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Purification and Characterization of 3′-Phosphoadenosine-5′-Phosphosulfate:GalCer Sulfotransferase from Human Renal Cancer Cells[1]

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We have purified 3′-phosphoadenosine-5′-phosphosulfate:GalCer sulfotransferase [EC 2.8.2.11] from a human renal cancer cell line SMKT-R3 through a combination of affinity chromatographies using galactosylsphingosine, 3′,5′-bisphosphoadenosine and heparin as ligands. The purified sulfotransferase showed a specific activity of 1.2 μmol/min/mg, which is 300 times more than the highest activity among the enzyme preparations purified so far from other sources. Homogeneity of the purified sulfotransferase was supported by the facts that the enzyme preparation showed a single protein band with an apparent molecular mass of 54 kDa on reducing SDS-PAGE and that protein bands coincided with the enzyme activity on both native PAGE and nonreducing SDS-PAGE. GalCer was the best acceptor for the purified enzyme. LacCer, GalAAG, and GalDG were also good acceptors. GlcCer, Gg3Cer, Gg4Cer, Gb4Cer, and nLc4Cer did serve as acceptors although the relative activities were low. On the other hand, the enzyme could not act on Gb3Cer, which possesses α-galactoside at the nonreducing terminus. Neither galactose nor lactose served as an acceptor. These observations suggest that the sulfotransferase prefers β-glycoside, especially β-galactoside, at the nonreducing termini of sugar chains attached to a lipid moiety.

Key words: affinity chromatography, biosynthesis of glycosphingolipids, renaturation, substrate specificity, sulfatide.

Sulfoglycolipids are a class of acidic glycolipids containing sulfate esters on their oligosaccharide chains, and are responsible for some of the negative charge on the cell surface. Sulfoglycolipids are suggested to be implicated in a variety of physiological functions through their specific binding to several proteins including laminin (*1, 2*), thrombospondin (*3*), von Willebrand factor (*4*), multicatalytic protease (*5*), amphoterin (*6*), and HGF (*7*).

Kidney is one of the tissues rich in sulfoglycolipids (for a review see Ref. *8*) and hence has been employed to study their biosynthesis (*9, 10*). In our previous study, it was demonstrated that human renal cell carcinoma tissue (*11*) and a cell line derived therefrom (*12*) had markedly increased glycolipid sulfotransferase activity, which was associated with an accumulation of sulfoglycolipids. The sulfotransferase level in the cell line was significantly enhanced through protein synthesis induced by the action of growth factors such as EGF (*13*), TGF-α (*14*), and HGF (*15*). These observations led us to assume that the involvement of sulfoglycolipids in physiological and pathological functions is regulated by the growth factors through changes in the gene expression of the sulfotransferase. Since a sulfotransferase gene has yet to be cloned, we undertook to purify the enzyme from a kidney tumor cell line to obtain a clue for gene cloning.

Although GalCer sulfotransferase has been purified from rat kidney (*10*) and testis (*16*) and mouse brain (*17*), the properties of the enzymes were different from each other and from that of the human enzyme in terms of molecular mass, specific activity, and other properties. The sulfotransferase purified from human renal cancer cells in the present study represented a 300-fold enriched specific activity compared with the highest activity among the enzyme preparations so far purified (*16*).

MATERIALS AND METHODS

Materials—[^{35}S]PAPS (72.5 GBq/mmol) was purchased from New England Nuclear. Unlabeled PAPS, PAP, N^6-(6-aminohexyl)-adenosine 3′,5′-diphosphate, and GalDG

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Abbreviations: BSA, bovine serum albumin; EGF, epidermal growth factor; GalAAG, galactosyl 1-alkyl-2-acyl-*sn*-glycerol; GalCer, galactosylceramide; GalDG, galactosyl diacylglycerol; GalSph, galactosylsphingosine; Gb3Cer, globotriaosylceramide; Gb4Cer, globotetraosylceramide; Gg3Cer, gangliotriaosylceramide; Gg4Cer, gangliotetraosylceramide; GlcCer, glucosylceramide; GM2, II^{3}NeuAcαGg$_3$Cer; GM1, II^{3}NeuAcαGg$_4$Cer; HGF, hepatocyte growth factor; LacCer, lactosylceramide; nLc4Cer, neolactotetraosylceramide; PAP, 3′,5′-bisphosphoadenosine; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PBS, phosphate-buffered saline; PLP, pyridoxal 5′-phosphate; SM2a, gangliotriaosylceramide sulfate, Gg3Cer II^3-sulfate; SM2b, gangliotriaosylceramide sulfate, Gg3Cer III^3-sulfate; SM3, lactosylceramide sulfate, LacCer II^3-sulfate; SM4, galactosylceramide sulfate, GalCer I^3-sulfate; TBS, Tris-buffered saline; TGF-α, transforming growth factor α.

were from Sigma; murine EGF, *Arthrobacter ureafaciens* sialidase, and bovine testis β-galactosidase from Boehringer Mannheim; NHS-activated HiTrap, heparin-Sepharose CL-6B, EAH-Sepharose 4B, ECH-Sepharose 4B, and DEAE Sephadex A-25 from Pharmacia; and DE-52 from Whatman. Precoated TLC plates (Silica Gel 60) were obtained from Merck. Other reagents were of analytical grade.

Glycosphingolipids were purified in our laboratory; GalCer and GM1 from bovine brain; GlcCer from a Gaucher's spleen; LacCer, Gb3Cer, Gb4Cer, and IV^3-NeuAcα-nLc4Cer from human erythrocytes; GM2 from a Tay-Sachs' brain. Gg3Cer, Gg4Cer, and nLc4Cer were prepared from GM2, GM1, and IV^{3}NeuAcα-nLc4Cer, respectively, using *A. ureafaciens* sialidase. GalAAG I^3-sulfate, seminolipid was a generous gift from Dr. Ineo Ishizuka, Teikyo University, Tokyo. GalAAG was prepared from GalAAG I^3-sulfate by solvolysis (*18*). GalSph was prepared from GalCer as described (*19*).

Cell Culture—SMKT-R3 cells established from surgically resected human renal cell carcinoma specimens (*20*) were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. Before harvest, the cells were incubated with 50 ng/ml of EGF for 12–24 h.

Protein Concentration—Protein concentrations were determined by either a dye-binding assay (Bio-Rad) or a bicinchoninic acid assay (Pierce Chemical), using bovine serum albumin as a standard.

Assay of GalCer Sulfotransferase—GalCer sulfotransferase activity was assayed as described previously (*21*) with a slight modification. The reaction mixture contained 5 nmol of GalCer, 0.5 μmol of $MnCl_2$, 1 nmol of [^{35}S]PAPS (100 cpm/pmol), 0.5 mg of Lubrol PX, 12.5 nmol of dithiothreitol, 0.25 μmol of NaF, 0.1 μmol of ATP, 20 μg of BSA, and enzyme protein in 25 mM Na cacodylate-HCl, pH 6.5, in a total volume of 50 μl. In the experiments to examine substrate specificity, 25 nmol of each test glycolipid was used as an acceptor. After incubation at 37°C for 30 min, the reaction was terminated with 1 ml of chloroform/methanol/water (30 : 60 : 8). The reaction product was isolated on a DEAE-Sephadex A-25 column and assayed for radioactivity using a liquid scintillation counter. The values were corrected for a blank value, which was obtained by using a reaction mixture devoid of the acceptor. One unit of the activity was defined as the amount of enzyme that transferred 1 μmol of sulfate per minute under the standard assay conditions.

Preparation of Affinity Columns—PLP-Sepharose was prepared as described previously (*22*).

HiTrap PAP was prepared by coupling of N^6-(6-aminohexyl)-adenosine 3′,5′-diphosphate to NHS-activated HiTrap according to the manufacturer's recommendations. The coupling yield assessed by measuring A_{260} of the coupling solution was 5.8 μmol/ml gel.

GalSph-Sepharose was prepared by coupling of GalSph to ECH-Sepharose 4B. Fifty micromoles of GalSph dissolved in 20 ml of ethanol was incubated with ECH-Sepharose 4B (10 ml of packed gel) at 70°C for 5 min. After addition of 200 mg of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, the suspension was incubated at 70°C for 3 h. Then the gel was filtered, washed and treated with monoethanolamine to block the functional groups that remained. Through these procedures a coupling efficiency of 2 μmol/ml gel was attained, as measured for galactose in the unreacted coupling solution.

Purification of GalCer Sulfotransferase from EGF-Treated SMKT-R3 Cells—Solubilization of SMKT-R3 cells: SMKT-R3 cells treated with 50 ng/ml EGF for 12–24 h were harvested, washed with PBS, and stored at −80°C until use. For use, 2.5×10^9 cells were thawed, suspended in the same volume of TBS and homogenized briefly with a Potter-type homogenizer. The homogenate was supplemented with the same volume of 2× solubilization buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 2% Lubrol PX, 40% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.02 mM E-64) and sonicated for 10 min on ice. After centrifugation at 100,000 × *g* for 1 h, the supernatant was dialyzed against buffer A (10 mM triethanolamine-HCl, pH 7.0, 10% glycerol, and 5 mM $MnCl_2$) overnight.

DE-52/heparin-Sepharose CL6B: The dialyzed material was centrifuged at 10,000 × *g* for 30 min to remove precipitates appearing during dialysis. No sulfotransferase activity was detected in the precipitates. The supernatant was applied to a DE-52 column (3 × 20 cm) the outlet of which was connected directly to a column (2 × 10 cm) of heparin-Sepharose CL6B that had been equilibrated with buffer B (10 mM triethanolamine-HCl, pH 7.0, 0.05% Lubrol PX, 10% glycerol, and 5 mM $MnCl_2$). The column was then washed with buffer B at a flow rate of 40 ml/h until the absorbance of the effluent at 280 nm became less than 0.02. After disconnection of the DE-52 column, the enzyme on the heparin-Sepharose column was eluted with 0.2 M NaCl in buffer C (20 mM triethanolamine-HCl, pH 7.0, 0.1% Lubrol PX, 20% glycerol, and 10 mM $MnCl_2$).

Affinity chromatography on GalSph-Sepharose: The enzyme-active fractions on heparin-Sepharose chromatography were pooled and dialyzed against buffer B. The dialyzate was applied at a flow rate of 5 ml/h to a column (1 × 10 cm) of GalSph-Sepharose, which had been equilibrated with buffer B. The column was then washed with buffer B until the eluate was essentially free of protein and the sulfotransferase was eluted with buffer C containing 0.1 M NaCl.

Affinity chromatography on HiTrap PAP: The eluate fractions on GalSph-Sepharose were pooled and dialyzed against 10 mM triethanolamine-HCl (pH 7.0) containing 10% glycerol and applied at a flow rate of 5 ml/h onto a column (1 × 10 cm) of PLP-Sepharose connected directly to a HiTrap PAP column (5-ml bed volume) which had been equilibrated with buffer D (10 mM triethanolamine-HCl, pH 7.0, 0.05% Lubrol PX, and 10% glycerol). The column was washed successively with buffer D and buffer B until the eluate was essentially free of protein, then the sulfotransferase was eluted with a linear gradient of 0–0.3 mM PAP in buffer D.

The enzyme-active fractions on HiTrap PAP chromatography were pooled and directly subjected to a second chromatography on a heparin-Sepharose column (0.3-ml bed volume) which had been equilibrated with buffer D. After washing of the column with buffer D, the sulfotransferase was eluted in 0.3-ml fractions with buffer C containing 0.3 M NaCl. Through this step, the enzyme preparation was concentrated to about one-fifth of the initial volume, and PAP, which was included in the enzyme preparation

from the preceding chromatography, was recovered in the flow-through fractions. The enzyme-active fractions were pooled and stored at −80°C in the presence of 20% glycerol.

Electrophoresis—Native PAGE was performed using a 5% slab-gel (1-mm width) under the conditions of Davis (*23*) except for the inclusion of 0.1% Lubrol PX and 10% glycerol in the gel and running buffer. Purified sulfotransferase (0.2 milliunits) was run in two separate lanes with a constant current of 8 mA for 8 h at 4°C. Under these conditions bromphenol blue was eluted at 5 h. After electrophoresis, one lane was cut off and stained with a silver-staining kit (Kanto Chemical, Tokyo). The other lane was sliced into 11 pieces (5 mm each) numbered from top to bottom, and each slice was cut into smaller pieces and left to incubate for 12 h at 4°C with 200 μl of extraction buffer (25 mM Na cacodylate-HCl, pH 6.4, 0.1 mM GalCer, 1% Lubrol PX, 0.25 mM dithiothreitol, 5 mM NaF, 2 mM ATP, 0.4 mg/ml BSA). After centrifugation (500×*g*, 1 min), 50 μl of the supernatant was incubated with 20 μM [^{35}S]PAPS (500 cpm/pmol) at 37°C for 1 h and assayed for the enzyme activity as described above.

SDS-PAGE was performed using an SDS-free 10–20% gradient slab-gel (84×90×1 mm, MULTI GEL, Daiichi Pure Chemicals, Tokyo) according to the method of Laemmli (*24*). Before electrophoresis, samples were heated at 60°C for 5 min in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 7% glycerol, 2% SDS, 0.005% bromphenol blue) in the presence or absence of 5% β-mercaptoethanol. When sulfotransferase activity was assayed following SDS-PAGE, samples were treated under nonreducing conditions. Renaturation of the enzyme following SDS-PAGE was performed essentially as described (*25*). The electrophoresis was done at room temperature with a constant current of 25 mA for 2 h. After separation, the gel was washed twice by shaking it gently in 250 ml of 50 mM triethanolamine-HCl buffer (pH 7.0) containing 20% 2-propanol for 30 min to remove the SDS attached to proteins. Then, part of the slab gel was cut off and stained with silver as described above. For enzyme assays, the sample-applied lane was sliced into 16 pieces (5 mm each) numbered from top to bottom, and each slice was cut into smaller pieces and left to incubate for 30 h at 4°C with 200 μl of a renaturation buffer (25 mM Na cacodylate-HCl, pH 6.4, 0.1 mM GalCer, 10 mM $MnCl_2$, 1% Lubrol PX, 0.25 mM dithiothreitol, 5 mM NaF, 2 mM ATP, 1 mg/ml BSA). After centrifugation (500×*g*, 1 min), 50 μl of the supernatant was incubated with 20 μM [^{35}S]PAPS (500 cpm/pmol) at 37°C for 2 h and assayed for enzyme activity as described above.

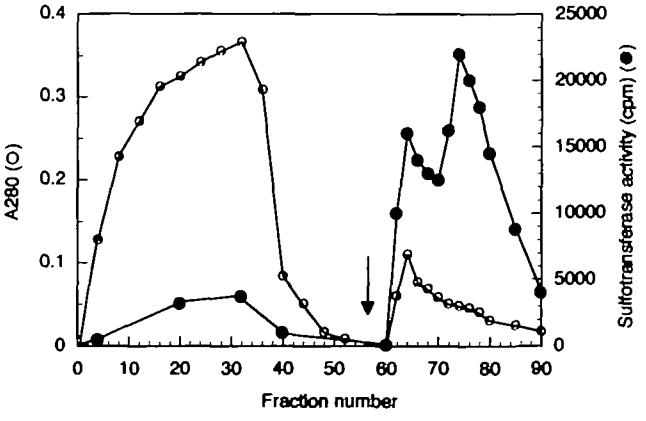

Fig. 1. **GalSph-Sepharose chromatography of PAPS:GalCer sulfotransferase of SMKT-R3 cells.** The enzyme-active fractions eluted from heparin-Sepharose were applied to a column of GalSph-Sepharose as described under "MATERIALS AND METHODS." After washing of the column with the equilibration buffer, the enzyme was eluted with the same buffer containing 0.1 M NaCl at the arrow. Each fraction (3 ml) was assayed for absorbance at 280 nm (○) and GalCer sulfotransferase activity (●). Fractions 71-90 were pooled, dialyzed, and subjected to PLP-Sepharose/HiTrap PAP chromatography.

RESULTS

Purification of GalCer Sulfotransferase from Human Renal Cancer Cells—GalCer sulfotransferase was purified from EGF-treated human SMKT-R3 renal cancer cells by a combination of affinity chromatographies on heparin-Sepharose, GalSph-Sepharose and HiTrap PAP. SMKT-R3 cells were directly solubilized by sonication with 1% Lubrol PX, a nonionic detergent. Since Lubrol PX does not interfere with measurement of the absorbance at a wavelength of 280 nm, this detergent was also employed for subsequent column chromatographies. The Lubrol extract was subjected to DE-52 chromatography after dialysis. The run-through fraction from the DE-52 column was directly applied to a heparin-Sepharose column. The sulfotransferase was firmly adsorbed to the heparin column and eluted with a recovery of more than 80% using 0.2 M NaCl. By this step, the sulfotransferase was concentrated 10-fold.

The eluate fraction from the heparin-Sepharose column was applied to a GalSph-Sepharose column (Fig. 1). On this chromatography, the sulfotransferase activity was eluted biphasically with a 0.1 M NaCl-containing buffer. The second peak was pooled and subjected to PLP-Sepharose/HiTrap PAP chromatography, and the first peak, with a

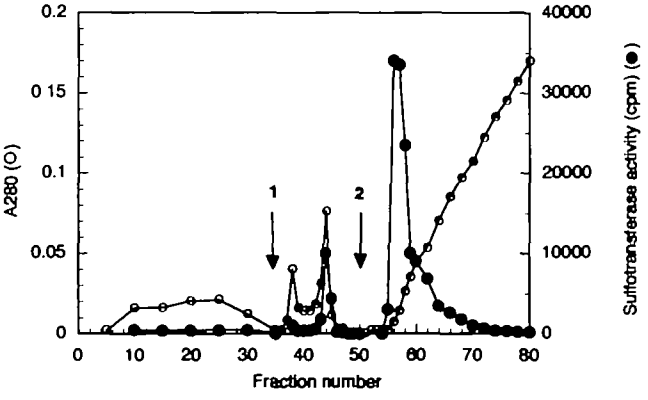

Fig. 2. **PLP-Sepharose/HiTrap PAP chromatography of PAPS: GalCer sulfotransferase of SMKT-R3 cells.** The dialysate of the effluent from the GalSph-Sepharose chromatography was applied to a PLP-Sepharose column connected to a HiTrap PAP column as described under "MATERIALS AND METHODS." After having been washed with the equilibration buffer, the columns were washed with the buffer containing 5 mM $MnCl_2$ (arrow 1) and the PLP-Sepharose column was disconnected. Then the sulfotransferase was eluted from the HiTrap PAP column with a linear gradient of 0-0.3 mM PAP (arrow 2). Each fraction (1.5 ml) was assayed for absorbance at 280 nm (○) and GalCer sulfotransferase activity (●). Fractions 56-62 were pooled and directly applied to the second heparin-Sepharose column.

small amount of the enzyme activity, was discarded. GalSph-Sepharose-chromatography enriched the enzyme 10-fold.

The sulfotransferase-active fractions were dialyzed against manganese-free buffer and subjected to PLP-Sepharose/HiTrap PAP chromatography (Fig. 2). The important feature of the HiTrap PAP chromatography was the washing step with $MnCl_2$-containing buffer. Through this washing, most nonspecific proteins that bound to the column, probably due to the chelating effect of PAP (*26*), were eluted, and then the sulfotransferase was eluted specifically with a low concentration of PAP as shown in Fig. 2 (note that the absorbances at 280 nm in the eluate after fraction 55 reflect the concentrations of PAP but not the protein amounts).

Table I summarizes the results of purification of GalCer sulfotransferase from EGF-treated SMKT-R3 cells. A 6,000-fold purification was obtained from the whole homogenates with an overall yield of 1%. The specific activity of the purified enzyme was 1.2 units/mg. The reaction product synthesized by the purified enzyme comigrated with authentic SM4 on thin-layer chromatography (Fig. 5).

The purified sulfotransferase showed a single broad band on native PAGE and this coincided with the enzyme activity (Fig. 3). In this experiment, 12% of the original enzyme activity was recovered from the gel. On reducing SDS-PAGE, the purified sulfotransferase showed a single protein band with an apparent molecular mass of 54 kDa (lane 1 in Fig. 4A), whereas under nonreducing conditions, a major 51 kDa band corresponding to the enzyme activity in the renaturation experiment and a minor 63 kDa band were observed (lane 3 in Fig. 4A). At present, the source of the 63 kDa band is unclear. The overall recovery of the sulfotransferase activity was approximately 0.1% in the renaturation experiment.

Substrate Specificity of GalCer Sulfotransferase from Human Renal Cancer Cells—The acceptor substrate specificity of GalCer sulfotransferase from SMKT-R3 cells was studied with a variety of glycolipids (Table II). GalCer was the best and LacCer was the second best acceptor under the employed conditions. GalAAG and GalDG also served to

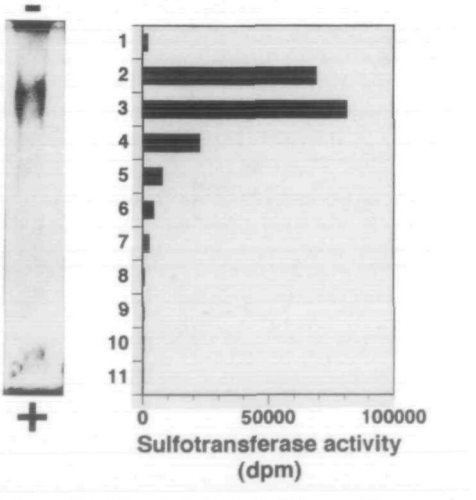

Fig. 3. **Native PAGE of PAPS:GalCer sulfotransferase of SMKT-R3 cells.** The sulfotransferase preparation obtained from the second Heparin-Sepharose chromatography (0.2 milliunit) was electrophoresed on two lanes as described under "MATERIALS AND METHODS." Then one lane of the gel was stained with silver (left) and the other was cut into 5-mm slices and assayed for the sulfotransferase activity (right). The vertical axis is adjusted to the length of the gel

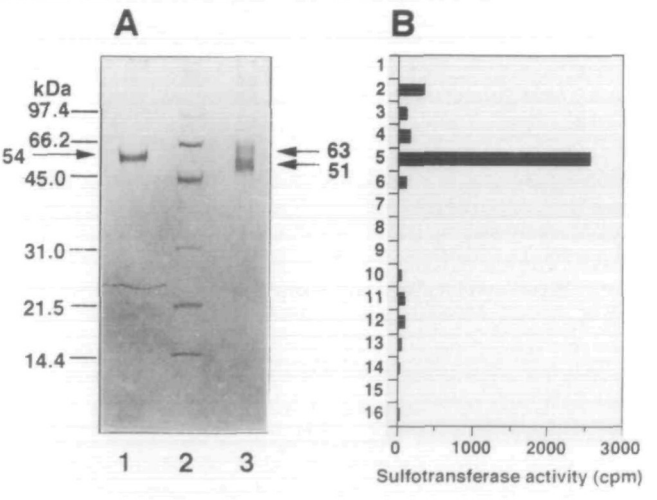

Fig. 4. **SDS-PAGE of PAPS:GalCer sulfotransferase of SMKT-R3 cells.** A: The sulfotransferase preparation (0.2 milliunit) from the second heparin-Sepharose chromatography was subjected to SDS-PAGE under reducing (lane 1) and nonreducing (lane 3) conditions together with molecular standard proteins (lane 2) and visualized with silver staining as described under "MATERIALS AND METHODS." Values on the left side indicate molecular masses of standard proteins: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). B: The purified enzyme preparation (0.2 milliunit) was treated under nonreducing conditions and applied to another lane of the gel. After electrophoresis, the lane was cut into 5-mm slices, renatured and assayed for the sulfotransferase activity as described under "MATERIALS AND METHODS." The vertical axis is adjusted to the length of the gel.

TABLE I. **Purification of PAPS:GalCer sulfotransferase from SMKT-R3 cells.** Results are shown for a typical preparation of the enzyme from 2.5×10^9 cells

Step	Volume (ml)	Protein (mg)	Specific activity[a] (units/mg)	Purification (-fold)	Yield (%)
Homogenate	320	2,430	0.0002	1	100
Lubrol extract	320	1,710	0.00028	1.4	99
DE-52/Heparin-Sepharose	110	140	0.003	15	85
GalSph-Sepharose	54	3.7	0.029	145	22
PLP-Sepharose/Hitrap PAP	11	ND[b]	ND	ND	3.9
Heparin-Sepharose	1.5	0.004	1.2	6,000	1.0

[a]One unit of activity is defined as 1 μmol of product formed per minute. [b]ND, not determined.

TABLE II **Acceptor requirement of GalCer sulfotransferase.**

Acceptor[a]	Structure	Relative activity (%)
GalCer	GalβCer	100[b]
LacCer	Galβ4GlcβCer	61
GalAAG	GalβAAG	21
GalDG	GalβDG	12
GlcCer	GlcβCer	5.9
Gb3Cer	Galα4Galβ4GlcβCer	0
Gb4Cer	GalNAcβ3Galα4Galβ4GlcβCer	<1
Gg3Cer	GalNAcβ4Galβ4GlcβCer	2.8
Gg4Cer	Galβ3GalNAcβ4Galβ4GlcβCer	3.2
nLc4Cer	Galβ4GlcNAcβ3Galβ4GlcβCer	<1

[a]The concentration of acceptor substrates was 500 μM [b]Twenty nanograms of purified enzyme was used for each assay. When GalCer was used as a substrate, 1 4 nmol of [^{35}S]sulfate was incorporated in 1 h The values represent the percentage of the activity compared with that of GalCer.

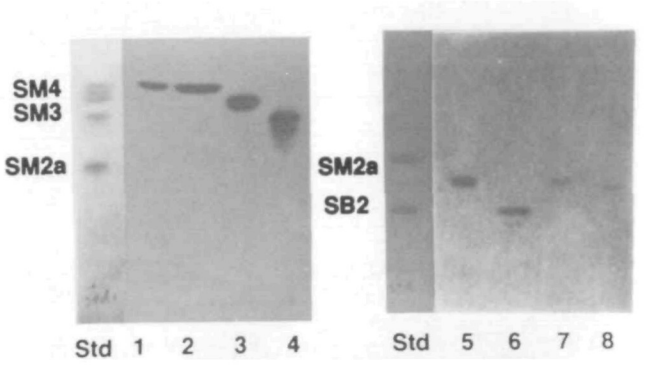

Fig 5 **Thin-layer chromatography of the reaction products using various acceptors.** GlcCer (lane 1), GalAAG (lane 2), GalCer (lane 3), LacCer (lane 4), Gg3Cer (lane 5), Gg4Cer (lane 6), Gb4Cer (lane 7), and nLc4Cer (lane 8) were incubated with [^{35}S]PAPS and the purified sulfotransferase for 4 h at 37°C, and the reaction products were isolated with a DEAE-Sephadex A-25 column followed by desalting with a SepPak C18 cartridge The desalted products were applied to a TLC plate and developed with chloroform/methanol/0.2% $CaCl_2$ (60 . 35 7 by volume), together with standard sulfolipids (Std) After development, the lanes of the standard were cut off and stained with the orcinol reagent Radioactive spots were detected by autoradiography (lanes 1-8)

some extent as acceptors. The purified sulfotransferase did act on GlcCer, Gb4Cer, Gg3Cer, Gg4Cer, and nLc4Cer, although the relative activities were less than 10% compared with the activity towards GalCer. The incorporation of [^{35}S]sulfate from PAPS into GlcCer, GalAAG, GalCer, LacCer, Gg3Cer, Gg4Cer, Gb4Cer, and nLc4Cer was confirmed by thin-layer chromatographic analysis of the reaction products (Fig. 5). On the other hand, Gb3Cer (possessing an α-galactoside at the nonreducing terminus) could not serve as an acceptor. Neither galactose nor lactose was a substrate for the sulfotransferase and they did not inhibit the synthesis of SM4 (data not shown).

Other Properties of GalCer Sulfotransferase from Human Renal Cancer Cells—NaCl enhanced the sulfotransferase activity at concentrations up to 0.1 M, but inhibited it at higher concentrations (data not shown). Inhibition by NaCl has been observed in rat brain GalCer sulfotransferase (*27*) and other glycoconjugate sulfotransferases (*27, 28*). The apparent K_m values of the purified sulfotransferase for GalCer and PAPS were 27 and 25 μM, respectively. The enzyme showed maximum activity between pH 6.5 and 7.0. The purified enzyme could be stored at −80°C in the presence of 20% glycerol without detectable loss of activity for at least 3 months.

DISCUSSION

The human renal cell line SMKT-R3 used in the present study has marked GalCer sulfotransferase activity of as much as 0.2 milliunits/mg in whole cell homogenates (*12*). From this cell line the sulfotransferase was purified 6,000-fold from the homogenate to apparent homogeneity as detected on reducing SDS-PAGE. The purified sulfotransferase showed a specific activity of 1.2 units/mg, comparable to that of two homogeneous sulfotransferases catalyzing sulfation of carbohydrates in proteoglycans (*29, 30*). The specific activity of the human sulfotransferase was much higher than those of the enzyme purified from rat testis (3.4 milliunits/mg) (*16*) and mouse brain (0.05 milliunit/mg) (*17*).

To purify the sulfotransferase, we employed a combination of chromatographic procedures including affinity chromatographies with immobilized analogs of the acceptor and donor substrates and conventional chromatographies. In a combined chromatography with DE-52/heparin-Sepharose, GalCer sulfotransferase passed through the DE-52 column. Cytosolic sulfotransferases involved in xenobiosis are generally acidic in nature (for example, see Ref. *31*) and they appeared to be retained on the first column. Similarly, chromatography on the acceptor analog, GalSph-Sepharose, seemed to eliminate the bulk of contaminating sulfotransferases. Mouse brain GalCer sulfotransferase is inhibited by PLP (*32*) and adsorbed on PLP-Sepharose (*17*). Indeed, PLP inhibited the human sulfotransferase competitively with respect to PAPS (*33*). However, preliminary experiments revealed that the human sulfotransferase, like the rat brain enzyme (*27*), did not bind to PLP-Sepharose but inert proteins did. Therefore, the PLP column was employed as a pre-column to reduce the load on the HiTrap PAP column. In regard to the inability of the human sulfotransferase to bind to PLP-Sepharose, it is most probable that blockage of the aldehyde group of PLP used for linking with the ligand carrier prevented the interaction with the enzyme, because the aldehyde group is essential for the interaction (*33*). The discrepancy of the behavior on PLP chromatography between the human renal cancer-cell enzyme and the mouse brain enzyme may reflect a difference of the enzyme molecules. The SMKT-R3 cell sulfotransferase had a high affinity for PAP, an analog of the donor, with a K_i value of the order of 10^{-5} M (*33*) and appeared to be effective for the purification, as with many types of sulfotransferases (*29-31*).

As to substrate specificity, the purified sulfotransferase had a relative activity of 61% towards LacCer compared with GalCer (Table II), the same ratio as when whole cell homogenate was used as an enzyme source (*12*). These findings strongly suggest that the same enzyme synthesizes both SM4 and SM3 in SMKT-R3 cells, supporting previous observations in substrate-competition experiments (*34, 35*). Sulfogalactosylglycerolipids are found in mammalian testis (*36*) and brain (*37*), and whether or not the same enzyme synthesizes both sulfoglycerolipids and sulfosphingolipids is an interesting question. Substrate-competition

studies suggested that GalAAG, GalCer, and LacCer are sulfated by a single enzyme in boar testis (*34*). Rat testicular galactolipid sulfotransferase favors GalCer rather than GalAAG *in vitro* (*16*). In the present study the human sulfotransferase did act on glycerolipids as well as sphingolipids, supporting the possibility that a single enzyme synthesizes both sulfolipids. However, whether the identical enzyme works in testis and renal cancer cells remains to be proved.

The purified sulfotransferase did act on GlcCer, Gb4Cer, Gg3Cer, Gg4Cer, and nLc4Cer, although the activities were low, indicating that the substrate specificity of the enzyme appeared to be relatively loose. An earlier study using whole cell homogenate of SMKT-R3 cells demonstrated that sulfotransferase from the cells acted on the nonreducing terminal *N*-acetylgalactosamine of Gg3Cer, but the activity of Gg3Cer sulfotransferase was approximately one-fortieth that of GalCer sulfotransferase (*38*). The reaction product using Gg3Cer as an acceptor migrated more slowly than SM2a, and seemed to be SM2b (Fig. 5). Considering that the relative activity towards Gg3Cer of the purified enzyme was comparable to that of the cell homogenate (Table II), the GalCer sulfotransferase may also catalyze the biosynthesis of SM2b in SMKT-R3 cells, although the possibility that other sulfotransferases act cannot be excluded. The relative activity towards Gg4Cer was higher than that towards Gg3Cer (Table II) in accordance with a previous observation using rat brain microsomes (*39*). In mammalian tissues Gg4Cer IV^3-sulfate has been found, but not Gg4Cer III^3-sulfate (*40*). Taken together, our results suggest that the nonreducing terminal galactose of Gg4Cer is sulfated by the purified sulfotransferase. Regarding the globo-series, Gb3Cer, possessing α-galactose at the nonreducing terminus, could not serve as an acceptor for the SMKT-R3 sulfotransferase, though Gb4Cer was able to. Considering that Gb4Cer IV^3-sulfate is found in human kidney (*18*), sulfate seemed to be transferred to the nonreducing terminal, β-*N*-acetylgalactosamine, of Gb4Cer. Similarly, also on nLc4Cer, sulfate may be transferred to the nonreducing terminal monosaccharides. Further study is needed to characterize the reaction products. On the other hand, neither galactose nor lactose was a substrate or a competitive inhibitor for the purified sulfotransferase, in contrast with a previous report using rat brain microsomal enzyme (*39*). The microsomal fraction might contain other sulfotransferases and cofactors. In conclusion, human renal cancer-cell sulfotransferase appears to prefer the nonreducing terminal β-glycoside, especially β-galactoside, of an oligosaccharide chain attached to a lipid moiety.

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