HUMAN GLYCOLIPID TRANSFER PROTEIN GENE (GLTP) EXPRESSION IS REGULATED BY Sp1 AND Sp3: INVOLVEMENT OF THE BIOACTIVE SPHINGOLIPID CERAMIDE*

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Running title: Regulation of Human GLTP Expression by Ceramide

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Glycolipid transfer protein (GLTP) accelerates glycolipid intermembrane transfer via a unique lipid transfer/binding fold (GLTP-fold) that defines the GLTP superfamily and is the prototype for functional GLTP-like domains in larger proteins, i.e. FAPP2. Human GLTP is encoded by the single-copy GLTP gene on chromosome 12 (12q24.11 locus), but regulation of GLTP gene expression remains completely unexplored. Herein, the ability of glycosphingolipids (and their sphingolipid metabolites) to regulate the transcriptional expression of GLTP via its promoter has been evaluated. Using luciferase and GFP reporters in concert with deletion mutants, the constitutive and basal (225bp; ~78% G+C) human GLTP promoters have been defined along with adjacent regulatory elements. Despite high G+C content, translational regulation was not evident by the mammalian target of rapamycin pathway. Four GC-boxes were shown to be functional Sp1/Sp3 transcription factor binding sites. Mutation of one GC-box was particularly detrimental to GLTP transcriptional activity. Sp1/Sp3 RNA silencing and mithramycin A treatment significantly inhibited GLTP promoter activity. Among tested sphingolipid analogs of glucosylceramide, sulfatide, ganglioside GM1, ceramide-1-phosphate, sphingosine-1-phosphate, dihydroceramide, sphingosine, only ceramide, a non-glycosylated precursor metabolite unable to bind to GLTP protein, induced GLTP promoter activity and raised transcript levels in vivo. Ceramide treatment partially blocked promoter activity decreases induced by Sp1/Sp3 knockdown. Ceramide treatment also altered the in vivo binding affinity of Sp1 and Sp3 for the GLTP promoter and decreased Sp3 acetylation. This study represents the first characterization of any gltp gene promoter and links human GLTP expression to sphingolipid homeostasis through ceramide.

Glycolipid transfer proteins (GLTPs) are small (~24 kDa), soluble, single-polypeptide proteins that selectively accelerate the intermembrane transfer of glycolipids (1). Compared to other lipid transfer/binding proteins, human GLTP exhibits a unique conformational fold (2-4), serves as the prototype and founding member of the GLTP superfamily (5,6), and has a membrane interaction domain that differs from the C1, C2, FYVE, PH, and PX domains found in many peripheral and amphitropic proteins (7-11). The GLTP-fold occurs widely among eukaryotes (12) and endows larger proteins, i.e. phosphoinositol 4-phosphate adaptor protein-2 (FAPP2) (13), with key functionality during synthesis of complex glycosphingolipids (GSLs), which serve as important signaling and structural components of raft microdomains in plasma membranes (14-16).

Despite the significance of the GLTP-fold, the in vivo function(s) of GLTP remains unsettled. GLTP resides in the cytoplasm (17,18), a favorable location for interaction with newly synthesized glucosylceramide (GlcCer) generated by GlcCer synthase on the cytoplasmic face of the Golgi (19,20). However, GlcCer destined for higher GSL synthesis is transferred through the Golgi by FAPP2, which contains a C-terminal GLTP-like domain, rather than by GLTP (13). RNAi knockdown of GLTP in the presence of the vesicle trafficking inhibitor, brefeldin A, suggests a role in GlcCer trafficking to the plasma membrane (21). Yet, docking of GLTP with ER-associated VAP proteins also appears possible as well as action as an intracellular glycolipid sensor involved in GSL homeostasis (1,17,18).

In the present study, our goal was to evaluate GLTP gene expression within the context of GSL metabolic homeostasis by determining if alterations in key sphingolipid metabolites trigger changes in GLTP transcription, as regulated by its previously uncharacterized GLTP gene promoter. Recently, we characterized human GLTP, a single-copy gene...
on chromosome 12 (12q24.11) (12). GLTP mRNA matures via classic cis-splicing into 5-exon transcripts, a highly conserved organizational pattern in therian mammals and other vertebrates (12). The discovery of an unusually G+C-rich CpG island in the 5'UTR of GLTP indicated possible regulation by transcriptional factors that bind to GC boxes, e.g. Sp1/Sp3 (22,23). The present study provides the first insights into human GLTP transcriptional regulation, including characterization of constitutive and basal GLTP promoter and adjacent regulatory regions. Promoter analyses using luciferase and GFP reporters as well as in vivo analyses by real-time PCR and other approaches show GLTP regulation via mechanistic participation of Sp1/Sp3 transcription factors in a manner influenced by ceramide, but not by related sphingolipid metabolites.

 Experimental Procedures

Cell culture conditions, promoter construct and deletion mutant preparation, epifluorescence microscopy, protein immunoprecipitation and Western blot analysis, and statistical analyses are detailed in on-line Supplemental Information.

5'-Rapid Amplification of cDNA Ends Assay (RACE)—Total RNA was isolated from HeLa cells using Trizol reagent (Invitrogen, Carlsbad CA). Transcriptional start sites were identified by FirstChoice RLM-RACE (Ambion Inc, Austin TX). Master-Amp™ Tth DNA polymerase and PCR enhancer (Epicentre, Madison WI) were used for 70°C reverse transcription. Herculase® II Fusion DNA Polymerase (Stratagene, La Jolla CA) supplemented with betaine (Sigma-Aldrich) was used for standard PCR amplification. Primer Ra1 and Ra2 were used for first- and second-round PCR amplifications. Reaction products were separated by 1.2% agarose gel electrophoresis before cloning in pGEM-T (Promega, Madison WI) and sequencing (Genewiz). Basal promoter (pGL3−350/+19) was used as template for amplification.

 Dual Luciferase Assays—HEK 293T, HeLa, and T47D cells, grown to ~60% confluency, were transfected with firefly luciferase construct (0.6 µg) and internal control Renilla luciferase construct, pRL-TK (20:1 ratio). Luciferase activities were measured after 48 h (Promega). pGL3-basic served as promoterless negative control.

 Electrophoretic Mobility Shift Assays (EMSA)—5'-Biotin-labeled, single-strand probes were synthesized by Sigma. Double-stranded oligonucleotide probes were generated by incubating equimolar amounts of complementary oligonucleotides in STE annealing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA) for 3 min at 95°C, slow cooling to room temperature, and storing (−20°C). EMSA reaction mixtures were incubated in 5Xbinding buffer (Promega) on ice for 10 min with or without unlabeled competitor, prior to adding end-labeled oligonucleotides for 20 min on ice. For supershift assays, HeLa cell nuclear extracts (Promega) were incubated on ice for 10 min with anti-Sp1 (PEP-2) and anti-Sp3 (D-20) antibody (Santa Cruz Biotech, CA) prior to addition of end-labeled oligonucleotides for 30 min on ice and electrophoresis on a 5% nondenaturing polyacrylamide gels in Tris-borate-EDTA buffer (0.5x) at 70V for 30 min at 4°C and then at 120V for ~60 min. Binding reactions were analyzed by transferring to Biodyne B pre-cut modified nylon membrane (Pierce, Rockford, IL) at 350mA for 60 min, cross-linking for 5 min using UV-light (312 nm bulb), and fixing at 80°C for 60 min before detection with biotin-labelled DNA using Light-shift electrophoretic mobility shift reagent (Pierce). Antibody-protein complexes were observed as supershifted or immunodepleted complexes.

 Chromatin Immunoprecipitation Assays (ChIP)—Chromatin isolated from HeLa cells was used in ChIP assays performed by manufacturer’s instructions (Upstate Biotech., Lake Placid NY). For amplification of GLTP promoter, primer pairs CH-1/CH-2 (first round) and CH-3/CH-4 (second round) were used for nested PCR. Primer pair Ne-1/Ne-2 (first and second PCR rounds), designed to amplify +489/+707 of human GLTP exon 5, served as control lacking Sp1/Sp3 binding sites. Herculase® Polymerase plus betaine, was used for PCR amplification. Cycling conditions were: 2 min at 98°C; 25 cycles of 98°C for 20s, 63°C for 20s, and 72°C for 30s; and final extension at 72°C for 3 min.
**RNA Interference**—eIF4E translation was silenced by transfecting HeLa cells with eIF4E siRNA (Cell Signaling, Danvers MA; 25nM) using TransIT-siQUEST™ (Mirus, Madison WI) and analyzing for exogenous protein expression level after 48 h. Sp1/Sp3 siRNA (Santa Cruz Biotech) were used to knockdown Sp1 or Sp3. Nonspecific siRNA served as control.

**Lipid Effects on GLTP Promoter Activity**—HeLa cells were transfected with different plasmid constructs for 24 h and then replenished with fresh medium. C6-ceramide, C6-dihydroceramide, C8-ceramide-1-phosphate, C18-sphingosine, C18-sphingosine-1-phosphate, C2-glucosylceramide, ganglioside GM1 or C12-3-sulfo-GalCer (Avanti Polar Lipids, Alabaster AL), dissolved in DMSO, were added to the medium to final concentrations of 5µM and 10 µM. Luciferase activity was measured using the dual luciferase reporter assay system 24 h after lipid treatment. To assess the regulatory role of acetylation, HeLa cells were transfected with pGL3(−1150/+19) and, after 24 h, treated with trichostatin A (TSA; dissolved in DMSO and added to medium to 100ng/ml final concentration), or with C6-ceramide (10µM) and TSA for 24 h. Luciferase activity was measured using the dual luciferase reporter assay system.

**Real-time RT-PCR**—Total RNA, isolated from HeLa cells using RNeasy Plus Mini-kits (Qiagen, Valencia, CA) and Trizol reagent, was reverse transcribed with Superscript III (Invitrogen). Real-time RT-PCR was performed using TaqMan Gene Expression assays (IDs = Hs00829505_g1 for human GLTP gene and Hs99999903_m1 for β-actin gene; Applied Biosystems, Foster City, CA).

**RESULTS**

**Human GLTP Transcriptional Start Sites**—RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) is designed to utilize only full-length, capped mRNA during amplification of cDNA, facilitating accurate identification of transcriptional start sites (TSS) (25,26). To identify the TSSs of human GLTP, we performed RLM-RACE PCR using mRNA from HeLa cells and GLTP-specific primers (Table S1). A single cDNA band of −400 bases was obtained and cloned into T vector for DNA sequencing (Fig S1). Ten randomly selected clones revealed TSSs located 26 bases (TSS1, 7 clones) and 24 bases (TSS2, 3 clones) from the translation initiating ATG codon of human GLTP (Fig. 1). Similar results were obtained with HEK 293T cells (data not shown), indicating that human GLTP can be transcribed from more than one start sites but that TSS1 represents the major start site.

**Human GLTP Promoter**—To define the human GLTP promoter, the −1150/+19 region (relative to major transcriptional start site) was inserted into promoterless phrGFP. Figure 2A shows that cells transfected with phrGFP(−1150/+19) became fluorescent after 48 h, but not when mock-transfected with empty phrGFP. Similar results were obtained in HeLa and HEK 293T cells, indicating a functional promoter in the −1150/+19 region of human GLTP gene.

Next, the basal and proximal regulatory regions of the GLTP promoter were characterized by constructing a series of luciferase reporter plasmids containing 5’ deletions of the putative (−1150/+19) promoter region and transfecting into HeLa, HEK 293T and T47D cells along with Renilla luciferase reporter as internal control. Deletion of sequence between −1150 and −350 increased transcriptional activity by 2- to 3-fold over pGL3(−1150/+19) in the different cell lines (Fig 2B), suggesting a negative regulatory region upstream of −350. In contrast, pGL3(−1150/+200) transcriptional activity was higher than that of pGL3(−1150/+19), consistent with a positive regulatory region between −350 and −126, is the GLTP basal promoter. Further analyses, involving 3’ deletions of the putative promoter, were performed in different cell lines (Fig 2C). Compared to pGL3(−350/+19), promoter activity for pGL3(−350/−154) decreased ~30% in HEK 293-T cells and ~55% in T47D cells. With pGL3(−350/−195), promoter activity also decreased in HeLa (~15%), HEK 293T (~30%) and T47D cells (~52%), suggesting important regulatory elements are located in the −350/−91 region.

**GLTP Expression Is Not Regulated by the Mammalian Target of Rapamycin (mTOR) Signaling Pathway**—A notable characteristic of human GLTP promoter is the extremely high G+C content, which is 79.54% for −416/+19 and 76.13% for the entire CpG island. Human GLTP also contains an extremely long first intron and the 5’ and 3’ regions of the first exon are highly G+C rich.
These features raised the issue of whether human GLTP falls into a gene class regulated at the translational level (27). With such mRNAs, the highly structured 5'-UTRs require eIF4E binding to the mRNA cap structure to mediate initiation of translation, thus facilitating efficient scanning and start codon recognition and enhancing translation of these mRNAs (27,28). As shown in Fig. 3A and 3B, neither RNAi suppression of eIF4E by 3- to 4-fold nor rapamycin treatment decreased GLTP levels despite phospho-4E-BP1 (Ser65) decrease, which enhances eIF4E and 4E-BP1 binding, preventing assembly into EIF4F complex and inhibiting cap-dependent translation. The results show that GLTP gene expression is not regulated by the mTOR signaling pathway.

Sp1 and Sp3 Bind to Multiple Sites in Human GLTP Promoter and Silencing Reduces Activity—Bioinformatics analyses indicated that the 225bp region (−350/−126) lacked canonical CCAAT and TATA boxes but contained consensus binding sites for various other transcription factors including Sp1/Sp3. To determine whether GC-boxes functioned as viable binding sites for Sp1 and Sp3, nuclear extracts from HeLa cells were analyzed by EMSA. As shown in Figure 4, bound biotin-labeled probe showed several labeled bands. Binding specificity was confirmed by the significantly decreased intensity resulting from incubation with 100-fold excess of unlabeled probe and the lack of intensity change by incubation with a 100-fold excess of unlabeled mutated probes. A major, low-mobility Sp1 complex was observed, which super-shifted upon addition of Sp1-specific antibody (Fig. 4). Furthermore, in the presence of Sp3-specific antibody, the super-shifted band became very faint, the intensity of the major complex decreased, and the lower Sp3 complex disappeared, indicating competitive binding by Sp3. When both Sp1 and Sp3 antibodies were added, the intensity of the major complex was dramatically decreased and the lower Sp3 complex again disappeared (Fig. 4). The binding affinity hierarchy for Sp1/Sp3 was: −191/−186 > −270/−265 > −219/−214 > −154/−149 based on staining density. Collectively, the EMSA analyses demonstrate that the GC-boxes located on the GLTP proximal promoter do serve as binding sites for Sp1 and Sp3.

The in vivo binding status of Sp1 and Sp3 was assessed by chromatin immunoprecipitation (ChIP). As shown in Figure 5A, human GLTP promoter was immunoprecipitated by either Sp1 or Sp3 antibody, but not by β-actin antibody. Plasmid controls as well as sheared and cross-linked input DNA served as templates for positive control bands (Fig. 5A). No signal was observed using a control primer pair specific for GLTP exon 5 region (Fig. 5A). The results clearly show in vivo binding of Sp1/Sp3 to the proximal region of GLTP promoter.

To directly assess the effect of Sp1/Sp3 on GLTP promoter activity, siRNA was used to knock down Sp1 and Sp3 expression (Fig. 5B). Down-regulation of Sp1/Sp3 expression resulted in 27~35% reduction in GLTP promoter activity (Fig. 5C). Treatment with mithramycin A, a drug that binds GC-rich regions of DNA and blocks Sp1 binding (29), also reduced GLTP promoter activity (Fig. 5C). Taken together, the data show regulation of human GLTP gene expression by Sp1/Sp3.

Sp1/Sp3 Binding Site Mutation at −219/−214 of GLTP Promoter Inhibits Transcriptional Activity—The contribution of representative Sp1/Sp3 binding sites were defined by site-directed mutagenesis. Because previous EMSA indicated that the mutant oligonucleotide duplexes could not compete for transcription factor binding, we concluded these mutant probes could not bind transcription factors Sp1/Sp3. As shown in Fig. 6A, site mutation of −219/−214 had a strong negative effect on GLTP promoter activity; whereas mutations at −270/−265, −191/−186 and −154/−149 had only mild effects on promoter activity. Interestingly, in T47D cells, significantly decreased GLTP promoter activity resulted from mutation at −191/−186, but not in HeLa cells and HEK 293T cells (Fig. 6A). BLAST searches of the GLTP 5’ flanking sequence of Homo sapiens and Macaca mulatta showed conservation of all four Sp1/Sp3 binding sites except for a single base substitution at the M4(−154/−149) binding site (Fig. 6B). Thus, the −219/−214 Sp1/Sp3 binding site, but not the −270/−265, −191/−186 and −154/−149 sites, is a key regulator of human GLTP promoter activity.

Ceramide Increases Human GLTP Promoter Activity & In Vivo Transcript Levels—Over the past decade, important signaling roles have emerged for nonglycosylated and glycosylated sphingolipids (15). To determine if various glycosylated and nonglycosylated sphingolipids regulate GLTP promoter activity, HeLa cells were transfected with pGL3(−1150/+19) and then incubated with either C6-ceramide, C6-dihydroceramide, C8-ceramide-1-phosphate, C18-sphingosine, C18-sphingosine-1-
phosphate, C<sub>6</sub>-glucosylerceramide, ganglioside GM<sub>1</sub> or C<sub>12</sub>-3-sulfo-GalCer. Fig. 7A shows that only C<sub>6</sub>-ceramide significantly increased pGL3(−1150/+19) luciferase activity (~20-47%). C<sub>6</sub>-ceramide treatment also: i) elevated GLTP transcript levels in vivo (Fig. 7B); ii) elevated endogenous long-chain ceramide levels (Fig. 7C); iii) mitigated decreases in GLTP promoter activity induced by Sp1/Sp3-siRNA (~58-61%) or by mithramycin A (~42-66%) (Figs. S2A & S2B).

Sp1 and Sp3 Involvement in Ceramide-Induced Up-Regulation of GLTP—Truncations of the GLTP promoter enabled mapping of the ceramide-response region. As shown in Fig. 8A, ceramide treatment increased the activity of pGL3(−284/+19) by ~20%, but had no effect on pGL3(−213/+19) or pGL3(−126/+19), suggesting that the −350/−213 region contains a ceramide response element. To gain further insights in vivo, the effect of ceramide treatment on expression levels of endogenous Sp1 and Sp3 was assessed, but no significant changes were observed (Fig. 8B). However, ChIP analyses revealed that ceramide treatment alters the in vivo binding affinity of Sp1 and Sp3 for GLTP, resulting in lower Sp1 binding but higher Sp3 binding (Fig. 8C). Because acetylation of Sp3 and Sp1 is known to regulate their binding (30-32) and ceramide treatment can alter Sp acetylation status (33, 34), we used trichostatin A (TSA) to increase Sp acetylation levels and found GLTP promoter activity to be diminished by ~60-65% (Fig. 8D). TSA alters Sp3/Sp1 acetylation by inhibiting lysine deacetylases, originally referred to as histone deacetylases (31, 35, 36). Ceramide treatment attenuated the TSA-induced loss of GLTP promoter activity (Fig. 8C) and significantly reduced in vivo levels of acetylated-Sp3 (Fig. 8E) without affecting acetylation status of Sp1 (Fig. S3). Collectively, the data show regulation of GLTP expression via Sp1/Sp3 by a complex mechanism that responds to elevated ceramide levels.

**DISCUSSION**

This investigation represents the first characterization of any gltp gene promoter and provides the first insights into the regulation of human GLTP gene expression. BLAST searches show the GLTP promoter to be highly conserved in primates, but less so in carnivora, cetartiodactyla and rodents, suggesting relatively recent and potentially important evolutionary developments within the human GLTP promoter. Our earlier study of GLTP gene organization, tissue transcript levels, and phylo-genetic/evolutionary relationships revealed orthologs in all vertebrate genomes and encoded within a highly conserved 5-exon/4-intron mRNA organizational pattern (12). In humans, two single-copy genes occur. The 12q24.11 gene (GLTP) accounts for all detectable GLTP transcript (12). In contrast, a complete GLTP ORF (94% homology) on human chromosome 11 (11p15.1) and present only in primates, is a transcriptionally-silent pseudogene (GLTPP1) based on methylation analyses of CpG islands and well-controlled PCR analyses. An unexpected outcome was the discovery of a 5′ UTR in GLTP with a CpG island unusually G+C rich. The high number of GC boxes revealed by on-line Transcription Element Search System (TESS) analysis of the GLTP promoter prompted our focus on regulation by Sp1/Sp3.

The very high G+C content (~76% for entire CpG island, including exon 1 ORF; ~80% for −416/+19) also raised the issue of whether GLTP might belong to a class of genes that are very efficiently transcribed (37) and translationally regulated in mammalian cells. In such genes, the G+C-rich 5′-UTRs are highly structured and require eIF4E binding to the mRNA cap to initiate translation (27, 28). However, GLTP levels were unaffected by RNAi knockdown of elf4E or by rapamycin treatment, indicating a lack of significant regulation by the mammalian target of rapamycin (mTOR) signaling pathway. The outcome could reflect the rather short 5′ UTR length of GLTP transcript. RLM-RACE PCR revealed more than one transcription start site, consistent with the observed lack of canonical CCAAT and TATA boxes (38, 39). Nonetheless, the major start site (TSS1) in 7 of 10 clones was only 26 bp upstream of the mRNA start codon. Such a short 5′ UTR length could help avoid highly structured conformation(s) and the need for elf4E involvement in mediating initiation of GLTP translation.

By luciferase reporter analyses, we demonstrated an 1169bp (−1150/+19) region relative to TSS1 to be transcriptionally active in HeLa, HEK 293T and T47D cells. 5′ and 3′ mutational deletion analyses suggested a negative regulatory region upstream of −350, a positive regulatory region (+19/+200), and a basal GLTP promoter (−350/−126). The 225bp core promoter is active in HeLa, HEK 293T and T47D cells. However, among these cells, differential regulation involving regions upstream of the core promoter may occur (Figs. 2 & 6). Future studies will be needed to elucidate the molecular
basis of the differences as well as the role of other predicted transcription factor sites (including other Sp1/Sp3 sites) in the regulation of GLTP promoter in a tissue-specific context.

What is clear is that the GLTP promoter can be regulated by Sp1/Sp3 as shown by the 27–35% reduction in activity by Sp1/Sp3 knockdown by RNAi or by mithramycin A treatment. Sp1 and Sp3 interaction with GLTP GC-boxes is evident in vitro and in vivo as assessed by EMSA and ChIP, respectively. Sp1 binding to GC-box elements is known to occur via zinc finger domains and enables RNA polymerase II binding to the transcription initiation site in TATA-boxless promoters (40-43) such as occurs in GLTP. More than 20 potential Sp1/Sp3 binding sites are predicted by the on-line TESS program within the −350/−213 region that also could contribute to the ceramide response. Our EMSA analyses establish the following hierarchy of binding affinity among the four sampled GC-boxes: (−191/−186 > −270/−265 > −219/−214 > −154/−149). However, GC-box mutation indicated that site −219/−214 is essential for maximal GLTP promoter activity.

Because Sp transcription factors regulate many housekeeping, tissue-specific, viral, and inducible genes, involvement of Sp1/Sp3 in GLTP promoter regulation is hardly surprising given its high G+C content. While Sp3 can either activate or repress, Sp1 more often activates gene promoters. For GLTP promoter, Sp1 and Sp3 both serve as activators, as indicated by RNAi knockdown and mithramycin A treatment. Adding to the complexity is the potential role of Sp1-like KLF proteins (44) and co-regulatory transcription activator factors (TAFs), able to bind to the glutamine and serine/threonine-rich regions of Sp1 proteins and be recruited to the multi-protein preinitiation complex during interaction with gene promoters. TAFs are known to confer cell-type-specific promoter selectivity, but their involvement in the regulation of the GLTP promoter remains to be determined (45,46). It is clear that the involvement of these other factor(s) in GLTP transcriptional regulation cannot be excluded.

We were surprised by the lack of GLTP promoter regulation by monoglycosylated ceramides (GlcCer or sulfatide) because of GLTP’s ability to transport simple glycosylated sphingolipids in vivo (21). Instead, nonglycosylated C6-ceramide induced significantly increased GLTP expression, an effect not duplicated by dihydroceramide, ceramide-1-phosphate, sphingosine or sphingosine-1-phosphate. Treatment of cells with C6-ceramide elevates endogenous long-chain ceramide levels by as much as 10-fold by 6 h while maintaining 5-fold elevation at 24 h (47). The elevation in endogenous ceramide is a consequence of remodeling by the sphingolipid salvage pathway (48). Treatment of cells with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of GlcCer synthase known to elevate endogenous ceramide levels (49-51), also increased GLTP promoter activity (Fig. S4A) and elevated transcript levels (Fig. S4B). However, treatment with fumonisin B1, which is known to reduce endogenous ceramide levels (52-54), did not affect human GLTP expression, as assessed by promoter (luciferase) activity (Fig. S4C) and GLTP transcript levels (Fig. S4D).

It is noteworthy that elevated ceramide not only up-regulates GLTP promoter activity but also mitigates decreases in promoter activity induced by Sp1/Sp3 knockdown. Interestingly, ceramide treatment does not alter endogenous levels of Sp1 and Sp3 but rather, their binding affinity for the GLTP promoter. Furthermore, in the case of Sp3, the altered binding affinity can be linked to ceramide-induced changes in acetylated Sp3 levels in vivo. Thus, our results suggest that ceramide treatment can affect the GLTP promoter in two ways: i) by altering Sp1/Sp3 binding affinity and ii) by altering Sp3 acetylation status. Our findings are supported by recent work showing that ceramide regulates transcriptional activity of human telomerase reverse transcriptase in A549 human lung adenocarcinoma cells by changes in Sp3 acetylation status (55). The ability of ceramide treatment to alter gene expression by promoter regulation has also been noted for human glucosylceramide synthase, c-myc, surfactant protein B, and matrix metalloproteinase 2 (e.g., 56-61).

From the standpoint of GSL metabolism, it is noteworthy that the promoter activity of the GlcCer synthase gene increases in response to C6-ceramide treatment by a mechanism involving Sp1 (52,54). A ceramide-stimulated increase in GlcCer synthase, involving coordinate elevation of GLTP gene expression, could provide an effective means for adjusting intracellular ceramide levels while maintaining cellular GSL homeostasis. Increased GLTP would be expected to compete with FAPP2, which normally transfers newly synthesized GlcCer from the cis medial Golgi using its pleckstrin homology.
(PH) domain to target the trans Golgi, the site of complex GSL synthesis (13). The lack of a PH domain in GLTP minimizes specific targeting to the trans Golgi. Thus up-regulation of GLTP could enable GlcCer to be siphoned away from the trans Golgi, preventing elevation of downstream GSL levels in the biosynthetic pathway, from lactosylceramide to complex gangliosides. In such a way, elevated GLTP levels could help to maintain complex GSL homeostasis during periods of elevated ceramide levels.

Implications. The link that we observe between sphingolipid homeostasis and GLTP expression via ceramide-responsive, Sp1/Sp3-mediated, transcriptional regulation of GLTP is an intriguing development, given the lack of effect elicited by other powerful sphingolipid signaling metabolites, i.e. ceramide-1-phosphate, sphingosine-1-phosphate, or sphingosine. The characterization of the human GLTP promoter and the fundamental insights into human GLTP transcriptional regulation undoubtedly will aid future elucidation of normal and pathological conditions involving GLTP expression.

REFERENCES


**FOOTNOTES**

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2Abbreviations: GLTP, glycolipid transfer protein; Sp1, specific protein-1; Sp3, specific protein-3; FAPP2, phosphoinositol 4-phosphate adaptor protein-2; GlcCer, glucosylceramide; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; GalCer, galactosylceramide; RT, reverse transcription; TSS, transcriptional start site; TSA, trichostatin A.

3GenBank Accession Number GU971358
FIGURE LEGENDS

Figure 1. Nucleotide sequence of the human GLTP promoter and the downstream region. The major transcriptional start site is indicated by the arrow at position +1. The coding sequence of the first exon is shown in uppercase letters with the corresponding amino acid sequence below. Sp1/Sp3 transcription factor binding sites are underlined. The predicted CpG island is boxed. Regions ≥ 15 bases in length with 100% G+C content are shaded.

Figure 2. Human GLTP promoter functional characterization. (A) Identification of GLTP promoter. Epifluorescence micrographs of HEK 293T cells transfected with phrGFP (−1150/+19), or phrGFP (negative control) for 48 h. DAPI, 4',6-diamidino-2-phenylindole (300nM) was used to stain the nucleus. The scale bar represents 20 µm. (B) 5’ deletion analysis of GLTP promoter in HeLa, HEK 293T, and T47D cells. Cotransfection of Renilla luciferase plasmid (pRL-TK) served as internal control for normalization of firefly luciferase activity. Numbering is relative to the major transcriptional start site. (C) 3’ deletion analysis of the (−350/+19) region of GLTP promoter in the same three cell lines. Control pGL3(−350/+19) values are set to 100 for all three cell lines. Luciferase activity was normalized to Renilla luciferase activity. Numbering is relative to the major transcriptional start site. Bars represent the means±SE of three to six determinations. *, p<0.05.

Figure 3. Analyses of translational regulation of GLTP gene expression. (A) Suppression of eIF4E by RNAi does not significantly decrease GLTP protein levels. 1. Control siRNA; 2. eIF4E-specific siRNA. (B) Rapamycin does not significantly inhibit human GLTP expression. HeLa cells were grown to ~60% confluency before adding rapamycin (100 nM in 0.1% DMSO) for 2, 4 and 6 h. Lysates were subjected to Western blot analysis using anti-GLTP, anti-Phospho-4E-BP1 (Ser65) and anti-β-actin. Lane 1: no treatment; Lane 2: DMSO (0.1%), 2 h; Lane 3: rapamycin, 2 h; Lane 4: DMSO (0.1%), 4 h; Lane 5: rapamycin, 4h; Lane 6: DMSO (0.1%), 6 h; Lane 7: rapamycin, 6 h. The density of GLTP protein bands was determined using the Quantity One program. eIF4E & GLTP levels were normalized to β-actin levels (internal control). Bars represent the means±SE of three to six determinations. **, p<0.01.

Figure 4. EMSA showing Sp1/Sp3 transcription factor binding to four sites in human GLTP promoter. Gel supershift analyses were performed with HeLa nuclear extracts and biotin-labeled probe. Competitive gel shift assays were performed in the presence of 100-fold molar excess of unlabelled double-strand oligonucleotides. Supershift analysis using specific antibodies anti-Sp1, anti-Sp3, and control assays were performed without antibody. The arrows show specific binding. Asterisks (*) indicate supershifted bands in B, C, D and E. (A) Locations of EMSA probes. Numbering is relative to the major transcriptional start site. Sp1/Sp3 transcription factor binding sites are underlined. Primers EM1, EM2, EM3, and EM4 are boxed. Asterisks (*) show mutated nucleotides position in primers M1, M2, M3, and M4. (B) EMSA for −270/−265 binding site. (C) EMSA for −219/−214 binding site. (D) EMSA for −191/−186 binding site. (E) EMSA for −154/−149 binding site.

Figure 5. Regulation of human GLTP promoter by Sp1 and Sp3 transcription factors. (A) Sp1 and Sp3 binding to GLTP promoter in vivo. ChIP assays were performed using DNA from HeLa cells as sources of human GLTP promoter and specific antibodies for Sp1, Sp3 or β-actin. GLTP promoter region (−350/−115) containing putative Sp1/Sp3 binding sites was amplified by nested PCR as described in Materials and Methods. Human GLTP exon 5 fragment (+489/+707), amplified using primer pair Ne-1/Ne-2, served as control lacking Sp1/Sp3 binding sites. Amplification controls were: cross-linked, sheared DNA prior to immunoprecipitation (input); plasmid carrying (−350/−115) or (+489/+707) was used as template (plasmid). (B) & (C) Downregulation of Sp1/Sp3 by siRNA knockdown reduces GLTP promoter activity. Cells transfected with pGL3(−1150/+19) were grown for 24 h, were transfected with Sp1/Sp3 siRNA (25 nM), and were grown for an additional 24 h before Western blot analysis and measurement of luciferase activity. (D) Mithramycin A treatment downregulates Sp1/Sp3 expression and decreases GLTP promoter activity. Cells transfected with pGL3 (−1150/+19) were grown 24 h, were treated
with mithramycin A (0, 100, 200 or 500 nM) for 24 h, and were analyzed for luciferase activity (normalized to Renilla luciferase activity). Vehicle, 0.1% DMSO. Bars show the means±SE of three to six determinations in HeLa cells. *, p<0.05; **, p<0.01.

Figure 6. Mutation of Sp1/Sp3 binding site at −219/−214 is detrimental to GLTP promoter activity. (A) Mutational analysis of the (−350/+19) region of human GLTP promoter. Luciferase activities obtained with binding site mutations correspond to M1 (−270/−265), M2 (−219/−214), M3 (−191/−186), and M4 (−154/−149), respectively. Control pGL3(−350/+19) values are set to 100 for all three cell lines and were normalized to Renilla luciferase activity. Bars represent the means±SE of three to six determinations. *, p<0.05; **, p<0.01. (B) Alignment of the GLTP partial promoter regions of Homo sapiens and Macaca mulatta showing the conservation of transcription factor binding sites. Numbering is relative to the major transcriptional start site.

Figure 7. Ceramide increases human GLTP promoter activity. Elevated endogenous ceramide level increases GLTP expression (A) Comparison of the effects of various sphingolipids on GLTP promoter activity. HeLa cells were transfected with pGL3(−1150/+19) and then treated with different lipids after 24 h. Luciferase activity was measured 24 h after lipids treatment and was normalized to Renilla luciferase activity. (B) Real-time PCR analyses of ceramide-induced up-regulation of GLTP transcript levels in vivo. Cells were grown to approximately 60% confluency and then were treated with 0 µM, 5 µM or 10 µM C₆ ceramide for 3, 6 or 12 h before harvesting for real-time RT-PCR analyses. GTLP mRNA levels were normalized to β-actin mRNA levels. (C) Effect of C₆-ceramide on endogenous ceramide levels. Cells were grown to approximatly 60% confluency and then Cells treated with 10 µM C₆-ceramide for 24 h, then were analyzed for their ceramide species content by HPLC-MS as described in the Supplemental Experimental Methods. Results were normalized to cell lipid phosphate. GTLP mRNA levels were normalized to β-actin mRNA levels. Cer, Ceramide; Bars represent the means±SE of three to six determinations, *, p<0.05 compared with cells treated with vehicle (0.1% DMSO), respectively. **, p<0.01.

Figure 8. Mechanistic link between Sp1/Sp3 and ceramide-induced up-regulation of human GLTP promoter activity. (A) Deletion analyses of GLTP promoter to localize ceramide response region. HeLa cells were transfected with different constructs for 24 h and then treated with C₆-ceramide (10 µM). Luciferase activity was measured 24 h after lipid treatment. *, p<0.05 compared with cells treated with vehicle (0.1% DMSO), respectively. Luciferase activity was normalized to Renilla luciferase activity. (B) Ceramide treatment does not alter the endogenous levels of Sp1 or Sp3. HeLa cells were grown with or without C₆-ceramide (10 µM) for 24 h. Sp1, Sp3 and β-actin levels in cell extracts were analyzed by Western blot analysis. (C) Ceramide treatment alters the Sp1 or Sp3 binding affinity to GLTP promoter. ChIP assays were performed using DNA from HeLa cells, treated with vehicle (DMSO) or C₆-ceramide (10 µM) for 24 h. Immunoprecipitation of DNA and Sp1/Sp3 complexes were performed using antibodies specific for Sp1 and Sp3. Amplification of GLTP promoter region(−350/−115) was performed as described in experimental procedures. (D) TSA-induced decrease in GLTP promoter activity is partially blocked by ceramide. HeLa cells were transfected with pGL3(−1150/+19) for 24 h and then treated with TSA (100 ng/ml) and C₆-ceramide (10 µM). After 24 h, luciferase activity was measured. *, p<0.05. (E) Ceramide decreases levels of acetylated Sp3. HeLa cells were grown with or without C₆-ceramide (10 µM) for 24 h. After immunoprecipitation with Sp3 antibody, acetylated Sp3 levels were determined by Western blot analysis (acetylated-lysine antibody). Specific binding is indicated by preclearing with beads containing no antibody. Vehicle, 0.1% DMSO; Cer, Ceramide. Bars represent the mean±SE of three to six determinations.
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

D

GLTP Promoter
(-350/-115)

GLTP gene
(+489/+707)

Control siRNA
Sp1 siRNA

Control siRNA
Sp3 siRNA

Control siRNA
Sp1 siRNA
Sp3 siRNA

Control siRNA
Sp1 siRNA
Sp3 siRNA

Relative Luciferase Activity (%)  

pGL3(-1150/+19)

Relative Luciferase Activity (%)  

pGL3(-1150/+19)

Vehicle
100nM
200nM
500nM

Mithramycin A

β-Actin

β-Actin

* * *
Figure 6
Figure 8
Human glycolipid transfer protein gene (GLTP) expression is regulated by Sp1 and Sp3: Involvement of the bioactive sphingolipid ceramide
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