Glycosphingolipid deficiency increases the sterol regulatory element-mediated gene transcription

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ABSTRACT

A major feedback mechanism of cholesterol in transcription of cholesterol metabolism-related genes is mediated by sterol regulatory element-binding protein (SREBP). Involvement of glycosphingolipids (GSLs) in the SREBP pathway is unknown. In this study, we examined the effects of GSL depletion on SRE-mediated gene transcription using GSL-defective cells. We found that the content of mature SREBP, the transcriptional active form, is increased in the GSL-defective cells. Transcription of SREBP target genes and cholesterol synthesis are also induced in the GSL-defective cells. These results indicate that GSL deficiency up-regulates the SREBP pathway, pointing out the regulatory role of GSL in cholesterol homeostasis.

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that play a crucial role in cellular cholesterol homeostasis. When cholesterol is supplemented to cells, precursor forms of SREBPs localize in ER where they form a complex with SCAP (SREBP cleavage-activating protein). Upon cholesterol depletion, the SREBP/SCAP complex is transported to the Golgi apparatus and SREBPs are processed by two proteases. The cleaved SREBP fragment (mature form) is then translocated to the nucleus. In nucleus, SREBPs bind to SREs, cis-acting elements found in the promoter region of a group of genes implicated cholesterol synthesis and lipid metabolism [1,2].

Sphingolipids are the major lipid components of the plasma membrane of mammalian cells. Sphingolipids share common ceramide backbone to which phosphocholine is conjugated in the case of sphingomyelin whereas various sugar chains are attached in glycosphingolipids (GSLs). Scheek et al. reported that the SREPB pathway is down-regulated when cells are treated with sphingo-

myelinase [3]. It is suggested that decreasing plasma membrane sphingomyelin content induce transport of cholesterol to the ER [4,5] and a subsequent suppression of SREBP cleavage [3]. The molecular mechanisms of sphingomyelinase-dependent cholesterol translocation are not clear. Sphingomyelin hydrolysis also results in the generation of ceramide. Worgall et al. showed that increasing ceramide through exogenous sphingomyelinase, addition of ceramide analogs, and inhibition of endogenous ceramidase decreased SRE-mediated gene transcription [6]. They also reported that de novo ceramide synthesis correlates with the SREBP pathway; that is, inhibition of ceramide synthesis decreases the cleavage of SREBP and SRE-mediated gene transcription [7]. In contrast, Lawler et al. showed that ceramide accelerates the maturation of SREBP during TNF-α induced neutral sphingomyelinase activation [8]. They also showed that the degradation of cell surface sphingomyelin by bacterial sphingomyelinase activates SREBP cleavage [8]. Compared to sphingomyelin and ceramide, little is known about the effect of GSLs on the SREBP pathways.

In this study, we examined the effects of GSL deficiency on SRE-mediated gene transcription. For this purpose, we employed GSL-defective melanoma mutant cells [9], GM95 cells. GM95 is deficient in ceramide glucosyltransferase (GlcT-1), which catalyzes the formation of glucosylceramide, the core structure of major GSLs. Present results showed that the content of mature SREBP is increased in the GSL-defective cells. Transcriptional activation of SREBP target genes and cholesterol synthesis are also induced in the GSL-defective cells. These results indicate that GSL deficiency up-regulates the SREBP pathway.

Materials and methods

Materials. [14C] Serine (165 mCi/mmol) was from Perkin-Elmer (Boston, MA). [1-14C]Acetic acid (54.7 mCi/ml) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Lipoprotein-deficient serum (LPDS), cholesterol, 25-hydroxycholesterol, lova-statin (mevinolin), and mevalonate were from Sigma (St. Louis, MO). Anti-SREBP2 antibody was kindly provided by Dr. T. Hama-kubo of Tokyo University.

Cells and cell culture. Mouse melanoma cell line, MEB4, its glyco-sphingolipid-deficient mutant GM95 [9] and the transfectant CG1, which stably expresses ceramide glucosyltransferase (GlcT-1), were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.z

Analysis of sphingolipid content. Cells grown in 6-cm dish were la-beled with 1 μCi/ml [14C]serine in DMEM containing 10% FCS for 2 days. The cells were washed with cold PBS and harvested by scrap-ing. Lipids were extracted by the method of Bligh and Dyer [10] and separated on TLC using a solvent of chloroform/methanol/methyl acetate/1-propanol/0.25% potassium chloride (25:10:25: 25:9). Distribution of radioactivity to each lipid was measured by BAS 2000 (FUJIFILM, Tokyo, Japan).

Immunoblot analysis of SREBP processing. MEB4, GM95, or CG1 cells were incubated for 18 h with 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol or 4 μM lovastatin and 100 μM mevalo-nate in 5% LPDS-containing medium. Cell fractionation was carried out as described previously [11,12]. Cells were suspended in 0.4 ml of buffer A (10 mM HEPES–KOH (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, and 0.25 M sucrose), passed through a 22-gauge needle 30 times, and centrifuged at 1000g for 7 min at 4 °C. The 1000g pellet was resuspended in 50 μl of buffer B (20 mM HEPES–KOH (pH 7.4), 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, and 1 mM sodium EGTA), rotated at 4 °C for 1 h, and centrifuged at 15,000g for 30 min at 4 °C. The supernatant from this spin was designated as the nuclear ex-tract. The supernatant from the original 1000g spin was used to pre-pare the membrane fraction by centrifugation at 100,000 g in a Beckman TLA 100.2 rotor. The resulting membrane pellets were resuspended in 50 μl SDS-lysis buffer. Fractionation buffers con-tained protease inhibitor mixture (5 μg/ml leupeptin, 5 μg/ml pep-statin, 0.25 mg/ml Pefabloc SC, 5 μg/ml aprotinin, and 25 μg/ml ALLN). Aliquots of proteins in nuclear extract and membrane frac-tion were subjected to SDS–PAGE followed by immunoblot analysis using anti-SREBP2 antibody.

Quantitative real-time PCR. Total RNA was prepared from the cells by using Trizol (GIBCO Invitrogen, Carlsbad, CA). First strand cDNA was synthesized from total RNA with random hexamer primers by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Specific primers for each gene were as de-scribed in supplemental Table 1. The real-time PCR contained, in a fi-nal volume of 20 μl, 4 ng of reverse-transcribed total RNA, 100 nM (250 nM in the case of cyclophilin) forward and reverse primers, and 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Fos-ter City, CA). PCRs were carried out by using the Applied Biosystems Prism 7900 Sequence Detection System. All reactions were done in triplicate. Cyclophilin mRNA was used as the invariant control.

Assay of de novo cholesterol synthesis. On day 0, the cells were plated in 6-cm dishes in DMEM containing 10% FCS. On day 1, the medium was replaced with DMEM containing 5% LPDS, 4 μM lovastatin, and 100 μM mevalonate. On day 2, the cells were la-beled with 0.5 μCi/ml [14C]acetic acid for 2 h. Cells were then washed twice with PBS. Lipids were extracted and separated on TLC using hexane/diethyl ether/acetic acid (80:20:1) as a solvent. Incorporation of [14C]acetic acid into [14C]cholesterol was quanti-fied by BAS 2000 (FUJIFILM).

Other methods. Protein concentration was determined using the Bradford or BCA assays.

Results

Selective decrease of glycosphingolipids (GSLs) in GM95 cells

GM95 is a melanoma cell mutant which is defective in ceramide glucosyltransferase (GlcT-I) and thus does not synthesize GSL. MEB4 is the parent cell of GM95 whereas CG1 is a transfectant of GlcT-I in GM95. In Fig. 1, we measured lipid composition of MEB4, GM95, and CG1 cells after cells were labeled with [14C]ser-ine. As previously reported [9], GM95 is deficient in GSLs, glucosyl-ceramide, and GM3. Ceramide content was similar among the three cell lines. The content of [14C]serine-labeled phosphatidyl-ethanolamine and phosphatidylserine was also similar among the three cell types. There was a significant increase of sphingomyelin in GM95 cells compared to MEB4 and CG1 cells as described [13].

GSL deficiency increases the SRE-mediated gene transcription

SREBPs are localized as inactive precursors in the ER. When cel-lular cholesterol level is decreased, SREBPs are transported to the Golgi apparatus and processed by proteases and the cleaved ma-ture SREBPs are translocated to the nucleus [1,2]. SREBP2 preferen-tially activates genes responsible for cholesterol synthesis and uptake whereas SREBP1 preferentially controls genes involved in lipogenesis [2,14]. In Fig. 2, we measured the precursor form of SREBP2 in cell membranes, and the cleaved mature form of SREBP2 in nuclear extracts. When the cholesterol content is depleted by incubating cells in the medium containing lipoprotein-deficient serum (LPDS) and lovastatin, a HMG-CoA reductase inhibitor, the content of the mature form of SREBP2 was increased in MEB4, GM95, and CG1 cells. However, the increase was not equal among these three cells. The increase was most significant in GM95 cells compared to MEB4 and CG1 cells that displayed comparable inten-sity. The precursor form of SREBP2 was also increased in GM95 cells, indicating that both precursor and cleaved forms of SREBP2 are increased by GSL deficiency.

The increase of SREBP2 cleavage suggests the activation of the SREBP pathway in GSL-deficient cells. We then determined mRNA
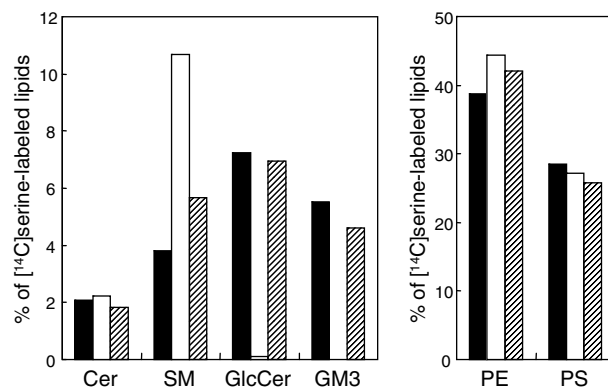
Fig. 1. GM95 cells are defective in glycosphingolipid biosynthesis. MEB4 (filled bar), GM95 (blank bar), or CG1 (hatched bar) cells were grown in the presence of [14C]serine for 2 days. Lipids were extracted and separated on TLC, and the content of sphingolipids, phosphatidylethanolamine, and phosphatidylserine was measured as described in Materials and methods. Data are expressed in percentage of the total [14C]serine-labeled lipids. Cer, ceramide; SM, sphingomyelin; GlcCer, glucosylcer-amide; PE, phosphatidylethanolamine; PS, phosphatidylserine.
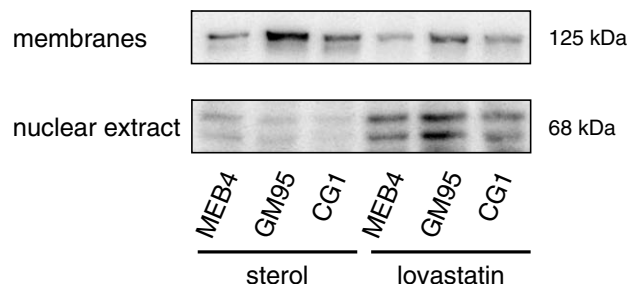
Fig. 2. Nuclear SREBP is increased in glycosphingolipid-deficient cells. MEB4, GM95, or CG1 cells were incubated for 18 h with 10 μg/ml cholesterol and 1 μg/ml of 25-hydroxycholesterol (sterol) or 4 μM lovastatin and 100 μM mevalonate (lovastatin) in 5% LPDS-containing medium. Aliquots of the nuclear extracts and membranes were subjected to SDS–PAGE and immunoblot analysis using anti SREBP2 antibody.

levels for SREBP2 target genes by quantitative real-time PCR (Fig. 3). We examined the genes preferentially activated by SREBP2 that are involved in cholesterol homeostasis: LDL receptor involved in LDL uptake, HMG-CoA synthase and HMG-CoA reductase that are required for cholesterol synthesis and SREBP2 itself. Fig. 3 showed that the transcription of these mRNA was dependent on cholesterol as previously reported. When cells were grown in FCS-containing medium, the mRNA levels for the SREBP2 target genes were low, and there was no significant difference among the three cells. Cholesterol depletion strongly enhance transcript of all genes examined. However, the increase was not equal. GM95 showed higher increase compared to the other two cells.

Cholesterol synthesis is increased in the GSL-deficient GM95 cells

In Fig. 4, cells were labeled with [¹⁴C]acetic acid, and cholesterol synthesis was determined. When cells were grown in FCS-containing medium, cholesterol synthesis was low and there was no significant difference in cholesterol synthesis among these cells. Cholesterol depletion of the cells increased cholesterol biosynthesis. The cholesterol synthetic rate in GM95 cells was twice of that in MEB4 and CG1 cells, in line with the increase in mRNA expression of the sterol biosynthesis genes (Fig. 3).
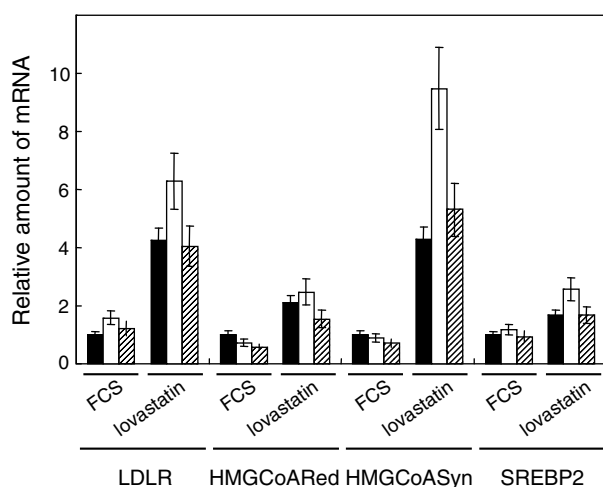
Fig. 3. SRE-mediated gene transcription is increased in glycosphingolipid-deficient cells. MEB4 (filled bar), GM95 (blank bar), or CG1 (hatched bar) cells were incubated for 18 h with 4 μM lovastatin and 100 μM mevalonate in 5% LPDS-containing medium or 10% FCS-containing medium. Total RNA was isolated from the cells and subjected to real-time RT-PCR quantification as described in Materials and methods.
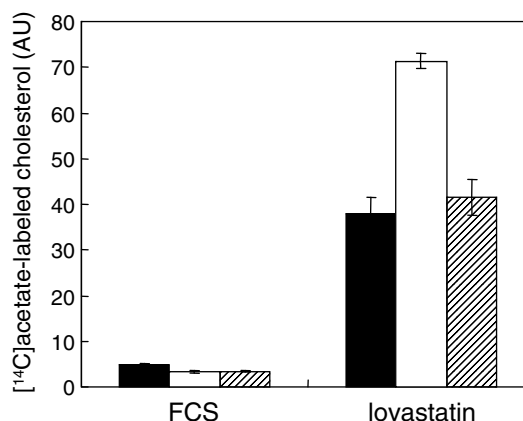
Fig. 4. Cholesterol synthesis is increased in glycosphingolipid-deficient cells. MEB4 (filled bar), GM95 (blank bar), or CG1 (hatched bar) cells were incubated for 18 h with 4 μM lovastatin and 100 μM mevalonate in 5% LPDS-containing medium or 10% FCS-containing medium. The cells were labeled with [¹⁴C]acetic acid for 2 h. Lipids were extracted and separated on TLC, and the incorporation of radioactivity into cholesterol was measured as described in Materials and methods. Results are the average of two independent experiments ± difference.

Discussion

In the present study, we showed that deficiency of glyco-sphingolipids (GSLs) increased the cleavage of SREBP2 and the transcript of its target genes mainly related to cholesterol homeostasis. We also showed that biosynthesis of cholesterol was increased in GSL-deficient cells.

The molecular mechanisms of the activation of SREBP by GSL deficiency are not clear. Bijl et al. also examined the effects of GSLs on SREBP activation using an inhibitor of GlcT-1 [15]. They found the inhibition of GSL synthesis leads to activation of SREBP target genes, but it is unknown how GSLs are involved in the SREBP pathway. Worgall et al. proposed that biosynthesis of ceramide is crucial in the maturation of SREBP [7]. However, in our system, the ceramide content is not changed in GM95 cells (Fig. 1). In GM95 cells, the content of sphingomyelin is increased. Previously Scheek et al. showed that the decrease of cell surface sphingomyelin by sphingomyelinase decreased the cleavage of SREBP [3]. It was suggested that the sphingomyelinase treatment leads to increased cholesterol translocation to the ER and a subsequent suppression of SREBP cleavage. If sphingomyelin but not GSLs retain cholesterol on the plasma membrane, our results may be explained by the trapping of cholesterol by high concentration of sphingomyelin, thus preventing the cholesterol traffic to the ER. However, cholesterol-specific dye filipin staining of the cells and the sensitivity of the cells to cholesterol-dependent toxin, amphotericin, were not affected by GSL deficiency (data not shown), suggesting that the content of the cell surface cholesterol is not significantly altered by GSL deficiency.

Whereas sphingomyelin is synthesized in the lumen of trans-Golgi [16–18], glucosylceramide is synthesized on the cytoplasmic leaflet of cis-Golgi and intermediate compartment between ER and cis-Golgi [19–21]. It has been reported that the deficiency of GSL alters the membrane traffic from the Golgi apparatus [22]. Thus, intriguing possibility is that the defect of GSLs affects the transport and/or cleavage of SREBP directly or indirectly. Further experiments are necessary to understand the role of GSLs on the SREBP pathway.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.030.

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