Molecular Cloning and Characterization of a Human β-Gal-3’-sulfotransferase That Acts on Both Type 1 and Type 2 (Galβ1–3/1–4GlcNAc-R) Oligosaccharides*

Received for publication, June 28, 2000, and in revised form, October 10, 2000
Published, JBC Papers in Press, October 11, 2000, DOI 10.1074/jbc.M005666200

A novel sulfotransferase gene (designated GP3ST) was identified on human chromosome 2q37.3 based on its similarity to the cerebroside 3′-sulfotransferase (CST) cDNA (Honke, K., Tsuda, M., Hirahara, Y., Ishii, A., Makita, A., and Wada, Y. (1997) J. Biol. Chem. 272, 4864–4868). A full-length cDNA was obtained by reverse transcription-polymerase chain reaction and 5′- and 3′-rapid amplification of cDNA ends analyses of human colon mRNA. The isolated cDNA clone predicts that the protein is a type II transmembrane protein composed of 398 amino acid residues. The amino acid sequence indicates 33% identity to the human CST sequence. A recombinant protein that is expressed in COS-1 cells showed no CST activity, but did show sulfotransferase activities toward oligosaccharides containing nonreducing β-galactosides such as N-acetyllactosamine, lactose, lacto-N-tetraose (Le4), lacto-N-neotetraose (nLe4), and Galβ1–3GalNAc-benzyl (O-glycan core 1 oligosaccharide). To characterize the cloned sulfotransferase, a sulfotransferase assay method was developed that uses pyridylaminated (PA) Le4 and nLe4 as enzyme substrates. The enzyme product using PA-Le4 as an acceptor was identified as HSO3-3Galβ1–3GalNAcβ1–3Galβ1–4Glc-PA by two-dimensional 1H NMR. Kinetics studies suggested that GP3ST is able to act on both type 1 (Galβ1–3GlcNAc-R) and type 2 (Galβ1–4GlcNAc-R) chains with a similar efficiency. In situ hybridization demonstrated that the GP3ST gene is expressed in epithelial cells lining the lower to middle layer of the crypts in colonic mucosa, hepatocytes surrounding the central vein of the liver, extravillous cytrophoblasts in the basal plate and septum of the placenta, renal tubules of the kidney, and neuronal cells of the cerebral cortex. The results of this study indicate the existence of a novel β-Gal-3′-sulfotransferase gene family.

Sulfated glycoconjugates, whose sulfate groups are biologically relevant, occur in a wide range of biological compounds (reviewed in Ref. 1), including glycoproteins, proteoglycans, glycolipids, and polysaccharides. The enzymes responsible for the sulfation of these compounds, sulfotransferases, utilize in common the sulfate donor 5′-phosphoadenosine 3′-phosphosulfate (PAPS).1

We recently reported on the purification of the glycolipid 3′-sulfotransferase (cerebroside sulfotransferase (CST); galactosylceramide sulfotransferase, EC 2.8.2.11) to homogeneity from human renal cancer cells (2) and the cloning of the human CST mRNA on the basis of the amino acid sequence of the purified enzyme (3). The deduced amino acid sequence shows no overall homology to other sulfotransferases, except for the PAPS-binding motifs, suggesting that CST has a different evolutionary origin (3).

Carbohydrate structures with 3′-sulfo-β-Gal linkages have been found in both N-glycans (4, 5) and O-glycans (6–13) of glycoproteins, and the β-Gal-3′-sulfotransferase activities responsible for the synthesis of these glycoproteins have been demonstrated (4, 13–16). The β-Gal-3′-sulfotransferase synthesizing O-glycans has been demonstrated to be different from CST (13). Thus far, of the sulfotransferases that act on glycoproteins, the molecular cloning of Gal-6′-sulfotransferase and GlcNac-6-sulfotransferase has been accomplished (17–20). However, no β-Gal-3′-sulfotransferase genes responsible for the biosynthesis of 3′-sulfo-β-Gal linkages in glycoproteins have been cloned. The fact that CST transfers a sulfate group to C-3 of the nonreducing terminal β-galactoside in glycolipids prompted us to undertake a search for a novel β-Gal-3′-sulfotransferase gene using CST cDNA sequence as a probe.

EXPERIMENTAL PROCEDURES

Materials—[35S]PAPS (72.5 GBq/mmol) was purchased from PerkinElmer Life Sciences. Unlabeled PAPS, lacto-N-fucopentaose I (Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc), lacto-N-fucopentaose II (Galβ1–3Fucα1–4GlcNAcβ1–3Galβ1–4Glc), lacto-N-fucopentaose III (Galβ1–4Fucα1–3GlcNAcβ1–3Galβ1–4Glc), N-acetyllactosaminase,

* This work was supported by Grant-in-aid 10178104 for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan. 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.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number AB040610.

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¶ The abbreviations used are: PAPS, 5′-phosphoadenosine 3′-phosphosulfate; CST, cerebroside sulfotransferase; Le4, lacto-N-neotetraose; nLe4, lacto-N-neotetraose; PA, pyridylaminated; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; MES, 2-(N-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography; DQF, double quantum filter; HO-HAHA, homonuclear Hartmann-Hahn spectroscopy; ROESY, rotational nuclear overhauser effect spectroscopy; MOPS, 3-(N-morpholino)propanesulfonic acid; PA-Le4-S, sulfated pyridylaminated lacto-N-neotetraose.
GalNAcO-benzyl, Galβ1–3GalNAcO-benzyl, Galβ1–4Gal, Galα1–4Gal, and methyl-β-Gal were from Sigma. Lacto-N-tetraose (Galβ1–3Galβ1–4Glc; Lc4) and lacto-N-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc; LNe4) were purchased from Seikagaku Kogyo (Tokyo, Japan). Methyl-α-Gal and lactose were from Nacalai Tesque (Kyoto, Japan). Galactosylceramide and lactosylceramide were prepared and analyzed in our laboratory as described previously (2). PA-Lc4 and PA-nLc4 were synthesized by pyridylation of lacto-N-tetraose and lacto-N-neotetraose, respectively, using a GlycoTAG reagent kit (Takara, Shiga, Japan) with an automated pyridylation apparatus (GlycoTAG, Takara).

Cloning of Human GP3ST cDNA—One microgram of total RNA isolated from various human tissues (OriGene Technologies, Rockville, MD) was reverse-transcribed with random hexamers and then subjected to PCR. PCR was carried out using Taq DNA polymerase and two pairs of primers: CF2 (5′-TTCCACCATCAGTTGCC-3′, corresponding to human GP3ST cDNA nucleotides 284–300 in Fig. 1A) and CR2 (5′-TGAGCCAGGCTGAACTT-3′, corresponding to nucleotides 490–506) and CF1 (5′-CCACGACCTTCTTAC-3′, corresponding to nucleotides 524–540) and CR1 (5′-GGACGGATGTTCTGCT-3′, corresponding to nucleotides 1302–1318), which are located in the putative exons of the CST-like candidate gene in the CEB1 cosmid (GenBankTM/EBI accession number AF048727). The PCR products were electrophoresed on 1.5% agarose gel. DNA fragments with the expected sizes of 229 and 212 bp, respectively, were excised and subcloned into pT7 Blue(R) and sequenced as described above. The determined sequences were identical to those in the CEB1 cosmid, corresponding to nucleotides 121–138 in Fig. 1.

Overexpression of Human GP3ST in COS-1 Cells and Sulfotransferase Activity—To obtain a large-scale GP3ST cDNA expression system, a Dye Terminator cycle sequencing method using a Dye Terminator cycle sequencing kit (Life Technologies, Inc.) was employed as the eluant in DEAE A25 column chromatography as a substitute for the organic solvent. The determined sequences were identical to those in the CEB1 cosmid, corresponding to nucleotides 1115–1134).

DNA Cloning of β-Gal-3′-sulfotransferase—To obtain a GP3ST cDNA clone that contained the entire open reading frame, RT-PCR was accomplished using total RNA from human colon (OriGene Technologies) that had been pre-cultured for 1 day in a 10-cm diameter dish to be transfected with 10 μg of pSV-GP3ST and 30 μl of LipofectAMINE (Life Technologies, Inc.). After 72 h, the cells were washed twice with 10 ml of cold phosphate-buffered saline, harvested with 1 ml of phosphate-buffered saline using a silicon scraper, centrifuged at 1500 rpm for 2 min, sonicated in 0.3 ml of ice-cold Tris-buffered saline containing 0.1% Triton X-100, and assayed for sulfotransferase activity according to the method for CST activity (2). When oligosaccharides were subjected to DEAE A25 column chromatography, as described above, the effluent corresponding to the peak was collected, eluted, and dried. Approximately 17 nmol of the reaction product, as evaluated by fluorescence intensity, was deuterium-exchanged; dissolved in 0.1 ml of water; and 50 μl of the dissolved solution was injected onto a TSKgel SuperQ-5PW column (7.5 × 75 mm; Tosoh) equipped with a Shimadzu LC-VP HPLC system. Elution was performed with a linear gradient of 0–0.2 mM ammonium acetate (pH 9.0) and monitored with a fluorescence spectrophotometer (excitation, 320 nm; emission, 400 nm).

Northern Blot Analysis—Samples containing 20 μg of total RNAs from human brain, heart, skeletal muscle, kidney, stomach, small intestine, colon, lung, liver, and testis (OriGene Technologies) were deparaffinized tissue sections were immersed in 0.2M HCl for 20 min and then digested with 100 μg/ml proteinase K at 37 °C for 20 min, followed by digestion with 4% paraformaldehyde. These slides were rinsed with 2 ml/mg glycine, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), and then defatted with chloroform and air-dried. After prehybridization with 50% deionized formamide and 2× SSC for 1 h at 45 °C, these slides were hybridized with 0.5 mg/ml antisense or sense probe in 50% deionized formamide, 2.5 mM EDTA (pH 8.0), 300 mM NaCl, 1× Denhardt's solution, 10%
dextran sulfate, and 1 mg/ml brewers' yeast tRNA at 45 °C for 48 h. After hybridization, the slides were washed with 50% formamide and 23S S S Cf o r1ha t4 5° Ca n ddigested with 10 mg/ml RNase A at 37 °C for 30 min. After sequential washing with 23SSC and 50% formamide at 45 °C for 1 h and 13SSC and 50% formamide at 45 °C for 1 h, the sections were subjected to immunohistochemistry for detection of the hybridized probes using an alkaline phosphatase-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals). The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in the presence of levamisole and 10% polyvinyl alcohol. A control study using the sense probe showed no specific reactivity.

**FIG. 1.** cDNA and deduced amino acid sequences of human GP3ST. A, the predicted amino acid sequence is indicated by the single-letter amino acid code below the nucleotide sequence. The putative transmembrane portion is double-underlined. The potential N-glycosylation site is indicated by an asterisk. The presumed PAPS-binding sites are boxed. B, shown is a comparison of the amino acid sequences of human GP3ST and human CST (hCST) (3). Asterisks below the sequences show identical residues.
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### RESULTS

**cDNA Cloning and the Predicted Protein Sequences of a Novel Sulfotransferase**—When the GenBank™/EMBL/DDBJ DNA Data Bank was searched for a sequence homologous to the coding region of the human CST gene (3), a cosmid clone containing the minisatellite CEB1 (GenBank™/EMBL accession number AF048727), located on human chromosome 2q37.3, included a significantly similar sequence (25). The CEB1 cosmid includes two exons with open reading frames that together cover 1076 base pairs of the human CST mRNA (25). To investigate the issue of whether the CEB1 candidate gene actually encodes a functional sulfotransferase, we elected to clone its cDNA from human tissues. First, the cDNA fragments LIKE-C1 (nucleotides 284–506 in Fig. 1A) and LIKE-C2 (nucleotides 524–540), which were found in the putative exons of the CEB1 candidate gene, were obtained by RT-PCR using total RNAs from various human tissues. Since the RT-PCR product was strongly detected in the colon (Fig. 5), we employed human colon as a source of mRNA for subsequent cDNA cloning. The 5'- and 3'-ends of the cDNA of the sulfotransferase candidate were obtained by RACE analyses as described under “Experimental Procedures.”

Fig. 1A shows the DNA and deduced amino acid sequences of the cloned cDNA (termed GP3ST). The deduced protein is a type II membrane protein composed of 398 amino acids with a molecular mass of 45,902 Da and has six potential N-glycosylation sites. Its sequence exhibits 33% identity to the human CST sequence (Fig. 1B) and contains PAPS-binding motifs (26), suggesting that the cDNA encodes a sulfotransferase.

**Substrate Specificity of GP3ST**—To investigate the issue of whether the isolated cDNA encodes a functional sulfotransferase and, if so, on what substrates the sulfotransferase acts, the cloned cDNA was inserted into a mammalian expression vector pSVK3 (pSV-GP3ST) and overexpressed in COS-1 cells. We performed a substrate specificity experiment using [35S]PAPS as the sulfate donor, various carbohydrate compounds as acceptors, and cellular lysates of pSV-GP3ST-introduced COS-1 cells as an enzyme source (Table I). A recombinant protein expressed in COS-1 cells showed no CST activity, but did show significant sulfotransferase activities toward oligosaccharides containing nonreducing β-galactosides such as N-acetyllactosamine, lactose, Lac4, and Galβ1–3GalNAc-benzyl (O-glycoside core 1 oligosaccharide). On the other hand, the COS-1 cells transfected with pSV-CST (3) or with pSVK3 alone did not show sulfotransferase activity toward any of the oligosaccharides examined (data not shown).

**Sulfotransferase Assay Using PA-Lc4 or PA-nLc4 as an Acceptor**—To further characterize the cloned sulfotransferase, we developed a novel sulfotransferase assay system using pyridylamminated oligosaccharides as acceptors. This system was originally developed for use in the structural analysis of oligosaccharides (27) and was later applied to glycosyltransferase assays (28). To separate the acidic products from the neutral substrates, anion-exchange chromatography was employed. A typical elution pattern of the reaction products when PA-Lc4 or PA-nLc4 was used as an acceptor is shown in Fig. 2. The arrows

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indicate the elution positions of the products. When COS-1 cell lysates transfected with pSV-GP3ST were used as an enzyme source, a robust peak was detected at the positions indicated by the arrows (Fig. 2, b and d), whereas COS-1 cells transfected with the pSVK3 vector alone showed no significant peak at the indicated positions (Fig. 2, a and c). The yield of GP3ST products was increased relative to incubation time (over a period of 2 h) in a linear fashion, as well as at enzyme concentrations up to 1 mg/ml (data not shown).

Identification of the Reaction Product—To investigate the position where the sulfate group was transferred, a 1H NMR analysis of the enzyme product was performed. For this, a large-scale reaction was performed using PA-Lc4 as a donor, and the product was isolated by sequential HPLC with anion-exchange chromatography and C18 reverse-phase chromatography as described under “Experimental Procedures.”

H1–H4 of the Gal residues in PA-Lc4 and the sulfation product (PA-Lc4-S) were assigned by DQF-COSY and two-dimensional HOHAHA. As shown in Fig. 3, the H2–H4 signals of the terminal Gal (IV) residue in PA-Lc4 were shifted to the lower field by 0.14, 0.67, and 0.38 ppm, respectively, indicating unequivocally that the hydroxyl group at C-3 was esterified by a sulfate. On the other hand, the signals of the internal Gal (II) residue in PA-Lc4-S remained unchanged relative to those in PA-Lc4. To investigate whether or not C-6 was sulfated, the H5 protons were assigned from the two-dimensional ROESY spectra in which the cross-peak between H1 and H5 was observed. The difference in chemical shift of the H5 proton in Gal (IV) was 0.06 ppm (Table II), which is typical for the sulfation at C-3 of the terminal residue (22, 29). The additional downfield shift by 0.2 ppm, which should result from the sulfation at C-6 (22, 30), was not observed. These results clearly show that GP3ST is a β-Gal-3'-sulfotransferase that transfers a sulfate group to C-3 of the nonreducing β-galactosyl residue.

Some Properties of GP3ST—The optimal pH was found to be between 6.0 and 6.5 (Fig. 4) when a MES buffer was used. The effect of divalent cations on GP3ST activity is summarized in Table III. GP3ST essentially has no requirement for divalent cations; and in fact, Cu²⁺ and Zn²⁺ strongly inhibited the sulfotransferase activity. The K_m values for PA-Lc4, PA-nLc4, and PAPS were found to be 220, 480, and 240 μM, respectively (Table IV). Although the K_m value for PA-nLc4 was higher than that for PA-Lc4, the V_max for PA-nLc4 was higher than that for PA-Lc4, and V_max/K_m values were nearly equal for PA-Lc4 and PA-nLc4, suggesting that GP3ST is able to act on both type 1 and type 2 chains with a similar efficiency.

Tissue-specific Expression of the GP3ST Gene—Northern blot and RT-PCR analyses using total RNAs from various human tissues consistently showed that GP3ST is ubiquitously transcribed (Fig. 5). Among the examined organs, GP3ST mRNA was found to be highly expressed in heart, stomach,
middle layer of the crypts in colonic mucosa, hepatocytes surrounding the central vein of the liver, extravillous cytrophoblasts in the basal plate and septum of the placenta, renal tubules of the kidney, and neuronal cells of the cerebral cortex.

**DISCUSSION**

We report herein the identification of a novel functional sulfotransferase gene (named GP3ST) based on the similarity of its DNA sequence to that of the CST gene (3). The primary structure of GP3ST showed an overall similarity to that of CST with 33% identity. GP3ST catalyzes the transfer of a sulfate group to C-3 of the nonreducing β-galactoside in oligosaccharides such as CST. The similarities of structure and function between CST and GP3ST argue that they are members of the β-Gal-3′-sulfotransferase family. Similarly, several isoforms of this carbohydrate sulfotransferase have been found, e.g. heparan-sulfate N-deacetylated/N-sulfotransferase (31–33), heparan-sulfate glucosaminyl-3-O-sulfotransferase (34, 35), heparan-sulfate 6-O-sulfotransferase (36), chondroitin-sulfate/keratan-sulfate 6-O-sulfotransferase (17, 37), and GlcNAc-6-sulfotransferase (18–20). The distinct regulation of gene expression and the different substrate specificities of these isoforms may give rise to the specificity and diversity of sulfated glycans that are often found in a particular cell type.

CST acts on various nonreducing terminal β-galactosides in glycolipids such as galactosylceramide, lactosylceramide, galactosyl-1-alkyl-2-acyl-sn-glycol, and galactosylacylglycol, but it does not act on oligosaccharides that are not associated with lipid (Ref. 2 and this study), suggesting that CST recognizes the nonreducing β-galactoside of an oligosaccharide chain attached to a lipid moiety. On the other hand, GP3ST acts only on the nonreducing terminal β-galactoside of an oligosaccharide without lipid, suggesting that this enzyme is involved in the biosynthesis of sulfated glycoproteins. The substrate specificity of GP3ST is similar to that of the mucin 3′-sulfotransferase activity in rat colonic mucosa (15), although GP3ST prefers N-acetyllactosamine to the O-glycan core 1 structure. Since the sulfation of core 1 prevents the branching reaction to form a core 2 structure (15), GP3ST may regulate the biosynthesis of core 2. GP3ST also acts on methyl-β-galactoside to some extent, like the mucin 3′-sulfotransferase in human respiratory mucosa (13). Since N-acetyllactosamine is a good substrate for GP3ST, it may catalyze the sulfation of the N-acetyllactosamine structures of N-glycans like the calf thyroid 3′-sulfotransferase (4). The issue of whether GP3ST actually acts on N-glycans, O-glycans, or both remains to be solved.

The reaction product of GP3ST, 3′-sulfo-Galβ1–3GlcNAcβ1–3Galβ1–4Glc, can be an oligosaccharide ligand for L-selectin in vitro (38). Furthermore, the 3′-sulfo-Lea (Galβ1–3[Fucα1–4]GlcNAc-R) and -LeX (Galβ1–4[Fucα1–3]GlcNAc-R) structures were revealed to be more potent ligands for L-selectin than 3′-sialyl-Lea and -LeX (38, 39), although they have been observed only in epithelia (9–11). Alternatively, the 3′-sulfated oligosaccharide chains on epithelia might be involved in the adhesion of microorganisms such as Helicobacter pylori (40). GP3ST might be involved in the biosynthesis of 3′-sulfo-Lea and -LeX epitopes. In the substrate specificity experiment (Table I), lacto-N-tetraose and lacto-N-neotetraose served as good substrates for the sulfotransferase, whereas neither lacto-N-fucopentaose II nor lacto-N-fucopentaose III served as a substrate. This result suggests that 3′-sulfation of the nonreducing terminal Gal residue occurs prior to the 3′-4'-fucosylation of the penultimate GlcNAc residue in the biosynthetic pathway of 3′-sulfo-Lea and -LeX structures, as 3′-sialylation occurs before the 3′-4'-fucosylation in the synthetic pathway of 3′-sialyl-Lea and -LeX (41, 42).

The expression of the GP3ST gene was found to be ubiqui-
tous, with a relatively high level in the colon, where a considerable amount of mucin is generated and secreted. GP3ST may be involved in the biosynthesis of sulfated mucin in the human colon. A decreased level of mucin sulfation has been observed in colon carcinomas and ulcerative colitis (43–46). This decrease in mucin sulfation is associated with reduced Gal-3 colon carcinomas and ulcerative colitis (43–46). This decrease in mucin sulfation is associated with reduced Gal-3

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