been partially separated from other proteins in 6–25% glycerol density gradients using either CHAPSO or NP-40.[11,12]

Conclusions

The purification of measurable quantities of GCS for studies of secondary structure, catalytic mechanism, inhibitor design, and Golgi localization will probably only be possible by the overexpression of GCS in a bacterial or eukaryotic cell system. The design of fusion proteins in which GCS is fused to tags (e.g., polyhistidine) for purification or to large hydrophilic carrier proteins for better solubility and decreased aggregation will likely facilitate GCS purification. The methods presented in this article on the stabilization, solubilization, and detection of GCS should continue to prove useful as these developments unfold.

[8] Analysis of Galactolipids and UDP-Galactose : Ceramide Galactosyltransferase

By HEIN SPRONG, GERRIT VAN MEER, and PETER VAN DER SLUIJS

Galactolipids and Galactosyltransferase

Glycosphingolipids form a highly polymorphic class of lipids, and several hundreds of the more than 2000 possible molecular species[1] have been characterized.[2] There are at least 20 different ceramide (Cer) backbones due to differences in sphingoid base, mostly sphingosine (4-sphingenine) and phytosphingosine (4-hydroxysphinganine), and acyl chain. The head groups can vary from 1 to 60 sugars. Glycosphingolipids in mammals can be subdivided into two major classes—galacto- and glucosphingolipids—based on the presence of Gal or Glc as the first sugar moiety. Most complex glycolipids are based on Gal β1-4 Glc β1-1 Cer, lactosylceramide (LacCer). Galactosylceramide (Gal β1-1 Cer or GalCer) serves as a precursor for a few simple glycolipids: the sulfatide SGalCer (SO_3-3 GalCer), galabiosylceramide (Gal α1-4 GalCer or Ga_2Cer), and the ganglioside sialo-GalCer (I^3NeuAc-GalCer or "G_{M4}"). Gal and SGal are also found on diglycerides: Gal β1-3 diacylglycerol (GalDAG), Gal β1-3 alkyl-acyl-glycerol (GalAAG), digalactosyldiglyceride, and seminolipid (SO_3-3 GalAAG).[3]

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Glycosphingolipids are enriched in the outer leaflet of the plasma membrane of most eukaryotic cells where they are thought to be involved in cell recognition and signaling.[1] Although glycosphingolipids constitute only a few mol% of the lipids in most membranes, they are major components of the myelin sheath,[4] where GalCer and SGalCer are involved in axonal insulation, myelin function, and stability.[5,6] The apical plasma membrane of epithelial cells in the gastrointestinal and urinary tracts is enriched in glycosphingolipids. In rodents these are typically glucolipids,[2,7] whereas in humans most are galactolipids.[8–10] Glycosphingolipids play a structural role in rigidifying and protecting the apical cell surface. Their role in sorting lipids and proteins to various membranes along the exocytotic and endocytotic transport routes is not fully understood.[7,11]

The foremost enzyme involved in the biosynthesis of galactosphingolipids is the UDP-galactose:ceramide galactosyltransferase, CGalT or GalT-1.[12] CGalT catalyzes the transfer of galactose from UDP-galactose to Cer yielding GalCer[13] and has a relatively promiscuous substrate specificity. Whether there are one or more CGalT enzymes with distinct specificity and cellular localization has been a controversial issue.[14–18] Importantly, knockout mice do not make GalCer,[5,6] showing that there is only one CGalT. *In vitro* studies demonstrated that partially purified CGalT from brain has a >15-fold preference for hydroxy fatty acid (HFA) over nonhydroxy fatty acid (NFA) containing Cer.[13,19] This has been confirmed for CGalT after transfection into CGalT-negative cells.[18,20] *In vivo,* however,

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CGalT is responsible for the galactosylation of HFA- as well as NFA-containing Cer. NFA–CGalT activity found previously in the Golgi[15,17,18] has now been demonstrated to be an *in vitro* activity of the Golgi ceramide glucosyltransferase (CGlcT).[21] CGalT is also responsible for the galactosylation of diglycerides.[18]

The localization of CGalT has long been enigmatic.[14,22–25] We have shown that the enzyme is exclusively localized to the endoplasmic reticulum (ER) by immunogold electron microscopy on ultrathin cryosections.[21] CGalT is a high mannose-type glycoprotein that is N-glycosylated at Asn-78 and Asn-333[26] and contains a putative carboxy terminal Lys–Lys–Val–Lys ER-retrieval signal.[20,27,28] Surprisingly, the conceptual translation product exhibits no amino acid sequence similarity with other glycosyltransferases. Instead, CGalT is related to the superfamily of UDP-glucuronosyltransferases.

Thus, while most glycosylation steps of sphingolipids occur in the Golgi complex, CGalT enzyme activity resides in the lumen of the ER (Fig. 1).[21] Cer is synthesized at the cytosolic surface and is sufficiently hydrophobic to diffuse freely across cellular membranes. How the other substrate, UDP-Gal, reaches the active center of CGalT is unclear. CHOlec8 cells, which are deficient in UDP-Gal import into the Golgi apparatus,[29] are also impaired in UDP-Gal import into the ER.[21] Whether UDP-Gal import in the ER and in the Golgi complex is mediated by the same or distinct UDP-Gal importers remains to be resolved. GalCer is converted to Ga_2Cer[30] and sulfatide[31] in the lumen of the Golgi, from where these products cannot reach the cytosolic surface.[17] In contrast, GalCer can translocate from the lumenal to the cytosolic leaflet of the ER membrane,[17] where it may interact with cytosolic galactose-binding lectins[32] or, in contrast to present dogma, may oligomerize and form microdomains in the cytosolic leaflet.

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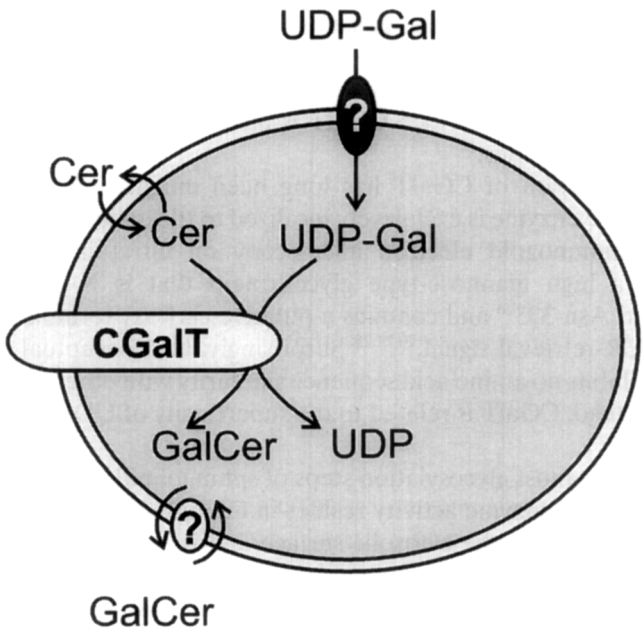

FIG. 1. Schematic organization of GalCer synthesis in the ER membrane.

Detection of CGalt by Its Products

Until recently, the presence of the CGalT could only be assessed via the presence of its products or by enzyme assay. GalCer and S-GalCer were discovered originally as major lipids in human brain by Thudichum in 1884,[33] whereas glycerol-based galactolipids were discovered by Carter *et al.*[34]

Chemical Detection of Galactolipids

Tissue can be analyzed for galactolipids chemically. Routinely, lipids are first extracted in chloroform/methanol (one-phase) at elevated temperatures for maximal yield. For sphingolipid analysis, glycerolipids are removed by alkaline hydrolysis, and acidic and neutral sphingolipids are separated by a DEAE column. Nonpolar lipids and sphingomyelin can then be removed by acetylation, column chromatography, and deacetylation. Next, the glycosphingolipids are subfractionated by thin-layer chromatography (TLC). Including dialysis steps and additional columns, this procedure may

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[34] H. E. Carter, P. Johnson, and E. J. Weber, *Annu. Rev. Biochem.* **34,** 109 (1965).

take two weeks.[35] A simplified analysis starts with a two-phase extraction,[36] after which the more polar lipids like sulfatides, which partition to some extent into the aqueous phase, can be recovered by adsorption to a reversed-phase cartridge. Lipids can be separated by two-dimensional TLC.[18,37] Separation of GalCer from GlcCer requires the use of borate-impregnated Whatman paper or TLC plates.[12,18,37–40] Spots are visualized classically by charring or staining by a variety of reagents.[12,35,39]

Galactolipids can be radiolabeled conveniently using galactose, acetate, fatty acid, and sulfate, whereas sphingolipids will be efficiently labeled also by serine, palmitate, sphingosine, sphinganine, or a ceramide containing a C_6(2-OH) chain (Figs. 2A and 2B). Fluorescent galactolipids can be produced from C_6-NBD-Cer, but more efficiently from C_6(2-OH)-NBD-Cer,[37,41] whereas C_6-NBD-DAG can be used to obtain C_6-NBD-GalDAG (Fig. 2C). Radiolabels and fluorescence are detected and quantitated by phosphorimaging or fluorography and scintillation counting[13,18,37] and by fluorimaging or fluorometry.[37]

Originally, galactolipids on TLC plates were identified by chemical determination of the sphingoid base or glycerol, fatty acid, galactose, or sulfate.[34] Often, sufficient information is obtained from comigration with standards, sensitivity of the lipid to enzymes such as α- or β-galactosidase, and in cell lines, after radiolabeling with specific precursors or treatment of the cells with inhibitors of glycolipid synthesis or sulfation.[18,37] The precise structure of a galactolipid can be obtained with mass spectrometry in combination with nuclear magnetic resonance (NMR) spectroscopy.[42] Whereas even one two-dimensional TLC separation of total lipids may yield galactolipid spots of sufficient purity to allow identification by mass spectrometry,[37] high-performance liquid chromatography (HPLC) remains the method of choice for this purpose.[43] Amounts in the picomole range can now be quantified with nano-electrospray tandem mass spectrometry.[44]

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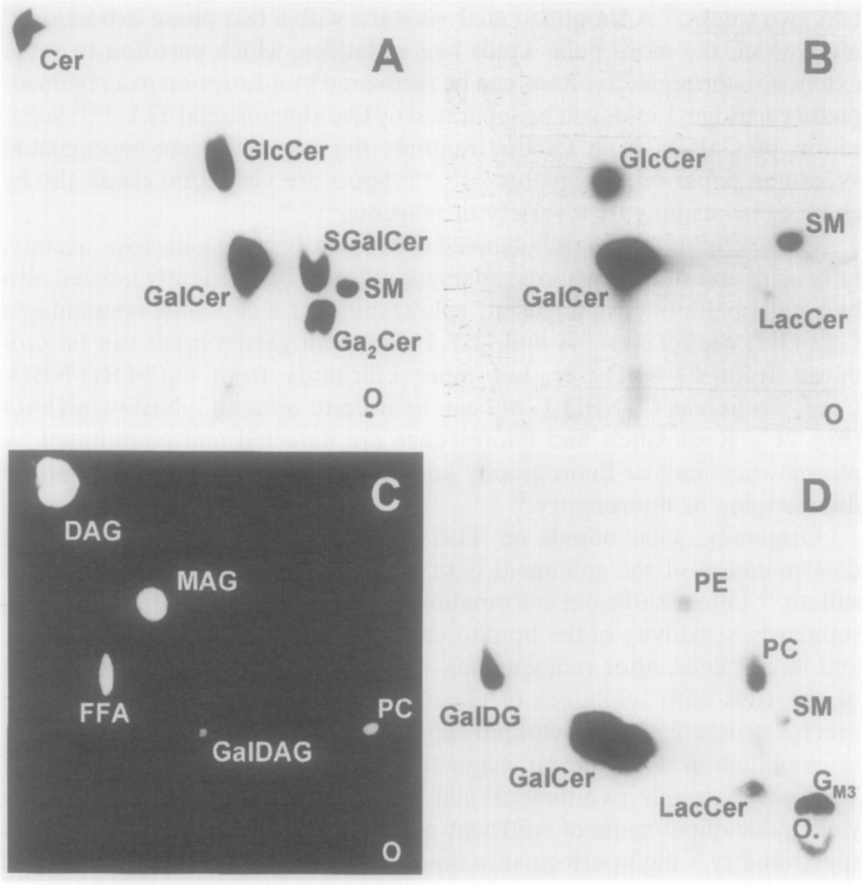

FIG. 2. Lipid synthesis in cell lines expressing CGalT. TLC analysis of the lipid products synthesized during 1 hr at 37° from C_6OH-[^{3}H]Cer in dog kidney MDCK II cells (A), in Chinese hamster ovary cells transfected with CGalT (CGalT-CHO) (B), and during 2 hr from NBD-DAG in CGalT-CHO cells (C).[1] (D) The fluorograph of CGalT-CHO lipids after an overnight incubation with [^{3}H]galactose [P. van der Bijl, G. J. Strous, M. Lopes-Cardozo, J. Thomas-Oates, and G. van Meer, *Biochem. J.* **317,** 589 (1996)]. FFA, C_6-NBD-hexanoic acid, free fatty acid; GalDG, sum of GalDAG and GalAAG; G_{M3}, sialo-LacCer; MAG, monoacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin. For solvents and further details, see text. Panel A was reproduced from the *J. Cell Biol.* **132,** 813 (1996) by copyright permission of The Rockefeller University Press. Panels C and D were reproduced with permission from the *Biochem. J.* **317,** 589 (1996). © Biochemical Society.

Often, a combination of the methods described here is required to define the precise galactolipid content of a sample.[18,37,45]

Immunological Detection of Galactolipids

Some lipids can be identified by antibody-overlay techniques.[46] Antibodies are available that recognize GalCer, GalDAG, GalAAG, Ga_2Cer, and their sulfated forms[47–59] with a degree of specificity.[60,61] A variation on this theme is the use of bacterial toxins recognizing GalCer,[62] the ectodomain of human immunodeficiency virus gp120 that recognizes GalCer and sulfatides,[63–70] or mammalian proteins that recognize sulfatides.[71–75] A common problem of these assays is their lack of specificity.

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Expression patterns of galactolipids may be established by immunolabeling methods. For light microscopy, a primary galactolipid-binding protein is visualized with fluorescently or otherwise labeled antibodies. For electron microscopy, protein A conjugated with colloidal gold is the detection method of choice. Because of the potential cross-reactivity of the galactolipid-binding protein, morphological techniques must always be confirmed by lipid analysis. Immunolabeling of (glyco)lipids is hampered by artifacts that include relocation and solubilization of the antigen during fixation with organic solvents and permeabilization with detergents. Immunolabeling of thawed cryosections may also result in the redistribution of lipid molecules. The best method so far is freeze substitution.[76,77] Glycolipids are thought to be enriched in patches in the membrane[7,11,77] However, antibody labeling may cluster glycolipids artificially, even after fixation. This can only be prevented by a second round of fixation after binding of the first antibody.[78]

Assays for CGalT Enzyme Activity

The enzyme activity producing GalCer was first demonstrated by Morell and Radin[13] and, since then, it has been characterized under numerous conditions. A technical problem is the difficulty of controlling the Cer concentration in the membrane containing CGalT as Cer is regulated tightly in the ER membrane *in vivo.*[79] Moreover, natural ceramides do not exchange efficiently between membranes *in vitro,* limiting the possibilities to manipulate Cer levels of isolated ER membranes. Cer has been supplied efficiently in detergent.[12,19,80] Detergent assays test enzyme activity under standard but nonphysiological conditions, as the ER membrane has been dissolved. Moreover, enzyme activity is reduced manyfold. Cer has also

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been presented from Celite[13] or phosphatidylethanolamine "membranes."[81] Disadvantages are the low efficiency, undefined local ceramide concentrations, and, in some cases, uncontrolled effects on the CGalT-containing membrane (e.g. by fusion). As an alternative, short chain ceramides provide a very efficient assay for enzyme activity in the ER membrane.[20,28,37,82,83] However, they yield indirect data on kinetics and substrate specificity.

Assay for CGalT Activity in Cells Using Short Chain Ceramides. The method used to detect CGalT enzyme activity is based on measuring the incorporation of fluorescent or radioactive, short chain Cer into GalCer. Because of the short fatty acyl chain, these ceramides and their products will display a higher off rate from membranes than the natural membrane lipids. For that reason, short chain lipid analogs can be efficiently presented to or depleted from membranes by a back-exchange against liposomes or bovine serum albumin (BSA) in the absence of detergent.[18,84] The reaction requires UDP-Gal, which, for *in vitro* studies, must be added exogenously. Lipids are extracted, separated by two-dimensional TLC, and quantitated by fluorescence of radioactivity.

Reagents

Phosphate-buffered saline (PBS; ice cold) containing 0.9 m*M* Ca^{2+} and 0.5 m*M* Mg^{2+}

Cell incubation mixture: Hanks' balanced salt solution, 20 m*M* HEPES–NaOH, pH 7.2, 1% (w/v) BSA (fraction V from Sigma, St. Louis, MO), and 35 n*M* of C_6OH-[^{3}H]Cer

Homogenization buffer (HB): 250 m*M* sucrose, 10 m*M* HEPES–NaOH, pH 7.2, and 1 m*M* EDTA

Reaction mixture: HB containing 2% (w/v) BSA, 4 m*M* UDP-glucose, 4 m*M* UDP-galactose, 4 m*M* $MgCl_2$, 4 m*M* $MnCl_2$, 1 μg/ml protease inhibitors, and 50 μM of NBD-Cer or NBD-DAG or 35 n*M* of C_6OH-[^{3}H]Cer

Ceramides: Fluorescent *N*-6(7-nitro-2,1,3-benzoxadiazol-4-yl)-aminohexanoylceramide (NBD-Cer) is obtained commercially (Molecular probes, Eugene, OR). The radiolabeled short chain ceramides hexanoyl-[^{3}H]Cer (C_6-[^{3}H]Cer) and 2-hydroxyhexanoyl-[^{3}H]Cer (C_6OH-[^{3}H]Cer) (800 MBq/μmol) are synthesized according to Ong and Brady.[18,85] Ceramides are dried from stock solutions in chloroform/

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methanol (2:1, v/v) under nitrogen, dissolved in ethanol (final concentration less than 0.2% v/v), and injected into BSA buffer under vortexing to yield the reaction mixture. This is incubated 30 min on ice, allowing BSA complexes of the ceramides to be formed prior to the addition of the enzyme source.

Fluorescent 1-palmitoyl-2,6(7-nitro-2,1,3-benzoxadiazol-4-yl)-aminohexanoyldiacylglycerol (NBD-DAG) is prepared from NBD-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) using phospholipase C[37]

TLC plates (Si60, Merck, Darmstadt, FRG) are dipped in 2.5% (w/v) boric acid in methanol[39] and dried prior to usage. Borate treatment is required to separate GlcCer and GalCer analogs.

All reactions and lipid extractions are performed in Corex or Pyrex glassware. Chromatography solvents are of Pro Analyse quality. All lipid stocks are stored in chloroform/methanol (2:1, v/v) at −20°. Solutions are stored under nitrogen and should be checked routinely for concentration and purity.

CGalT Source. Chinese hamster ovary (CHO) cells transfected with CGalT (CGalT-CHO cells[37]) are cultured in Eagle's minimum essential medium (MEM)-α (with nucleotides) with 10% fetal calf serum (FCS), 10 m*M* HEPES, and 500 μg/ml G418. To prepare a postnuclear supernatant (PNS), a 10-cm-diameter dish of CGalT-CHO cells is washed twice with ice-cold PBS and scraped gently in 1 ml ice-cold HB. Cells are pelleted and resuspended in 400 μl HB. The cells are homogenized by 12 to 14 passages through a 25-gauge needle and centrifuged for 15 min at 375*g* at 4° to remove nuclei and unbroken cells. Protein in the PNS is measured using the BCA assay (Pierce, Rockford, IL) and adjusted to 2 mg/ml with HB. In some cases, 0.4% (w/v) saponin is added to the PNS to permeabilize membranes during a 30-min incubation on ice prior to the experiment. MDCK II cells are grown as monolayers in MEM with 10 m*M* HEPES and 5% FCS.

Incubation. A 3-cm dish of CGalT-CHO cells or a 24-mm filter with MDCK cells is incubated with 1 ml cell incubation mixture. When PNS is used, 1 volume of reaction mixture is added to the PNS and the samples are incubated for 1 or 2 hr at 37°. The reaction is stopped by transferring the samples to an ice bath and by starting the lipid extraction.

Lipid Analysis. Lipids from cells, media, or PNS are extracted by a two-phase extraction.[36] The aqueous solution used for the phase separation contains 20 m*M* acetic acid and (for radiolabeled lipids) 120 m*M* KCl. An additional chloroform wash of the upper (aqueous) phase is performed. The organic (lower) phase is dried under N_2 at 37°, and the lipids are applied to borate-treated TLC plates using chloroform/methanol (2:1,

v/v). Thin-layer chromatography plates are developed in the first dimension using chloroform/methanol/25% (v/v) NH_4OH/water (65:35:4:4, v/v) and in chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v) in the second dimension. Fluorescent spots are quantitated using a STORM imager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software. Alternatively, spots are detected under UV, scraped, and extracted from the silica in 2 ml chloroform/methanol/20 m*M* acetic acid (1:2.2:1, v/v) for 30 min. After pelleting the silica for 10 min at 2000*g*, NBD fluorescence in the supernatant is quantified in a fluorometer at 470/535 nm using the appropriate controls and after calibration of the fluorometer using the Raman band of water at 350/397 nm. Radiolabeled spots are detected by fluorography after dipping the TLC plates in 0.4% (v/v) 2,5-diphenyl oxazole in 2-methylnaphthalene with 10% (v/v) xylene.[86] Preflashed film (Kodak X-Omat S, France) is exposed to the TLC plates for several days at −80°. The radioactive spots are scraped from the plates and the radioactivity is quantified by liquid scintillation counting in 0.3 ml Solulyte (J.T. Baker Chemicals, Deventer, The Netherlands) and 3 ml of Ultima Gold (Packard Instrument Company, Downers Grove, IL).

Results. The results of this assay are highly reproducible. In dog kidney MDCK cells, C_6OH-[^{3}H]Cer is converted to GalCer, Ga_2Cer, and SGalCer, whereas GlcCer and sphingomyelin are also formed (Fig. 2A). In contrast, transfection of CHO cells with rat CGalT results in a shift from incorporation into GlcCer and sphingomyelin to the production of C_6OH-[^{3}H]GalCer (Fig. 2B). In homogenates from both cell types, CGalT has a great preference for ceramides containing a 2-OH fatty acid.[13,19,37] Interestingly, tissues expressing high CGalT activity also contain high levels of 2-OH fatty acids. GalCer produced in CGalT-CHO cells contained exclusively nonhydroxy fatty acids,[37] which suggests that in the genome CGalT and the enzymes responsible for the synthesis of 2-OH fatty acids are coordinately controlled. This is apparently also the case for the α1,4-galactosyltransferase responsible for the synthesis of Ga_2Cer and the sulfotransferase synthesizing SGalCer. In contrast to the parental CHO cells, CGalT-CHO cells synthesized GalDAG from C_6-NBD-DAG (Fig. 2C) and a mixture of GalDAG and GalAAG from [^{3}H]galactose (Fig. 2D).

It should be noted that cellular factors may influence the CGalT activity measured. For example, the synthesis of GalCer is dependent on UDP-Gal import into the lumen of the ER. Some cell lines, such as CHOlec8 cells, have an impaired UDP-Gal import. A PNS of CGalT-CHOlec8 cells displayed low CGalT activity. This activity could be restored by permeabilizing membranes prepared from CGalT-CHOlec8 cells with saponin, sug-

[86] W. M. Bonner and J. D. Stedman, *Anal. Biochem.* **89,** 247 (1978).

gesting that the ER in CHOlec8 cells does not import UDP-Gal. These cells are known to lack the Golgi UDP-Gal transporter,[29] suggesting that the two transporter activities may reside within the same protein.

Enzyme assays have suggested the existence of two CGalTs with different intracellular locations (see earlier discussion). In our own studies, this finding was caused by an artifact of the CGalT assay. After the observation that the second CGalT activity had many properties in common with CGlcT in the Golgi,[17,37] a comparison between CGalT-negative cells that did or did not express CGlcT demonstrated that CGlcT can synthesize GalCer when assayed in the absence of UDP-Glc.[21] Similar observations were made using a purified CGlcT.[87] In the presence of UDP-Glc (as in living cells), UDP-Gal was essentially competed out. Alternatively, GalCer synthesis by CGlcT can be inhibited by a specific CGlcT inhibitor, such as D-threo-PDMP.[88]

Detection of CGalT Protein in Cells

Until recently, the characteristics of the CGalT could only be addressed by measuring its activity in isolated subcellular fractions (Ref. 17 and references therein). Although antibodies have been available for some time,[83] only the antibodies raised against recombinant CGalT have facilitated analysis of the protein. Histidine-tagged fusion proteins representing different regions of rat CGalT were used to generate rabbit polyclonal antisera that specifically recognize different lumenal regions of rat CGalT.[21] CGalT antisera work well for Western blotting, immunoprecipitation, and immunofluorescence microscopy. Cross-reactivity in other species has not been tested yet.

To study the properties of CGalT in cultured cells, newly synthesized proteins are labeled metabolically with radioactive amino acids and are chased with unlabeled amino acids for various time periods. Now different aspects of CGalT can be studied in more detail, such as its biosynthetic maturation and its membrane topology. Assays for analysis of its co- and posttranslational modifications can also be found elsewhere.[27] Radiolabeled CGalT is isolated by immunoprecipitation, followed by separation on sodium dodecyl sulfate–polyacrylamide (SDS–PAA) gels and analysis by phosphorimaging.

Reagents

Depletion medium: Cysteine- and methionine-free minimum essential medium (MEM α, Sigma, M3786), 20 m*M* HEPES, pH 7.3, at 37°

[87] P. Paul, Y. Kamisaka, D. L. Marks, and R. E. Pagano, *J. Biol. Chem.* **271,** 2287 (1996).
[88] J.-i. Inokuchi, K. Momosaki, H. Shimeno, A. Nagamatsu, and N. S. Radin, *J. Cell. Physiol.* **141,** 573 (1989).

Pulse medium: Depletion medium containing 250 μCi/ml Tran[^{35}S]-label (>1000 Ci/mmol; ICN, Costa Mesa, CA) at 37°

Chase medium: MEM supplemented with 5 m*M* methionine, 5 m*M* cysteine, and 20 m*M* HEPES, pH 7.4, at 37°

Stop buffer: PBS, 20 m*M* *N*-ethylmaleimide (NEM), ice cold. An alkylating agent, such as NEM or iodoacetamide, should be included in the stop and lysis buffer to prevent artificial formation of disulfide bonds.

Lysis buffer: PBS, 0.5% (v/v) Triton X-100 (TX-100), 1 m*M* EDTA, 20 m*M* NEM, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml of aprotinin, chymostatin, leupeptin, pepstatin A (ice cold). Because alkylating agents and protease inhibitors have short half-lives in aquous solutions, they should be added to buffers immediately prior to use.

Wash buffer: 150 m*M* NaCl, 2 m*M* EDTA, 100 m*M* Tris–HCl, pH 8.3, 0.1% (w/v) SDS, 0.5% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate

Homogenization buffer

TE: 20 m*M* Tris–HCl, pH 6.8, 1 m*M* EDTA

4× reducing sample buffer: 800 m*M* Tris–HCl, pH 6.8, 12% (w/v) SDS, 40% (v/v) glycerol, 4 m*M* EDTA, 0.01% (w/v) bromophenol blue, 300 m*M* dithiothreitol

Biosynthetic Processing of CGalT. CGalT-CHO cells grown in 6-cm tissue culture dishes are rinsed with PBS and once with depletion medium. To deplete cellular cysteine and methionine levels, cells are starved for 30 min in depletion medium. Cells are labeled in pulse medium for 5 min at 37°. Cells are rinsed with chase medium once and are incubated at 37° in chase medium. To follow biosynthetic processing of CGalT, the cells are put on ice after different periods of time, washed with stop buffer, and incubated for 20 min with stop buffer on ice. Cells are lysed in PBS, 1% (v/v) TX-100 and are centrifuged at 14,000*g* for 10 min at 4°. Cleared lysates are subjected to immunoprecipitation.

Protease Protection Assay. For a protease protection assay, CGalT-CHO cells are labeled metabolically for 15 min as described, followed by a chase period of 10 min, and PNS as described earlier. Fifty microliters 1 ($\pm$50 μg) PNS is incubated with 0.1 mg/ml of proteinase K or trypsin (pretreated with L-1-tosylamide-2-phenylethyl chloromethyl ketone) for 60 min at 10° in the presence or absence of 0.5% (w/v) saponin. The digestion should be performed in a small volume in order to keep the total amount of protease as low as possible. Samples are transferred to ice and the reaction is stopped by adding PMSF (2.5 mg/ml), leupeptin (0.25 mg/ml), aprotinin (0.25 mg/ml), pepstatin A (0.25 mg/ml), and trypsin inhibitor (1.0 mg/ml) to the indicated concentrations. Membranes are solubilized in 0.5%

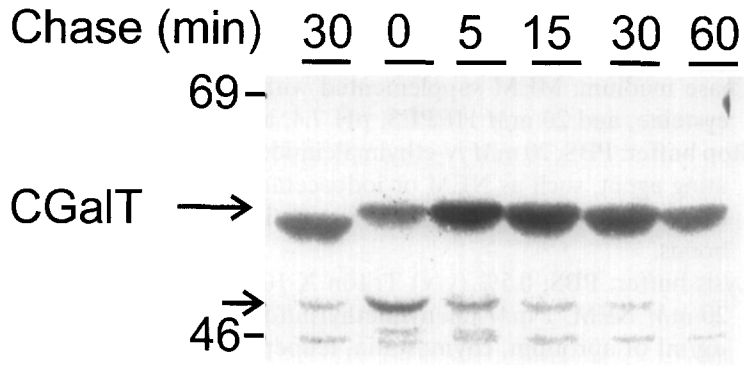

FIG. 3. Maturation of CGalT. CGalT-CHO cells were pulse labeled for 5 min with Tran^{35}S-labeled amino acids and were then, $t = 0$, chased for different time intervals. After cell lysis, CGalT was imunoprecipitated with antiserum 635. Proteins were resolved under reducing conditions by SDS–polyacrylamide gel electrophoresis on a 10% gel. Please note the small shift in mobility of the mature CGalT (large arrow) and the disappearance of the immature CGalT of 50 kDa (small arrow) in time. The $t = 30$ sample was run twice to facilitate comparison with the $t = 0$ sample.

(v/v) TX-100, and CGalT is immunoprecipitated from the detergent lysates in the presence of protease inhibitors.

Immunoprecipitation. Protein A–Sephacryl CL4B beads are washed five times with ice-cold PBS, 0.5% (w/v) BSA and are incubated with anti-CGalT rabbit serum 635[21] for at least 1 hr at 4°. Beads are pelleted by centrifugation at 14,000*g* for 1 min at 4°. The supernatant is removed and the pellet is resuspended in ice-cold PBS, 0.5% (w/v) BSA. Cell lysates are incubated with the 60 μl of 10% beads for at least 1 hr at 4°. Beads are pelleted and washed three times with wash buffer. Eventually, the beads are resuspended in 30 μl TE, and 10 μl 4× reducing sample buffer is added. Samples are incubated for 5 min at 95° and centrifuged briefly at 14,000*g*. Samples are separated by SDS–PAA gel electrophoresis.[89] Gels are dried onto Whatman 3MM filter paper and exposed to a phosphor-imaging screen.

Results. Immature CGalT appears as a 50-kDa precursor protein that is N-glycosylated rapidly, resulting in a band of 54 kDa. A small but significant shift to a higher mobility form of CGalT occurred in the first hour after the pulse. This shift represents processing of N-linked oligosaccharides in the ER (Fig. 3).

The predicted molecular mass of the CGalT is approximately 64 kDa, and several studies describe an apparent molecular mass of 50–70 kDa.[6,26,27] We, however, consistently detected mature CGalT in different assay sys-

[89] U. K. Laemmli, *Nature* **227,** 680 (1970).

tems and in distinct cell lines as a band with an apparent molecular mass of 54 kDa.[21] In order to obtain sufficient resolution to separate mature and newly synthesized CGalT, we used 7.5 or 10% SDS polyacrylamide gels. Possibly the high content of hydrophobic amino acids in the lumenal portion of CGalT is responsible for the anomalous behavior of the protein on SDS–PAA gels.

Important questions to be solved in the near future are the coordinate transcriptional regulation of the enzymes involved in galactosphingolipid synthesis and the unraveling of the cellular functions of each of the various products.

[9] Assay of Lactosylceramide Synthase and Comments on Its Potential Role in Signal Transduction

By SUBROTO CHATTERJEE

Introduction

Lactosylceramide synthase (GalT-2) catalyzes the transfer of galactose from UDP-galactose to glucosylceramide (GlcCer) to form lactosylceramide via the following reaction:

$$\text{UDP-[}^{14}\text{C or }^{3}\text{H]Gal} + \text{GlcCer} \xrightarrow[\text{Mg}^{2+}\text{ and/or m}^{2+}]{\text{GalT-2}} \text{Gal}\beta\text{1-4GlcCer} + \text{UDP}$$

An essential feature of this galactosyltransferase is the requirement for manganese ions and detergent for optimal activity. Most, if not all, GalT-2 activity has been associated with the Golgi apparatus. However, evidence indicates that some enzymatic activity may also be associated with the cell membrane.[1,2]

Increases in GalT-2 activity have been associated with cell fusion in small Fu-1 cells, atherosclerotic tissue, and polycystic kidney diseased cells, as well as in renal cancer. This is accompanied by the accumulation of large amounts of LacCer in these tissues.[3–10] Interestingly, atherosclerotic tissue

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