Identification and characterization of the human Gb3/CD77 synthase gene promoter

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Hemolytic uremic syndrome (HUS) is triggered by verotoxin (VT) produced by the Escherichia coli O157 strain. Several studies have demonstrated that VT induces endothelial cell (EC) death via the VT receptor globotriaosylceramide (Gb3/CD77) leading to this symptom. Inflammatory mediators which are produced as a result of E. coli O157 infection, increase the expression level of Gb3 in EC. Therefore increased expression of Gb3 is considered as a progression step for HUS. The increased expression of Gb3 is due to the transcriptional upregulation of Gb3/CD77 synthase gene (Gb3S, also known as α1,4-galactosyltransferase gene), the mechanism of which still remains unknown. To understand the transcriptional machinery and to elucidate the onset mechanism of HUS, we cloned and characterized the human Gb3S promoter. A modified 5'-RACE was used to determine the transcriptional initiation site, which revealed the presence of a TATA-less GC-rich sequence in the proximal region. Promoter activity measured using a luciferase assay demonstrated that the GC-rich sequence is necessary for the basal transcriptional activity, and two silencer elements located 5'-upstream of this GC-rich region regulated the transcriptional level. Furthermore, we found that the GC-rich sequence contained three potential Sp1 binding sites and that all three Sp1 binding elements acted as positive regulators. Since Sp1 is an inducer of several genes in the presence of the inflammatory cytokines in EC, our results suggest that the transcriptional regulation of the Gb3S gene by Sp1 might affect the VT sensitivity of EC and HUS progression.

Keywords: Escherichia coli O157/glycosphingolipid/α1,4-galactosyltransferase/hemolytic uremic syndrome/promoter

Introduction

Diversity of glycosphingolipids is mainly due to the presence of various structures of sugar chains. In the mammalian cells, a unique α1,4-galactose structure is found only in the globotriaosylceramide (Gb3/CD77) or its derivatives (Hakomori et al. 1971), but in not in any other glycans. Synthesis of Gb3 is mediated by the Gb3/CD77 synthase gene (Keusch et al. 2000; Kojima et al. 2000; Steffensen et al. 2000), which codes for the enzyme lactosylceramide α1,4-galactosyltransferase (EC 2.4.1.228). In human, Gb3 is found in several tissues, especially abundantly in the kidney (Matka 1964). Gb3 is known as the P^k blood group antigen on erythrocyte (Marcus et al. 1981) and is also known as the CD77 antigen, which was previously characterized as Burkitt lymphoma-associated antigen or germinal center B-cell differentiation antigen (Wiels et al. 1981). Its role as the receptor for Verotoxin (VT, also called Shiga-like toxin), produced by Escherichia coli O157 strain, is also well characterized (Jacewicz et al. 1986; Lingwood et al. 1987). VT specifically binds to Gb3 via its B-subunit and induces host cell death. The effect of VT on endothelial cell (EC) leads to circulatory disturbances and tissue damages and induces the onset of hemolytic uremic syndrome (HUS) (Noris and Remuzzi 2005). HUS is associated with acute renal failure, thrombocytopenia, and hemolytic anemia. Consciousness disorder is also known as a HUS-related symptom and is a major risk factor leading to the death of a patient. Previously, we created a Gb3S null mutant mice and demonstrated that the VT-induced tissue damage completely depended on the expression of its receptor Gb3 in vivo and also that the main target of VT was the Gb3 on the endothelial cells (Okuda et al. 2006). Gb3 is heterogeneously expressed on the endothelial cell surface, and its expression is increased by inflammatory mediators, such as LPS and TNF-α (van de Kar et al. 1992; Obrig et al. 1993). This inflammatory mediator-induced increase in the Gb3 expression is due to the transcriptional upregulation of the Gb3S gene (Stricklett et al. 2005; Okuda et al. 2006), which has been considered as a reason for the progression of HUS. However, the molecular basis of this mechanism still remains unknown. In this report, we identified and characterized the human Gb3S promoter and revealed that the transcriptional factor Sp1 (Dyman and Tjian 1983) is a key factor that regulated the Gb3S expression. The 5′-flanking region of the Gb3S gene was located at chromosome 22q13, and this region included two silencer elements and a promoter element containing three Sp1 binding sites. Furthermore, we used site-directed mutagenesis and gel mobility shift assay to assess the role of Sp1 in Gb3S promoter activity. Our results showed that the Sp1 binding sites contributed to the cell-specific expression of the Gb3S gene, indicating that the Sp1 activity is important for the Gb3S transcription.

Results

Identification of the 5′-flanking region of human Gb3/CD77 synthase gene

We used the human cervix carcinoma HeLa cells as the host for our experiments because thin-layer chromatography (TLC) analysis of glycolipids revealed that Gb3 and its derivatives were abundant in these cells (Figure 1A). In contrast, acidic glycolipids were hardly detected (Figure 1B), suggesting that they are minor components in these cells. We next performed RNA
Fig. 1. TLC analysis of glycolipids from HeLa cells. (A) TLC of neutral glycolipids from HeLa cells was visualized by orcinol-H2SO4. Lane 1, standard neutral glycolipids and lane 2, glycolipids from HeLa cells. Lane 3 shows TLC immunostaining of lane 2 with the anti-Gb3 antibody (38.13). (B) TLC of acidic glycolipids from HeLa cells (lane 3) was visualized by resorcinol. The bovine brain ganglioside (lane 1) and GM3 (lane 2) were used as standard markers. These experiments were performed with a solvent system consisting of chloroform/methanol/water (60:35:8).

Fig. 2. Identification of the transcriptional initiation site of the Gb3S gene. (A) RNA ligase-mediated 5′-RACE. Total RNA was prepared from HeLa cells and the 5′-capping structure was replaced with a RNA oligonucleotide by using a series of enzymatic reactions, which was subsequently used for dual PCR amplification with Gb3S gene-specific primers. First PCR was performed using the GRP-1 and GSP-1 primers and second PCR was performed using the GRP-2 and GSP-2 primers. The amplified product was electrophoresed on 1.5% agarose gel and visualized by EtBr staining: lane 1, 100 bp standard DNA marker and lane 2, the second PCR product. (B) The nucleotide sequence of the second PCR product. Capital fonts and bold fonts represent the sequence of the second PCR product and the RNA oligonucleotide sequence, respectively. Arrows indicate the sequences of the corresponding primers, arrowheads indicate the splice junctions, and a solid black box indicates the ATG start codon. (C) Genomic organization of the Gb3S gene. Boxes represent exons and the solid bar indicates the coding region. Respective positions of the GSP-1 and GSP-2 primers used in this experiment are shown using long arrows.

Fig. 3. Nucleotide sequence and structural organization of the Gb3S gene promoter region. Putative binding sites for the transcriptional factors are underlined. The transcriptional initiation site is shown in italic. Solid black boxes, GC box; the unfilled box, exon-1.

40 bp from this transcriptional start site included the entire 5′-end sequence of the Gb3S full-length cDNA, which was cloned from various organs and was reported in the Genbank, suggesting that this region is a major transcriptional initiation site of the human Gb3S gene. According to the NCBI Genome Blast search result (GenBank accession number AL049757), the Gb3S gene is derived from three exons (Figure 2C) and is located at chromosome 22q13. The genomic organization of the human Gb3S was also previously published (Iwamura et al. 2003). We amplified the 5′-flanking region of the Gb3S gene from the HeLa cell DNA by PCR, and the entire DNA sequence of the 5′-flanking region is shown in Figure 3. The proximal region of the transcriptional initiation site includes four GC boxes but lacks typical basal elements such as TATA and CCAAT boxes. In addition, there were four places where the DNA sequence of the HeLa clone differed from the existing human genome sequence found in NCBI. The clone from the HeLa cells contained an insertion of a G residue at position 95 upstream (position –95) from the transcription initiation site and a mutation of A–G was found at position –151. In addition, compared to the existing information in the human genome database, the DNA sequence
of the HeLa clone lacked three T bases in a poly(T) containing stretch (position −1331 to −1356) and revealed two additional A bases in a poly(A) containing stretch (position −1394 to −1422). The mutation found at position −151 was previously reported as a SNP related to the P1/P2 phenotype of the P antigen system (Iwamura et al. 2003), suggesting that the genotype of HeLa cell originated in Japanese individuals. Next, we searched for potential transcriptional factor binding sites in the 5′-flanking region using an algorithm (MATCH™, http://www.gene-regulation.com) and a command: “cut-off selection for the matrix group to minimize false positives.” Based on this analysis, we identified consensus binding sequences for MZF1, AML1a, E47, Oct-6, and Sp1 in the 5′-flanking region of the Gb3S gene as shown in Figure 3.

Characterization of regulatory elements of Gb3S gene promoter

To determine the apparent promoter activity of the 5′-flanking region of Gb3S, the entire fragment (−1893 bp to +84 bp) was cloned into the luciferase reporter vector pML, the resultant plasmid (pML−1893/+84) was transfected into the HeLa cells, and a luciferase assay was performed. As shown in Figure 4, the luciferase activity in HeLa cells transfected with pML−1893/+84 plasmid was much higher than in the cells transfected with the control pML plasmid (empty vector), suggesting that this 5′-flanking region indeed contained promoter activity. To determine the role of the putative regulatory elements that were identified in this 5′-flanking fragment, we generated several 5′- and 3′-deletion constructs and analyzed their promoter activities. First, we noted that the promoter activities associated with the deletion plasmids pML−845/+84 and pML−133/+84 were two times higher than the plasmid constructs containing longer upstream regions, suggesting that the −1207 bp to −846 bp and −222 bp to −134 bp regions contain silencer elements.

Among all the constructs tested, the pML−133/+84 construct showed the highest promoter activity. Deletion of the region from −133 bp to −60 bp ablated the promoter activity of the pML−133/+84, whereas deletion of the region from −60 bp to +84 bp retained, albeit to a lesser extent, the promoter activity of the pML−133/+84. These results suggest that the region between −133 bp and −60 bp contains one or more elements positively regulating the Gb3S transcription.

Sp1 is a positive transregulator of Gb3S promoter

As shown in Figure 3, there are three putative consensus Sp1 binding sites in the −133 bp to −60 bp region. To examine whether Sp1 acts on this region as a transactivator, we altered all three putative Sp1 binding sites (designated as Sp1a, Sp1b, and Sp1c in Figure 5A), one at a time, by mutagenesis and examined the effect of each mutation on the promoter activity. Thus, the following point mutations were introduced to individually alter each Sp1 consensus sequence in the pML−133/+84: Sp1a, C-109A; Sp1b, C-104A; and Sp1c, G-86T (Figure 5A). The promoter activities of the Sp1a C-109A, Sp1b C-104A, and Sp1c G-86T mutants were approximately 75%, 75%, and 65%, respectively, of that of the wild type (Figure 5B). We further confirmed the role of these consensus Sp1 binding sites on the promoter activity by deletion analysis. Thus, deletion of the Sp1c site (construct Δ−90/−81) reduced the promoter activity in a manner as the Sp1c G-86T site-specific mutant, whereas deletion of both Sp1a and Sp1b sites (construct Δ−99/+84) very significantly reduced the promoter activity (Figure 5B). Together, these results suggest that all three Sp1 binding sites affect the Gb3S promoter activity. In order to demonstrate that the Sp1 protein regulates the Gb3S promoter activity, we examined the effects of several Sp1 inhibitors on the promoter activity. Mithramycin A is known to act on the DNA sequence of the consensus Sp1 binding site and inhibits the promoter activity.
Fig. 5. Role of putative Sp1 binding sites on the promoter activity. Panel A, top: positions of three putative Sp1 consensus binding sites (named here as Sp1a, Sp1b, and Sp1c) in the $-133/+84$ construct (top of the panel) and bottom: point mutations (C-109A, C-104A, and G-86T; marked using solid black box) created by site-directed mutagenesis to individually alter each one of these three Sp1 binding sites. Panel B, luciferase activity expressed by the three Sp1 site-directed mutants and three deletion mutants ($/Delta1$ $-90/-81$, $-99/+84$, $-59/+84$) lacking partly or completely the three Sp1 binding sites. The deletion mutant $/Delta1$ $-90/-81$ lacks the bases $-90$ to $-81$ in the corresponding Sp1c site, deletion mutant $-99/+84$ lacks both Sp1a and Sp1b sites, and deletion mutant $-59/+84$ lacks all three Sp1 sites. Relative luciferase activity expressed by each mutant is expressed as a percentage of the activity expressed by the $-133/+84$ construct. Error bars: mean ± SE, $n = 4$.

activity (Ray et al. 1989; Blume et al. 1991). On the other hand, streptozotocin (STZ) and 2-deoxy-D-glucose (2-DG) are known to inhibit the transcriptional activity of Sp1 by increasing the $\alpha$-linkage of N-acetylglucosamine to Sp1 activation domain (Yang et al. 2001; Kang et al. 2003). As expected, mithramycin A, STZ, and 2-DG inhibited the $Gb3S$ promoter activity in a dose-dependent manner (Figure 6). Next, we confirmed direct interaction between the Sp1 and three consensus Sp1 binding sites using the gel mobility shift assay. For this purpose, the HeLa cell nuclear extract was incubated with either the biotinylated oligonucleotide probe-1, which included both the Sp1a and Sp1b binding site sequences, or the biotinylated oligonucleotide probe-2, which included the Sp1c binding site sequence, and subsequently each mixture was analyzed by polyacrylamide gel electrophoresis (PAGE) (Figure 7). Migration of the oligonucleotide probe was retarded (Figure 7, lanes 2 and 5, both panels indicated by arrowheads) as compared to the samples where the assay mixture was incubated with either excess

Fig. 6. Effect of various Sp1 inhibitors on the promoter activity. pML$-133/+84$ plasmid was transiently transfected into the HeLa cells and the transfected cells were then treated with indicated concentrations of a given Sp1 inhibitor (mithramycin A, STZ or 2-DG) for 16 h. Following the treatment with the inhibitor, luciferase activity in the culture medium was measured as described in Material and methods. Results shown represent the relative luciferase activity (as percentage of the untreated control). Error bars: mean ± SE, $n = 2$.

Fig. 7. Binding of Sp1 to its consensus binding sites in the $Gb3S$ promoter. HeLa cell nuclear extracts were incubated with a biotinylated oligonucleotide probe (probe-1) encompassing both Sp1a and Sp1b sites (position $-117$ bp to $-96$ bp, left panel) or a biotinylated oligonucleotide probe (probe-2) encompassing the Sp1c site (position $-95$ bp to $-74$ bp, right panel), and the mixture was subsequently analyzed by a gel shift assay (lanes 2–6) as described in Material and methods. To absorb the nonspecific background, the nuclear extract was preincubated with the 1.75 pmol of unlabeled probes carrying point mutations in the Sp1 consensus sequences (Mut-probe), and then used for the gel shift assay as described above (lane 4). Competition assay experiments using a nonlabeled Sp1-consensus oligonucleotide (Competitor, lane 3) or an anti-Sp1 antibody (lane 6) were performed as described in Material and methods. After electroblotting these samples on nylon membranes, the biotinylated probes were detected with the avidin–biotin–HRP complex.
of unlabeled Sp1 consensus oligonucleotide (Figure 7, lane 3, both panels) or with anti-Sp1 antibody (Figure 7, lane 6, both panels). The retarded band was observed even when the nuclear extract was preabsorbed with a mutant oligonucleotide probe carrying point mutation in the Sp1 consensus sequence to eliminate any nonspecific binding (Figure 7, lane 4, both panels). The intensity of the retarded band was reduced by pretreatment of the mutant probes. This result should be due to the remaining Sp1 binding capability of mutant probes because Sp1 consensus sequences were slightly retained and excessively added to absorb the nonspecific background completely. These results indicate that Sp1 binds specifically to these consensus binding sites on the Gb3S promoter and positively regulates the promoter activity.

Correlation between the activation of the Gb3S promoter and Gb3 expression in cells

The properties of the identified Gb3S promoter were further characterized in Gb3-positive (HeLa, NCCIT) and Gb3-negative (SK-MEL-28) cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that the Gb3S transcripts were barely expressed in the SK-MEL-28 cell (Figure 8B, upper panel), which is consistent with the absence of Gb3 in these cells, as its synthesis is controlled by the transcriptional factor Sp1. The construct pML−1893/+84 showed the high level of promoter activity in both the Gb3-positive HeLa and NCCIT cells but quite low in SK-MEL-28 cells, which was reduced to the background level (Figure 8A). Even the construct pML−133/+84 that lacked the silencer domains showed weak promoter activity in Gb3-negative cells (Figure 8A). These results suggest that the −133 bp to −60 bp region is essential for the Gb3S promoter activity. Thus, we expected that the expression of Gb3 would be determined by the expression level of Sp1 in the cells. Although the expression level of the Sp1 mRNA was similar in all three cells (Figure 8B, middle panel), the relative expression of the Sp1 protein in the nuclear extracts of the Gb3-positive cells was to some extent higher than that of the Gb3-negative cells. A transcriptional regulator KLF4, which is known as a synergistic coactivator of Sp1 (Brembeck and Rustgi 2000; Higaki et al. 2002), was also found to be expressed at a higher level in the Gb3-positive cells than in the Gb3-negative cells. Taken together, these results suggest that the expression of Gb3 in the cells depends on the promoter activity of the −133 bp to −60 bp region of the Gb3S gene and its expression is regulated by Sp1 and some other factors.

Discussion

This study described the structure of the Gb3S promoter and offered information on mechanisms regulating this promoter activity. Here, we identified a single transcription initiation site of the Gb3S gene from the HeLa cell mRNA. The neighboring bases from this transcriptional start site included the entire 5′-end sequence of the Gb3S full-length cDNAs, cloned from various organs and reported in the Genbank, suggesting that this region is a major transcriptional initiation site of the human Gb3S gene. Our analysis of the 5′-flanking region of the Gb3S gene led to the localization of the promoter element and also suggested that this promoter acts as the principal promoter for the Gb3S gene in human tissues.

The Gb3S promoter lacks the TATA and CCAAT boxes that were commonly found in the mammalian glycolipid glycosyltransferase genes (Ichikawa et al. 1998; Kim et al. 2002; Zeng et al. 2003; Sato and Furukawa 2004). Instead, we found a highly GC-rich sequence in the proximal promoter, which is controlled by the transcriptional factor Sp1. Similar promoter structure was found in the glucosylceramide synthase gene (Ichikawa et al. 1998) and also in the β-1,4-galactosyltransferase V gene (Sato and Furukawa 2004), which is known to synthesize highly branched N-glycans (Sato and Furukawa 2004). Importance of the β-1,4-galactosyltransferase gene in lactosylceramide synthesis has also been reported (Kolmakova and Chatterjee 2005). These genes, which are involved in the Gb3 synthesis pathway, have similar promoter structure and are regulated by Sp1. Thus, the transcription factor Sp1 plays an important role in the Gb3 synthesis.

We found a correlation between the Gb3S promoter activity and Gb3 expression in our test cells. Since the Sp1 protein seems to be mainly involved in regulating this promoter activity, we believe that activation or silencing of the Gb3S promoter by Sp1 and its cofactors may play a part in the cell-specific expression of Gb3S. In the present study, we found silencer elements in two places of the 5′-flanking region of the Gb3S promoter (Figure 4). Since these silencer elements are unlikely to influence the cell-specific expression of Gb3S, they may constantly control the promoter activity at low levels in all cells. A SNP related to P2 phenotype of the blood group P antigen system was also found in the 5′-flanking region of Gb3S. This SNP correlated with the decrease of transcriptional levels of Gb3S (Iwamura et al. 2003); however, we were unable to find any function of this SNP in the Gb3S promoter activity (Figure 4).
The strength of basic transcription activity of the *Gb3S* promoter through the Sp1 or Sp1 consensus sequence seemed to be important for the cell-specific expression of *Gb3S* (results shown in Figure 8A). Although the relative expression level of the Sp1 mRNA was similar in both Gb3-positive and Gb3-negative cells, the relative expression level of the Sp1 protein was higher in the Gb3-positive cells than in the Gb3-negative cells. It was previously reported that postranslational modifications of Sp1, such as glycosylation (Zhang et al. 2003) or sumoylation (Spengler and Brattain 2006), regulate degradation of this protein in cells. These modifications may contribute to the cell-specific activation of *Gb3S* promoter by Sp1. On the other hand, certain transcriptional factors that are known to interact with Sp1 might also play a role in regulating the cell-specific activation of the target gene promoter. In this regard, it is noteworthy that the transcription factor KLF4, which was abundantly expressed in Gb3-positive cells (Figure 8C), was previously shown to modulate the Sp1 activity by interacting with it. For example, KLF4 was shown to act as a synergistic coactivator of Sp1 on the keratin 19 gene (Brembeck and Rustgi 2000) or laminin gamma1 chain gene promoter (Higaki et al. 2002), but was also shown to inhibit the activity of other promoters by direct interaction with the Sp1 protein (Zhang et al. 1998) or via the Sp1 binding domain on other gene promoter (Shie et al. 2000). In the case of the *Gb3S* promoter, KLF4 might act as a coactivator.

In EC, Sp1 is known to play a role as a gene inducer under inflammatory condition. Hamanaka et al. (1992) showed that the expression level of the LDL receptor gene in EC was enhanced by TNF-α even though there was no NF-κB motif on the promoter of this gene and that the induction of the LDL receptor gene by TNF-α was mediated via Sp1. Similar results were also obtained with the VEGF receptor 2 gene (Zhang et al. 1998) or via the Sp1 binding domain on other gene promoter (Shie et al. 2000). In the case of the *Gb3S* promoter, KLF4 might act as a coactivator.

Material and methods

Cells culture

HeLa cell and SK-MEL-28 cell were obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. NCCIT human teratocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA), which were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

Glycolipid extraction, TLC, and TLC immunostaining

Glycolipid extraction and TLC immunostaining were performed as described (Furukawa et al. 1985). Briefly, total lipids from 1 × 10⁶ cells were sequentially extracted with the 2:1, 1:1, and 1:2 mixtures (v/v), respectively, of chloroform and methanol. Contaminating phospholipids were hydrolyzed with mild alkaline treatment, and the glycolipids were subsequently purified by using Sep-Pack C18 cartridge (Waters, Milford, MA). Acidic and neutral glycolipids were separated by column chromatography using DEAE Sephadex A-25 (SIGMA, St. Louis, MO) and Iatrobeads 6RS-8060 (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively. Purified glycolipids were analyzed on HPTLC plates (Merck, Darmstadt, Germany) with a solvent system consisting of chloroform/methanol/water (60:35:8, v/v/v). Glycolipids were visualized by resorcinol (for acidic glycolipids) or orcinol-H₂SO₄ (for neutral glycolipids). TLC immuno-staining was performed using an anti-Gb3 monoclonal antibody 38.13 (Immunotech, Marseille, France), and antibody binding was detected using an ABC kit™ (Vector Laboratories, Burlingame, CA) and SuperSignal™ West Dura Extended Duration Substrate (Pierce, Rockford, IL).

Identification of the 5'-end of the *Gb3S* mRNA

The 5'-end of *Gb3S* mRNA was determined by RNA ligase-mediated 5'-RACE. This experiment was carried out using a GeneRacer™ Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Briefly, the 5'-cap structure of HeLa cell mRNAs was replaced with a RNA oligonucleotide (5'-CGA CUG CAG CAC GAG GCC ACU GAC AUG UGA AGG AGU AGA AA-3') by using a series of enzyme reactions. After reverse transcribing with oligo (dT) primer, the *Gb3S* cDNA was amplified by dual PCR using *Gb3S* gene-specific primers. First round of PCR was carried out using the GeneRacer™ 5' Primer (GRP-1) and GSP-1 (5'-TGG CGG GCC CCT CAC AAC AAG TAC A-3') reverse primer. Second round of PCR was performed using the GeneRacer™ 5’ Nested Primer and GSP-2 (5'-GAA GCC GAT GAT GAA CAG GGT GCA GA -3') primer. Corresponding positions of the primers on the *Gb3S* gene are depicted in Figure 2.

Construction of plasmids

The 5'-flanking region of the *Gb3S* gene was isolated by PCR using the HeLa cell genomic DNA as a template and the following amplification primers: 5'-TGA GTC GAC TCA GCT CTT GGA GGG GCA ACA-3' and 5'-GGG GCC ACA AAT GTC GCC TTC AGA ACA-3'. To measure promoter activity of the 5'-flanking region of the *Gb3S* gene, the amplified DNA was digested with the restriction enzymes SalI and BamHI, recognition sites for which were internally located in the fragment, and the digested DNA was subcloned into the pMet-Luciferase (pML) reporter vector (Clontech, Mountain View, CA). This DNA insert consisted of −1893 bp to +546 and −462/+84 were created by digesting the pML−1893/+546 and pML−462/+84 with HindIII/EcoRI and EcoRI/BamHI, respectively, and subcloning the respective DNA fragments into the pML reporter vector. Other deletion fragments were generated by PCR using the pML−1893/+546 plasmid DNA as a template. A common 3'-primer, 5'-GCT AAG CTT AGC ACG CTG AGC TCG GTA-3', and the following 5'-primers were used for the generating the following deletion fragments: for the −1207/+48 fragment, 5'-GAG TTT CAT CAT GTT GGC CA-3'; for the −845/+54 fragment, 5'-GGG CAC CCA TGT AGT GCC A-3'; for the −657/+54 fragment, 5'-GAC ATG CTT AGA ACA TGG CA-3'; for the −222/+54 fragment, 5'-GGA CTG...
GGG ACT GTC CGC A; for the −133/+84 fragment, 5′-ACC CGC AGG GTA GGT CGG GA-3′; for the −99/+84 fragment, 5′-CGG CGC TGA CCC CGC CCC GGG CCG GA-3′; and for the −59/+84 fragment, 5′-TCC CGC CGG GCC CCA GGC ACT-3′. Since these PCR products contained the full-length sequence of the pML plasmid, the 5′- and 3′-ends were ligated using T4 DNA ligase to obtain the following deletion plasmids: pML−1207/+84, pML−845/+84, pML−657/+84, pML−222/+84, pML−133/+84, pML−99/+84, and pML−59/+84. Deletion plasmids pML−133−/−60 and pMLΔ−90−/−81 were also prepared following the similar protocol except for using the pML−133/+84 plasmid DNA as a template and the following combination of primers: for the −133−/−60 fragment, 5′-GGA TTC ACC GGT CGC CAC CAT-3′ and 5′-GGC AGC ACC GCC CCT-3′ and for the Δ−90−/−81 fragment, 5′-GGC GGC AGG GGC GGT GCC CCT-3′ and 5′-TCA GGC CCG GGG CGG GGT CAG C-3′. Site-directed mutagenesis of the putative Sp1 sites in pML−133/+84 (shown in Figure 5) was performed by PCR using the following primer sets: for C-109A mutation, 5′-AGG TCG GGA GAG GGG CCG GCC GCC GCT-3′ and 5′-AGC GCC GCC CCC CTC CCG GCC TCC CGA CCT-3′; for C-104A mutation, 5′-GTC GGG ACG GCC GGG GAG GGG C-3′ and 5′-GTA CGG CCC CCC CCC CTC CCG GCC TCC CGA CCT-3′; and for C-86T mutation, 5′-GCC GAC CCC TCC CCC GGG CCG CGG A-3′ and 5′-TCC CGG CCG GGG AGG GGT CAG C-3′.

All PCR experiments were performed using the PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan). The nucleotide sequence of the DNA insert in each plasmid construct was verified by sequencing.

Transfection and luciferase assay
0.4 µg of each plasmid was transfected into 2 × 10⁵ cells (HeLa, NCCIT and SK-MEL-28) with lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions and then incubated for 16 h (HeLa) or 48 h (NCCIT and SK-MEL-28). To provide an internal control, we used 0.2 µg of the pSEAP2-control vector (Clontech), which contained the secreted form of the human placental alkaline phosphatase (SEAP) under the SV40 promoter, for cotransfection. Since the reporter proteins (luciferase and central alkaline phosphatase (SEAP) under the SV40 promoter, (Clontech), which contained the secreted form of the human plasmid DNA was isolated by electrophoresis at 90mV constant voltage for 1 h. After blotting, the membranes were incubated either with the anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) or anti-KLF4 antibody (H-180, Santa Cruz Biotechnology), and bound antibody was detected using the ABC kit™ (Vector Laboratories) and SuperSignal™ West Dura Extended Duration Substrate (Pierce). Unlabeled double-stranded oligonucleotide probes carrying point mutations in the Sp1 consensus sequences (oligonucleotides for probe-1: 5′-GGG ACG GCC GGG GAG GGG CGG CGG C-3′ and 5′-GCC GCC CCC CGC CCG TCC C-3′; oligonucleotides for probe-2: 5′-GTC GAC CCC CTC CCG GCC TCC CGA CCT-3′ and 5′-TCA GGC CCG GGG CGG GGT CAG C-3′) were used for the absorption assays. For competition assays, the HeLa nuclear extract was incubated with 1.75 pmol of nonlabeled Sp1 consensus oligonucleotide (Promega) or 1 µg anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology, CA) for 60 min at 4°C in the presence of the binding buffer before the addition of the biotin-labeled probes.

Western blot analysis
Nuclear proteins were extracted from HeLa, NCCIT, and SK-MEL-28 cell by using NE-PERTM Nuclear and Cytoplasmic Extraction Regents (Pierce) according to manufacturer’s instructions. Nuclear proteins (3 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (SuperSep™ 5–20% Gel, Wako, Osaka, Japan) and the separated proteins were transferred from the gel onto the Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) by electrophoresis at 90mV constant voltage for 1 h. After blotting, the membranes were incubated either with the anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) or anti-KLF4 antibody (H-180, Santa Cruz Biotechnology), and bound antibody was detected using the ABC kit™ (Vector Laboratories) and SuperSignal™ West Dura Extended Duration Substrate (Pierce).

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Conflict of interest statement
None declared.

Abbreviations
EC, endothelial cell; 2-DG, 2-deoxy-D-glucose; DMEM, Dulbecco’s modified Eagle’s minimal essential medium; Gb3, globotriaosylceramide (Galα1,4Galβ1,4Glc—Ceramide); GM3, NeuAco2,3Galβ1,4Glc—ceramide; HUS, hemolytic uremic
syndrome; LDL, low-density lipoprotein; LPS, lipopolysaccharide; RT-PCR, real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; STZ, streptozotocin; TLC, thin-layer chromatography; TNF, tumor necrosis factor; VT, verotoxin; VEGF, vascular endothelial growth factor.

References


