Molecular Cloning and Expression of Two Distinct Human Chondroitin 4-O-Sulfotransferases That Belong to the HNK-1 Sulfotransferase Gene Family*

Using an expression cloning strategy, the cDNA encoding the human HNK-1 sulfotransferase (HNK-1ST) has been cloned. During this cloning we found that HNK-1ST and other Golgi-associated sulfotransferases cloned before share homologous sequences including the RDP motif (Ong, E., Yeh, J.-C., Ding, Y., Hindskaul, O., and Fukuda, M. (1998) J. Biol. Chem. 273, 5190–5195). Using this conserved sequence in HNK-1ST as a probe, we identified two expressed sequence tags in EST data base which have 31.6 and 30.7% identity with HNK-1ST at the amino acid levels. Expression of these two full-length cDNAs failed to form HNK-1 glycan nor to add sulfate to CD34 or NCAM. Surprisingly, proteins expressed by these cDNAs transferred sulfate to the C-4 position of N-acetylgalactosamine in chondroitin and desulfated deraman sulfate, thus we named these two enzymes, chondroitin 4-O-sulfotransferase 1 and -2 (C4ST-1 and C4ST-2). Both C4ST-1 and C4ST-2, however, did not form 4,6-di-O-sulfated N-acetylgalactosamine when chondroitin sulfate C was used as an acceptor. Moreover, analysis of 35S-labeled deraman sulfate formed by C4ST-1 indicate that sulfate preferentially took place in GlcA→GalNAc unit than in IdoA→GalNAc unit, suggesting that 4-O-sulfation at N-acetylgalactosamine may precede epimerization of glucuronic acid to iduronic acid during deraman sulfate biosynthesis. Northern analysis demonstrated that the transcript for C4ST-1 is predominantly expressed in peripheral leukocytes and hematopoietic tissues while the C4ST-2 transcript is more widely expressed in various tissues. These results indicate C4ST-1 and C4ST-2 play complementary roles in chondroitin and deraman sulfate synthesis in different tissues.

Sulfate groups in carbohydrates play important roles in conferring highly specific functions on glycoproteins, glycolipids, and proteoglycans (1–3). Expression of certain sulfated carbohydrates is spatially and temporally controlled, thereby providing developmental regulation of those functions displayed by such sulfation. One of these sulfated glycans is the HNK-1 glycan (4, 5). The functional significance of HNK-1 glycan was first recognized as an antigen involved in peripheral demyelinating neuropathy. The structural analysis of glycolipids reactive with the autoantibodies led to the discovery that the HNK-1 epitope is sulfo-3GlcAβ1→3Galβ1→4GalNAcβ→R (6, 7).

Subsequently, HNK-1 glycan has been found in a number of neural cell adhesion molecules, including NCAM, myelin-associated glycoprotein, L1, contactin, and Po (5, 8–11). Using monoclonal antibodies or isolated carbohydrates, various laboratories reported that HNK-1 glycan is involved in cell-cell and cell-substratum interactions (12, 13). In one study, a non-sulfated form of HNK-1 precursor glycan did not facilitate neurite outgrowth as opposed to a functional, intact HNK-1 glycan (13). These results, combined together, suggest that HNK-1 glycan plays critical roles in development, in particular during neural cell development.

The HNK-1 carbohydrate is synthesized by the addition of a sulfate to β1,3-glucuronylated N-acetyllactosamine, GlcA-β1→3Gal-β1→4GalNAc-R (4). Recently, we and others cloned the cDNA encoding HNK-1 sulfotransferase using an expression cloning strategy (14, 15). During this cloning, we discovered that the newly cloned HNK-1 sulfotransferase and other Golgi-associated sulfotransferases cloned before share a common sequence motif, which includes ZZRPXXXZ, where X and Z denote any amino acid and hydrophobic amino acids, respectively (14). Subsequently, it was revealed that this sequence motif is a part of the binding site for 3'-phosphate group of the donor substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (16, 17). Most recent studies showed that the arginine residue (Arg) in the RDP motif is involved in hydrogen bonding to 3'-phosphate group while aspartic acid (Asp) and proline (Pro) residues participate in the core structure of the 3'-phosphate-binding site by residing in a tight turn of the polypeptides (17, 18). In addition, the amino acid sequences responsible for binding to 5'-phosphosulfate are conserved among different sulfotransferases (17).

The presence of the above weak but discernible similarity among different sulfotransferases suggested a possibility that other sulfotransferases may be identified by their similarity to sulfotransferases cloned already. In fact, we and others cloned the cDNA encoding L-selectin ligand sulfotransferases that add

* This work was supported by National Cancer Institute Grants P01CA71932 and CA33895. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The nucleotide sequences reported in this paper has been submitted to GenBank with accession number AF239820 for C4ST-1 and AF239822 for C4ST-2.

The abbreviations used are: GlcA, D-glucuronic acid; IdoA, L-iduronic acid; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; EST, expressed sequence tag; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; HNK-1ST, HNK-1 sulfotransferase; C4ST, chondroitin 4-O-sulfotransferase; CSST, chondroitin 6-O-sulfotransferase; HPLC, high performance liquid chromatography.

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a sulfate to the 6-position of N-acetylgalactosamine, which is eventually converted to 6-sulf-o sialyl Lewis X, NeuNac2→3-Galβ1→4[sulfu→6-Fuc1→3GlcNAc]β1→6R (19–21). This cloning was achieved by searching the EST data base for cDNAs related to chondroitin sulfate 6-O-sulfotransferase (22) and keratan sulfate Gal-6-O-sulfotransferase (23).

In the present study, we first describe the isolation of two isofoms of cDNAs by screening the EST data base for cDNAs related to the human HNK-1 sulfotransferase (14). The expression of full-length cDNAs unexpectedly revealed that these cDNAs encode novel chondroitin 4-O-sulfotransferases, adding a sulfate to 4-position of N-acetylgalactosamine residues in chondroitin and desulfated dermanatan sulfate. Moreover, we found that these two chondroitin 4-sulfotransferases exhibit diverse tissue distribution, indicating that these two enzymes play complementary roles in different tissues.

**EXPERIMENTAL PROCEDURES**

**Isolation of cDNAs Encoding Chondroitin 4-O-Sulfotransferases**—In HNK-1ST, the conserved motif, IVRDPFERL, was used as a probe to search dbEST using the TBLASTN program. Initially, two query genes AA310375 and AA233362 were identified, which had 50% in 50 amino acids and 69% in 26 amino acids identity with HNK-1ST, respectively.

After blast search for a sequence homologous to AA310375, AA744877 was identified. AA744877 is a cDNA prepared from germinal center B lymphocytes. Sequence analysis of this cDNA, obtained from Genome Systems (St.Louis, MS), revealed that this cDNA encodes a protein consisting of 352 amino acids. The cDNA also contains 5'-untranslated sequence (150 base pairs) and 3'-protein consisting of 352 amino acids. The cDNA also contains 5'-untranslated sequence (150 base pairs) and 3'-untranslated sequence (330 base pairs). The cDNA insert was digested with HindIII and XhoI and cloned into the same sites of pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA), resulting in pcDNA3.1-C4ST-1 (the name of C4ST-1 was given after the determination of acceptor specificity).

The second gene was initially identified in AA233362 and AA777237 derived from the human NT2 cell line and SS20w fetal liver/spleen. Since these two clones lacked the 5'-region, 5'-rapid amplification of cDNA ends was carried out using poly(A) RNA from human lymph nodes (CLONTECH, Palo Alto, CA). However, a new EST sequence, AA182585, which contained the full coding sequence was released in the meantime. This cDNA was thus excised from AA182585 with BsrHI and XhoI and cloned into the same sites of pcDNA3.1/Hygro, resulting in pcDNA3.1-C4ST-2.

pcDNA-HNK1ST harboring the cDNA encoding a human HNK-1 sulfotransferase was cloned as described previously (14). The cDNA encoding chondroitin 6-O-sulfotransferase (C6ST) was cloned by reverse transcriptase-PCR using poly(A) RNA isolated from mouse embryonic cells by oligo(dT) primers previously used in this PCR correspond to nucleotides 67 to 49 (1-3 encode for the initiation methionine) and nucleotides 1518-1522, respectively (24), and also contained XhoI and HindIII sites, respectively. The PCR products were cloned into pBluescript by TA cloning. The resultant cDNA was excised by XhoI and HindIII and cloned into the same sites of pcDNA3.1/Zeo, resulting in pcDNA3.1-C6ST. pcDNA-GlAT-P encoding 6,β-glucurononyltransferase that forms the HNK-1 precursor glycan (25) was cloned as described before (14).

**Sulfotransferase Assay**—CHO cells were transfected with pcDNA3.1-C4ST-1, pcDNA3.1-C4ST-2, pcDNA3.1-C6ST, or pcDNA -HNK-1ST using LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD). Sixty-two h after transfection, the cells attached to plates were washed with phosphate-buffered saline, scraped, and homogenized in 10 mM Tris-HCl, pH 7.2, containing 0.5% Triton X-100, 0.25 mM sucrose, a protease inhibitor mixture, and 1 mM aprotinin as described previously (22). The homogenate was mixed well by rotation for 1 h, then centrifuged at 10,000 × g for 15 min. The supernatant derived from the transfected CHO cells and mock-transfected CHO cells were used as the enzyme source.

Chondroitin sulfate 4- and 6-O-sulfotransferase and heparan sulfate sulfotransferase activities were assayed as described previously (26). Briefly, the reaction mixture (50 μl) contained 50 mM imidazole-HCl, pH 6.8, 0.005% protamine chloride, 2 mM dithiothreitol, 50 μg of acceptor glycosaminoglycans, 2 μM [35S]PAPS (about 5 × 106 cpm), and 25 μl of an enzyme solution. After incubation for 1 h at 37 °C, the reaction mixture was boiled for 2 min, then 0.1 volume of 4 N potassium acetate and 3 volumes of ethanol were added. The reaction products were precipitated by brief centrifugation, and subjected to Sephadex G-25 gel filtration in 0.1 M NH4HCO3 to separate high molecular weight products from the remaining unreacted [35S]PAPS and degradation products.

Chondroitin sulfotransferase assay, 0.05% protamine chloride instead of 0.005% protamine chloride was added in the same reaction mixture described above (26). For keratan sulfate sulfotransferase assay, the reaction mixture (50 μl) contained 50 mM imidazole-HCl, pH 6.4, 10 mM CaCl2, 2 mM dithiothreitol, 50 μg of keratan sulfate, 2 μM [35S]PAPS (about 5 × 106 cpm), and 25 μl of an enzyme solution (23). The reaction products were purified as described above. HNK-1ST cDNA, which includes the above motif, was thus used as a probe to search dbEST using the TBLASTN program. Initially, two query genes AA310375 and AA233362 were identified, which had 50% in 50 amino acids and 69% in 26 amino acids identity with HNK-1ST, respectively.

Two Distinct Novel Chondroitin 4-O-Sulfotransferases

**RESULTS**

**Isolation of cDNAs Encoding Chondroitin 4-O-Sulfotransferases (C4STs)**—Comparison of the amino acid sequences of cloned sulfotransferases demonstrated that there is a weak but discernible homologous sequence motif among Golgi-associated sulfotransferases (14). In particular, the RDP sequence motif was conserved among those sulfotransferases compared. By searching the EST data base for a novel cDNA related to HNK-1ST, two distinct cDNA sequences were found to have...
homology to the HNK-1ST sequence. The first cDNA (AA744877 in dbEST) encodes an open reading frame of 1,059 base pairs, predicting a protein of 352 amino acid residues (41,488 Da), which we subsequently termed C4ST-1 (Fig. 1). The second cDNA (AA182585 in dbEST) encodes an open reading frame of 1,245 base pairs, predicting a 414-amino acid residue protein (48,348 Da), which we subsequently termed C4ST-2 (Fig. 2). The cDNAs encoding C4ST-1 and C4ST-2 were cloned into pcDNA3.1/Hygro, resulting in pcDNA3.1-C4ST-1 and pcDNA3.1-C4ST-2, respectively.

The comparison of the amino acid sequences of C4ST-1 and C4ST-2 with HNK-1ST reveals the following points (Fig. 3). The sequences of the cytoplasmic segment and the transmembrane/anchoring domain are not strongly similar among these sulfotransferases, while the sequences are highly homologous to each other in the catalytic domains. There are four regions where sulfotransferases are highly homologous. The first two are the 5'-phosphosulfate-binding and 3'-phosphate-binding sites, respectively (Fig. 3). The third and fourth regions (A and B in Fig. 3) have not been reported before, but probably correspond to two a-helical domains near the carboxy-terminal ends (34).

As a whole, the amino acid sequence of C4ST-1 is more homologous to that of C4ST-2 (41.6% identity) than that of HNK-1ST (31.6% identity), while HNK-1ST and C4ST-2 share 30.7% identity. None of the other amino acid sequences in the data base showed significant homology to these three sulfotransferases, while the sequences are highly homologous to each other in the catalytic domains. There are four regions where sulfotransferases are highly homologous. The first two are the 5'-phosphosulfate-binding and 3'-phosphate-binding sites, respectively (Fig. 3). The third and fourth regions (A and B in Fig. 3) have not been reported before, but probably correspond to two a-helical domains near the carboxy-terminal ends (34).

Expression of C4ST-1 and C4ST-2—To determine the acceptor specificity of C4ST-1 and C4ST-2, pcDNA3.1-C4ST-1, pcDNA3.1-C4ST-2, and control pcDNA3.1 were separately transfected into CHO cells. Sulfotransferase activity in cell extracts from the transfected cells was determined using various acceptors. First, neither C4ST-1 nor C4ST-2 exhibited activity toward the HNK-1ST precursor acceptor GlcAlp1→3Galβ1→4GlcNAcβ1→octyl. In contrast to HNK-1ST, C4ST-1 and C4ST-2 failed to express the HNK-1 antigen when transiently expressed in Lec2 cells. The resulting HNK-1 precursors produced no activity with any of the acceptors tested (data not shown). In addition, neither C4ST-1 nor C4ST-2 increased [35S]sulfate incorporation into NCAM- or CD34-human IgG chimeric protein in the presence or absence of β1,3-galactosyltransferase (25) or core 2 β1,6-N-acetylgalactosaminyl transferase (36) carried out as described previously (20) (data not shown). We then tested various glycosaminoglycans as acceptors. As shown in Fig. 4, C4ST-1 and C4ST-2 incorporated [35S]sulfate to chondroitin, chondroitin 4-O-sulfate (chondroitin sulfate A), chondroitin 6-O-sulfate (chondroitin sulfate C), and desulfated dermatan sulfate. In contrast, C4ST-1 and C4ST-2 did not incorporate [35S]sulfate to dermatan sulfate, desulfated N-sulfated heparin, or desulfated and N-acetylated heparin or keratan sulfate (lower figures in Fig. 4).

When the activity of C6ST was assayed, C8ST incorporated [35S]sulfate into chondroitin, chondroitin sulfate A, chondroitin sulfate C, and keratin sulfate, as expected (22). On the other hand, HNK-1ST did not show any detectable activity toward these glycosaminoglycan acceptors (Fig. 4). These results indicate that newly cloned C4ST-1 and C4ST-2 are sulfotransferases that add sulfate(s) to chondroitin sulfate, and desulfated dermatan sulfate.

Identification of Reaction Products—The above results showed that both C4ST-1 and C4ST-2 utilized almost identical acceptors, but did not show if C4ST-1 and C4ST-2 added sulfate to the 4- or 6-position of N-acetylgalactosamine or the 2-position of β-glucuronic acid.

To determine the structures of the sulfated products derived from chondroitin, we took advantage of the fact that isomers of sulfated disaccharide units produced by chondroitinase ABC can be separated by SAX-10 HPLC. As shown in Fig. 5A, almost all of the products by C4ST-1 eluted at the position of ΔDi-4S. The peak corresponding to ΔDi-4S released sulfate after treatment with chondro-4-sulfatase (Fig. 5B), but not with chondro-6-sulfatase (Fig. 5C). These results, combined together, indicate that C4ST-1 incorporated a sulfate to the 4-position of N-acetylgalactosamine in chondroitin. The products derived from chondroitin sulfate A or C showed a prominent peak corresponding to ΔDi-4S after chondroitinase ABC digestion, but did not contain any disulfated disaccharide (Fig. 5D), indicating that C4ST-1 adds a sulfate group only when neither glucuronic acid nor N-acetylgalactosamine in the acceptors contain a sulfate group. The amount of ΔDi-6S was almost the same as that observed in control experiments, indicating that 6-O-sulfation was due to an endogenous enzyme (Fig. 5A). The products from C4ST-2 were analyzed in an identical manner. These results are very similar to those described for C4ST-1 (Fig. 5, E-H).

Sulfation of Dermatan Sulfate by C4ST-1 and C4ST-2—Both C4ST-1 and C4ST-2 incorporated [35S]sulfate to desulfated dermatan sulfate (Fig. 4). To determine how C4ST-1 and C4ST-2 act on dermatan sulfate, [35S]-labeled products obtained from desulfated dermatan sulfate were digested with chondroitinase ABC. HPLC analysis of the digested material showed that C4ST-1 produced ΔDi-4S, indicating that C4ST-1 added a sul-
fate to the 4-position of N-acetylgalactosamine in desulfated dermatan sulfate (Fig. 6A). No disulfated disaccharide was detected. Almost identical results were obtained for C4ST-2 (data not shown). To further delineate the acceptor specificity of C4ST-1, the 35S-labeled products were digested with chondroitinase AC I, which cleaves only N-acetylgalactosaminyl linkage to 4-glucuronic acid. The results demonstrated that approximately one-fourth of the total radioactivity was released as ΔDi-4S and the rest eluted in later fractions (Fig. 6B). After digestion with chondro-4-sulfatase, the peak correspond-
ing to ΔDi-4S disappeared and a prominent free sulfate ion peak appeared instead (Fig. 6C). However, no significant change in larger 35S-labeled oligosaccharides, eluted after 24.5 min, was observed, indicating that chondro-4-sulfatase did not release sulfate from oligosaccharides larger than disaccharides. Digestion of the same material by chondro-6-sulfatase, on the other hand, barely changed the elution profile (Fig. 6D), being consistent with the above conclusions that C4ST-1 incorporated [35S]sulfate to the 4-position of N-acetylgalactosamine.

To determine the nature of larger oligosaccharides obtained after chondroitinase AC I treatment, the same sample analyzed in Fig. 6D was subjected to Bio-Gel P-4 gel filtration. The results showed that approximately one-fourth of the total radioactivity eluted at ΔDi-4S and approximately 10% of total radioactivity eluted as tetrasaccharide and hexasaccharide (Fig. 6E). Chondroitinase AC I can release ΔDi-4S only from GlcA-GalNAc(6S) that is flanked by GlcA-GalNAc units (29). These results suggest that [35S]sulfate was incorporated into GlcA-GalNAc unit.

To corroborate the above experiments, intact [35S]sulfate-labeled dermatan sulfate was directly digested by chondroitinase B, which cleaves a sulfated N-acetylgalactosaminyl linkage to iduronic acid flanked by IdoA-GalNAc units (30, 31). The results demonstrated no release of [35S]labeled ΔDi-4S or ΔDi-6S-labeled oligosaccharides (Fig. 6F). These results combined together indicate that C4ST-1 and most likely C4ST-2 preferentially incorporate a sulfate at the 4-position of N-acetylgalactosamine.

C4ST-1 and C4ST-2 Are Differentially Expressed in Various Tissues—To determine the expression of C4ST-1 and C4ST-2 transcripts in various tissues, Northern and dot blot analysis was carried out. Gel fractionated blot (Fig. 7) and dot blot (Fig. 8) analyses show that the C4ST-1 transcript is highly expressed in spleen, thymus, peripheral blood leukocytes, lymph node, bone marrow, lung, and placenta. In contrast, the transcripts of C4ST-2 are expressed more ubiquitously (Fig. 7), but significantly more in spinal cord, heart, thyroid, pituitary gland, adrenal gland, small intestine, spleen, peripheral blood leukocytes, thymus, lung, fetal kidney, fetal spleen, and fetal lung on the dot blot (Fig. 8). These results show that C4ST-1 is mostly expressed in leukocytes and hematopoietic tissues, while C4ST-2 is widely expressed in various tissues, including...
endocrine organs and nervous systems.

Chromosomal Mapping of the C4ST-1 and C4ST-2 Genes—To determine the chromosomal localization of C4ST-1 and C4ST-2 genes, PCR analysis was carried out using the Stanford G3 RH panel. PCR primers were chosen from coding regions of C4ST-1 and C4ST-2 genes, and based on the criteria that PCR products showed the same molecular weight when C4ST-1 or C4ST-2 cDNA or genomic DNA was used as a template, but not using hamster genomic DNA. This analysis placed C4ST-1 between D12S1607 and D12S360, thus mapping the gene to the q23 region of chromosome 12. Similarly, the C4ST-2 gene was placed between D7S2563 and D7S2521, mapping the gene to the p22 region of chromosome 7.

DISCUSSION

The present study describes the isolation of novel cDNAs encoding chondroitin 4-O-sulfotransferase by searching the EST data bases for cDNAs homologous to the human HNK-1ST (14). HNK-1ST adds a sulfate to the 3-position of glucuronic acid, which in turn is attached to the 3-position of galactose in N-acetyllactosamine. C4ST, on the other hand, adds a sulfate to the 4-position of N-acetylgalactosamine, which is in turn attached to the 4-position of glucuronic acid. These results are striking since these two acceptors are rather dissimilar. The β-glucuronyl residue in HNK-1 glycan is at the nonreducing terminal. In contrast, C4ST apparently acts on an already elongated chondroitin chain since no preferential addition to shorter acceptors has been noticed when the products were analyzed by gel filtration (data not shown, see also Ref. 26). The hydroxyl groups in both C-3 of glucuronic acid and C-4 of N-acetylgalactosamine are projected above their respective pyranose rings in their normal conformations (37). It is tempting to speculate that the active sites of both HNK-1ST and C4ST may approach the acceptor from above the plane of GlcAβ1→3Galβ1→4GlcNAcβ1→R (for HNK-1ST) and

FIG. 6. Analysis of 35S-labeled dermatan sulfate obtained after incubation with C4ST-1. A–D, HPLC analysis of 35S-labeled dermatan sulfate after digestion with chondroitinase ABC (A), chondroitinase AC I (B), chondroitinase AC I followed by chondro-4-sulfatase (C) or chondro-6-sulfatase (D) (closed circles). The elution positions of ΔDi-0S (E), ΔDi-4S (F), ΔDi-6S (G), free sulfate ion (SO₄²⁻), and ΔDi-diS₆ (H) are shown. In A and B, open circles denote the radioactivity obtained from mock experiments. E and F, Bio-Gel P-4 gel filtration analysis of 35S-labeled dermatan sulfate after digestion with chondroitinase AC I followed by chondro-6-sulfatase (E) or digestion with chondroitinase B followed by chondro-6-sulfatase (F). The elution positions of free sulfate ion (SO₄²⁻), ΔDi-4S (G), tetrasaccharides (A), and hexasaccharides (B) obtained after digestion of chondroitin sulfate A with chondroitinase ABC, and void volume (V₀) are shown.

FIG. 7. Northern analysis of C4ST-1 and C4ST-2 transcripts. Each lane contained 2 µg of poly(A)⁺ RNA. The blots were hybridized with the appropriate 32P-labeled C4ST cDNAs. Each blot contained four or eight lanes and was run separately. The migration positions of molecular markers are shown at the left. The positions of the transcripts are indicated by arrowheads.
Fig. 8. Dot blot analysis of C4ST-1 and C4ST-2 transcripts. Human RNA Master Blot™ shown at the far left was sequentially hybridized to 32P-labeled human C4ST-1 or C4ST-2 cDNA.

Fig. 9. Schematic representation of phylogenetic tree of Golgi-associated carbohydrate sulfotransferases. Amino acid sequences predicted from cloned cDNAs are compared using the Clustal W method with PAM250 residue weight table. The following sequences are compared: human heparan sulfate N-glucosaminyl 3-O-sulfotransferase, hu HS3ST-1 (53), -2, -3A, and -3B (54); human heparan sulfate N-deacetylase/sulfotransferase, hu HSNDST-1, -2, and -3 (55–58); human chondroitin N-acetylgalactosamine-6-O-sulfotransferase, hu C6ST (35); human keratan sulfate N-galactose-6-O-sulfotransferase, hu KSST (23); human N-acetylgalactosamine-6-O-sulfotransferase, hu GlcNAc6ST (38); human intestinal N-acetylgalactosamine-6-O-sulfotransferase, hu I-GlcNAc6ST (38); human L-selectin ligand sulfotransferase, hu LSST (20, 21); mouse L-selectin ligand sulfotransferase, mo LSST (20, 21); human HNK-1 sulfotransferase, hu HNK-1ST (14); human chondroitin N-acetylgalactosamine-4-O-sulfotransferase, hu C4ST-1 and -2 (present study); human dermatan/chondroitin uronyl-2-O-sulfotransferase, hu CS/DS2ST (60); human heparan sulfate 2-O-sulfotransferase, hu HS2OST (61); human galactosylceramide N-Gal-3-O-sulfotransferase, hu GalCerST (62); mouse heparan sulfate N-sulfoglucosamine-6-O-sulfotransferase, mo HS6OST-1, -2, and -3 (63).

It is noteworthy that C4ST-1 and C4ST-2 share only 41.8% identity at the amino acid levels, but share a common catalytic property. C4ST-1 and C4ST-2, however, are much more homologous to each other in the vicinity of 5'-phosphosulfate and 3'-phosphate binding sites (Fig. 3). Moreover, C4ST-1 and C4ST-2 apparently share common structural domains toward the carboxyl-terminal regions (A and B in Fig. 3). These regions do not share homology with other sulfotransferases (34) and further studies are necessary to determine their roles.

Fig. 9 illustrates the phylogenetic relationship of cloned Golgi-associated sulfotransferases that add a sulfate on carbohydrate acceptors. The results clearly indicate that C4ST-1, C4ST-2, and HNK-1ST form a gene family distinct from the rest of the sulfotransferase gene families. The members within the same gene family depicted in Fig. 9 catalyze identical or similar reactions, except for one case. LSST, I-GlcNAc6ST, GlcNAc6ST, C6ST, and KSST form a gene family whose acceptor specificities are not clearly related to each other. The cDNAs (GlcNAc6ST, LSST, and I-GlcNAc6ST) encoding a sulfotransferase that adds a sulfate to the 6-position of N-acetylgalactosamine at the nonreducing terminal were identified in EST data base for their homology to C6ST or KSST (19–21, 38). In contrast, C6ST and KSST add a sulfate on the 6-position of N-acetylgalactosamine or galactose on already elongated substrates (22, 23). These results, combined together with the results obtained in the present study, indicate that it is possible to identify cDNAs encoding enzymes that utilize very different acceptors from those utilized by a protein whose cDNA was used as a probe. Further studies will be significant to determine the three-dimensional structures of these sulfotransferases bound to acceptors in order to test if these enzymes approach their acceptors from above the plane of the acceptors.

The present study demonstrated that both C4ST-1 and C4ST-2 act much more efficiently on non-sulfated chondroitin or desulfated dermatan sulfate than chondroitin sulfate A, chondroitin sulfate C, or dermatan sulfate (Fig. 4). No desulfated disaccharide was released after chondroitinase ABC digestion of reaction products derived from chondroitin sulfate C (Fig. 5). These results indicate that C4ST-1 and C4ST-2 add sulfate only on unsulfated N-acetylgalactosamines.

The present study also demonstrated that C4ST-1 and C4ST-2 act much more efficiently on non-sulfated chondroitin or desulfated dermatan sulfate than chondroitin sulfate A, chondroitin sulfate C, or dermatan sulfate (Fig. 4). No desulfated disaccharide was released after chondroitinase ABC digestion of reaction products derived from chondroitin sulfate C (Fig. 5). These results indicate that C4ST-1 and C4ST-2 add sulfate only on unsulfated N-acetylgalactosamines.

The present study also demonstrated that C4ST-1 and C4ST-2 add a sulfate to the 4-position of N-acetylgalactosamine residues in dermatan sulfate, which had been chemically desulfated (Figs. 4 and 6). The detailed analysis of dermatan sulfated by C4ST-1 revealed the following points. Even though glucuronic acid residues are minor components in the dermatan sulfate, at least one-fourth of the total radioactivity was detected in ΔDi-4S when released by chondroitinase AC I digestion. In this case, ΔDi-4S was released only when 4-sulfated N-acetylgalactosamine are positioned between two glucuronic
acids. This finding suggests that C4ST-1 acts on N-acetylgalactosamine residues next to glucuronic acid. If C4ST-1 transfers a sulfate to N-acetylgalactosamine linked to iduronic acid as efficiently as to N-acetylgalactosamine linked to glucuronic acid, more 35S-labeled oligosaccharides would be released by chondroitinase B digestion than by chondroitinase AC I digestion. However, ΔDi-4S was hardly released after chondroitinase B digestion (Fig. 6). These results, combined together, indicate that the GlcA→GalNAc unit is a much better acceptor for C4ST-1 (and most likely for C4ST-2 as well) than the IdoA→GalNAc unit.

The results obtained in the present study are similar to those obtained on C4ST purified from a rat chondrosarcoma cell line (26). However, the C4ST in that study added a sulfate more on desulfated dermatan sulfate (porcine skin) and the products were highly susceptible to chondroitinase AC II, which cleaves IdoA residues next to glucuronic acid. If C4ST-1 transfers acids. This finding suggests that C4ST-1 acts on dermatan sulfate containing large amounts of IdoA→GalNAc(4S). However, if the same enzyme contributes to form both chondroitin sulfate and dermatan sulfate, several factors may determine the expression of these synthesized molecules to chondroitin sulfate or dermatan sulfate. When an enzyme is epimerized, it converts a glucuronic acid to an iduronic acid, and this reaction is accelerated by the sulfation of N-acetylgalactosamine introduced by C4ST. In contrast, in the absence of an epimerase, no dermatan sulfate is formed. Thus, the presence of an epimerase should be a main regulator, but another possibility needs to be considered. The results of in vitro enzyme assay showed that the sulfotransferase activity of C4ST to chondroitin and dermatan sulfate changes depending upon the concentration of proteamine chloride in the reaction mixture (Ref. 26 and the present study). The concentration of proteamine chloride also affects the activity of chondroitin 6-O-sulfotransferase including its substrate specificity (43). These findings suggest that the environmental factors affecting the activity of C4ST might contribute to the regulation of the chondroitin sulfate and dermatan sulfate biosynthesis. It is also possible that another sulfotransferase preferentially acting on IdoA→GalNAc is involved in dermatan sulfate biosynthesis. Further studies will be significant to clarify these points.

While we were preparing this manuscript, the mouse counterpart of C4ST-1 was reported (44). The human and mouse C4ST-1 have 96.0% identity at the amino acid levels. The expression profile of the mouse C4ST is slightly different from that of human C4ST-1 in that it is mainly expressed in the brain and kidney. The mouse C4ST-1, however, apparently exhibits almost the same substrate specificity as the human C4ST-1 and C4ST-2 (44). The transcripts of human C4ST-1 and C4ST-2 are differentially expressed in various tissues. The C4ST-1 transcript is predominantly expressed in peripheral blood leukocytes and hematopoietic tissues such as bone marrow and spleen, while the C4ST-2 transcript is more widely expressed, including in the pituitary gland, adrenal gland, spinal cord, small intestine, spleen, and lung (Fig. 7 and 8). These results indicate that C4ST-1 and C4ST-2 may play complementary roles in different tissues.

Chondroitin sulfate proteoglycans have been found in the brain and have been shown to play roles in neural cell adhesion and neurite outgrowth (45–48), and neural cell migration (49). Chondroitin sulfate is also present in blood cells and has been shown to be involved in interaction with CD44 (50, 51) and L-selectin (52). C4ST-1 and C4ST-2 cloned in the present study will be powerful tools to determine the roles of chondroitin sulfate in these various biological systems.

Acknowledgments—We thank the members of our laboratories for useful discussions, and Susan Wynant and Risa Tabata for organizing the manuscript.

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Two Distinct Novel Chondroitin 4-O-Sulfotransferases
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J. Biol. Chem. 2000, 275:20188-20196.
doi: 10.1074/jbc.M002443200 originally published online April 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002443200

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