J. Biochem. 124, 171-178 (1998)

Functional Expression of Human Golgi CMP-Sialic Acid Transporter in the Golgi Complex of a Transporter-Deficient Chinese Hamster Ovary Cell Mutant[1]

Nobuhiro Ishida, Miyuki Ito, Shigemi Yoshioka, Ge-Hong Sun-Wada, and Masao Kawakita[2]

Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 18-22, Honkomagome 3-chome, Bunkyo-ku, Tokyo 113-8613

Received for publication, February 18, 1998

We recently described the cloning of putative human CMP-sialic acid transporter (hCST) cDNA [Ishida, N. *et al.* (1996) *J. Biochem.* 120, 1074-1078]. The hCST cDNA coded for a hydrophobic protein with an amino acid sequence showing a high degree of similarity (92% identity) to that of murine CMP-sialic acid transporter. In this report, we demonstrate that hCST corrects the CMP-sialic acid transporter-deficient phenotype of CHO-derived Lec2 cells, as judged from the recovery of WGA-sensitivity by transformants, and the recovery of CMP-sialic acid transporting ability by microsomal vesicles prepared from them. A peptide antibody against the C-terminus of the hCST protein detected the cDNA products expressed in the microsomes of the transformants. The subcellular localization of the hCST protein in the Golgi membrane was demonstrated by immunofluorescence microscopy, using the hCST-specific antibody. These results clearly indicate that hCST cDNA encodes the human CMP-sialic acid transporter protein. Plasma membrane-selective permeabilization combined with immunofluorescence microscopy provided strong evidence that the C-terminus of the human CMP-Sia transporter is exposed to the cytosol on the outer surface of the Golgi membrane.

Key words: CMP-sialic acid transporter, Golgi apparatus, immunofluorescence microscopy, nucleotide-sugar transporter, peptide antibody.

Nucleotide sugar transporters are localized in the membranes of the Golgi apparatus, and provide various glycosyltransferases in the Golgi lumen with appropriate substrates for their reactions (*1-4*). In the past few years, several nucleotide sugar transporter genes have been cloned (for a recent review, see Ref. *5*). These include those of the human and *Schizosaccharomyces pombe* UDP-galactose (UDP-Gal) transporters (*6-8*), the murine and hamster CMP-sialic acid (CMP-Sia) transporters (*9, 10*) and their putative human homologue (*7*), the *Kluyveromyces lactis* UDP-GlcNAc transporter (*11*), the *Leishmania donovani* and *Saccharomyces cerevisiae* GDP-mannose transporters (*12, 13*), and the putative human, murine and rat nucleotide-sugar transporters for an as yet unidentified substrate (*7*).

The products of these cDNAs exhibit 40-60% similarities to each other, and are predicted to be intrinsic membrane proteins with multiple membrane-spanning domains (*5-7*). They were expressed in the Golgi membranes when the cDNAs were introduced into pertinent transporter-deficient mutant cells (*9, 10, 12, 14*), and the membranes of these cells were shown to recover the nucleotide sugar-transporting activity (*7, 8, 11-13*). More recently, human UDP-Gal transporter and murine CMP-Sia transporter cDNAs were independently reported to be expressed heterologously in the yeast, *S. cerevisiae*, and in consequence, the microsomal membranes of the recombinant yeast acquired the ability to transport the respective nucleotide sugars (*15, 16*). These lines of evidence indicate that these cDNAs encode the nucleotide sugar transporter proteins *per se* instead of regulatory or auxiliary factors, particularly since the yeast does not possess intrinsic transporters for either UDP-Gal or CMP-Sia.

In one of our previous reports we described the cloning of a putative human CMP-Sia transporter (hCST) cDNA (*7*). This assignment was based on the fact that the hCST cDNA potentially coded for a protein whose amino acid sequence showed close similarity (92% identity) to that of murine CMP-Sia transporter. The assignment thus has to be verified based on more direct evidence. The results of experiments in this direction, which led us to conclude that the hCST cDNA does code for the human CMP-Sia transporter localized in the Golgi complex, are described in this report.

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan, and grants from Suntory Co., Japan, and Taisho Pharmaceutical Co., Japan.

[2] To whom correspondence should be addressed. Tel: +81-3-3823-2101 (Ext. 5285), Fax: +81-3-3823-2965, E-mail: kawakita@rinshoken.or.jp

Abbreviations: ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; Gal, galactose; HA, influenza virus hemagglutinin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; Sia, sialic acid; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Materials—The radioactive substrates, CMP-[9-^{3}H]sialic acid (33.2 Ci/mmol) and UDP-[4,5-^{3}H]galactose (48.3 Ci/mmol), were purchased from NEN Life Science Products. A human adult liver cDNA pool (Clontech Human Liver 5′-RACE-Ready cDNA) was purchased from Clontech Laboratory (Palo Alto, CA).

Cell Culture—CHO-K1 wild type cells, and Lec2 (ATCC CRL1736) and Lec8 (ATCC CRL1737) mutants (*17*) were maintained in minimum essential medium α (MEM-α) (Gibco/BRL, Gaithersberg, MD) supplemented with 10% fetal calf serum (FCS) (designated below as the standard medium). Transfection of expression vectors was carried out using Lipofectin reagent (Gibco/BRL), following the manufacturer's instructions. Stable transformants were selected in the standard medium containing 1 mg/ml G418.

DNA Sequencing—The nucleotide sequences of both strands of PCR products were determined by the dideoxy chain termination method (*18*) using a Dye terminator cycle sequencing FS ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA) or a Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham International plc, Buckinghamshire, England) with an ABI Prism A377 sequencer (Perkin-Elmer Applied Biosystems). At least three independent clones from PCR reactions were sequenced in order to exclude sequence errors introduced by the PCR.

Hemagglutinin Tag—hCST with a C-terminal influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA) was prepared by PCR using a primer, NI346 (5′-TTAGGCGTAGTCAGGGACGTCGTAAGGGTACACACCAATAACTCTCTC-3′), that contained a sequence for introducing the coding sequence of the HA tag (underlined), as explained under "RESULTS."

Assessment of Lectin Sensitivity—The same number of cells of each clone (10^4 cells in 0.1 ml of culture medium) was initially inoculated into the standard medium containing various concentrations of wheat germ agglutinin (WGA) (EY Laboratories, San Mateo, CA), and then the cells were grown for 3 days in a 96-well culture plate. The relative numbers of viable cells were compared by means of the tetrazolium-reduction method using a CellTiter96 assay kit (Promega) by measuring the absorbance at 550 nm according to the manufacturer's instructions.

Fluorescence-Activated Cell Sorting (FACS) Analysis—About 10^7 cells grown in the standard medium were detached from culture dishes by trypsin treatment, washed twice with 3 ml of ice-cold PBS, and then incubated on ice for 30 min in 50 μg/ml FITC conjugated WGA or GS-II (EY Laboratories) in 0.1 ml of PBS containing 1% BSA and 0.02% NaN_3. The FITC-labelled cells were washed three times with 3 ml of ice-cold PBS, and then analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA) to determine the fluorescence intensity. The instrument was equipped with an argon-ion laser which was operated at 488 nm with 15 mW energy. Emission from FITC was measured at 530 nm. Cells were passed through flow cytometers and the fluorescence intensity in 20,000 events was analyzed using the CELLQuest software (Becton Dickinson).

Preparation of Microsomal Membrane Vesicles—A microsomal fraction was prepared as described previously with slight modification (*14*). Cells were harvested, washed with an ice-cold 10 mM HEPES-Tris buffer (pH 7.4) containing 0.25 M sucrose, and then suspended in 4 volumes of a solution comprising 0.25 M sucrose, 10 mM HEPES-Tris buffer (pH 7.4), 1 mM EDTA, and 1 μg each of leupeptin, aprotinin and pepstatin A. The cells were disrupted twice with a homogenizer with a rotor stator generator probe (Physcotron Micro Homogenizer NS-310E; Niti-on, Chiba) at the speed setting of 10 for 30 s. The post-mitochondrial supernatant obtained through successive centrifugations of the homogenate at 1,000 $\times g$ for 5 min and 7,700 $\times g$ for 5 min was further centrifuged at 100,000 $\times g$ for 70 min to recover microsomal membrane vesicles in the pellet. The microsomal membranes were suspended in a solution comprising 0.25 M sucrose and 10 mM HEPES-Tris (pH 7.4), and then stored frozen at −80°C. The protein concentration of the vesicle preparation was determined with a BCA kit (Pierce, Rockford, IL).

Nucleotide-Sugar Transport Assay—The uptake of CMP-Sia and UDP-Gal was measured as described (*14*). The transport reaction was started by mixing 50 μl of microsomal vesicles (1 mg/ml) with an equal volume of a 2-times concentrated reaction medium to obtain a 100 μl reaction mixture comprising 0.25 M sucrose, 10 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 0.5 mM dimercaptopropanol and 1 μM CMP-[^{3}H]Sia or UDP-[^{3}H]Gal (6,400 Ci/mol). The samples were incubated for 1 min at 30°C unless otherwise specified, and then the reaction was stopped by 10-fold dilution with an ice-cold stop solution comprising 0.25 M sucrose, 10 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, and 1 μM non-radioactive CMP-Sia or UDP-Gal, depending on the radioactive substrate used. The entire reaction mixture was poured onto an Advantec Toyo A020A025A nitrocellulose filter (Advantec Toyo, Tokyo). The filter was washed three times with 1 ml of the ice-cold stop buffer and dried, and then the radioactivity remaining on the filter was determined. To assess the nonspecific adsorption of the radioactive substrate, reactions at 0°C were stopped immediately after the addition of the substrates. The radioactivity remaining on the nitrocellulose membrane filter under these conditions, that ranged from 16 cpm to 49 cpm, was subtracted as the background value.

Peptide-Antibody—A peptide corresponding to the 17 amino acid residues from the hCST C-terminus (*7*) was synthesized and rabbit anti-serum against the peptide conjugated with keyhole limpet hemocyanin was prepared by a commercial firm (Bio-Synthesis, Inc., Lewisville, TX). The IgG fraction was prepared from the crude anti-serum on an immobilized protein A column (Ampure PA; Amersham) according to the manufacturer's instructions. Specific antibodies (anti-hCST peptide antibodies) were enriched from the IgG fraction by affinity chromatography, using a column of AF-NH_2 Toyopearl 650 (Tosoh, Tokyo) coupled with the antigen peptide as described (*14*).

Western Blotting Analysis—The samples were fractionated on a 12% SDS-PAGE gel and then electrotransferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk and 0.5% Tween 20 in Tris-buffered saline (TBS), consisting of 20 mM Tris-HCl (pH 7.6) and 0.14 M NaCl, the membrane was incubated with the anti-hCST antibodies or rat anti-HA monoclonal antibody (mAb) 3F10 (Boehringer Mannheim) at room temperature for 1 h. The binding of the antibodies

was detected as chemiluminescence using HRP-conjugated protein A (1:20,000) (Amersham) or HRP-conjugated anti-mouse IgG antibodies (1:1,000) (Bio-Rad, Hercules, CA) with an ECL detection kit (Amersham).

Immunofluorescence—Cells were cultured on a Lab-Tek 8-well chamber glass slide (Nalge Nunc International, Naperville, IL), fixed with cold methanol for 6 min, and then incubated in PBS containing 0.2% gelatin (PBSG) for 30 min. The cells were incubated with primary antibodies in PBSG for 1 h. The Golgi 58-kDa protein was immunolocalized with mAb 58K-9 (Sigma).

Selective permeabilization of cells using streptolysin O (SLO) (Sigma) was performed according to the method described by Otto and Smith (*19*). The cells were washed with PBS and then fixed in 2% formaldehyde-PBS for 30 min at room temperature. The fixed cells were then incubated for 10 min at 4°C with SLO-PBS (200 U/ml), which had been pre-activated by incubation with 10 mM dithiothreitol (DTT) in PBS for 10 min at 0°C. Unbound SLO was removed by washing twice with ice-cold PBS and then the cells were incubated at 37°C for 20 min in 10 mM DTT in PBS. The cells were blocked with PBSG at room temperature for 30 min. The SLO-treated cells were then incubated with anti-HA mAb 3F10 and a rabbit polyclonal antibody against α-mannosidase II (*20*), kindly provided by K. Moremen (University of Georgia, Athens), in PBSG with or without 0.2% saponin for 45 min at room temperature. For the detection of specific binding of the antibodies, the cells were washed three times with PBSG, and then incubated with FITC-conjugated anti-rabbit IgG antibodies (Vector Laboratories, Barlingame, CA) and Cy3-conjugated anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Fluorescence labelling was visualized by laser scanning confocal microscopy (model MRC-600; Bio-Rad).

Northern Blot Analysis—Human hCST cDNA (about 880 bp) was prepared by PCR from pMKIT-neo-hCST using primers NI323 and NI274 (Fig. 1). Human β-actin cDNA was obtained from Clontech. Hybridization with a human multiple tissue Northern blot was carried out as recommended by the manufacturer (Clontech). Radioactivity was captured and visualized with Fuji BAS2000 (Fuji Photo Film, Tokyo).

RESULTS

Construction of the Expression Vectors—The complete open reading frame (ORF) for human putative CMP-Sia transporter (Fig. 1) was constructed using clone 44875 cDNA [The I.M.A.G.E. Consortium (*21*)], and a PCR fragment obtained by the same procedure as described (*7*) using NI273 and NI323 (Fig. 1) as the primers and a human adult liver cDNA pool as the template. The PCR fragment was ligated at the *Bst*EII site with the clone 44875 cDNA. During this process, we found an allelic variation, that is, ACG for Thr45 in the clone 44875 to ACC in the human cDNA pool. The mutation was silent like those in other human genes (*22*).

In order to facilitate detection of the recombinant protein, we also constructed an epitope-tagged human putative CMP-Sia transporter ORF with an influenza virus HA protein epitope at its C-terminus. A PCR primer, NI346, was designed to replace the termination codon of hCST ORF with the coding sequence for the HA-tag followed by a new termination codon. The codons occurring frequently in human genes were chosen, those scarcely observed in yeast being excluded. PCR involving NI272 and NI364 was performed to prepare the C-terminal portion of hCST cDNA with the HA-tag (Fig. 1). The intact 3′ region of hCST cDNA including the C-terminus and 3′-UTR was replaced with the PCR product utilizing the *Pst*I restriction site (Fig. 1).

The ORFs with and without the epitope-tag were inserted into a mammalian expression vector, pMKIT-neo, as described previously (*6, 7*) to yield pMKIT-neo-hCST and pMKIT-neo-hCST-cHA, respectively. The stable transformants, Lec2/hCST and Lec2/hCST-cHA, were selected for G418 resistance. CHO-K1 and Lec2 cells were also transfected with the pMKIT-neo control vector, and the resultant transformants, CHO-K1/vec and Lec2/vec cells, respectively, served as references for the biological and biochemical analyses below.

WGA Sensitivity—To determine whether or not the hCST cDNA corrects the mutant phenotype of Lec2 cells, the transformants were subjected to the WGA sensitivity assay. This lectin recognizes terminal sialic acid residues of cell-surface glycoconjugates, and binds to cells to exert its cytotoxic effect (*23, 24*). Figure 2 shows the results for representative transformant clones, namely CHO-K1/vec, Lec2/vec, Lec2/hCST, and Lec2/hCST-cHA. Lec2 cells are deficient in CMP-Sia transport activity, and their surface glycoconjugates lack terminal sialic acid residues (*25*). Lec2/vec cells therefore showed resistance to the cytotoxic effect of WGA. On the other hand, wild type CHO-K1/vec cells were highly sensitive to WGA, their growth being arrested at concentrations as low as 3 μg/ml. The introduction of either the hCST or hCST-cHA ORF abolished the resistance of Lec2 cells to WGA, resulting in

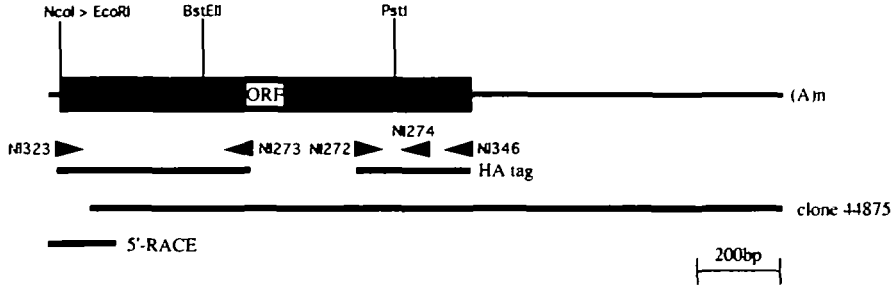

Fig. 1. **Construction of the open reading frame for the putative human CMP-Sia transporter with and without the HA tag.** The regions amplified by PCR to construct the hCST open reading frame (ORF) and the HA-tagged ORF are shown together with the PCR primers utilized. An *Eco*RI-*Nco*I adaptor was introduced at the *Nco*I site located just before the initiating methionine codon to facilitate insertion into pMKIT-neo. The portion of the hCST cDNA covered by clone 44875 and that determined by 5′-RACE in a previous work (*7*) are also indicated.

WGA sensitivity very close to that of CHO-K1/vec cells. This strongly suggests that the sialylated cell surface glycoconjugates were recovered by these transformant cells.

Lectin Binding—The binding of FITC-labelled lectins to cells was assessed by fluorescence-activated cell sorting (FACS) to obtain further information on the structures of the carbohydrate chains on the cell surface. As shown in Fig. 3, the binding capacity of Lec2 cells as to WGA was significantly reduced as compared to that of CHO-K1 cells (Fig. 3, A and B). The Lec2/hCST cells recovered the WGA-binding to the same level as that observed in the wild type cells (Fig. 3C). This is consistent with the results shown above.

We also analyzed the binding of *Griffonia simplicifolia* lectin GS-II. GS-II recognizes the terminal GlcNAc residues of cell-surface carbohydrate chains (*24, 26*). GS-II did not bind to either CHO-K1, Lec2, or Lec2/hCST cells (Fig. 3, E-G), while it efficiently bound to Lec8 cells (Fig. 3H). This is as expected, since cell-surface complex sugar chains should have terminal sialic acid residues in CHO-K1 and Lec2/hCST cells, and terminal galactose in Lec2 cells, respectively (*25*). On the other hand, Lec8 cells are known to be defective in UDP-Gal transport activity and should have truncated cell-surface glycans with GlcNAc at their termini (*27*). Lec8 cells bound a greater amount of WGA than Lec2 cells did, but significantly less than CHO-K1 cells did (Fig. 3D). This probably reflects the fact that WGA also recognizes terminal GlcNAc, although less efficiently than the terminal sialic acid residues (*23*).

Nucleotide Sugar Transporting Activities of Microsomal Vesicles—As described above, we observed the recovery of the wild type structure of surface glycans in Lec2 cells on introduction of the hCST gene. The CMP-Sia transporting activity of microsomal vesicles was measured to determine whether the recovery of sugar chains with terminal sialic acids is correlated with the recovery of the CMP-Sia transporting activity or not. Figure 4 indicates that the membrane vesicles from Lec2 cells lacked the ability to transport CMP-Sia (Fig. 4A, column 2), while their UDP-Gal transporting ability was comparable to that of

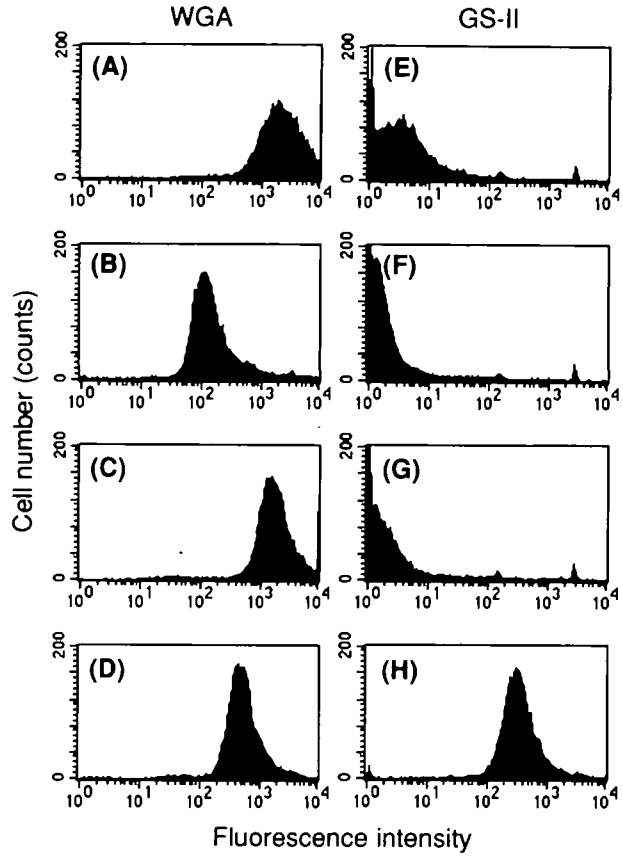

Fig. 3. **FACS analysis of lectin binding.** CHO-K1 (A, E), Lec2 (B, F), Lec2/hCST (C, G), and Lec8 (D, H) cells were stained with FITC conjugated to WGA (A-D) or GS-II (E-H). Fluorescence intensity was measured by FACS as described under "MATERIALS AND METHODS."

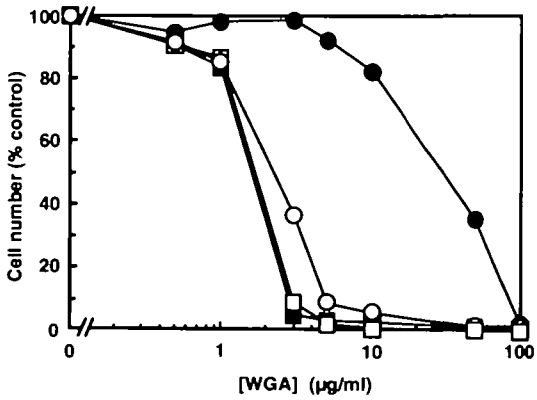

Fig. 2. **Recovery of WGA-sensitivity to the wild type level on introduction of human CST cDNAs into Lec2 cells.** The same number of cells (10^4 cells) of each clone was initially inoculated into the growth medium containing various concentrations of WGA, as indicated. After incubation for 3 days, the relative numbers of viable cells were compared by the dye-reduction method and are presented as the percentages of those of control cultures grown in the absence of the lectin. ■, CHO-K1/vec; ●, Lec2/vec; □, Lec2/hCST; ○, Lec2/hCST-cHA.

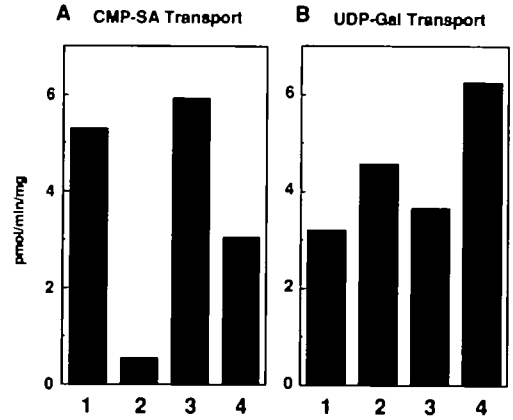

Fig. 4. **Recovery of CMP-Sia transporting activity by microsomal vesicles prepared from Lec2 transformants with human CST cDNAs.** Microsomal vesicles (50 μg/assay) were prepared from CHO-K1/vec (1), Lec2/vec (2), Lec2/hCST (3), and Lec2/hCST-cHA (4) cells, respectively. Assays were performed as described under "MATERIALS AND METHODS." The amount of radioactivity retained on the filter at 0°C at 0 min incubation (background) was subtracted from the corresponding experimental values.

vesicles derived from the wild type CHO-K1 cells (Fig. 4B). Upon the introduction of either hCST or hCST-cHA cDNA into Lec2 cells, the membrane vesicles derived from the transformants, Lec2/hCST or Lec2/hCST-cHA, recovered the CMP-Sia transporting activity (Fig. 4A, column 3 and 4).

The CMP-Sia transport reaction was temperature-dependent, so CMP-Sia was not accumulated in the membrane vesicles at all at 0°C (data not shown). The membrane vesicles from Lec2/hCST-cHA cells seemed to have lower CMP-Sia transporting activity than those from CHO-K1 cells. This may be in accordance with the observation that Lec2/hCST-cHA cells showed slightly lower sensitivity to WGA cytotoxicity as compared to that of CHO-K1 cells (Fig. 2). The addition of the HA tag at the C-terminus of the transporter may somewhat, but not critically, decrease the transporting activity.

Expression of the hCST Products in the Microsomal Membranes of the hCST-Transformants—Immunoblotting analysis was performed to determine whether or not the recovery of the transporting activity is accompanied by the presence of the recombinant proteins in the microsomal vesicles. Figure 5 shows that the anti-hCST peptide antibody recognized approximately equivalent amounts of 29 and 30.5 kDa proteins specifically expressed in Lec2/hCST cells and Lec2/hCST-cHA cells, respectively. The difference in mobility between these two proteins is consistent with the increase in the molecular mass due to the addition of the HA tag expected for the latter. The observed molecular mass of the HA-tagged hCST protein was also consistent with that of the HA-tagged murine CMP-Sia transporter protein reported by Berninsone *et al.* (*16*). As shown in Fig. 5B, the anti-HA mAb specifically recognized the 30.5 kDa protein expressed in Lec2/hCST-cHA cells. This observation further confirmed that the expression of the products of the introduced genes was detected by these antibodies.

Immunofluorescence Microscopy—To investigate the subcellular distribution of the transporter, immunofluorescence-microscopic analysis was performed as previously (*14*), utilizing the anti-hCST peptide antibody. Lec2/hCST cells were double-stained with the anti-hCST peptide antibody and monoclonal antibody 58K-9, an anti-Golgi 58-kDa protein antibody (*28, 29*). The Golgi region was distinctly identified on immunofluorescent staining with monoclonal antibody 58K-9 (Fig. 6B). The same organelle was clearly recognized by the anti-hCST peptide antibody (Fig. 6A), indicating that the hCST protein expressed in Lec2 cells is localized in the Golgi apparatus. The localization of the HA-tagged hCST protein was also investigated. Exactly overlapping regions were immunolabelled by both the anti-HA monoclonal antibody and the anti-hCST peptide antibody (Fig. 6, C and D), suggesting that the HA-tagged hCST protein is correctly localized in the Golgi apparatus.

C-Terminal Topology of the Human CMP-Sia Transporter in the Golgi Membrane—Streptolysin O (SLO) is a bacterial toxin produced by *Streptococcus pyogenes*. SLO may be conveniently utilized to selectively permeabilize the plasma membrane, since it generates pore complexes, which have sufficient diameters to allow the passage of larger proteins such as antibodies (*30*), in cholesterol-containing plasma membranes leaving the organellar membranes intact. The toxin was first bound at 4°C to the

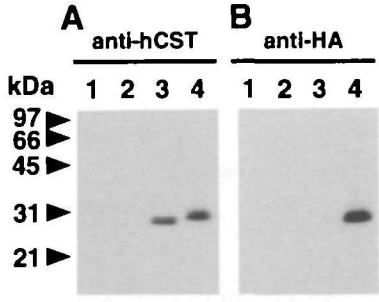

Fig. 5. **Expression of hCST products in microsomes from the transformants.** Microsomal membrane vesicles (40 μg) prepared as described under "MATERIALS AND METHODS" were fractionated on a 12% SDS-PAGE gel. The proteins were transferred to PVDF membranes, and then reacted with the anti-hCST peptide polyclonal antibody (1:1,000) (A), or the anti-HA mAb (1:2,000) (B). Binding of the antibodies was detected as chemiluminescence using an ECL detection system (Amersham). The sources of the membrane vesicles were: lane 1, CHO-K1/vec cells; lane 2, Lec2/vec cells; lane 3, Lec2/hCST cells; and lane 4, Lec2/hCST-cHA cells.

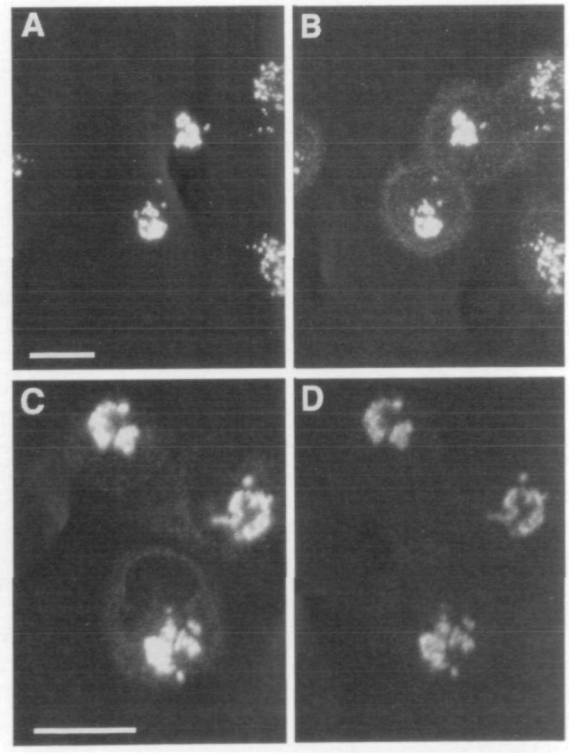

Fig. 6. **Subcellular localization of hCST proteins.** Lec2 cells were stably transfected with pMKIT-neo-hCST or pMKIT-neo-hCST-cHA encoding human CMP-Sia transporter (Lec2/hCST) (A-B) or human CMP-Sia transporter tagged with the HA epitope at its C-terminus (Lec2/hCST-cHA) (C-D), respectively. Double-labelling of the hCST protein (A, D), and either the Golgi 58-kDa protein (B) or HA epitope (C) was performed with the rabbit anti-hCST peptide antibody (1:100) and the anti-Golgi 58-kDa protein mAb 58K-9 (1:100) or the anti-HA mAb 3F10 (1:100), followed by anti-rabbit Ig antibody-FITC (1:80) and anti-mouse Ig antibody-Cy3 (1:100), as described under "MATERIALS AND METHODS." The shape of the cells can be observed in (B) as the background fluorescence. Bar: 10 μm.

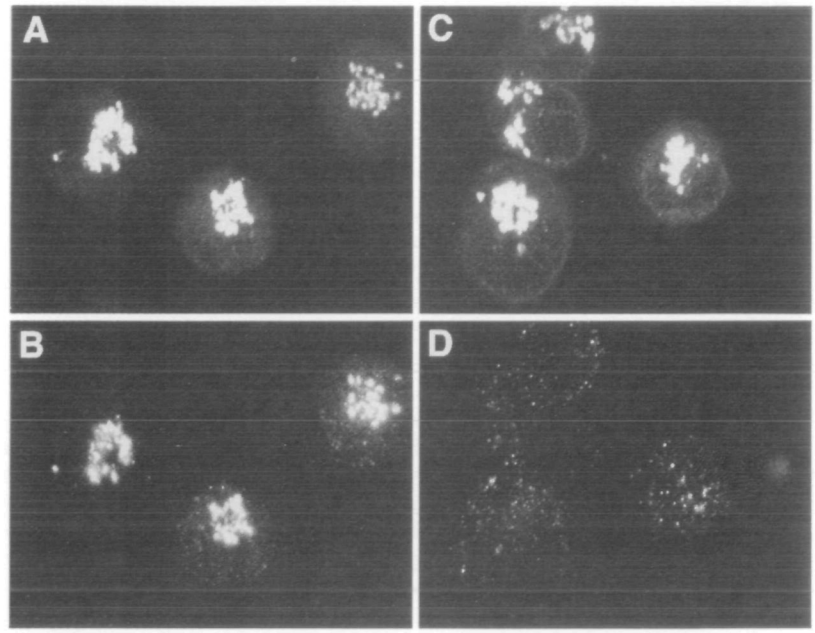

Fig. 7. **C-Terminal topology of the human CMP-Sia transporter.** Lec2/hCST-cHA cells were first treated with SLO to selectively permeabilize the plasma membrane, and then further incubated with (A-B) or without (C-D) saponin to nonselectively permeabilize all the cellular membranes. Cells were double-stained with the anti-HA mAb 3F10 (1:100) (A, C) and anti-mannosidase II polyclonal antibody (1:1,000) (B, D). Detection of the specific binding of the antibodies was achieved as described in the legend to Fig. 6.

surface of intact cells without causing permeabilization. The excess toxin was removed at this stage, and subsequently pore formation was induced by simply raising the temperature to 37°C. The selectivity of SLO is markedly improved by means of this two-step procedure.

The orientation of the C-terminus of hCST relative to the Golgi membrane was examined by immunofluorescence staining using SLO or a saponin as permeabilizing reagent. The immunofluorescence staining with an antibody against the catalytic domain of α-mannosidase II was used as a control for Golgi lumenal localization (*20*). When SLO-permeabilized Lec2/hCST-cHA cells were further treated with saponin to permeabilize the internal membranes, the antibodies against Golgi α-mannosidase II clearly detected their specific antigen to give strong labelling in the Golgi region (Fig. 7B). The same organelle was clearly recognized by the anti-HA monoclonal antibody (Fig. 7A), again confirming that the HA-tagged hCST protein expressed in Lec2 cells is correctly localized in the Golgi apparatus. On the other hand, when the plasma membranes of Lec2/hCST-cHA cells were selectively permeabilized with SLO, the antibodies against Golgi α-mannosidase II failed to show any specific labelling (Fig. 7D). In contrast, in the same SLO-treated cells, the anti-HA monoclonal antibody gave clear and intense Golgi-specific staining (Fig. 7C). These results clearly indicate that the C-terminus of the hCST protein is exposed to the cytosol on the external side of the Golgi membrane.

Distribution of the Human CMP-Sia Transporter in Human Tissues—As it was demonstrated above that the hCST gene product represents the CMP-Sia transporter located in the Golgi apparatus, we examined the expression of the gene in various human tissues. As shown in Fig. 8, the ubiquitous expression of the hCST mRNA was evident. A single band corresponding to 2.0 kb was observed for all the tissues examined (Fig. 8, arrow). We could not observe the band corresponding to the minor 1.4 kb band detected in mouse by Eckhardt *et al.* (*9*) for human tissues.

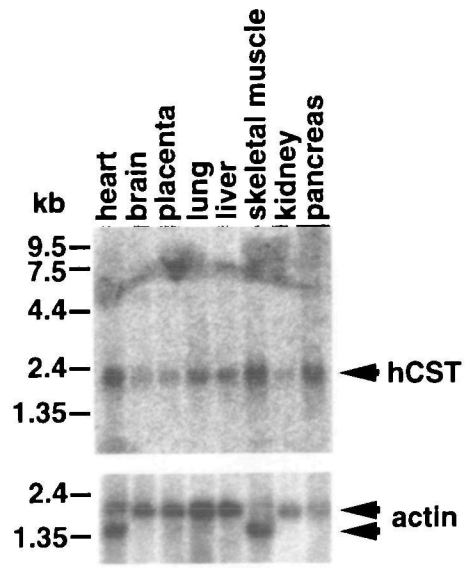

Fig. 8. **Expression of the CMP-Sia transporter transcripts *in vivo*.** Poly(A)$^+$ RNAs (2 μg/lane) from human adult tissues were probed with a hCST cDNA or a human β-actin cDNA. The positions of marker RNAs and hybridized transcripts (arrows) are indicated. In heart and skeletal muscle, the muscle form of actin of 1.6-1.8 kbases long was also observed (*33, 34*).

DISCUSSION

We recently reported the cloning of several hUGT-related genes (*7*). We had noticed the partial sequences of these genes while analyzing dbEST, a database widely recognized for its usefulness (*31*), and used them to obtain the full sequences of these genes. hCST cDNA was one of the thus found and cloned cDNAs. Homology analysis revealed that the hCST gene exhibited a high degree of similarity (92% identity in terms of the amino acid sequence of the coding

region) with the murine CMP-Sia transporter gene (*9*), and it was highly likely that the former represented the human homologue of the latter (*7*).

In this report, we showed that hCST cDNA, when introduced into a CMP-Sia transporter-deficient cell line, Lec2, was able to correct the structural defects of surface oligosaccharides, as judged from the binding (Fig. 3) as well as the cytotoxic effect of WGA on the transformants (Fig. 2). We also demonstrated that the cDNA encodes a protein that is expressed in the Golgi membranes of a transformant cell line, Lec2/hCST (Figs. 5 and 6), and concomitantly renders the membranes competent as to CMP-Sia transport (Fig. 4). Based on these results, we conclude that the hCST gene encodes the human CMP-Sia transporter protein. The identification of the human CMP-Sia transporter cDNA has thus finally been completed. Our previous works revealed the molecular characteristics of human UDP-Gal transporter (*6, 7, 14, 15*). Human CST therefore represents the second example of a human nucleotide-sugar transporter characterized so far in considerable molecular detail. Exact information concerning the human CST structure will be invaluable for future biomedical studies.

CMP-Sia transporting activity has not been directly demonstrated so far in microsomal membranes obtained from CST-transformants of mammalian cells deficient in the transporter. The microsomal vesicles obtained in this study from hCST-transformed Lec2 cells showed high CMP-Sia transporting activity, *i.e.* approximately 10 times as high as that of those from murine CST-transformed yeast cells on an equivalent protein basis (*16*) (Fig. 4). The expression system described here will prove useful in future analysis of the biochemical mechanisms underlying the transport.

Experiments involving streptolysin O clearly indicated that the C-terminus of the hCST protein in the Golgi membrane faced the cytosol (Fig. 7). We have obtained similar results also for the UDP-Gal transporter expressed in mouse Had-1 cells (Sun-Wada, G.-H., unpublished observation). This orientation relative to the membrane is consistent with the model proposed previously for the UDP-Gal transporter (*5, 6*). On the other hand, Ma *et al.* reported that the C-terminus of the *Leishmania* GDP-mannose transporter faced the Golgi lumen (*12*). The structures of nucleotide sugar transporters may be somewhat different between mammals and protozoa.

Recent progress in the molecular cloning of nucleotide-sugar transporters has opened the door for penetrating studies on various aspects of the transporters, including their structure-function relationships, their modes of action, and developmental and tissue-specific control of their expression and its physiological implication (*5*). The determinant of the substrate specificity is one of the critical issues that is entirely obscure at present. We now have two well defined transporters, human UDP-Gal transporter and human CMP-Sia transporter, in hand (*6, 7, 14*). These can be expressed in the Golgi membranes of cultured cells, where they should be localized in intact tissues, and are easily detected by specific antibodies, as clearly demonstrated here and in one of our recent studies (*14*). Moreover, they are mutually related, exhibiting approximately 60% similarity in terms of the amino acid sequence (*7*). We may therefore readily construct chimeric molecules from these transporters, and examine their activity and substrate specificity, using appropriate transporter-deficient mutant cells such as Had-1, Lec8, and Lec2 cells (*24, 25, 27, 32*). Such studies are currently in progress in this laboratory, and will provide us with valuable information on the structure-function relationships of the nucleotide-sugar transporter molecules.

We are grateful to Drs. K. Maruyama and K. Moremen for kindly providing the pMKIT-neo vector and α-mannosidase II antiserum, respectively.

REFERENCES

1. Hirschberg, C.B. and Snider, M.D. (1987) Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu. Rev. Biochem.* **56**, 63-87
2. Hirschberg, C.B. (1996) Transporters of nucleotides and nucleotide derivatives in the endoplasmic reticulum and Golgi apparatus in *Organellar Ion Channels and Transporters* (Clapham, D.E. and Ehrlich, B.E., eds.) pp. 105-120, The Rockefeller University Press, New York
3. Driouich, A., Faye, L., and Staehelin, L.A. (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *Trends Biochem. Sci.* **18**, 210-214
4. Varki, A. (1998) Factors controlling the glycosylation potential of the Golgi apparatus. *Trends Cell Biol.* **8**, 34-40
5. Kawakita, M., Ishida, N., Miura, N., Sun-Wada, G.-H., and Yoshioka, S. (1998) Nucleotide sugar transporters: Elucidation of their molecular identity and its implication for future studies. *J. Biochem.* **123**, 777-785
6. Miura, N., Ishida, N., Hoshino, M., Yamauchi, M., Hara, T., Ayusawa, D., and Kawakita, M. (1996) Human UDP-galactose translocator: Molecular cloning of a complementary DNA that complements the genetic defect of a mutant cell line deficient in UDP-galactose translocator. *J. Biochem.* **120**, 236-241
7. Ishida, N., Miura, N., Yoshioka, S., and Kawakita, M. (1996) Molecular cloning and characterization of a novel isoform of the human UDP-galactose transporter, and of related complementary DNAs belonging to the nucleotide-sugar transporter gene family. *J. Biochem.* **120**, 1074-1078
8. Tabuchi, M., Tanaka, N., Iwahara, S., and Takegawa, K. (1997) The *Schizosaccharomyces pombe gms1*$^+$ gene encodes an UDP-galactose transporter homologue required for protein galactosylation. *Biochem. Biophys. Res. Commun.* **232**, 121-125
9. Eckhardt, M., Muhlenhoff, V., Bethe, A., and Gerardy-Schahn, R. (1996) Expression cloning of the Golgi CMP-sialic acid transporter. *Proc. Natl. Acad. Sci. USA* **93**, 7572-7576
10. Eckhardt, M. and Gerardy-Schahn, R. (1997) Molecular cloning of the hamster CMP-sialic acid transporter. *Eur. J. Biochem.* **248**, 187-192
11. Abeijon, C., Robbins, P.W., and Hirschberg, C.B. (1996) Molecular cloning of the Golgi apparatus uridine diphosphate-*N*-acetylglucosamine transporter from *Kluyveromyces lactis*. *Proc. Natl. Acad. Sci. USA* **93**, 5963-5968
12. Ma, D.Q., Russell, D.G., Beverley, S.M., and Turco, S.J. (1997) Golgi GDP-mannose uptake requires *Leishmania LPG2*: A member of a eukaryotic family of putative nucleotide-sugar transporters. *J. Biol. Chem.* **272**, 3799-3805
13. Dean, N., Zhang, Y.B., and Poster, J.B. (1997) The *VGR4* gene is required for GDP-mannose transport into the lumen of the Golgi in the yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 31908-31914
14. Yoshioka, S., Sun-Wada, G.-H., Ishida, N., and Kawakita, M. (1997) Expression of the human UDP-galactose transporter in the Golgi membranes of murine Had-1 cells that lack the endogenous transporter. *J. Biochem.* **122**, 691-695
15. Sun-Wada, G.-H., Yoshioka, S., Ishida, N., and Kawakita, M. (1998) Functional expression of the human UDP-galactose transporter in the yeast *Saccharomyces cerevisiae*. *J. Biochem.* **123**, 912-917
16. Berninsone, P., Eckhardt, M., Gerardy-Schahn, R., and Hirsch-

berg, C.B. (1997) Functional expression of the murine Golgi CMP-sialic acid transporter in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 12616-12619
17. Stanley, P. and Siminovitch, L. (1977) Complementation between mutants of CHO cells resistant to a variety of plant lectins. *Somatic Cell Genet.* **3**, 391-405
18. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
19. Otto, J.C. and Smith, W.L. (1994) The orientation of prostaglandin endoperoxide synthases-1 and -2 in the endoplasmic reticulum. *J. Biol. Chem.* **269**, 19868-19875
20. Moremen, K.W., Touster, O., and Robbins, P.W. (1991) Novel purification of the catalytic domain of Golgi α-mannosidase II. Characterization and comparison with the intact enzyme. *J. Biol. Chem.* **266**, 16876-16885
21. Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M.B. (1996) The I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression. *Genomics* **33**, 151-152
22. Ishida, N., Fujita, H., Fukuda, Y., Noguchi, T., Doss, M., Kappas, A., and Sassa, S. (1992) Cloning and expression of the defective genes from a patient with δ-aminolevulinate dehydratase porphyria. *J. Clin. Invest.* **89**, 1431-1437
23. Bhavanandan, V.P. and Katlic, A.W. (1979) The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. *J. Biol. Chem.* **254**, 4000-4008
24. Hara, T., Hattori, S., and Kawakita, M. (1989) Isolation and characterization of mouse FM3A cell mutants which are devoid of Newcastle disease virus receptors. *J. Virol.* **63**, 182-188
25. Deutscher, S.L., Nuwayhid, N., Stanley, P., Briles, E.I., and Hirschberg, C.B. (1984) Translocation across Golgi vesicle membranes: a CHO glycosylation mutant deficient in CMP-sialic acid transport. *Cell* **39**, 295-299
26. Ebisu, S. and Goldstein, I.J. (1978) *Bandeiraea simplicifolia* lectin II in *Methods in Enzymology* (Ginsburg, V., ed.) Vol. 50, pp. 350-354, Academic Press, New York
27. Deutscher, S.L. and Hirschberg, C.B. (1986) Mechanism of galactosylation in the Golgi apparatus. A Chinese hamster ovary cell mutant deficient in translocation of UDP-galactose across Golgi vesicle membranes. *J. Biol. Chem.* **261**, 96-100
28. Bloom, G.S. and Brashear, T.A. (1989) A novel 58-kDa protein associates with the Golgi apparatus and microtubules. *J. Biol. Chem.* **264**, 16083-16092
29. Lahtinen, U., Hellman, U., Wernstedt, C., Saraste, J., and Pettersson, R.F. (1996) Molecular cloning and expression of a 58-kDa *cis*-Golgi and intermediate compartment protein. *J. Biol. Chem.* **271**, 4031-4037
30. Gravotta, D., Adesnik, M., and Sabatini, D.D. (1990) Transport of influenza HA from the trans-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins. *J. Cell Biol.* **111**, 2893-2908
31. Marra, M.A., Hillier, L., and Waterston, R.H. (1998) Expressed sequence tags-ESTablishing bridges between genomes. *Trends Biochem. Sci.* **14**, 4-7
32. Hara, T., Endo, T., Furukawa, K., Kawakita, M., and Kobata, A. (1989) Elucidation of the phenotypic change on the surface of Had-1 cell, a mutant cell line of mouse FM3A carcinoma cells selected by resistance to Newcastle disease virus infection. *J. Biochem.* **106**, 236-247
33. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J., and Kirschner, M.W. (1980) Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. *Cell* **20**, 95-105
34. Pari, G., Jardine, K., and McBurney, M.W. (1991) Multiple CArG boxes in the human cardiac actin gene promoter required for expression in embryonic cardiac muscle cells developing in vitro from embryonal carcinoma cells. *Mol. Cell. Biol.* **11**, 4796-4803