Molecular Cloning and Expression of a Novel Chondroitin 6-O-Sulfotransferase*

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A novel human chondroitin 6-O-sulfotransferase, designated C6ST-2, was identified by BLAST analysis of expressed sequence tag using the sequence of a previously described human chondroitin 6-O-sulfotransferase (C6ST-1) as a probe. The new cDNA sequence revealed an open reading frame coding for a protein of 486 amino acids with a type II transmembrane protein topology. The amino acid sequence displayed 24% identity to the human C6ST-1, and the highest sequence identity was found in the COOH-terminal catalytic domain. The expression of a soluble recombinant form of the protein in COS-1 cells produced an active sulfotransferase with marked specificity for polymer chondroitin. In contrast, keratan sulfate and oligosaccharides containing the Galβ1→4GlcNAc sequence, which are good acceptor substrates for the C6ST-1, hardly served as acceptors. The identification of the reaction product indicated that the enzyme is a novel chondroitin 6-O-sulfotransferase (C6ST-2) that mainly transfers sulfate to N-acetylgalactosamine. The coding region of C6ST-2 was contained in a single exon and localized to chromosome Xp11. Northern blot analysis of human brain poly(A)^+ RNA revealed a single transcript of 2.4 kilobase pairs. Reverse transcription-polymerase chain reaction analysis showed that C6ST-2 is developmentally regulated in various tissues with expression persisting through adulthood in the spleen. Thus, we demonstrated the redundancy in chondroitin 6-O-sulfotransferases capable of forming chondroitin 6-sulfate, which is important for understanding the mechanisms leading to specific changes in the sulfation profile of chondroitin sulfate chains in various tissues during development and malignant transformation.

Chondroitin sulfates are synthesized as proteoglycans that can be expressed on the surfaces of most cells and in extracellular matrices that are covalently linked to a wide range of core protein families. Chondroitin sulfate proteoglycans are increasingly implicated as important regulators of many biological processes, such as cell migration and recognition, extracellular matrix deposition, and morphogenesis (for reviews see Refs. 1 and 2). Growing evidence indicates that many of their functions are associated with the sulfated glycosaminoglycan (GAG)^3 moieties (for a review see Ref. 3).

Chondroitin sulfate GAG has a linear polymer structure that possesses repetitive, sulfated disaccharide units containing glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). These sulfated GAGs are generated by a family of sulfotransferases that transfer sulfate from its high energy donor PAPS to the C-2 or C-3 position of GlcA residues or to the C-4 or C-6 position of GalNAc residues. Sulfotransferases generate considerable structural diversity by transferring sulfate with remarkable specificity for the underlying oligosaccharide substrate (for a review see Ref. 4). Chondroitin sulfate GAGs considerably change in the position and degree of sulfation during normal embryonic development, growth, and malignant transformation (3, 5–7). The regulated expression of sulfated GAGs appears to be dependent on many factors including the availability of PAPS to the Golgi lumen; various competing sulfotransferases; and co-localization of appropriate acceptors, sulfotransferases, and PAPS transporters within a particular Golgi cisterna. However, the most important determinant of sulfated GAGs expression is probably the regulated expression observed for each member of the sulfotransferase gene family (7).

Based on known sulfated structures for chondroitin sulfate, the sulfotransferase gene family has been estimated to consist of at least four independent gene products described above, although it is possible that more sulfotransferases with different specificities exist. In terms of chondroitin 6-O-sulfotransferase (C6ST) that catalyzes the transfer of sulfate from PAPS to the C-6 position of the GalNAc residue, only one orthologous gene, designated C6ST-1, has been cloned to date from chickens, mice, and humans (8–10), despite the growing number of sulfotransferase cDNAs homologous to C6ST-1 that have been cloned (11–14). To search for additional members of the sulfotransferase gene family involved in chondroitin sulfate biosynthesis, the C6ST-1 protein sequence was used to screen the translated data base of expressed sequence tags (EST). Here, we describe the cloning of a human cDNA encoding a novel chondroitin 6-O-sulfotransferase, designated C6ST-2, with high specificity for polymer chondroitin.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB037187.

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‡ The abbreviations used are: GAG, glycosaminoglycan; bp, base pair(s); C6ST, chondroitin 6-O-sulfotransferase; EST, expressed sequence tags; GalNAc, N-acetyl-d-galactosamine; GlcA, d-glucuronic acid; Gn6ST, N-acetylgalucosamine-6-sulfotransferase; kb, kilobase(s); Δ′HexA, 4,5-unsaturated hexuronic acid or 4-deoxy-a-L-threo-hex-4-ene-pyranoxyuronic acid; HPLC, high performance liquid chromatography; KSGal6ST, keratan sulfate Gal-6-O-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.
Cloning of a Second Chondroitin 6-O-Sulfotransferase

EXPERIMENTAL PROCEDURES

Materials—35S]PAPS and unlabeled PAPS were purchased from NEN Life Science Products and Sigma, respectively. Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), various chondroitin sulfate isomers, desulfated keratan sulfate from bovine cornea, completely desulfated N-sulfated heparin from porcine intestine, four unsaturated standard disaccharides derived from C6ST-1 was a gift from K. Yoshida (Seikagaku Corporation). Superdex75m Peptide HR10/30 and HITRAP™ desalting columns were obtained from Amersham Pharmacia Biotech. A commercial human brain Multiple Tissue Northern blot II (CLONTECH) membrane was used for the analysis. 2 μg of polyadenylated RNA was loaded in each lane. The membrane was probed with a gel purified, radiolabeled (>1 × 106 cpm/μg), 1.3-kb PCR fragment amplified as described above.

Expression Levels of the Enzyme in Human Tissues—Commercial human multiple tissue cDNA panels (CLONTECH) were used for the analysis. The manufacturer normalizes each cDNA sample against six housekeeping genes. To verify this, we determined the level of amplification of the glyceraldehyde-3-phosphate dehydrogenase, whose transcript is always present in the tissues at a constant level (17). Using the normalized cDNA input, we performed the amplification of a transcript, using a serial number of cycles (27, 30, and 33 cycles) to find the conditions for a semiquantitative amplification. The best results were obtained by carrying out 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 35 s in a total volume of 50 μl using the 5′ and 3′ primers described above, which were designed to span the intron in the novel sulfotransferase gene to discriminate a PCR product amplified from cDNA from, if any, one amplified from contaminating genomic DNA. A 10-μl aliquot of the amplified PCR products were then visualized by electrophoresis on a 1.0% agarose gel containing ethidium bromide. To verify that the amplified DNA were derived from the novel sulfotransferase mRNA, the amplified fragments were gel purified, subcloned into the pGEM®-T Easy vector and sequenced. The nucleotide sequences of the amplified DNAs were identical to that of the novel sulfotransferase cDNA (data not shown).

RESULTS

Molecular Cloning of a Human cDNA Homologous to C6ST-1—we found a short sequence of 494 bp (GenBank™ accession number A1088880) through an EST approach in which we screened human EST data bases for sequences that were homologous to a human C6ST-1 (10). The EST clone from human EST data bases conformed to the Kozak consensus sequence for initiation (18), predicting that the protein has a type II transmembrane topology characteristic of many Golgi-localized sulfotransferases.
and glycosyltransferases cloned to date (Fig. 1). An additional characteristic feature in the amino acid sequence of the newly cloned gene was a cluster of six consecutive arginine residues in the cytoplasmic tail, which was located three residues from the amino terminus (Fig. 1).

Data base searches indicated that the amino acid sequence displayed 24, 27, 40, 27, and 27% identity to human C6ST-1 (10), human keratan sulfate Gal-6-O-sulfotransferase (KSGal6ST) (11), human N-acetylglucosamine 6-O-sulfotransferase (Gn6ST) (12), human high endothelial cell N-acetylglucosamine 6-O-sulfotransferase (HEC-Gn6ST) (13), and human intestinal N-acetylglucosamine 6-O-sulfotransferase (I-Gn6ST) (14), respectively. The highest sequence identity was found in the COOH-terminal catalytic domain, in which three highly conserved motifs (I–III), reported in previous studies (13, 14), were observed (Fig. 2). Motifs I and II contained elements that conformed to the recently described consensus binding motifs for the high energy sulfate donor, PAPS, which were predicted from the x-ray crystallographic analyses of estrogen sulfotransferase (20) and N-sulfotransferase (21). These elements are found in all sulfotransferases cloned to date. Notably, six invariant cysteines were observed in the catalytic domain. Comparison of the primary structure of the newly cloned gene product and the five other cloned sulfotransferases indicates that the C6ST-2 sequence is the longest of those for the six enzymes, which range from 386 to 486 amino acids in length (Fig. 2). This sequence conservation strongly suggests that the newly cloned gene product is a novel sulfotransferase.

Genomic Organization and Chromosomal Localization—Comparison of the newly cloned cDNA sequence with the genome sequence deposited in the Human Genome Project Data Base revealed the genomic structure and the chromosomal localization of the newly cloned gene. The gene spans over 24 kb, consists of at least two exons, and exhibits an intron-less coding region as shown in Fig. 3. Three highly conserved regions named motifs I–III, including putative PAPS binding sites (motifs I and II), are indicated by arrows.

Fig. 1. Nucleotide and deduced amino acid sequences of the novel sulfotransferase cDNA. The putative membrane spanning domain and four potential N-glycosylation sites are marked by asterisks. The presumptive polyadenylation signal ATTAAA is boxed. The sequences are numbered relative to the translation initiation site, which begins at the first in-frame ATG codon. The location of an intron is indicated by an arrowhead.

Fig. 2. Comparison of the predicted amino acid sequence of the human novel sulfotransferase (C6ST-2), C6ST-1, KSGal6ST, HEC-Gn6ST, and I-Gn6ST. The predicted amino acid sequences were aligned using the GENETYX-MAC (version 10) computer program. Black and shaded boxes indicate that the predicted amino acid in the alignment is identical among all six and more than any four sequences, respectively. The amino acid sequence of the newly cloned C6ST-2 displayed 24, 27, 40, 27, and 27% identity to human C6ST-1 (10), human KSGal6ST (11), human Gn6ST (12), human HEC-Gn6ST (13), and human I-Gn6ST (14), respectively. There are two regions in which more than four consecutive amino acid clusters are identical among the six sequences. Gaps introduced for maximal alignment are indicated by dashes. Three highly conserved regions named motifs I–II, including putative PAPS binding sites (motifs I and II), are indicated by arrows.
sequences (data not shown). This gene is located on human chromosome Xp11.

Expression of a Soluble Form of the Novel Sulfotransferase and Characterization as C6ST-2—To facilitate the functional analysis of the putative sulfotransferase, a soluble form of the protein was generated by replacing the first 63 amino acids of the putative sulfotransferase with a cleavable insulin signal sequence and a protein A IgG-binding domain as described under “Experimental Procedures,” and then the soluble putative sulfotransferase was expressed in COS-1 cells as a recombinant enzyme fused with the protein A IgG-binding domain. The fused enzyme expressed in the medium was absorbed on IgG-Sepharose beads to eliminate endogenous sulfotransferases, and then the enzyme-bound beads were used as an enzyme source. The bound fusion protein was assayed for sulfotransferase activity using a variety of GAG acceptor substrates. As shown in Table I, activity was detected with polymer chondroitin and desulfated keratan sulfate, although the latter showed 20-fold less sulfate incorporation. In contrast, no activity was detected with various chondroitin sulfate isoforms or completely desulfated N-sulfated heparin. No detectable sulfotransferase activity was recovered by the affinity purification from a control pSVL transfection sample. These findings clearly indicate that the expressed protein is a sulfotransferase with marked specificity for polymer chondroitin.

To identify the sulfotransferase reaction products, polymer chondroitin was labeled with [35S]sulfate by incubation with [35S]PAPS as a sulfate donor and the enzyme-bound beads as an enzyme source. The bound fusion protein was assayed for sulfotransferase activity using a variety of GAG acceptor substrates under the incubation conditions described under “Experimental Procedures.” As shown in Fig. 4, the digest yielded quantitatively a single [35S]-labeled peak at the position of Δ4,5HexAα1–3GalNAc(6-O-sulfate) (panel A), which was shifted to the position of inorganic sulfate by subsequent digestion with chondro-6-O-sulfatase (panel B) but not with chondro-4-O-sulfatase (panel C). These findings indicate that the sulfate was incorporated exclusively into the GalNAc C-6 position in a nonsulfated disaccharide unit, GlcAβ1–3GalNAc. Thus, the enzyme was identified as a novel chondroitin 6-O-sulfotransferase, designated C6ST-2 with marked specificity for a GlcAβ1–3GalNAc sequence.

To distinguish the substrate specificity of C6ST-2 from that of previously cloned C6ST-1, the recombinant soluble C6ST-2 was compared with recombinant human C6ST-1 (10) for their utilization of a variety of GAG acceptor substrates as well as various compounds structurally related to sulfated GAGs including GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. As shown in Tables I and II, C6ST-2 mainly utilized polymer chondroitin consisting of a nonsulfated disaccharide unit, GlcAβ1–3GalNAc, whereas C6ST-1 utilized various substrates including polymer chondroitin, various chondroitin sulfate isoforms, desulfated keratan sulfate, and inorganic sulfate as well as chondroitin sulfate (8, 10, 23). Thus, although

FIG. 3. Genomic organization of the human novel sulfotransferase (C6ST-2) gene. Nod1 (N), Pst I (P), and BamHI (B) restriction sites are shown as vertical lines. Exon regions are denoted by boxes. A closed box represents the coding sequence, and open boxes denote the 5′- and 3′-untranslated sequences. The translation initiation codon (ATG) and the termination codon (TAG) are also shown. The black horizontal bar denotes the intron.

FIG. 4. Identification of the novel sulfotransferase reaction products. Enzymatic reactions were carried out using polymer chondroitin as an acceptor substrate under the incubation conditions described under “Experimental Procedures.” The reaction products were separated from [35S]PAPS and its degradation products by gel filtration chromatography on a HiTRAPTM desalting column and were quantified by liquid scintillation counting. Relative rates for each enzyme are calculated as percentages of the incorporation obtained with polymer chondroitin. The incorporation was 7,342 dpm for C6ST-2 and 58,402 dpm for C6ST-1. The values represent the averages of two independent experiments, where the two series of experiments gave essentially identical results.

TABLE I

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>C6ST-2 %</th>
<th>C6ST-1 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chondroitin sulfate A</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>Chondroitin sulfate B</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Chondroitin sulfate C</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Chondroitin sulfate D</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Chondroitin sulfate E</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Desulfated keratan sulfate</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>CDSNS-heparin*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* CDSNS-heparin, completely desulfated N-sulfated heparin.

The recombinant C6ST-1 or -2 was incubated for 1 h with 100 μg each of the various acceptor substrates listed in the table under the conditions described under “Experimental Procedures.” The reaction products were separated from [35S]PAPS and its degradation products by gel filtration chromatography on a HiTRAP™ desalting column and were quantified by liquid scintillation counting. Relative rates for each enzyme are calculated as percentages of the incorporation obtained with polymer chondroitin. The incorporation was 7,342 dpm for C6ST-2 and 58,402 dpm for C6ST-1. The values represent the averages of two independent experiments, where the two series of experiments gave essentially identical results.
both enzymes utilized the GlcAβ1-3GalNAc sequence as their best substrates, C6ST-2 exhibited a more strict specificity, exhibiting negligible sulfate incorporation into galactosides that are good acceptor substrates for C6ST-1. These findings indicate that the two chondroitin 6-O-sulfotransferases differ in their substrate specificity and that C6ST-2 mainly transfers sulfate to the C-6 position of GalNAc in a nonsulfated disaccharide unit, GlcAβ1-3GalNAc.

Expression Pattern of C6ST-2—Northern blot analysis of mRNA demonstrated a single band of 2.4 kb for all human brain tissues examined (Fig. 5). The expression of the C6ST-2 gene was next determined in various human tissues using PCR-based methods with normalized cDNA pools. A single amplified DNA of the expected size (1328 bp) was obtained from most cDNA preparations of the 18 adult and 8 fetal human tissues examined, although the amounts of the amplified cDNAs varied considerably (Fig. 6). Notably, expression was not detected in adult skin and appears to be developmentally regulated in various tissues with expression persisting through adulthood in the spleen.

**DISCUSSION**

We cloned the second chondroitin 6-O-sulfotransferase, C6ST-2, which is homologous to but distinct from previously cloned C6ST-1. The two chondroitin 6-O-sulfotransferases exhibit distinct but overlapping acceptor substrate specificities as shown in Tables I and II. Polymer chondroitin is the best substrate for both enzymes, yet C6ST-2 exhibits a more strict specificity, exhibiting negligible sulfate incorporation into other substrates. Although no chondroitin 6-O-sulfotransferase with the specificity demonstrated for C6ST-2 has been reported so far, it has been suggested that at least one such sulfotransferase might exist, based on the observation that C6ST-1 knockout mice synthesize a small amount of chondroitin 6-sulfate and the mice are apparently normal and viable through adulthood (24). This hypothesis has now been confirmed by the cDNA cloning of C6ST-2. It is likely that additional distinct chondroitin 6-O-sulfotransferases exist. For example, because neither C6ST-1 nor -2 transferred sulfate to the C-6 position of a GalNAc residue in a monosulfated disaccharide unit, GlcAβ1-3GalNAc(4-O-sulfate), another chondroitin 6-O-sulfotransferase responsible for the synthesis of a disulfated disaccharide unit, GlcAβ1-3GalNAc(4-O-disulfate) probably exists. Likewise, a chondroitin 6-O-sulfotransferase that transfers sulfate to a monosulfated disaccharide unit, GlcA(2-O-sulfate)β1-3GalNAc to form GlcA(2-O-sulfate)β1-3GalNAc(6-O-sulfate) may also exist. The presence of multiple sulfotransferase isoforms has been reported for heparan sulfate D-glucosaminyl 3-O-sulfotransferases, where four homologous enzymes with distinct but overlapping acceptor specificities have been identified (25). Furthermore, three heparan sulfate D-glucosaminyl 6-O-sulfotransferases have recently been reported (26).

The recombinant C6ST-2 showed high specificity toward polymer chondroitin (Table I). No other chondroitin sulfate isoforms were utilized by the enzyme despite these chondroitin sulfate isoforms containing a small but significant amount of a nonsulfated disaccharide unit, GlcAβ1-3GalNAc. In strong contrast, the recombinant C6ST-1 utilized the nonsulfated disaccharide unit, GlcApβ1-3GalNAc in these isoforms (Ref. 10 and Table I). These findings suggest that the prior sulfation of the acceptor substrates strongly influences subsequent sulfations especially by C6ST-2. Thus, it will be interesting to determine how a preceding sulfation of saccharide residues on the reducing end and/or the nonreducing side(s) of saccharide sequences influences the 6-O-sulfation of the penultimate GalNAc using sulfated hexasaccharides such as GlcAβ1-3GalNAc(4-O- or 6-O-sulfate)β1-4GlcAβ1-3GalNAcβ1-4GlcAβ1-3GalNAc(4-O- or 6-O-sulfate) when such hexasaccharides become available.

C6ST-2 may provide functional redundancy with C6ST-1, as implied by the C6ST-1 knockout experiment that showed no apparent anomalies (24). Alternatively or in addition, C6ST-2 may play a unique role in the expression of the sulfation pattern of chondroitin sulfate in some tissues in view of the different specificity (Tables I and II) and distinct tissue expres-

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**FIG. 5.** Northern blot analysis of the C6ST-2 in human brain tissues. Northern blots with RNA from various human brain tissues were hybridized with a probe for C6ST-2, as described under “Experimental Procedures.”

**TABLE II**

Comparison of the oligosaccharide acceptor specificity of C6ST-1 and -2

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Sulfo transferase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C6ST-2</th>
<th>C6ST-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAcβ1-0-CH3</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GlcNAcβ1-3GlcNAcβ1-3Glcβ1-4GlcNAc</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The values represent the averages of two independent experiments, where the two series of experiments gave essentially identical results. 
<sup>b</sup>ND, not detected (<0.01 pmol/ml medium/h).

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**FIG. 6.** Differential expression of the C6ST-2 gene in various human tissues. The procedures used are described under “Experimental Procedures.” The arrow indicates the expected position for a PCR product.
sion of C6ST-1 and -2. Each gene exhibits a unique tissue-specific pattern of expression. In fetal tissues, abundant expression of the C6ST-2 gene was seen in the heart, lung, skeletal muscle, and spleen. Particularly striking is the abundant expression of the C6ST-2 gene in the adult spleen, with modest expression in the lung, pancreas, ovary, peripheral blood leukocytes, and small intestine (Fig. 6). In contrast, human C6ST-1 exhibited abundant expression in the adult heart, placenta, skeletal muscle, and thymus and very little in adult lung and peripheral blood leukocytes (23). Furthermore, a deficiency in chondroitin 6-O-sulfotransferase activity is reportedly associated with a heritable form of spondyloepiphyseal dysplasia (27, 28), suggesting that the chondroitin 6-O-sulfotransferase appears to be essential for normal skeletal development. Therefore, the products of the genes may not provide functional back-up in all cells. More detailed characterization of the C6ST-1 knockout mice will be required. In addition, generation and analysis of C6ST-2 knockout mice will provide further insights into the possible distinct functions of these genes.

Data base searches indicated that the amino acid sequence of C6ST-2 displayed 24, 27, 27, and 27% identity to the human C6ST-1 (10), KSGal6ST (11), Gn6ST (12), high endothelial cell Gn6ST (HEC-Gn6ST) (13), and intestinal Gn6ST (I-Gn6ST) (14), respectively, showing that they constitute a family of highly conserved enzymes. Although C6ST-2 displayed 24, 27, 40, 27, and 27% identity to the human C6ST-2 displayed 24, 27, 40, 27, and 27% identity to the human KSGal6ST, Gn6ST, and C6ST-2, indicating that these genes are localized on different human chromosomes, 11p11.1-p11.2, 7q31, and Xp11 (Refs. 11 and 12 and this study), respectively, despite the significant homology in nucleotide and amino acid sequences observed among the three genes. These findings strongly suggest that the three sulfotransferases diverged from an ancestor gene early in evolution. It remains to be determined whether the three other sulfotransferase genes, C6ST-1, HEC-Gn6ST, and I-Gn6ST, are likewise dispersed in the human genome.

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