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Glycosylation of Amphipathic Lactoside Primers with Consequent Inhibition of Endogenous Glycosphingolipid Synthesis[1]

Hideki Nakajima, Yoshiaki Miura,[2] and Tatsuya Yamagata[3]

Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midoriku, Yokohama 226-8501

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The incubation of amphipathic lactosides with cultured cells was found to prime the glycosylation of lactosides whose oligosaccharide structures were exactly the same as those of glycosphingolipids produced by cells: B16 melanoma cells produced α2-3 sialylated lactosides; and PC12 cells, Galα1-4- and Galα1-3Galα1-4lactosides. Analysis of the cell-associated glycosylated products indicated that C16 series lactoside primers function 5-6 times more efficiently as acceptors than C12 series primers. The glycosylated lactosides were also secreted into the culture medium. Lactoside primers with longer hydrophobic chains hampered the release of glycosylated products from cells. The presence of an *N*-acyl chain in the lipophilic moiety of primers suppressed the secretion of glycosylated products. Owing to its overall availability, lactosides with the C12 alkyl chain were glycosylated 2-3 times more than C16 series lactosides and 1.4 times more than lactosides with the C12 acyl chain. C8-lactosides did not function as primers under the conditions of this study, but they were found to be the best acceptors for sialic acid transfer with the soluble enzyme fraction. The incubation of cells with 10 μM *N*-hexadecanoylaminoethyl-β-*O*-lactoside caused a 30% decrease in endogenous GM3 of B16 cells and a 34% decrease in Gb3Cer synthesis of PC12 cells. The results of the present study demonstrate that lactoside primers serve as an efficient means to inhibit endogenous glycosphingolipids in studies to clarify glycosphingolipid functions.

Key words: *N*-acyl chain, glycoside primer, glycosphingolipid, glycosyltransferase, lactoside, lactosyl ceramide.

Glycosphingolipids (GSLs) are situated mainly on the outer leaflet of plasma membranes of higher vertebrates and may be essential for cell recognition, proliferation, motility, and cell-substrate interactions in biological processes such as cell differentiation, embryogenesis, inflammation, and carcinogenesis (*1-3*). GSL functions, though studied extensively, are not yet fully understood. To increase our understanding, attention should be directed to approaches that include disruption of GSL synthesis through application of synthetic glycoside analogs to cells. The synthesis of sialylα2-3galactosylβ1-4xyloside was noted subsequent to incubating CHO cells with 4MU-β-xyloside (*4*). Octyl-β-glucoside also produced GSL-like molecules (*5*). The effects of β-xyloside were not GSL-specific, and β-glucoside failed to inhibit GSL biosynthesis. GSL synthesis occurs almost entirely via lactosyl ceramide (LacCer) in animal cells, and the lactosyl structure is unique to GSLs. Thus, in this study, lactose was chemically modified (*6*) to render it amphipathic, in order to facilitate the incorporation of amphipathic lactoside primers into cells to promote

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[2] Present address: The Burnham Institute, La Jolla Cancer Research Center, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA.

[3] To whom correspondence should be addressed at Japan Institute of Leather Research, 1-1 Senju-Midoricho, Adachiku, Tokyo 120-8601. Tel: +81-3-3888-5111 (Ext. 2383), Fax: +81-3-3870-9631, E-mail: tcyamagata@pop16.odn.ne.jp

Abbreviations: C8alkylLac, *n*-octyl-β-*O*-lactoside; C10alkylLac, *n*-decyl-β-*O*-lactoside; C12alkylLac, *n*-dodecyl-β-*O*-lactoside; C14alkylLac, *n*-tetradecyl-β-*O*-lactoside; C16alkylLac, *n*-hexadecyl-β-*O*-lactoside; C8amideLac, *N*-octanoyl-aminoethyl-β-*O*-lactoside; C12amideLac, *N*-dodecanoyl-aminoethyl-β-*O*-lactoside; C14amideLac, *N*-tetradecanoyl-aminoethyl-β-*O*-lactoside; C16amideLac, *N*-hexadecanoyl-aminoethyl-β-*O*-lactoside; pNp, *p*-nitrophenyl; 4MU, 4-methylumbelliferyl; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; CHO, Chinese hamster ovary; TI/DF, Dulbecco's modified Eagle medium and Ham's F12 (1:1) containing 30 nM SeO_2, 5 μg/ml transferrin, and 5 μg/ml insulin; PBS(−), phosphate-buffered saline without calcium and magnesium ions; FBS, fetal bovine serum; GSL, glycosphingolipid; HPTLC, high performance thin layer chromatography; GAG, glycosaminoglycan; GM3, NeuAcα2-3LacCer; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; GlcCer, glucosyl ceramide; LacCer, lactosyl ceramide; PSL, photo-stimulated luminescence; SA, sialic acid; Gb3Cer, globotriaosylceramide; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; Cer, ceramide; C2-Cer, *N*-acetylsphingosine; C6-Cer, *N*-hexanoylsphingosine; Le^x, $Lewis^x$.

GSL oligosaccharide synthesis. Subsequent to the temperature-dependent incorporation of amphipathic primers into B16 cells, sialylα2-3lactosides were obtained, whose oligosaccharide structures were exactly the same as that of GM3, the main ganglioside synthesized by B16 cells (*7*). Glycosylation of the primers also caused inhibition of endogenous GM3 synthesis, and the glycosylated primers were found to be situated on the cell surface membrane. The rate of uptake of primers by cells, the capacity for saccharide elongation, and the extent of accumulation on the cell surface appeared to depend on the hydrophobic structure. Amphipathic compound features were thus studied in detail, with special attention to the priming activity.

The present paper reports that LacCer analogs, each with a single lipophilic chain, are incorporated into cells and function as primers for GSL-specific glycosylation. Glycosylated C16 series lactosides accumulated more within cells than glycosylated C12 lactosides, while C12 series lactosides were more readily available as glycoside primers whose glycosylated products could be recovered from the medium fraction. The presence of amide bonds in hydrophobic moieties was noted to promote the association of glycosylated products with cells.

EXPERIMENTAL PROCEDURES

Materials—B16 and GM95 cells were obtained from Riken Cell bank (Tsukuba) and PC12 cells by courtesy of Dr. T. Amano (Mitsubishi Kasei Institute of Life Sciences, Tokyo). DMEM was from Dainippon Pharmaceutical. A mixture of DMEM and Ham F12 (1:1), fetal bovine serum (FBS), and horse serum were from Gibco BRL; and insulin and transferrin were from Wako Pure Chemical, Tokyo. [^{14}C]Galactose (9.25 MBq) and CMP-[^{14}C]NeuAc (370 kBq) were from Amersham; and tritiated sodium borohydride (1.0 Gbq) was from New England Nuclear-Du Pont. Neuraminidase from Newcastle disease virus [EC 3.2.1.18] was from Oxford GlycoSystems. Galactose oxidase [EC 1.1.3.9], peroxidase [EC 1.11.1.7], and α-galactosidase from coffee beans [EC 3.2.1.22] were from Sigma; and β-*N*-acetylhexosaminidase from jack bean [EC 3.2.1.52] was from Seikagaku, Tokyo. SepPak C18 was from Waters. HPTLC (Silica gel 60) and preparative TLC plates were from Merck, Darmstadt, Germany. The lactosides (*n*-octyl-β-*O*-lactoside, *n*-decyl-β-*O*-lactoside, *n*-dodecyl-β-*O*-lactoside, *n*-tetradecyl-β-*O*-lactoside, *n*-hexadecyl β-*O*-lactoside, *N*-octanoyl-aminoethyl-β-*O*-lactoside, *N*-dodecanoyl-aminoethyl-β-*O*-lactoside, *N*-tetradecanoyl-aminoethyl-β-*O*-lactoside, and *N*-hexadecanoyl-aminoethyl-β-*O*-lactoside) were prepared as described (*6*). All other chemicals were of the highest purity available.

Cell Culture—The B16 and GM95 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were detached through application of 0.025% trypsin-EDTA and passaged every 5 days. PC12 cells were grown in DMEM containing 10% FBS, 5% horse serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The cells were detached by vigorous pipetting, passaged every three days, and maintained in humidified atmosphere of 5% CO_2 air at 37°C.

Effects of Lactoside on Cell Growth—Inocula of 3.0×10^3 B16 cells were seeded into each well of a 96-well culture plate containing 50 μl of serum-free TI/DF and incubated for 6 h. Then lactosides were added at various concentrations and incubation was continued for a specified period. During the last 2 h of incubation, a cell counting kit (Dojindo, Kumamoto) was added to each well for measurement of absorbancy at 415 nm, with the reference wavelength at 660 nm.

Preparation of ^{3}H-Labeled Lactosides—Lactosides were tritiated with galactose oxidase-NaB^3H_4 as previously described (*8*) with slight modification. Each lactoside (10 mg) was dissolved in 2 ml of 50 mM phosphate buffer, pH 7.0, containing 5 mg of taurodeoxycholate and galactose oxidase (100 units) and incubated at 37°C for 1 day with gentle shaking. Then an extra portion of galactose oxidase (50 U) and peroxidase (1 mg) were added, and incubation was continued for 1 day. Following lyophilization, the lactosides were extracted with chloroform/methanol (2 : 1, by volume), dried under a N_2 gas stream, and each was dissolved in 1 ml tetrahydrofuran. Tritiated sodium borohydride (148 MBq) was added, and the reaction tube was shaken gently overnight at room temperature. Then 1 mg cold sodium borohydride was added to terminate the reaction, and the reaction mixture was left in the dark for 1 h. Thereafter, 10 ml water were added and each labeled lactoside was isolated using a SepPak C18 column and purified by preparative TLC. Radioactivity was measured using a liquid scintillation spectrometer (Beckman). The specific activities of C16alkylLac, C16amideLac, C12alkylLac, C12amideLac, C8alkylLac, and C8amideLac were determined based on radioactivity and densitometric quantification of compounds on a HPTLC plate as 487.3, 1,391, 710.2, 2,541, 734.7, and 383.1 Bq/nmol, respectively. Densitometric measurement was then conducted with a Bio 1D image analyzer (Vilber Lourmat, France) after spraying the HPTLC plates with orcinol-H_2SO_4.

Incubation of Cells with Radiolabeled Lactosides—Inocula of 2.0×10^6 cells were seeded into 100-mm culture dishes containing 5 ml of medium and incubated overnight. This was followed with washing with TI/DF to remove serum, and incubation with 5 μM ^{3}H-labeled lactosides in serum-free TI/DF for a specified time at 37°C. After incubation, the culture media were collected and cells were washed with PBS(−), harvested with 0.25% EDTA in PBS(−), and centrifuged at $1,000\times g$ for 10 min. The cell pellet thus obtained was resuspended in 1.5 ml of PBS(−), and 50-μl aliquots were used for protein determination (*9*) and radioactivity quantification. The lipids were extracted from remaining cells with chloroform/methanol (2 : 1, by volume), then with chloroform/isopropanol/water (7 : 11 : 2, by volume) in a sonicated bath. Lipids from the culture media were purified using a SepPak C18 column and analyzed by HPTLC with chloroform/methanol/0.2% aqueous calcium chloride (5 : 4 : 1, by volume) as the solvent. A BAS 2000 bioimage analyzer (Fuji Film) was used to detect and quantify radioactivity on the HPTLC plate.

Glycosylated Lactoside Identification—All lipid fractions were developed on the HPTLC plate, and the position of each tritium-labeled glycosylated lactoside was noted and quantified with the BAS 2000 bioimage analyzer. All glycosylated products were scraped from the plate and extracted with chloroform/methanol (2 : 1) for digestion with glycosidases. The reaction mixtures, containing 2 mU

sialidase from Newcastle disease virus or 5 mU α-galactosidase from coffee bean in 50 μl of 50 mM sodium acetate buffer, pH 5.5, and 0.1 mg/ml sodium taurodeoxycholate, or 5 mU β-*N*-acetylhexosaminidase from jack bean in 50 μl of 50 mM sodium acetate buffer, pH 5.0, and 0.1 mg/ml sodium taurodeoxycholate, were incubated at 37°C for 24 h. The digestion was terminated by immersing the reaction tubes in ice-cold water. The glycosides were purified using a SepPak C18 column, separated by HPTLC, and visualized with the BAS 2000 bioimage analyzer.

Acceptor Capacity of Amphipathic Lactosides In Vitro—GM95 cells (2.0×10^6 cells) cultured as above were suspended in PBS(−), homogenized with a sonicator (Sonics & Materials, 6 times 10 s each at 10 s intervals), and centrifuged at $1,000\times g$ for 10 min. The supernatant was recentrifuged at $11,000\times g$ for 20 min, and the supernatant was centrifuged again at $105,000\times g$ for 60 min. The Golgi vesicle fraction thus obtained was resuspended in 100 mM cacodylate buffer, pH 6.0, containing 5 mM $MgCl_2$ and 0.3% Triton CF-54, and protein concentration was determined (*9*). The reaction mixture containing 50 μl of 100 mM cacodylate buffer, pH 6.0, 5 mM $MgCl_2$, 0.3% Triton CF-54, 5 mM CMP-NeuAc, 0.5 mM lactoside, 3.7 kBq CMP-[^{14}C]NeuAc, and 50 μg of protein was incubated at 37°C for 3 h, and the reaction was terminated by immersion

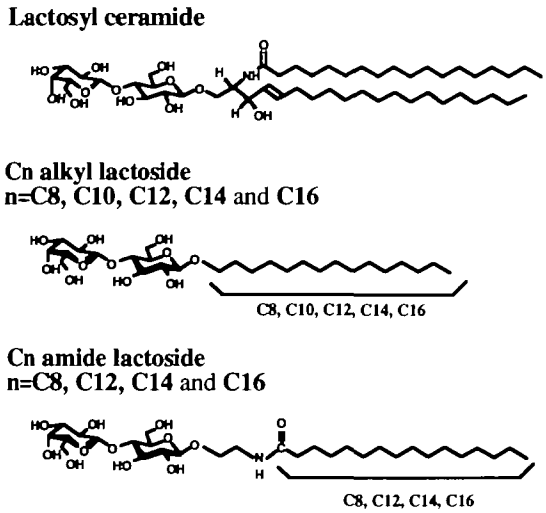
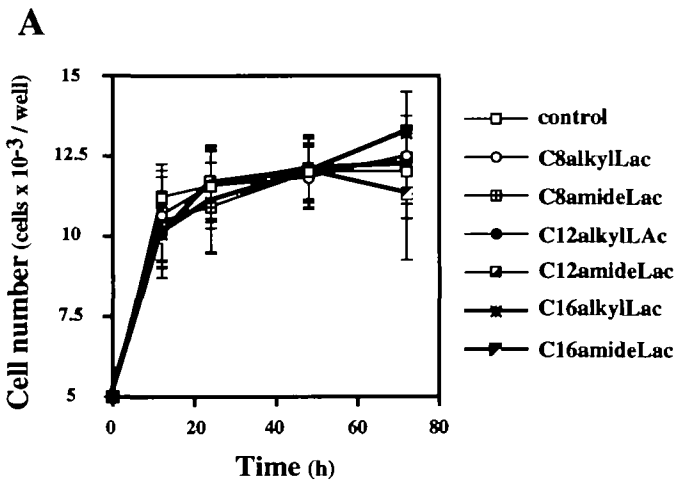


Fig. 1. **Structures of amphipathic lactoside primers and LacCer.** Lactosides with a simple alkyl chain are abbreviated as Cn alkylLac, and those with an *N*-acyl chain as Cn amideLac. Alkyl or *N*-acyl chain carbon number is indicated by the prefix.



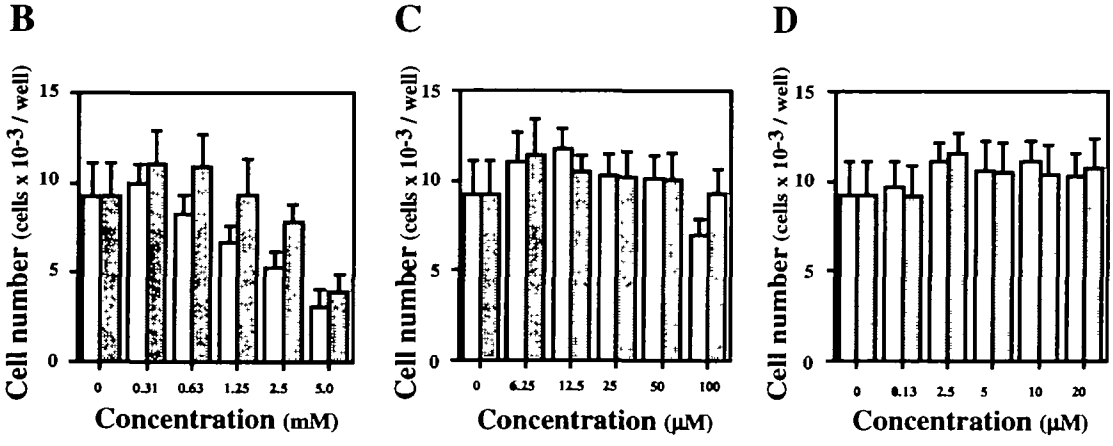


Fig. 2. **Effects of amphipathic lactoside primers on B16 cell growth.** Inocula of 3.0×10^3 cells B16 cells were seeded in wells of 96-well culture plates and cultured in serum-free TI/DF containing 10 μM lactoside for a specified period. Panel A shows cell growth with time; B, C, and D show cell growth at different concentrations of C8, C12, and C16 series lactosides, respectively. The cell number was determined with a cell counting kit (Dojindo).

of the reaction tubes in ice-cold water. The reaction mixture was lyophilized, separated by HPTLC, and analyzed with the BAS 2000 bioimage analyzer. Product radioactivity was expressed as PSL.

Effects of Lactoside on the Synthesis of Endogenous GSLs—B16 cells cultured in a 100-mm-diameter culture dish were washed with TI/DF and incubated in 5 ml of serum-free TI/DF containing 10 μM C16amideLac and [^{14}C]galactose (18.5 kBq/ml) for 3 h. Likewise, PC12 cells were incubated in 5 ml of serum-free TI/DF containing 100 μM C12amideLac or 10 μM C16amideLac in the presence of [^{14}C]galactose (18.5 kBq/ml) for 24 h. The cells were then washed with PBS(−), harvested, resuspended in 1.5 ml PBS(−), and protein was determined. Lipids of the cell fraction were analyzed as above.

RESULTS

Effects of Amphipathic Lactoside Primers on Cell Growth—B16 melanoma cells were cultured in the presence of amphipathic lactoside primers (Fig. 1) at various concentrations for a specified period as shown in Fig. 2A. The growth of cells cultured for three days in the presence of 10 μM primers was the same as that of the control (Fig. 2A). None of the primers appeared to express toxic activity toward the cells up to the following concentrations: C8alkylLac, 0.31 mM; C8amideLac, 1.25 mM; C12alkylLac, 50 μM; C12amideLac, 100 μM; C16alkylLac, 20 μM; C16amideLac, 20 μM (Fig. 2, B, C, and D). Owing to the insolubility of the C12 and C16 series primers, their effects on cell viability at more than 100 and 20 μM, respectively, could not be determined. *p*-Nitrophenyl-β-xyloside at 2 mM had no adverse effect on cells (*10, 11*), but the presence of octyl-β-glucoside at a concentration of more than 5 mM resulted in cell lysis (*12, 13*).

Amphipathic Lactoside Primers as Acceptors for Saccharides in Cells—To determine the fates of amphipathic lactoside primers taken up by cells, tritium-labeled C16amideLac was added to cultured B16 melanoma cells expressing GM3 at high levels. It was considered that the incorporated lactoside primers would serve as acceptors of sialic acid. The B16 cells cultured overnight in the presence of FBS were washed with culture medium lacking FBS, then cultured in the absence of FBS but with 5 μM [6′-^{3}H]-C16amideLac for 24 h. Analysis by HPTLC of the lipid extracts of the B16 cells indicated a band corresponding to the position of a putative glycosylated lactoside (X1 in Fig. 3A). One sialyl residue appeared attached to the lactoside primer, judging from its mobility on the HPTLC plate. Lane 2 in Fig. 3A is the autoradiograph of the extract of B16 cells labeled with [^{14}C]galactose and shows radioactivity not to be associated with compound X1. ^{3}H-labeled X1 was extracted from the HPTLC plate, treated with Newcastle disease virus neuraminidase (specific to α2-3 and α2-8 linked sialic acid), and analyzed by HPTLC. Figure 3A, lane 4, shows the radioactivity of X1 to have shifted to the position corresponding to the parent lactoside primer. The C16amideLac primer would thus appear to function as an acceptor for sialic acid, which would in turn produce only α2-3 sialylated lactoside with exactly the oligosaccharide structure of GM3. The glycosylated lactoside confirmed in the previous study (7) using endoglycoceramidase (*14-16*) is consistent with the present results.

An experiment similar to the above was conducted using PC12 cells expressing high Gb3Cer and Galα1-3Gb3Cer (*17*). The incubation of PC12 cells with 5 μM [6′-^{3}H]-C16amideLac for 24 h led to the production of two putative glycosylated lactosides, X2 and X3, as shown in Fig. 3B, lane 5. [^{14}C]Galactose-labeled PC12 cells showed C16amideLac to move to a position quite close to Gb3Cer, and X2 to move close to Galα1-3Gb3Cer. These ^{3}H-labeled compounds were extracted, purified, and subjected to enzymatic digestion and HPTLC analysis as shown in Fig. 3B. In brief, following the digestion of X2 with α-galactosidase from green coffee beans, a band at the position of C16amideLac primer appeared, but no such effect by β-*N*-acetylhexosaminidase was noted (data not shown), indicating X2 to possibly be Galα1-4lactoside. X3 also gave rise to a lactoside primer when treated with α-galactosidase, but not with β-*N*-acetylhexosaminidase. The fact that PC12 cells produce Gb3Cer and Galα1-3Gb3Cer (*17*), and that the relative positions of these two compounds on the HPTLC plate were exactly the same as those of X2 and X3, indicate that X3 is Galα1-3Galα1-4lactoside. It is

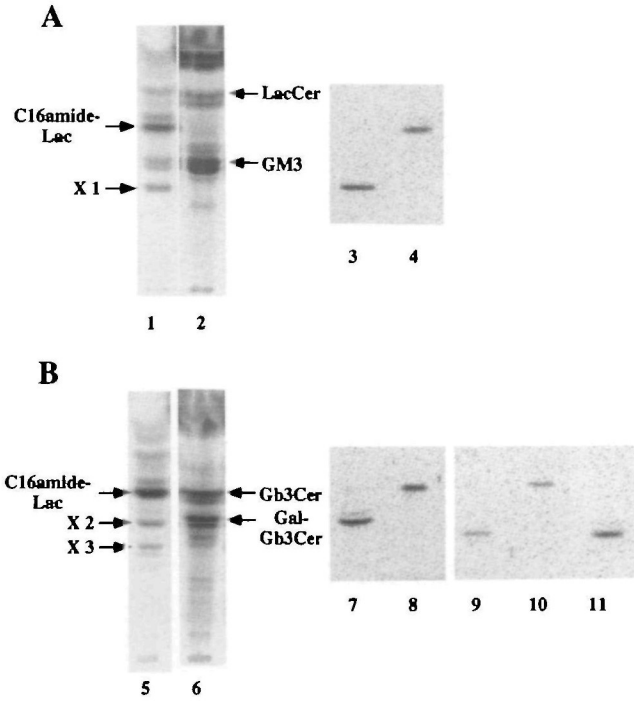


Fig. 3. **Glycosylated lactosides produced by adding amphipathic lactoside primers to cells.** B16 or PC12 cells were incubated for 24 h in TI/DF with 5 μM [6′-^{3}H]C16amideLac, washed with ice-cold PBS(−), and harvested. Lipids were extracted, separated by HPTLC, and analyzed with a BAS 2000 bioimage analyzer. The glycosylated products were purified as described in "EXPERIMENTAL PROCEDURES," digested with glycosidase, and analyzed. Panel A shows glycosylation products of B16 cells, with X1 representing the glycosylation product from the added primer. Panel B shows those of PC12 cells, with X2 and X3 as glycosylated products. Lane 1, B16 cells cultured with 5 μM ^{3}H-labeled C16amideLac; lane 2, B16 cells cultured with [^{14}C]galactose (37 kBq/ml); lane 3, glycosylated product, X1; lane 4, Newcastle disease virus sialidase treatment of X1; lane 5, PC12 cells cultured with 5 μM ^{3}H-labeled C16amideLac; lane 6, PC12 cells cultured with [^{14}C]galactose (37 kBq/ml); lane 7, glycosylated product, X2; lane 8, α-galactosidase treatment of X2; lane 9, glycosylated product, X3; lane 10, α-galactosidase treatment of X3; lane 11, β-*N*-acetylhexosaminidase treatment of X3.

significant that there were no other bands on the HPTLC plate moving slower than the parent primer and thus, the lactoside primers may be used only for cell-specific GSL synthesis.

As shown in Fig. 3, A and B, radioactivity associated with the [6′-^{3}H]lactoside primer at the start of the experiment was expressed on the HPTLC plate as several bands corresponding not only to the glycosylated lactoside but LacCer and GM3 as well (in Fig. 3A). Accordingly, some lactoside primers may, following their incorporation, undergo degradation in endosome-lysosomes, and radioactive monosaccharide may be used again for the synthesis of glycoconjugates including GSLs.

Glycosylated Lactosides in the Cell and Medium Fractions—Lipid fractions were recovered using SepPak C18 from culture media containing amphipathic lactoside primers and analysis by HPTLC indicated glycosylated lactosides to have also been present. Based on the specific radioactivity of each lactoside and the amounts of lactoside primers added to each cell culture (25 nmol in 5 ml), the amounts of glycosylated lactosides accumulated within the cell and culture medium fractions could be determined.

Figure 4A clearly shows that, during a 6 h period, more C16 series primers than C12 series primers were glycosylated and accumulated within cells: the amount of glycosylated C16amideLac (22 pmol) was 6.3 times that of C12amideLac (3.5 pmol); glycosylated C16alkylLac (18 pmol) was 5.1 times that of C12alkylLac (3.5 pmol); and glycosylated C16amideLac was 1.2 times that of C16alkylLac. In contrast to intracellular accumulation, glycosylated lactoside accumulation was greatest in the medium of B16 cells cultured with C12alkylLac (846 pmol), followed by that in the medium with C12amideLac (404 pmol), as shown in Fig. 4B. Glycosylated lactosides were generated in smaller amounts from C16alkylLac (203 pmol) and C16amideLac (189 pmol) (Fig. 4B). Essentially the same findings were noted after culture for 24 or 48 h (Fig. 5, A and B). Glycosylated lactosides associated with cells increased rapidly up to 24 h and slightly thereafter (Fig. 5A). In culture media, glycosylated lactosides increased to plateau levels at 24 h and remained steady for the subsequent 24 h (Fig. 5B). Of 25 nmol lactosides added to cells, 0.6 nmol (2.5% of the added primer), 0.8 nmol (3.0%), 1.4 nmol (5.5%), and 1.9 nmol (7.5%) were glycosylated from C16amideLac, C16alkylLac, C12amideLac, and C12alkylLac, respectively, in 48 h. C12 series lactosides after glycosylation are thus more easily secreted or eliminated from the cells than C16 series lactosides. The greater use of C12 series primers for glycosylation may be due to the preference of sialyltransferase for C12 series lactosides over C16 series primers or the higher rate of uptake of C12 series primers into cells compared to C16 series primers. C12alkylLac utilization in 48 h was 1.4

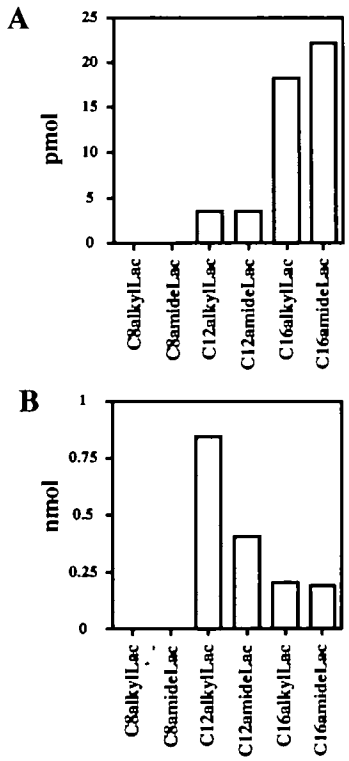


Fig. 4. **Sialylated lactosides in the cell and medium fractions.** B16 cells were incubated in serum-free TI/DF with 5 μM [^{3}H]amphipathic lactosides for 6 h, and lipids accumulated within cells were prepared as described. Lipids from the culture medium were purified by SepPak C18 columns. Radioactivity corresponding to the sialylated lactoside on the HPTLC plate was quantified using known amounts of radioactive lactosides developed on the same plate. Panel A shows the results of the analysis of the cell fractions; and B shows those for the medium fractions.

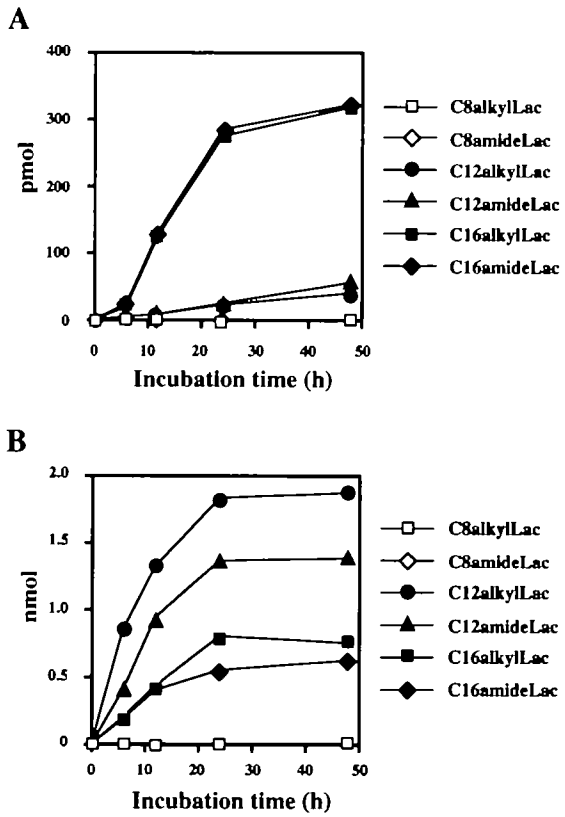


Fig. 5. **Sialylated lactoside production with time.** B16 cells were incubated in serum-free TI/DF with 5 μM [^{3}H]lactoside primers for a specified time and sialylated products in the cell and medium fractions were quantified. Panel A shows sialylated lactosides accumulated within cells; and B shows accumulation in the media.

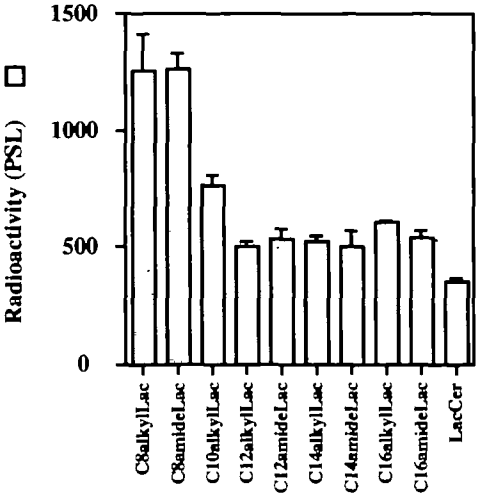


Fig. 6. ***In vitro* sialylation of amphipathic lactoside primers.** The reaction was conducted in 50 μl of 100 mM cacodylate buffer, pH 6.0, containing 5 mM $MgCl_2$, 0.3% Triton CF-54, 5 mM CMP-NeuAc, 0.5 mM lactoside, 3.7 kBq CMP-[^{14}C]NeuAc, and cell homogenate (50 μg protein) for 3 h; and it was terminated by immersing the reaction tubes in ice-cold water. Reaction mixtures were lyophilized and analyzed by HPTLC. Radioactivity of the glycosylated products was expressed as PSL.

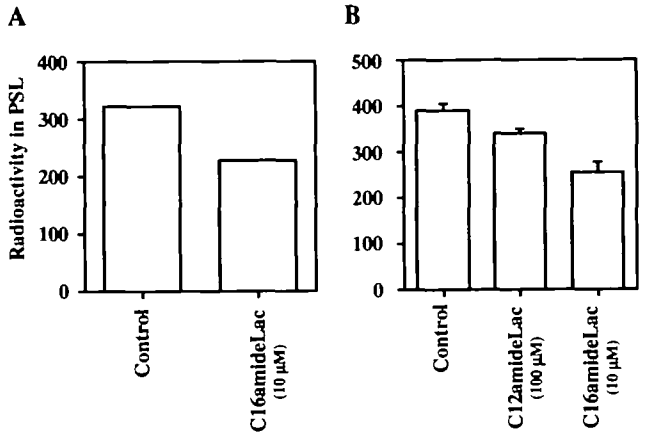


Fig. 7. **Suppression of endogenous GSL synthesis by primers.** Panel A shows radioactivity associated with GM3 of B16 cells, and Panel B that with Gb3Cer of PC12 cells. B16 and PC12 cells were incubated for 3 and 24 h, respectively, in serum-free TI/DF in the absence (control) and presence of 10 μM C16amideLac or 100 μM C12amideLac with 18.5 kBq/ml [^{14}C]galactose. Lipids were extracted from the cells and resolved by HPTLC. Radioactivity associated with GM3 and Gb3Cer was measured. Each value is the average of three independent experiments.

times that of C12amideLac, and C16alkylLac utilization 1.3 times that of C16amideLac, thus showing that amide bonds result in lower use of lactoside primers.

In Vitro Amphipathic Lactoside Primer Acceptor Activity toward α2-3 Sialyltransferase—The above findings demonstrate that not all lactoside primers are used equally by cells, and this prompted an individual assessment of acceptor capacity *in vitro*. A homogenate of GM95 cells devoid of GSL expression (*18*) was prepared as in "EXPERIMENTAL PROCEDURES" to serve as the source of sialyltransferase. One significant advantage of GM95 cells is that the cell lysate contains no LacCer, which might compete with amphipathic primers for sialic acid. Figure 6 clearly shows radioactive sialic acid to be significantly transferred onto C8 series lactoside primers, followed by C10alkylLac primer. Lower acceptor activity of roughly 40% that of C8 series lactosides was noted for primers with hydrophobic tails longer than C12 or longer. The lowest acceptor activity was found for LacCer. The results obtained *in vitro* appear at variance with the data for cultured cells, and primer participation in glycosylation by cells may thus be determined by their affinity for plasma membranes but not sialyltransferase. The sialylation of lactoside previously noted at α2-3 and α2-6 (*19*) was seen in this study only at α2-3 (Fig. 3, also refer to Ref. 7). α2-6 Sialyltransferase activity in the enzyme preparation was not measured, but GM95 cells would probably not have α2-6 sialyltransferase activity.

Hindrance of Endogenous GSL Synthesis by Amphipathic Lactoside Primers—The lactoside primers, C16amideLac and C16alkylLac, were previously shown to replace by 40 and 10%, respectively, endogenous GM3 on the cell surface of B16 melanoma cells cultured for 48 h, as determined by "back exchange" (*7*). To clarify the effects of lactoside primers on endogenous GSL synthesis biosynthetically, B16 cells were incubated with ^{14}C-labeled galactose in the presence and absence of C16amideLac for 3 h. The radioactivity of GM3 produced in the presence of the lactoside primer was 70% that generated in its absence (Fig. 7A), demonstrating its inhibition of endogenous GM3 synthesis. When PC12 cells were incubated with ^{14}C-labeled galactose for 24 h, the presence of 100 μM C12amideLac and 10 μM C16amideLac reduced endogenous Gb3Cer synthesis to 87 and 66% of the control (Fig. 7B), respectively, indicating that the primer suppresses endogenous Gb3Cer synthesis.

DISCUSSION

β-Xyloside, first used to study glycosaminoglycan (GAG) by Suzuki and his associates (*20*), was shown to function as an acceptor in GAG elongation and serve effectively to inhibit proteoglycan synthesis. This primer inhibited endogenous chondroitin sulfate synthesis, but with alteration in the aglycon constituent, also that of heparan sulfate proteoglycan (*21, 22*). Amphipathic α-*N*-acetylgalactosaminides inhibited *O*-glycan synthesis (*23*), and benzyl-β-*N*-acetylglucosaminide in CHO cells was shown to prime lactosamine synthesis (*24*). Acetylated naphthalene methanol disaccharide was recently used to promote the synthesis of sialylLe^x and inhibit the cell expression of sialylLe^x (*25*). The disaccharide head group is hydrophilic and thus the disaccharide would not be incorporated into cells by diffusion. Peracetylation of hydroxy groups appeared to enable the disaccharide to be taken up by cells (*26*).

β-Xyloside inhibited not only proteoglycan but also glycolipid synthesis (*4*). At 1 mM concentration 4MU-β-xyloside was found to yield sialylα2-3galactosylβ1-4xylosylβ-MU in CHO cells as well as other glycosylated β-xylosides whose structures were surmised based on the linkage structure of GAG with the core protein. Octyl-β-glucoside was glycosylated in CHO and melanoma cells to produce a GM3-like molecule (*5*). These primers, nonethe-

less, were not always specifically brought into the GSL synthetic pathway, and consequently there was no distinctive inhibition of GSL biosynthesis.

LacCer is a key substance in the synthetic route for nearly all GSLs, and thus a series of artificial glycolipids consisting of a lactosyl moiety and an alkyl chain of a given length (named alkylLac) were synthesized for use in a study to clarify GSL functions after disrupting GSL synthesis. Some compounds possessed an amide group at the position where this group would normally be situated in intact GSL (named amideLac) (*6*). These lactosides were previously glycosylated in B16 cells following their incorporation (*7*), and in this study the relationship between lactoside structure and priming activity was examined in detail. Following their administration to B16 cells highly expressing GM3, these lactoside primers underwent glycosylation to produce α2-3 sialylated compounds (Fig. 3A). In rat pheochromocytoma PC12 cells rich in Gb3Cer and Galα1-3Gb3Cer (*17*), the glycosylation of lactoside primers yielded the same sugar units of Gb3Cer and Galα1-3Gb3Cer, *i.e.*, α1-4 galactosylated and 3-*O*-(α-Gal)α1-4 galactosylated compounds (Fig. 3B). These were recovered as the sole glycosylated products from the cell and medium fractions, thus indicating that the lactoside primers may be incorporated into cells, conveyed to the Golgi apparatus, then taken up into the GSL biosynthetic machinery to serve as acceptors; but that they are not involved in the synthesis of other glycoconjugate species. A GlcCer analog having a sulfur in place of oxygen in the linkage between glucose and the aglycon, and NBD-GlcCer have been used to assess their priming activity (*27*). NBD in the aglycon moiety reduced the use of NBD-GlcCer in glycosylation compared to that of the GlcCer analog possessing a native structure except for the linkage molecule specified above. The glycosylated NBD-compound was released at more than 50% into the medium, indicating the structure of lipid moiety to affect the rate of incorporation and glycosylation of compounds and the fates of glycosylated compounds.

The same was noted in the present study on the relationship between lipid structure and primer glycosylation. Analysis of cell-associated lactosides indicated that C16 series lactoside primers were glycosylated by cells to 5.1-6.3 times the extent of C12 series primers (Fig. 4A). No C8 series primers were used for glycosylation at 10 μM in this study. Examination of the medium confirmed C12 series lactoside primer use to be 2.1-4.4 times that of C16 series lactosides for glycosylation by cells (Figs. 5 and 6). The amounts of overall glycosylated lactosides were determined and the probability of glycosylation was found to increase in the order C16amideLac (2.5% in 48 h)< C16alkylLac (3.0%)<C12amideLac (5.5%)<C12alkylLac (7.5%), indicating that C12 series primers have better priming activity than C16 series primers. On the other hand, a C16 primer had stronger inhibitory activity than a cognate C12 primer, as shown in Fig. 7. Glycosylation of primers causes primer molecules to become hydrophilic, particularly in the case of C12 series primers; and thus more glycosylated C12 lactosides may be released from the cell membrane into the medium compared to C16 series products, which may reside in the membrane (*7*), leading to the inhibition of GSL synthesis.

In previous work, C6-Cer was glycosylated more than C2-Cer by B16 melanoma cells, and glycosylated ceramides were quickly liberated from the cells (*28*). Ceramide is intrinsically noxious to cells, and thus it may be detoxified by glycosylation. Whatever the reasons for glycosylation, it is fascilitated by the hydrophobic nature of acceptors. C6-NBD-Cer was previously used to synthesize sphigomyelin and cerebroside by Chinese hamster V79 fibroblasts (*29*), but more complex GSLs failed to be produced, possibly owing to the presence of NBD. C6-NBD-GM1 was incorporated into cultured hippocampal neuronal cells but was not glycosylated to higher gangliosides or degraded to GM2 or GM3 (*30*), indicating that NBD in the aglycon restricts metabolism and possibly the sites compounds may occupy.

The amount of cell-associated sialylated C16amideLac was 1.2 times that of C16alkylLac (Fig. 4A). Amide bonds would thus appear to promote the interactions of glycosylated lactosides with plasma membrane constituents, thereby rendering primers with amide bonds capable of closer association with the plasma membrane than primers with alkyl chains. This possibility is supported by previous finding by differential calorimetry that 1,2-dimyristoylamide-1,2-deoxyphosphatydylcholine has a peak corresponding to hydrogen bonds that stabilize the lipid bilayer through the formation of a hydrogen bond belt (*31*). Lafrance *et al.* demonstrated by transmission and attenuated total reflectance infrared spectroscopy that, in membranes made from 1,2-dihexadecanoylamide-1,2-deoxyphosphatidylethanolamine, ester C=O and amide N-H groups are linked by intermolecular hydrogen bonds (*32*). AmideLacs, particularly those of C16, may settle more compactly on the plasma membrane than alkylLacs, perhaps assisted by hydrogen bonds with other cell surface molecules, in addition to hydrophobic interactions *via* acyl chains. This in turn would reduce the turnover of amideLacs.

LacCers having different lipid moiety structures were used to examine lipid effects on sialylation *in vitro* (*33*). Increase of hydrophobicity decreased glycosylation capacity. Pohlentz *et al.* reported C8-lactoside to be sialylated the most by the homogenate of rat liver Golgi vesicles (*19*), followed by C10 alkyl lactoside and those with longer alkyl chains. This is consistent with the present study using the cell homogenate derived from GM95 cells (Fig. 6). Why octyl lactoside is a better acceptor of sialic acid with solubilized enzyme is not understood at present, though in this study using living cells, octyl lactoside was hardly used by B16 melanoma cells. Triton CF-54 was used as detergent in both *in vitro* studies, and the most appropriate lactoside structures serving as acceptors for sialyltransferase may differ according to the detergent. Sialylation *in vitro* appears unaffected not only by size but also the presence of the amide bond in the lipophilic moiety, and thus sialyltransferase extruding into the Golgi lumen may have been designed in such a way as to recognize only the lactosyl residue also extending into the lumen. The greater use of C12 series lactoside primers than C16 series primers for glycosylation may be an indication that the former are more easily incorporated into cells than the latter.

Lactoside primers inhibited endogenous glycosphingolipid biosynthesis (Fig. 7, also Ref. 7). D-PDMP and its analogs (*34*) and *N*-butyldeoxygalactonojirimycin (*35*) have been used to inhibit GSL biosynthesis. These compounds inhibit the synthesis of GlcCer, the first glycosylation step in GSL biosynthesis, which suppresses the syn-

thesis of all downstream GSLs, and accordingly it becomes difficult to clarify the functions of individual GSL species. The inhibition of GlcCer synthesis causes ceramide, possibly a bioactive molecule (*36-38*), to accumulate in the cell (*39*). Inhibitors capable of suppressing the synthesis of different GSL species should thus be established (*40*). Gluco-4-epoxyl-4-C-methylene glycosyl ceramide inhibited the synthesis of LacCer, following administration to chick embryonic neuronal cells, but the galacto-4-epoxyl-4-C-methylene glycosyl ceramide had no such effect. These compounds inhibit GSL synthesis by restraining the glycosyltransferase reaction, but the lactoside primers in the present study exert their action by bypassing GSL chain elongation. β-Xylosides (*4, 24*) and octyl-β-glucoside (*5*) caused saccharide chain elongation on added primers, as also noted for the primers in this study. But still these primers were not GSL-specific.

Lactoside primers served as acceptors for α2-3 sialic acid (B16) and α1-4 galactose (PC12), and thus it should be possible to produce glycosylation inhibitors by modifying the saccharide acceptor hydroxyl group into a deoxy or methoxy group (*41*). C16amideLac at 10 μM inhibited endogenous Gb3Cer of PC12 cells by 34%, while C12-amideLac at 100 μM did so by only 13% (Fig. 7B), even though the two primers have essentially the same acceptor capability *in vitro* study as shown in Fig. 6. It follows, then, that cells in culture should be used to evaluate primer and inhibitor structures.

LacCer analogs having a single lipophilic chain are clearly shown by the present study to be incorporated into cells to prime cell type-specific GSL synthesis in the Golgi apparatus, the extent depending on the size and structures of lipophilic tails of the lactosides. The use of lactoside primers does not lead to the accumulation of harmful ceramide in cells, and thus they may be used to disrupt cell GSL expression, thereby greatly facilitating examination of the roles of GSLs.

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REFERENCES

1. Hakomori, S. and Kannagi, R. (1983) Glycosphingolipids as tumor-associated and differentiation markers. *J. Natl. Cancer Inst.* **71**, 231-251
2. Hakomori, S. (1986) Glycosphingolipids. *Sci. Am.* **254**, 44-53
3. Yamakawa, T. (1996) A reflection on the early history of glycosphingolipids. *Glycoconj. J.* **13**, 123-126
4. Freeze, H.H., Sampath, D., and Varki, A. (1993) Alpha- and beta-xylosides alter glycolipid synthesis in human melanoma and Chinese hamster ovary cells. *J. Biol. Chem.* **268**, 1618-1627
5. Pörtner, A., Etchison, J.R., Sampath, D., and Freeze, H.H. (1996) Human melanoma and Chinese hamster ovary cells galactosylate *n*-alkyl-beta-glucosides using UDP gal: GlcNAc beta-1,4 galactosyltransferase. *Glycobiology* **6**, 7-13
6. Miura, Y., Arai, T., and Yamagata, T. (1996) Synthesis of amphiphilic lactosides that possess a lactosylceramide-mimicking *N*-acyl structure: Alternative universal substrates for endo-type glycosylceramidases. *Carbohydr. Res.* **289**, 193-199
7. Miura, Y. and Yamagata, T. (1997) Glycosylation of lactosylceramide analogs in animal cells: Amphipathic disaccharide primers for glycosphingolipid synthesis. *Biochem. Biophys. Res. Commun.* **241**, 698-703
8. Suzuki, Y. and Suzuki, K. (1972) Specific radioactive labeling of terminal *N*-acetylgalactosamine of glycosphingolipids by the galactose oxidase-sodium borohydride method. *J. Lipid Res.* **13**, 687-690
9. Lowly, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
10. Sobue, M., Habuchi, H., Ito, K., Yonekura, H., Oguri, K., Sakurai, K., Kamohara, S., Ueno, Y., Noyori, R., and Suzuki, S. (1987) β-D-Xylosides and their analogues as artificial initiators of glycosaminoglycan chain synthesis. *Biochem. J.* **241**, 591-601
11. Farach, M.C., Tang, J.-P., Decker, G.L., and D., C.D. (1988) Differential effect of *p*-nitrophenyl-D-xylosides on mouse blastocysts and uterine epithelial cells. *Biol. Reprod.* **39**, 443-455
12. Shiao, Y.J., Chen, J.C., and Wang, C.T. (1989) The solubilization and morphological change of human platelets in various detergents. *Biochim. Biophys. Acta* **980**, 56-68
13. Carson, S.D. (1996) Manifestation of cryptic fibroblast tissue factor occurs at detergent concentrations which dissolve the plasma membrane. *Blood Coagul. Fibrinolysis* **7**, 303-313
14. Ito, M. and Yamagata, T. (1989) Endoglycoceramidase from *Rhodococcus* species G-74-2. *Methods Enzymol.* **179**, 488-496
15. Ito, M. and Yamagata, T. (1986) A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids. *J. Biol. Chem.* **261**, 14278-14282
16. Ito, M. and Yamagata, T. (1989) Purification and characterization of glycosphingolipid-specific endoglycosidases (endoglycoceramidases) from a mutant strain of *Rhodococcus* sp. Evidence for three molecular species of endoglycoceramidase with different specificities. *J. Biol. Chem.* **264**, 9510-9519
17. Shimamura, M., Hayase, T., Ito, M., Rasilo, M.L., and Yamagata, T. (1988) Characterization of a major neutral glycolipid in PC12 cells as III^{3}Gal alpha-globotriaosylceramide by the method for determining glycosphingolipid saccharide sequence with endoglycoceramidase. *J. Biol. Chem.* **263**, 12124-12128
18. Ichikawa, S., Nakajo, N., Sakiyama, H., and Hirabayashi, Y. (1994) A mouse B16 melanoma mutant deficient in glycolipids. *Proc. Natl. Acad. Sci. USA* **91**, 2703-2707
19. Pohlentz, G., Trimborn, M., and Egge, H. (1994) Sialylation of *n*-alkyllactosides, galactosides and glucosides by sialyltransferases from rat liver Golgi vesicles. *Glycobiology* **4**, 625-631
20. Okayama, M., Kimata, K., and Suzuki, S. (1973) The influence of *p*-nitrophenyl β-D-xyloside on the synthesis of proteochondroitin sulfate by slices of embryonic chick cartilage. *J. Biochem.* **74**, 1069-1073
21. Lugemwa, F. and Esko, J.D. (1991) Estradiol β-D-xyloside, an efficient primer for heparan sulfate biosynthesis. *J. Biol. Chem.* **266**, 6674-6677
22. Fritz, T.A., Lugemwa, F.N., Sarkar, A.K., and Esko, J.D. (1994) Biosynthesis of heparan sulfate on beta-D-xylosides depends on aglycone structure. *J. Biol. Chem.* **269**, 300-307
23. Kuan, S.-F., Byrd, J.C., Basbaum, C., and Kim, Y.S. (1989) Inhibition of mucin glycosylation by aryl-*N*-acetyl-α-galactosaminedes in human colon cancer cells. *J. Biol.Chem.* **264**, 19271-19277
24. Neville, D.C., Field, R.A., and Ferguson, M.A. (1995) Hydrophobic glycosides of *N*-acetylglucosamine can act as primers for polylactosamine synthesis and can affect glycolipid synthesis *in vivo*. *Biochem. J.* **307**, 791-797
25. Sarkar, A.K., Fritz, T.A., Taylor, W.H., and Esko, J.D. (1995) Disaccharide uptake and priming in animal cells: inhibition of sialyl Lewis^x by acetylated Gal beta 1→4GlcNAc beta-*O*-naphthalenemethanol. *Proc. Natl. Acad. Sci. USA* **92**, 3323-3327
26. Sarkar, A.K., Rostand, K.S., Jain, R.K., Matta, K.L., and Esko, J.D. (1997) Fucosylation of disaccharide precursors of sialyl lewis^x inhibit selectin-mediated cell adhesion. *J. Biol. Chem.* **272**, 25608-25616
27. Schwarzmann, G., Hofmann, P., Putz, U., and Albrecht, B. (1995) Demonstration of direct glycosylation of nondegradable glucosylceramide analogs in cultured cells. *J. Biol. Chem.* **270**, 21271-21276

28. Komori, H. and Ito, M. (1995) Conversion of short-chain ceramides to short-chain ceramide GM3 in B16 melanoma cells. *FEBS Lett.* **374**, 299-302
29. Lipsky, N.G. and Pagano, R.E. (1983) Sphingolipid metabolism in cultured fibroblasts: Microscopic and biochemical studies employing a fluorescent ceramide analogue. *Proc. Natl. Acad. Sci. USA* **80**, 2608-2612
30. Sofer, A., Schwarzmann, G., and Futerman, A.H. (1996) The internalization of short acyl chain analogue of ganglioside GM1 in polarized neurons. *J. Cell Sci.* **109**, 2111-2119
31. Sunamoto, J., Goto, M., Iwamoto, K., Kondo, H., and Sato, T. (1990) Synthesis and characterization of 1,2-dimiristoylamino-1,2-deoxyphosphatidylcholine as an artificial boundary lipid. *Biochim. Biophys. Acta* **1024**, 209-219
32. Lafrance, C.-P., Blochet, J.-É, and Pézolet, M. (1997) *N*-Acylphosphatidylethanolamines : Effect of the *N*-acyl chain length on its orientation. *Biophys. J.* **72**, 2559-2568
33. Kadowaki, H. and Grant, M.A. (1994) Mechanism of GM3 ganglioside synthesis. Kinetic study of rat liver CMP-*N*-neuraminate:lactosylceramide alpha 2,3-sialyltransferase employing 19 molecular species of lactosylceramide. *J. Biol. Chem.* **269**, 14931-14938
34. Abe, A., Inokuchi, J., Jimbo, M., Shimeno, H., Nagamatsu, A., Shayman, J.A., Shukla, G.S., and Radin, N.S. (1992) Improved inhibitors of glucosylceramide synthase. *J. Biochem.* **111**, 191-196
35. Platt, F.M., Neises, G.R., Karlsson, G.B., Dwek, R.A., and Butters, T.D. (1994) *N*-Butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect *N*-linked oligosaccharide processing. *J. Biol. Chem.* **269**, 27108-27114
36. Bielawska, A., Linardic, C.M., and Hannun, Y.A. (1992) Ceramide-mediated biology. Determination of structural and stereospecific requirements through the use of *N*-acyl-phenylamino-alcohol analogs. *J. Biol. Chem.* **267**, 18493-18497
37. Kolesnick, R. and Golde, D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77**, 325-338
38. Okazaki, T., Domae, N., Bell, R.M., and Hannun, Y. (1994) Ceramide, a sphingolipid second messenger. *Trends Glycosci. Glycotechnol.* **6**, 278-285
39. Abe, A., Radin, N.S., Shayman, J.A., Wotring, L.L., Zipkin, R.E., Sivakumar, R., Ruggieri, J.M., Carson, K.G., and Ganem, B. (1995) Structural and stereochemical studies of potent inhibitors of glucosylceramide synthase and tumor cell growth. *J. Lipid Res.* **36**, 611-621
40. Zacharias, C., van Echten Deckert, G., Plewe, M., Schmidt, R.R., and Sandhoff, K. (1994) A truncated epoxy-glucosylceramide uncouples glycosphingolipid biosynthesis by decreasing lactosylceramide synthase activity. *J. Biol. Chem.* **269**, 13313-13317
41. Hindsgaul, O., Kaur, K.J., Srivastava, G., Blaszczyk-Thurin, M., Crawley, S.C., Heerze, L.D., and Palcic, M.M. (1991) Evaluation of deoxygenated oligosaccharide acceptor analogs as specific inhibitors of glycosyltransferases. *J. Biol. Chem.* **266**, 17858-17862