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Up-regulation of ceramide glucosyltransferase during the differentiation of U937 cells

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Junya Aida[1], Syouta Higuchi[1], Yoshie Hasegawa[1], Michiyo Nagano-Ito[1], Yoshio Hirabayashi[2], Aoi Banba[1], Takamitsu Shimizu[1], Asako Kikuchi[1], Mayuko Saga[1] and Shinichi Ichikawa[1,*]

[1]Laboratory for Animal Cell Engineering, Niigata University of Pharmacy and Applied Life Sciences (NUPALS), 265-1 Higashijima, Akiha-ku, Niigata-shi Niigata 956-8603 and [2]Laboratory for Molecular Membrane Neuroscience, Brain Science Institute (BSI), The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-01, Japan

*Shinichi Ichikawa, Laboratory for Animal Cell Engineering, Niigata University of Pharmacy and Applied Life Sciences (NUPALS), 265-1 Higashijima, Akiha-ku, Niigata-shi Niigata 956-8603, Japan. Tel: +81 250 25 5114, Fax: +81 25025 5115, email: shin@nupals.ac.jp

The phorbol ester tetradecanoylphorbol acetate (TPA) induces promyelocytic leukaemia cells to differentiate to macrophage-like cells *in vitro*. During the course of this differentiation, the cells adhere to the bottom of the culture dish, a process that requires an increase in cell surface glycosphingolipids (GSLs). We examined the cellular content of glucosylceramide (GlcCer), the simplest of the GSLs, in a TPA-treated leukaemia cell line, U937. Following TPA treatment, we observed a 3.5-fold increase in GlcCer levels that was caused by enhanced activity of ceramide glucosyltransferase (GlcT-1), which catalyses ceramide glycosylation. Furthermore, in TPA-treated cell GlcT-1 amounts were increased at both the mRNA and protein levels. We also found decreased activity of lactosylceramide synthase in TPA-treated cells, which could also contribute to the increase in cellular GlcCer content.

Keywords: adhesion/ceramide/differentiation/glucosylceramide/leukaemia.

Abbreviations: DMSO, dimethyl sulfoxide; GlcCer, glucosylceramide; GM3, sialyl lactosyl ceramide; GSL, glycosphingolipid; LacCer, lactosyl ceramide; RT–PCR, reverse transcription–polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Ceramide glucosyltransferase (UDP-glucose ceramide glucosyltransferase, GlcT-1, EC.2.4.1.80) catalyses the first glycosylation step of glycosphingolipid (GSL) synthesis. cDNA cloning of human and mouse GlcT-1 revealed that the protein is composed of 394 amino acids (44.9 kDa) (*1*, *2*). GlcT-1 transfers glucose from UDP-glucose to the lipid ceramide to form GlcCer, which serves as a source for over 300 GSLs (*3*, *4*). These GSLs play important roles in various cellular processes including cell recognition, growth and differentiation (*5*). The lipid substrate of GlcT-1, ceramide, serves as a second messenger for various cellular events, including apoptosis (*6*), and GlcT-1 controls its intracellular content (*7*). Recently, GlcT-1 was also suggested to be implicated in insulin resistance of lipid cells (*8*) and cancer cell drug resistance (*9*).

GSLs, in addition to sphingomyelin, also play important roles in cellular adhesion to substrata. Promyelocytic leukaemia U937 and HL-60 cells have the potential to differentiate into macrophage-like cells following tetradecanoylphorbol acetate (TPA) treatment (*10*). One of the phenotypic changes that accompany differentiation is adherence to a culture dish. In addition, HL-60 cells have increased GlcCer and GM3 content during differentiation (*11*, *12*). Chung *et al.* (*13*) demonstrated that the GM3 synthase promoter is stimulated via protein kinase C/extracellular signal-regulated kinases-dependent cAMP responsive element activation during the course of differentiation, and contributes to the increase of GM3. It was further demonstrated that D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a competitive inhibitor of GlcT-1, blocked TPA-induced adherence of HL-60 cells (*11*). Although PDMP blocked cell adherence, it did not inhibit differentiation as determined by enzyme markers and cell morphology (*11*). This result emphasizes the importance of GSLs for cell adherence. Another sphingolipid, sphingomyelin, is also involved in adherence. Dressler *et al.* (*14*) showed that sphingomyelin is necessary for adherence during HL-60 cell differentiation. Both GSLs and sphingomyelin play roles in the adherence of other cell types, including the B16 mouse melanoma cell line, which requires either GSLs or sphingomyelin for adherence to a dish (*15*). Here, we show that GlcT-1 is up-regulated at the mRNA and protein levels during the course of U937 differentiation, resulting in increased amounts of GlcCer.

Materials and Methods

Materials

TPA was obtained from Sigma (Tokyo, Japan). A fluorescent substrate for GlcT-1, 6-{((N-7-Nitrobenz-2-oxa-1,3-diazol-4yl)-amino)caproyl}sphingosine (C_6-NBD-Cer) was purchased from Invitrogen (Carlsbad, CA, USA). C_6-NBD-LacCer and C_6-NBD-GlcCer were from Matreya, LLC (Pleasant Gap, PA, USA). A monoclonal antibody to GM3, M2590, was obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). A horseradish peroxidase conjugated anti-mouse immunoglobulin M polyclonal antibody was from Funakoshi (Tokyo, Japan). All other reagents used were of analytical grade.

Cell line and culture conditions

The human promyelocytic leukaemia cell line U937 DE-4, a subline of U937, was obtained from RIKEN cell bank (Tsukuba, Japan) and used throughout the experiments (*10*). The cells were grown in humidified 5% CO_2 at 37°C in DMEM medium containing 10% fetal bovine serum.

Differentiation of U937 cells into macrophage-like cells

To induce differentiation, TPA (10 mg/ml in DMSO) was added to the medium at a final concentration of 10 ng/ml. The differentiation into macrophage-like cells was evaluated by monitoring non-specific esterase activity using α-naphthyl butyrate (Sigma) as a substrate 48 h after TPA addition (*16*).

Lipid analyses

Total lipids were extracted from the cells with $CHCl_3/CH_3OH$, 2 : 1 (vol/vol) and evaporated to dryness. The dried lipids were then dissolved in a small volume of $CHCl_3/CH_3OH$, 2 : 1 (vol/vol), and chromatographed on silica gel 60 TLC plates (Merck, Darmstadt, Germany) in $CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4 (vol/vol). GSLs on the plate were visualized by spraying 0.2% orcinol in 2 M sulphuric acid followed by heating at 120°C on a hot plate.

GM3 was analysed by TLC immunostaining (*17*). Total lipids were applied onto a Polygram SIL G plate (Macherey & Nagel, Germany) and developed in $CHCl_3/CH_3OH/H_2O$, 5 : 4 : 1 (vol/vol). GM3 was detected by indirect immunostaining using the Konica Immunostain HRP-1000 kit (Seikagaku Corporation, Tokyo, Japan).

For quantification of GlcCer and GM3, TLC images were processed using NIH ImageJ (Rasband, 1997–2005) software.

Enzyme assay

Enzyme activities of GlcCer-metabolizing enzymes were measured using NBD labelled fluorescent substrates. After enzyme reactions, the lipids were extracted with $CHCl_3/CH_3OH$, 2 : 1 (vol/vol) and applied to silica gel TLC plates. NBD lipids were then separated in the same solvent used for the lipid analysis chromatography and visualized by UV-B illumination. To quantify enzyme products, TLC images were processed with NIH ImageJ software. Reaction conditions for each enzyme assay are as described below.

GlcT-1 activity was measured according to Lipsky and Pagano (*18*) with slight modifications (*1*). A reaction mixture (50 µl) composed of 20 mM Tris–HCl (pH 7.5), 500 µM UDP-Glucose, 10 µl liposomes [C_6-NBD-Cer (0.5 µg) and lecithin (5 µg) in water] and 30 µg cell protein was incubated at 25°C for 2 h.

The LacCer synthase reaction was performed using the method of Nishie *et al.* (*19*) with slight modifications. A reaction mixture (50 µl) composed of 50 mM HEPES buffer (pH 7.5), 200 µM UDP-Galactose, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 µl liposomes [C_6-NBD-GlcCer (0.5 µg) and lecithin (5 µg) in water] and 60 µg cell protein was incubated at 30°C for 12 h.

For glucocerebrosidase assays, a reaction mixture (50 µl) composed of 50 mM sodium acetate buffer (pH 5.2), 10 µl liposomes [C_6-NBD-GlcCer (0.5 µg) and lecithin (5 µg) in water] and 50 µg cell protein was incubated at 37°C for 30 min.

Klotho-related protein (neutral glycoceramidase) reactions were carried out according to Hayashi *et al.* (*20*) with slight modifications. A reaction mixture (50 µl) composed of 50 mM MES buffer (pH 6.0), 0.08% deoxycholate, 10 µl liposomes [C_6-NBD-GlcCer (0.5 µg) and lecithin (5 µg) in water] and 7 µg cell protein was incubated at 37°C for 30 min.

Reverse transcription-polymerase chain reaction

Total RNA was purified from the cells using TRIzol Reagent (Invitrogen). The cDNA was synthesized from total RNA (2.5 µg) using MMLV reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed with the Expand High Fidelity PCR system (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instruction with the cDNA as a template. Glyceraldehyde-3-dehydrogenase (G3PDH) primers were used for amplification of G3PDH cDNA as a normalization control. The following primers and thermal cycling conditions were used.

GlcT-1:
GlcT-1 forward primer: 5′-ATGATGATCCAGCCATTGATGTA TGT-3′
GlcT-1 reverse primer: 5′-TCAATTTCTCAGTTTCAATCCAGA ATG-3′
94°C for 1 min, 61°C for 1 min and 68°C for 2 min, 29 cycles.
G3PDH:
G3PDH forward primer: 5′-TCTTTTGCGTCGCCAGCCGAGC-3′
G3PDH reverse primer: 5′-GAAGGCTGGGGCTCATTTGCAGG-3′
94°C for 1 min, 67°C for 1 min and 68°C for 2 min, 18 cycles.
Glucocerebrosidase:
GBA forward primer: 5′-TACTGTGACTCCTTTGACCCC-3′
GBA reverse primer: 5′-CAGGTCTGGTGGTAGATGTCT -3′
94°C for 1 min, 57°C for 1 min and 68°C for 2 min, 24 cycles.
Klotho-related protein:
KLrP forward primer: 5′-AAAAGGCCCTTGTGTCTGGGA-3′
KLrP reverse primer: 5′-AAAAGCAGACGCCTCTCCTGT-3′
94°C for 1 min, 57°C for 1 min and 68°C for 2 min, 30 cycles.
β-1,4-galactosyltransferase V (LacCer synthase):
B4GALT5 forward primer: 5′-TGCTCAGGTTTATGAGCAGGT-3′
B4GALT5 reverse primer: 5′-ATAGTTGCGATCACTTTCCGG-3′
94°C for 1 min, 57°C for 1 min and 68°C for 2 min, 26 cycles.
β-1,4-galactosyltransferase VI (LacCer synthase)
B4GALT6 forward primer: 5′-GTACAAGCTCGAGGTATAATG-3′
B4GALT6 reverse primer: 5′-AGGTTGTGTGCCAGTCTGTTC-3′
94°C for 1 min, 57°C for 1 min and 68°C for 2 min, 26 cycles.
GM3 synthase (Sialyltransferase I):
SAT1 forward primer: 5′-TTTGGGTTCCGGAAGTTCTCC-3′
SAT1 reverse primer: 5′-CGAAGTAGTGCAAAGGTGTTC-3′
94°C for 1 min, 57°C for 1 min and 68°C for 2 min, 27 cycles.
For semi-quantitative analysis of amplified cDNA fragments, agarose gel images were processed with NIH ImageJ software.

Promoter analysis

To search for transcription factor binding sites, the promoter sequence of the human GlcT-1 gene was subjected to TFSEARCH program (Yutaka Akiyama, http://mbs.cbrc.jp/research/db/TFSEARCHJ.html) (*21*).

Western blot analysis

Generation of anti-GlcT-1 antibody and western blot analysis of GlcT-1 were performed according to Watanabe *et al.* (*22*).

Reporter gene assay

The 1.3 and 2.4 kb human GlcT-1 promoter fragments were amplified by PCR using a pair of hPF1 and hPR1 primers (1.3 kb), or a pair of hPF2 and hPR1 primers (2.4 kb). RPCI11.C DNA carrying the GlcT-1 gene (Life Technologies Japan, Tokyo Japan) was used as a template.

hPF1: 5′-ATCTCGAGTTGAGTACTAGCATCTTCTTG-3′ (between positions −1407 and −1387 when A of the initiator ATG is numbered as 0)
hPF2: 5′-ATCTCGAGACTATAGGTGCCATAACCATG-3′ (between positions −2428 and −2408 when A of the initiator ATG is numbered as 0)
hPR1: 5′-ATAAGCTTGGGAGGCGAGAGGAAGGT-3′ (between positions −98 and −115 when A of the initiator ATG is numbered as 0).

The amplified DNA fragments were double digested with *Xho*I and *Hind*III, and cloned into the pGL4.17 vector carrying the luciferase gene (Promega, Tokyo Japan). The resulting constructs were transfected into U937 cells using the Lipofectamine 2000 transfection reagent (Invitrogen) and selected with 400 µg/ml G418 for two weeks. Luciferase activity was measured by a luminometer using the Luciferase Enzyme Assay System (Promega).

DNA manipulation

DNA manipulation was carried out according to Sambrook and Russell (*23*).

Protein assay

Total protein amounts were assayed using the BCA protein assay reagent kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Results

Forty-eight hours after treatment with TPA (10 ng/ml), >90% of the U937 cells adhered to the dish and differentiated into macrophage-like cells as assessed by non-specific esterase activity (data not shown). Adherence began 8 h after treatment initiation and most of the cells had adhered within 20 h. Since GSLs are known to stimulate adhesion of cells to a dish, we analysed the lipid content of cell lysates from the TPA-treated cells after 20 h treatment. The major GSLs in U937 cells were GlcCer and GM3. LacCer levels were too low to be detectable. As shown in Fig. 1A and B, TPA treatment increased GlcCer and GM3 levels to 3.5- and 1.3-times, respectively, higher than that seen in untreated cells. We postulated that this increased GSL content was due to enhanced GlcT-1 activity and therefore we directly assessed GlcT-1 enzyme activity. As expected, GlcT-1 activity in TPA-treated cell lysates was ~2-fold higher than that of untreated control cells (Fig. 2A and B). The same level of activation was observed for lysates obtained from cells 48 h after treatment (data not shown). To determine if the observed activity increase was caused by an activator in TPA-treated cells, we mixed cell lysates from TPA-treated and TPA-untreated cells and estimated the enzyme activity. The GlcT-1 activity of the mixture was essentially the average of the individual treated and untreated lysates (Fig. 3A and B), indicating that no activator was present in TPA-treated cell lysates. Since the increased GlcT-1 activity was likely not caused by alterations in enzyme properties, we estimated the GlcT-1 mRNA levels by semi-quantitative RT–PCR. As expected, mRNA levels were much higher in TPA-treated cells [Fig. 4A(a) and A(b)], whereas no difference was seen in G3PDH mRNA expression [Fig. 4B(a) and B(b)]. Thus, we examined transcriptional activation of human GlcT-1 promoter using a reporter gene assay. Fragments from human GlcT-1 promoter region (1.3 kb and 2.4 kb) were ligated to the luciferase gene and stably transfected into U937 cells (Fig. 5A). Unexpectedly, luciferase activities of the stable transfectants were not altered when cell lysates were tested 20 h after TPA treatment (Fig. 5B). In addition, luciferase activities were not increased, but rather decreased 48 h after TPA addition (data not shown). Since the elevated GlcT-1 mRNA level could be due to a reduction in the mRNA degradation, we analysed GlcT-1 mRNA degradation. The *de novo* synthesis of mRNA was blocked by actinomycin D and decreases in GlcT-1 mRNA

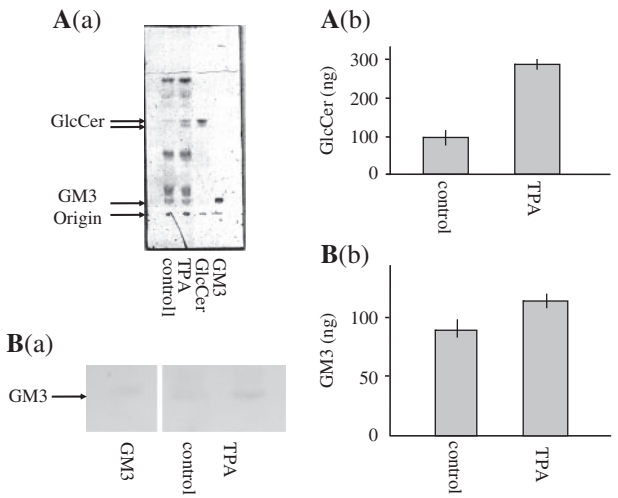

Fig. 1 GSLs in TPA-treated U937 cells were analysed by silica gel TLC. The cells were treated with 10 ng/ml TPA for 20 h. A(a) After separation by TLC, GSLs were visualized with orcinol/sulphuric acid reagent. The amount of cells corresponding to 600 μg protein content was used for analysis. A(b) GlcCer was quantitated after TLC separation. Data represent the means from three experiments and the bars indicate SD. The GlcCer content of untreated cells corresponding to 600 μg cellular protein is 95 ng. B(a) TLC immunostaining of GM3 with M2590 anti-GM3 mAb. The amount of cells corresponding to 100 μg protein content was used for analysis. B(b) GM3 was quantitated after TLC separation. Data represent the means of three experiments and bars indicate SD. The GM3 content of untreated cells corresponding 100 μg cellular protein is 91 ng. Control, untreated cells; TPA, cells treated with 10 ng/ml TPA for 20 h.

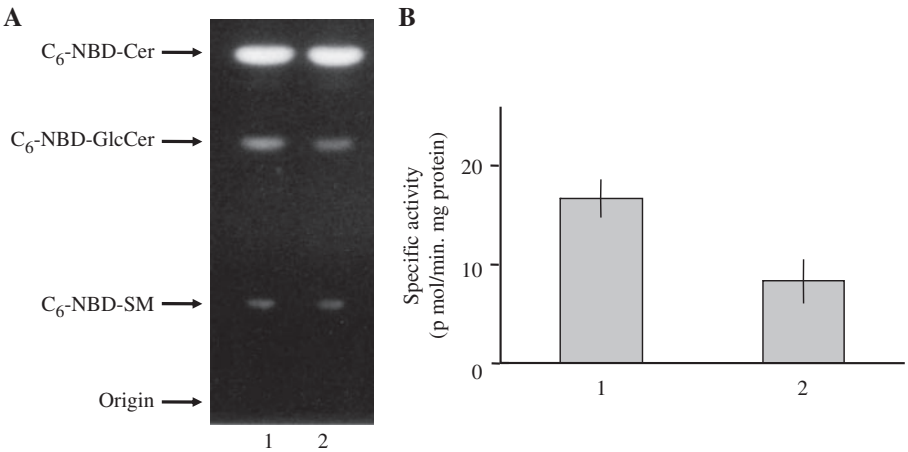

Fig. 2 GlcT-1 activity in cell lysates from TPA-treated U937 cells. (A) TLC analysis of GlcT-1 product as catalysed by TPA-treated U937 cell lysates. The cells were treated with 10 ng/ml TPA for 20 h. Cell lysates (30 μg protein) were used as the enzyme source. Lane 1, TPA-treated cells; lane 2, untreated control; SM, sphingomyelin. (B) The amount of C_6-NBD-GlcCer generated by the enzyme reaction. Column 1, TPA-treated cells; column 2, untreated control. The specific activity of GlcT-1 in untreated control cells is 8.4 p mol/min. mg protein.

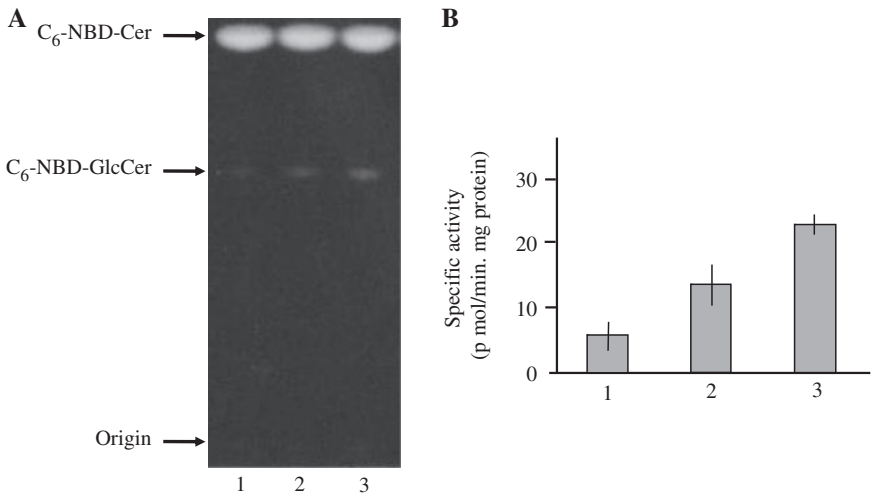

Fig. 3 The effect of TPA-treated cell lysates on GlcT-1activity. Equal amounts of cell lysates from TPA-treated and untreated cells were mixed and the activity was measured. The cells were treated with TPA (10 ng/ml) for 20 h. (A) TLC analysis of GlcT-1 as catalysed by cell lysates. Lane 1, untreated cells (30 μg protein); lane 2, mixture of TPA-treated and TPA-untreated cells (15 μg protein each, total 30 μg protein); lane 3, TPA-treated cells (30 μg protein). (B) The amount of C_6-NBD-GlcCer generated by the enzyme reaction. Column 1, untreated cells (30 μg protein); column 2, mixture of TPA-treated and TPA-untreated cells (15 μg protein each, total 30 μg protein); column 3, TPA-treated cells (30 μg protein). Data are the means of three experiments with SD shown. The specific activity of GlcT-1 in untreated cell lysates is 5.8 p mol/min. mg protein.

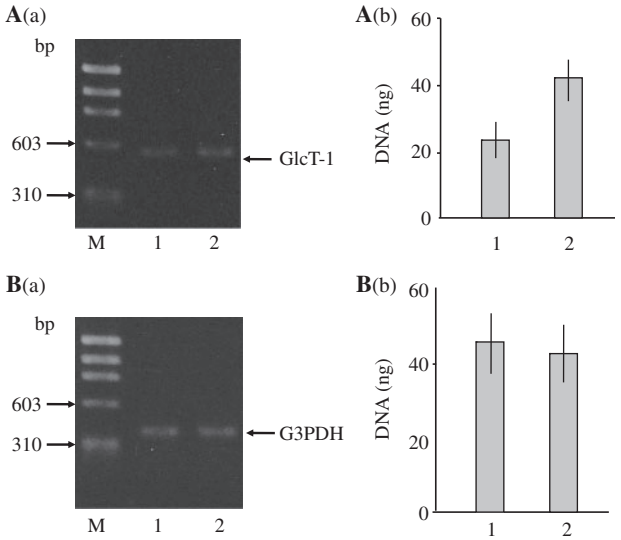

Fig. 4 GlcT-1 mRNA expression in TPA-treated U937 cells was estimated by RT–PCR. PCR products were subjected to 1% agarose gel electrophoresis. The cells were treated with 10 ng/ml TPA for 20 h. A(a) lane M, ϕX174/HaeIII digest; lane 1, untreated control; lane 2, TPA-treated cells. A(b) Semi-quantitative analysis of GlcT-1 mRNA. Column 1, untreated control; column 2, TPA-treated cells. B(a) Expression of G3PDH mRNA as a normalization control. Lane M, ϕX174/HaeIII digest; lane 1, untreated control; lane 2, TPA-treated cells; B(b) Semi-quantitative analysis of G3PDH mRNA. Column 1, untreated control; column 2, TPA-treated cells; G3PDH, glyceraldehyde 3-phosphate dehydrogenase. Data are the means of three experiments and bars indicate the SD.

levels in TPA-treated and TPA-untreated cells were compared. The degradation rate was faster for TPA-treated cells (data not shown), indicating that the increase of cellular GlcT-1 mRNA content was not caused by a reduced mRNA degradation rate. We also examined GlcT-1 protein content in the TPA-treated cells by western blot analysis and found that GlcT-1 levels were increased (Fig. 6).

Since increased GlcCer levels might also be caused by alterations in other GlcCer-metabolizing enzymes, we analysed the mRNA levels and activities of those enzymes. As shown in Fig. 7, mRNA expression of the LacCer synthases, β-1,4-galactosyltransferse V (B4GALT5) (*24*) and β-1,4-galactosyltransferase VI (B4GALT6), (*25*) were dramatically decreased. On the other hand, a large increase in GM3 synthase mRNA was observed as was also reported previously for HL-60 cells (Fig. 7) (*13*). In addition, we examined mRNA levels of the GlcCer-degrading enzymes, glucocerebrosidase and Klotho-related protein and detected a slight and moderate increase in mRNAs for glucocerebrosidase and Klotho-related protein, respectively (Fig. 8). Since alterations in these mRNA levels of the enzymes were observed, we next measured their enzyme activities. LacCer-synthesizing activity, comprised of β-1,4-galactosyltransferse V and β-1, 4-galactosyltransferase VI, was measured and a large decrease in activity was detected in TPA-treated cells (Fig. 9). On the other hand, glucocerebrosidase and Klotho-related protein activities were increased by 1.7- and 2.4-fold, respectively (Fig. 10).

Discussion

For macrophage-like cells, both GSLs and sphingomyelin play important roles in cellular adherence to the substratum (*11*, *14*). Although the detailed mechanism is not clear, lipid rafts containing these lipids may serve as platforms for cell adhesion molecules such as integrins. Since the increase of GlcCer was much greater than that of GM3, GlcCer might be the most important lipid involved in this adhesion. Previously, Kan *et al.* (*11*) demonstrated that the

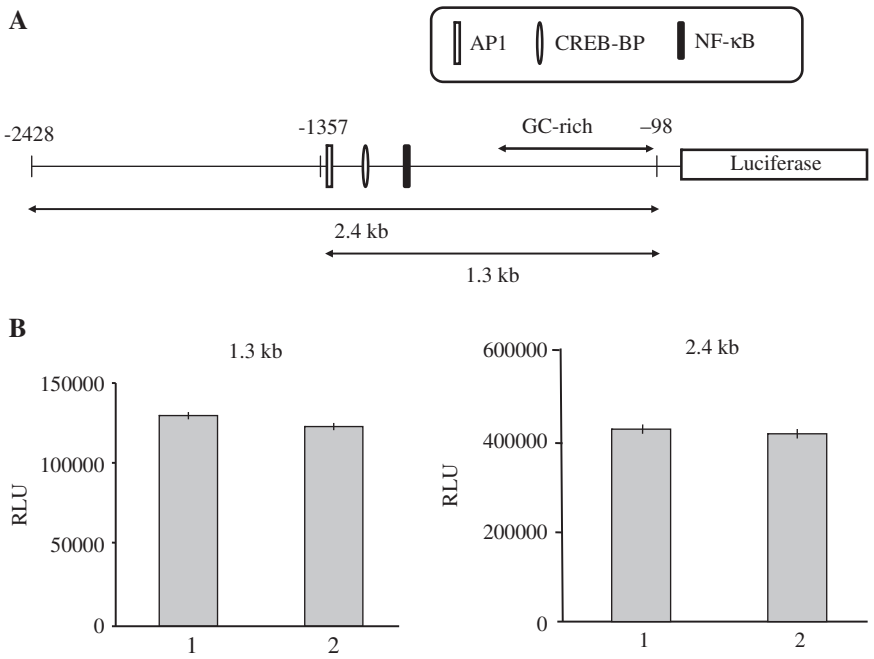

Fig. 5 Human GlcT-1 promoter activity in TPA-treated U937 cells. (A) Schematic diagram of a chimeric promoter construct composed of the GlcT-1 promoter and luciferase gene. Transcription factor binding sites and the GC-rich region containing putative Sp1 binding sites are indicated. (B) GlcT-1 promoter activity of TPA-treated cells. U937 cells (2×10^6) carrying the chimera of the GlcT-1 promoter and luciferase gene were seeded in 10 ml DMEM containing 10% FBS and 10 ng/ml TPA and incubated at 37°C under 5% CO_2 for 20 h. The cells were harvested and lysed in Cell Culture Lysis Buffer (Promega). The cell lysate (30 μg protein in 10 μl) was then mixed with 50 μl luciferase substrate (Promega) and the luminescence was measured by a luminometer. Lane 1, untreated control; lane 2, TPA-treated cells. Data are the means of three experiments and bars indicate SD. RLU: relative light unit.

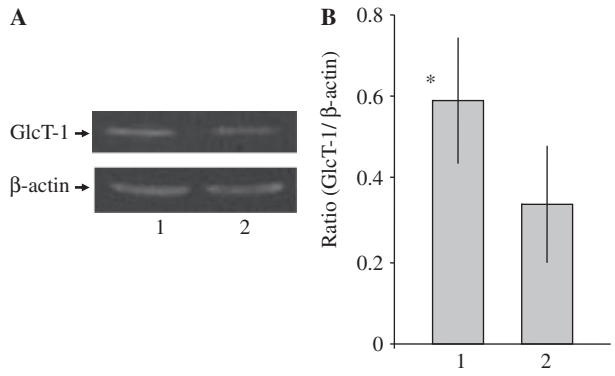

Fig. 6 Expression of GlcT-1 protein in TPA-treated U937 cells analysed by western blot analysis. The cells were treated with 10 ng/ml TPA for 20 h. The cell lysates (50 μg each) from untreated and TPA-treated cells were analysed. Expression of β-actin was also analysed as a normalization control. (A) lane 1, TPA-treated cells; lane 2, untreated control. (B) Histogram shows the ratio of GlcT-1/β-actin after pixel quantification. Column 1, TPA-treated cells; column 2, untreated control. Data are the means of three experiments presented with SD bars. $*P < 0.01$.

GlcT-1 inhibitor PDMP inhibits adherence during TPA-induced macrophage-like differentiation of HL-60 cells. They also observed an increase in cellular GSLs, including GlcCer and GM3. Chung *et al.* (*13*) showed that GM3 synthase is up-regulated during the differentiation of HL-60 cells. Although the involvement of GSLs in adhesion has been established, the mechanisms responsible for this increase have not been fully elucidated. Thus, we examined the mechanism underlying the increase of GSLs, including GlcCer, during cellular differentiation. In our studies of U937 cells, the increase in GlcCer was due to increased GlcT-1 activity. We assume that the activation of GlcT-1 stimulates cellular adhesion via this increased total GSL content.

Increased activity of GlcT-1 has been reported previously for normal differentiation of human keratinocytes (*22*) and neuronal differentiation of the pheochromocytoma cell line PC12 (*26*). In the case of PC12 differentiation induced by TPA, the cells up-regulate expression of the proto-oncogene c-*fos* and its protein product, c-Fos. c-Fos can directly bind to and activate GlcT-1 (*26*). Since the up-regulation of c-Fos expression during U937 differentiation was reported previously (*27*), we analysed the c-Fos content in TPA-treated cells under our experimental conditions and confirmed that c-Fos levels did indeed increase (data not shown). However, the increase in GlcT-1 activity is presumably not due to direct interaction of c-Fos and GlcT-1 because cell lysates from TPA-treated cells did not stimulate the activity in untreated cell lysates (Fig. 3A and B), although it is also possible that there was insufficient free c-Fos present in the TPA lysate to activate GlcT-1. On the other hand, RT–PCR revealed an increase in GlcT-1 mRNA in TPA-treated cells [Fig. 4A(a) and (b)]. Kitamura *et al.* (*28*) investigated mRNA profiles in the cytoplasmic fraction of U937 cells before and after TPA stimulation using microarrays with 15107 oligonucleotide probes and detected a 2.7-fold increase of GlcT-1 mRNA, which supports our RT–PCR data. Since this increase could be the consequence of enhanced

transcriptional activity, we examined GlcT-1 promoter activity using a reporter gene assay. Both mouse (*29*) and human GlcT-1 promoters contain multiple Sp1 binding sites as basal elements and several putative regulatory elements, including NF-κB and cAMP responsive element-binding protein (CRE-BP) binding sites. In addition to those elements, the human GlcT-1 promoter contains a site for AP1 binding (Fig. 5A). c-Fos is known to bind to the AP1 motif to stimulate transcription and CRE-BP is also known to be activated by TPA. Therefore, we examined the promoter activity of a 1.3 kb human GlcT-1 promoter fragment containing these motifs. Unexpectedly, no enhancement of luciferase activity was observed in the TPA-treated cells (Fig. 5B), suggesting that the putative AP1, CRE-BP and NF-κB motifs found in this 1.3 kb promoter region are not functional and the sequence responsible for the enhanced mRNA synthesis was not present in this fragment. Since sequences further upstream from this promoter fragment could be responsible for the enhanced GlcT-1 activity, we examined a 2.4 kb promoter fragment carrying an additional 1.1 kb of 5′ sequences that lie upstream of the 1.3 kb fragment. Again, no enhancement of luciferase activity was observed. This result indicates that the regulatory sequences do not exist in this 2.4 kb promoter fragment. Sequences further upstream from this 2.4 kb fragment are unlikely to be responsible for the increase in GlcT-1 mRNA because this region lies too distal from the GC-rich region containing putative Sp1 binding sites. In addition, the increase in GlcT-1 mRNA was not due to a reduced rate of mRNA degradation (data not shown). This discrepancy is presumably caused by differences between exogenous and authentic (endogenous) promoters. The local change of the chromatin structure around the authentic GlcT-1 promoter might be responsible for the increase in GlcT-1 mRNA and this change during cell differentiation could enhance the accessibility of the promoter to transcription factors and/or RNA polymerase II. So far, the mechanism of the GlcT-1mRNA increase is not entirely clear and requires further study.

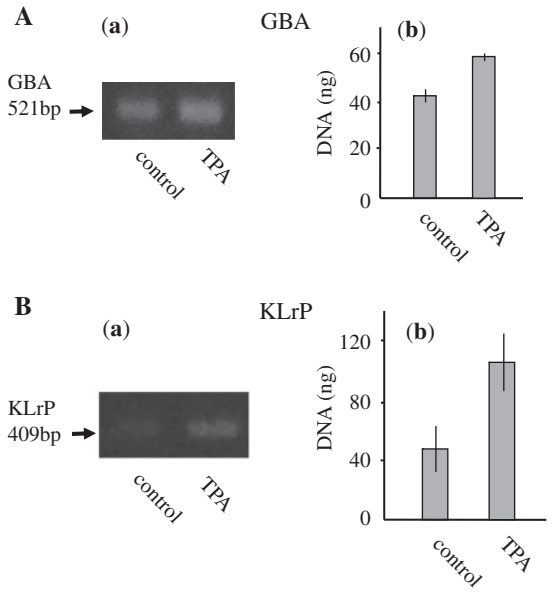

Fig. 8 Increased mRNA expression levels of enzymes involved in GlcCer degradation in TPA-treated U937 cells. (A and B) mRNA expression levels of enzymes involved in GlcCer degradation. The mRNA contents were estimated by RT–PCR. (a) PCR products were subjected to 1% agarose gel electrophoresis. (b) Semi-quantitative analysis of mRNAs. Data are the means of three experiments and bars indicate SD. Control, untreated cells; TPA, cells treated with 10 ng/ml TPA for 20 h; GBA, glucocerebrosidase; KLrP, Klotho-related protein.

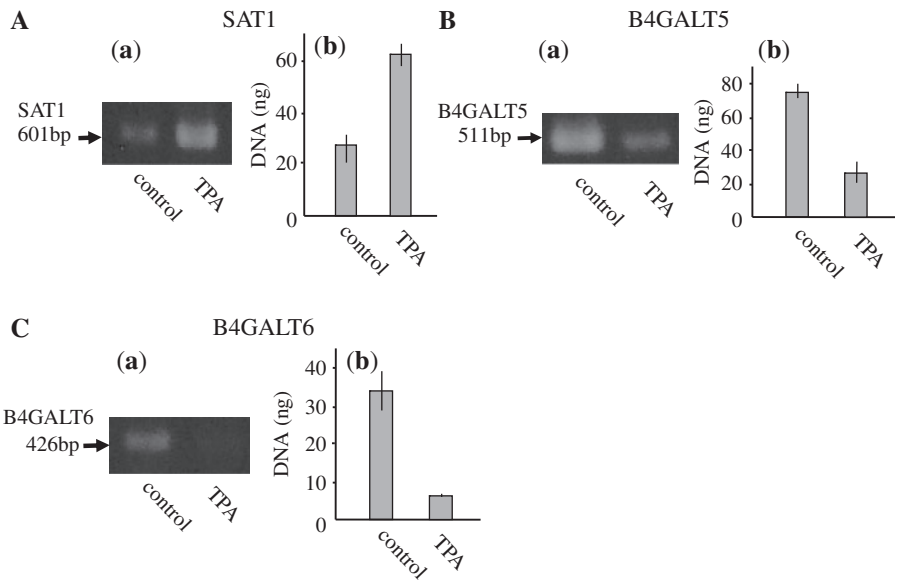

Fig. 7 Expression of glycosyltransferase mRNAs in TPA-treated U937 cells. (A–C) mRNA expression levels of glycosyltransferases involved in GSL synthesis in TPA-treated U937 cells. The mRNA contents were estimated by RT–PCR. (a) PCR products were subjected to 1% agarose gel electrophoresis. (b) Semi-quantitative analysis of mRNAs. Data are the means of three experiments and bars indicate SD. Control, untreated cells; TPA, cells treated with 10 ng/ml TPA for 20 h; B4GALT5, β-1,4-galactosyltransferase V; B4GALT6, β-1,4-galactosyltransferase VI; SAT1, GM3 synthase. B4GALT5 and B4GALT6 are LacCer synthases.

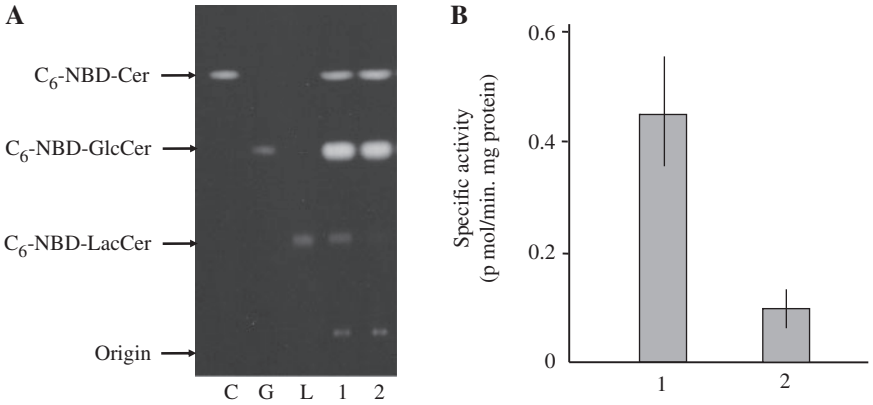

Fig. 9 LacCer synthase activity in cell lysates from TPA-treated U937 cells. (A) TLC analysis of LacCer synthase-product catalysed by cell lysates from TPA-treated U937 cells. The cells were treated with 10 ng/ml TPA for 20 h. Cell lysates (60 μg protein) were used as the enzyme source. Lane C, C_6-NBD-Cer; lane G, C_6-NBD-GlcCer; lane L. C_6-NBD-LacCer; lane 1, untreated control; lane 2, TPA-treated cells. (B) The amount of C_6-NBD-LacCer generated by the enzyme reaction. Column 1, untreated control; column 2, TPA-treated cells. The specific activity of LacCer synthase in untreated control cells is 0.45 p mol/min. mg protein. LacCer synthase activity is comprised of β-1,4-galactosyltransferase V and β-1,4-galactosyltransferase VI activities.

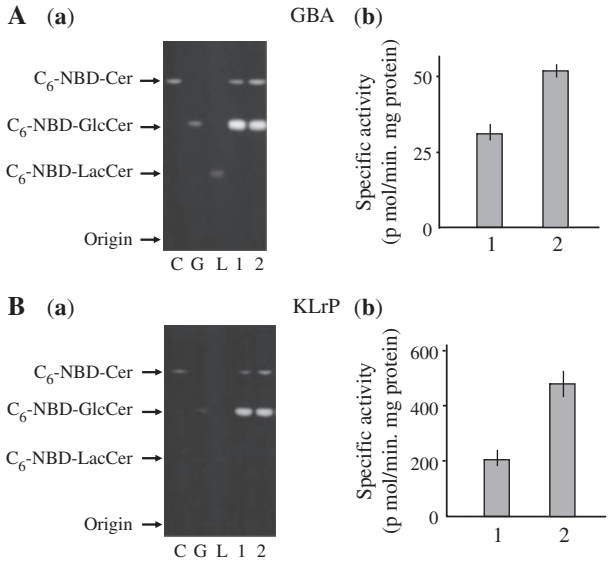

Fig. 10 Increased activity of GlcCer-degrading enzymes in cell lysates from TPA-treated U937 cells. (A and B) Activity of GlcCer-degrading enzymes in cell lysates from TPA-treated U937 cells. Cells were treated with 10 ng/ml TPA for 20 h. GBA, glucocerebrosidase; KLrP, Klotho-related protein. (a) TLC analyses of the enzyme products. Lane C, C_6-NBD-Cer; lane G, C_6-NBD-GlcCer; lane L. C_6-NBD-LacCer; lane 1, untreated control; lane 2, TPA-treated cells. (b) The amount of C_6-NBD-Cer generated by enzyme reactions. Column 1, untreated control; column 2, TPA-treated cells. The specific activities of glucocerebrosidase and Klotho-related protein in untreated control are 30.7 and 206 p mol/min. mg protein, respectively.

Since the increase in cellular GlcCer content might be caused by alterations in other GlcCer-metabolizing enzymes, we analysed mRNA levels and activities of those enzymes. We found decreases in mRNA levels and activity of LacCer synthase in TPA-treated cells (Figs 7 and 9). As GlcCer is a substrate for LacCer synthase, the decrease in LacCer synthase activity might also contribute to the increased GlcCer content in TPA-treated cells. Unexpectedly, we observed large increases in mRNA levels and activities of the GlcCer-degrading enzymes glucocerebrosidase and Klotho-related protein in TPA-treated cells (Figs 8 and 10). Why these increases in enzyme activity did not reduce cellular GlcCer content is unclear, but lysosomal localization of glucocerebrosidase could require GlcCer to be transported to lysosomes for degradation. Glucocerebrosidase also requires other proteins such as saposins for its activation *in vivo*, which may not have been available in sufficient amounts. Klotho-related protein is located in the cytosol and GlcCer is also synthesized on the cytoplasmic side of the Golgi apparatus. GlcCer, however, is translocated to the lumenal side of the organelle by flippase activity. Presumably, this translocation is rapid and most of GlcCer may be translocated in advance of the enzyme degradation.

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Conflict of interest

None declared.

References

1. Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K.I. -P.J., and Hirabayashi, Y. (1996) Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl Acad. Sci. USA* **93**, 4638–4643
2. Ichikawa, S., Ozawa, K., and Hirabayashi, Y. (1998) Molecular cloning and expression of mouse ceramide glucosyltransferase. *Biochem. Mol. Biol. Int.* **44**, 1193–1202
3. Basu, S., Kaufman, B., and Roseman, S. (1968) Enzymatic synthesis of ceramide-glucose and ceramide-

lactose by glycosyltransferases from embryonic chicken brain. *J. Biol. Chem.* **243**, 5802–5804
4. Ichikawa, S. and Hirabayashi, Y. (1998) Glucosylceramide synthase and glycosphingolipid synthesis. *Trends Cell Biol.* **8**, 198–202
5. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**, 97–130
6. Hannun, Y.A. (1994) The sphingomyelin cycle and second messenger function of ceramide. *J. Biol. Chem.* **269**, 3125–3128
7. Komori, H., Ichikawa, S., Hirabayashi, Y., and Ito, M. (1999) Regulation of intracellular ceramide in B16 melanoma cells: biological implication of ceramide glycosylation. *J. Biol. Chem.* **274**, 8981–8987
8. Aert, J.M., Ottenhoff, R., Powlson, A.S., Grefhorst, A., van Eijk, M., Dubbelhuis, P.F., Aten, J., Kuipers, F., Serlie, M.J., Wennekes, T., Sethi, J.K., O'Rahilly, S., and Overkleeft, H.S. (2007) Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes* **56**, 1341–1349
9. Liu, Y.Y., Han, T.Y., Giuliano, A.E., and Cabot, M.C. (2001) Ceramide glucosylation potentiates cellular multidrug resistance. *FASEB J.* **15**, 719–730
10. Ralph, P., Moore, M.A., and Nilsson, K. (1976) Lysozyme synthesis by established human and murine histiocytic lymphoma cell line. *J. Exp. Med.* **143**, 1528–1533
11. Kan, C.C. and Kolesnick, N. (1992) A synthetic ceramide analog, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, selectively inhibits adherence during macrophage differentiation of human leukemia cells. *J. Biol. Chem.* **267**, 9663–9667
12. Nojiri, H., Takaku, F., Tetsuka, T., Motoyoshi, K., Miura, Y., and Saito, M. (1984) Characteristic expression of glycosphingolipid profiles in the bipotential cell differentiation of human promyelocytic leukemia cell line HL-60. *Blood* **64**, 534–541
13. Chung, T.W., Choi, H.J., Lee, Y.C., and Kim, C.H. (2005) Molecular mechanism for transcriptional activation of ganglioside GM3 synthase and its function of differentiation of HL-60 cells. *Glycobiology* **15**, 233–244
14. Dressler, K.A., Kan, C.C., and Kolesnick, R.N. (1991) Sphingomyelin synthesis is involved in adherence during macrophage differentiation of HL-60 cells. *J. Biol. Chem.* **266**, 11522–11527
15. Hidari, K.I.-P.J., Ichikawa, S., Fujita, T., Sakiyama, H., and Hirabayashi, Y. (1996) Complete removal of sphingolipids from the plasma membrane disrupts cell-substratum adhesion. *J. Biol. Chem.* **271**, 14636–14641
16. Li, C.Y., Lam, K.W., and Yam, L.T. (1973) Esterases in human leukocytes. *J. Histochem. Cytochem.* **21**, 1–12
17. Higashi, H., Fukui, Y., Ueda, S., Kato, S., Hirabayashi, Y., Matsumoto, M., and Naiki, M. (1984) Sensitive enzyme-immunostaining and densitometric determination on thin-layer chromatography of N-glycolylneuraminic acid-containing glycosphingolipids, Hanganutziu-Deicher antigens. *J. Biochem.* **95**, 1517–1520
18. Lipsky, N.G. and Pagano, R.E. (1985) Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucocerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. *J. Cell Biol.* **100**, 27–34
19. Nishie, T., Hikimochi, Y., Zama, K., Fukusumi, Y., Ito, M., Yokoyama, H., Naruse, C., Ito, M., and Asano, M. (2010) β4-galactosyltransferase-5 is a lactosylceramide synthase essential for mouse extra-embryonic development. *Glycobiology* **20**, 1311–1322
20. Hayashi, Y., Okino, N., Kakuta, Y., Shikanai, T., Motohiro, T., Narimastu, H., and Ito, M. (2007) Klotho-related protein is a novel cytosolic neutral β-glycosylceramidase. *J. Biol. Chem.* **282**, 30889–30900
21. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A.E., Kel, O.V., Ignatieva, E.V., Ananko, E.A., Podkolodnaya, O.A., Kolpakov, F.A., Podkolodny, N.L., and Kolchanov, N.A. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Res.* **26**, 364–370
22. Watanabe, R., Wu, K., Paul, P., Marks, D.L., Kobayashi, T., Pittelkow, M.R., and Pagano, R.E. (1998) Up-regulation of glucosylceramide synthase expression and activity during human keratinocyte differentiation. *J. Biol. Chem.* **273**, 9651–9655
23. Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: Laboratory Manual*. 3rd edn, Cold Spring Harbor Laboratory Press, Plainview, NY
24. Kumagai, T., Sato, T., Natsuka, S., Kobayashi, Y., Zhou, D., Shinakai, T., Hayakawa, S., and Furukawa, S. (2010) Involvement of murine β-1.4-galactosyltransferase V in lactosylceramide synthesis. *Glycoconj. J* **27**, 685–695
25. Nomura, T., Takizawa, M., Aoki, J., Arai, H., Inoue, K., Wakisaka, E., Yoshizuka, N., Imokawa, G., Dohmae, N., Takio, K., Hattori, M., and Matsuo, M. (1998) Purification cDNA cloning, and expression of UDP-Gal: glucosylceramide β-1,4-galactosyltransferase from rat brain. *J. Biol. Chem.* **273**, 13570–13577
26. Crespo, P.M., Silvestre, D.C., Gil, G.A., Maccioni, H.J.F., Daniotti, J.L., and Caputto, B.L. (2008) c-Fos activates glucosylceramide synthase and glycolipid synthesis in PC12 cells. *J. Biol. Chem.* **283**, 31163–31171
27. Brazilay, J., Kushtai, G., Plaksin, D., Feldman, M., and Eisenbach, L. (1987) Expression of major histocompatibility class I genes in differentiating leukemia cells is temporally related to activation of c-fos proto-oncogenes. *Leukemia* **1**, 198–204
28. Kitamura, H., Nakagawa, T., Takayama, M., Kimura, Y., Hijikata, A., and Ohara, O. (2004) Post-transcriptional effects of 12-myristate 13-acetate on transcriptome of U937 cells. *FEBS Lett.* **578**, 180–184
29. Ichikawa, S., Ozawa, K., and Hirabayashi, Y. (1998) Molecular cloning and characterization of the mouse ceramide glucosyltransferase gene. *Biochem. Biophys. Res. Commun.* **253**, 707–711