Rapid Communication J. Biochem. 122, 691-695 (1997)

Expression of the Human UDP-Galactose Transporter in the Golgi Membranes of Murine Had-1 Cells That Lack the Endogenous Transporter[1]

Shigemi Yoshioka, Ge-Hong Sun-Wada, Nobuhiro Ishida, 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

Received for publication, July 17, 1997

In our previous study, we demonstrated that UDP-galactose transporter cDNAs (hUGT1 and hUGT2) were able to complement the genetic defect of murine Had-1 cells that were deficient in the UDP-galactose transporter, and that the microsomal vesicles isolated from Had-1-transformants, which were obtained through transfection with these cDNAs, had recovered the ability to uptake UDP-galactose [Ishida, N. *et al.* (1996) *J. Biochem.* 120, 1074-1078]. In this report, we describe the preparation of peptide antibodies that recognize the hUGT isozymes, and the detection of hUGT proteins expressed in the transformants. The occurrence of the endogenous hUGT1 protein in HeLa cells was also detected. Using the hUGT1-specific antibodies, the subcellular localization of hUGT1 in the Golgi membrane was demonstrated by immunofluorescence microscopy and subcellular fractionation. These studies led us to develop a simple procedure, based on Percoll density gradient centrifugation, for preparing functional Golgi vesicles from the hUGT1-transformed Had-1 cells, that will facilitate future biochemical analyses of the UDP-galactose transporter for the elucidation of its structure-function relationship.

Key words: Golgi apparatus, immunofluorescence microscopy, nucleotide-sugar transporter, peptide antibody, UDP-galactose transporter.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

Nucleotide sugar transporters are localized in the membranes of the Golgi complex, and deliver the substrates for glycoconjugate biosynthesis from the cytosol to the intra-Golgi compartments (*1*). Several groups including ours recently identified cDNA clones that encode putative nucleotide sugar transporters of man (*2, 3*) and other mammals (*4, 3*), protozoa (*5*), and yeast (*6, 7*), based on their ability to complement the genetic defects of nucleotide sugar transporter-deficient mutant cells and on their structural similarity. The cDNAs code for hydrophobic proteins that are predicted to be intrinsic membrane proteins with multiple membrane-spanning domains with varying degrees of similarity to each other (*3*). Furthermore, in some of these cases, pertinent nucleotide sugar transporting activity was detected in Golgi-enriched membrane vesicles (*3, 5-7*), while in some the products tagged with a hemagglutinin epitope were immunocytochemically detected in the Golgi complex region (*4, 5*), in parallel with the expression of a cDNA product in transporter-deficient cells.

As to the human UDP-Gal transporter (hUGT) isoforms (hUGT1 and hUGT2), which we described in our recent publications, we showed that the microsome fraction obtained from Had-1-derived cells, that had recovered a lectin-sensitivity spectrum similar to that of the parental FM3A cells as the result of transfection with hUGT1 and hUGT2 cDNAs, had acquired the UDP-Gal transporting activity that was lacking in the microsomes similarly isolated from Had-1 cells (*2, 3*). To establish further that these cDNAs indeed code for the transporter proteins *per se* and also to better characterize the proteins produced from the cDNAs, we have now attempted to show that the products of these cDNAs are located exclusively in the Golgi membranes recovered from UGT-positive transformant cells.

To detect the products of hUGT1 and hUGT2 cDNAs, peptide antibodies were raised in rabbits. Specifically, two peptides representing the C-terminal portions of hUGT1 and hUGT2 with sequences of 377RGDLITEPFLPKSVL-VK393 (hUGT1 peptide) and 381LITEPFLPKLLTKVK-GS396 (hUGT2 peptide), respectively, were utilized. An extra Cys residue was added at the N-terminus of each peptide in order to couple it to keyhole limpet hemocyanin (Sigma, St. Louis, MO, USA) *via* 4-(maleimidobenzoyl)-*N*-hydroxysuccinimide (Pierce, Rockford, IL, USA). The resulting conjugates were used to immunize New Zealand White rabbits. The IgG fraction was prepared from crude antisera using an immobilized protein A column (Ampure PA; Amersham, Buckinghamshire, UK). Specific antibodies were enriched from the IgG fraction using affinity

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and grants from Suntory Co., Japan, and Kirin Brewery 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; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

columns, namely ones of AF-Amino Toyopearl (Tosoh, Tokyo) coupled with the antigen peptides, according to the procedure described previously (*8*).

The specificity of the purified antibodies was assessed by means of an enzyme-linked immunosorbent assay using the hUGT1 and hUGT2 peptides as solid-phase antigens (Table I). The antibody against the hUGT1 peptide (anti-hUGT1 antibody) reacted exclusively with the hUGT1 peptide, with practically no cross-reaction with the hUGT2 peptide, although a part of the amino acid sequence is shared by the hUGT1 and hUGT2 peptides. The antibody against the hUGT2 peptide (anti-hUGT2 antibody) showed significant cross-reaction with the hUGT1 peptide due to the common sequence in the two peptides.

The specificities of the anti-hUGT1 and anti-hUGT2 antibodies thus being defined, we next prepared microsomal vesicles from FM3A, Had-1, Had-1-hUGT1-GS-II-3 (Had-1/hUGT1), and Had-1-hUGT2-GS-II-G5 (Had-1/hUGT2) cells (*2, 3*), and examined whether the products of the hUGT cDNAs were detected in these vesicles. Figure 1 shows that the anti-hUGT1 antibody detected a 36 kDa protein on immunoblots of Had-1/hUGT1 microsomal proteins. The anti-hUGT2 antibody detected a specific band on those of Had-1/hUGT2 microsomal proteins. While the anti-hUGT2 antibody preferentially detected a 36 kDa protein in Had-1/hUGT2 cells, it also reacted with that observed in Had-1/hUGT1 cells, indicating that the antibody cross-reacted with the hUGT1 protein. The binding of these proteins with the antibodies was effectively quenched by an appropriate antigen-peptide, verifying the specificity of the detection. Neither peptide antibody gave a positive signal with immunoblots of FM3A or Had-1 microsomes, which indicates that the murine UGT protein is not recognized by anti-hUGT antibodies. These results definitely indicate that the products of hUGT1 and hUGT2 cDNAs are expressed in the microsomal membranes obtained from the respective transformant cells.

TABLE I. **Determination of the relative preference of the peptide antibodies for the peptide antigens.** An enzyme-linked immunosorbent assay was carried out to compare the relative affinities of the peptide antibodies for the peptides used to raise the antibodies, as follows: the wells of microtiter plates (Corning #25801) were coated with 0.1 ml of the hUGT1 peptide or hUGT2 peptide (10 μg/ml) in phosphate-buffered saline (PBS) for 1 h at room temperature. The wells were blocked with 0.2 ml of 5% skimmed milk and 0.02% Tween 20 in Tris-buffered saline (TBS), consisting of 20 mM Tris-HCl (pH 7.6) and 0.14 M NaCl, for 90 min at room temperature, and then washed three times with 0.2 ml of 0.02% Tween 20 in TBS. A serial dilution of anti-hUGT1 or anti-hUGT2 antibodies in PBS containing 1% BSA was added to each well, and then the plate was incubated at room temperature for 1 h. The antibodies were removed, and the wells were washed as before. To each well was added 0.1 ml of HRP-labeled protein A (Amersham) in PBS containing 1% BSA at 1:2,500 dilution, and then the plate was kept at room temperature for 1 h. The wells were washed 3 times as above, and then 0.1 ml of a H_2O_2-2,2′-azino-di[3-ethyl-benzothiazoline sulfonate(6)] (ABTS) solution (KPL, Gaithersburg, MD, USA) was added to each well. After keeping the plate for 30 min at room temperature, the absorbance at 405 nm was measured. The highest dilution factors at which the absorbance exceeded 0.7 are indicated for each antibody and peptide set.

	hUGT1 peptide	hUGT2 peptide
Anti-hUGT1	1:8,000	<1:500
Anti-hUGT2	1:4,000	1:8,000

The hUGT1 protein, which is recognized by the anti-hUGT1 antibody, was clearly discernible in the microsomal membranes of HeLa cells (Fig. 1E). Although the anti-hUGT2 antibody also detected specific protein(s) with the same mobility (data not shown), we can not determine at present whether hUGT2 is expressed in HeLa cells or not, since the anti-hUGT2 antibody cross-reacts with the hUGT1 protein. The mobility of the hUGT1 and hUGT2 proteins expressed in the transformant cells on SDS-PAGE was identical with that of the intrinsic hUGT1 protein in HeLa cells, but was considerably greater than that expected from their calculated molecular weights (*2, 3*). While the reason for this is unclear at present, Kanamori *et al.* made a similar observation for the acetyl-CoA transporter (*9*), and the murine CMP-sialic acid transporter expressed in *Saccharomyces cerevisiae* also behaved similarly (*10*).

To investigate the subcellular localization of the transporter, immunocytochemical analysis of the hUGT1 isoform was carried out, utilizing the anti-hUGT1 antibody. Had-1/hUGT1 cells were double-stained with the anti-hUGT1 antibody and monoclonal antibody 58K-9, an anti-Golgi 58-kDa protein antibody (Sigma) (*11, 12*). The Golgi region was distinctly identified on immunofluorescent staining using monoclonal antibody 58K-9 (Fig. 2C), and the same organelle were recognized by the anti-hUGT1 antibody (Fig. 2B), indicating that the hUGT1 protein expressed in Had-1 cells is localized in the Golgi apparatus.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

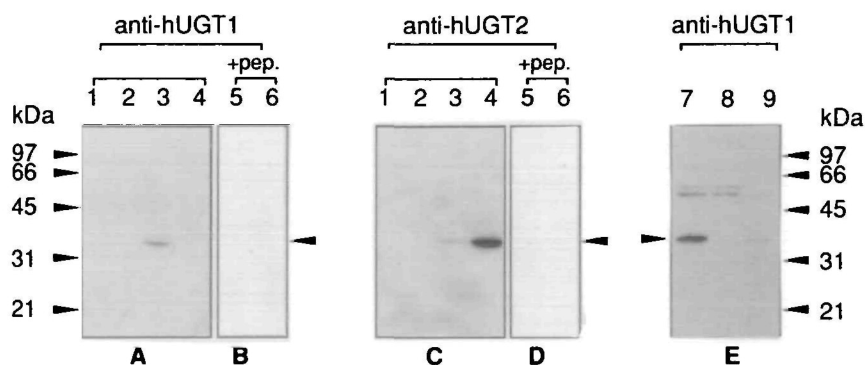

Fig. 1. **Expression of hUGT1 and hUGT2 in microsomes obtained from the transformants and HeLa cells.** The samples were fractionated on a 12% SDS-PAGE gel, and then electrotransferred to a polyvinylidene difluoride membrane. After blocking with TBS containing 5% skimmed milk and 0.5% Tween 20, the membrane was incubated with anti-hUGT1 (A, E) or anti-hUGT2 (C) antibodies (1:2,000). For peptide competition, the anti-hUGT1 and anti-hUGT2 antibodies were preincubated with 0.2 mg/ml hUGT1 peptide (B) and hUGT2 peptide (D), respectively, at room temperature for 2 h. Binding of the antibodies was detected through chemiluminescence using HRP-conjugated protein A (Amersham) and an ECL detection kit (Amersham). The post-mitochondrial supernatant (Lanes 7 and 8) and microsomal vesicles (Lanes 1-6, and 9) were prepared from FM3A (Lane 1), Had-1 (Lane 2), Had-1/hUGT1 (Lanes 3, 5, and 7), Had-1/hUGT2 (Lanes 4, 6, and 8), and HeLa (Lane 9) cells, respectively, as described previously (*3*).

The immunofluorescence observed for HeLa cells also revealed the colocalization of the hUGT1 protein and the Golgi 58-kDa protein (Fig. 2, E and F). It is particularly significant that the hUGT1 protein naturally occurring in cells was demonstrated to be localized in the Golgi membranes.

The Golgi-localization of the hUGT1 protein was also examined by subcellular fractionation of Had-1/hUGT1 cells. A post-mitochondrial supernatant prepared from Had-1/hUGT1 cells was layered on top of 20% Percoll, followed by centrifugation at 27,000 rpm for 50 min in a Beckman 60Ti rotor. The fractions were assayed for NADPH-cytochrome *c* reductase (*13*), an ER marker, β-1,4-galactosyltransferase (*14*), a *trans*-Golgi marker, and UDP-Gal transporting (*3*) activities. The amount of the hUGT1 protein was estimated by immunoblot analysis using the anti-hUGT1 antibody. Figure 3 shows that the occurrence of the hUGT1 protein was closely correlated with the presence of the UDP-Gal transporting activity in individual fractions. It is also obvious that the distribution of the UDP-Gal transporting activity over the fractions was coincident with that of β-1,4-galactosyltransferase activity. In contrast, NADPH-cytochrome *c* reductase activity is distributed separately from these ones.

These results indicate that the products of hUGT1 and hUGT2 cDNAs, the hUGT proteins, are localized in the Golgi membranes. In previous studies, we already demonstrated that their occurrence in the microsomal vesicles recovered from Had-1 or its hUGT transformants determines whether the vesicles are competent in transporting UDP-Gal or not (*3*). We may thus conclude that the hUGT proteins represent the major, if not all, part of the Golgi UDP-Gal transporter molecule. At least, the proteins determine the substrate specificity of the transport apparatus. The results presented above also provide direct evidence of the subcellular localization of the transporter in the Golgi membrane, which has been suggested based mainly on the results of experiments involving Golgi-enriched microsomal fractions (*15*).

Fraction 6 in Fig. 3 was enriched in β-1,4-galactosyl-

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

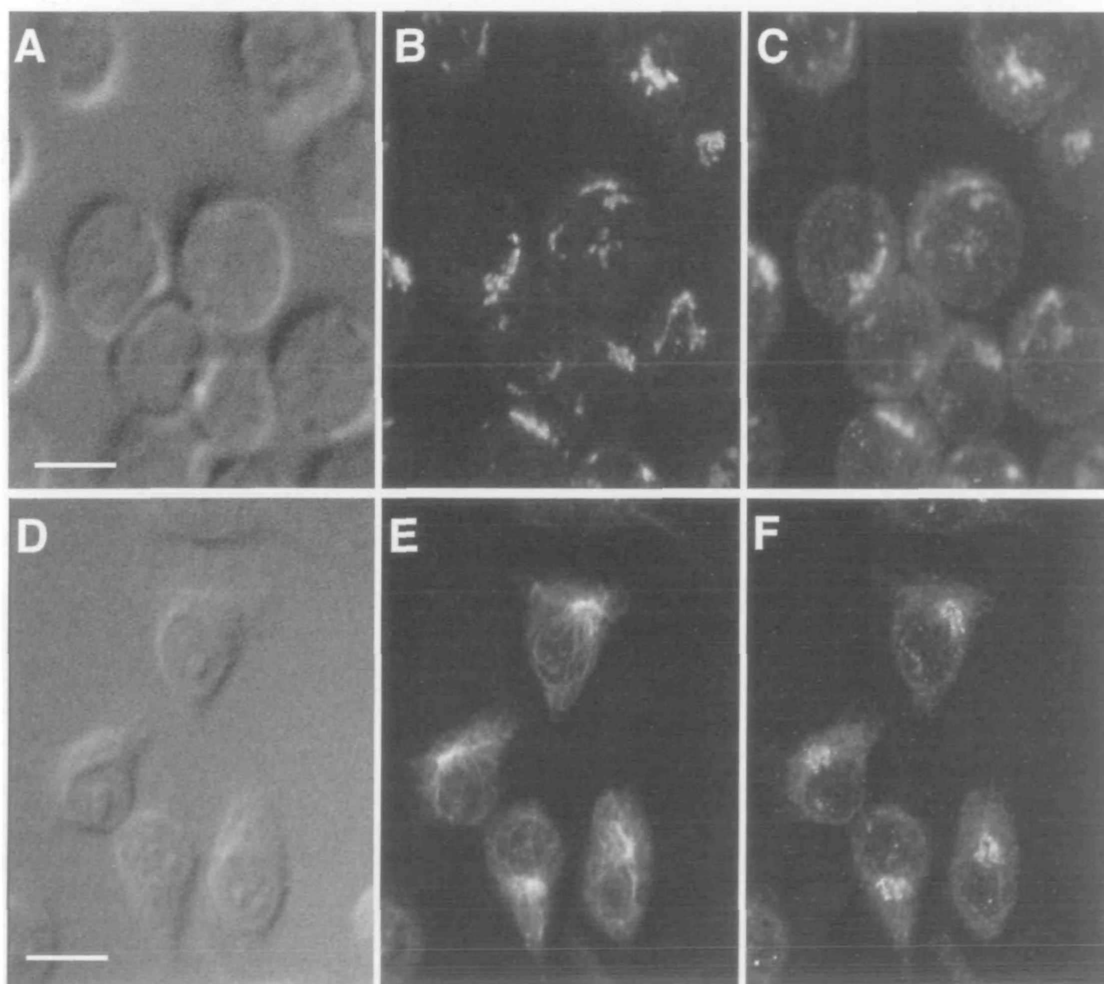

Fig. 2. **Localization of hUGT1 with a Golgi marker 58-kDa protein by immunofluorescence microscopy.** Had-1/hUGT1 (A–C) and HeLa (D–F) cells were fixed in methanol at −20°C, and then doubly labeled with affinity-purified rabbit anti-hUGT1 (B, E) and mouse anti-Golgi 58-kDa protein (C, F) antibodies according to the procedure described previously (*11*). Primary antibodies were used at 1:50 dilution in PBS containing 0.2% gelatin. FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG antibodies were obtained from Vector Laboratories (Barlingame, CA, USA), and Jackson Immuno Research Laboratories (West Grove, PA, USA), respectively. The preparation was mounted and viewed by laser scanning confocal microscopy (model MRC-600; BioRad Labs, Hercules, CA). A and D: Nomarski images. Bar, 10 μm (A–C); 20 μm (D–F).

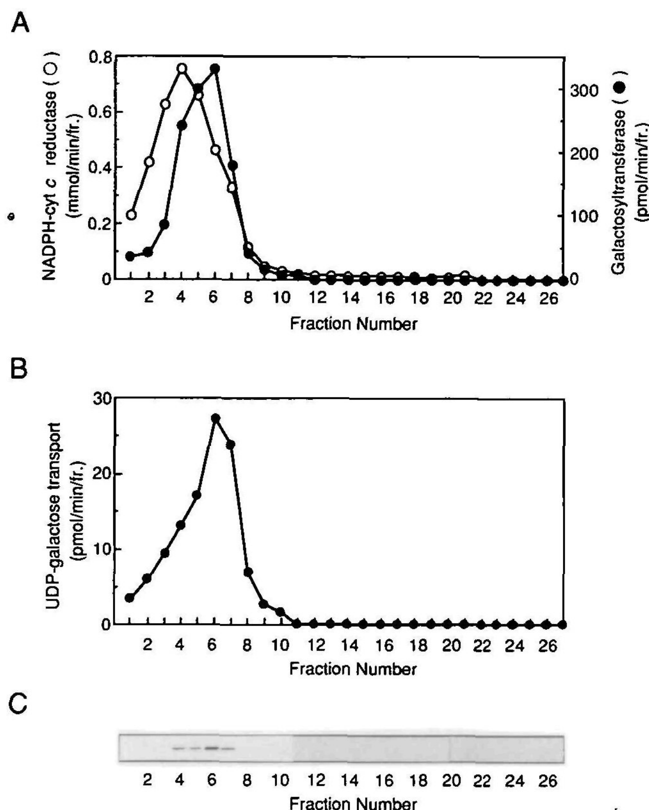

Fig. 3. **Fractionation of post-mitochondrial membrane vesicles derived from Had-1/hUGT1 cells by Percoll density gradient centrifugation.** A post-mitochondrial supernatant was prepared from 4×10^8 Had-1/hUGT1 cells as described in Fig. 1. A 3-ml aliquot of the supernatant was layered on top of 32 ml 20% Percoll containing 10 mM HEPES-Tris (pH 7.4), 0.25 M sucrose, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin A, followed by centrifugation at 27,000 rpm for 50 min in a Beckman 60Ti rotor. After the centrifugation, 1.2-ml fractions were collected from the top of the tube. A: The NADPH-cytochrome c reductase (○) and β-1,4-galactosyltransferase (●) activities were assayed in 5- and 20-μl aliquots of each fraction, respectively, as described (*13, 14*). B: The UDP-Gal transporting activity was assayed as described before with slight modification (*3*). Briefly, the transport reaction was started by mixing 50 μl of each fraction with an equal volume of a 2-times concentrated reaction medium to obtain a 100 μl reaction mixture containing 0.25 M sucrose, 10 mM HEPES-Tris (pH 7.4), 1 mM $MgCl_2$, 0.5 mM dimercaptopropanol, and 1 μM UDP-[^{3}H]Gal (6,400 Ci/mol). The samples were incubated for 1 min at 30°C, and then the reaction was stopped by 10-fold dilution with an ice-cold stop solution comprising 0.25 M sucrose, 10 mM HEPES-Tris (pH 7.4), 1 mM $MgCl_2$, and 1 μM nonradioactive UDP-Gal. The entire reaction mixture was poured onto an Advantec Toyo A045A025A nitrocellulose filter (Advantec Toyo, Tokyo). The filter was washed 3 times with 1 ml of the ice-cold stop buffer, and then dried. The radioactivity remaining on the filter was determined. C: Immunoblot analysis of the hUGT1 protein was carried out as in Fig. 1 on a 10-μl aliquot of each fraction, using the anti-hUGT1 antibody.

transferase activity by approximately 2- and 3-fold, as compared to the crude microsomal membranes and the post-mitochondrial supernatant, respectively. The Golgi-enriched membrane fraction is very well preserved, since the activity of β-1,4-galactosyltransferase, whose catalytic site is on the lumenal side of the membrane, was only detectable in the presence of Triton X-100, as shown in Fig. 4. This indicates that the membrane vesicles retained the correct sidedness as was found in the intact Golgi apparatus. Figure 4 also shows that the uptake of UDP-Gal was not observed in the presence of detergents such as Triton X-100. Thus, the uptake of UDP-Gal by the Golgi membrane fractions represents the net transport of the substrate into sealed vesicles across the membranes. We have thus developed a simple procedure for preparing functionally well preserved Golgi membranes using hUGT1-transformants of Had-1 cells. This will significantly facilitate future biochemical analyses for elucidation of the structure-function relationships of nucleotide sugar transporters including the UDP-Gal transporter.

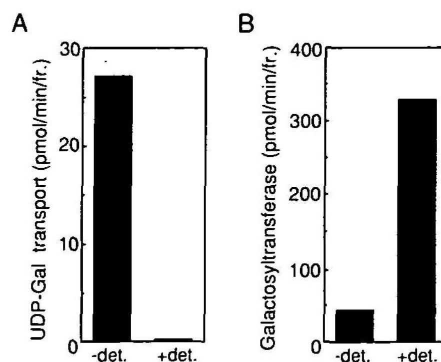

Fig. 4. **Effects of Triton X-100 on the UDP-Gal transport and the β-1,4-galactosyltransferase activities of isolated Golgi vesicles.** Fraction 6 in Fig. 3 was saved, and 50- and 20-μl aliquots of it were used to measure the UDP-Gal transport (A) and β-1,4-galactosyltransferase (B) activities, respectively. The reaction was performed as in Fig. 3 with or without 0.2% Triton X-100 (+/− det.).

We are grateful to Dr. Hiroyuki Aizawa, The Tokyo Metropolitan Institute of Medical Science, for his helpful advice on confocal microscopy.

REFERENCES

1. Abeijon, C., Mandon, E.C., and Hirschberg, C.B. (1997) Transporters of nucleotide sugars, nucleotide sulfate and ATP in the Golgi apparatus. *Trends Biochem. Sci.* **22**, 203-207
2. 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
3. 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
4. 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
5. 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
6. 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
7. 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

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

8. Yoshioka, S., Suzuki, T., and Kawakita, M. (1997) Analysis of the distribution of Na^+/H^+ exchanger isoforms among the plasma membrane subfractions of bovine kidney cortex: Reevaluation of methods for fractionating the brush-border and the basolateral membranes. *J. Biochem.* **122**, 641-646
9. Kanamori, A., Nakayama, J., Fukuda, M.N., Stallcup, W.B., Sasaki, K., Fukuda, M., and Hirabayashi, Y. (1997) Expression cloning and characterization of a cDNA encoding a novel membrane protein required for the formation of *O*-acetylated ganglioside: a putative acetyl-CoA transporter. *Proc. Natl. Acad. Sci. USA* **94**, 2897-2902
10. Berninsone, P., Eckhardt, M., Gerardy-Schahn, R., and Hirschberg, C.B. (1997) Functional expression of the murine Golgi CMP-sialic acid transporter in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 12616-12619
11. 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
12. 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
13. Omura, T. and Takesue, S. (1970) A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome *c* reductase from rat liver microsomes. *J. Biochem.* **67**, 249-257
14. Verdon, B. and Berger, E.G. (1983) Galactosyltransferase in *Methods of Enzymatic Analysis*, 3rd ed. (Bergmeyer, H.U., ed.) Vol. III, pp. 374-381, Verlag Chemie, Weinheim
15. 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

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012