Molecular Cloning and Expression of cDNA Encoding Human 3’-Phosphoadenylylsulfate:galactosylceramide 3’-Sulfotransferase*

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We have isolated a cDNA clone encoding human 3’-phosphoadenylylsulfate:galactosylceramide 3’-sulfotransferase (EC 2.8.2.11). Degenerate oligonucleotides, based on amino acid sequence data for the purified enzyme, were used as primers to amplify fragments of the gene from human renal cancer cell cDNA by the polymerase chain reaction method. The amplified cDNA fragment was then used as probe to screen a human renal cancer cell cDNA library. The isolated cDNA clone contained an open reading frame encoding 423 amino acids including all of the peptides that were sequenced. The deduced amino acid sequence predicts a type II transmembrane topology and contains two potential N-glycosylation sites. There is no significant homology between this sequence and either the sulfotransferases cloned to date or other known proteins. Northern blot analysis demonstrated that a 1.9-kilobase mRNA was unique to renal cancer cells. When the cDNA was inserted into the expression vector pSVK3 and transfected into COS-1 cells, galactosylceramide sulfotransferase activity in the transfected cells increased from 8- to 16-fold over that of controls, and the enzyme product, sulfated, was expressed on the transformed cells.

Sulfoglycolipids are a class of acidic glycolipids containing sulfate esters on their oligosaccharide chains which were originally found in the human brain by Thudichum (1). Sulfoglycolipids are abundant in myelin, spermatozoa, kidney, and small intestine (for review, see Ref. 2) and have been implicated in a variety of physiological functions through their interactions with extracellular matrix proteins, cellular adhesive receptors, blood coagulation systems, complement activation systems, cation transport systems, and microorganisms (for a review, see Ref. 3). The addition of sulfate ester is catalyzed by a sulfotransferase with PAPS1 serving as the sulfate donor.

We have demonstrated that GalCer sulfotransferase activity is remarkably enhanced in human renal cell carcinoma (4, 5) and that the sulfotransferase level in cancer cells is raised by the action of epidermal growth factor (6), transforming growth factor-α (7), and hepatocyte growth factor (8). Furthermore, tyrosine kinases have been shown to be involved in the expression of sulfotransferase in cancer cells (9). Previously, we purified GalCer sulfotransferase to apparent homogeneity from human renal cancer cells (10). Not only GalCer but also lactosylceramide, galactosyl 1-alkyl-2-acyl-sn-glycerol, and galactosyl diacylglycerol served as good acceptors for the purified enzyme. Now we have cloned a cDNA encoding the human GalCer sulfotransferase on the basis of the partial amino acid sequence of the purified enzyme. This is the first report on gene cloning of glycolipid sulfotransferase.

EXPERIMENTAL PROCEDURES

Amino Acid Sequencing of Peptides Derived from GalCer Sulfotransferase—GalCer sulfotransferase was purified from 1 × 10^9 cells from a human renal cell carcinoma cell line, SMKT-R3 (11) using a method described previously (10). The purified sulfotransferase (10 μg) was reduced and S-pyridylethylated. The treated enzyme was applied to a reversed phase HPLC column (Cosmosil 5C4-AR-300, 4.6 × 50 mm, Nacalai tesque, Japan) and eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Following digestion of the isolated enzyme with lysylendopeptidase (Achromobacter protease I, Wako, Japan), the hydrolysate was subjected to another reversed phase HPLC column (Aquapore OD-300, 7 μm, 1.0 × 250 mm, Applied Biosystems) and eluted with the solvent system described above. The NH2-terminal sequence analysis of separated peptides was performed by automated Edman degradation using a protein sequencer (Applied Biosystems 492).

Oligonucleotides and Polymerase Chain Reaction—Based on the amino acid sequence of peptides (Table I) derived from the purified GalCer sulfotransferase, degenerate oligonucleotides of both sense and antisense strands were synthesized with deoxyxinoine substitution (Applied Biosystems 392) as indicated in Table II. These oligonucleotides served as primers for RT-PCR analysis using total RNA from SMKT-R3 cells. A reverse transcriptase reaction was performed at 37 °C for 1 h using 50 pmol of oligo(dT) primers, 2 μg of total RNA, a 0.5 mM concentration of each dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, and 200 units of Moloney murine leukemia virus reverse transcriptase (SuperScript II, Life Technologies, Inc.) in a final volume of 20 μl. The reaction mixture of the following PCR contained 4-μl aliquots of the reverse transcriptase reaction solution, 100 pmol of each primer, 0.25 mM concentration of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 1.25 units of Taq polymerase (Perkin-Elmer) in a final volume of 50 μl. The reactions were subjected to 35 cycles of denaturation at 94 °C for 30 s, annealing at 45–55 °C for 30 s, and extension at 72 °C for 1–2 min. After agarose electrophoresis of the PCR products, DNA fragments were excised, subcloned into pT7Blue vector (Novagen), and sequenced as described below.

cDNA Library of Human Renal Cancer Cells—Total RNA was extracted from SMKT-R3 cells, and poly(A)+ RNA was purified with oligo(dT) latex (Oligo-ex-T30, Roche). Double-stranded cDNA was synthesized using a cDNA synthesis kit (SuperScript Choice System, Life
The amino acid sequences are written using the single letter code. X indicates the amino acid residues that could not be assigned. The underlined amino acid residues indicate discrepancies from those predicted by the cDNA sequence.

| Peptide | Amino acid sequence |
|---------|---------------------|
| P1      | TASSTTLNLRFQGK      |
| P2      | KFESMMAK            |
| P3      | SILEYLNK            |
| P4      | LGSDGG              |
| P5      | LNAKNDXVP           |
| P6      | HRLK                |
| P7      | FIGFLEX             |

Technologies, Inc.). Briefly, the first cDNA strand was synthesized by reverse transcription of the poly(A)^+ RNA with oligo(dT) primers. After synthesis of the second strand, the double-stranded cDNA was ligated with an EcoRI adapter. Then, the EcoRI-adapted cDNA was ligated to EcoRI-digested agt10 and subsequently packaged in vitro (Ready-To-Go Lambda Packaging Kit, Pharmacia Biotech Inc.).

**Oligonucleotide Probe and Southern Blot Analysis**—Based on the sequence of the PCR products using primer sets 1Sd and 1A, a hybridization buffer containing 2 pmol/ml digoxigenin-labeled oligonucleotide probe, 5 × SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% N-laurylsarcosine, and 0.02% SDS. The detection procedure was carried out with a DIG luminescent detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Isolation of cDNA Clones from the Human Renal Cancer Cell cDNA Library**—Approximately 2 × 10^9 recombinant phages were screened by plaque hybridization with a digoxigenin-labeled RNA probe (see "Results") which had been synthesized using a DIG RNA labeling kit with T7 RNA polymerase (Boehringer Mannheim). Hybridization was carried out at 50 °C overnight with a nylon membrane (Boehringer Mannheim) in a hybridization buffer containing 1 ng of digoxigenin-labeled RNA probe/cm² of membrane, 50% formamide, 5 × SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% N-laurylsarcosine, and 0.02% SDS. Six λ phage clones were picked up from positive plaques, and the inserted cDNAs were subcloned into pBluescript (Toyobo, Tokyo, Japan) by EcoRI digestion.

**DNA Sequencing**—The subcloned DNAs were sequenced by the dideoxy chain termination method using Taq DNA polymerase (dye terminator cycle sequencing kit, Perkin-Elmer) with a DNA sequencer (Applied Biosystems 373A).

**Northern Blot Analysis**—Ten μg of total RNA from SMKT-R3, THP-1 (human monocytic leukemia), GOTO (human neuroblastoma), and HT-1080 (human fibrosarcoma) cells were denatured in 50% (v/v) formamide, 6% (v/v) formaldehyde, 20 mM MOPS (pH 7.0) at 65 °C, electrophoresed in a 1% agarose gel containing 6% formaldehyde, and transferred to a nylon membrane (Boehringer Mannheim). A digoxigenin-labeled DNA probe was synthesized from the 0.64-kilobase SacI fragment of pBS-ICST1 (nucleotides 425–1065 in Fig. 1) using a DIG high prime kit (Boehringer Mannheim). The membrane was hybridized with the DNA probe at 50 °C. Other methods were the same as those used for the plaque hybridization.

Expression of GalCer Sulfotransferase cDNA in COS-1 Cells—pBS-lcST1 was digested with EcoRI, and the inserted DNA was ligated into the EcoRI site of expression vector pSVSK3 (Pharmacia). The direction of the inserted sequence was determined by the restriction enzyme map. COS-1 cells (2 × 10⁵) precultured for 1 day in a 35-mm-diameter dish were transfected with 1 μg of plasmid DNA and 5 μl of LipofectAMINE (Life Technologies, Inc.). After 72 h, the cells were washed twice with 2 ml of cold Tri-buffered saline, and incubated with 0.2 ml of Tri-buffered saline containing 0.1% Triton X-100 using a silicon scraper, sonicated on ice, and assayed for GalCer sulfotransferase activity (12) and protein concentration (BCA protein assay kit, Pierce).

To examine the expression of sulfatide on the COS-1 cells transfected with the cDNA, the cells (1 × 10⁶) were transfected with 0.5 μg of plasmid DNA and 1 μl of LipofectAMINE in a Lab-Tek chamber slide (Nunc Inc.). After 48 h, the cells were washed with phosphate-buffered saline, fixed in 1% paraformaldehyde in phosphate-buffered saline, and incubated with an anti-sulfatide monoclonal antibody, SulphI (13), followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Zymed Laboratories Inc.). Each incubation was for 45 min. Labeled cells were washed in Vectashield mounting medium (Vector Laboratories). A Zeiss Axioshot with epi-illumination for fluorescence was used for fluorescence and phase microscopy.

**RESULTS**

**Amino Acid Sequence Determination and Preparation of cDNA Probe by PCR**—To determine a partial amino acid sequence, purified GalCer sulfotransferase was digested with lysylendopeptidase, and the hydrolysates were isolated on a reversed phase HPLC column. Amino acid sequences determined for seven peptides are shown in Table I. Based on the amino acid sequences of three peptides, P1, P2, and P3, we synthesized degenerate oligonucleotides for sense and antisense primers (Table II). To reduce the primer combinations, deoxyinosine was substituted in positions where the codon degeneration exceeded 2, and the frequently used codon, CTX, was employed for leucine. For each serine residue, we prepared primer combinations of two codon types: TCX and AGCT. Oligonucleotides 1Sa–d and 1A were synthesized on the basis of the amino- and the carboxyl-terminal sequences, respectively, of P1. Four possible pairs of sense (1Sa–15d) and antisense (1A) primers were first used in RT-PCR analysis with total RNA from human renal cancer cells, SMKT-R3, as the template. The pair of 15d and 1A primer sets produced a cDNA fragment of 47 bp, corresponding to the length estimated from the amino acid sequence of P1. When the fragment was subcloned and sequenced, the deduced amino acid sequence coindexd with that of P1. Then, we synthesized a degenerate oligonucleotide probe (OP1) based on the sequence of the 47-bp cDNA fragment for Southern hybridization of RT-PCR products. In the RT-PCR products using primer sets of 2Sa and 3A, a band of 600 bp was hybridized with the OP1 probe. Based on sequencing analysis, the 600-bp fragment included the sequence encoding P1 and P4, suggesting that the fragment was a part of

| Oligonucleotide | Nucleotide sequence |
|-----------------|---------------------|
| 1Sa             | 5'-ACIGCITCTICTCATTCT-3' |
| 1Sb             | 5'-ACIGCITCTICTCATTCT-3' |
| 1Sc             | 5'-ACIGCITCATTCTATCT-3' |
| 1Sd             | 5'-ACIGCITCATTCTATCT-3' |
| 1A              | 5'-TTTGGCCACAAAAGAAGA-3' |
| 2Sa             | 5'-AACCCCTGCATTCTTAT-3' |
| 2Sb             | 5'-AACCCCTGCATTCTTAT-3' |
| 2Ab             | 5'-TGGGATATATTAGTT-3' |
| 3S              | 5'-ATTTTCGTTTATGTT-3' |
| 3A              | 5'-TTATAGATTATATTGTTA-3' |

**TABLE II**

| Oligonucleotide primers for the cDNA clone isolation experiments using PCR |
|-------------------------------------------------|
| Degenerate oligonucleotides 1–3 were derived from the sequences of peptides P1–P3, respectively; S indicates sense, and A indicates anti-sense directions. In the case of sequences containing serine residue(s), oligonucleotide combinations possessing either TCX or AGT/C as the codon for each serine residue were synthesized (1Sa-15d, 2Sa-2Sb, and 2Sb-2Ab). Nucleotide I indicates deoxyinosine. |
the target gene. However, we later recognized that the 600-bp fragment lacked nucleotides 304–425 and 825–1291 sequences of the cloned cDNA (Fig. 1A) and contained an aberrant sequence of 70 nucleotides. The reason for the discrepancy is not clear at present.

Screening of cDNA Library of Human Renal Cancer Cells—We have screened a λgt10 cDNA library made from SMKT-R3 cells using a digoxigenin-labeled RNA probe synthesized from the above described 600-bp cDNA fragment. Six positive clones were isolated from 2 × 10^6 plaques and subcloned into pBluescript. The nucleotide sequence of the largest cDNA insert (1.8 kilobases), termed pBS-hCST1, was determined.

cDNA and Predicted Protein Sequence of the GalCer Sulfo-
transferase—DNA sequencing analysis revealed that the cDNA consisted of 1791 nucleotides with a putative initiator codon at 204 and a TGA stop codon at 1473, having an open reading frame encoding 423 amino acid residues with a molecular mass of 48,763 Da. A putative polyadenylation signal was located at nucleotide 1761 followed by poly(A) tracts. The 1801-bp cDNA length is highly consistent with the mRNA size observed in SMKT-R3 cells (Fig. 2), indicating that the cDNA is nearly full-length. The deduced amino acid sequence contained two potential N-linked glycosylation sites (Fig. 1A). Since the renal cancer GalCer sulfotransferase bound to lectin columns such as concanavalin A- and wheat germ agglutinin-agarose, the enzyme may be modified by N-linked oligosaccharide chain(s). If the enzyme protein possesses two N-linked oligosaccharide chains, the molecular mass will agree with that of the purified protein, which is 54 kDa, observed on SDS-polyacrylamide gel electrophoresis (10). The deduced amino acid sequence in-

FIG. 1. Nucleotide and deduced amino acid sequences of the human GalCer sulfotransferase and hydropathy plot of the protein. Panel A, the predicted amino acid sequence is indicated by the single letter amino acid code below the nucleotide sequence. The positions of the seven peptide sequences obtained by digestion of the purified sulfotransferase are underlined with a single continuous line. Asterisks indicate potential N-glycosylation sites. The putative transmembrane hydrophobic domain is underlined with double continuous lines. Panel B, the hydropathy plot was calculated by the method of Kyte and Doolittle (27) with a window of 11 amino acids.
cDNA Cloning of Human Glycolipid Sulfotransferase

We have cloned a cDNA that encodes human GalCer sulfotransferase. Several lines of evidence indicate that the cloned cDNA corresponds to the GalCer sulfotransferase purified previously from human renal cancer cells: (a) the predicted sequence of the protein contains all seven peptides obtained from the purified enzyme protein; (b) when the cDNA was introduced into a eukaryotic expression vector and transfected into COS-1 cells, the enzyme activity expressed exceeded that in controls by 8–16-fold; and (c) the characteristics of the predicted protein are consistent with those of the purified protein in terms of molecular mass and membrane localization.

Sulfonation is an important pathway in the metabolism of many drugs, xenobiotics, hormones, and neurotransmitters. Sulfotransferases involved in this process are cytosolic enzymes and a number of cDNA clones coding for these sulfotransferases have been isolated (for a review, see Ref. 14). These cytosolic sulfotransferases, including plant flavonol sulfotransferases, show considerable homology and have been classified into three families on the basis of their amino acid sequences (14). However, Golgi sulfotransferases functioning in the sulfonation of glycosaminoglycans, N-deacetylase/N-sulfotransferase (15–17) and chondroitin 6-sulfotransferase (18), have little homology with the cytosolic sulfotransferases. There is no significant homology between N-deacetylase/N-sulfotransferase and chondroitin 6-sulfotransferase (18). The present GalCer sulfotransferase showed homology to neither the cytosolic sulfotransferases nor the Golgi sulfotransferases. These observations suggest that GalCer sulfotransferase has

**Discussion**

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an evolutionary origin different from that of the other sulfotransferases.

Based on a modification with pyridoxal phosphate, we suggested previously that lysine residue(s) may be involved in the PAPS binding site of human GalCer sulfotransferase (19). Hashimoto et al. (15) noted a putative PAPS binding motif in sulfotransferases, GXXGXXK, which resembles the P-loop nucleotide binding motif for ATP- and GTP-binding proteins, and this motif is actually conserved near the COOH terminus of many sulfotransferases cloned to date (20). However, there was no such sequence in GalCer sulfotransferase. The conserved motif, YPKSGT(T/N)W, which is located in the NH2-terminal portion of cytosolic sulfotransferases and bacterial sulfotransferases and has been suggested to be a PAPS binding site (21), was not present in GalCer sulfotransferase. Another candidate for the PAPS binding motif, LEKCGR, which has been demonstrated in arylsulfotransferase IV by affinity labeling with ATP dialdehyde (22), was also absent from GalCer sulfotransferase. Accordingly, we plan to continue the search for another PAPS binding motif in GalCer sulfotransferase.

Since there is only one in-frame ATG codon upstream from the sequence of the P2 peptide, which is the most upstream peptide obtained, in the open reading frame, we identified the initiation codon a priori. Compared with the consensus eukaryotic translation initiation sequence (23), the most important purine, which is at the −3 position, is conserved. The putative hydrophobic transmembrane domain of GalCer sulfotransferase contains 23 amino acid residues with cationic borders characteristic of type II transmembrane proteins. From the nucleotide sequence of the cDNA, we deduced the amino acid sequence of GalCer sulfotransferase, showed it to consist of 423 amino acids, and calculated a molecular mass of 48,763 Da. Considering the two N-glycosylation sites, the 54-kDa purified human enzyme (10) appears to reflect the size of the mature enzyme. However, the sizes of the GalCer sulfotransferases from rat kidney (24) and testis (25), 64 and 56 kDa, respectively, are too large. On the other hand, the 31 kDa of the mouse brain enzyme (26) is too small. Although the reason for these discrepancies is unknown, possibilities include species differences and the existence of isoforms.

On Northern analysis, transcripts of GalCer sulfotransferase were detected only in SMKT-R3 cells. When we measured the sulfotransferase activity of various human cell lines, this sulfotransferase was detectable only in renal cancer cells (5). These observations suggest that the high sulfotransferase activity in renal cancer cells is based on a high level of the gene expression. Future studies will be directed at elucidating the regulatory mechanisms of sulfotransferase expression in human renal cancer cells. It is also of particular interest to ascertain what causes the tissue-specific expression of sulfoglycolipids. In addition, experimental manipulation of sulfotransferase expression may clarify the physiological role of sulfoglycolipids in various biological processes such as myelination and fertilization.

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