 °C. The samples were centrifuged, and the supernatants were applied to a DEAE-Sephacel column (GE Healthcare) that was equilibrated with 50 mM Tris-HCl, pH 7.5. After washing with 10 column volumes of 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, GAG-rich fractions were eluted with 50 mM Tris-HCl, pH 7.5, 2 M NaCl. The eluates were precipitated by the addition of 3 volumes of 95% ethanol containing 1.3% potassium acetate. Finally, the precipitate was dissolved in distilled water.

**Structural Analysis of CS Chain**—To examine the disaccharide compositions of CS, an aliquot of the extracted GAGs was separated from low molecular weight proteins and disaccharides by filtration using Ultrafree-MC (5,000 molecular weight cut-off). Unfiltered GAGs in the filter cups were treated with 30 milliunits of chondroitinase ABC in 25 μl of 50 mM Tris-HCl, pH 7.5, 0.04% BSA for 2 h at 37 °C, and filtered for a second time using Ultrafree-MC (5,000 molecular weight cut-off). Unsaturated disaccharides in the filtrates were analyzed according to Toyoda’s method (18) with a slight modification of elution conditions. To examine the chain length of CS, an aliquot of the isolated GAGs was treated with the heparitinase (Seikagaku Corp.) mixture and labeled with [3H]sodium borohydride (PerkinElmer Life Sciences). Briefly, 10 μl of sample source was reacted with 8.4 pmol of [3H]sodium borohydride (12 nCi/pmol) for 3 h at room temperature, treated with 2 μl of 2 M CH₃COOH, and neutralized with 2 μl of 2 M NaOH. After removal of free [3H]sodium borohydride by precipitation using 3 volumes of 95% ethanol containing 1.3% potassium acetate, the labeled sample was applied to a Superose 6 column (GE Healthcare) equilibrated in 0.2 M NaCl, followed by scintillation counting of each fraction.

**Western Blotting**—The cartilage PG was extracted from tibial cartilage of newborn mice using 4 M guanidine hydrochloride method (19). The samples of fragmented cartilage depleted of surrounding tissues were treated with 4 M guanidine hydrochloride in 20 mM Tris-HCl, pH 8.0, supplemented with a proteinase inhibitor mixture (Roche Applied Science). PG was then extracted for 24 h at 4 °C with gently rotating. The extract was subjected to dialysis against Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and digested with chondroitinase ABC after determination of protein concentration. Deglycosylated proteins (5 μg) by chondroitinase ABC treatment were separated on 10% SDS-PAGE, and then proteins were transferred to a Hybond ECL (GE Healthcare) membrane. After blocking with 5% skimmed milk for 1 h at room temperature, membrane was blotted with the following primary antibodies: anti-aggrecan mAb (1C6), antimouse collagen II Ab (R&D Systems, Minneapolis, MN), and anti-β-actin mAb (Sigma-Aldrich). The specific signal of the primary antibody was developed with horseradish peroxidase.
Csgalnact1-deficient Mice Display Dwarf Phenotype

conjugated to each secondary antibody of anti-mouse IgG (GE Healthcare) and anti-sheep IgG (Jackson ImmunoResearch Laboratories) using Western Lighting Plus-ECL (PerkinElmer Life Sciences) and Hyperfilm ECL (GE Healthcare).

RESULTS

Generation of Conditional Csgalnact1−/− Mice—We generated conditional Csgalnact1 mutant mice using the Cre-loxP systems. A targeting vector was constructed in which exon 1, containing the 5′-untranslated region (UTR) and encoding a translational start site followed by the transmembrane domain, was flanked with loxP sequences (Fig. 1B). We confirmed homologous recombination between the targeting vector and the Csgalnact1 locus in C57BL/6J mouse ES cells by PCR and Southern hybridization analyses (data not shown). Chimeric mice were obtained by microinjecting Csgalnact1fllox/+ ES cells into mouse blastocyst, and the resulting animals were crossed with C57BL/6J mice to achieve germ line transmission of the floxed allele. Heterozygotes (Csgalnact1+/−) harboring the null allele were successfully generated by mating male Csgalnact1fllox/+ with female Ayu1-Cre mice, a general deleter Cre recombinase-expressing transgenic line (17).

Then, homozygotes (Csgalnact1−/−) were generated by subsequent interbreeding of Csgalnact1+/−. The genotype of Csgalnact1−/− mice harboring the null allele was confirmed by a PCR method using tail genomic DNA to amplify a 433-bp product from the wild-type (WT) allele and a 258-bp product from the Csgalnact1-null allele (Fig. 1C). Further confirmation was obtained by Southern hybridization using tail genomic DNA (Fig. 1D). The transcripts of Csgalnact1 and another initiation enzyme for CS synthesis, Csgalnact2, were measured using brain and cartilage cDNAs of WT and Csgalnact1−/− littermates by real-time PCR. The level of Csgalnact2 transcript was the same between WT and Csgalnact1−/−, although the Csgalnact1 transcript was undetectable in Csgalnact1−/− mice (Fig. 1E, and see Fig. 7). These results demonstrate successful disruption of Csgalnact1 gene in the C57BL/6J background.

Csgalnact1−/− Mice Exhibit Dwarfism—The mutant mice developed normally and were fertile. Intercrosses between the heterozygotes gave rise to 49 WT, 118 heterozygous, and 55 homozygous mice, nearly proportional to the expected frequency of 1:2:1. However, the homozygote mice showed a significantly slower growth rate (~10% reduction) compared with that of their WT littermates (Fig. 2). Despite the smaller size, male and female Csgalnact1−/− mice had a normal lifespan and fertility resulting in viable offspring by inbreed mating.

To confirm the dwarfism, we measured the bone length of Csgalnact1−/− and WT mice at 2 weeks of age. When skeletal preparations of humerus double-stained with Alizarin red and Alcian blue were observed (Fig. 3A), both humerus and tibia of Csgalnact1−/− were found to be clearly shorter than that of their WT littermates. When measured, the length of both humerus and tibia were significantly reduced in Csgalnact1−/− mice (Fig. 3B).

We further performed histological analysis of the growth plate at 2 weeks old (Fig. 3C). At this stage, the secondary ossification center is formed, and the growth plate contains columns of proliferative and hypertrophic chondrocytes. The tibial growth plate of 2-week-old Csgalnact1−/− mice exhibited a slight disorganization of chondrocyte columns and a decrease in proliferative chondrocytes. The length of the growth plate was measured by microscopy. The growth plate of Csgalnact1−/− mice was shorter than that of WT (441 ± 10 μm and 307 ± 6 μm for WT and Csgalnact1−/−, respectively).

Growth Plate Structure Is Altered in Csgalnact1−/− Embryos at E18.5—Although the dwarfism of Csgalnact1−/− mice was not apparent by gross appearances a few days after birth, we reasoned that slight changes resulting in dwarfism had occurred at an early stage in development. To determine when these changes are initiated in Csgalnact1−/− mice, we performed an analysis at the embryonic stage. As shown in Fig. 4A, no abnormalities were apparent in the whole skeleton, skull, forelimb, hind limb, lumbar spine, and digits after double staining with Alizarin red and Alcian blue.

The short bones of Csgalnact1−/− mice are likely to be due to impairment of endochondral ossification. Thus, we performed histological analysis of the growth plate, which is essential for this process. The growth plate was found to contain columns of chondrocytes at resting, proliferative, and hypertrophic stages. The ECM was mainly in the resting and proliferative zones. Humerus cartilage of Csgalnact1−/− at E18.5 exhibited a decreased ECM volume and densely packed proliferative chondrocytes. Moreover, the chondrocyte columns were slightly disorganized by comparison with those of WT (Fig. 4B and C). Specifically, the proliferative chondrocyte layer of Csgalnact1−/− was shorter than WT, although the hypertrophic chondrocyte layer was similar in length (Fig. 4D).

We further examined the chondrocyte proliferation by in vivo BrdU incorporation. The number of chondrocytes that incorporated BrdU was similar between Csgalnact1−/− and WT (data not shown). These observations indicate that the main abnormality in the Csgalnact1−/− growth plate is a decrease in ECM volume, resulting in short chondrocyte columns. Nonetheless, chondrocyte proliferation and differentiation are almost normal.

Analysis of CS in Cartilages—Next, we performed biochemical analysis of CS chains in cartilage. Initially, it was confirmed that both Csgalnact1 transcripts were expressed in all cartilage samples from WT mice. Csgalnact1 transcript could not be detected in any of the cartilage samples derived from Csgalnact1−/− mice (Fig. 5A). The chain length and disaccharide composition of CS were determined using cartilage extracts from humerus, tibia, and rib cartilage of WT and Csgalnact1−/− mice as described under “Experimental Procedures.” The elution profile of gel filtration chromatography revealed a peak at fraction 30 in WT and a peak at fraction 28 in Csgalnact1−/− (Fig. 5B). Based on the elution pattern, we estimated that the amount of CS in Csgalnact1−/− cartilage was ~50% of that in WT (Fig. 5B). This decrease in the amount of CS was more pronounced in the tibia than in the humerus and rib samples. Analysis of disaccharide composition showed a similar sulfation ratio for C0S (HexA-GalNAc),...
C4S (HexA-GalNAc(4S)) and C6S (HexA-GalNAc(6S)). The relative amounts of C0S, C4S, and C6S in the various samples were as follows: humerus, 11.7%, 85.5%, and 2.8% for WT and 5.6%, 92.1%, and 2.3% for \textit{Csgalnact1(H1)} \textit{H1}/\textit{H1}/\textit{H1} ; tibia, 10.4%, 86.5%, and 3.1% for WT and 6.7%, 90.5%, and 2.8% for \textit{Csgalnact1(H1)} \textit{H1}/\textit{H1}/\textit{H1} ; rib, 2.8%, 96.2%, and 1.1% for WT and 6.3%, 92.1%, and 1.6% for \textit{Csgalnact1(H1)} \textit{H1}/\textit{H1}/\textit{H1}, respectively (Fig. 5C). These data confirm that the amount of CS in \textit{Csgalnact1(H1)} \textit{H1}/\textit{H1}/\textit{H1} cartilage is reduced by ~50%, although the chain length and disaccharide composition are similar to those of WT.

Both Aggrecan and Link Protein-1 Are Decreased in \textit{Csgalnact1(H1)} \textit{H1} Cartilage—Although our biochemical analysis revealed ~50% decrease in the amount of CS, this decrease alone may not be responsible for the decreased ECM volume observed in \textit{Csgalnact1(H1)} \textit{H1} cartilage. Thus, we examined the major ECM molecules of cartilage, including aggrecan, LP-1/Hapln-1, and collagen II.

Using anti-aggrecan mAb, 1C6, \textit{Csgalnact1(H1)} \textit{H1} humerus cartilage immunostained at E18.5 more faintly than that of WT (Fig. 6, A–D). Aggrecan in WT was mainly localized in the interterritorial zone, whereas in \textit{Csgalnact1(H1)} \textit{H1} it was localized in the pericellular zone (Fig. 6, C and D). An anti-LP-1 mAb, 8A4, immunostained \textit{Csgalnact1(H1)} \textit{H1} humerus cartilage at E18.5 more faintly than WT (Fig. 6, E and F). Indeed, the relative staining intensity was similar to that observed for aggrecan. In contrast, when collagen II was immunostained, both \textit{Csgalnact1(H1)} \textit{H1} and WT cartilage showed a similar level of intensity, although the immunostained area was reduced in \textit{Csgalnact1(H1)} \textit{H1} (Fig. 6, G and H). Hyaluronan staining in cartilage also gave a similar level of intensity (data not shown).

Characterization of Aggrecan in Cartilage—Next, we determined which process was affected in the decreased deposition of aggrecan and LP-1. When transcriptional levels were investigated in tibia by quantitative real-time PCR, the levels of aggrecan, LP-1/Hapln-1, \textit{Adams} 1, and \textit{Adams} 5 in \textit{Csgalnact1(H1)} \textit{H1} were found to be similar to those of WT (Fig. 7A). We then performed Western blot analysis of aggrecan.
Csgalnact1-deficient Mice Display Dwarf Phenotype

**Figure 3. Csgalnact1 deficiency causes bone growth defects.** A, double staining with Alizarin red and Alcian blue of upper limb skeleton of the WT (n = 6) and Csgalnact1^+/− (n = 3) littermates at 2 weeks old. Scale bar, 1 mm. B, statistically significant differences of humerus and tibia length of the WT (n = 3) and Csgalnact1^+/− (n = 3) littermates. *, p < 0.05. Error bars, S.E.

When a PG extract from tibial cartilage was treated with chondroitinase ABC and applied to Western blot analysis, the anti-aggreca n mAb 1C6 detected two bands at >300 kDa i.e. a band at ~200 kDa and a band at ~100 kDa in both Csgalnact1^+/− and WT. WT samples gave a major band at the highest molecular mass, representing full-length aggrecan core protein. In contrast, Csgalnact1^+/− samples showed a major band at ~200 kDa, with slightly greater signal intensity than that of WT. Moreover, the signal intensity of bands at >300 kDa was substantially diminished by comparison with WT (Fig 7B), indicating degradation of aggrecan core protein. These results strongly suggest that the decrease in the level of aggrecan in the ECM of Csgalnact1^+/− cartilage is mainly due to increased aggrecan degradation.

**DISCUSSION**

In this study, we generated and analyzed mice lacking Csgalnact1 gene expression. The mutant mice exhibited slight dwarfism due to a minor impairment of endochondral ossification. Histological examinations revealed a short prolifera-

tive layer with mild disorganization of chondrocyte columns and substantially decreased ECM. Biochemical analysis showed that the amount of CS was only ~50% that of normal mice. Intriguingly, the levels of processed forms of aggrecan core protein were substantially increased in Csgalnact1^+/− cartilage. These results suggest that CSGalNAcT-1 contributes to cartilage formation and subsequent endochondral ossification. Moreover, CSGalNAcT-1 is involved in the metabolism of aggrecan.

CS and chondroitin are present in many organisms including Caenorhabditis elegans and Drosophila melanogaster. Whereas mammals have six CS glycosyltransferases, C. elegans has only two, which are orthologous to CSS1/ChSy1 and CSS2/ChPF. Bacterial strain K4 synthesizes chondroitin modified with fructose, which is catalyzed by a dual glycosyltransferase KfoC (20). The fact that three glycosyltransferases, Ext1, Ext2, and Ext3 (21), are involved in heparan sulfate biosynthesis suggests that CS glycosyltransferases were evolutionarily duplicated from three ancestral enzymes to six. The presence of three pairs of glycosyltransferases, together with their rather ubiquitous expression patterns, strongly suggests functional redundancy and difficulty in acquiring a phenotype in knock-out mice by targeting a single enzyme. Nonetheless, our observation that the absence of CSGalNAcT-1 causes a decrease in the level of CS in cartilage by as much as ~50%, despite the presence of all the other enzymes, clearly indicates a functional selectivity of CSGalNAcT-1. Indeed, these results further support our previous hypothesis that CSGalNAcT-1 is a key enzyme for CS biosynthesis in cartilage (15). Because we have not observed an initiation activity in CSS1/ChSy1 and CSS3/ChSy2, it is likely that CS chain initiation is operated by CSGalNAcT-1 and -2. The expression level of Csgalnact2 was ~50% that of Csgalnact1 in humerus and tibial cartilages and brain in WT mice, and the level of the CS in Csgalnact1^+/− mice was ~50 and ~30% (data not shown), respectively. This correlation suggests that both CSGalNAcT-1 and -2 cooperatively contribute to CS biosynthesis and CSGalNAcT-2 alone does not fully compensate CS chain initiation in the absence of CSGalNAcT-1 in these tissues.

Our analysis demonstrated that although the levels of CS in Csgalnact1^+/− cartilage decreased to ~50% that of normal mice, the CS chain length remained similar or slightly increased. This observation implies that CSGalNAcT-1 catalyzes chain initiation and does not contribute to chain elongation, consistent with our previous results (15). When we overexpressed Csgalnact1 in chondrocytic cells, the amount of CS increased by ~2.2-fold, but the chain length remained unaltered. In Csgalnact1^+/− cartilage, Csgalnact2, which had stronger chain elongation activity than Csgalnact1 in vitro (14), might be able to use excess UDP-GalNAc as a donor substrate more efficiently, resulting in a slight increase in chain length.

Histological and immunohistochemical analyses demonstrated a marked reduction of the ECM, especially of aggrecan and L.P.-1, in Csgalnact1^+/− cartilage. Interestingly, aggrecan was localized to the pericellular region in Csgalnact1^+/− cartilage but was diffuse in the WT ECM. Western blotting experiments showed an increase in the level.
of processed forms of aggrecan and a decrease in the amount of intact core protein. These observations suggest that CS chains attached to the core protein protect them from enzymatic digestion by proteinases. Indeed, GAGs such as CS and keratan sulfate attached to aggrecan core protein affect the manner of cleavage by proteinases (22). Although various matrix metalloproteinases, a disintegrin and metalloproteinase with a thrombospondin motif (ADAMTS)-1, -4, -5, -8, -9, -15 and m-calpain, have been shown to cleave the aggrecan core protein at specific sites (23–26), the mechanism of physiological aggrecan metabolism has not been fully elucidated. Determination of the cleavage sites may lead to identification of proteinases that participate in normal aggrecan metabolism.

FIGURE 4. Phenotype of the Csgalnact1−/− mice at E18.5. A, skeletal preparation of WT and Csgalnact1−/− mice. E18.5 embryos were stained with Alizarin red and Alcian blue. Whole body, skull, forelimb, hind limb, lumbar spine, and digit are shown. B and C, histological analysis in the humerus cartilage of WT and Csgalnact1−/− embryos. Masson’s trichrome (MT), Alcian blue (AB), and hematoxylin and eosin (HE) staining are shown. Scale bars, 200 μm (B) and 100 μm (C). D, width of chondrocyte layer. PC and HC refer to proliferative chondrocyte and hypertrophic chondrocyte, respectively. Error bars, S.E.
To date, several gene knock-out mice exhibiting impaired biosynthesis of CS and aggrecan have been reported. For example, knock-out mice of \textit{Slc35d1}, which encode a nucleotide-sugar transporter transporting UDP-GlcUA and UDP-GalNAc into the endoplasmic reticulum (27), show a marked decrease in CS and die at the embryonic stages. Gene-trapped knock-out mice of \textit{C4st1}, which encodes chondroitin-4-sulfotransferase involved in 4-O-sulfation of the chondroitin chain, show 85% reduction of CS and exhibit severe chondrodysplasia (28). In their cartilage, aggrecan is immunostained in the pericellular region, similarly to that of \textit{Csgalnact1\textsuperscript{-/-}} cartilage. These results strongly suggest that CS chains play an important role in the transportation of aggrecan from the cell surface to the interterritorial zone. Aggrecan knock-out mice display cartilage matrix deficiency (\textit{cmd/cmd}) (29, 30), and \textit{LP-1-null} mice (31) demonstrate a good correlation of the decrease in aggrecan aggregate with severity of skeletal phenotype. Mice \textit{cmd/cmd} exhibit dwarfism and marked chondrodysplasia and die at the perinatal period, and their growth plates lack chondrocyte columns. \textit{LP-1-null} mice exhibit a similar but milder phenotype than \textit{cmd/cmd} mice. Specifically, the growth plate of \textit{LP-1-null} mice contains disorganized chondrocyte columns and a decrease in aggrecan deposition to \(\sim 25\%\) that of WT. \textit{Cmd} heterozygotes show a slight dwarfism and cervical intervertebral disc herniation.
at 1 year of age, where aggrecan deposition is reduced to ~80%. The dwarfism of Csgalnact1−/− mice is more conspicuous than cmd heterozygotes and much less pronounced than Ctrl1-null mice. Thus, the deposition level of aggrecan that contributes to endochondral ossification appears to be ~80% and much >25%, although Csgalnact1−/− cartilage contains 50% less CS and aggrecan is partly fragmented. It is still unclear how the decreased CS level causes dwarfism. We speculate that the decreased ECM volume, together with the reduction of aggrecan deposition, disabled the alignment of an adequate number of proliferative chondrocytes that participate in endochondral ossification. Many signaling molecules and growth factors, such as PTHrP (32, 33), Ihh (34), TGF-β, and BMP (35, 36), have been shown to regulate the differentiation and apoptosis of growth plate chondrocytes. Although these signaling pathways may be altered in Csgalnact1−/− cartilage, it is likely that such changes are too small to be observed.

Acknowledgment—We thank Dr. K. Araki, Kumamoto University, for providing Ayu1-Cre transgenic mice.

REFERENCES
1. Watanabe, H., Yamada, Y., and Kimata, K. (1998) J. Biochem. 124, 687–693
2. Kimi, C., Chen, L., Wu, Y. J., Yee, A. J., and Yang, B. B. (2002) Cell Res. 12, 19–32
3. Hardingham, T. E., and Fosang, A. J. (1992) FASEB J. 6, 861–870
4. Dityatev, A., Brückner, G., Dityateva, G., Grosche, J., Kleene, R., and Schachner, M. (2007) Dev. Neurobiol. 67, 570–588
5. Ishii, M., and Maeda, N. (2008) J. Biol. Chem. 283, 32610–32620
6. Muramatsu, T. (2002) J. Biochem. 132, 359–371
7. Kitagawa, H., Uyama, T., and Sugahara, K. (2001) J. Biol. Chem. 276, 38721–38726
8. Yada, T., Sato, T., Kaseyama, H., Gotoh, M., Iwasaki, H., Kikuchi, N., Kwon, Y. D., Togayachi, A., Kudo, T., Watanabe, H., Narimatsu, H., and Kimata, K. (2003) J. Biol. Chem. 278, 39711–39725
9. Yada, T., Gotoh, M., Sato, T., Shionyu, M., Go, M., Kaseyama, H., Iwasaki, H., Kikuchi, N., Kwon, Y. D., Togayachi, A., Kudo, T., Watanabe, H., Narimatsu, H., and Kimata, K. (2003) J. Biol. Chem. 278, 30235–30247
