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Biochemistry

Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis

(glucosylceramide/glucocerebroside/glucosylceramide synthase/melanoma)

SHINICHI ICHIKAWA*†, HISAKO SAKIYAMA‡, GEN SUZUKI§, KAZUYA I.-P. JWA HIDARI*, AND YOSHIO HIRABAYASHI*

*Laboratory for Glyco-Cell Biology, Frontier Research Program, The Institute of Chemical and Physical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-01, Japan; and ‡Division of Physiology and Pathology, and §Division of Clinical Research and Radiation Health, National Institute of Radiological Science, 4-9-1 Anagawa, Chiba-shi, Chiba 260, Japan

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ABSTRACT **We have isolated a cDNA encoding human ceramide glucosyltransferase (glucosylceramide synthase, UDP-glucose:*N*-acylsphingosine D-glucosyltransferase, EC 2.4.1.80) by expression cloning using as a recipient GM-95 cells lacking the enzyme. The enzyme catalyzes the first glycosylation step of glycosphingolipid synthesis and the product, glucosylceramide, serves as the core of more than 300 glycosphingolipids. The cDNA has a G+C-rich 5′ untranslated region of 290 nucleotides and the open reading frame encodes 394 amino acids (44.9 kDa). A hydrophobic segment was found near the N terminus that is the potential signal-anchor sequence. In addition, considerable hydrophobicity was detected in the regions close to the C terminus, which may interact with the membrane. A catalytically active enzyme was produced from *Escherichia coli* transfected with the cDNA. Northern blot analysis revealed a single transcript of 3.5 kb, and the mRNA was widely expressed in organs. The amino acid sequence of ceramide glucosyltransferase shows no significant homology to ceramide galactosyltransferase, which indicates different evolutionalary origins of these enzymes.**

Glycosphingolipids (GSLs) are a group of membrane components that have the lipid portion embedded in the outer plasma membrane leaflet and the sugar chains extended to the outer environment. GSLs are present in essentially all animal cells. Several lines of evidence suggest the importance of GSLs in various cellular processes such as differentiation, adhesion, proliferation, and cell–cell recognition (for review, see ref. 1).

Ceramide glucosyltransferase [(GlcT-1); glucosylceramide (GlcCer) synthase, UDP-glucose:ceramide β1-1′ glucosyltransferase, EC 2.4.1.80] catalyzes the first glycosylation step of GSL biosynthesis, the transfer of glucose from UDP-Glc to ceramide (2). The product, GlcCer, serves as the core structure of more than 300 GSLs. GlcCer has also been shown to stimulate proliferation of a variety of cells (for review, see ref. 3).

GlcT-1 was originally found by Basu *et al.* (2) from embryonic chicken brain. Properties of the enzyme, however, have not been studied satisfactorily because of the difficulty in assaying and purifying the enzyme. Thus far, only limited data have been published on GlcT-1: successful solubilization of the enzyme from rat Golgi fraction (4), the different properties of liver and brain enzymes (5), and a specific inhibitor of the enzyme (6). Papers from several laboratories have demonstrated that the synthesis of GlcCer occurs at the cytosolic surface of the Golgi apparatus, whereas other glycosylation reactions of GSL synthesis take place at the lumenal side of the organelle (7–9). Although the topology of the lactosylceramide formation, the next step of the synthesis, remains unknown and controversial, recent studies suggest the lumenal orientation of the synthase (10, 11). Thus, GlcCer must be translocated before it can be used as the precursor of other GSLs. Another unique feature of GlcT-1 is the substrate specificity. Apparently, GlcT-1 is involved only in glycolipid synthesis since no β-glucosyl residues have been found in either glycoproteins or the lipid precursors. This hypothesis is also supported by the fact that the GlcT-1-defective mutant showed normal protein glycosylation patterns (12).

For detailed studies of GlcT-1, isolation of the cDNA was essential. In the previous studies, we have isolated and characterized a mouse melanoma B16 mutant, GM-95, deficient in GlcT-1 (12, 13). The mutant allows us to isolate GlcT-1 cDNA in combination with the expression cloning methodology (14). In this paper, we report on the molecular cloning of GlcT-1, and the characterization of recombinant GlcT-1 expressed in the mutant cells and in *Escherichia coli*.

MATERIALS AND METHODS

Materials. Anti-sialyllactosylceramide (GM3), monoclonal antibody (mAb) M2590 (IgM) (15) was purchased from Meiji Seika (Tokyo). Anti-mouse IgM goat IgG (μ chain specific) was obtained from Organon. Fluorescent-labeled anti-mouse gamma globulin was purchased from Antibodies Inc. Lipofectin and G418 were from Life Technologies Oriental (Tokyo). The pET system was from Takara Shuzo (Kyoto) (16). Homobifunctional linker, bis(sulfosuccinimidyl) suberate was obtained from Pierce. 6-{[(N-7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl}sphingosine (C_6-NBD-Cer) was from Molecular Probes. pPSVE-PyE plasmid, which carries the early region of polyoma virus, was a generous gift from M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA) (17). All other reagents were of analytical grade.

cDNA Library. A cDNA library of a human melanoma cell line SK-Mel-28 was a gift from M. Fukuda. The library was prepared from poly(A)$^+$ RNA by the method of Seed and Aruffo (14) using pcDNA I (Invitrogen) as a mammalian expression vector. The library contained 2.5×10^6 independent clones in *E. coli* strain MC1061/P3.

Cell Lines and Culture Conditions. GM-95, a B16 melanoma mutant that does not express GlcT-1, was isolated from

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Abbreviations: GSL, glycosphingolipid; GlcCer, glucosylceramide or glucocerebroside; GM3, sialyllactosylceramide; GlcT-1, ceramide; glucosyltransferase; CGT, ceramide galactosyltransferase; mAb, monoclonal antibody; C_6-NBD-Cer, 6-{[(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl}sphingosine; FCS, fetal calf serum.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. D50840).

†To whom reprint requests should be addressed.

MEB-4 cells (12, 13). MEB-4 cells, a subline of B16 cells, do not express a melanoma-specific antigen recognized by mAbs M562 and M622, but express another melanoma antigen recognized by mAb M2590 (anti-GM3, IgM) (12). GM-95-PyT, a recipient cell line for transient GlcT-1 expression, was established by cotransfection of GM-95 with pSV2*neo* (18) and pPSVE-PyE (17) plasmids followed by selection with G418. The cells were maintained in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS).

Isolation of a GlcT-1 Encoding cDNA by Complementation. GM-95-PyT cells (2×10^7) were washed with K-PBS$^-$ (30.8 mM NaCl/120.7 mM KCl/8.1 mM Na_2HPO_4/1.46 mM KH_2PO_4), and suspended in 400 μl of K-PBS$^-$ supplemented with 5 mM $MgCl_2$ (K-PBS$^+$). Plasmid DNAs (100 μg, 2.5×10^6 independent clones) were added to this, dissolved in 400 μl K-PBS$^+$, and the mixture was kept on ice 10 min. Cells were then transferred to 0.4-cm cuvette exposed to a 300-V pulse with a capacitance of 960 μF by a Gene Pulsar (Bio-Rad) (19) and replaced on ice. After 10 min, the cell suspension was diluted with 5 ml of cold serum-free DMEM, incubated at 25°C for 30 min, inoculated into dishes, and cultured at 37°C in DMEM supplemented with 20% FCS. A total of 1×10^8 cells were transfected as above and cultured in five 15-cm culture dishes.

To enrich GM3-expressing transfectants, a panning procedure was performed (20). Sixty hours after transfection, the cells were detached from the plate using 5 mM EDTA in PBS and resuspended at a concentration of 5×10^7 cells/ml in PBS/EDTA/NaN_3 (PBS containing 5% fetal bovine serum/0.02% sodium azide/0.5 mM EDTA). The cells were incubated with an anti-GM3 mAb M2590 (20 μg/ml) for 2 h. After incubation, the cells were washed twice with ice-cold PBS, diluted 1:5 with PBS containing 50 mM Hepes buffer (pH 8.3) and 0.2 mM bis(sulfosuccinimidyl) suberate, and kept on ice for 30 min. The cells were then washed twice with ice-cold PBS, resuspended in 10 ml of PBS/EDTA/NaN_3, and distributed to five 6-cm panning plates coated with anti-mouse IgM goat IgG. After 4 h of incubation at 25°C, nonadherent cells were removed by gentle washing with PBS/EDTA/NaN_3. The plasmids were rescued from adherent cells by the method of Hirt (21) and introduced into *E. coli* MC1061/P3 by electroporation. The plasmids were prepared from 500 ml of 2× YT (22) and another round of panning was performed as described above. After the second round of panning, 500 *E. coli* clones were divided into 32 pools, and plasmids were prepared from each pool by the minipreparation method (22). They were introduced into GM-95-PyT cells cultured in 24-well plates by the DEAE-dextran method (23) and the cells were incubated for 60 h. The GlcT-1 activity was measured in the cells harvested from each well. Two pools were shown to have the enzyme activity. The 62 individual *E. coli* clones from the positive pools were examined for the activity as described above, and finally GlcT-1 cDNA was isolated. The clone was designated pCG-1.

Stable Expression of GlcT-1 in GM-95 Cells. GM-95 cells were plated in 10 cm culture dishes in 10 ml of DMEM supplemented with 10% FCS and incubated until they reached 50% confluency. The medium was replaced by 10 ml of serum-free DMEM, and a mixture of pCG-1 (60 μg), pSV2*neo* (6 μg), and lipofectin (150 μg) in 300 μl of water was added (24). As a control, the pcDNA I vector and pSV2*neo* were transfected. The next day, the cells were subjected to selection by G418 (800 μg/ml). After 2 weeks, more than 100 colonies were obtained from each plate. The cells were pooled, replated, and maintained for 2 months in the medium containing G418. The lipid composition and reactivity of the transfectants with M2590 were examined in the mixture population.

Expression of GlcT-1 in *E. coli*. The GlcT-1 cDNA excised from pCG1 with *Eco*RI was cloned into the *Eco*RI site of Bluescript KS vector (Stratagene) to locate the *Bam*HI site at the 3′ end (pCG-2). An *Nde*I site was introduced at the ATG codon of the GlcT-1 open reading frame by the standard PCR method (25) using primers 5′-ATCATATGGCGCTGGAC-CTGGC-3′ and 5′-CAATCTAGCATCAACATTTGGATA-3′. The resulting 341-bp fragment was digested with *Hin*dIII and cloned into the *Sma*I–*Hin*dIII site of pUC119. After amplification, the fragment was recovered by digesting with *Kpn*I and *Hin*dIII. The fragment was then ligated to the 1.1-kb *Kpn*I–*Hin*dIII fragment of pCG-2, resulting in pCG-3. The full-length cDNA with *Nde*I and *Bam*HI sites from pCG-3 was then cloned into *E. coli* expression vector pET3a (16). The resulting plasmid pET-CG-1 was transformed into the *E. coli* strain BL21(DE3) (16). For the expression of the cloned GlcT-1, *E. coli* cells harboring the plasmid were grown in NZCYM medium (22) at 37°C. When cell density reached $OD_{600} = 0.25$ isopropyl β-thiogalactopyranoside was added to a final concentration of 1 mM and the cells were incubated for an additional 5 h. After the incubation the cells were harvested and disrupted by sonication. For the enzyme assay, 150 μg of cell lysate was added to each reaction mixture and the mixture was incubated for 7 h.

Nucleotide Sequence Determination. The plasmid containing human GlcT-1 cDNA was treated with exonuclease III and mung bean nuclease to construct nested deletion clones of various lengths (26). Nucleotide sequences were determined in both directions by the dideoxynucleotide chain-termination method using either *Buca*BEST (Takara Shuzo) (27) or the cycle sequencing methods using *Taq* DNA polymerase. Applied Biosystems model 373A and Pharmacia A.L.F. DNA sequencers were used for the analyses. Homology searches against entire databases (GenBank and EMBL for DNA and PIR and Swiss-Prot for proteins, update of April 1, 1995) were performed using GENEBRIGHT (Hitachi Software Engineering) (28). A portion of the analysis was performed by Takara Shuzo.

DNA Manipulation. DNA manipulation was carried out as described in ref. 22.

Flow Cytometry. Cells were incubated for 2 h on ice with M2590 (20 μg/ml in PBS/EDTA/NaN_3) and then for 3 h on ice with fluorescein isothiocyanate labeled anti-mouse Ig. Fluorescent intensity was analyzed by FACStar (Becton Dickinson).

Lipid Analyses. Cells were scraped at subconfluency, washed twice with PBS, and lyophilized. Total lipids were extracted from the cells with 20 vol of $CHCl_3/CH_3OH$, 2:1 (vol/vol), filtered, and evaporated to dryness. The lipids were then redissolved in a small volume of $CHCl_3/CH_3OH$, 2:1 (vol/vol), and chromatographed on a precoated silica-gel TLC plate (Merck) in $CHCl_3/CH_3OH/H_2O$, 65:25:4 (vol/vol). GSLs were visualized with orcinol/H_2SO_4 reagent.

Enzyme Assay. GlcT-1 activity was assayed according to the method of Lipsky and Pagano (29) with slight modifications. C_6-NBD-Cer, a synthetic fluorescent substrate (50 μg), and lecithin (500 μg) were mixed in 100 μl of ethanol and the solvent was evaporated. Water (1 ml) was added and the mixture was sonicated to form liposomes. A standard reaction mixture (100 μl), composed of 20 mM Tris·HCl (pH 7.5), 500 μM UDP-Glc, 20 μl of liposomes, and 50 μg of cell protein was incubated at 30°C for 4 h. After the incubation, lipids were extracted and applied on silica gel 60 plates (Merck). NBD lipids were separated in the same solvent used for the chromatography and visualized by UV illumination.

Protein Assay. Proteins were assayed using the Micro BCA Protein Assay reagent kit (Pierce) (30).

Isolation of mRNA and Northern Blot Analysis. Poly(A)$^+$ RNA from each cell line was isolated by the Fast Track mRNA Isolation kit (Invitrogen) according to the manufacturer's instructions. Two micrograms of poly(A)$^+$ RNA was subjected to electrophoresis on a 1% agarose gel containing formaldehyde, and transblotted onto nylon membrane (Hybond-N, Amersham) (22). A premade membrane was used for the

analysis of mRNA from various tissues (Human Multiple Tissue Northern Blot, Clontech). The 1.1-kb *Hin*dIII–*Xho*I cDNA fragment of pCG-1 was labeled with [α-^{32}P]dCTP [6000 Ci/mmol, Amersham; 1 Ci = 37 GBq) by the Multiprime DNA labeling system (Amersham) and used as a probe. Hybridization was carried out at 42°C for 24 h in 5× SSPE [1× SSPE contains 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), and 1 mM EDTA] containing 50% formaldehyde/2% SDS/10× Denhardt's solution/100 μg/ml of salmon sperm DNA, and ^{32}P-labeled probe. After hybridization, the membrane was washed with 2× SSC containing 0.5% SDS and 0.1× SSC, each time at 50°C for 40 min. This was followed by autoradiography.

RESULTS

Molecular Cloning of a cDNA Encoding GlcT-1. To clone a cDNA encoding GlcT-1 that participates in GSL synthesis, we used a mammalian expression cloning system developed by Seed and Aruffo (14). The cDNAs from an SK-Mel-28 library in the expression vector pcDNA I were introduced into GM-95-PyT cells, GM-95 cells stably expressing polyoma large T antigen. The recipient cells allow episomal replication of transfected plasmids. Although GM-95-PyT cells are deficient in GlcT-1, other enzymes involved in GSL synthesis remain active. Thus, the complementation of the GlcT-1 cDNA restores the expression of GlcCer, lactosylceramide, and GM3 (Fig. 1). Three days after transfection, cells expressing GM3 were selected by panning as described in *Materials and Methods*. Because the binding between the M2590 and GM3 was not strong enough to hold the cells on the plate, the bound mAb were cross-linked with cell surface proteins by a bifunctional cross-linker. Plasmids were isolated from the adherent cells and electroporated into *E. coli* for further amplification. After the second round of amplification, *E. coli* colonies were divided into subpools and screened by a sibling selection based on the enzyme assay. Finally, a single clone, pCG-1, was isolated. pCG-1 was found to contain an insert 1.6 kb in length.

Transient Expression of GlcT-1 Activity in GM-95-PyT Cells. GM-95-PyT cells transfected with pCG-1 were assayed for GlcT-1 activities using C_6-NBD-Cer as a substrate. As shown in Fig. 2, GlcT-1 activity was detected in GM-95-PyT cells transfected with pCG-1 (lane 9) but not in those transfected with pcDNA I vector alone (lane 7). The production of C_6-NBD-GlcCer was UDP-Glc dependent (lane 10). GlcT-1 activity was obviously detected in SK-Mel-28 cells (lane 1), from which the library was derived and in MEB-4 (lane 3). A small amount of C_6-NBD-GlcCer was synthesized in the lysates from these two cell lines without an addition of UDP-Glc because they had endogenous UDP-Glc (lanes 2 and 4).

Restoration of GSL Expression in the Mutant Cells by GlcT-1 cDNA. Expression of GSLs was first analyzed by flow

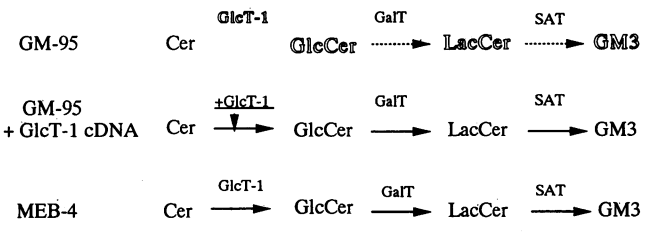

FIG. 1. The synthetic pathway of GSLs. GM-95 cells do not express any GSLs due to the deficiency of GlcT-1. MEB-4 cells are the parental cells of GM-95 expressing a normal pattern of GSLs. Transfection of GlcT-1 cDNA into GM-95 cells restores expression of all GSLs. The origins of the cells are explained in *Materials and Methods*. Open letters indicate enzymes and GSLs deficient in GM-95 cells. Broken arrows are reactions that do not proceed in GM-95 cells due to the deficiency of substrates. Cer, ceramide; GalT, UDP-galactose:glucosylceramide galactosyltransferase; SAT, CMP-sialic acid:lactosylceramide sialyltransferase.

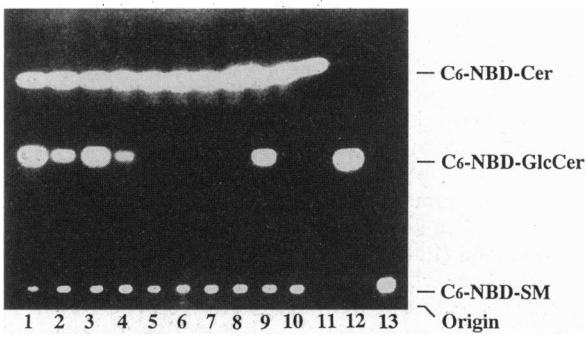

FIG. 2. TLC analysis of GlcT-1 product as catalyzed by cell lysates. The enzyme reaction was carried out according to the method of Lipsky and Pagano (29) with slight modification. The NBD-labeled enzyme product was visualized with UV irradiation. Lanes: 1, SK-Mel-28 cells; 2, SK-Mel-28 cells with no UDP-Glc addition; 3, MEB-4 cells; 4, MEB-4 cells with no UDP-Glc addition; 5, GM-95-PyT cells; 6, GM-95-PyT cells with no UDP-Glc addition; 7, GM-95-PyT cells transfected with pcDNA I; 8, GM-95-PyT cells transfected with pcDNA I, with no UDP-Glc addition; 9, GM-95-PyT cells transfected with pCG-1; 10, GM-95-PyT cells transfected with pCG-1, with no UDP-Glc addition; 11, C_6-NBD-Cer (100 pmol); 12, C_6-NBD-GlcCer (100 pmol); 13, C_6-NBD-SM (100 pmol). SM represents sphingomyelin.

cytometry using anti-GM3 mAb. Although a significant shift of main peak and a small population of strongly stained cells were observed in GM-95-PyT cells transfected with pCG-1, the percentage of positive population was relatively small (Fig. 3*A*, c). This was mainly due to the low transfection efficiency of GM-95-PyT cells (data not shown). Therefore, we attempted to make GM-95 cells expressing GlcT-1 stably. GM-95 cells were cotransfected with pCG-1 and pSV2*neo* and selected with G418. More than 100 colonies were obtained. The cells were mixed and expanded for GSL analysis without cloning. For GM3 expression 75% of the cells were positively stained with the antibody and 15% were stained stronger than the parental cells (Fig. 3*B*, b). GSLs of the neomycin-resistant cells were further analyzed by TLC followed by orcinol/H_2SO_4 reaction (Fig. 4). GlcCer and GM3 were detected in the total lipid extracted from GM-95 cells carrying pCG-1 (lane 5) but not in that from the cells carrying pcDNA I (lane 4).

Expression of Enzymatically Active GlcT-1 in *E. coli*. There was a possibility that pCG-1 encodes a regulatory protein required for the expression of GlcT-1 activity but not the enzyme itself. To exclude this possibility, we expressed the enzyme in *E. coli* that is known to have neither GlcT-1 nor GSLs. As shown in Fig. 5, the GlcT-1 activity was detected in the cells transformed with pET-CG-1 (lane 3) and the production of C_6-NBD-GlcCer was UDP-Glc dependent (lane 4). No GlcT-1 activity was detected in the cells that carried pET3a vector alone (lane 1). These data indicate that pCG-1 encodes GlcT-1.

Primary Structure of GlcT-1 cDNA. Nucleotide sequence of the 1.6-kb cDNA was determined by the dideoxynucleotide chain termination method (27). The cDNA had a G+C-rich 5′-untranslated region of 290 nucleotides and a long open reading frame that started from the first ATG. G+C content decreased dramatically after the first ATG. The deduced amino acid sequence of the open reading frame showed that it consisted of 394 amino acids with a calculated M_r of 44,853 daltons (Fig. 6A). Homology searches against currently available protein and nucleic acid databases (Swiss-Prot and PIR for proteins, GenBank and EMBL for nucleic acid) were carried out. However, no significant homologies with GlcT-1 were found, indicating that GlcT-1 is a novel protein. The G+C-rich sequence that precedes the initiation codon is presumably a part of CpG island sequence that surrounds promoters of housekeeping genes (32). Genes that contain the

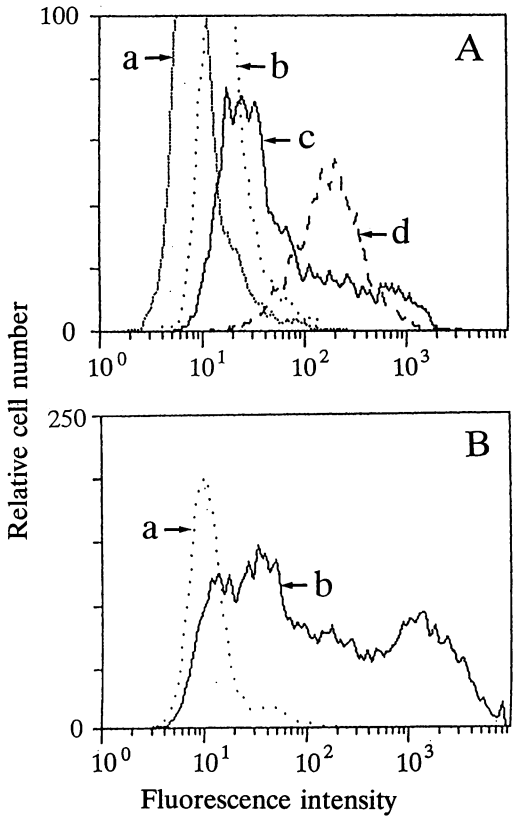

FIG. 3. Flow cytometry of the GlcT-1-deficient cells transfected with pCG-1. Cells were incubated for 2 h on ice with M2590 (20 μg/ml) and then for 3 h on ice with fluorescein isothiocyanate-labeled anti-mouse Ig. Fluorescent intensity was analyzed by FACStar (*A*) Transient expression: a, GM-95-PyT cells; b, GM-95-PyT cells transfected with pcDNA I; c, GM-95-PyT cells transfected with pCG-1; d, MEB-4 cells. (*B*) Stable expression: a, GM-95 cells cotransfected with pcDNA I and pSV2*neo*; b, GM-95 cells cotransfected with pCG-1 and pSV2*neo*.

sequence are usually essential for cell viability and are expressed in most cells. The hydropathy plot analysis (31) revealed the presence of a hydrophobic segment near the N terminus that is the potential signal-anchor sequence (amino acids 11-32) (Fig. 6*B*). The analysis by the PSORT (33) program also predicted the type III (type Ib) (for review, see ref. 34) membrane protein structure for the enzyme, which has a single

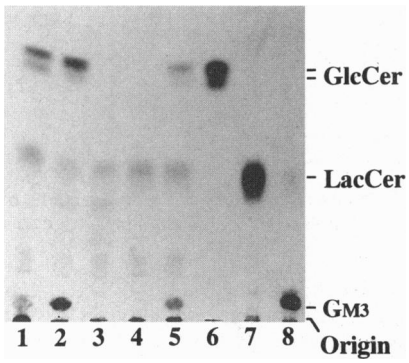

FIG. 4. TLC analysis of GSLs in the stable transfectants. Total lipids from 10^6 cells were spotted onto a silica-gel plate and developed with a solvent [$CHCl_3/CH_3OH/H_2O$, 65:25:4 (vol/vol)]. GSLs were visualized with orcinol/H_2SO_4 reagent. Lanes: 1, SK-Mel-28 cells; 2, MEB-4 cells; 3, GM-95 cells; 4, GM-95 cells cotransfected with pcDNA I and pSV2*neo*; 5, GM-95 cells cotransfected with pCG-1 and pSV2*neo*; 6, GlcCer (2 μg); 7; lactosylceramide (1 μg); 8, GM3 (1 μg).

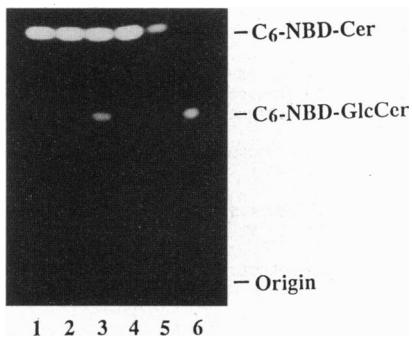

FIG. 5. Expression of recombinant GlcT-1 activity in *E. coli*. Lanes: 1, pET3a/BL21(DE3); 2, pET3a/BL21(DE3) with no UDP-Glc addition; 3, pET-CG-1/BL21(DE3); 4, pET-CG-1/BL21(DE3) with no UDP-Glc addition; 5, C_6-NBD-Cer (40 pmol); 6, C_6-NBD-GlcCer (40 pmol).

transmembrane segment near the N terminus including an uncleavable signal sequence and a long cytoplasmic tail (amino acids 32–394). However, the considerable hydrophobicity was detected in the regions close to the C terminus, which may interact with the membrane (Fig. 6*B*).

Northern Blot Analysis of GlcT-1 mRNA. The expression of GlcT-1 mRNA in various melanoma cell lines and human tissues was analyzed by Northern blot analysis (Fig. 7*A*). A single transcript of GlcT-1 with the size of 3.5 kb was detected in SK-Mel-28 [Fig. 7*A* (a), lane 1] and MEB-4 cells [Fig. 7*A* (a), lane 2]. Due to the deficiency of GlcT-1 activity, a very faint band was observed in GM-95 cells [Fig. 7*A* (a), lane 3]. As shown in Fig. 7*B* (a), GlcT-1 mRNA was found in all tissues examined: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (lanes 1–8, respectively). The results were in good agreement with a ubiquitous expression of GSLs. Human glyceraldehyde-3-phosphate dehydrogenase cDNA was used as a control [Fig. 7*A* (b) and 7*B* (b)]. As previously reported, the strong expression of glyceraldehyde 3-phosphate dehydrogenase mRNA was observed in skeletal muscle and heart (35).

DISCUSSION

In previous papers, we reported the isolation of the GlcT-1-negative mutant GM-95 (12, 13). Here we have described the isolation of a cDNA which encodes GlcT-1 by expression cloning using the mutant cells as a recipient. The isolated cDNA encoded GlcT-1 but not a regulatory factor of the enzyme, because *E. coli* transformed with the gene produced the active enzyme. From the nucleotide sequence of the cDNA, we deduced the amino acid sequence of GlcT-1 and showed that it consists of 394 amino acids and has a calculated molecular mass of 44,853 daltons. Homology searches of protein and nucleic acid databases indicate that GlcT-1 is a novel protein. On the other hand, the enzyme with a similar catalytic activity, ceramide galactosyltransferase (CGT), has recently been cloned (36). The sequence analysis of CGT revealed that the enzyme was homologous to glucuronyltransferases, which are involved in drug metabolism. Even though GlcT-1 and CGT catalyze similar reactions, no significant sequence homology was found. In addition, the occurrence of the glucuronyltransferases is at the lumenal side of the endoplasmic reticulum, but GlcT-1 is at the cytoplasmic side (37). These observations may suggest that these two enzymes have different evolutionary origins.

Among glycosyltransferases involved in GSL synthesis, GlcT-1 is the only enzyme that catalyzes the reaction at the cytoplasmic side of the Golgi apparatus. The topology of the enzyme is of great interest because it may serve as "flippase" as well as glucosyltransferase. Hydropathy plot analysis re-

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-290                                                                (5') GAGGCGAACCGGAGCGCG  -271
-270 GCCGCGGTCGCCCCGACCAGAGCCGGGAGACCGCAGCACCCGCAGCCGCCCGCGAGCGCGCCGAAGACAGCGCGCAGGCGAGAGCGCGCG  -181
-180 GGCGGGGGCGCGCAGGCCCTGCCCGCCCCTTCCGTCCCCACCCCCCTCCGCCCTTTCCTCTCCCCACCTTCCTCTCGCCTCCCGCGCCCC   -91
 -90 CGCACCGGGCGCCCACCCTGTCCTCCTCCTGCGGGAGCGTTGTCCGTGTTGGCGGCCGCAGCGGGCCGGGCCGGTCCGGCGGGCCGGGGG    -1

   1 ATGGCGCTGCTGGACCTGGCCTTGGAGGGAATGGCCGTCTTCGGGTTCGTCCTCTTCTTGGTGCTGTGGCTGATGCATTTCATGGCTATC     90
   1 M A L L D L A L E G M A V F G F V L F L V L W L M H F M A I                                    30

  91 ATCTACACCCGATTACACCTCAACAAGAAGGCAACTGACAAACAGCCTTATAGCAAGCTCCCAGGTGTCTCTCTTCTGAAACCACTGAAA  180
  31 I Y T R L H L N K K A T D K Q P Y S K L P G V S L L K P L K                                   60

 181 GGGGTAGATCCTAACTTAATCAACAACCTGGAAACATTCTTTGAATTGGATTATCCCAAATATGAAGTGCTCCTTTGTGTACAAGATCAT  270
  61 G V D P N L I N N L E T F F E L D Y P K Y E V L L C V Q D H                                   90

 271 GATGATCCAGCCATTGATGTATGTAAGAAGCTTCTTGGAAAATATCCAAATGTTGATGCTAGATTGTTTATAGGTGGTAAAAAAGTTGGC  360
  91 D D P A I D V C K K L L G K Y P N V D A R L F I G G K K V G                                   120

 361 ATTAATCCTAAAATTAATAATTTAATGCCAGGATATGAAGTTGCAAAGTATGATCTTATATGGATTTGTGATAGTGGAATAAGAGTAATT  450
 121 I N P K I N N L M P G Y E V A K Y D L I W I C D S G I R V I                                  150

 451 CCAGATACGCTTACTGACATGGTGAATCAAATGACAGAAAAAGTAGGCTTGGTTCACGGGCTGCCTTACGTAGCAGACAGACAGGGCTTT  540
 151 P D T L T D M V N Q M T E K V G L V H G L P Y V A D R Q G F                                  180

 541 GCTGCCACCTTAGAGCAGGTATATTTTGGAACTTCACATCCAAGATACTATATCTCTGCCAATGTAACTGGTTTCAAATGTGTGACAGGA  630
 181 A A T L E Q V Y F G T S H P R Y Y I S A N V T G F K C V T G                                  210

 631 ATGTCTTGTTTAATGAGAAAAGATGTGTTGGATCAAGCAGGAGGACTTATAGCTTTTGCTCAGTACATTGCCGAAGATTACTTTATGGCC  720
 211 M S C L M R K D V L D Q A G G L I A F A Q Y I A E D Y F M A                                  240

 721 AAAGCGATAGCTGACCGAGGTTGGAGGTTTGCAATGTCCACTCAAGTTGCAATGCAAAACTCTGGCTCATATTCAATTTCTCAGTTTCAA  810
 241 K A I A D R G W R F A M S T Q V A M Q N S G S Y S I S Q F Q                                  270

 811 TCCAGAATGATCAGGTGGACCAAACTACGAATTAACATGCTTCCTGCTACAATAATTTGTGAGCCAATTTCAGAATGCTTTGTTGCCAGT  900
 271 S R M I R W T K L R I N M L P A T I I C E P I S E C F V A S                                  300

 901 TTAATTATTGGATGGGCAGCCCACCATGTGTTCAGATGGGATATTATGGTATTTTTCATGTGTCATTGCCTGGCATGGTTTATATTTGAC  990
 301 L I I G W A A H H V F R W D I M V F F M C H C L A W F I F D                                  330

 991 TACATTCAACTCAGGGGTGTCCAGGGTGGCACACTGTGTTTTTCAAAACTTGATTATGCAGTCGCCTGGTTCATCCGCGAATCCATGACA 1080
 331 Y I Q L R G V Q G G T L C F S K L D Y A V A W F I R E S M T                                  360

1081 ATATACATTTTTTTGTCTGCATTATGGGACCCAACTATAAGCTGGAGAACTGGTCGCTACAGATTACGCTGTGGGGGTACAGCAGAGGAA 1170
 361 I Y I F L S A L W D P T I S W R T G R Y R L R C G G T A E E                                  390

1171 ATCCTAGATGTATAACTACAGCTTTGTGACTGTATATAAAGGAAAAAAGAGAAGTATTATAAATTATGTTTATATAAATGCTTTTAAAAA 1260
 391 I L D V end                                                                                 394

1261 TCTACCTTCTGTAGTTTTATCACATGTATGTTTTGGTATCTGTTCTTTAATTTATTTTTGCATGGCACTTGCATCTGTGAAAAAAAA(3') 1347
```

B

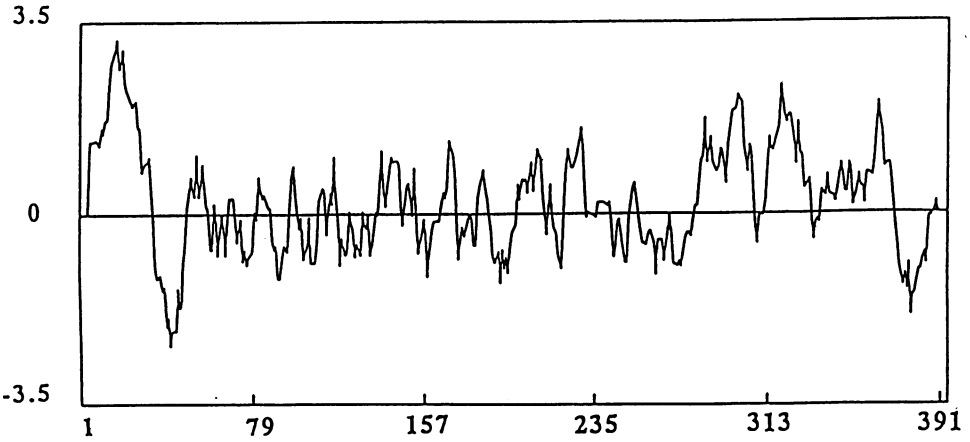

FIG. 6. (*A*) Nucleotide sequence and deduced amino acid sequence of GlcT-1. Putative signal-anchor sequence is underlined. (*B*) Hydropathy plot of the predicted amino acid sequence analyzed by the method of Kyte and Doolittle with a window of 10 (31). Positive values represent increased hydrophobicity.

vealed a single strongly hydrophobic segment near the N terminus; this is the putative signal-anchor sequence. In addition, considerable hydrophobicity was detected in the regions close to the C terminus; these may interact with the membrane. The PSORT program (33) predicted the type III (type Ib) (for review, see ref. 34) structure for the enzyme. Type III proteins extend a short N-terminal segment to the lumenal side and have a single transmembrane segment and a long cytosolic tail. This result supports the cytoplasmic orientation of GlcT-1. However, detailed empirical study using the cDNA is necessary to confirm the topology of the enzyme.

The ubiquitous expression of GlcT-1 mRNA and 5′ C+G-rich sequence suggests that the gene is essential for cell viability. However, our previous study (12) showed that GSLs are not essential for B16 cell growth *in vitro*. The elucidation of the discrepancy requires further investigation. The requirement of GSLs for cell growth was discussed previously by Radin (for review, see ref. 38).

We have previously reported differences in cellular properties between GM-95 and parental cells (12, 13). As for morphology, GM-95 cells show a typical fibroblastic shape while the parental cells showed irregular shape and tended to

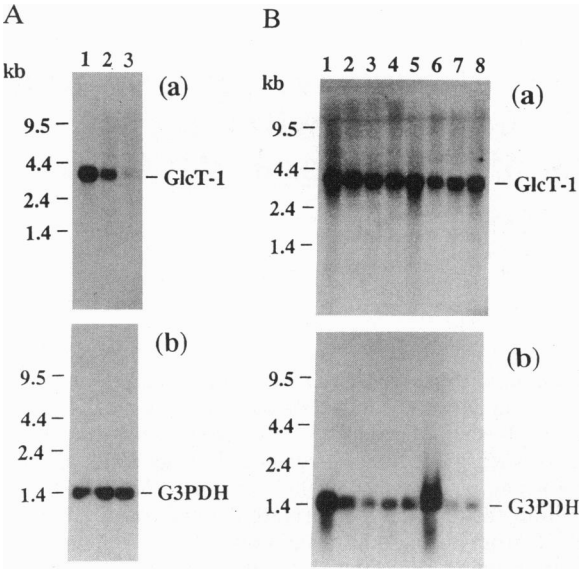

FIG. 7. Northern blot analysis of poly(A)$^+$ RNAs from the melanoma cell lines and human tissues. Poly(A)$^+$ RNA were subjected to electrophoresis on 1% agarose gel containing formaldehyde, and transblotted onto nylon membrane. A premade membrane was used for the analysis of mRNA from human tissues (Human Multiple Tissue Northern Blot, Clontech). The 1.1 kb-*Hin*dIII–*Xho*I cDNA fragment of pCG-1 was labeled with [α-^{32}P]dCTP by the Multiprime DNA labeling system (Amersham) and used as a probe. Position of GlcT-1 mRNA is a 3.5 kb [*A* (a) and *B* (a)]. (*A*) The melanoma cell lines [2 μg of poly(A)$^+$ RNA]: lane 1, SK-Mel-28; lane 2, MEB-4; lane 3, GM-95. (*B*) Human tissues [2 μg of poly(A)$^+$ RNA]: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. Hybridization with human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was carried out as control experiments [*A* (b) and *B* (b)]. Strong bands were observed in skeletal muscle and heart as reported previously (35).

overlap each other. The morphology, however, was not reverted by the expression of GlcT-1. The result suggests that the presence of GSLs in the membrane does not affect the morphology. The effect of GlcT-1 expression on other cellular properties remains to be solved.

The final goal of our study is to find biological roles of GSLs. GlcCer, the product of GlcT-1, is a pivotal GSL that serves as a precursor for numerous number of GSLs. These GSLs including gangliosides are believed to have impotant roles in various cellular processes. With the cDNA of GlcT-1, it might be possible to create knock-out mice (39) and various types of cell deficient in an entire group of GSLs except galactosphingolipids. The mice and cells would be invaluable tools for the elucidation of the GSL functions.

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