Synthesis of non-hydroxy-galactosylceramides and galactosyldiglycerides by hydroxy-ceramide galactosyltransferase

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Galactosylceramide (GalCer) is the major glycolipid in brain. In order to characterize the activity of brain UDPgalactose:ceramide galactosyltransferase (CGalT), it has been stably expressed in CGalT-negative Chinese hamster ovary (CHO) cells. After fractionation of transfected cells, CHO-CGT, on sucrose gradients, the activity resides at the density of endoplasmic reticulum and not of Golgi. A lipid chromatogram from CHO-CGT cells revealed two new iodine-staining spots identified as GalCer, since they comigrate with GalCer standards, can be metabolically labelled with [^{3}H]galactose, are recognized by anti-GalCer antibodies, and are resistant to alkaline hydrolysis. A third [^{3}H]galactose lipid was identified as galactosyldiglyceride. In the homogenate CGalT displays a 25-fold preference for hydroxy fatty acid-containing ceramides. Remarkably, endogenous GalCer of transfected cells contains exclusively non-hydroxy fatty acids: fast atom bombardment and collision-induced dissociation mass spectrometric analysis revealed mainly $C_{16:0}$ in the lower GalCer band on TLC and mainly $C_{22:0}$ and $C_{24:0}$ in the upper band. Our results suggest that CGalT galactosylates both hydroxy- and non-hydroxy fatty acid-containing ceramides and diglycerides, depending on their local availability. Thus, CGalT alone may be responsible for the synthesis of hydroxy- and non-hydroxy-GalCer, and galactosyldiglyceride in myelin.

INTRODUCTION

High levels of glycosphingolipids are present in myelin [1] and epithelia of the intestine and kidney, where they are present predominantly in the apical plasma membrane [2]. Galactosylceramide (GalCer) is the major glycosphingolipid in the myelinating tissue of the nervous system. In myelin sheaths, consisting of approximately 70% lipid and 30% protein, GalCer and its sulphated derivative [sulphatide (SGalCer)] account for 25–30% of total lipids [1]. The ceramide part of GalCer is polymorphic. In mammalian myelin its sphingosine (4-sphingenine) backbone is N-acylated with roughly equal amounts of 2-hydroxy fatty acids (HFA) and non-hydroxy fatty acids (NFA), and contains a high proportion of very-long-chain fatty acids (mainly C_{24} [3–5]).

While in most epithelia glucosylceramide (GlcCer) and its derivatives are the major glycosphingolipids, in parts of the human gastrointestinal tract [6] and in human kidney epithelial cells [7] GalCer is the major monohexosyl sphingolipid. In the dog kidney cell line MDCK, where GalCer accounts for 14% and GlcCer for 24% of total glycosphingolipids [8], GalCer has a ceramide backbone composed of sphingosine and mainly non-hydroxy fatty acids (C_{16}, C_{20}, C_{24} [9,10]), while GalCer in bovine kidney also contains phytosphingosine (4-hydroxysphinganine) and mainly hydroxy fatty acids [11,12].

UDPgalactose:ceramide galactosyltransferase (CGalT) catalyses the transfer of galactose to ceramide, yielding GalCer. Whether one single or two separate enzymes (EC 2.4.1.45 and EC 2.4.1.47) are responsible for the synthesis of HFA- and NFA-GalCer *in vivo* is a controversial issue (see [13]). The major enzyme in neural tissues has a preference for HFA-ceramides *in vitro*, and has thus been referred to as HFA-ceramide galactosyltransferase or HFA-CGalT [14–18]. Recently, the cDNA encoding this CGalT has been cloned from rat brain [19–21]. The mRNA is expressed in oligodendrocytes and Schwann cells during myelination [22,23]. When transfected into COS7 cells the enzyme shows a preference for HFA-ceramides as substrates for galactosylation *in vitro* [23].

In the present study we characterize the substrate specificity of the enzyme encoded by the cloned cDNA [21,23], and investigate whether the HFA-CGalT can be responsible for the synthesis of NFA-GalCer *in vivo*. We have thus stably transfected the cDNA encoding the enzyme into CHO cells, which do not possess GalCer, but only GlcCer, lactosylceramide [LacCer (Galβ1-4Glcβ1-1Cer)] and the ganglioside NeuAcα2-3Galβ1-4Glcβ1-1Cer (G_{M3}) [24]. CHO cells expressing CGalT are able to synthesize both HFA- and NFA-GalCer *in vitro*, at a subcellular site that co-localizes with the endoplasmic reticulum (ER) on a sucrose gradient. However, *in vivo*, CGalT-transfected CHO cells synthesize exclusively NFA-GalCer and the glycerolipid galactosyldiglyceride.

EXPERIMENTAL

Materials

UDP-Gal, UDP-Glc, ceramides (bovine brain type III and IV), BSA (fraction V), GalCer (bovine brain type I and II), Gal-DAG (DAG is 1,2-diacylglycerol), β-galactosidase (jack beans) and Protein A were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L,D-threo-l-phenyl-2-decanoylamino-3-morpho-

Abbreviations used: AAG, 1-alkyl-2-acyl-glycerol; {C_6}, hexanoyl; {C_6OH}, D-2-hydroxy hexanoyl; Cer, ceramide; CHO, Chinese hamster ovary; CHO-WT, wild-type CHO cells; CID, collision-induced dissociation; CGalT, UDPgalactose:ceramide galactosyltransferase; CGlcT, UDPglucose:ceramide glucosyltransferase; DAG, 1,2-diacylglycerol; DG, diglyceride (AAG + DAG); dh, dihydro; ER, endoplasmic reticulum; FAB, fast atom bombardment; FCS, fetal calf serum; Ga_2Cer, Galα1-4Galβ1-1Cer; GalCer, galactosylceramide, Galβ1-1Cer; GlcCer, glucosylceramide, Glcβ1-1Cer; G_{M3}, NeuAcα2-3Galβ1-4Glcβ1-1Cer; HFA, hydroxy fatty acyl; LacCer, lactosylceramide, Galβ1-4Glcβ1-1Cer; MEM, Eagle's minimum essential medium; NBD, 7-nitrobenz-2-oxa-1,3-diazole; NFA, non-hydroxy fatty acyl; SGalCer, sulphatide; SM, sphingomyelin.

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lino-1-propanol was from Matreya (Pleasant Gap, PA, U.S.A.), and pronase E (3640 units/mg) from Merck (Darmstadt, Germany). Geneticin (G418), Lipofectin reagent and cell culture media were from Gibco (Paisley, U.K.). Cell-culture plastics were from Costar (Cambridge, MA, U.S.A.). UDP-[4,5-^{3}H-(*N*)]galactose (1600 MBq/μmol) and D-erythro-[3-^{3}H]sphingosine (800 MBq/μmol) were from NEN DuPont (Boston, MA, U.S.A.), and D-[1-^{3}H]galactose (255 MBq/μmol) and L-[3-^{3}H]serine (481 MBq/μmol) from Amersham (Amersham, U.K.). The radiolabelled short-chain ceramides Cer{[^{14}C]C_6} (0.3 MBq/μmol; {C_6}, hexanoyl), [^{3}H]Cer{C_6} and [^{3}H]Cer-{C_6OH} (800 MBq/μmol; {C_6OH}, D-2-hydroxy hexanoyl) and [^{3}H]dihydro (dh)-Cer{C_6OH} (200 MBq/μmol) were synthesized as described [25]. The D-stereoisomer of the {C_6OH} ceramides was used. Cer{C_6-NBD} (NBD, 7-nitrobenz-2-oxa-1,3-diazole) was purchased from Molecular Probes (Eugene, OR, U.S.A.). DAG{C_6-NBD} was synthesized from PC{2-C_6-NBD} (PC, phosphatidylcholine) (Avanti Polar Lipids, Pelham, AL, U.S.A.) using phospholipase C [26].

Cells

Wild-type Chinese hamster ovary cells (CHO-WT), obtained from P. Rottier (Utrecht University, Utrecht, The Netherlands), were cultured in Eagle's minimum essential medium (MEM)-alpha (with nucleotides) with 10 % fetal calf serum (FCS). CHO-CGT cells were cultured in MEM-alpha, supplemented with 10 % (v/v) FCS, 10 mM Hepes and 500 μg/ml G418. MDCK II cells were grown as monolayers in MEM with 10 mM Hepes and 5 % (v/v) FCS. D6P2T cells [27], a gift from S. E. Pfeiffer (University of Connecticut Health Center, Farmington, CT, U.S.A.), were cultured in Dulbecco's modified Eagle's medium (high glucose) with 5 % FCS. Oligodendrocytes were isolated from rat pups and cultured [28].

Construction of CGalT/pcDNA3

The CGalT insert was digested from the pCMX vector (obtained from N. Schaeren-Wiemers, Friedrich Miescher Institute, Basel, Switzerland [21,23]) with the restriction enzymes *Hin*dIII and *Xba*I, purified from agarose gel using GeneCleanII (BIO 101 Inc., La Jolla, CA, U.S.A.), and ligated into the vector pcDNA3 (Invitrogen, San Diego, CA, U.S.A.). Plasmids were grown in *Escherichia coli* strain JA 221, and identified by *in vitro* translation: cDNA was translated for 90 min at 30 °C in a reticulocyte lysate system (Promega, Madison, WI, U.S.A.) using [^{35}S]-methionine (ICN, Costa Mesa, CA, U.S.A.). The protein products were analysed by SDS/10 %-PAGE and a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Transfection of CHO cells

CHO cells (60 % confluent) on two 6-cm dishes were washed and transfected with 20 μg of DNA and 20 μl of Lipofectin reagent (1 mg/ml) in 2 ml of serum-free medium, according to the manufacturer's instructions. After 18 h in the CO_2 incubator the medium was replaced by MEM-alpha with 10 % FCS. Two days later the cells were passaged and cultured in MEM-alpha, supplemented with 10 % FCS, 10 mM Hepes and 500 μg/ml G418 to select for stably transfected cells. After 9 days in the presence of G418, cells were passaged and cloned by limiting dilution on 15-cm dishes. Colonies were picked up using cloning cylinders.

Incubation of intact cells with ceramides

BSA-complexes of short-chain ceramides were prepared by injection of 10 μl of an ethanolic solution of the ceramide into 1 ml of Hanks' balanced salt solution, without bicarbonate/10 mM Hepes, pH 7.4, containing 1 % BSA. CHO, D6P2T and MDCK II cells on 3-cm dishes, or oligodendrocytes (4 days in culture) on 6-cm dishes, were washed with PBS and incubated with 50 nM [^{3}H]dh-Cer{C_6OH} as BSA-complexes in 1 ml (3-cm dish) or 4 ml (6-cm dish) at 37 °C. Cell density and incubation time (1 or 2 h) were variable between experiments but did not influence the relative amounts of products synthesized. Lipids were extracted [29] from media and cells after scraping, and separated on borate-impregnated TLC plates in two dimensions: (1) chloroform/methanol/25 % NH_4OH/water (65:35:4:4, by vol.); and (2) chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol.). Radiolabelled spots were visualized by fluorography, identified, and quantified as before [25]. Retention factors in the first and second direction are: sphingomyelin (SM), 0.32 and 0.14; GalCer, 0.34 and 0.37; GlcCer, 0.51 and 0.40.

Galactosyltransferase activity

CGalT activity was measured essentially as before [23]. Cells on a 9-cm dish were rinsed with ice-cold PBS, scraped in 400 μl of buffer A (120 mM potassium glutamate/15 mM KCl/5 mM NaCl/0.8 mM $CaCl_2$/2 mM $MgCl_2$/2 mM $MnCl_2$/1.6 mM EGTA/20 mM Hepes/KOH, pH 7.2), and homogenized with ten strokes of a tight-fitting Dounce homogenizer. Fractions (100 μl) were incubated for 1 h at 37 °C in the presence of 1 mM UDP-Gal with BSA-complexes of [^{3}H]labelled short-chain ceramides (35 nM) with or without 8 mM CHAPS, or diglyceride (20 μM DAG{C_6-NBD}) without detergent, in a final volume of 150 μl. Lipids were extracted and separated by two-dimensional TLC as above. The synthesis of all products mentioned was linear with time during 1 h at 37 °C. GalCer synthesis was linear with ceramide concentration up to substrate concentrations that were 100-fold higher (3.5 μM) than those used here.

For galactosyltransferase assays with long-chain ceramides, 60 μg of HFA- or NFA-ceramide from bovine brain (in chloroform/methanol, 2:1, v/v) was mixed with 735 μg of CHAPS and evaporated to dryness. The mixture was suspended in 50 μl of buffer A with 1 mM UDP-Gal and 18.5 kBq of UDP-[^{3}H]galactose, and added to 100 μl of cell homogenate. After 1 h at 37 °C, the lipids were extracted, separated on TLC in chloroform/methanol/25 % NH_4OH (65:25:4, by vol.) and visualized and quantified as above. Detergent is required to solubilize long-chain ceramide precursors.

Immunoblotting of lipids on thin-layer chromogratograms

Lipids were immunoblotted on thin-layer chromatograms by a method modified from Magnani et al. [30]. After extraction [29], cellular lipids were separated by TLC in chloroform/methanol/25 % NH_4OH (65:25:4, by vol.) on aluminium-backed silica sheets (Merck, Darmstadt, Germany) that had been pre-run in the same solvent. The sheets were then dried and soaked for 1 min in hexane containing 0.4 % poly-isobutylmethacrylate (Aldrich, Brussels, Belgium), diluted from a 2.5 % stock in chloroform. After drying under hot air for 15 min, the sheets were blocked in Blotto [5 % milk powder (w/v) in 50 mM Tris/HCl, pH 7.8 containing 2 mM $CaCl_2$] for 30 min. Subsequently, one of the anti-GalCer monoclonal antibodies O1 ([31]; IgM, a gift from J. Trotter, University of Heidelberg,

Heidelberg, Germany) or Ranscht ([32]; IgG3, a gift from D. Koppenaal, Nijmegen, The Netherlands) was applied to the sheet in a small plastic bag which was incubated on a rocker for 1 h at room temperature. Sheets were rinsed four times with Blotto, and incubated with 0.1 μg/ml ^{125}I-Protein A, which had been ^{125}I-labelled to 2×10^6 c.p.m./μg using Iodobeads (Pierce, Rockford, IL, U.S.A.) and separated from free $^{125}I^-$ on a Sephadex G-25 column. After four washes with Blotto and two washes with ice-cold PBS, the sheets were air-dried. Radioactivity was detected with a PhosphorImager.

Labelling cellular lipids with [^{3}H]galactose or [^{3}H]serine

Cells (80 % confluent) on 6-cm dishes were incubated with 2 ml of D-[1-^{3}H]galactose (185 kBq/ml) or L-[3-^{3}H]serine (74 kBq/ml) in fresh culture medium for 20 h in a CO_2 incubator. Lipids were extracted and analysed by TLC.

Alkaline hydrolysis and galactosidase treatment

For mild alkaline hydrolysis [33] and galactosidase treatment, silica spots were scraped from the plates and lipids were extracted from the silica in chloroform/methanol/20 mM acetic acid (1:2.2:1, by vol.). For galactosidase treatment, lipids were mixed with 250 μg of sodium taurocholate in chloroform/methanol (2:1, v/v), dried, and redissolved in 400 μl of 84 mM Na_2HPO_4/58 mM citric acid/2 mM EDTA, pH 4.2. β-Galactosidase (0.15 unit in 30 μl) was added and the mixture was incubated for 24 h at 37 °C. Lipids were extracted and identified as above.

Mass spectrometry

Fast atom bombardment (FAB) mass spectra were obtained using MS1 of a JEOL JMS-SX/SX 102A tandem mass spectrometer operated at + or −10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA, using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett-Packard HP 9000 data system running JEOL COMPLEMENT software. Collision-induced dissociation (CID) mass spectra were recorded using the same machine, with helium as the collision gas in the third field-free region collision cell, at a pressure sufficient to reduce the parent ion to one-third of its original intensity.

Permethylation was carried out as previously described [34] and the permethylated products were recovered by extraction into dichloromethane. Samples for mass-spectrometric analysis were dissolved in 10 μl of methanol, and 1.5 μl aliquots of sample solution were loaded into a matrix of monothioglycerol (negative-ion mode) or *m*-nitrobenzyl alcohol (positive-ion mode, permethyl derivatives).

Isolation of membranes and subfractionation on sucrose gradients

Crude ER/Golgi membranes were prepared [35] and cell subfractionation was carried out as described [36]. Cells were washed in PBS, scraped from a 9-cm dish into 750 μl of 15 mM KCl/1.5 mM $MgCl_2$/10 mM Hepes/NaOH, pH 7.2, and homogenized using a Dounce homogenizer. After centrifugation (10 min at 375 g_{max} and 4 °C), the postnuclear supernatant was layered on top of a linear sucrose gradient (0.7–1.5 M sucrose in 1 mM EDTA/10 mM Hepes/NaOH, pH 7.2) and centrifuged in a SW41 rotor for 3 h at 180000 g_{av} and 4 °C. Fractions (1 ml) were collected and stored at −20 °C. Sphingolipid synthesis was assayed in each gradient fraction by incubating 250 μl for 1 h at 37 °C with 100 μl of an incubation mixture in buffer A (see above) containing 109 nM [^{3}H]dh-Cer{C_6OH} and 1 mM UDP-Gal for HFA-GalCer synthesis, 20 μM Cer{[^{14}C]C_6} and 1 mM UDP-Gal for NFA-GalCer and SM synthesis, and 17 μM Cer{C_6-NBD} and 1 mM UDP-Glc for GlcCer synthesis. SM synthesis profiles were identical for all three ceramides used and were independent of the presence of UDP-Glc or UDP-Gal. Lipids were extracted and analysed by two-dimensional TLC as above. Fluorescent NBD-lipids were quantified as described previously [26].

RESULTS

CGalT cDNA encodes CGalT

The CGalT cDNA was subcloned into the *Hin*dIII and *Xba*I sites of the expression vector pcDNA3. The protein product of CGalT/pcDNA3 was identified by *in vitro* translation in a reticulocyte lysate system. A protein with an apparent molecular mass of about 60 kDa was synthesized (not shown), as expected for CGalT (58 kDa [19]). A clear shift to lower mobility on the

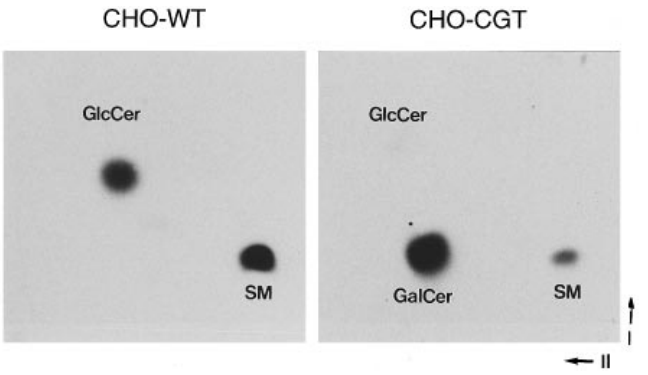

Figure 1 Transfection of CHO cells with ceramide galactosyltransferase generates the capacity to synthesize GalCer from exogenous HFA-ceramide

CHO-WT cells or CHO-CGT cells were incubated with the short-chain HFA-ceramide [^{3}H]dh-Cer{C_6-OH}, 50 nM for 1 h at 37 °C, after which the lipids were extracted and separated by two-dimensional TLC on borate-impregnated plates. The autoradiographs show the relevant part of the TLC plates (for retention factors see the Experimental section). I, First dimension; II, second dimension.

Table 1 Sphingolipid synthesis from short-chain HFA-ceramide in intact cells

Cells on dishes were incubated for 1–2 h at 37 °C with the short-chain HFA-ceramide [^{3}H]dh-Cer{C_6OH}. Lipids are presented as a percentage of total lipid products formed (n = 4–6). (—), no visible spot on film; Ga_2Cer and SGalCer were identified as described elsewhere [25].

	Products (% of total)				
Cell type	SM	GlcCer	GalCer	Ga_2Cer	SGalCer
CHO-WT	45	55	—	—	—
CHO-CGT	8	1	91	—	—
MDCK II	7	10	58	13	12
D6P2T	11	4	37	—	48
Oligodendrocytes	11	2	71	—	15

Table 2 Lipids formed from short-chain HFA- and NFA-ceramides in cell homogenates

Homogenates from equal numbers of cells were incubated for 1 h at 37 °C with 35 nM [^{3}H]Cer{C_6OH} (HFA) or [^{3}H]Cer{C_6} (NFA) in buffer A, 1 mM UDP-Gal and 8 mM CHAPS. The amount of GlcCer synthesized is not presented because, as no UDP-Glc had been added, the concentration of UDP-Glc was undefined. The results of one typical experiment (out of three) are shown. Data represent Bq of [^{3}H] minus background (< 2 Bq). Detergent was added to exclude the possibility that importing UDP-Gal into the GalCer-synthesizing compartment limits GalCer synthesis. However, this turns out not to be a problem as GalCer synthesis from short-chain ceramide in the absence of CHAPS is higher than in its presence (results not shown).

	[^{3}H]Ceramide incorporation (Bq)				
	SM		GalCer		
Cell type	HFA	NFA	HFA	NFA	HFA/NFA
CHO-WT	112	201	28	16	2
CHO-CGT	222	457	400	17	24
MDCK II	137	269	335	12	28

gel was observed after *in vitro* translation in the presence of microsomes, in agreement with the reported glycosylation of the protein (to 64 kDa [19]). CHO-WT cells were transfected and CGalT/pcDNA3-positive cells were selected by growing in the presence of G418 for 1 week. GalCer synthesis in homogenates was assayed in the presence of UDP-Gal using a ceramide carrying a short hydroxy fatty acid, [^{3}H]dh-Cer{C_6OH}, as substrate. GalCer was the major [^{3}H]product in the surviving cell population. After cloning the cells, clone CHO-CGT retained its CGalT expression through more than 15 passages.

In CHO-WT cells the short-chain HFA-ceramide [^{3}H]dh-Cer{C_6OH} is converted into SM and GlcCer; no GalCer is observed (Figure 1, Table 1). In transfected cells (CHO-CGT), GalCer accounts for more than 90% of the HFA-ceramide products. CHO-CGT does not synthesize substituted galactosphingolipids, such as SGalCer and Ga_2Cer (Galα1-4Galβ1-1Cer). In this, they differ from the CGalT-positive cell types MDCK II, D6P2T and oligodendrocytes, where GalCer and its derivatives comprise 85% of total HFA-ceramide products. When compared with SM synthesis, the expression level of CGalT in CHO-CGT is similar to that observed in the other cell types (Tables 1 and 2).

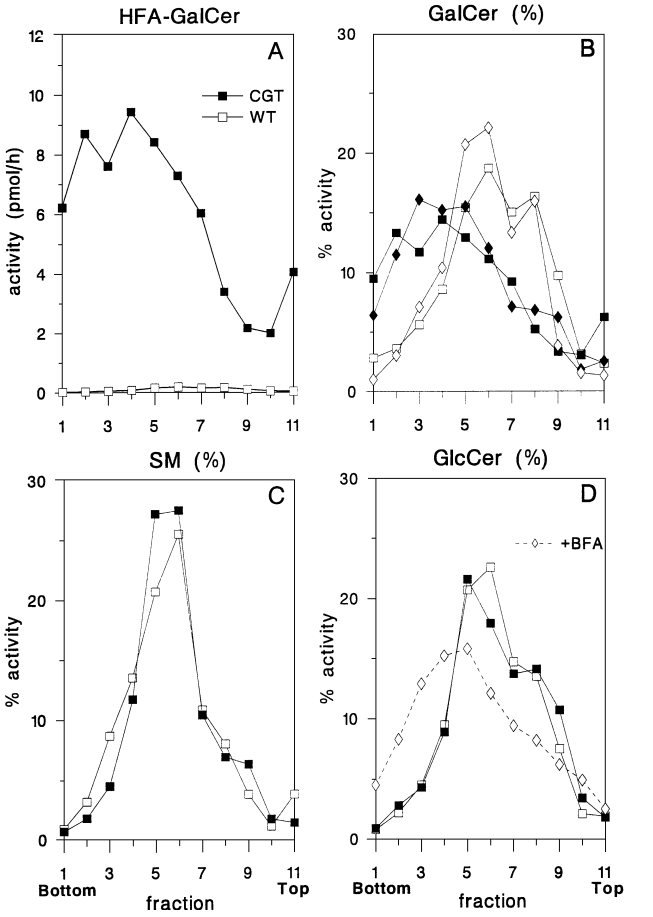

Figure 2 Localization of CGalT, CGlcT and SM synthase activities on a sucrose gradient

A post-nuclear supernatant of transfected (CGT, closed symbols) or wild-type (WT, open symbols) CHO cells was fractionated on a linear sucrose gradient. Fractions, collected from the bottom (1.5 M, fraction 1) to the top (0.7 M, fraction 11), were assayed for synthesis of HFA-GalCer (**A**, **B**, ■, □), NFA-GalCer (**B**, ◆, ◇), SM (**C**), and GlcCer (**D**), as described in the Experimental section. Enzyme activities in each fraction are expressed as pmol of HFA-GalCer formed per h (**A**) or as a percentage of the total enzyme activity in the gradient (**B**, **C** and **D**). ◇, in (**D**), represents GlcCer synthesis after pretreatment of the cells for 30 min at 37 °C with 1 μg/ml brefeldin A (BFA) to locate the ER on the gradient. Total synthesis in the gradients of CHO-CGT/CHO-WT, expressed as a percentage of substrate added: (**B**) HFA-GalCer, 15.0/0.2; NFA-GalCer, 0.8/0.1; (**C**) SM, 1.5/0.5; and (**D**) GlcCer, 3.4/1.5; + BFA, 1.3.

CGalT prefers HFA- over NFA-ceramides as substrates for galactosylation *in vitro*

In contrast to the efficient synthesis of GalCer from HFA-ceramide, only small amounts of GalCer are synthesized when CHO-CGT cells are incubated with short-chain NFA-ceramide (results not shown). To study the substrate preference of CGalT in more detail, short-chain HFA- and NFA-ceramides were tested in an enzyme assay in the presence of detergent (Table 2). In CHO-CGT cells, 25 times more GalCer is synthesized from the HFA- than the NFA-ceramide, in line with data obtained from the partially purified enzyme (see the Introduction). In an analogous experiment (results not shown), when a CHO-CGT homogenate was incubated with long-chain HFA- or NFA-ceramides in the presence of UDP-[^{3}H]galactose, 21 times more HFA- than NFA-GalCer was synthesized. This is very similar to the results from the rat Schwann cell line D6P2T, where a ratio of HFA:NFA-GalCer of 23 was obtained (results not shown).

CGalT co-fractionates with the ER in CHO-CGT cells

The C-terminal amino acids of the CGalT, -KKVK [19], contain the consensus ER retrieval signal -KKXX [37]. Together with the high mannose content of the enzyme and the homology with the ER glucuronyltransferases [19], this strongly suggests that the enzyme is located in the lumen of the ER and not in the Golgi. Indeed, after subfractionation of CHO-CGT cells on sucrose gradients, galactosylation of both HFA- and NFA-ceramides is detected (Figures 2A and 2B, closed symbols) at a higher density than the Golgi enzymes SM synthase and UDPglucose:ceramide glucosyltransferase (CGlcT; Figures 2C and 2D, closed symbols). The CGalT activity co-fractionates with ER, as defined by the location of the CGlcT after brefeldin A treatment (Figure 2D, open diamonds). Brefeldin A has been shown to redistribute Golgi enzymes, including CGlcT, from the Golgi to the ER [35,36].

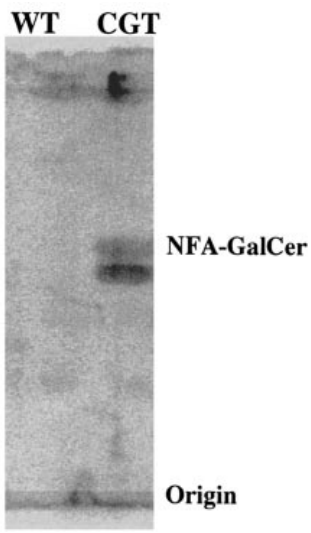

Figure 3 Immunolabelling of galactolipids on TLC

Lipids, extracted from a 3-cm dish of wild-type CHO-WT cells (WT) or CGalT-transfected cells (CGT) were separated by TLC in chloroform/methanol/25% NH_4OH (65:25:4, by vol.). The TLC plate was incubated with the monoclonal antibody O1, followed by an incubation with ^{125}I-Protein A and imaging by means of the PhosphorImager. The signal at the top of the CGT lane is an artefact.

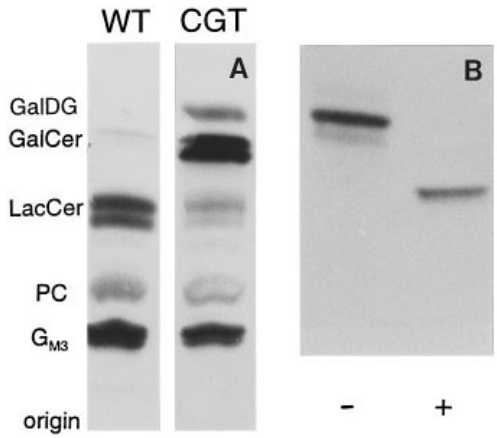

Figure 4 Analysis of [^{3}H]galactose-labelled lipids

Cells were incubated overnight with [^{3}H]galactose. (**A**) Lipids from CHO-WT and CHO-CGT were extracted and separated on TLC in chloroform/methanol/0.22% $CaCl_2$ (60:35:8, by vol.). The autoradiograph shows the area between the origin and the front. ^{3}H-incorporation: 2.7 kBq (WT) and 3.3 kBq (CGT). In CHO-WT, LacCer and G_{M3} contain 32 ± 4% and 53 ± 1% of the total radiolabel, while in CHO-CGT 62 ± 11% of the radiolabel is found in GalCer plus GalDG, 9 ± 4% in LacCer and 21 ± 6% in G_{M3} ($n = 3$); PC, phosphatidylcholine. (**B**) Deacylation of GalDG (−) by mild alkaline hydrolysis yields a lysolipid product (+), identifying the major part of GalDG as galactosylalkylacylglycerol (GalAAG). GalDG had a higher turnover than GalCer: labelling of CHO-CGT for 16, 24 or 48 h with [^{3}H]galactose (185 kBq/0.7 nmol per 3-cm dish) yielded equal amounts of [^{3}H]GalCer, while GalDG after 48 h was only about 10% of the GalDG after 16 h.

In contrast to the absence of GalCer synthesis in intact CHO-WT cells (Figure 1; Table 1), a small amount of GalCer is synthesized during incubation of a cell homogenate with short-chain ceramides and UDP-Gal (Table 2). However, when this minor activity in CHO-WT cells (Figure 2A, open symbols) is plotted as a percentage of total CGalT activity in these cells (Figure 2B, open symbols; HFA, squares; NFA, diamonds), it co-localizes with the CGlcT activity (Figure 2D). Various other pieces of evidence support the notion that the GalCer in wild-type CHO homogenates is synthesized by CGlcT: in incubations for 90 min at 37 °C, (1) addition of 1 mM UDP-Glc almost completely blocks GalCer synthesis in CHO cell homogenates with a minor effect on GalCer synthesis in CHO-CGT; (2) 50 μM of the specific CGlcT inhibitor PDMP [38] blocks GalCer synthesis in wild-type CHO cells, with far less effect on GalCer synthesis in CHO-CGT; and (3) treatment of isolated membranes with Pronase E [2 h, 37 °C at a Pronase-to-sample protein ratio of 1:1 (w/w)], which inactivates CGlcT [39], completely abolishes CGalT activity in CHO-WT cells, but not in CHO-CGT.

In order to assess whether a proportion of the CGalT is present on the surface of CHO-CGT, cells were incubated with 30 nM [^{3}H]Cer{C_6-OH} at 10 °C for 3 h in the presence of UDP-Gal, UDP-Glc and BSA. New lipid products synthesized on the cell surface are selectively extracted into the medium by the BSA [26]; 22% of the newly synthesized SM, but no GlcCer and GalCer, was detected in the medium of CHO-CGT cells. CHO-CGT cells do deliver newly synthesized GalCer to the cell surface at 37 °C, since when intact transfected cells are incubated with 40 nM [^{3}H]dh-Cer{C_6OH} or 15 μM Cer{[^{14}C]C_6} for 90 min at 37 °C in the presence of 1% BSA, approx. 75% of the radiolabelled SM, 25% of the GlcCer and 20% of the GalCer are recovered in the BSA-medium, reflecting their arrival at the cell surface.

CHO cells transfected with CGalT synthesize endogenous GalCer and galactosyldiglyceride

Iodine staining of the lipids isolated from CHO-CGT cells after TLC in alkaline solvent reveals two bands not observed in wild-type cells. Both are recognized by the monoclonal anti-GalCer antibodies Ranscht (results not shown) and O1 (Figure 3). The upper band co-migrates with NFA-GalCer from bovine brain (which contains mainly long-chain fatty acids, $C_{24:0}$ and $C_{24:1}$), while the lower band runs somewhat faster than HFA-GalCer from bovine brain. CHO-WT cells are negative (Figure 3), as is a CGalT-transfected clone that has lost CGalT activity, but remained G418 resistant (results not shown).

When CHO-WT cells are labelled overnight with [^{3}H]galactose, two of the major labelled glycolipid bands co-localize with dihexosylceramides (Figure 4). Their sensitivity to β-galactosidase (not shown) identifies these lipids as various species of LacCer (Galβ1-4Glcβ1-1Cer). The [^{3}H]galactosylated lipid closer to the origin co-migrates with G_{M3}, a major glycolipid in CHO cells [24,33]. After transfection, the level of LacCer decreases dramatically and three new lipid bands appear in transfected cells (Figures 4 and 5). The two spots with the lower R_F values are identified as GalCer, as they (1) co-localize with GalCer standards in both TLC systems, (2) are labelled in the presence of [^{3}H]serine, suggesting a sphingoid backbone (Figure 5D), and (3) are insensitive to alkaline hydrolysis (results not shown).

The faster migrating spot of the [^{3}H]galactosylated lipids from CHO-CGT co-localizes with GalDAG in both solvent systems (Figures 4 and 5). Indeed, it is degraded by β-galactosidase (not shown) and alkaline hydrolysis. Because alkaline hydrolysis yields a band at the location of a lysolipid (Figure 4B), a proportion of the species in the GalDG spot must correspond to a galactosylalkylacylglycerol (GalAAG). Independent evidence that CGalT uses diglycerides as a substrate for galactosylation was obtained from incubations of cell homogenates with a short-chain fluorescent DAG. CHO-WT cell homogenates use DAG{C_6-NBD} for the synthesis of several glycerolipids (Figure

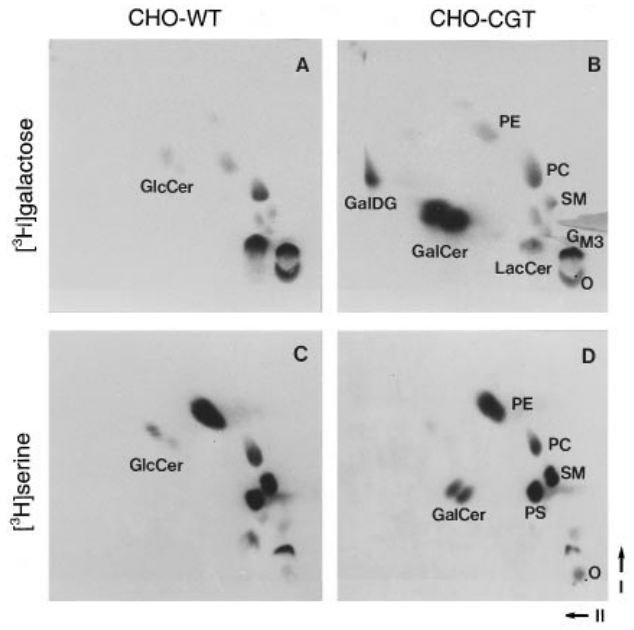

Figure 5 Lipid chromatogram of [^{3}H]galactose-and [^{3}H]serine-labelled cells

CHO-WT (**A**, **C**) or CHO-CGT cells (**B**, **D**) were labelled overnight with [^{3}H]galactose (**A**, **B**) or [^{3}H]serine (**C**, **D**). Lipids were separated by two-dimensional TLC. In CHO-CGT (**B**, **D**) labelled GalCer and GalDG co-localize with GalCer and GalDAG standards. GalCer and GalDG are absent from wild-type cells, which in contrast display more intense labelling of GlcCer, LacCer and G_{M3} (**A**, **C**). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; O, origin. I, First dimension, II, second dimension.

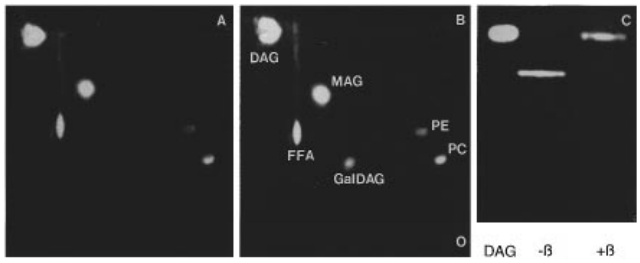

Figure 6 Analysis of fluorescent lipids formed from DAG{C_6-NBD}

Homogenates of CHO-WT (**A**) or CHO-CGT (**B**) cells were incubated with DAG{C_6-NBD} for 2 h at 37 °C. The extracted lipids were separated by two-dimensional TLC. Compared with wild-type cells, a new spot was observed in the CHO-CGT homogenates. (**C**) After treatment of the new lipid with β-galactosidase ($+\beta$) the breakdown product co-migrates with DAG{C_6-NBD} on TLC in chloroform/methanol/0.22% $CaCl_2$ (60:35:8, by vol.), confirming the lipid to be GalDAG{C_6-NBD}. DAG, diacylglycerol (and, in A and B, triacylglycerol); FFA, free fatty acids; MAG, most likely monoacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; O, origin.

6A). In transfected cells a new lipid appears (Figure 6B) that is identified as GalDAG{C_6-NBD}, following β-galactosidase treatment (Figure 6C).

Endogenous GalCer in transfected cells exclusively contains non-hydroxy fatty acids

Iodine-staining, immunoblotting and labelling with [^{3}H]serine and [^{3}H]galactose demonstrated the presence of two endogenous GalCer bands in transfected cells. In a preliminary experiment, the area of the two-dimensional TLC plate containing the two spots was extracted and the recovered glycolipid mixture was analysed using FAB-MS in the negative-ion mode. The spectrum obtained (not shown) contains M-H$^-$ pseudomolecular ions indicating the presence of monohexosylsphingolipids bearing a mixture of different non-hydroxy fatty acids. The major ion (m/z 698) indicates the presence of a component with a $C_{16:0}$ fatty acyl chain, while much less intense ions correspond to the presence of $C_{16:1}$ (m/z 696), $C_{22:0}$ (m/z 782), $C_{24:2}$ (m/z 806), $C_{24:1}$ (m/z 808) and $C_{24:0}$ (m/z 810) fatty acyl-containing species as minor components of the mixture. No ions can be identified to indicate the presence of even very minor quantities of hydroxy fatty acid-containing glycosphingolipids in the mixture.

An attempt was made to recover the glycosphingolipids from the two GalCer spots separately, following TLC fractionation of the mixture and iodine staining of the chromatogram to reveal their location. The spots were marked and allowed to decolorize when the silica was removed from the TLC plates and extracted. The two extracts were examined using negative-ion mode FAB-MS. The spectrum of spot A (closest to the origin) contains a very intense M-H$^-$ pseudomolecular ion for the $C_{16:0}$ fatty acyl-containing species (at m/z 698), together with a very minor amount of the $C_{22:0}$-containing species (m/z 782). In contrast, the spectrum obtained from spot B indicates a heterogeneous mixture. The most intense ion is again observed at m/z 698, corresponding to an M-H$^-$ pseudomolecular ion for the $C_{16:0}$-containing species, although this ion is much less intense than in the spectrum obtained from spot A. In addition, ions of roughly comparable intensities are observed at m/z 782, 806, 808 and 810 for analogous species bearing $C_{22:0}$, $C_{24:2}$, $C_{24:1}$ and $C_{24:0}$ fatty acyl chains. Neither fraction gives rise to any ions that could indicate the presence of hydroxy fatty acid-containing species in the extracts of the two spots. Since the sum of the different structures identified following separation of the glycosphingolipid mixture and iodine staining is similar to that identified without separation or iodine staining, we conclude that the separation does not result in a failure to recover any of the components, and that iodine staining does not result in any significant destruction or chemical modification.

In order to obtain confirmation of the fatty acid and long-chain base structures present in the glycosphingolipids in the two spots, the extracts of both were subjected to permethylation, followed by positive-ion FAB-MS analysis. The pseudomolecular ion regions of the resulting spectra of the two preparations contain intense ions corresponding to $[M+Na]^+$, weak $[M+H]^+$ ions and intense ions for $[M+H\text{-MeOH}]^+$ for the species identified in the negative-ion spectra of the underivatized samples. In addition, an intense fragment ion, designated the Z_o ion [40], is observed for each component. This ion arises by cleavage between the glycosidic oxygen and the sphingolipid portion of the molecule (see Figure 7). Further information on the structure of the sphingolipid portion can be obtained on fragmentation of this ion by colliding it with a collision gas and recording the resulting CID tandem mass spectrum [40]. The Z_o ion (at m/z 548) from the major $C_{16:0}$ fatty acyl-containing component in the spectrum of permethylated spot A was analysed in this way (Figure 7). Very similar data were obtained from the analogous experiment carried out on the same component in spot B. The fragmentation proceeds as described [40] and is summarized in the fragmentation scheme (Figure 7). The fragment ions confirm the presence of sphingosine ($C_{18:1}$) as the long-chain base, and demonstrate clearly that it bears a $C_{16:0}$ fatty acyl chain. Similar experiments were carried out on the Z_o ions arising from the $C_{22:0}$ (m/z 632) and $C_{24:0}$ (m/z 660) fatty acyl-bearing species. The spectra obtained (results not shown) allow

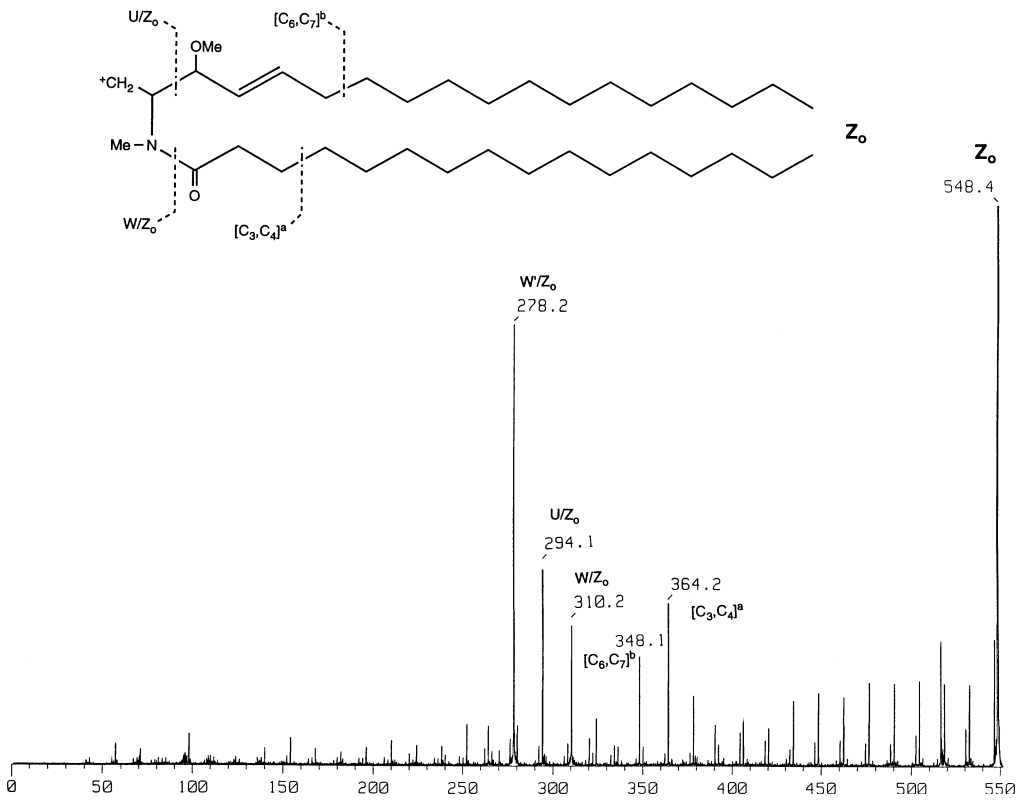

Figure 7 CID mass spectrometric analysis of the major GalCer produced by transfected cells

CID tandem mass spectrum obtained from the Z_0 ion of the permethylated $C_{16:0}$ fatty acyl-containing GalCer following two-dimensional TLC separation. Structurally diagnostic fragment ions are indicated and the fragmentations giving rise to them are shown. The ion designations follow the proposals of Perreault and Costello [40] in which W′ indicates a W ion from which the elements of methanol have been eliminated, and [a] and [b] denote fragmentations in the fatty acyl chain and long- chain base respectively. Methyl groups introduced chemically during permethylation are indicated by Me.

the presence of sphingosine and the expected fatty acyl chains to be confirmed. CID mass spectrometric analysis of the Z_0 ions of the remaining minor components is not possible, due to the very low intensities of these ions.

General properties of transfected and CHO-WT cells

To detect whether transfection with the CGalT influences the physiology of the cells, we compared lipid composition, growth rate and morphology of transfected cells and wild-type cells. The phospholipid composition of the CHO cells is not significantly changed after transfection. Quantification of phospholipids separated on TLC and expressed as a percentage of total phospholipids [9] yields the following numbers for transfected cells (compared with those for wild-type cells): phosphatidylinositol, 5% (8%); phosphatidylserine, 7% (7%); SM, 12% (13%); phosphatidylcholine, 55% (52%); and phosphatidylethanolamine, 20% (20%). Transfected and CHO-WT cells show similar growth curves: after a 1:20 surface dilution from a confluent dish, confluency was reached in 3 days. No differences were detected in the size and shape of the cells using light microscopy (results not shown).

DISCUSSION

The present study extends our previous findings on the substrate specificity of cloned CGalT transiently transfected into COS7 cells [23]. Because wild-type COS7 cells have a low basal CGalT activity, we have now used CHO cells that synthesize no GalCer when intact. Transfection and cloning resulted in a stable CGalT-expressing cell line, CHO-CGT.

After transfection, GalCer is a major lipid in CHO-CGT, appearing as two prominent iodine-staining spots on TLC that are not present in lipid extracts of CHO-WT cells. The spots co-localize with GalCer standards in different solvent systems, bind monoclonal antibodies directed against GalCer (Figure 3), and are labelled with [^{3}H]galactose. [^{3}H]Galactose-labelled LacCer synthesized in CHO-WT cells is almost absent from CHO-CGT (Figures 4 and 5). Since G_{M3} is still formed, this reduction is not due to a block in the biosynthetic pathway but to competition for UDP-Gal or ceramide. Although the glycolipid pattern changes dramatically, CGalT expression does not alter the phospholipid composition, growth rate or morphology of the CHO cells. In the transfected cells CGalT seems restricted to the ER (Figure 2), as it is in oligodendrocytes [19].

CGalT substrate-specificity and products *in vivo*

The cloned gene product CGalT has a preference for HFA-ceramides, as has already been described for whole tissue homogenates [14,15] and for purified enzyme [41]. In the present study, we have compared short- and long-chain ceramides in order to investigate the substrate preference of CGalT transfected into CHO cells. In all situations, the enzyme preferentially transfers galactose to HFA-ceramides. When HFA- and NFA-ceramides are used, the ratio HFA:NFA-GalCer is 24 for short-

chain ceramides (Table 2) and 21 for long-chain ceramides in CHO-CGT.

Because of the pronounced preference of CGalT for HFA-ceramides, the two endogenous GalCer spots observed in CHO-CGT lipid extracts (Figures 3–5) were expected to contain a significant proportion of hydroxy fatty acids. Surprisingly, mass spectrometric analysis reveals exclusively non-hydroxy fatty acids in both spots. *In vivo*, the cells with the highest production of GalCer are the myelin-forming oligodendrocytes. They contain roughly equal levels of HFA-and NFA-GalCer, and so far it is unclear whether these species are synthesized by one or by two different enzymes [13]. Our present data, showing that HFA-ceramide galactosyltransferase or HFA-CGalT [14–18] produces exclusively NFA-GalCer under certain conditions, demonstrate that the CGalT can, by itself, account for all GalCer in myelin. The observation that the HFA:NFA ratio of GalCer produced from long-chain ceramides by the rat Schwann cell-line D6P2T is identical with that in CHO cells transfected with the CGalT from rat brain (23 versus 21), suggests that one and the same CGalT is alone responsible for the GalCer synthesis in myelin, and that the relative amounts of HFA-GalCer and NFA-GalCer synthesized depend on the availability of HFA- and NFA-ceramides in these cells.

Oligodendrocytes have an active α-hydroxylation machinery [42]. Interestingly, the α-hydroxylation of fatty acids seems to be closely linked to the synthesis of HFA-ceramide and HFA-GalCer, as free hydroxy fatty acids and HFA-ceramide have not been detected in brain [42,43] or kidney [11]; the latter apparently also displays α-hydroxylation as its GalCer contains mainly HFA [11,12]. Although the kidney-derived MDCK cells contain only low levels of HFA-GalCer [10], their CGalT displays the same preference for HFA-ceramide as the myelin enzyme (Table 2) and is localized in the ER [35], suggesting that epithelial cells and myelin express the same CGalT.

CGalT synthesizes galactosyldiglycerides

In addition to GalCer, CGalT-transfected CHO cells synthesize galactosyldiglycerides (GalDG: GalDAG plus GalAAG), detected using [^{3}H]galactose labelling (Figures 4 and 5). This was confirmed by an *in vitro* galactosyltransferase assay using short-chain DAG (Figure 6). The fact that GalDG in transfected cells is not recognized by monoclonal antibodies against GalCer (Figure 1), which recognize GalDG [44,45], is most probably due to the much lower level of GalDG than of GalCer in these cells; this is also observed on iodine staining (results not shown) and [^{3}H]galactose labelling (Figures 4 and 5). Co-expression of GalCer and GalDG is a familiar theme in myelination [46–48]. Galactoglycerolipids (GalDAG, GalAAG and their sulphated derivatives) comprise but a minor fraction of total galactolipid in myelinating tissue (less than 10 % [3]). These galactoglycerolipids are actively synthesized when myelination starts but, as we have observed, their metabolic half-life is much shorter than that of GalCer and SGalCer. As a consequence, their levels in the rat central nervous system rise initially and then decline during post-natal development [49]. Further evidence for co-expression of GalCer and GalDG comes from studies of the myelin-deficient mutant Jimpy mouse, in which levels of GalCer and GalDG are both reduced [46]. The ability of CGalT to catalyse the synthesis of GalDG, not previously reported, suggests that the ceramide-binding site of CGalT is not very specific. Interestingly, SM synthase can also use both ceramide and diglyceride as a substrate, since it transfers a phosphocholine head-group from one to the other [26,50].

Conclusion

This study shows that HFA-CGalT catalyses the synthesis of HFA- and NFA-GalCer and GalDG in intact CHO cells, implying that galactolipid synthesis depends on the local availability of the various substrates to one and the same enzyme. The expression of a single specific CGalT in CHO cells, in the absence of enzymes that convert GalCer into more complex glycosphingolipids, will facilitate the characterization of the transport steps of GalCer from its site of synthesis to the cell surface. In addition, the controversial role of GalCer as a potential receptor for the human immunodeficiency virus (HIV) can now be elucidated separately from that of sulphatide [51]. Finally, the establishment of stable cell lines expressing different levels of GalCer holds great potential for investigating the influence of glycosphingolipid levels on intracellular protein transport and targeting, and on a variety of other sphingolipid-related cellular processes.

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REFERENCES

1 Morell, P., Quarles, R. H. and Norton, W. T. (1994) in Basic Neurochemistry: Molecular, Cellular and Medical Aspects (Siegel, G. J., Agranoff, B. W., Albers, R. W. and Molinoff, P. B., eds.), pp. 117–143, Raven Press, New York
2 Simons, K. and van Meer, G. (1988) Biochemistry **27**, 6197–6202
3 Nonaka, G. and Kishimoto, Y. (1979) Biochim. Biophys. Acta **572**, 432–441
4 Svennerholm, L., Boström, K., Fredman, P., Jungbjer, B., Månsson, J.-E. and Rynmark, B.-M. (1992) Biochim. Biophys. Acta **1128**, 1–7
5 Agranoff, B. W. and Hajra, A. K. (1994) in Basic Neurochemistry: Molecular, Cellular and Medical Aspects. (Siegel, G. J., Agranoff, B. W., Albers, R. W. and Molinoff, P. B., eds.), pp. 97–116, Raven Press, New York
6 Natomi, H., Saitoh, T., Sugano, K., Iwamori, M., Fukayama, M. and Nagai, Y. (1993) Lipids **28**, 737–742
7 Carter, H. E., Johnson, P. and Weber, E. J. (1965) Annu. Rev. Biochem. **34**, 109–142
8 Brändli, A. W., Hansson, G. C., Rodriguez-Boulan, E. and Simons, K. (1988) J. Biol. Chem. **263**, 16283–16290
9 Hansson, G. C., Simons, K. and van Meer, G. (1986) EMBO J. **5**, 483–489
10 Niimura, Y. and Ishizuka, I. (1986) J. Biochem. (Tokyo) **100**, 825–835
11 Karlsson, K.-A., Samuelsson, B. E. and Steen, G. O. (1973) Biochim. Biophys. Acta **316**, 317–335
12 Karlsson, K.-A. (1982) in Biological Membranes (Chapman, D., ed.), Vol. 4, pp. 1–74, Academic Press, London
13 Vos, J. P., Lopes-Cardozo, M. and Gadella, B. M. (1994) Biochim. Biophys. Acta **1211**, 125–149
14 Morell, P. and Radin, N. S. (1969) Biochemistry **8**, 506–512
15 Basu, S., Schultz, A. M., Basu, M. and Roseman, S. (1971) J. Biol. Chem. **246**, 4272–4279
16 Basu, M., De, T., Das, K. K., Kyle, J. W., Chon, H. C., Schaeper, R. J. and Basu, S. (1987) Methods Enzymol. **138**, 575–607
17 Koul, O., Singh, I. and Jungalwala, F. B. (1988) J. Neurochem. **50**, 580–588
18 Sato, C., Black, J. A. and Yu, R. K. (1988) J. Neurochem. **50**, 1887–1893
19 Schulte, S. and Stoffel, W. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10265–10269
20 Stahl, N., Jurevics, H., Morell, P., Suzuki, K. and Popko, B. (1994) J. Neurosci. Res. **38**, 234–242
21 Schaeren-Wiemers, N., Schaefer, C., Valenzuela, D. M., Yancopoulos, G. D. and Schwab, M. E. (1995) J. Neurochem. **65**, 10–22
22 Garbay, B. and Cassagne, C. (1994) Dev. Brain Res. **83**, 119–124
23 Schaeren-Wiemers, N., van der Bijl, P. and Schwab, M. E. (1995) J. Neurochem. **65**, 2267–2278
24 Warnock, D. E., Lutz, M. S., Blackburn, W. A., Young, W. W., Jr., and Baenziger, J. U. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 2708–2712
25 van der Bijl, P., Lopes-Cardozo, M. and van Meer, G. (1996) J. Cell Biol. **132**, 813–821
26 van Helvoort, A. L. B., van 't Hof, W., Ritsema, T., Sandra, A. and van Meer, G. (1994) J. Biol. Chem. **269**, 1763–1769
27 Bansal, R. and Pfeiffer, S. E. (1987) J. Neurochem. **49**, 1902–1911

28 van der Pal, R. H. M., Vos, J. P., van Golde, L. M. G. and Lopes-Cardozo, M. (1990) Biochim. Biophys. Acta **1051**, 159–165
29 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
30 Magnani, J. L., Spitalnik, S. L. and Ginsburg, V. (1987) Methods Enzymol. **138**, 195–207
31 Sommer, I. and Schachner, M. (1981) Dev. Biol. **83**, 311–327
32 Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. and Seifert, W. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2709–2713
33 Ladenson, R. C., Monsey, J. D., Allin, J. and Silbert, D. F. (1993) J. Biol. Chem. **268**, 7650–7659
34 de Lederkremer, R. M., Lima, C., Ramirez, M. J., Ferguson, M. A. J., Homans, S. W. and Thomas-Oates, J. (1991) J. Biol. Chem. **266**, 23670–23675
35 Burger, K. N. J., van der Bijl, P. and van Meer, G. (1996) J. Cell Biol. **133**, 15–28
36 Strous, G. J., van Kerkhof, P., van Meer, G., Rijnboutt, S. and Stoorvogel, W. (1993) J. Biol. Chem. **268**, 2341–2347
37 Nilsson, T. and Warren, G. (1994) Curr. Opin. Cell Biol. **6**, 517–521
38 Radin, N. S. and Inokuchi, J.-i. (1991) Trends Glycosci. Glycotechnol. **3**, 200–213
39 Jeckel, D., Karrenbauer, A., Burger, K. N. J., van Meer, G. and Wieland, F. (1992) J. Cell Biol. **117**, 259–267

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40 Perreault, H. and Costello, C. E. (1994) Org. Mass Spectrom. **29**, 720–735
41 Neskovic, N. M., Roussel, G. and Nussbaum, J. L. (1986) J. Neurochem. **47**, 1412–1418
42 Kishimoto, Y., Akanuma, H. and Singh, I. (1979) Mol. Cell. Biochem. **28**, 93–105
43 Radin, N. S. (1994) Neurochem. Res. **19**, 533–540
44 Bansal, R., Warrington, A. E., Gard, A. L., Ranscht, B. and Pfeiffer, S. E. (1989) J. Neurosci. Res. **24**, 548–557
45 Gadella, B. M., Gadella, T. W. J., Jr., Colenbrander, B., van Golde, L. M. G. and Lopes-Cardozo, M. (1994) J. Cell Sci. **107**, 2151–2163
46 Deshmukh, D. S., Inoue, T. and Pieringer, R. A. (1971) J. Biol. Chem. **246**, 5695–5699
47 Inoue, T., Deshmukh, D. S. and Pieringer, R. A. (1971) J. Biol. Chem. **246**, 5688–5694
48 Schmidt-Schultz, T. and Althaus, H. H. (1994) J. Neurochem. **62**, 1578–1585
49 de Haas, C. G. M. and Lopes-Cardozo, M. (1995) Int. J. Dev. Neurosci. **13**, 447–454
50 Marggraf, W.-D. and Kanfer, J. N. (1984) Biochim. Biophys. Acta **793**, 346–353
51 McAlarney, T., Apostoloski, S., Lederman, S. and Latov, N. (1994) J. Neurosci Res. **37**, 453–460