UDP-galactose:ceramide galactosyltransferase (CGaT) 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 have raised antibodies against CGaT 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 CGaT 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. CGaT 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 CGaT 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 CGaT in vivo in the lumen of the endoplasmic reticulum.

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

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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).

CGaT was recently cloned (16–18), and knockout studies have shown that there is only one GalCer-synthesizing enzyme in the brain (1, 3). CGaT 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 CGaT 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 CGaT and its membrane topology. We raised antibodies against CGaT and show that it is a class I integral membrane

¹ The abbreviations used are: GalCer, galactosylceramide; GlcCer, glucosylceramide; ER, endoplasmic reticulum; CGaT, 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.

25880 This paper is available on line at http://www.jbc.org
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 synthesize 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 CGaIT localization.

**Experimental Procedures**

**Materials**—Reagents used in this study were from commercial sources and described in previous papers originating from this laboratory (31, 32, 33, 34).

**Cell Culture and Transfection**—Chinese hamster ovary (CHO) cells, CHoLe8 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). CHoLe8 cells were transfected with CGaITpcDNA3 (23) using the calcium phosphate procedure (32). Stable cell lines were obtained by subcloning individual colonies. Positive clones were selected by measuring CGaIT enzyme activity as described (23). Transfected CGaIT-CHO (23) and CGaIT-CHOe8 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 isobutylmethylxanthate (Fluka, Buchs, Switzerland) 14-16 h prior to all experiments (33).

**Plasmid Construction**—Specific regions of CGaIT (Fig. 1) were amplified in PCR reactions using CGaITpcDNA3 (23) as template and the following primer sets: for 635, 5′-CGG GAT CAA GGA GGA G-3′ (forward) and 5′-GGG AAT TCA TCA TTG TGC CGC CAA TATG-3′ (reverse); for 636, 5′-GGG AAT TCA TCT GGG TGA ACC AGG-3′ (forward); and 5′-GGG AAT TCA TCT GGG TGA ACC AGG-3′ (reverse); for 638, 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (forward) and 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (reverse); for 639, 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (forward) and 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (reverse); for 640, 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (forward) and 5′-GGG ATC CTA TCT GCC ATC CAA AAG-3′ (reverse). PCR products were ligated between the BamHI and EcoRI sites of pRSSET-A (Invitrogen, Leek, The Netherlands). A C-myc epitope was spliced between Seq69 and Leu20 of CGaIT by separately amplifying the 5′ and 3′ region of its cDNA in PCR reactions using CGaITpcDNA3 as template and the following primer sets: for the 5′ region, 5′-CGT CCC TCA GAA TGT AGT CTT GTC AGA AGA ATG-3′ (forward) and 5′-GGG GAT CCC TCA GAA TGT AGT CTT GTC AGA AGA ATG-3′ (reverse); PCR products were ligated between the EcoRI and SpeI sites of 5′-CGCTGGAAGATATAGGTCGGGTATCC (forward) and 5′-GGG GAT CCC TCA GAA TGT AGT CTT GTC AGA AGA ATG-3′ (reverse); for the 3′ region, 5′-GGG CAT CAA GAG CAT TCT CAT GCA TCA CAA GGA GGA GGA GGA G-3′ (forward) and 5′-GGG CAT CAA GAG CAT TCT CAT GCA TCA CAA GGA GGA GGA GGA G-3′ (reverse); PCR products were ligated into pGMBlot (Promega, Madison, WI). The 5′ region was released with EcoRV and SpeI and ligated between the EcoRV and SpeI sites of the 3′ region of pGMBlot easy, a CGaIT expression vector that was released with HindIII and XhoI and inserted in pcDNA3 (Invitrogen). A CGlCt cDNA was generously provided by Yoshio Hirabayashi and ligated in the NotI site of pcDNA3 to produce CGlCtpcDNA3. All constructs made by PCR were confirmed by sequencing both strands (34).

**Antibodies**—pRSSET-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, Valencia, 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 CGaIT was immunoprecipitated from the supernatant.

**Glycosphingolipid Synthesis**—Cells were homogenized, and post-nuclear supernatant was prepared for various periods of time at 37 °C with 1% bovine serum albumin, 2 mM UDP-Glc, 2 mM UDP-Gal, 2 mM MgCl2, 2 mM MnCl2, and 50 μM C6-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 detected using ultraviolet light and viewed with ImageQuant software.

**Metabolic Labeling**—CGaIT-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 Tran35S-label for 5 or 15 min at 37 °C. Cells were washed and chased at 37 °C in growth medium containing 30 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 CGaIT was immunoprecipitated from detergent lysates as described below. HeLa cells were depleted as described for CGaIT-CHO cells and labeled for 45 min with 150 μCi/ml Tran35S-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 × 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 × sucrose, 1 ml of 1.25 × 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 × sucrose interface and used for blot purification of antibodies against CGaIT.

**Protease Protection Assay**—Fifty μl (50 μg) of post-nuclear supernatant labeled with a GST-CGalT-CHO fusion protein incubated with 0.1 mg/ml of proteinase K or trypsin for 30 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 CGaIT was immunoprecipitated from the detergent lysates in the presence of protease inhibitors.

**Immunoprecipitation and Endoglycosidase H Digestion—Antibodies were prebound to protein A-Sepharose 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 mM 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% Skim milk, and a 1:1000 dilution of the primary antibody. Blots were washed, gently scraped in 250 mM sucrose, and postnuclear supernatants were prepared for various periods of time at 37 °C with 1% bovine serum albumin, 2 mM UDP-Glc, 2 mM UDP-Gal, 2 mM MgCl2, 2 mM MnCl2, and 50 μM C6-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, 35).

**Immunofluorescence Microscopy**—Cells were grown on coverslips to 40–60% confluency. The cells were fixed with 3% formaldehyde; quenched in PBS, 50 mM NH4Cl; 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 CGaIT, the mouse monoclonal antibody 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**—CGaIT was synthesized from CGaITpcDNA3 in a coupled T7 RNA polymerase transcription-translation system (Promega) in the presence of rabbit reticulocyte lysate, dog pancreas microsomes (Promega), and Tran35S-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 CGaIT was immunoprecipitated from the supernatant.
microscope using separate filters for each fluorochrome viewed: for 5-((4,6-dichlorotriazin-2-yl)amino)fluorescein, \( \lambda_{ex} = 488 \) nm and \( \lambda_{em} = 515 \) LP; for indocarbocyanine, \( \lambda_{ex} = 568 \) nm and \( \lambda_{em} = 585 \) 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 0.1M 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. 1A). Antisera were first tested for their ability to immunoprecipitate CGalT that was synthesized in an 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.

![Image](https://example.com/image1.png)

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

Ultrastructural Localization of CGaIT—Although the confocal immune fluorescence experiments suggested extensive overlapping distributions of CGaIT and PDI, this technique does not have the required resolution to unambiguously demonstrate that CGaIT colocalized with the ER marker. We therefore performed immunogold electron microscopy with the CGaIT antibody on ultrathin cryosections prepared from CGaIT-CHO cells. As shown in Fig. 5A, the CGaIT 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. 5B) and the plasma membrane. Double label experiments also revealed extensive colocalization of CGaIT with PDI (Fig. 5C). Occasionally we observed colabeling of CGaIT and PDI in tubulovesicular structures at the cis-face of the Golgi complex. We next quantitatively addressed the distribution of CGaIT on these cryosections, the results of which are shown in Fig. 5D. 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 CGaIT does not move beyond the ER. We also performed immunolabeling of cryosections from the D6P2T cells; however, expression of CGaIT was too low to discriminate specific labeling from background (not shown).

Membrane Topology of CGaIT—Having localized CGaIT to the ER, we next determined the membrane topology of CGaIT in this compartment. CGaIT-CHO cells were metabolically labeled with Tran-S-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. CGaIT 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. 6A, from which we estimated that protease treatment resulted in removal of a 4-kDa fragment from CGaIT. Because the CGaIT antibody was raised against the amino-terminal portion of the protein (Fig. 1A), we concluded that this part of CGaIT 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, CGaIT was completely degraded (Fig. 6A), showing that it is intrinsically susceptible to trypsin and proteinase K.

CGaIT Is a Dual Specificity Enzyme in Vitro—In vitro substrate specificity assays have shown that CGaIT has a marked preference for hydroxy fatty acid ceramide when compared with nonhydroxy fatty acid ceramide (15, 22, 24). In vivo CGaIT, 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 vivo assay converted the well characterized short chain fluorescent model substrate NBD-Cer to NBD-GalCer. In contrast to the ER CGaIT, the CGaIT 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 CGaIT 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.

CGaIT Requires Translocation of UDP-Gal into the ER—The predicted membrane topology of CGaIT and the results of the protease protection assays suggested that the active site of CGaIT is oriented toward the lumen of the ER. Therefore, CGaIT enzyme activity should require UDP-Gal import. To test this requirement, postnuclear supernatants were prepared from CGaIT-CHO cells and the CGaIT-CHOlec8 cell line, which
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. 7B). 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. 7A), 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. 7C, 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 transporter 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. 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-ylamino)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.
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

**Fig. 6. Membrane topology of CGalT.** CGalT-CHO cells were labeled with Tran35S-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.

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**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.
Ceramide Galactosyltransferase

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          | pmol/mg protein |
|----------------|---------------|--------------------|-----------------|
|                |               | No UDP-sugars     | UDP-Gal         | UDP-Glc         | Both UDP-sugars |
| MEB4           | Sphingomyelin | 52                 | 52              | 27              | 35              |
| GM95           | GlcCer        | ND                 | 35              | 413             | 406             |
| SM             | GlcCer        | ND                 | 35              | ND              | 2               |
| SM             | GalCer        | ND                 | ND              | ND              | ND              |
| GM95-CGlcT     | SM            | ND                 | 53              | 54              | 59              |
| GM95-CGlcT     | GlcCer        | 17                 | 22              | 85              | 76              |
| GM95-CGlcT     | GalCer        | ND                 | 5               | ND              | 1               |

* 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.](image)

In vivo and in vitro 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 confined to the membrane, it is possible that some CGalT is associated with the ER, it is likely 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 also showed 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 cytosolic surface of the Golgi apparatus. It is unlikely that CGalT 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 CGalT 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-
Ceramide Galactosyltransferase

The Ceramide Galactosyltransferase (CGalT) enzyme is essential for the formation of galactolipids in epithelial cells and myelin. CGalT synthesizes galactosylceramide (GalCer) and glycosylceramide (GlcCer) by catalyzing the transfer of galactose from UDP-galactose to ceramide. This reaction is crucial for the development and function of the central nervous system, as GalCer and GlcCer are abundant in the myelin sheath of neurons.

GalCer and GlcCer are synthesized on the cytosolic side of the endoplasmic reticulum (ER) by the ER enzymes GalCer synthase (CGalT1) and GlcCer synthase (CGalT2), respectively. These enzymes are integral membrane proteins with multiple transmembrane domains. The specific functions of GalCer and GlcCer are diverse, ranging from structural components of myelin and cell membranes to regulators of cell signaling pathways.

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