A role for glycosphingolipids in protein sorting
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Chapter 2

Analysis of galactolipids and UDP-galactose:ceramide galactosyltransferase

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Introduction

Glycosphingolipids form a highly polymorphic class of lipids and several hundreds of the more than 2000 possible molecular species (107) have been characterized (108). There are at least 20 different ceramide (Cer) backbones due to differences in sphingoid base, mostly sphingosine (4-sphinganine) and phytosphingosine (4-hydroxysphinganine), and acyl chain. The headgroups can vary from 1-60 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 B1-4 Glc B1-1 Cer, lactosylceramide. Galactosylceramide (Gal B1-1 Cer or GalCer) serves as a precursor for few simple glycolipids: the sulfatide SGalCer (SO₃⁻3 GalCer), galabiosylceramide (Gal α₁-4 GalCer, and the ganglioside sialo-GalCer (I⁻³NeuAc-GalCer or GM₄). Gal and SGal are also found on diglycerides: Gal B1-3 diacylglycerol (GalDAG), Gal B1-3 alkyl-acyl-glycerol (GalAAG), digalactosyldiglyceride, and seminolipid: SO₃⁻3 GalAAG (109).

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 (107). While glycosphingolipids constitute only a few mol% of the lipids in most membranes, they are major components of the myelin sheath (110), where GalCer and SGalCer are involved in axonal insulation, myelin function, and stability (111, 112). The apical plasma membrane of epithelial cells in the gastro-intestinal and urinary tracts is enriched in glycosphingolipids. In rodents these are typically glucolipids (18, 108), whereas in humans most are galactolipids (113-115). 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 exocytic and endocytic transport routes is not fully understood (18, 116).

The foremost enzyme involved in the biosynthesis of galactosphingolipids is the UDP-galactose:ceramide galactosyltransferase, GaT-1 (117). GaT-1 catalyses the transfer of galactose from UDP-galactose (UDP-Gal) to Cer yielding GalCer (118) and has a relatively promiscuous substrate specificity. Whether there are one or more GaT-1 enzymes with distinct specificity and cellular localization has been a controversial issue (119-123). Importantly, knock-out mice do not make GalCer (111, 112), showing there is only one GaT-1. In vitro studies demonstrated that partially purified GaT-1 from brain has >15 fold preference for 2-hydroxy fatty acid- over non-hydroxy fatty acid containing Cer (118, 124). This has been confirmed for GaT-1 after transfection into GaT-1-negative cells (123, 125). In vivo, however, GaT-1 is responsible for the galactosylation of 2-hydroxy fatty acid- as well as non-hydroxy fatty acid containing Cer. The GaT-1 activity, specific for non-hydroxy fatty acid containing Cer, which was previously found in the Golgi (120, 122, 123), has now been demonstrated to be an in vitro activity of the Golgi UDP-glucose:ceramide glucosyltransferase (CGlcT; chapter 3). GaT-1 is also responsible for the galactosylation of diglycerides (123, 126).

The localization of GaT-1 has long been enigmatic (119, 127-130). Recently, we showed that the enzyme was exclusively localized to the ER by immunogold electron microscopy on ultrathin cryosections (chapter 3). GaT-1 is a high-mannose type glycoprotein that is N-glycosylated at Asn 78 and Asn 333 (131) and contains a putative carboxy terminal Lys-Lys-Val-Lys ER-retrieval signal (125, 132, 133). Surprisingly, the conceptual translation product exhibits no amino acid sequence similarity with other glycosyltransferases. Instead, GaT-1 is related to the superfamily of UDP-glucuronosyltransferases.

Thus, while most glycosylation steps of sphingolipids occur in the Golgi complex, the GaT-1 enzyme activity resides in the lumen of the endoplasmic reticulum (Figure 1; chapter 3). 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 GalT-1 is unclear. CHOlec8 cells, which are deficient in UDP-Gal import into the Golgi apparatus (134), are also impaired in UDP-Gal import into the endoplasmic reticulum (chapter 3 and 4). 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 galabiosyl-ceramide (135) and sulfatide (136) in the lumen of the Golgi, from where these products cannot reach the cytosolic surface (122). In contrast, GalCer can translocate from the lumenal to the cytosolic leaflet of the ER membrane (122), where it may interact with cytosolic galactose binding lectins (137), or, in contrast to present dogma, may oligomerize and form microdomains in the cytosolic leaflet.

Figure 1: Schematic organization of GalCer synthesis in the ER membrane  
For details see text.

Detection of GalT-1 by its products

Until recently, the presence of the GalT-1 could only be assessed via the presence of its products or by enzyme assay. GalCer and S-GalCer were originally discovered as major lipids in human brain by Thudichum in 1884 (138), whereas glycerol-based galactolipids were discovered by Carter et al. (139).

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. Non-polar lipids and sphingomyelin can then be removed by acetylation, column chromatography, and deacetylation. Next, the glycosphingolipids are subfractionated by TLC. Including dialysis steps and additional columns, this procedure may take two weeks (140). A simplified analysis starts with a two-phase extraction (141), 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 (123, 142). Separation of GalCer from GlcCer requires the use of
borate-impregnated Whatman paper or TLC plates (20, 117, 123, 142-144). Spots are classically visualized by charring or staining by a variety of reagents (117, 140, 144).

Galactolipids can be conveniently radiolabeled by using galactose, acetate, fatty acid, and sulfate, whereas the sphingolipids will be efficiently labeled also by serine, palmitate, sphingosine, sphiangamine or a ceramide containing a C6(2-OH) chain (Figure 2A, B). Fluorescent galactolipids can be produced from NBD-Cer, but more efficiently from (2-OH)NBD-Cer(123, 145), while NBD-DAG can be used to obtain NBD-GalDAG (Figure 2C). Radiolabels and fluorescence are detected and quantitated by phosphorimaging or fluorography and scintillation counting, and fluorimaging or fluorometry (118, 123, 142).

Originally, galactolipids on TLC plates were identified by chemical determination of the sphingoid base or glycerol, fatty acid, galactose, or sulfate (139). Often, sufficient information is obtained from co-migration with standards, sensitivity of the lipid to enzymes like 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 (123, 142). The precise structure of a galactolipid can be obtained with mass spectrometry in combination with NMR spectroscopy (146). While even one 2D-TLC separation of total lipids may yield galactolipid spots of sufficient purity to allow identification by mass-spectrometry (123), HPLC remains the method of choice for this purpose (147). Amounts in the pmol range can now be quantified with nano-electrospray tandem mass spectrometry (148). Often, a combination of the methods described here is required to define the precise galactolipid content of a sample (123, 142, 149).

**Immunological detection of galactolipids**

Some lipids can be identified by antibody-overlay techniques (150). Antibodies are available that recognize GalCer, GalDAG, GalAAG, galabiosylceramide, and their sulfated forms (151-163), with a degree of specificity (164, 165). A variation on this theme is the use of bacterial toxins recognizing GalCer (166), the ectodomain of of human immunodeficiency virus gp120 that recognizes GalCer and sulfatides (167-174), or mammalian proteins that recognize sulfatides (175-179). A common problem of these assays is their lack of specificity.

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 artefacts 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 redistribution of lipid molecules. The best method so far is freeze-substitution (20, 60). Glycolipids are thought to be enriched in patches in the membrane (18, 60, 116). 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 (180).

**Assays for GalT-1 enzyme activity**

The enzyme activity producing GalCer was first demonstrated by Morell and Radin (118) and, since then, it has been characterized under numerous conditions. A technical problem is the difficulty to control the Cer concentration in the membrane containing GalT-1 as Cer is tightly regulated in the ER membrane *in vivo* (181). Moreover, natural ceramides do not efficiently exchange between membranes *in vitro*, limiting the possibilities to manipulate Cer levels of isolated ER membranes. Cer has been efficiently supplied in detergent (117, 124, 182).
Detergent assays test enzyme activity under standard but non-physiological conditions, as the ER membrane has been dissolved. Moreover, enzyme activity is reduced many-fold. Cer has also been presented from Celite (118) or phosphatidylethanolamine "membranes" (183). Disadvantages are the low efficiency, undefined local ceramide concentrations, and, in some cases, uncontrolled effects on the GalT-1-containing membrane (by fusion, for example). As an alternative, short-chain ceramides provide a very efficient assay for the enzyme activity in the ER membrane (123, 125, 133, 184, 185). However, they yield indirect data on kinetics and substrate specificity.

Assay for GalT-1 activity in cells using short-chain ceramides

The method to detect GalT-1 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 BSA, in the absence of detergent (142, 186). The reaction requires UDP-Gal, which, for *in vitro* studies, must be added exogenously. Lipids are extracted, separated by 2D-TLC, and quantitated by fluorescence of radioactivity.

**Reagents**

Reaction mixture: HB containing 2% w/v BSA, 4 mM UDP-Glc, 4 mM UDP-Gal, 4 mM MgCl₂, 4 mM MnCl₂, 1 µg/ml protease inhibitors, and 50 µM of NBD-Cer or NBD-DAG, or 35 nM of C₆OH-[^3H]Cer.

Cell incubation mixture: Hanks' Balanced Salt Solution, 20 mM Hepes-NaOH, pH 7.2, 1% w/v bovine serum albumin (BSA; fraction V from Sigma, St. Louis, MO), and 35 nM of C₆OH-[^3H]Cer.

Homogenization buffer (HB): 250 mM sucrose, 10 mM Hepes-NaOH, pH 7.2, 1 mM EDTA.

Ceramides: Fluorescent N-6(7-nitro-2,1,3-benzoaxadiazol-4-yl)-aminohexanoyl-ceramide (NBD-Cer) was obtained commercially (Molecular probes, Eugene, OR). The radiolabeled short-chain ceramides hexanoyl[^3H]Cer (C₆[^3H]Cer) and 2-hydroxyhexanoyl[^3H]Cer (C₆OH-[^3H]Cer; 800 M bq/µmol), were synthesized according to Ong and Brady (142, 187). Ceramides were dried from stock solutions in chloroform/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 was incubated 30 min on ice allowing BSA-complexes of the ceramides to be formed prior to addition of the enzyme source. Fluorescent 1-palmitoyl-2-6(7-nitro-2,1,3-benzoaxadiazol-4-yl)-aminohexanoyl-diacylglycerol (NBD-DAG) was prepared from NBD-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) using phospholipase C (123). TLC-plates (Si60, Merck, Darmstadt, FRG) were dipped in 2.5% w/v boric acid in methanol (144), and dried prior to usage. Borate treatment is required to separate GlcCer from GalCer analogs. All reactions and lipid extractions were performed in Corex or Pyrex glassware. Chromatography solvents were of Pro Analysie quality. All lipid stocks are stored in chloroform/methanol (2:1, v/v) at −20°C. Solutions are stored under nitrogen and should be checked routinely for concentration and purity.

**GalT-1 source**

Chinese hamster ovary (CHO) cells transfected with GalT-1 (GalT-1-CHO cells; 123) were cultured in Eagle's minimum essential medium (MEM)-alpha with nucleotides, 10% fetal calf serum, 10 mM Hepes, and 500 µg/ml G418. To prepare a postnuclear supernatant (PNS), a 10 cm diameter dish of GalT-1-CHO cells is washed twice with ice-cold PBS, and gently scraped 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 375g at 4°C to remove nuclei and unbroken cells. Protein in the PNS was measured using the BCA assay (Pierce, Rockford, IL) and adjusted to 2 mg/ml with HB. In some cases, saponin is added to 0.4% w/v to the PNS to permeabilize membranes during an incubation of 30 min on ice prior to the experiment. Madin-Darby canine kidney type II (MDCK II) cells were grown as monolayers in MEM with 10 mM Hepes and 5% FCS.

**Incubation**

A 3 cm dish of GalT-1-CHO cells or a 24 mm filter with MDCK cells is incubated with 1 ml cell incubation mixture. When PNS is used, one volume of reaction mixture is added to the PNS and the samples are incubated for 1 or 2 h at 37°C. 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 (141). The aqueous solution used for the phase separation contains 20 mM acetic acid and (for radiolabeled lipids) 120 mM KCl. An additional chloroform wash of the upper (aqueous) phase is performed. The organic (lower) phase is dried under N$_2$ at 37°C and the lipids are applied to borate-treated TLC plates using chloroform/methanol (2:1, v/v). TLC plates are developed in the first dimension using chloroform/methanol/25% v/v NH$_4$OH/water (65:35:4:4, v/v), and in the second dimension in chloroform/acetic acid/water (50:20:10:10:5, v/v). Fluorescent spots are quantitated using a STORM 860 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 mM acetic acid (1:2:2:1, v/v) for 30 min. After pelleting the silica for 10 min at 2,000g, NBD-fluorescence in the supernatant is quantified in a fluorometer at 470 nm/535 nm using the appropriate controls and after calibration of the fluorometer using the Raman band of water at 350 nm/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 (188). Preflashed film (Kodak X-Omat S, France) is exposed to the TLC plates for several days at -80°C. 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, USA).

**Results**

The results of this assay are highly reproducible. In dog kidney MDCK cells C$_6$OH-[3H]Cer is converted to GalCer, galabiaosylceramide, and SGalCer, while also GlcCer and sphingomyelin are being formed (Figure 2A). In contrast, transfection of CHO cells with rat GalT-1 results in a shift from incorporation into GlcCer and sphingomyelin to production of C$_6$OH-[3H]GalCer (Figure 2B). In homogenates from both cell types, GalT-1 has a great preference for ceramides containing a 2-OH fatty acid (118, 123, 124). Interestingly, tissues expressing high GalT-1 activity also contain high levels of 2-OH fatty acids. GalCer produced in GalT-1-CHO cells contained exclusively non-hydroxy fatty acids (123), which suggests that in the genome GalT-1 and the enzymes responsible for the synthesis of 2-hydroxy fatty acids are coordinately controlled. This is apparently also the case for the α1-4 galactosyltransferasen responsible for the synthesis of galabiaosylceramide and the sulfotransferase synthesizing SGalCer. In contrast to the parental CHO cells, GalT-1-CHO cells synthesized GalDAG from NBD-DAG (Figure 2C), and a mixture of GalDAG and GalAAG from [3H]galactose (Figure 2D).
It should be noted that cellular factors may influence the GalT-1 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 GalT-1-CHOlec8 cells displayed low GalT-1 activity. This activity could be restored by permeabilizing membranes prepared from GalT-1-CHOlec8 cells with saponin suggesting that the ER in CHOlec8 cells does not import UDP-Gal. These cells are known to lack the Golgi UDP-Gal transporter (134), suggesting that the two transporter activities may reside within the same protein.

Figure 2: Lipid synthesis in cell lines expressing GalT-1
TLC analysis of the lipid products synthesized A: during 1 h at 37°C from C6OH-[3H]Cer in dog kidney MDCK II cells, B: in Chinese hamster ovary cells transfected with GalT-1 (GalT-1-CHO), and C: during 2 h from NBD-DAG in GalT-1-CHO cells. D: Panel shows the fluorograph of GalT-1-CHO lipids after an overnight incubation with [3H]galactose. FFA, NBD-fatty acid; GalDG, sum of GalDAG and GaAAG; Ga2Cer, galabiosylceramide; MAG, monoacylglycerol. See also: Abbreviations. For solvents and further details, see text. Panels A, C and D were reproduced with permission (123, 142).
Enzyme assays have suggested the existence of two GalT-1s with different intracellular locations (see above). In our own studies this finding was caused by an artefact of the GalT-1 assay. After the observation that the second GalT-1 activity had many properties in common with CGlcT in the Golgi (122, 123), a comparison between GalT-1 negative cells that did or did not express CGlcT demonstrated that CGlcT can synthesize GalCer when assayed in the absence of UDP-Glc (chapter 3). Similar observations were made using a purified CGlcT (189). 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-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (190).

### Detection of GalT-1 protein in cells

Until recently, the characteristics of the GalT-1 could only be addressed by measuring its activity in isolated subcellular fractions (122, and references therein). Although antibodies have been available for some time (185), only the recent antibodies raised against recombinant GalT-1 have facilitated analysis of the protein. Histidine-tagged fusion proteins representing different regions of rat GalT-1 were used to generate rabbit polyclonal antisera that specifically recognize different luminal regions of rat GalT-1 (chapter 3). The GalT-1 antisera work well for Western blotting, immunoprecipitation, and immuno-fluorescence microscopy. Cross reactivity in other species has not been tested yet.

To study the properties of GalT-1 in cultured cells, newly synthesized proteins are metabolically labeled with radioactive amino acids and chased with unlabeled amino acids for various time periods. Now different aspects of GalT-1 can be studied in more detail, such as its biosynthetic maturation and its membrane topology. Assays for analysis of its co- and post-translational modifications can also be found elsewhere (132). Radiolabeled GalT-1 is isolated by immunoprecipitation, followed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysis by phosphorimaging.

### Reagents

**Depletion medium:** Cysteine- and methionine-free minimum essential medium (MEM alpha, Sigma, M3786), 20 mM Hapes pH 7.3, at 37°C.

**Pulse medium:** Depletion medium containing 250 μCi/ml Tran[35S]label (> 1,000 Ci/mmol; ICN, Costa Mesa, CA), at 37°C.

**Chase medium:** MEM supplemented with 5 mM methionine, 5 mM cysteine, and 20 mM Hapes pH 7.4, at 37°C.

**Stop buffer:** PBS, 20 mM N-ethylmaleimide, ice-cold. An alkylating agent, such as N-ethylmaleimide 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 mM EDTA, 20 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1μg/ml of aprotinin, chymostatin, leupeptin, and peptstatin A, ice-cold. Because alkylating agents and protease inhibitors have short half lives in aqueous solutions, they should be added to buffers immediately prior to use.

**Wash buffer:** 150 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl pH 8.3, 0.1% w/v SDS, 0.5% w/v Nonidet P40, 0.5% w/v sodiumdeoxycholate.

**HB:** Homogenization buffer: see above.

**TE:** 20 mM Tris-HCl pH 6.8, 1 mM EDTA.

**4x sample buffer:** 800 mM Tris-HCl pH 6.8, 12% w/v SDS, 40% v/v glycerol, 4 mM EDTA, 0.01% w/v bromophenol blue, 300 mM dithiothreitol.

24
Biosynthetic processing of GalT-1

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

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Figure 3: Maturation of GalT-1

GalT-1-CHO cells were pulse-labeled for 5 min with Tran[^35S]labeled amino acids and were then, $t=0$, chased for different time intervals. After cell lysis, GalT-1 was immunoprecipitated with antisera 635. Proteins were resolved under reducing conditions by SDS-PAGE on a 10% gel. Please note the small shift in mobility of the mature GalT-1 (large arrow) and the disappearance of the immature GalT-1 of 50 kDa (small arrow) in time. The $t=30$ sample was run twice to facilitate comparison with the $t=0$ sample.

Protease protection assay

For a protease protection assay GalT-1-CHO cells were metabolically labeled for 15 min as described, followed by a chase period of 10 min, and a PNS was prepared as above. Fifty μl (± 50 μg) PNS was 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°C 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 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), pepstatin A (0.25 mg/ml) and trypsin inhibitor (1.0 mg/ml) to the indicated concentrations. Membranes were solubilized in 0.5% v/v TX-100 and GalT-1 was immunoprecipitated from the detergent lysates in the presence of protease inhibitors.

Immunoprecipitation

Protein A-Sepharyl CL4B beads were washed 5 times with ice-cold PBS, 0.5% w/v BSA and incubated with anti GalT-1 rabbit serum 635 (chapter 3) for at least 1 h at 4°C. Beads were pelleted by centrifugation at 14,000g for 1 min at 4°C. Supernatant was removed and the pellet was resuspended in ice-cold PBS, 0.5% w/v BSA. Cell lysates were incubated with the 60 μl of 10% beads for at least 1 h at 4°C. Beads were pelleted and washed three times with wash buffer. Eventually, the beads were resuspended in 30 μl TE and 10 μl 4x sample buffer was added. Samples were incubated for 5 min at 95°C, and centrifuged briefly at 14,000g. Samples
were separated by SDS-PAGE (191). Gels were dried onto Whatman 3MM filter paper and exposed to a phosphor imaging screen.

**Results**

Immature GalT-1 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 GalT-1 occurred in the first h after the pulse. This shift represents processing of N-linked oligosaccharides in the ER (Figure 3).

The predicted molecular weight of the GalT-1 is approximately 64 kDa, and several studies describe an apparent molecular mass of 50-70 kDa (111, 131, 132). We, however, consistently detected mature GalT-1 in different assay systems and in distinct cell lines as a band with an apparent molecular weight of 54 kDa (chapter 3). In order to obtain sufficient resolution to separate mature and newly synthesized GalT-1, we used 7.5% or 10% SDS polyacrylamide gels. Possibly the high content of hydrophobic amino acids in the lumenal portion of GalT-1 is responsible for the anomalous behavior of the protein on SDS-PAGE 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.