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UDP-Galactose:Ceramide Galactosyltransferase Is a Class I Integral Membrane Protein of the Endoplasmic Reticulum*

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UDP-galactose:ceramide galactosyltransferase (CGalT) transfers UDP-galactose to ceramide to form the glycosphingolipid galactosylceramide. Galactosylceramide is the major constituent of myelin and is also highly enriched in many epithelial cells, where it is thought to play an important role in lipid and protein sorting. Although the biochemical pathways of glycosphingolipid biosynthesis are relatively well understood, the localization of the enzymes involved in these processes has remained controversial. We here have raised antibodies against CGalT and shown by immunocytochemistry on ultrathin cryosections that the enzyme is localized to the endoplasmic reticulum and nuclear envelope but not to the Golgi apparatus or the plasma membrane. In pulse-chase experiments, we have observed that newly synthesized CGalT remains sensitive to endoglycosidase H, confirming the results of the morphological localization experiments. In protease protection assays, we show that the largest part of the protein, including the amino terminus, is oriented toward the lumen of the endoplasmic reticulum. CGalT enzyme activity required import of UDP-galactose into the lumen of the endoplasmic reticulum by a UDP-galactose translocator that is present in the Golgi apparatus of CHO cells but absent in CHOlec8 cells. Finally, we show that CGalT activity previously observed in Golgi membrane fractions *in vitro*, in the absence of UDP-glucose, is caused by UDP-glucose:ceramide glucosyltransferase. Therefore all galactosylceramide synthesis occurs by CGalT *in vivo* in the lumen of the endoplasmic reticulum.

Glycosphingolipids are enriched in the outer membrane leaflet of the plasma membrane of most eukaryotic cells, where they play a structural role in rigidifying and protecting the cell surface. A remarkable property of glycosphingolipids is found in the myelin sheath of Schwann cells where galactosylceramide (GalCer)[1] and sulfatide are involved in axonal insulation, myelin function, and stability (1–3). The enormous diversity in glycosidic structure of glycosphingolipids suggests specific roles of individual glycosphingolipids in cell physiology. Glycosphingolipids are involved in a variety of cellular processes including differentiation, cell-cell interaction, transmembrane signaling (4–6), and internalization of bacterial toxins (7) and viruses (8). Furthermore, glycosphingolipids are thought to play a key role in the sorting of lipids and proteins to the apical plasma membrane domain of polarized epithelial cells (9, 10).

Although the biochemical pathways of glycosphingolipid synthesis are relatively well established, the intracellular localization and the topology of the enzymes involved in these pathways are incompletely understood. Until recently, these questions have largely been addressed by measuring the activity of these enzymes in isolated subcellular fractions (11–14). The usefulness of such approaches, however, is limited, because removal of contaminating membranes has the caveat of selecting a subfraction of the membrane of interest. Alternatively, enzymes associated with a given intracellular compartment may dissociate from accessory factors, resulting in diminished activity or specificity. As a consequence, the localization of some of the glycosphingolipid-synthesizing enzymes is not clear. One of these enzymes is the UDP-galactose:ceramide galactosyltransferase. It catalyzes the transfer of galactose from UDP-galactose (UDP-Gal) to ceramide, yielding GalCer (15).

CGalT was recently cloned (16–18), and knockout studies have shown that there is only one GalCer-synthesizing enzyme in the brain (1, 3). CGalT contains a carboxy-terminal KKVK sequence that may act as an endoplasmic reticulum (ER) retrieval signal, and the lack of complex glycosylated oligosaccharide chains is consistent with an ER localization (19). Still, a vast body of controversial results concerning the intracellular localization of CGalT has been reported. Biochemical enzyme assays on semipurified membranes and immunocytochemistry suggest that GalCer synthesis occurs in the Golgi complex and ER (13, 20–23) and plasma membrane (24–27).

As part of our ongoing efforts to define the molecular mechanism of glycosphingolipid-mediated intracellular protein and lipid sorting, we here investigated the cellular location of CGalT and its membrane topology. We raised antibodies against CGalT and show that it is a class I integral membrane

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[1] The abbreviations used are: GalCer, galactosylceramide; GlcCer, glucosylceramide; ER, endoplasmic reticulum; CGalT, UDP-galactose:ceramide galactosyltransferase; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; Endo H, endoglycosidase H; PAGE, polyacrylamide gel electrophoresis; NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); PDI, protein-disulfide isomerase; CGlcT, UDP-glucose:ceramide glucosyltransferase.

protein that is localized to the ER but not to the Golgi complex or the plasma membrane. Importantly, we found that CGlcT (28) in addition to GlcCer also synthesizes GalCer from a short chain ceramide *in vitro* when assayed in the presence of UDP-Gal, without UDP-glucose (UDP-Glc). This explains many of the ambiguities previously observed for CGalT localization.

EXPERIMENTAL PROCEDURES

Materials—Reagents used in this study were from commercial sources and described in previous papers originating from this laboratory (13, 23, 29, 30).

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells, CHOlec8 cells (ATCC, Rockville, MD), and HeLa cells were cultured as described before (31). D6P2T cells, MEB4 cells, and CGlcT-deficient GM95 cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Transient expression in HeLa cells was done with recombinant vaccinia T7 RNA polymerase, and protein expression was analyzed 5 h after infection (31). CHOlec8 cells were transfected with CGalTpcDNA3 (23) using the calcium phosphate procedure (32). Stable cell lines were obtained by subcloning individual colonies. Positive clones were selected by measuring CGalT enzyme activity as described (23). Transfected CGalT-CHO (23) and CGalT-CHOlec8 cells were cultured in α-minimal essential medium containing 10% fetal calf serum and 0.5 mg/ml geneticin. Protein expression was induced by 5 mM sodium butyrate (Fluka, Buchs, Germany) 14–16 h prior to all experiments (33).

Plasmid Construction—Specific regions of CGalT (Fig. 1) were amplified in PCR reactions using CGalTpcDNA3 (23) as template and the following primer sets: for 635, 5′-CGG GAT CCA AAA TCA TCA TTG TGC CGC CAA TATG-3′ (forward) and 5′-GGG AAT TCA TCA TTG GGG TCA ACC AGT AGC AG-3′ (reverse); for 636, 5′-CGG GAT CCC CTG CTG AAG TCG GAG CGC CTG-3′ (forward) and 5′-GGG AAT TCG TTA GCA ATG TCT TCT GAC AGA TAC-3′ (reverse); for 637, 5′-CGG ATC CAG AAA AGT CAA AAG TCT GTT CTA G-3′ (forward) and 5′-GGG AAT TCA TTT TAC CTT TTT TTC ATG TTT AAT ATG-3′ (reverse); for 638, 5′-CGG GAT CCGT CAA GTA TCT GTC AGA AGA CAT TGC-3′ (forward) and 5′-GGG AAT TCG AAC GGA GGT GAT GGG CTC C-3′ (reverse). PCR products were ligated between the *Bam*HI and *Eco*RI sites of pRSET-A (Invitrogen, Leek, The Netherlands). A c-Myc epitope was spliced between Ser^{69} and Leu^{70} of CGalT by separately amplifying the 5′ and 3′ region of its cDNA in PCR reactions using CGalTpcDNA3 as template and the following primer sets: for the 5′ region, 5′-CGT CAA TGG GAG TTT GTT TTG GCA C-3′ (forward) and 5′-CTC TTC CGA TAT CAG CTT CTG TTC CTC GCT GTA GTG ATT AGA TGG GTC AAT GTC TC-3′ (reverse); for the 3′ region, 5′-GAG GAA CAG AAG CTG ATA TCG GAA GAG GAT CTA CTC CAG CGA TAC CCA GGG-3′ (forward) and 5′-GGT CAA GGA AGG CAC GGG GGA G-3′ (reverse). PCR products were ligated into pGEMT-easy (Promega, Madison, WI). The 3′ region was released with *Eco*RV and *Spe*I and ligated between the *Eco*RV and *Spe*I sites of 5′ region pGEMT-easy. CGalTmyc was released with *Hin*dIII and *Xba*I and inserted in pcDNA3 (Invitrogen). A CGlcT cDNA was generously provided by Yoshio Hirabayashi and ligated in the *Not*I site of pcDNA3 to produce CGlcTpcDNA3. All constructs made by PCR were confirmed by sequencing both strands (34).

Antibodies—pRSET-A constructs were transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI) and used for fusion protein production. Fusion proteins were insoluble and purified under denaturing conditions on nickel-nitrilotriacetic acid columns (Qiagen, Leusden, The Netherlands) according to the vendor's instructions. His_6-CGalT fusion proteins were dialyzed against PBS and injected into New Zealand White rabbits. Microsomal proteins prepared from CGalT-CHO cells were separated on preparative 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes and used for affinity purification of antibodies. The mouse monoclonal antibodies 1D3 against PDI and CTR433 against a Golgi protein (35) were generous gifts of Stephen Fuller and Michel Bornens, respectively. The mouse monoclonal antibody 9E10 against the Myc epitope was described previously (36).

In Vitro Transcription and Translation—CGalT was synthesized from CGalTpcDNA3 in a coupled T7 RNA polymerase transcription-translation system (Promega) in the presence of rabbit reticulocyte lysate, dog pancreas microsomes (Promega), and $Tran^{35}S$-label (ICN, Costa Mesa, CA) as described previously (37). The translation mixture was diluted with PBS containing 1% Triton X-100 and spun for 10 min at 13,000 rpm in a microcentrifuge at 4 °C, after which CGalT was immunoprecipitated from the supernatant.

Metabolic Labeling—CGalT-CHO cells were washed with PBS and methionine- and cysteine-free minimal essential medium containing 20 mM Hepes, pH 7.4 (pulse medium). The cells were subsequently incubated for 30 min in pulse medium and labeled with 250 μCi/ml $Tran^{35}S$-label for 5 or 15 min at 37 °C. Cells were washed and chased at 37 °C in growth medium containing 5 mM methionine, 5 mM cysteine, and 20 mM Hepes, pH 7.4. After different periods of chase time, the cells were lysed in PBS, 1% TX-100, and CGalT was immunoprecipitated from detergent lysates as described below. HeLa cells were depleted as described for CGalT-CHO cells and labeled for 45 min with 150 μCi/ml $Tran^{35}S$-label. Cells were next detergent-lysed and processed for immunoprecipitation.

Cell Fractionation—Cells were washed, gently scraped in 250 mM sucrose, 10 mM Hepes, 1 mM EDTA-NaOH, pH 7.2 (homogenization buffer), and broken by 12–14 passages through a 25-gauge needle. A postnuclear supernatant was prepared by centrifugation for 15 min at $375 \times g$. Protein concentrations were adjusted to 1.0 mg of protein/ml using the BCA assay (Pierce). For some experiments, postnuclear supernatants were layered over 1 ml of 0.4 M sucrose, 1 ml of 1.25 M sucrose in homogenization buffer and centrifuged for 30 min at 50,000 rpm in a SW60 rotor. Membranes were retrieved from the 0.4/1.25 M sucrose interface and used for blot purification of antibodies against CGalT.

Protease Protection Assay—Fifty μl (50 μg) of post-nuclear supernatant prepared from metabolically labeled CGalT-CHO cells was incubated with 0.1 mg/ml of proteinase K or trypsin for 60 min at 10 °C in the presence or absence of 0.5% saponin. Samples were transferred to ice, and the reaction was stopped by adding phenylmethylsulfonyl fluoride (2.5 mg/ml), leupeptin (0.25 mg/ml), aprotinin (0.25 mg/ml), and pepstatin A (0.25 mg/ml) to the indicated final concentrations. Membranes were solubilized in 0.5% saponin, and CGalT was immunoprecipitated from the detergent lysates in the presence of protease inhibitors.

Immunoprecipitation and Endoglycosidase H Digestion—Antibodies were prebound to protein A-Sepharcyl CL4B beads, and immunoprecipitations were done exactly as described (37). Immunoprecipitates were resuspended in 50 μl of endoglycosidase H (Endo H) buffer (50 mM sodium citrate, pH 5.5, 20 mM EDTA, 0.1 M 2-mercaptoethanol, 0.1% SDS containing 1 μg/ml of chymostatin, leupeptin, aprotinin, pepstatin and 0.5 mM phenylmethylsulfonyl fluoride). Samples were split into two equal aliquots, one of which received 3 milliunits of Endo H, and both tubes were incubated for 6 h at 30 °C and processed for SDS-PAGE.

SDS-PAGE and Western Blot—After the addition of 4× reducing Laemmli sample buffer, samples were heated for 5 min at 95 °C and resolved by SDS-PAGE on 10% minigels. Gels were analyzed by fluorography or a STORM860 PhosphorImager using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For Western blotting, polyvinylidene difluoride transfers were blocked for 90 min in PBS, 5% Protifar (Nutricia, Zoetermeer, The Netherlands), 0.2% Tween 20 (blotto). Primary antibody incubations were done for 60 min in blotto. Detection was with horseradish peroxidase-conjugated goat anti-rabbit IgG using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). For quantitative Western blots, detection was done with ^{125}I-protein A and PhosphorImager analysis with ImageQuant software.

Glycosphingolipid Synthesis—Cells were homogenized, and postnuclear supernatant was prepared as described above. Unless stated otherwise, postnuclear supernatants were incubated for various periods of time at 37 °C with 1% bovine serum albumin, 2 mM UDP-Glc, 2.0 mM UDP-Gal, 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 50 μM C_6-NBD-ceramide (NBD-Cer). At the end of the incubation period, lipids were extracted as described (38). Samples were dried under nitrogen and applied to TLC plates using chloroform/methanol (2:1, v/v). Fluorescent lipids were separated by two-dimensional thin layer chromatography, identified by comparison with standards, and quantitated as described (23, 38).

Immunofluorescence Microscopy—Cells were grown on coverslips to 40–60% confluency. The cells were fixed with 3% paraformaldehyde; quenched in PBS, 50 mM NH_4Cl; and incubated for 1 h in PBS, 0.5% bovine serum albumin, 0.1% saponin (blocking buffer). The cells were labeled with affinity-purified antibody 635 against CGalT, the mouse monoclonal antibody 1D3 against PDI, or the mouse monoclonal antibody CTR433 against a Golgi protein. After 30 min, the coverslips were washed for four periods of 10 min with blocking buffer and counterstained for 15 min with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein-labeled goat anti-rabbit IgG and indocarbocyanine-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The coverslips were mounted in Mowiol and examined with a Leica confocal microscope (Leica, Heidelberg, Germany) attached to a Leica

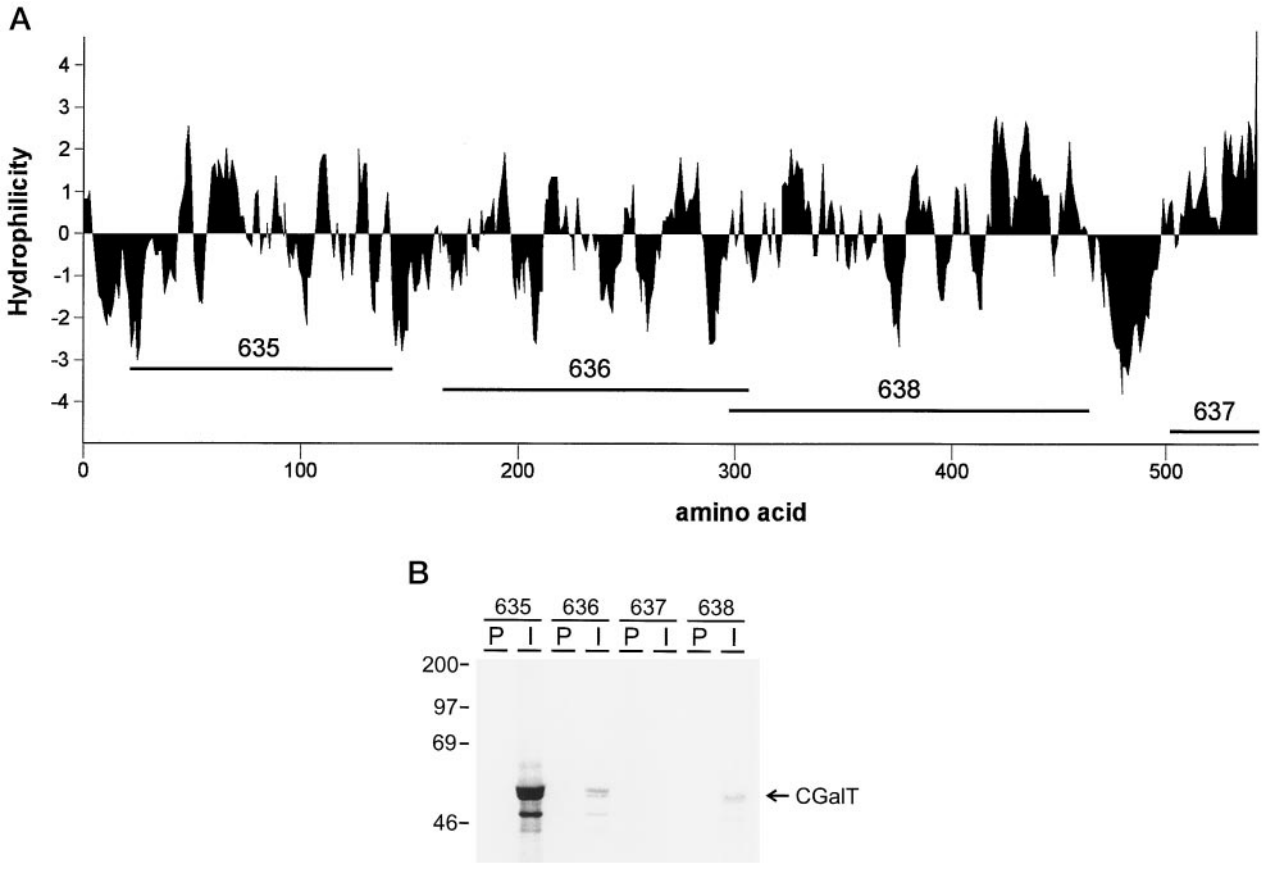

FIG. 1. **Generation of antisera against CGalT.** *A*, hydrophilicity profile of the translated cDNA sequence of CGalT generated by the method of Kyte and Doolittle with a seven-residue moving window. *Horizontal bars below* the hydrophilicity plot denote parts of CGalT that were expressed as His-tagged fusion proteins and used for antibody production in rabbits. *B*, ^{35}S-labeled CGalT was produced from CGalTpcDNA3 in a coupled *in vitro* transcription-translation system in the presence of dog pancreas microsomes. Expression products were immunoprecipitated with antisera 635–638 (*I*), or the corresponding preimmune sera (*P*) and separated on a 10% SDS-polyacrylamide gel.

microscope using separate filters for each fluorochrome viewed: for 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein, λ_{ex} = 488 nm and λ_{em} = 515 LP; for indocarbocyanine, λ_{ex} = 568 nm and λ_{em} = 585 LP. Singly labeled cells were examined to exclude the possibility that bleed-through occurred for the given confocal conditions. Images were imported into Adobe Photoshop and printed on a Tektronix dye sublimation printer.

Immunoelectron Microscopy—For immunogold electron microscopy, cells were fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After 2 h at room temperature, the cells were scraped, embedded in 10% gelatin, and stored for ultracryotomy as described (48). Cryosections were prepared as described (49) and labeled with rabbit CGalT antibody, and with the monoclonal 1D3 antibody against PDI, followed by protein A gold. For single labeling of CGalT, we used 10- or 15-nm protein A gold. For double label experiments, CGalT was detected with 15-nm protein A gold, and PDI was detected with 5-nm protein A gold. A swine anti-mouse antibody was used to enhance binding of protein A gold to sections labeled with monoclonal antibodies. The intracellular distribution of gold label was quantitated by counting the gold particles associated with identifiable organelles on sections prepared from CGalT-CHO cells (n = 10). Data were corrected for nonspecific labeling of sections prepared from nontransfected control CHO cells (n = 10).

RESULTS

Characterization of CGalT Antibodies—To avoid difficulties associated with the use of enzyme assays on partially purified cell fractions for localizing CGalT, we first raised antibodies against the protein in rabbits. For this purpose, we expressed four regions of CGalT as polyhistidine-tagged fusion proteins in *E. coli*. Three fusion proteins contained parts of the predicted luminal domain and one the putative cytoplasmic domain of the protein (Fig. 1*A*). Antisera were first tested for their ability to immunoprecipitate CGalT that was synthesized in an *in vitro* transcription translation system in the presence of microsomes. As shown in Fig. 1*B*, three of the four antisera recognized CGalT expression products, whereas the corresponding preimmune sera did not. The antibodies detected at least three bands with different mobility in SDS-polyacrylamide gels. The two lowest bands probably represent immature forms of the protein caused by incomplete *N*-glycosylation or degradation products. The band with a molecular mass of approximately 54 kDa most likely is mature CGalT, since it has the same molecular weight as the major band we observed on Western blots of whole cell lysates and in pulse-chase experiments (see below).

Expression of CGalT in Transfected and Wild Type Cells—The novel antibodies allowed us to characterize the molecular features of CGalT in more detail. To obtain cells with high expression levels of CGalT, we generated stable cell lines by transfecting CHO (23) and CHOlec8 cells with the CGalTpcDNA3. CHO cells were selected as recipients because they do not express endogenous GalCer (39) and therefore represented an ideal background for our studies. Both CHO and CHOlec8 cells transfected with CGalT (CGalT-CHO and CGalT-CHOlec8) expressed a protein with a molecular mass of approximately 54 kDa that was recognized by antiserum 635 on Western blots (Fig. 2). Two other antisera (636 and 638) that reacted with CGalT by immunoprecipitation (Fig. 1*B*) also efficiently recognized the 54-kDa protein (not shown). In the nontransfected control CHO and CHOlec8 cell lines, this band was not detectable. Other studies describe an apparent molecular mass of 50–70 kDa (1, 18, 19), whereas the molecular mass of the conceptually translated CGalT cDNA is 60 kDa.

To rule out the possibility that the 54-kDa band was due to

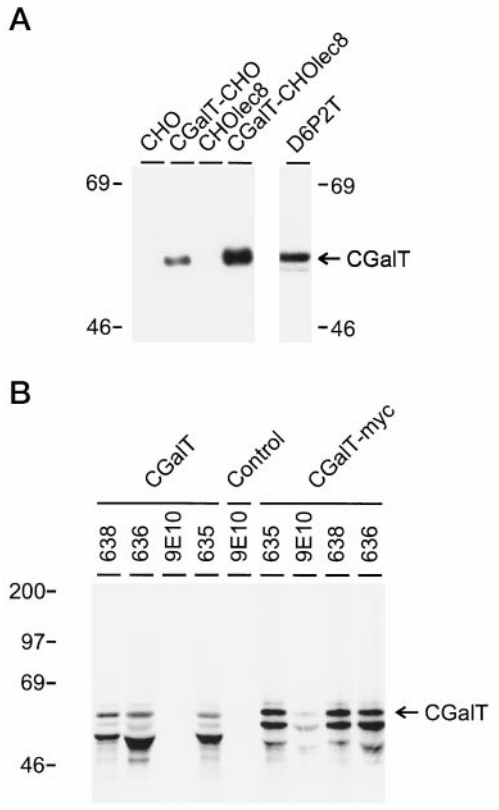

FIG. 2. **Expression of CGalT.** Postnuclear supernatants of D6P2T and CGalT-transfected CHO and CHOlec8 cells were resolved on 10% SDS-polyacrylamide gels followed by Western blot detection with antiserum 635. A prominent band in the CGalT-transfected cells comigrated with the 54-kDa band seen in the rat oligodendrocyte D6P2T cell line (*A*). HeLa cells were transfected with CGalTpcDNA3 or CGalTmycpcDNA3 or mock-transfected with recombinant vaccinia virus. Five h after transfection, the cells were metabolically labeled with Tran^{35}S-label, and CGalT was immunoprecipitated with antibodies 635, 636, and 638 against CGalT or the 9E10 antibody against the Myc epitope tag (*B*). The position of molecular weight standards is indicated on the *left*.

some peculiarity of CHO cells or our antibodies, we analyzed the presence of endogenous CGalT in D6P2T cells, and we transfected Myc-tagged CGalT in a human cell line. The rat oligodendrocyte D6P2T cell line (40) contains high levels of GalCer; accordingly, we expected appreciable expression levels of CGalT. As shown in Fig. 2*A*, the 54-kDa band that was seen in the CHO transfectants expressing rat CGalT also represented the major form of CGalT in this nontransfected oligodendrocyte cell line. From quantitative Western blots, we estimated that initially the expression in CGalT-CHO and CGalT-CHOlec8 cells was 4 and 13 times the endogenous CGalT in the D6P2T cells, respectively. As a second control experiment, we transiently expressed CGalT and the epitope-tagged version CGalTmyc in HeLa cells using the recombinant vaccinia T7 RNA polymerase system. Transfected cells were metabolically labeled for 45 min, and CGalT was immunoprecipitated with the 9E10 antibody against the epitope tag or antibodies against CGalT. As shown in Fig. 2*B*, the 9E10 antibody immunoprecipitated, although less efficiently, the same bands as the rabbit antibodies that were raised against different regions of CGalT. Upon comparing the bands immunoprecipitated from the CGalT and CGalTmyc lysates with the rabbit antibodies, it is clear that insertion of the Myc sequence altered the immunoprecipitation efficiency of some of the forms. The Myc sequence was inserted in the region to which antibody 635 was raised, possibly causing conformational changes in the putative luminal domain that may affect the epitopes seen by the rabbit antibodies. We therefore concluded that the apparent molecular mass of CGalT was ~54 kDa in our gel system. When the same samples were run on 12.5% (instead of 10%) SDS-polyacrylamide gels, the immunoprecipitated CGalT bands shifted to 62 kDa (not shown).

Biosynthesis and Processing of CGalT—To investigate the intracellular fate of CGalT, CGalT-CHO cells were pulse-labeled for 5 min with Tran^{35}S-labeled amino acids and then chased for different periods of time. Besides some background bands (Fig. 3), the same 54-kDa band was immunoprecipitated by antibody 635 as detected on the Western blots. The antisera 636 and 638 also immunoprecipitated this band (not shown), and we therefore interpreted it as CGalT. A small but significant shift to a higher mobility form of CGalT occurred in the first hour after the pulse. Since this shift did not occur after Endo H treatment (see below), it represented processing of *N*-linked oligosaccharides of CGalT. Quantitative analysis of the 54-kDa band during the chase period showed that the half-life of CGalT was about 4 h. We next assessed the rate of CGalT degradation under steady state conditions in CGalT-CHO cells in the presence of 10 mM cycloheximide to inhibit protein synthesis. From the analysis of CGalT levels by quantitative Western blot (not shown), we calculated a very similar half-life, confirming the results of the pulse-chase analysis.

The intracellular transport of CGalT was further analyzed by assaying the acquisition of resistance to Endo H cleavage of its *N*-linked oligosaccharide side chains. It has already been shown by lectin blotting that the protein is a high mannose glycoprotein, although not all of the predicted *N*-glycosylation sites appear to be used (18). *N*-Glycosylated proteins whose transport is arrested in the ER or resident ER proteins containing high mannose sugars are susceptible to Endo H digestion, whereas glycoproteins that are transported beyond the medial Golgi generally possess *N*-glycans that are resistant to Endo H (41). It is shown in Fig. 3 that CGalT remained Endo H-sensitive even with chase times up to 4 h. To exclude the possibility that very slow transport through the early biosynthetic pathway retarded the majority of newly synthesized CGalT in an Endo H-sensitive form, we also addressed its Endo H sensitivity after an overnight chase. For this purpose, we labeled CGalT-CHO cells for 30 min and chased the protein for a period of 16 h. As shown in Fig. 3, CGalT remained Endo H-sensitive, suggesting that it is not transported through the Golgi complex.

Confocal Immune Fluorescence Microscopy—The finding that CGalT remained Endo H-sensitive suggested that the protein is not transported beyond the ER and possibly the *cis*-Golgi. The translated CGalT cDNA sequence contains a carboxyl-terminal KKVK sequence that may act as an ER retrieval signal, which also suggests that CGalT is located in compartments of the early biosynthetic pathway. To extend this observation to the morphological level, we next performed double label confocal immune fluorescence microscopy on the CGalT-transfected CHO cells using antibodies against CGalT, PDI (a resident protein of the ER lumen (42) and intermediate compartment), and a Golgi marker, CTR433 (35).

As shown in Fig. 4, the localization of CGalT (*A*, *green*) nearly completely overlapped with the diffuse reticular ER and nuclear envelope distribution of PDI (*B*, *red*). The Golgi complex was not labeled with the CGalT antibodies in these cells because the staining patterns of CGalT (*D*, *red*) and CTR 433 (*E*, *green*) were mutually exclusive. To highlight areas of colocalization, we merged the CGalT and PDI channels in Fig. 4*C*, where codistribution in the same optical section is seen as a *yellow* signal. In contrast, the different localizations of CGalT and CTR433 images did not produce yellow images (*F*). To

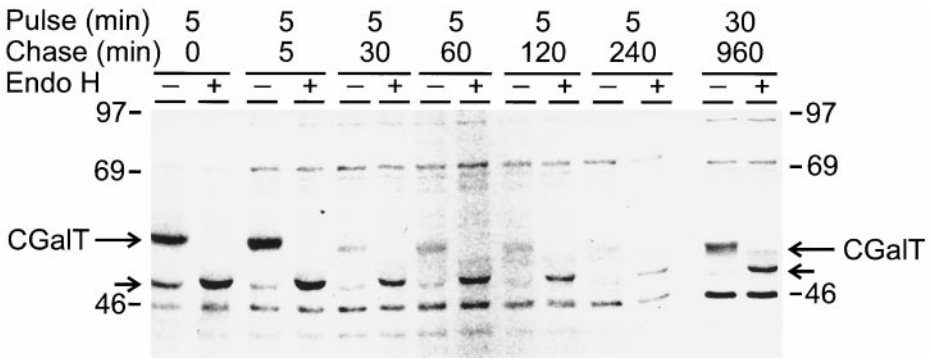

FIG. 3. **Transport and processing of CGalT.** CGalT-CHO cells were pulse-labeled for 5 min with Tran^{35}S-labeled amino acids and then chased for different time intervals up to 4 h. After cell lysis, CGalT was immunoprecipitated with antiserum 635, and immunoprecipitates were treated with Endo H as described under "Experimental Procedures." Proteins were resolved under reducing conditions by SDS-PAGE on a 7.5% gel.

ascertain that the labeling of CGalT in the CGalT-CHO cells was specific, we labeled nontransfected control CHO cells lacking endogenous CGalT with CGalT and PDI antibodies. As can be seen in Fig. 4*G* (*green* channel), labeling with the CGalT antibody yielded a very faint signal in the CHO cells, while the staining pattern with the PDI antibody (*H*, *red*) was identical in the CGalT-CHO and CHO cells. In the oligodendrocytic D6P2T cell line having high levels of endogenous CGalT, we also found extensive colocalization of endogenous CGalT (*J*, *green*) and PDI (*K*, *red*). Importantly, CGalT labeling was not present on the plasma membrane and Golgi apparatus in the D6P2T cell line, suggesting that CGalT does not localize to these compartments and is restricted to the ER.

Ultrastructural Localization of CGalT—Although the confocal immune fluorescence experiments suggested extensive overlapping distributions of CGalT and PDI, this technique does not have the required resolution to unambiguously demonstrate that CGalT colocalized with the ER marker. We therefore performed immunogold electron microscopy with the CGalT antibody on ultrathin crysosections prepared from CGalT-CHO cells. As shown in Fig. 5*A*, the CGalT antibody heavily decorated the intracellular membranes in the perinuclear area. Most of the labeling occurred on ER cisternae and the nuclear envelope. In accordance with the confocal immune fluorescence experiments, we did not observe labeling of the Golgi apparatus (Fig. 5*B*) and the plasma membrane. Double label experiments also revealed extensive colocalization of CGalT with PDI (Fig. 5*C*). Occasionally we observed colabeling of CGalT and PDI in tubulovesicular structures at the *cis*-face of the Golgi complex. We next quantitatively addressed the distribution of CGalT on these crysosections, the results of which are shown in Fig. 5*D*. About 70% of the gold label was associated with the ER, and 23% was associated with the nuclear envelope, whereas the labeling of plasma membrane and Golgi apparatus was essentially negligible. These results confirm and extend the data from the light microscopy experiments in which we showed that CGalT does not move beyond the ER. We also performed immunolabeling of cryosections from the D6P2T cells; however, expression of CGalT was too low to discriminate specific labeling from background (not shown).

Membrane Topology of CGalT—Having localized CGalT to the ER, we next determined the membrane topology of CGalT in this compartment. CGalT-CHO cells were metabolically labeled with Tran^{35}S-label for 10 min and subsequently incubated for 5 min in chase medium to allow completion of nascent chains. Postnuclear supernatants were incubated for 1 h with proteases in the presence or absence of detergent. CGalT was then immunoprecipitated with the 635 antibody. In the absence of saponin, treatment with proteinase K or trypsin resulted in a truncated protein. The results of this experiment are shown in Fig. 6*A*, from which we estimated that protease treatment resulted in removal of a 4-kDa fragment from CGalT. Because the CGalT antibody was raised against the amino-terminal portion of the protein (Fig. 1*A*), we concluded that this part of CGalT must be present in the lumen of sealed membranes in this experiment. When protease digestion was done in the presence of saponin to permeabilize the membranes, CGalT was completely degraded (Fig. 6*A*), showing that it is intrinsically susceptible to trypsin and proteinase K.

CGlcT Is a Dual Specificity Enzyme in Vitro—*In vitro* substrate specificity assays have shown that CGalT has a marked preference for hydroxy fatty acid ceramide when compared with nonhydroxy fatty acid ceramide (15, 22, 24). *In vivo* CGalT, however, galactosylates both nonhydroxy fatty acid and hydroxy fatty acid ceramides, depending on their local availability (23). Interestingly, although CHO cells do not produce GalCer, membranes from these cells in an *in vitro* assay converted the well characterized short chain fluorescent model substrate NBD-Cer to NBD-GalCer. In contrast to the ER CGalT, the CGalT activity fractionated at the density of Golgi membranes of CHO and Madin-Darby canine kidney cells was inhibited by UDP-Glc, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol and was protease-sensitive (13, 23).

Because the Golgi-associated CGalT activity shared several characteristics with CGlcT, we suspected that CGlcT might in fact be responsible for NBD-GalCer synthesis *in vitro*. To address this question, postnuclear supernatants prepared from the CGlcT-negative GM95 cell line (28) were incubated with NBD-Cer and UDP-Gal, and glycolipids produced in this assay were analyzed. As shown in Table I, GM95 cells did not synthesize NBD-GlcCer as expected. Interestingly, they also did not produce the NBD-GalCer product. In contrast, in the MEB4 cell line from which the GM95 cell line was derived, we observed appreciable NBD-GalCer synthesis in the presence of UDP-Gal. To show that the GlcCer-deficient phenotype of GM95 cells is due to the absence of only CGlcT, we transfected GM95 cells with CGlcT and assayed synthesis of NBD-GlcCer and NBD-GalCer in postnuclear supernatants prepared from these cells. In addition to NBD-GlcCer, we also found considerable synthesis of NBD-GalCer in the presence of UDP-Gal (Table I). These results suggested that CGlcT could act as a dual specificity enzyme *in vitro*. This idea was tested by directly comparing the amount of synthesized products when equimolar UDP-Gal and UDP-Glc were added to postnuclear supernatants of MEB4 cells. As shown in Table I, GlcCer as well as GalCer were produced in this experiment, but GalCer synthesis was dramatically reduced in the presence of UDP-Glc.

CGalT Requires Translocation of UDP-Gal into the ER—The predicted membrane topology of CGalT and the results of the protease protection assays suggested that the active site of CGalT is oriented toward the lumen of the ER. Therefore, CGalT enzyme activity should require UDP-Gal import. To test this requirement, postnuclear supernatants were prepared from CGalT-CHO cells and the CGalT-CHOlec8 cell line, which

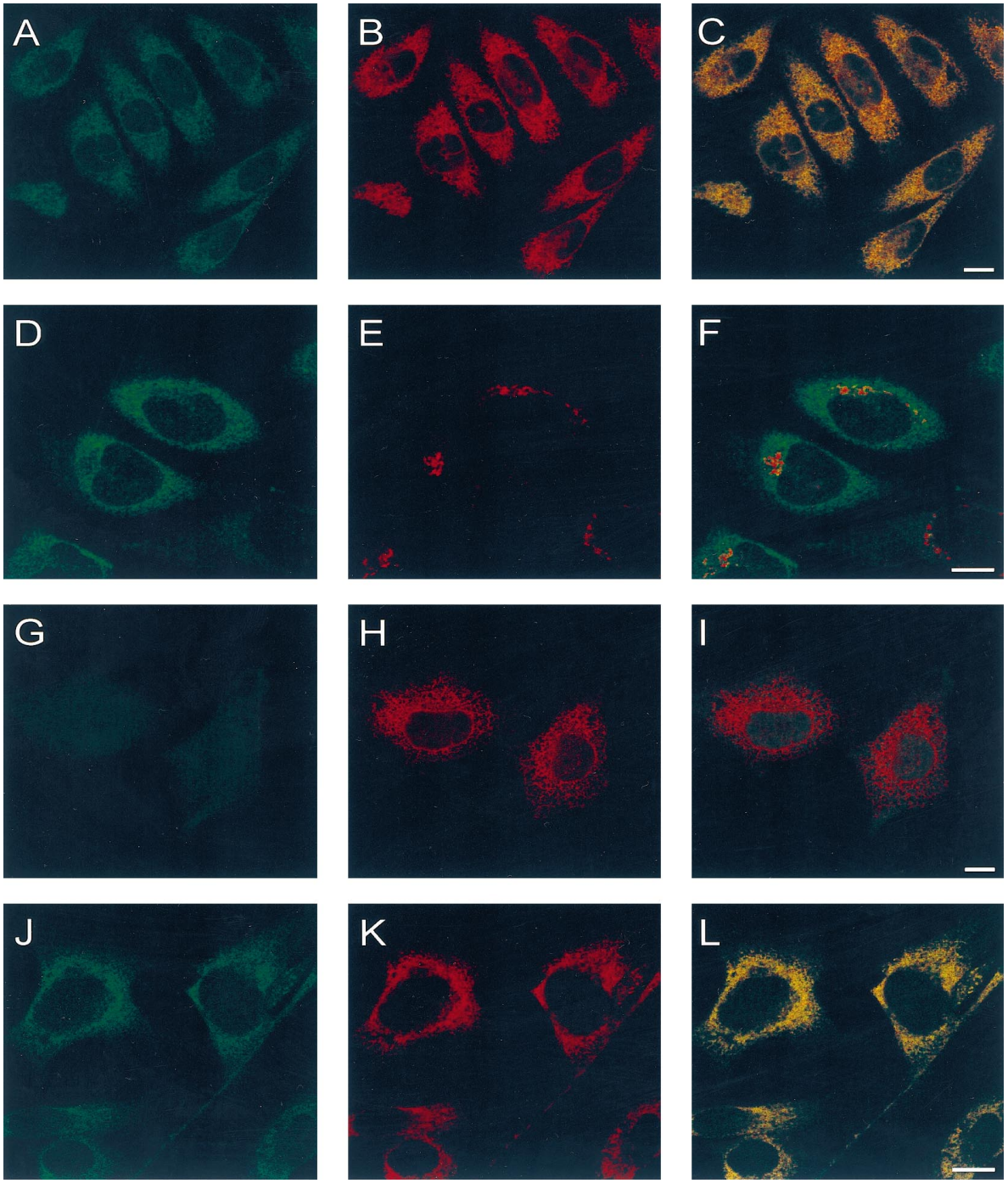

FIG. 4. **Confocal immunofluorescence microscopy of CGalT.** CGalT-CHO cells (*A–F*), nontransfected CHO control cells (*G–I*), and D6P2T cells (*J–L*) were labeled with rabbit anti CGalT antibody (*A*, *D*, *G*, and *J*) and mouse anti PDI (*B*, *H*, and *K*) or mouse anti-Golgi (*E*) antibody and counterstained with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein-labeled goat anti-rabbit (*A*, *D*, *G*, *J*) and indocarbocyanine-labeled goat anti-mouse antibodies (*B*, *E*, *H*, and *K*). Areas of overlapping distributions in the same optical section appeared as *yellow* in the merged images (*C*, *F*, *I*, and *L*). *Bar*, 10 μm.

is deficient in UDP-Gal import into the Golgi apparatus (43). An enzyme assay with NBD-Cer and UDP-Gal in the absence or presence of saponin to render membranes permeable to UDP-Gal showed that the specific activity of both postnuclear supernatants was comparable (Fig. 7*B*). In contrast, when the enzyme assay was carried out on intact membranes, the CGlcT activity in CGalT-CHOlec8 cells was much lower than in the CGalT-CHO cells (Fig. 7*A*), which suggests that UDP-Gal import was limiting.

Finally, we investigated the dependence of NBD-GalCer synthesis on UDP-Gal concentration. If UDP-Gal import is limiting for GalCer synthesis, saturation kinetics would be predicted for NBD-GalCer formation in the CGalT-CHO cells. As shown in Fig. 7*C*, NBD-GalCer synthesis indeed increased over a UDP-Gal concentration range that occurs in cytosol. In contrast, a much lower, nearly linear increase was observed in the CGalT-CHOlec8 cells, which was probably caused by leakage of the membranes or by CGalT activity of CGlcT as discussed above. Subtraction of this background signal from the CGalT-CHO data points shows that saturation of the UDP-Gal translocator already occurred at 0.5 mM UDP-Gal. Thus NBD-GalCer synthesis is critically dependent on an active UDP-Gal transporter. Interestingly, our results also document that the UDP-Gal translocator, which originally was identified in the

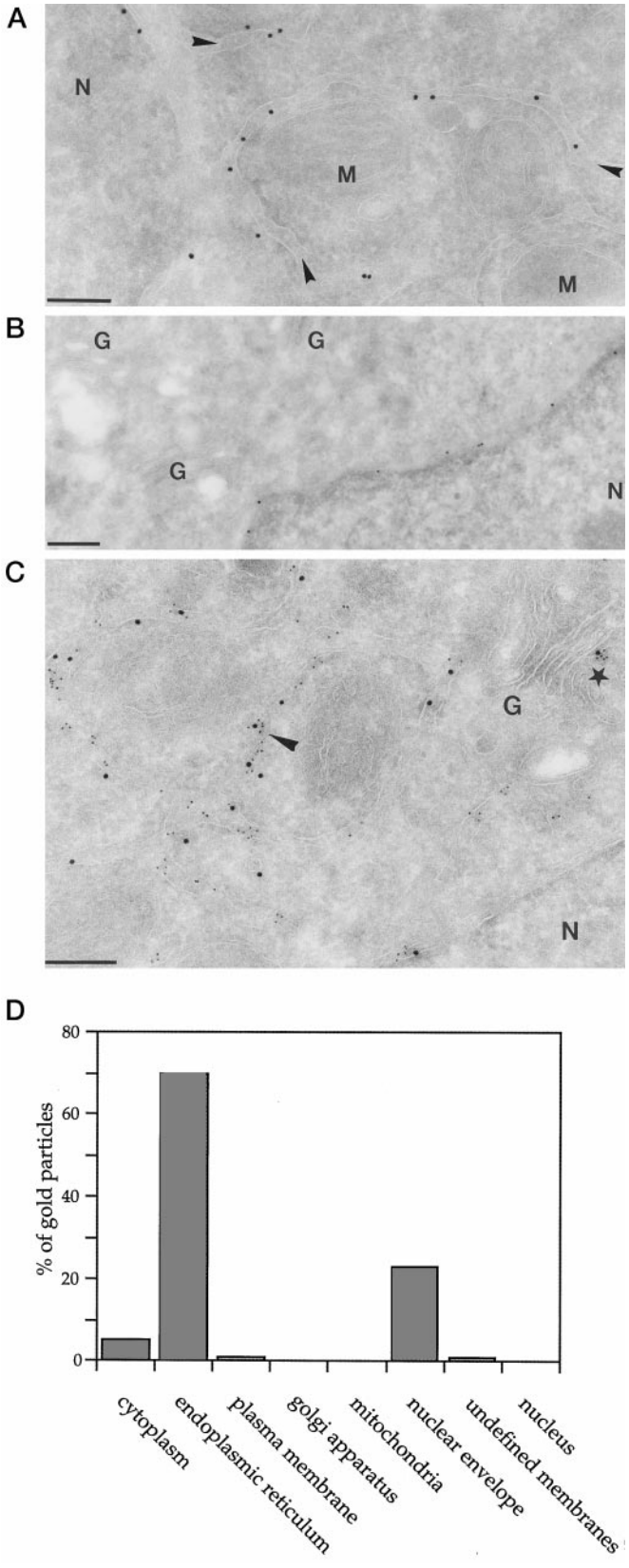

FIG. 5. **Ultrastructural localization of CGalT.** Ultrathin cryosections of CGalT-CHOlec8 cells were incubated with antibody against CGalT and PDI and labeled with 10-nm protein A gold. *A*, most of the label on these sections was associated with the ER (*arrowhead*) and nuclear envelope. *B*, note the absence of CGalT labeling of the Golgi apparatus (*G*), nucleus and mitochondria (*M*). *C*, CGalT colocalized with PDI to the ER. In this experiment, PDI and CGalT detection was done with 5 and 15 nm protein A gold, respectively. Occasionally, colocalization of CGalT and PDI is seen in tubulovesicular structures at the *cis*-side of the Golgi complex (*asterisk*). *D*, the distribution of CGalT immunoreactivity in CGalT-CHO cells was quantitated from sections of 10 individual cells (1500 gold particles counted) and corrected for nonspecific labeling (300 gold particles counted) on sections prepared from nontransfected control CHO cells. *Bar*, 200 nm.

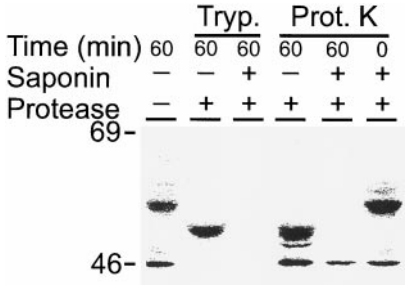

FIG. 6. **Membrane topology of CGalT.** CGalT-CHO cells were labeled with Tran^{35}S-label for 15 min and chased for 5 min. Postnuclear supernatants were incubated for 0 and 60 min at 10 °C with trypsin (*Tryp.*) or proteinase K (*Prot. K*) in the presence or absence of 0.5% saponin. CGalT was immunoprecipitated with antiserum 635 and analyzed by 7.5% SDS-PAGE.

Golgi apparatus, is required and sufficient for UDP-Gal import into the lumen of the ER to serve with NBD-Cer as substrates for CGalT.

DISCUSSION

The availability of high affinity antibodies against CGalT has greatly facilitated the analysis of the protein by allowing us to rigorously establish its biosynthesis and maturation, intracellular localization, and membrane topology. CGalT was not detectable by Western blot analysis in CHO and CHOlec8 cells using the CGalT antibodies, extending previous observations that CHO cells do not contain GalCer. Using antibodies raised against three different regions of CGalT, we consistently detected mature CGalT in four different expression systems as a band with an apparent molecular mass of 54 kDa. In order to separate mature and newly synthesized CGalT, we used 10% SDS-polyacrylamide gels. This caused a downward shift of 10 kDa in apparent molecular mass as compared with analyzing the protein on 12.5% gels and with the size of the protein recently reported by others (19). The resolution of the latter separating system is insufficient to visualize the relatively small differences in molecular weight during biosynthesis of the protein. Importantly, the immunoreactive 54-kDa band was also detected with the 9E10 antibody in cells transfected with CGalTmyc, and we identified it in the nontransfected rat Schwann cell line D6P2T expressing high levels of GalCer. Possibly, the high content of hydrophobic amino acids in the luminal portion of CGalT is responsible for the anomalous behavior of the protein on SDS-polyacrylamide gels.

CGalT is synthesized as a 60-kDa precursor protein that reached its mature form within a period of 60 min. The small decrease in molecular weight that we observed by SDS-PAGE during the first 30 min after the pulse showed that the protein was subject to oligosaccharide trimming in the ER. This was confirmed in Endo H-treated samples in which we found that CGalT remained Endo H-sensitive and where no such decrease occurred. The fact that CGalT did not become Endo H-resistant even after a 16-h chase showed that the protein does not pass through the Golgi apparatus. A quantitative morphological analysis at the ultrastructural level ascertained that CGalT is retained within the ER and the nuclear envelope. Using this sensitive technique, we did not detect CGalT in other intracellular compartments including the Golgi apparatus.

CGalT behaved as a type I transmembrane protein. Protease

TABLE I
GalCer synthesis by CGlcT in vitro

Postnuclear supernatants prepared from MEB4 cells, CGlcT-deficient GM95 cells, and CGlcT-transfected GM95 cells were incubated with 50 μM NBD-Cer and 2 mM UDP-Gal, 2 mM UDP-Glc, or 2 mM of both for 1 h at 37 °C. NBD-lipids were analyzed as described under "Experimental Procedures" and expressed in pmol/mg of protein. Data are means of two independent experiments (n = 4). S.D. was less than 2%.

Cells	NBD-lipid	Condition			
		No UDP-sugars	UDP-Gal	UDP-Glc	Both UDP-sugars
		pmol/mg protein			
MEB4	Sphingomyelin	52	52	27	35
	GlcCer	35	35	413	406
	GalCer	ND[a]	35	ND	2
GM95	SM	62	63	64	69
	GlcCer	ND	ND	ND	ND
	GalCer	ND	ND	ND	ND
GM95-CGlcT	SM	53	53	54	59
	GlcCer	17	22	85	76
	GalCer	ND	5	ND	1

[a] ND, not detectable; a fluorescence signal corresponding to less than 0.5 pmol.

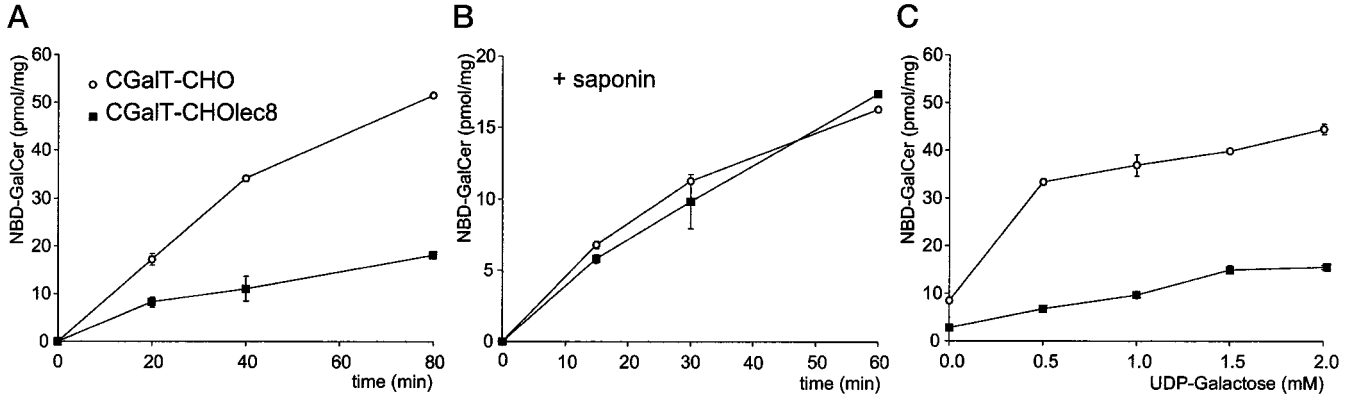

FIG. 7. **CGalT activity is dependent on UDP-Gal translocation in the ER.** Postnuclear supernatants prepared from CGalT-CHO cells (*open symbols*) and CGalT-CHOlec8 cells (*closed symbols*) were incubated with NBD-Cer and UDP-Gal for different periods of time in the absence (*A*) or presence of saponin (*B*), and NBD-GalCer synthesis was analyzed. Synthesis of NBD-GalCer is dependent on UDP-Gal concentration (*C*). When the experiment was carried out in the presence of NBD-GlcCer, synthesis of lactosylceramide was virtually lost in CHOlec8 (not shown), as reported for untransfected CHOlec8 cells (13). Although we showed that CGalT-CHOlec8 cells expressed about 3 times more CGalT than the CGalT-CHO cells, the transfected cells gradually lose expression as we previously observed for the CGalT-CHO cells. This explains why the CGalT enzyme activity of the CGalT-CHOlec8 postnuclear supernatant in the presence of saponin was not directly related to the expression of the protein. UDP-Glc was included in the assay to inhibit NBD-GalCer synthesis via CGlcT (see Table I).

treatment of intact membranes produced a truncated protein with a molecular mass of about 50 kDa, which could still be immunoprecipitated with an antibody directed against the amino-terminal portion of the protein, suggesting that the amino terminus was intact and oriented toward the lumen of the organelle. Thus, a carboxyl-terminal region of 4 kDa appeared to be exposed to the cytosol, which is in excellent agreement with the length of the predicted cytoplasmic tail of 49 amino acids and the presence of an arginine and various lysines close to the membrane. The carboxyl terminus also contains the -KKVK sequence that most likely retains CGalT in the ER by acting as a cytosolic ER retrieval signal (44). Although most of the CGalT labeling on the cryosections was associated with the ER, it is possible that some CGalT is present in the intermediate compartment between the ER and the *cis*-Golgi. From here, it could be recycled back to the ER by retrograde transport.

Because we showed here that CGalT is localized to the ER, the previously reported CGalT activity in Golgi fractions (13, 21, 23, 45) must be accounted for by a different enzyme. We here found that the CGlcT-deficient cell mutant GM95 not only lacked the ability to synthesize GlcCer but also failed to synthesize GalCer under low UDP-Glc conditions *in vitro*. Retransfection of CGlcT in this cell line restored the ability to synthesize GlcCer and, *in vitro*, GalCer. Thus, *in vitro* in the absence of UDP-Glc, CGlcT is capable of transferring Gal from UDP-Gal to ceramide. The ceramide galactosyl transferase mechanism of CGlcT seems to be unrelated to that of CGalT, since CGlcT and CGalT do not share significant sequence homology and they are localized to cellular compartments with entirely different redox and ionic compositions. CGalT is exposed to the interior of the ER, whereas CGlcT most likely resides on the cytoplasmic surface of the Golgi apparatus. It is unlikely that CGlcT synthesizes GalCer *in vivo*, since all mammalian cells express CGlcT, but no GalCer is observed unless the cells express CGalT as well. The K_m of CGlcT for UDP-Glc is at least 200 times lower than for UDP-Gal when assayed *in vitro*.

Synthesis of GalCer in membranes from CGalT-CHOlec8 cells was greatly stimulated as compared with control cells when the membranes were permeabilized with saponin upon the addition of UDP-Gal. As CGalT-CHOlec8 cells are deficient in the UDP-Gal translocator in the Golgi apparatus (43), we conclude that the active site of CGalT must reside on the luminal side of the ER and that the Golgi UDP-Gal translocator is responsible for the translocation of UDP-Gal into the ER as well. Because antibodies against this translocator are not available, its intracellular distribution can only be inferred from functional *in vitro* assays. This question may soon be solved, since the cDNA of a UDP-Gal translocator that complements the genetic defect of cells with a phenotype similar to that of CHOlec8 has been cloned (50).

In contrast to all other glycosyltransferases identified in glycosphingolipid synthesis, CGalT is located in the ER and not in the Golgi. It is most closely related to the glucuronyltrans-

ferase family of ER enzymes (16). The presence of newly synthesized GalCer in the luminal leaflet of the ER membrane is potentially interesting in terms of domain formation and sorting of (glycosylphosphatidylinositol-anchored) proteins. However, because lipids rapidly translocate across the ER membrane, GalCer has access to the cytosolic surface.

Interestingly, CGlcT, the other enzyme generating a monoglycosylceramide, synthesizes its product GlcCer on the cytosolic surface and not, like all others, to the lumen, of the Golgi. While GalCer and GlcCer may translocate across the Golgi membrane to serve as substrates for higher glycolipid synthesis (13), the finding of a GalCer and GlcCer transfer protein (46, 47) suggests that these glycolipids can reach the cytosolic surface of other membranes where they may fulfill yet unknown functions. In addition, subsequent specific translocation across the apical membrane of epithelial cells (30) could contribute to epithelial lipid polarity, the enrichment of glycolipids on the apical cell surface.

In conclusion, we have shown that rat CGalT is a type I integral membrane protein exclusively localized to the endoplasmic reticulum. In mice, the enzyme is essential for the formation of functional myelin (1, 3). In humans, the enzyme is responsible for high levels of galactolipids in numerous epithelia as well. It will be interesting to see how the specific features of CGalT relate to its functions and those of its product GalCer in the organism.

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UDP-Galactose:Ceramide Galactosyltransferase Is a Class I Integral Membrane Protein of the Endoplasmic Reticulum

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