REGULATION OF GALACTOSYLCERAMIDE BIOSYNTHESIS

A Thesis
Presented to
The Academic Faculty

By

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In Partial Fulfillment
Of the Requirements for the Degree
Masters of Science in Biology

Georgia Institute of Technology

August 2006
Regulation of Galactosylceramide Biosynthesis

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Date Approved: July 10, 2006
ACKNOWLEDGEMENTS

This project would not have been possible without the support of many people. I would like to thank my advisor, Dr. Merrill, for his help, guidance, and patience. Also thanks to my committee members, Dr. Sewer and Dr. Sullards, for their time and support. Thank you to the entire Dr. Merrill lab for assistance and support, especially to Jeremy Allegood and Elaine Wang.
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SPT, Serine palmitoyltransferase
ER, endoplasmic reticulum
3KSR, 3-ketosphinganine reductase
Lass, Logevity assurance gene
DES1, dihydroceramide desaturase 1
DES2, dihydroceramide desaturase 2
SM, sphingomyelin
SMase, sphingomylinase
aSMase, lysosomal acid sphingomylinase
sSMase, zinc ion-dependent secretory sphingomyelinase
nSMase, neutral magnesium ion-dependent Smase
bSMase, alkaline Smase; GlcCer, glucosylceramide
GalCer, galactosylceramide
Cer-1-P, ceramide-1-phosphate
SMS1, sphingomyelin synthase 1
SMS2, sphingomyelin synthase 2
CERK, ceramide kinase
CGlcT, UDP-Glucose:Ceramide Glucosyltransferase
UDP-Glc, UDP-glucose
CGalT, UDP-Galactose:Ceramide Galactosyltransferase
UDP-Gal, UDP-galactose
UGT1, UDP-galactose transporter 1
UGT2, UDP-galactose 2
APC, active protein C
PS, phosphatidylserine
MDR, multi-drug resistance
GM4, sialosylgalactosylceramide
HIV, human immunodeficiency virus
HEK293, human embryonic kidney cells
SPT1/2, human embryonic kidney cells stably overexpressing SPT1 and SPT2
LC-ESI MS/MS, liquid chromatography electrospray tandem mass spectrometric
BFA, Brefeldin A
GSD, glycosphingolipid storage diseases
LSD, lysosomal storage diseases
PDMP, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
QRT-PCR, quantitative real-time PCR
HB, homogenization buffer
NBD-C₆-Cer, NBD-C₆-ceramide
So, sphingosine
Sa, sphinganine
HPLC, high performance liquid chromatography;
SUMMARY

An important branchpoint of mammalian sphingolipid metabolism occurs at the step where ceramides are glycosylated to glucosylceramide (GlcCer) versus galactosylceramide (GalCer), which are precursors of all mammalian glycosphingolipids. Relatively few studies have focused on this branchpoint because these monohexosylceramides are somewhat difficult to resolve chromatographically and because molecular biology tools have only recently become available to follow expression of these genes. The goal of this thesis is to better understand the mechanisms of cell regulation determining galactosylceramide synthesis.
Sphingolipids are found in all eukaryotes and some prokaryotes and are important in membrane function, cellular structure, regulation of cell growth, differentiation and apoptosis (2-4), and serve as receptors for toxins and viruses on the cell surface (5). Sphingolipids are a highly diverse class of lipids, composed of a sphingoid base with a fatty acid attached through an amide bond. Sphingoid bases vary in alkyl chain lengths (14-22 carbon atoms), degrees of saturation (at carbons 4 and 5), hydroxyl group positions (at carbons 4 and 6), double bonds in alkyl chains (in other positions), and branching methyl groups (ω-1 and ω-2) (6). The most common sphingoid bases in mammalian sphingolipids are sphingosine ((2S,3R4E)-2-aminooctadec-4-ene-1,3,-diol), sphinganine (dihydro-sphingosine) and 4-hydroxy-sphinganine ((2S,3S,4R)-2-2aminooctadecane-1,3,4-triol, also called phytosphingosine) (6).

Ceramides are formed when a n-acyl fatty acid is attached to a sphingoid base via an amide bond. The diversity of ceramides is due to the variation in alkyl chain length (14-30 carbons), in degree of saturation of fatty acid, and in presence of absence of hydroxyl group on the α or ω carbon atoms. Ceramides are the lipid backbone moiety of over 300 complex sphingolipids (6). Sphingolipids are formed via two routes, by de novo biosynthesis, and recycled components derived from turnover of complex species.

**De novo biosynthesis**

Serine palmitoyltransferase (SPT) catalyzes the initial step in de novo sphingolipid biosynthesis, the condensation of L-serine with palmitoyl coenzymeA,
yielding 3-ketosphinganine (7). SPT is a heterodimer of SPTLC1 and SPTLC2 and is located in the endoplasmic reticulum (ER), with an active site facing the cytoplasm (7-9). Activity has been found in all mammalian tissue and cell types, and is abundant in kidney, liver, and lung. SPT is regarded as the rate limiting step in sphingolipid biosynthesis (7), and its activity increases in response to inflammation, stress, and apoptotic stimuli (10,11).

3-ketosphinganine is reduced by 3-ketosphinganine reductase (3KSR) to form sphinganine in an NADPH-dependent manner. 3KSR protein is predicted to have three transmembrane domains; the N-terminus is in the lumen of the ER, whereas the active site residue and the C-terminus are in the cytosol (12). All cell types have detectable 3KSR activity, with high levels in the lung, kidney, stomach, and small intestine (12).

Sphinganine is converted to dihyrdroceramide by (dihydro)ceramide synthase, by the addition of a fatty acyl CoA. (Dihydro)ceramide synthase is located in the ER with an active site facing the cytosol, and is not a single enzyme, but a family of enzymes from a family of genes, the Longevity assurance gene family (Lass) (13-16).

Dihydroceramide is converted to ceramide by the incorporation of a 1 4,-5 trans-double bond by dihydroceramide desaturase 1 (DES1) or a 4-hydroxyl group by dihydroceramide desaturase 2 (DES2) (17-19). DES1 and DES2 are located in the ER, with their active sites facing the cytosol (20). These enzymes are important in cell survival and function because they convert dihydroceramide, which is not currently thought to be an effective inducer of apoptosis, (21) to ceramide, which is able to induce apoptosis (22).
Turnover sphingolipids also generates ceramide

Ceramide and sphingoid bases can be generated through recycling of the components from the breakdown of complex sphingolipids (23,24). Ceramide can be synthesized from sphingomyelin (SM) by sphingomylinase (SMase) which catalyzes the hydrolysis of both golgi and plasma membrane sphingomyelin to form ceramide (25). Currently there are five characterized sphingomylinases; lysosomal acid sphingomylinase (aSMase), zinc ion-dependent secretory sphingomyelinase (sSMase), neutral magnesium ion-dependent SMase (nSMase), magnesium ion-dependent nSMase, and alkaline SMase (bSMase) (26). Ceramide can be generated from the breakdown of glycosphingolipids by cerebrosidase activity, for instance, glucosylceramide (GlcCer) and galactosylceramide (GalCer) beta-glycosidase linkage is hydrolyzed by glucocerebrosidase (27) and by galactocerebrosidase (28), respectively. Ceramide-1-phosphate (Cer-1-P) can by hydrolyzed to ceramide by a unique phosphatase, Cer-1-P phosphatase (29).

Ceramide trafficking and metabolites

Ceramide, as explained above, is synthesized on the cytosolic ER, while all complex sphingolipids, with the exception of GalCer (30), are synthesized at the Golgi and plasma membrane (31-33). Ceramide can be transported from the ER to the Golgi apparatus through vesicular transport, although the exact mechanism for vesicular transport is currently unclear (34). The main pathway for transportation of ceramide from the ER to the Golgi apparatus is non-vesicular transport, through ATP-cytosol dependent, or ATP-cytosol independent routes (35-37). The key factor in non-vesicular
transport in ER-to-Golgi ceramide transport is CERT, which contains a domain that interacts with the Golgi, a domain that interacts with ER, and a (StAR)-related lipid transfer domain (37,38). Ceramide can be trafficked to the Golgi for synthesis of sphingomyelin, ceramide-1-phosphate, and glucosylceramide, and into the ER lumen for galactosylceramide synthesis.

Ceramide is metabolized to SM at the Golgi by sphingomyelin synthase 1 (SMS1) and at the plasma membrane by sphingomyelin synthase 2 (SMS2) (31-33). Sphingomyelin synthase catalyzes the transfer of phosphorylcholine from phosphatidylycholine onto the 1-hydroxyl of the ceramide backbone (23,24). SM is preferentially located in the outer leaflet of the plasma membrane where it is involved in membrane rigidity, signaling and protein transport (39-44).

Ceramide-1-phosphate is synthesized when ceramide is phosphorylated by a unique lipid kinase, ceramide kinase (CERK) (45-47). The first known biological function of Cer-1-P was stimulating DNA synthesis and induction of proliferating-cell nuclear antigen (48) and has since been discovered to play roles in a wide variety of biological activities, including phagocytosis (49,50), inflammatory response (51,52), and calcium mobilization (53-56). In macrophages CIP inhibits apoptosis, blocks DNA fragmentation and caspase activation, and blocks acid Smase activity thereby preventing accumulation of ceramides (57).

**Glycosphingolipids**

Ceramide can also be glycosylated; biosynthesis of glycosphingolipids begins with the condensation of a carbohydrate moiety to the 1 position of the ceramide
backbone. The hexose may either be a glucose or galactose, resulting in GlcCer or GalCer, respectively. GlcCer is synthesized on the cytosolic side of the Golgi apparatus; catalyzed by UDP-Glucose:Ceramide Glucosyltransferase (CGlcT) utilizing ceramide and UDP-glucose (UDP-Glc) (58-60). GalCer is synthesized in the lumen of the endoplasmic reticulum; and is catalyzed by UDP-Galactose:Ceramide Galactosyltransferase (CGalT) utilizing ceramide and UDP-Galactose (UDP-Gal) (61,62). UDP-Gal is transported to the luminal ER by UDP-Galactose transporter 2 (UGT2), this transporter is a splice variant of UGT1, containing an ER locating dilysine motif (KVKAS) (30). UGT1 transports UDP-Gal into the Golgi for synthesis of complex sphingolipids such at lactosylceramide (30).

UDP-Glucose:Ceramide Glucosyltransferase consists of a strongly hydrophobic Golgi anchor segment near the N-terminus, (63) and a catalytic C-terminal located in the cytoplasm (64). CGalT mRNA is ubiquitously expressed across cell types, suggesting that it is essential for cellular function, but studies have shown that it is not required for cell growth in vitro (65). However, CGlcT knock out is embryonic lethal, embryonic cells were able to proceed into primitive stages of cell division/differentiation, but ultimately did not survive (66).

UDP-Galactose:Ceramide Galactosyltransferase contains a 4 kDa ER retrieval signal KVKK at its C-terminal end (61), and the active N-terminal site faces the luminal ER (61). CGalT is highly expressed in oligodendrocytes and Schwann cells, with a pattern that matches that of myelination (67), and the disruption in CGalT leads to unstable myelin and progressive paralysis in mouse models (68).
Glucosylceramide is a biologically active molecule, and is the precursor to lactosylceramide, which in turn is the precursor to the largest glycosphingolipid family (6). GlcCer stimulates cell proliferation (69,70) and cellular differentiation (71,72), specifically in neuronal cells (73-75). GlcCer is involved in Ca$^{2+}$ mobilization through interaction with a Ca$^{2+}$ channel in the ER (76), and enhancement of active protein C (APC) activity by stereo-specifically binding APC, and increasing its affinity for phosphatidylserine (PS) (77). GlcCer synthesis is protective against the affects of ceramide-induced cellular stress, through the reduction of ceramide (78). Conversely, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), is an inhibitor of CGlcT and increases the cellular pool of ceramide (79). Controversy exists regarding the role of GlcCer in multi-drug resistance (MDR) of cancer cells, as discussed later.

GalCer is the precursor to a smaller family of glycosphingolipids than GlcCer, including sulfatide and sialosylgalactosylceramide (GM4), and a major component of the myelin sheath and contributes to neuronal structure and signal conduction (67,80). Lack of GalCer results in tremors, neuronal conduction deficits, and eventual paralysis (81). GalCer is important in lipid and protein sorting in lipid enriched detergent insoluble domains, specifically in epithelial cells (82,83), and is also a cell surface receptor for pathogens including the gp120 subunit of the human immunodeficiency virus (HIV) (84-89).

**Partitioning of Ceramides**

Sphingolipid biosynthesis is a complex process, involving not only relative activity of enzymes, but subcellular localization of enzymes and substrates, and little is
known about the regulating mechanisms of ceramide partitioning to its metabolites (90). One approach to understanding a branchpoint is to determine the consequences of increasing precursors. To explore the question of what effect an increase of precursors will have on ceramide partitioning, human embryonic kidney cells (HEK293) cells stably overexpressing SPT1 and STP2 (SPT1/2) (a gift from J.K. Uhlinger, Johnson and Johnson Pharmaceutical Research & Development, Raritan, New Jersey 08869) were obtained. SPT1/2 cells, analyzed with liquid chromatography electrospray tandem mass spectrometric method (LC-ESI MS/MS) (91), synthesize an increase of ceramide and monohexosylceramide in comparison with HEK293 cells. The goal of this thesis is to characterize the increase of monohexosylceramide, as GalCer or GlcCer; and understand why the overexpression of the SPT1/2 induce these changes.

In addition to providing basic information about sphingolipid biochemistry, a clearer understanding of ceramide partitioning regulation can be useful in understanding the roles of GalCer and GlcCer in glycosphingolipid storage diseases, multidrug resistant cancers, involvement in neuronal cell growth and differentiation, blood coagulation, and HIV-1 attachment to host cells.

**Glycosphingolipid Storage Diseases**

Glycosphingolipid storage diseases (GSD) are a group of lysosomal storage diseases (LSDs) which are caused by aberrant catalytic activity of lysosomal hydrolase’s responsible for the breakdown of complex sphingolipids (92). All GSDs, with the exception of Gauchers Type 1, have a neurological effect, emphasizing the important role
of glycosphingolipids role in neuronal cell growth and differentiation (93,94). Discussed here are the GSDs which affect ceramide, SM, GlcCer and GalCer.

Farber, a rare auto-recessive disorder, results from defective acid ceramidase (95). Acid ceramidase is the enzyme that catalyzes the hydrolysis of ceramide into sphingosine and fatty acid (96). Farber disease is marked by ceramide accumulation in the liver, spleen and lung, and symptoms include subcutaneous nodules, deformed joints, hoarseness due to larynx involvement, and nervous system dysfunctions, resulting in a premature death (95).

Niemann-Pick A & B, are auto-recessive disorder, and result from deficient sphingomyelinase, which leads to the accumulation of SM (97). Type A Niemann-Pick has progressive and severe neurodegeneration with death in infancy, while Type B Niemann-Pick has less severe neurodegeneration but includes growth retardation and frequent respiratory infections (98). Type A and Type B forms both manifest with foam cell (cells containing lipids in small vacuoles) infiltration and abnormally enlarged intestines (98).

The most common GSD is Gauchers, an auto-recessive disorder arising from defective β-glucosidase, resulting in the accumulation of GlcCer, specifically in cells of macrophage lineage (99). Gauchers Type 1 is strictly a macrophage disorder and lacks neuronal involvement (100). Gauchers Type 2 is the acute nueropathic form, manifesting in oculomoter abnormalities and brainstem involvement, leading to premature death (101). Gauchers Type 3 is late onset neurological symptoms of Type 2 (99).

Krabbe, an auto-recessive disorder, is the accumulation of GalCer due to defective β-galactosidase (102). The consequences of GalCer accumulation are almost
exclusively neuronal, as GalCer is highly concentrated in myelin and in cerebral blood vessels (103). The clinical variability (death in infancy to non-fatal through adulthood) is not well matched with biological consequences such as genetic mutations or GalCer concentrations, the causes of differing severity still remains to be full explained.

**Multi-Drug Resistant Cancer Cells association with Glucosylceramide**

Controversy exists regarding the role of GlcCer in multi-drug resistance (MDR) of cancer cells. Some evidence exists that accumulation of GlcCer causes MDR, for example the multi-drug resistant breast cancer cell line, MCF-7AdrR, accumulates GlcCer to a greater extent than the non-multi-drug resistant breast cancer cell line MCF-7 (104). The transformation of MCF-7 cells with CGlcT confers resistance to chemotherapeutic agents (105). In agreement with these studies, decreasing GlcCer synthesis reverses drug resistance; treatment with antisense oligodeoxyribonucleotides to CGlcT gene, and threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (CGlcT inhibitor) resensitized MCF-7-AdrR cells (106-108).

In contrast, these resulted where not replicated when a different cell line was tested. For example, in GM95 cells, a cell line lacking glycosphinglipid synthesis, transfection with CGlcT did not increase the cells resistance to chemotherapeutic agents (109). It has also been shown that other inhibitors of CGlcT, N-alkylated iminosugars such as N-alkyldideoxygalactonojirimycin, do not have the same affect on MDR cells MES-SA/DX-5 as PDMP (110). This study suggests that PDMP has a secondary effect other than inhibiting CGlcT that allows it to reverse drug resistance.
Glucosylceramide involved in Neuronal cell proliferation and differentiation

Depletion of glycosphingolipids, stemming from inhibition of sphingolipid synthesis, significantly affects axonal growth (71), and knock out of CGlcT is lethal in mice (111). GlcCer synthesis is required for stage 3 neuronal growth, and is required for stimulation of axonal growth by basic fibroblast growth factor and laminin (73,75), and synthesis is activated when axonal growth is stimulated by a downstream target of GlcCer, basic fibroblast growth factor CTP:phosphocholine cytidylytransferase (CTT) (112,113). GlcCer affects calcium homeostasis in neurons, modulates agonist-induced Ca$^{2+}$ release from microsomes by modulating Ryanodine receptor, a major Ca$^{2+}$ channel in the ER (76).

Glucosylceramide role in blood coagulation

Blood coagulation is regulated by the delicate balance between coagulation and anticoagulation reactions (77). The enzymes for these reactions must form multicomponent complexes on lipid membrane surfaces for the procoagulant and the anticoagulant reactions to occur at a physiologically significant rate (114). GlcCer increases the anticoagulant activity of APC by binding to APC, which increases APC’s affinity for negatively charged phospholipid vesicles containing phosphatidylserine (PS) (77).

Galactosylceramide involvement in myelination

GalCer is the major component of myelin sheath (25%), and is essential in neuronal growth, differentiation, and myelin formation (115). In the myelin sheath,
GalCer supports the structure, stability, and curvature of the membrane bilayer (116). GalCer supports intermembrane stabilization by divalent cation linkages of its carbohydrate group with sulfatide, its metabolite, which is also abundant in neuronal cells (117-119). Implantation of cells secreting anti-GalCer caused inhibition of myelination in rat spinal cord development, and treatment of oligodendrocytes with anti-GalCer caused distorted membrane morphology (120,121). Mice with no GalCer show oligodendrocytes with development delays; the mice do form myelin, but it is thin and structurally abnormal, causing tremors, paralysis, and premature death (80,115,122).

**Galactosylceramide and HIV-1**

GalCer is an alternative receptor for human immunodeficiency virus (HIV-1), which usually attaches to host cells via CD4 and HIV envelope protein gp120 interaction (84,123). CD4 negative cells such as fibroblast and colorectal mucosal epithelial cells are susceptible to HIV-1 infection through gp120 associating with GalCer to translocate across cellular membranes (84,124). In CD4 positive cells GalCer appears to play a stabilization role in the binding of HIV-1 to CD4 and its co receptors CXCR4 and CCR5, as changes in GalCer synthesis can modulate HIV-1 entry into CD4 positive cells (125).
CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Human embryonic kidney 293 cells (Hek293) (ATCC, Manassa, VA) and Human embryonic kidney 293 overexpressing SPTLC1 and SPTLC2 (STP1/SPT2) (a gift from Uhlinger et al), were maintained in Dulbecco Modified Eagle Medium with Ham's F12 Nutrient Mixture (DMEMF12) (Invitrogen, Carlsbad, Ca) media supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), and 1.2 g/l sodium bicarbonate. The cells where incubated at 37°C in a humidified incubator with an atmosphere containing 5% CO₂.

Brefeldin Treatment

Brefeldin-A (BFA) powder (Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol and stored at -20°C. Cells where seeded at 2.5x10^6 cells per 10 ml dish and grown for 2 days. Cells were treated with 5 μM BFA and incubated 0, 1, 3, or 6 h. To harvest cells for mass spectrometric analysis, media was removed and the cells were washed twice with ice cold PBS. Cells where scraped in 1 ml of PBS, pelleted, and resuspended in 0.5 ml methanol. For normalization, protein for each sample was evaluated using BCA (bicinchoninic acid) protein assay per manufacturers instructions (Pierce, Rockford, IL).

RNA isolation and Quantitative RT-PCR confirming gene expression

RNA was isolated using Stratagene Absolute Miniprep mRNA kit (Stratagene, La Jolla, CA) according to the manufacturer’s directions. Complementary DNA was
synthesized using TaqMan Reverse Transcriptase reagents (Applied Biosystems, Foster City, CA), using random hexamers and mRNA (0.2 μg/μl). Genes of interest were amplified using quantitative real-time PCR (QRT-PCR) and detected using the SYBR green 1 dye (Applied Biosystems, Foster City, CA). The QRT-PCR procedure was done in a total reaction volume of 25 μl, containing 3 μl cDNA, 1.5 μl (5 μmol/l) each of the forward and reverse primers (Invitrogen, Carlsbad, CA), 12.5 μl SYBR Breen 1 PCR master mix (Applied Biosystems, Foster City, CA) and 6.5 μl Rnase-free H2O. CGalT sequences are forward 5’ AGAAGGCTTTCCGAAATTAC3’; reverse 5’AGTATAACAAGGCAGCACCA3’ (amplifies a fragment 188 bp long) having a melting temperature of 54°C. QRT-PCR primers for CGlcT sequences are, forward 5’ CTGCCACCTTAGAGCAGGTA3’; reverse 5’ TCTTGGCAATGTACTGAGC3’ (amplifies a fragment 166 bp long) having a melting temperature of 56°C. UGT1 sequence forward 5’ACTCGTCATTGGTGGCTGTCT3’; reverse 5’TACCTCCACCAGCACTGACT3’ (amplifies a fragment 194 bp long) having a melting temperature of 58°C. UGT2 sequence forward 5’ ACTCGTCATTGGTGGCTGTCT3’; reverse 5’CTAGGAACCCCTTCACCTTG3’ (amplifies a fragment 203 bp long) having melting temperature of 58°C. β-actin sequence forward 5’ TCCTGTGGCATCCACGAAACT3’; reverse 5’ GAACATGGCATTTGCGGAGAT3’ having a melting temperature of 58°C. The mixtures were preheated at 95°C for 10 min, then cycled 40 times at 95°C for 20 sec, 54°C (CGalT, CGlcT) or 56°C (UGT1, UGT2) or 58°C (for β-actin) for 40 sec, and 72°C for 40 sec, and then 72°C for 1 h, in an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, Ca). All data were normalized using β-actin.
**Enzymatic Assay**

The enzymatic assay was adapted from (126), cells in 10 ml dishes where washed twice with ice cold PBS and scraped gently in 1 ml of ice cold PBS. Cells were pelleted and resuspended into 400 μl of homogenization buffer (250 μM sucrose, 10 μM HEPes NaOH pH 7.2, 1 μM EDTA), and homogenized by sonication for 30 sec (sonication repeated 3 times). The cell mixture was centrifuged for 15 min at 400 g and 4°C to remove nuclei and unbroken cells for isolation of postnuclear supernate (PNS). Protein in the PNS was measured using the BCA assay and adjusted to desired protein concentration in homogenization buffer (HB). Equal volume of reaction mixture ( HB containing 2% (w/v) BSA, 4 μM UDP-Glc or UDP-Gal, 4 μM MgCl₂, 4 μM MnCl₂, 1 mg/ml protease inhibitor, 50 μM NBD-C₆-Ceramide (NBD-C₆-Cer)) was added to the adjusted PNS, and incubated at 37°C for desired time. The reaction was stopped by transfer into cold methanol. The samples were analyzed by High Performance Liquid Chromatography using CH₃OH: H₂O: H₃PO₄ (800:200:5) (v:v:v) at a flow rate of 1 ml/min and a Nova Pak C₁₈ 4 micron cartridge (Millipore Corporation, Milford, Ma).

**Extraction for LC-MS/MS Analysis**

Cells were scraped from cell culture dish with rubber policeman in 1 ml of ice cold methanol, pelleted, and resuspended in 0.5 ml of cold methanol and transferred into a glass tube. Internal standards (500 pmol) and 250 μl of choloform, were added and the samples were sonicated for 45 sec, and then incubated overnight at 48°C in a heating block. The tubes were then allowed to cool and 75 μl of 1 M KOH in methanol was
added, the samples were sonicated, and subsequently incubated for 2 h at 37°C. Samples were cooled to room temperature and 0.4 ml of sample was transferred to a new test tube to be used for long chain base separation by reverse-phase LC. The remaining sample was neutralized with 3 μl of glacial acetic acid. Further extraction was performed by the addition 1 ml of chloroform and 2 ml water, followed by vortex and sonication. Samples were centrifuged and the upper layer carefully removed by pasteur pipette, leaving the fraction containing ceramides and complex sphingolipids. Solvent from both groups (the half for long chain base separation and the remaining half) was removed by speed-vac-type concentrator.

**Liquid Chromatography Electrospray Tandem Mass Spectrometry of Sphingolipids**

All data was collected using a Perkin Elmer Series 200 MicroPump system consisting of two pumps and an autosampler. These were coupled to a PE Sciex API 3000 triple quadrupole tandem mass spectrometer equipped with a turbo ion-spray source.

The monohexosylceramides were separated by normal phase HPLC using a Supelco 2.1 mm i.d. x 25 cm LC-Si column at a flow rate of 1.5 ml/min. Isocratic mobile phase consisted of CH₃CN:CH₃OH:CH₃COOH (97:2:1) (v:v:v) with 5 mM ammonium acetate. Before every run the column was equilibrated for 1.5 min prior to injection. After sample injection, the column was eluted for 8 min. The column was then washed for 1.5 min.

In the API 3000 triple quadrupole mass spectrometer, dry N₂ at 500°C was used as the nebulizing gas at a flow rate of 6 liters/min. The ionspray needle was held at 5500
V, and the orifice and ring voltages were kept low (40 V and 220 V, respectively) to prevent collisional decomposition of molecular ions prior to entry into the first quadrupole. MRM transitions were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. N₂ was used to collisionally induce dissociations in Q2, which was offset from Q1 by 30-40 V. For example, for the GlcCers, these transitions occur at m/z 700.7/264.4, 728.7/264.4, 756.7/264.4, 784.8/264.4, 810.9/264.4, and 812.9/264.4, which correspond to GlcCers with a d18:1 sphingoid base and C16:0, C18:0, C20:0, C22:0, C24:1, and C24:0 fatty acids, respectively. The dwell time was 25 ms for each transition with an interchannel delay of 5 ms intertransition. Quantitation was achieved by spiking the samples prior to extraction with the synthetic d18:1/C12:0 internal standards.

Prior to building an MRM for each cell type the variation in fatty-acyl chain length is determined, which allows MRM transitions to be tailored for the major N-acyl species. This is usually accomplished by precursor ion scans of m/z 184.4, the structure specific fragmentation indicative of SM. SM was chosen because they are usually abundant and are indicative of both sphingosine (So) and sphinganine (Sa) based species. For example, in HEK 293 cells, the major SM are C16:0, C18:0, and C24:1.

**Cerezyme**

Cells were prepared as described for LC-MS/MS analysis, with the exception that no internal standards were added for cerezyme analysis. Cells were resuspended in 0.5 ml of potassium phosphate buffer at a pH of 5.5, and incubated for 30 min at 37°C vortexing at 5 min intervals. Samples were subsequently sonicated and treated with 50
μM of cerezyme (a gift from Futterman, Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel) dissolved in 100 μl of water.

**Semi-intact cell assay**

Semi-intact cells were prepared as previously published by Funakoshi et al (127). HEK293 and SPT1/2 cells were seeded at 2.5x10^6 and grown for 2 days. The cells were washed twice with ice cold hypotonic buffer (10 mM HEPES-KOH pH 7.2, 15 mM KCl, 0.1 mM MgCl_2_), and incubated for 10 min in 5 ml of hypotonic buffer. The hypotonic buffer was removed, and the cells were scraped in 5 ml of Hepes/KCl (25 mM HEPES-KOH pH7.2, 115 mM KCl), and centrifuged at 250 g at 4°C for 5 min. Pellets of semi intact cells were washed with 5 ml of Hepes/KCl, and resuspended in 200 μl of Hepes/KCl. BCA analysis was used to measure protein of the semi intact cells and protein concentration was adjusted to 1 mg/ml. Equal volume of reaction mixture (H/KCl buffer, 4 μM UDP-Glc or UDP-Gal, 1 mg/ml protease inhibitor, 50 mM NBD-C_6_Cer) was added to post nuclear protein. The reaction was then incubated at 37°C for the desired time, and stopped by transferring into cold methanol. The samples were analyzed by HPLC using CH_3OH: H_2O: H_3PO_4 (800:200:5) (v,v,v) at a flow rate of 1 ml/min and a Nova Pak C_{18} 4 micron cartridge (Millipore, Billerica, Ma).
CHAPTER 3: RESULTS

**Glucosylceramide and Galactosylceramide separation by liquid chromatography electrospray tandem mass spectrometric method**

Fragmentation and ionization efficiencies for equal n-acyl chain length GlcCer and GalCer do not differ as they are isobaric, therefore to elucidate quantitative differences chromatographic separation was required. Normal phase HPLC allows distinction of GlcCer and GalCer by retention on HPLC (91). First the separation was verified using synthetic standards (Figure 1A), then it was determined that biological background did not preclude the separation of GalCer and GlcCer (Figure 1B). Verification of separation with cellular background was achieved by treatment with glycocerebrosidase, (cerezyme is an analogue of the human enzyme (beta)-glucocerebrosidase) which catalyzes the hydrolysis of glucosylceramide to glucose and ceramide (128). Cerezyme treatment resulted in a stereo specific cleavage which resulted in loss of the earlier peak in LC-MS/MS chromatagrams of biological samples, confirming that the earlier eluting peak of the pair of MRM's was indeed glucosylceramide (Figure 1C).
Figure 1. Normal phase LC-MS/MS separation of monohexoylceramides
Normal phase LC-MS/MS of A. 250 pmol of d18:1/16:0 GlcCer and GalCer synthetic standards B. SPT1/2 cells d18:1/16:0 GlcCer and GalCer C. SPT1/2 cells treated with cerezyme
Monohexosylceramide in HEK and SPT1/2 overexpression cells

GalCer and GlcCer were analyzed in HEK 293 and SPT1/2 cells, analyzed via HPLC-ESI MS/MS method. As displayed in figure 2, HEK293 and SPT1/2 cells synthesize GlcCer ranging in chain length C16-C26 and varying in saturation (C24:0 – C24:1, C26:0 – C26:1). In HEK293 cells, the amount of C16 GlcCers is 432 +/- 30 pmol/mg of protein, which is within 10% difference to C24:1 479 +/-43 pmol/mg of protein, and 15% difference in C24:0, 372 +/- 6 pmol/mg of protein (Figure 2A). However in SPT1/2 cells, C16:0 GlcCer is 615 +/- 37 pmol/mg of protein, which is 80% less than C24:1 and C24:0, 1127 +/- 124 and 1857 +/- 223 pmol/mg of protein respectively (Figure 2C). In HEK293 cells, the amount of C16:0 is 432 +/- 30 pmol/mg of protein, which is more than 5 times higher than the amount of C18:0 GlcCer, 78 +/- 9 pmol/mg of protein (Figure 2A), whereas in SPT1/2 cells, C16:0 and C18:0 differ by only 10%, 615 +/- 37 pmol/mg of protein and 554 +/- 44 pmol/mg of protein (Figure 2C). Therefore, GlcCer C16:0 is the species that changes the most in its proportion to the other species, increasing less in proportion in SPT1/2 cells compared with HEK293 cells. Thus it can be seen that SPT1/2 cells not only have an increase in total GlcCer, but also a change in the distribution of chain lengths. GalCer chain length C16:0 is the species that increases the most in SPT1/2 cells versus HEK293 cells; SPT1/2 cells have 73 +/- 5 pmol/mg of protein of GalCer, (Figure 2D) which is approximately a 70 fold increase (Figure 2B).
Figure 2. Comparison of monohexosylceramides in HEK293 and SPT1/2 cells
Shown are the amounts of GlcCer and GalCer of varying fatty acid chain lengths (16, 18, etc) and analyzed by LC-ESI MS/MS as described in the text. Each sample was analyzed in triplicate and are given as the mean +/- the SD. A. HEK293 GlcCer B. HEK293 GalCer C. SPT1/2 GlcCer D. SPT1/2 GalCer
**CGaT and CGlcT Expression in HEK293 and SPT1/2 cells**

Several explanations could account for the lack of detectable GalCer in HEK293 cells; first, it is possible that the gene for galactosylceramide synthase is not expressed. In order to address this hypothesis, the relative abundance of CGaT and CGlcT expression in HEK293 cells and SPT1/2 cells was evaluated utilizing CGlcT and CGaT primers and QRT-PCR, as described in materials and methods. In HEK293 cells there is little difference in CGaT and CGlcT, and in SPT1/2 cells there is 5 fold difference in CGaT expression compared to CGlcT (Figure 3A). There does not appear to be any difference in the amount of CGaT mRNA in HEK293 and SPT1/2 cells. Therefore, perhaps this decrease in CGlcT mRNA in SPT1/2 cells, rather than an increase in CGaT, could play a role in the appearance of GalCer in SPT1/2 cells.

**CGaT and CGlcT Enzymatic Activity in HEK293 and SPT1/2 cells**

The expression of the CGaT synthase mRNA gene does not ensure CGaT activity, therefore the activity of GalCer synthase and GlcCer synthase was measured through an *in vitro* assay utilizing NBD-C₆-Cer. NBD-Cer is thought to be used by the cells in a similar way the cells use natural ceramides (129), making it a useful tool to detect and follow sphingolipid metabolism. The production of NBD-GalCer and NBD-GlcCer were measured using high performance liquid chromatography with detection of the metabolites by fluorescence. When permeabilized HEK293 and SPT1/2 cells incubated with only NBD-C₆-Cer do not synthesize NBD-hexosylceramide, however, when HEK293 and SPT1/2 cells were incubated with UDP-Glc and NBD-C₆-Cer therefore NBD-GalCer and NBD-GlcCer distinction is determined by whether the cells
were incubated with UDP-Glc or UDP-Gal, respectively. HEK293 and SPT1/2 cells incubated with UDP-Glc or UDP-Gal and NBD-C₆-Cer synthesized NBD-GlcCer and NBD-GalCer, respectively, was evident (Figure 3B). Using this assay, the activities of CGlcT in HEK293 and SPT1/2 cells are comparable (2.5 +/- .7 and 2.1 +/- .3 nmol/min/mg of protein) (Figure 3B). The same is true for CGalT in HEK293 and SPT1/2 cells, which were 1.0 +/- .3 and .8 +/- .3 nmol/min/mg of protein, respectively (Figure 3B). These results illustrate that HEK293 and SPT1/2 cells not only express GalCer synthase mRNA, but also that GalCer synthase in vitro.
Figure 3. Measurement of mRNA and in vitro enzymatic activity for CGlcT and CGalT in HEK293 and SPT1/2 cells

A. Relative mRNA (versus β-actin) for CGlcT and CGalT analyzed by QRT-PCR

B. In vitro enzymatic activity using permeabilized HEK293 and SPT1/2 cells analyzed by incubation with NBD-C₆-Cer and either UDP-Glc or UDP-Gal and analyzed by HPLC with fluorescent detection of the products.
Substrate Availability in the HEK293 cells

Another possibility for the lack of detectable GalCer in HEK293 cells is that CGaIT is located in the lumen of the ER, therefore, perhaps it does not have access to the substrates ceramide or UDP-Gal (61). To test this hypothesis, we took advantage of the known existence of transporters in the Golgi because ceramide must be transported from the cytoplasmic to the luminal face of the Golgi for sphingomyelin synthesis (32,33); furthermore, the Golgi contains a UGT1 that transfers UDP-Gal from the cytosol to the Golgi matrix for synthesis of complex glycosphingolipids such as lactosylceramide (130). Since, BFA treatment induces tabule formation of the Golgi and subsequent fusion with the ER, allowing enzymes and substrates in the ER access to enzymes and substrates localized in the Golgi (131-133); thus, the cells were incubated with BFA to determine if mixing these transporters with the enzymatic machinery in the ER would result in greater GalCer synthesis. When HEK293 cells were treated with 2 μM BFA for 1 h, they now synthesize GalCer, with the predominent species being C16 (165 +/- 36 pmol/mg of protein) (Figure 1B). GalCer synthesis increased further with a higher concentration of BFA and with a longer time of incubation (Figure 4B and 4D). GlcCer decreased in HEK293 cells incubated with 2 μM BFA for 1 h, and for comparison with the C16 GalCer, the amount of the C16 GlcCer was initially 290 +/- 69 pmol/mg of protein and decreased to 199 +/- 36 pmol/mg of protein, and continued to decrease with use of a higher BFA concentration and longer incubation time (Figure 4A and 4C). The finding that HEK293 cells synthesize GalCer upon fusion of the ER and Golgi by treatment with BFA, suggests that provision of more of the components that are needed for GalCer synthesis (ceramide and UDP-Gal) can strongly influence whether this lipid is made, and
hence the most likely reason that there is a lack of GalCer synthesis in HEK293 cells (in absence of BFA) is that the enzyme does not have access to the necessary substrates.
Figure 4. The effect of BFA on the amounts of monohexosylceramides in HEK293 cells

HEK293 cells were incubated with the shown concentration of BFA and the GlcCer and GalCer were measured by LC-ESI MS/MS. A. Cells incubated for 1 h. B. Cells incubated for 6 h. Control cells were incubated for the same amount of time, but with no BFA.
UGT1 and UGT2 expression in HEK293 and SPT1/2 cells

UDP-Gal is transported into the ER by UDP-Gal transporter isoform UGT2, which has an ER localizing di-lysine motif that distinguishes it from UGT1, which is the UDP-Gal transporter for the Golgi (30). The relative abundance of expression UGT1 and UGT2 mRNA was studied by QRT-PCR utilizing primers designed for the unique C-terminus ends of UGT1 and UGT2 (Figure 5). The UGT2 mRNA expression is not higher in SPT1/2 cells than in HEK293 cells. These results demonstrate, at least by the criterion of mRNA expression, that HEK293 cells have as much mRNA for the ER isoform of the UDP-Gal transporter as do SPT1/2 cells, which is suggestive that UDP-galactose transport into the ER is not limiting.
Figure 5. Relative amounts of UGT1 and UGT2 mRNA in HEK293 and SPT1/2 cells

Total mRNA was isolated from HEK293 cells and SPT1/2 cells and sequence specific primers were used to determine the relative amounts of UGT1 and UGT2 mRNA (relative to β-actin) by QRT-PCR.
Evaluation of possible differences in substrate availability in HEK293 and SPT1/2 cells using semi-intact cells

The protocol that has been used up to this point to assay for GalCer synthase involves sonication of the cells to disrupt ER structure and ensure that the enzyme has access to its co-substrates. To determine if substrate availability may be limiting when the enzyme is assayed under conditions where the ER is more intact, a method for preparing semi-intact cells method (127). This semi-intact method sheers outer membrane but leaves intracellular membranes intact, which would allow NBD-Cer and UDP-Gal or UDP-Glc direct access to enzymes with active sites on the cytosolic leaflet of the ER and Golgi (i.e. CGlcT), whereas, they will first have to pass through the membranes of these intracellular organelles to gain access to enzymes in the lumen (i.e. CGalT).

Thus, in these experiments, enzymatic activity was measured with permeabilized (semi-intact) HEK293 and SPT1/2 cells that had been both permeabilized and sonicated to break intracellular membranes (Figure 6). There was little difference in the amount of NBD-GalCer that was synthesized by semi-intact versus semi-intact and sonicated SPT1/2 at a number of different time points of incubation cells displayed in semi-intact cells in comparison with sonicated cells (Figure 6A). HEK293 semi-intact and sonicated cells synthesized similar NBD-GalCer nmol/mg of protein at 10 min, however at 20 min the amount was 45% higher for sonicated cells (implying that the synthesis in the synthesis in the semi-intact cells was lower due to a more limited access of the enzyme to this substrate), and this elevation was also seen at 30 min (Figure 6D). In contrast, neither SPT1/2 nor HEK293 cells displayed a difference in synthesis of NBD-GlcCer in
semi-intact cells in comparison with sonicated cells (Figure 6A and 6C), indicating the added substrates (NBD-C6-Cer and UDP-Glc) have access to the CGlcT via the cytosol of the cell.
Figure 6. Measurement of \textit{in vitro} Enzymatic activity of CGalT and CGlcT in semi-intact and sonicated cells

In vitro enzymatic activity using semi-intact and sonicated HEK293 and SPT1/2 cells by incubation with NBD-C6-Cer and either UDP-Glc or UDP-Gal analyzed by HPLC 

\textbf{A}. NBD-GlcCer in SPT1/2 cells 

\textbf{B}. NBD-GalCer in SPT1/2 cells 

\textbf{C}. NBD-GlcCer in HEK293 cells 

\textbf{D}. NBD-GalCer in HEK293 cells
Galactosylceramide synthesis in other cell lines

The effect of BFA on GalCer synthesis was also examined in three additional cell lines that did not contain detectable GalCer under basal conditions as measured by LC-MS/MS. In all cases, treatment with 5 μM BFA for 6 h resulted in considerable increase in GalCer. For HeLa cells, the predominant GalCer species were C24:1 and C24:0 (87 +/- 19 and 106 +/- 34 pmol/mg of protein, respectively) (Figure 7A); for HepG2 cells, the predominant species was C24:1 (230 +/- 19 pmol/mg of protein) (Figure 7B); and for HL-60 cells, the major species was C16 GalCer (81 +/- 4 pmol/mg of protein) (Figure 7C). These results illustrate that the ability to synthesize GalCer exists in a range of cells in which GalCer is not normally detected.
Figure 7. The effect of BFA on GalCer in Hela, HepG2, and HL-60 cells
Shown are amounts of GalCer of varying chain lengths. Cells were incubated with 5 μM concentration of BFA for 6 h, analyzed by LC-ESI MS/MS as described in the text A. Hela cells B HepG2 cells C. HL-60 cells control cells were incubated for 5 h with no BFA
CHAPTER 4: DISCUSSION

The goal of this thesis is to characterize the increase in monohexosylceramides found in HEK293 cells that have been stably transfected with SPTLC1 and SPTLC2 (and termed STP1/2 cells) to increase the rate of ceramide and, presumably, downstream sphingolipid and biosynthesis. Many laboratories have studied the regulation of ceramide biosynthesis (7,12,20,31,33,34,61,90,134); however, little is known about how the partitioning of ceramide into the downstream glycosphingolipids GlcCer and GalCer are regulated (34,90). SPT1/2 cells offer a unique opportunity to study the effects of increasing ceramide biosynthesis and determining the consequences of ceramide partitioning.

Characterization of the Monohexosylceramides of HEK293 and SPT1/2 cells

Analysis of the monohexosylceramides of HEK293 and SPT1/2 cells by LC-ESI-MS/MS revealed that SP1/2 cells not only synthesize more monohexosylceramides than HEK293 cells, but SPT1/2 cells synthesize GalCer, a metabolite not normally produced in HEK293 cells. The molecular subspecies of GlcCer in both HEK293 and SPT1/2 cells had fatty acyl side chains ranging from C16:0 to C26:0, with the most abundant species being C16, C24:1, and C24:0 (Figure 2A). SPT1/2 cells have higher amounts of GlcCer than HEK293 cells, and the largest elevations are in C24:1 and C24:0 subspecies, which increase by 57% and 80%, respectively (Figure 2A and 2C). This differential change also reflected a shift in fatty acid chain length distribution in GlcCer of HEK293 versus SPT1/2 cells: in HEK293 cells, the proportions of C16 were nearly the same, whereas in
SPT1/2 cells, the proportion of C16 GlcCer was much lower than C24:1 and C24:0 (Figure 2A and 2C).

As noted, HEK293 cells synthesize no detectable GalCer (Figure 2B) but there are significant amounts in SPT1/2 cells (predominantly as the C16 and C18 subspecies) (Figure 2D). It is noteworthy that the most abundant species of GalCer in SPT1/2 cells (C16 GalCer) has the same ceramide backbone as the subspecies of GlcCer that decreased in SPT1/2 cells versus HEK293 cells. Therefore, perhaps one reason for the lower proportion of C16 GlcCer is that a portion of the C16 ceramide is being diverted to GalCer synthesis. Thus, the question is whether this shift in metabolism is due to upregulation of the enzyme that adds the galactose head group to ceramide (CGalT) and to some other factor, such as ceramide transport since it is known that this is synthesized on the cytosolic side of the ER (9,20,135) and transported predominantly through a non-vesicular, CERT dependent mechanism (37,38,136) to the Golgi apparatus, with the synthesis of GlcCer being thought to occur in the Golgi or en route to this organelle.

Expression and activity of CGalT and CGlcT in HEK293 and SPT1/2 cells

GalCer is synthesis is catalyzed by CGalT (61,62) and GlcCer synthesis is catalyzed by CGlcT (58-60). The lack of detectable GalCer in HEK293 cells could be due to lack of genetic expression of GalCer synthase. QRT-PCR analysis revealed that HEK293 cells express a comparable amount of CGalT mRNA to the CGalT mRNA expressed in SPT1/2 cells (Figure 3A). The lack of GalCer in HEK293 cells is therefore not due to lack of expressed CGalT. SPT1/2 cells express 85% less CGlcT in comparison with HEK293 cells. This decrease mRNA expression could be a regulatory in
transcription due to the influx of the precursor ceramide, and/or an abundance of the product GlcCer.

Expression of CGalT does not assure activity of GalCer synthase in HEK293 cells. A useful tool in detecting sphingolipid biosynthesis is NBD-Ceramide, which is trafficked and metabolized in similar way as natural ceramide (129). An in vitro assay providing the cells with NBD-Cer and UDP-Gal or UDP-Glc was used to analyze the activity of CGalT and CGlcT in HEK293 and STP1/2 cells. HEK293 cells synthesized NBD-GalCer when provided with NBD-Cer and UDP-Gal at a rate similar to the rate of NBD-GalCer synthesized by the SPT1/2 cells (Figure 3B). HEK293 and SPT1/2 cells synthesized NBD-GlcCer at a similar rate, but a rate that was more than twice that of NBD-GalCer synthesis (Figure 3B). This would be consistent with the finding that more GlcCer was synthesized in the cells than GalCer (Figure 2). Therefore, HEK293 cells not only express CGalT, but CGalT was active when provided with its substrates.

**Substrate Availability**

The HEK293 cells contain a functional CGalT, therefore, the absence of enzyme per se is not likely to be responsible for the absence of GalCer in these cells. The next most likely possibility is that the enzyme does not have access to UDP-Gal and/or ceramide because it is located in the lumen of the ER whereas these substrates are made in the cytosol and cytosolic leaflet of the ER, respectively (61). Unfortunately, relatively little is known about transport of these substrates into the ER, in contrast to the Golgi apparatus, which clearly has the ability to transport ceramide to luminal face of the Golgi (as seen by SM synthesis occurring in the lumen of the Golgi) (32,33) and the Golgi also
contains a UDP-Gal transporter (UGT1) that is used to transport UDP-Gal for synthesis of complex glycosphingolipids such as lactosylceramide (130). If substrate availability in the ER was the limiting factor for GalCer synthesis, treatment of the cells with BFA to merge the ER and Golgi should relieve the limitations, and this was clearly seen in HEK293 cells as well as several other cell lines.

Lack of availability of substrates in the ER for GalCer synthesis could be either due to lack of ceramide or to lack of UDP-Gal. Recently it has been discovered that the UDP-Gal transporter has two isoforms, one localized to the ER, and one localized to the Golgi (30). UDP-Gal is transported into the ER by UDP-Gal transporter UGT2, which has an ER localizing di-lysine motif (30). UGT2 is not a post-translational modification of UGT1, but was rather a splice variant (30), therefore, unique primers could be designed for UGT1 and UGT2. It was shown that HEK293 cells express UGT2 mRNA, they express more UGT2 than SPT1/2 cells (Figure 5A).

**Endoplasmic reticulum permeability**

This study so far has shown that HEK293 cells express GalCer synthase gene, and that this enzyme is functional is functional in intact cells when given access to its substrates, as seen in NBD-Cer *in vitro* assay and in BFA treated HEK293 cells. The evidence to date is most consistent with the lack of GalCer production in HEK293 cells where the activity was increased (but only for HEK293 cells and the production of GalCer) when semi-intact cells (which are known to have intact ER and Golgi) (127) were also sonicated to disrupt the intracellular membranes. This difference was not seen in SPT1/2 cells, for which there was no significant difference in NBD-GalCer production.
between the semi-intact and the sonicated cells (Figure 6A). The similarity of NBD-GalCer synthesis in SPT1/2 semi-intact and permeabilized cells suggests that GalCer synthesis in SPT1/2 is not due to a transporter in the ER that allows for substrate access to CGalT. If SPT1/2 cells contained a transporter that HEK293 cells lack, sonication would have increased the substrates access to CGalT, and therefore increased GalCer synthesis. However, no change in GalCer synthesis was observed, indicating that the ER of SPT1/2 cells were already permeable to GalCer substrates, and therefore sonication did not change substrate availability.

A permeable, or leaky, ER membrane could be a result of the increase of ceramide in SPT1/2 cells, because recent studies have implicated ceramide in membrane rearrangements that result in leaky membranes (137). Ceramides can influence flux by, lateral segregation of ceramide-rich domains in plane of the membrane due to their shape and high melting temperature (138). Ceramides promote negative curvature in lipid monolayers (138), segregate into ceramide-rich domains and, due to its higher melting temperature than SM, increases membrane rigidity when the rest of the membrane is fluid (138,139). These characteristics cause the co-existence of highly ordered and disordered domains, and the interface between these domains could allow release of vesicular contents (140,141).

In HEK293 cells there was an increase in NBD-GalCer synthesis when the cells were broken compared to the semi-intact cells at 20 and 30 min of incubation of semi-intact cells with NBD-C6-Ceramide (Figure 6D). This difference indicates that ER membranes in HEK293 cells were not leaky.
This study has shown that SPT1/2 cells synthesize not only more GlcCer than HEK293 cells, but also that they synthesize GalCer, a monohexosylceramide not synthesized in HEK293 cells. HEK293 cells have a functional galactosylceramide synthase, and synthesize GalCer when given access to substrates UDP-galactose and ceramide. Therefore, the lack of synthesis of GalCer in HEK293 is due to lack of substrate availability, but probably not the UDP-Gal substrate because HEK293 cells express UGT2, the UDP-Gal transport for the ER. Because semi-intact SPT1/2 cells showed no increase in NBD-GalCer synthesis after the cells were sonicated (versus HEK293 cells, which did increase in NBD-GalCer), this indicates that for SPT1/2 cells, the ER membrane may be more permeable to the substrates for GalCer. This may be due to the increase of ceramide in SPT1/2 cells because ceramides have the ability to disrupt membrane stability and allow efflux across membranes (138,139).

It surprising to us that every cell line that was tested (Hela, HepG2, and HL-60 cells) that do not produce GalCer under basal conditions displayed a dramatic increase in GalCer upon treatment with BFA – and as surprising, is that this reagent has been used in countless studies by other labs to study protein trafficking, synthesis, and structural changes in the cell (142) without noticing this effect. It is unclear why it is so common for cells to have a significant amount of “latent” CGalT activity even though GalCer is not normally being synthesized, however, this implies that cells may use GalCer synthesis as a response to a change in ER structure or function that need to be responded to by formation of this metabolite (for example, this may protect cells from accumulation of toxic amounts of ceramide). This should be explored in future studies.
This thesis investigated the regulation of the ceramide branchpoint to GlcCer versus GalCer beginning with studies of cells that have increased our basic biochemical knowledge about the regulation of GalCer and GlcCer, but also possibly sphingolipids in general, which could lead to useful findings about the biological process controlled by these compounds, such as neuronal cell growth and differentiation, myelination, and blood coagulation. Further studies of the regulation of ceramide partitioning into GalCer and GlcCer might also be helpful in diagnosis, treatment and prevention of glycosphingolipid storage diseases, multi-drug resistance cancers, and HIV-1 infection.
REFERENCES


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