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Expression machinery of GM4: the excess amounts of GM3/GM4S synthase (ST3GAL5) are necessary for GM4 synthesis in mammalian cells

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Abstract The ganglioside GM4 is a sialic acid-containing glycosphingolipid mainly expressed in mammalian brain and erythrocytes. GM4 is synthesized by the sialylation of galactosylceramide (GalCer), while the ganglioside GM3 is synthesized by the sialylation of lactosylceramide (LacCer). Recently, the enzyme GM3 synthase was found to be responsible for the synthesis of GM4 *in vitro* and *in vivo*, yet the mechanism behind GM4 expression in cells remains unclear. In this study, we attempted to establish GM4-reconstituted cells to reveal the regulation of GM4 synthesis. Interestingly, GM4 was not detected in RPMI 1846 cells expressing LacCer, GalCer, and GM3. Similarly, GM4 was not detected in CHO-K1 cells, even when such cells expressing LacCer and GM3 were stably transfected with the GalCer synthase (GalCerS) gene. GM4 became detectable only when the GM3/GM4 synthase (GM3/GM4S, ST3GAL5) gene was overexpressed in either RPMI 1846 or CHO-K1/GalCerS cells. A mutant of the B16 melanoma cell line, GM-95, lacks GlcCer and LacCer, due to an absence of GlcCer synthase, but carries endogenous LacCer synthase and GM3/GM4S. GalCer became detectable after transfection of GalCerS into GM95 cells, but the GM95/GalCerS reconstituted cells did not express GM4, indicating that competition between the substrates LacCer and GalCer for GM3/GM4S does not cause the failure of GM4 synthesis. These results suggest that the expression machinery of GM4 under physiological conditions is independent from that of GM3.

Keywords Gangliosides · GM4 · GM3 · Glycosyltransferase · Sialyltransferase · ST3GAL5

Abbreviations

GalCer	Galactosylceramide
LacCer	Lactosylceramide
GalCerS	GalCer synthase
GM3/GM4S	GM3/GM4 synthase
ER	Endoplasmic reticulum
GlcCer	Glucosylceramide
GlcCerS	GlcCer synthase
LacCerS	LacCer synthase
TLC	Thin-layer chromatography

Introduction

Gangliosides, glycosphingolipids containing sialic acids, exist in the outer leaflet of the plasma membrane and are involved in a variety of cell processes. For example, the ganglioside GM3 is involved in the insulin resistance of type 2 diabetes [1–4], and GM3 levels are increased in the adipose tissue in a typical rodent model of obesity [1]. GM3 accumulation results in the dissociation of the insulin receptor from caveolae and leads to the insulin resistance [5, 6]. Another ganglioside, GM4, is expressed on the epithelial cells of the intestinal tract in red sea bream (Pagrus major) where it functions as a receptor for the pathogen that causes vibriosis [7]. In mammals, GM4 is reportedly expressed in the white matter,

Highlights - First establishment of GM4-reconstituted cells by overexpression of ST3Gal5 gene.

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myelin, and astrocytes of human brain [8–10], but in general, the function and physiological significance of GM4 is still not clear.

Glycosphingolipid synthesis begins with ceramide formed in the endoplasmic reticulum (ER) (Fig. 1). In the GM3 synthesis pathway, the glucosylation of ceramide by glucosylceramide synthase (GlcCerS) occurs on the cytosolic leaflet of the cis-Golgi membrane, forming glucosylceramide (GlcCer). GlcCer is transported to the trans-Golgi compartment by FAPP2, and then translocated to the luminal leaflet [11]. GlcCer is then converted to lactosylceramide (LacCer), which is the acceptor substrate for GM3 synthesis. Thus, the sequential biosynthesis of GM3 from GlcCer is believed to occur in the lumen of the trans-Golgi compartment. In contrast, in the GM4 synthesis pathway, the galactosylation of ceramide by galactosylceramide synthase (GalCerS) occurs in the lumen of the ER, forming galactosylceramide (GalCer) (Fig. 1). GalCerS is a type I membrane protein that has an ER retention signal in its cytoplasmic tail and localizes in the ER [12]. UDP-galactose, the donor substrate for GalCer synthesis, is imported into the ER lumen as the UDP-galactose transporter localizes in the ER by interacting with GalCerS [12]. Subsequently, GalCer is transported to the trans-Golgi compartment from the ER, although the detailed mechanism for this step is unknown.

In mammals, ST3Gal5 is a common sialyltransferase active in generating GM3 and GM4 from LacCer and GalCer, respectively [13], so here we designate this enzyme GM3/GM4 synthase (GM3/GM4S, ST3GAL5). GM3/GM4S is a type II membrane protein with three isoforms (M1-, M2-, M3-GM3/GM4S) [14]. M2- and M3-GM3/GM4S are localized in the Golgi apparatus, whereas M1-GM3/GM4S is mainly localized in the ER due to a retrograde transport signal (Arginine [R]-based motif) in its cytoplasmic tail [14]. However, unlike GalCer synthesis, both GM3 and GM4 synthesis should occur in the trans-Golgi since the CMP-sialic acid transporter localizes exclusively in the Golgi and not in the ER [15].

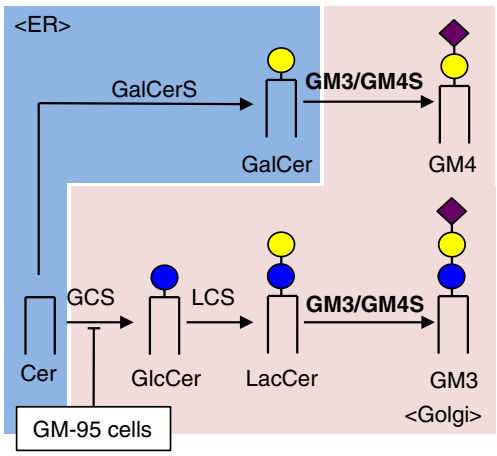

Fig. 1 The biosynthesis pathways of GM3 and GM4. GM3/GM4S transfers a sialic acid to LacCer and GalCer to generate GM3 and GM4, respectively. Mouse B16 melanoma GM-95 cells are glycosphingolipid-deficient mutants that lack GlcCerS (GCS) activity. A blue circle, yellow circle, and purple diamond indicate glucose, galactose, and a sialic acid, respectively. LCS denotes galactosyltransferase (LacCerS), which catalyzes LacCer synthesis

In the presented study, to examine the expression machinery of GM3 and GM4 in cells, we have attempted to establish GM4-reconstituted cells using several cell lines. Interestingly, overexpression of the GM3/GM4S gene was necessary to detect GM4 in cells expressing GalCer and endogenous GM3/GM4S. These results suggest that the regulation of the production and/or degradation of GM4 are different from those of GM3.

Material and methods

Materials

RPMI 1846 and GM-95 cells were kindly provided by Dr. M. Ito (Kyushu University, Hukuoka, Japan) and Dr. Y. Hirabayashi (Riken Brain Science Institute, Wako, Japan), respectively. Anti-GM4 antibody (rabbit IgG), LacCer, GalCer, and GlcCer were purchased from Matoreya (Pleasant Gap, PA). GM3 was also purchased from Matoreya. GM4 (d18:1-C18:0) was purchased from Wako (Osaka, Japan). Thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). CSII-CMV-MCS, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev plasmids were provided by the DNA Bank, RIKEN BioResource Center (Ibaraki, Japan).

Plasmids

The pNF4 plasmid encoding GalCer synthase GalCerS was constructed using cDNA prepared from mouse kidney and primers 5′-GGATCCACCATGAAGTCTTACACTC CATATTTCATGC-3′ and 5′-TCATCTCACCTTCTT TTCGTGTTTAACAC-3′. The resulting fragments were cloned into a pGEM-T Easy vector (Promega, Madison, WI) to generate pNF2. The 1.6-kb BamHI-NotI fragment of pNF2 was then cloned into the BamHI-NotI site of a pcDNA3.1 Zeo(+) vector (Life Technologies, Carlsbad, CA) to generate pNF4.

To produce a lentivirus, the pFS311 (GM3/GM4S/CSII-CMV-RfA) was constructed using pcDNA3.1 Zeo(+)-GM3/GM4S [16] and primers 5′-CACCATGAGAAGAC CCAGCTTGTTAATAAAAGA-3′ and 5′-TTCAGTGG ATGCCGCCGCTGAGGTCCTC-3′. The resulting fragments were cloned into a pENTR/D-TOPO vector (Life Technologies) to generate pSU212. The CSII-CMV-MCS plasmid was converted to a Gateway® destination vector (CSII-CMV-RfA) using the Gateway® vector conversion system (Life Technologies). The GM3/GM4S gene of pSU212 was inserted into

the lentiviral expression vector, CSII-CMV-RfA, using the LR clonase reaction to generate pFS311.

Cell culture

Hamster melanoma RPMI 1846 cells, Chinese hamster ovary CHO-K1 (CHO) cells, mouse melanoma GM-95 cells, and human kidney 297 T cells were cultured in RPMI 1640 (Wako), F-12 HAM (Wako), low-glucose DMEM (Wako), and high-glucose DMEM (Life Technologies) medium, respectively, each supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 and GM-95 cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Stable transfectants were selected in the same medium used for culture in the presence of zeocin (Life Technologies).

Preparation of lentiviral vectors

Twenty-four hours before transfection, 293 T cells (0.5×10^6) were seeded in a poly-lysine coated 60-mm dish. The 293 T cells were transfected with 3 μg of pFS311 (GM3/GM4 synthase/CSII-CMV-RfA), 1.5 μg of pCAG-HIVgp, and 1.5 μg of pCMV-VSV-G-RSV-Rev plasmids using Lipofectamine 2000. The cells were incubated for 24 h at 37 °C, then the medium was replaced with 4 mL of high-glucose DMEM containing 10 μM Forskolin. Approximately 32 h after transfection, the culture temperature was shifted from 37 °C to 32 °C to maintain virus stability. The lentivirus-containing supernatant was harvested 48 h after transfection and centrifuged at 200 x *g* for 3 min to remove living cells. The lentiviral supernatant was either used immediately for experiments or snap-frozen in liquid nitrogen and stored at −80 °C for later applications. For the lentiviral transduction of RPMI 1846, CHO-K1/GalCerS, or GM-95/GalCerS cells, 4 ml of the lentiviral supernatant was added to 0.25×10^6 cells in a 60-mm dish, and the cells were cultured at 32 °C. After 24 h, the culture temperature was shifted from 32 °C to 37 °C, and the cells were incubated for 48 h.

Lipid analysis

Cells were washed twice with PBS, and total lipids were extracted from the cells with chloroform/methanol (1/1 and 1/2 v/v, successively). Samples were equalized for protein concentration, which was determined using a BCA kit (Pierce Chemical, Rockford, IL). Briefly, the total lipid extract was dissolved in chloroform/methanol/water (30/60/8, v/v/v), passed through a DEAE-Sephadex A-25 column (0.8 × 4.5 cm, acetate form), and eluted with another five volumes each of the 30/60/8 (neutral lipid fraction) and chloroform/methanol/1 M aqueous Na acetate (30/60/8) (acidic lipid fraction). The solvent was evaporated to dryness, and esters were cleaved with methanolic 0.5 M NaOH for 1 h 40 °C. The solution was neutralized with 1 M acetic acid in methanol and diluted with 6 ml 50 mM NaCl solution then desalted using a Sep-Pack C18 reverse-phase cartridge (Waters). Neutral lipid and acidic lipid fractions were separated by TLC using chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5, v/v/v/v/v) and chloroform/methanol/0.5 % $CaCl_2$ (55/45/10, v/v/v), respectively, then detected with orcinol-sulfuric acid reagent.

TLC immunostaining

TLC immunostaining was performed as described [17] with slight modifications. Briefly, GM4 (standard) and the acidic lipid fraction were spotted on a TLC plate and separated with chloroform/methanol/0.2 % $CaCl_2$ (55/45/10, v/v/v). The dry plate was dipped in cyclohexane containing 0.1 % (w/v) polyisobutylmethacrylate for 60 s to air-dry and then blocked by incubation in PBS containing 1 % bovine serum albumin at room temperature for 1 h. The plate was incubated with a polyclonal anti-GM4 antibody (rabbit IgG) in PBS at room temperature for 2 h. The plate was then washed five times with PBS, and the plate was incubated with horseradish peroxidase-conjugated anti-rabbit Fab fragment at room temperature for 1 h. Labeling was detected using an ECL kit (GE Healthcare Bio-Sciences, Piscataway, NJ). The polyclonal antibody specifically recognizes GM4 but not other GSLs such as GM3 and GalCer.

Immunofluorescence microscopy

GM3/GM3S overexpressed cells were cultured on coverglass and then fixed for 15 min with 3.7 % formaldehyde in PBS at room temperature. After rinsing with PBS, the cells were permeabilized by 0.5 % SDS in PBS, treated with Image-iT FX Signal Enhancer (Life Technologies) for 30 min, and then incubated for 1 h with 1:100 dilutions of anti-GM3/GM4S (9129) [14] and anti-GM130 (BD Bioscience, Franklin Lakes, NJ) antibodies. After washing three times in PBS, the cells were incubated for 30 min with 1:200 dilutions of Alexa 488-conjugated anti-rabbit IgG antibodies (Life Technologies) and Alexa 594-conjugated anti-mouse IgG antibodies (Life Technologies). Coverslips were washed in PBS three times, mounted on glass slides using ProLong Gold antifade reagent (Life Technologies), and analyzed by fluorescence microscopy FV1000 (Olympus, Tokyo, Japan).

Results

GM4 synthesis in cell lines expressing LacCer, GalCer, and endogenous GM3/GM4 synthase

Hamster melanoma RPMI 1846 cells reportedly express GalCer on their cell surface [13]. However, since GM3 and

GM4 synthesis activities *in vitro* are barely detectable in the lysates of these cells [13], we had assumed that RPMI 1846 cells express only the neutral glycosphingolipids GlcCer, LacCer, and GalCer (Fig. 1). To confirm the glycosphingolipid composition of RPM1 1846 cells, we performed a lipid analysis using TLC. Neutral and acidic lipid fractions were separated from the total lipid extracts of RPMI 1846 cells using DEAE-Sephadex A-25 column, and each fraction was analyzed by TLC (Fig. 2a). As expected, GlcCer, LacCer, and GalCer were detected in the neutral lipid fraction. Surprisingly, GM3 and unidentified bands were also detected in the acidic lipid fraction. These bands reacted with an anti-GM3 antibody (M2590) by TLC-immunostaining (data not shown), suggesting that the unidentified bands might be sialylparaglobosides. GM4 was not detected in RPMI 1846 cells although the cells express GalCer and endogenous GM3/GM4S.

Next, we attempted to establish GM4-reconstituted cells using Chinese hamster CHO-K1 cells expressing GlcCer, LacCer and GM3, but not expressing GalCer due to the lack of GalCerS. When CHO-K1 cells were stably transfected with the GalCerS gene, bands corresponding to GalCer appeared during TLC analysis (Fig. 2b left panel). However, as observed in RPMI 1846 cells, GM4 was not detected in the acidic lipid fraction of the CHO-K1/GalCer (Fig. 2b right panel). These results suggest that the expression of both the GalCer substrate and endogenous GM3/GM4S is not sufficient to induce GM4 expression in cultured cells.

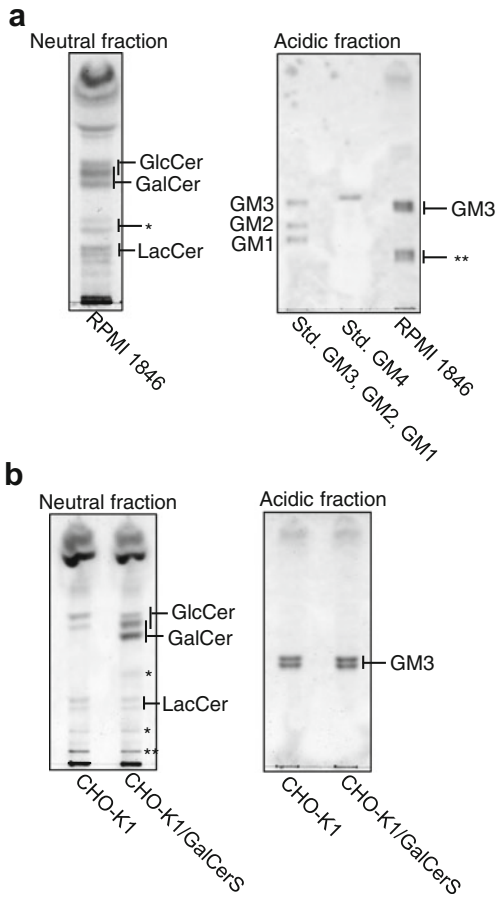

Fig. 2 Endogenous GM3/GM4S is not sufficient for GM4 expression *in vivo*. **a**. Glycosphingolipids (neutral and acidic fractions) extracted from RPMI 1846 cells, corresponding to 2 mg of protein, were separated on TLC plates and stained with orcinol-sulfuric acids. **b**. CHO cells were stably transfected with GalCerS using Lipofectamine 2000. Glycosphingolipids (neutral and acidic fractions) extracted from CHO or CHO/GalCerS cells, corresponding to 1.5 mg of protein, were separated on TLC plates and stained with orcinol-sulfuric acid reagent. A *single asterisk* indicates unidentified neutral glycosphingolipids. A *double asterisk* indicates unidentified acidic glycosphingolipids, which were M2590 (anti-GM3 monoclonal antibody)-positive

The effect of GM3/GM4S overexpression on GM4 synthesis

The cell surface expression of GM4 detected by anti-GM4 antibody is markedly increased in RPMI 1846 cells upon transient transfection of the GM3/GM4S gene [13]. We analyzed the cellular levels of GM4 in RPMI 1846 cells stably transfected with GM3/GM4S (using a lentivirus expression system) by separating lipids on TLC plates and detecting them with orcinol-sulfuric acid reagent and by TLC immunostaining (Fig. 3a and b). In the neutral fraction, the amounts of GalCer, GlcCer, and LacCer in RPMI 1846 cells expressing GM3/GM4S (RPMI 1846/GM3/GM4S cells) were similar to those in the original RPMI 1846 cells (Fig. 3a left panel). In the acidic fraction, faint bands were apparent that corresponded to GM4 (just above the GM3) in RPMI 1846/GM3/GM4S cells, while the amount of GM3 was unchanged (Fig. 3a right panel). TLC immunostaining with anti-GM4 antibodies confirmed the presence of GM4 (Fig. 3b).

We next investigated whether the overexpression of GM3/GM4S affects the GM4 synthesis in CHO-K1/GalCerS cells. The lipid composition of the CHO-K1/GalCerS/GM3/GM4S cells was similar to that of the CHO-K1/GalCerS cells. Although GM4 bands were not detected using orcinol-sulfuric acid reagent in the acidic fraction from the CHO-K1/GalCerS/GM3/GM4S cells (Fig. 3c), TLC immunostaining with anti-GM4 antibodies did reveal GM4 in the transfected cells (Fig. 3d). Taken together, these results indicate that overexpression of the GM3/GM4S gene is required to express GM4 in both RPMI 1846 and CHO-K1/GalCerS cells.

GalCer, which is an acceptor in GM4 synthesis, is produced in ER. However, since CMP-sialic acid, which is a donor in GM4 synthesis, is transported into lumenal side at Golgi apparatus by CMP-sialic acid transporter, GM4 synthesis would also occur in Golgi apparatus such as GM3 synthesis. Thus, the GalCer synthesis ought not to affect the localization of overexpressed GM3/GM4S in Golgi apparatus. Accordingly, we performed indirect immunofluorescence microscopic analysis to determine the subcellular localization of M3-GM3/GM4S expressed in RPMI1846, CHO-K1 and

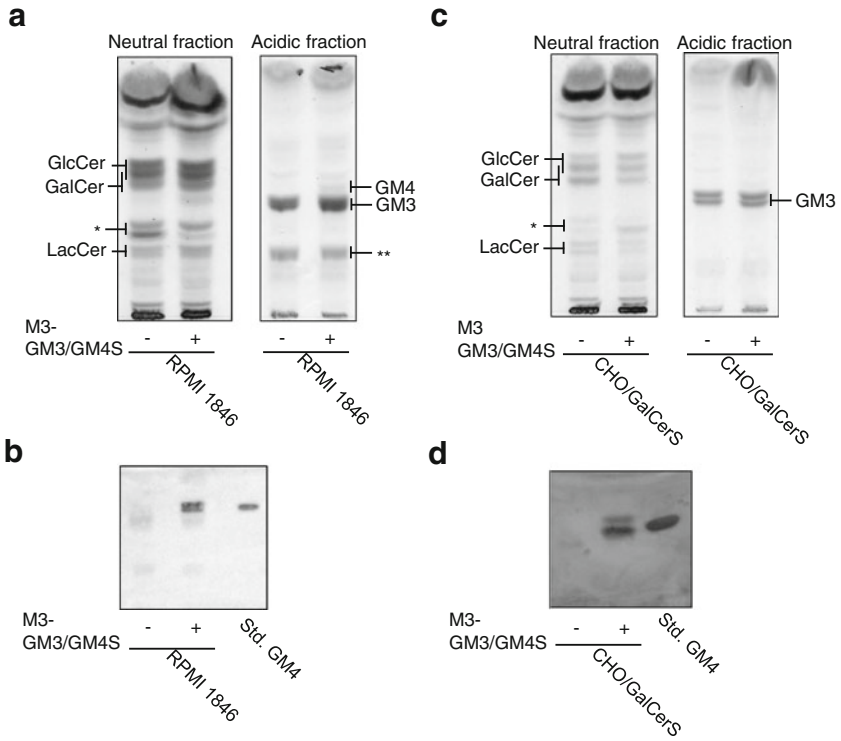

Fig. 3 Overexpression of the GM3/GM4S gene induces GM4 expression in RPMI 1846 and CHO/GalCerS cells. RPMI 1846 and CHO/GalCerS cells were transfected with (+) GM3/GM4S using a lentivirus expression system. A *minus sign* indicates no transfection. **a**. Glycosphingolipids (neutral and acidic fractions) extracted from RPMI 1846 (−) or RPMI 1846 (+) cells, corresponding to 2 mg of protein, were separated on TLC plates and stained with orcinol-sulfuric acid reagent. A *single asterisk* indicates unidentified neutral glycosphingolipids. The *double asterisk* indicates unidentified acidic glycosphingolipids (M2590-positive bands). **b**. The acidic lipid fractions from cells in A were analyzed by immunostaining with using an anti-GM4 polyclonal antibody. **c**. Glycosphingolipids (neutral and acidic fractions) extracted from CHO/GalCerS (−) or CHO/GalCerS (+) cells, corresponding to 1.5 mg of protein, were separated on TLC plates and stained with orcinol-sulfuric acid reagent. The *asterisk* indicates unidentified neutral glycosphingolipids. **d**. The acidic lipid fractions from cells in C were analyzed by immunostaining with anti-GM4 polyclonal antibody

CHO-K1/GalCerS cells. In all cell lines, M3-GM3/GM4S localized to a compact juxtanuclear reticulum and partially colocalized with GM130, a cis-Golgi marker proteins (Fig. 4), suggesting that GM4 synthesis occurs in Golgi apparatus.

Possibility of competition between the substrates LacCer and GalCer in GM4 synthesis

Since GM3 and GM4 are produced by the same enzyme (GM3/GM4S), differences in the affinity of GM3/GM4S toward the two substrates, LacCer and GalCer, may be responsible for the amounts of GM3 and GM4 produced in RPMI 1846 and CHO/GalCerS cells. If the failure of GM4 expression in these cells is caused by the competition of LacCer and GalCer for the enzyme, then cells expressing only GalCer and not LacCer, should synthesize GM4 more efficiently. We employed GM-95 cells, a mutant clone derived from mouse B16 melanoma cells [18], to examine this possibility. While GM-95 cell lysates exhibit LacCer and GM3 synthesis activity *in vitro*, the intact cells do not express GlcCer, LacCer, or GM3 due to a deletion of GlcCer synthase (GlcCerS) (Fig. 1). Indeed, studies have shown that GM-95 cells express LacCer and GM3 after introduction of the GlcCerS gene [19]. Accordingly, to establish cells expressing a single substrate, GalCer, GM-95 cells were stably transfected with the GalCerS gene, and the lipid composition of the resultant GM-95/GalCerS cells was analyzed by TLC. As shown Fig. 5a, the GM-95/GalCerS cells expressed only GalCer and not GlcCer or LacCer. As a result, GM4 was not detectable in the acidic fraction of the GM-95/GalCerS cells, although endogenous GM3/GM4S is present in these cells. This result indicates that the failure of GM4 synthesis observed in RPMI 1846 and CHO-K1/GalCerS cells is not caused by a competition between substrates. Moreover, overexpression of the GM3/GM4S gene by the lentivirus expression system induced GM4 synthesis in GM-95/GalCer cells similar to that observed in RPMI 1846 and CHO-K1/GalCer cells (Fig. 5a and b), suggesting that an excess amount of GM3/GM4S is required to detect GM4 in mammalian cells.

Discussion

In mammals, small amounts of GM4 are known to be expressed in brain and erythrocytes [8–10, 20], but the

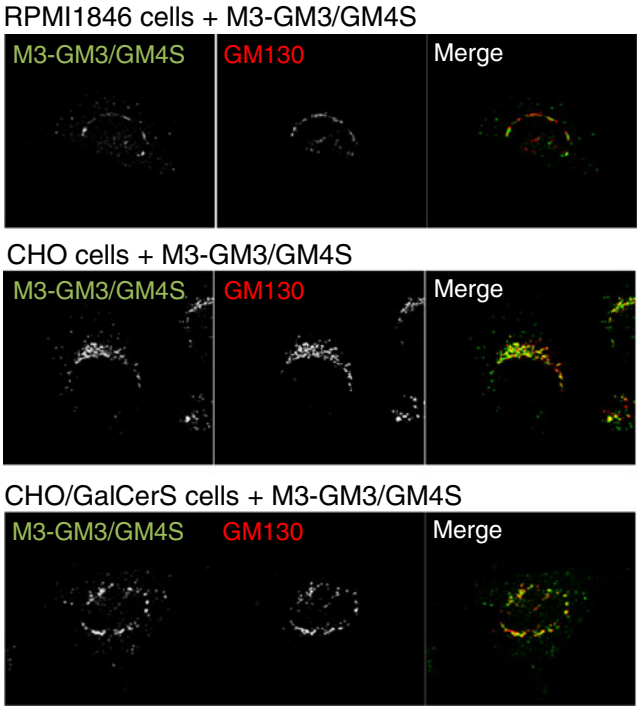

Fig. 4 Subcellular localization of overexpressed M3-GM3/GM4S in RPMI1846, and CHO, and CHO/GalCerS cells. The cells were fixed, permeabilized with 0.5 % SDS in PBS, and stained with anti-GM3/GM4S antibodies and Alexa 488-conjugated anti-rabbit IgG. Cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were incubation with antibodies against GM130 for 1 h, followed by incubation with Alexa 594-conjugated anti-mouse IgG for 30 min. Merged images are shown in the right panels

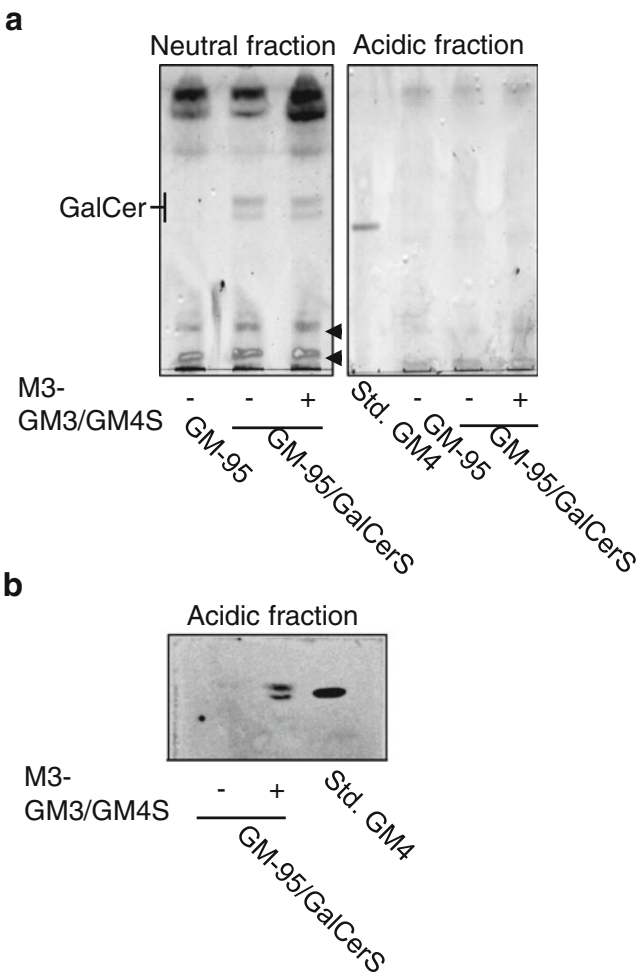

Fig. 5 GM4 expression is not affected by GM3 synthesis machinery. GM-95 cells were stably transfected with GalCerS using Lipofectamine 2000, and the resultant GM-95/GalCer cells were transfected with (+) GM3/GM4S using a lentivirus expression system. A *minus sign* indicates no transfection. **a**. Glycosphingolipids (neutral and acidic fractions) extracted from GM-95 (−), GM-95/GalCerS (−), or GM-95/GalCerS (+) cells, corresponding to 1 mg of protein, were separated on TLC plates and stained with orcinol-sulfuric acid reagent. The *arrowheads* indicate unidentified neutral glycosphingolipids. **b**. TLC immunostaining of GM-95/GalCerS (−) or GM-95/GalCerS (+) cells using anti-GM4 polyclonal antibody

regulation of GM4 synthesis remains unknown. Recently, several groups, including ours, independently found that GM3S is also involved in GM4 synthesis [13, 21]. Accordingly, we have investigated how the production and degradation of GM4 are regulated at the cellular level. In the presented study, we attempted to establish GM4-reconsituted cells using hamster melanoma RPMI 1846, Chinese hamster CHO-K1, and mouse melanoma GM-95 cells. Interestingly, GM4 was not detected by TLC immunostaining with anti-GM4 antibody in RPMI 1846 or CHO-K1/GalCerS cells, both of which express GalCer, LacCer, and GM3. Since GM4 was not detected in GM-95/GalCerS cells expressing GalCer and GM3/GM4S but not LacCer, the lack of GM4 synthesis is not caused by the competition between the acceptor substrates LacCer and GalCer. GM4 was detectable only when excess amounts of the GM3S/GM4S gene were introduced in these cells.

What is implied by these results regarding the expression mechanisms of GM4? One possibility is that GM4 synthesis is not efficient compared with GM3 synthesis. Since GM3/GM4S forms a complex with LacCer synthase (LacCerS) in the Golgi apparatus [22], GlcCer transported by FAPP2 is rapidly converted to GM3 *via* LacCer. If GlcCer is selectively transported to the compartment of the LacCerS-GM3/GM4S complex, these synthase complexes will proceed to synthesize GM3 more effectively. On the other hand, GalCer, which is a substrate in GM4 synthesis by GM3/GM4S, is produced in the ER and transported to the Golgi apparatus [12]. It is not clear whether GalCer is transported efficiently to the compartment of GM4 synthesis since the details behind the mechanism of transporting GalCer into Golgi have not been determined. Moreover, overexpression of GM3/GM4S was necessary to detect GM4 in several cell lines expressing GalCer. Under this condition, the amount of free-GM3/GM4S, which is not associated with LacCerS, may have increased. Therefore, this result may suggest that GM4 is synthesized only by

free GM3/GM4S and that the expression machinery of GM4 is independent from that of GM3.

Another possibility is that the stability of GM4 is remarkably lower than that of GM3. GM3 and the ganglioside GM1, which are generally regarded as microdomain markers, exist in distinct microdomians in the native cell membrane [23]. Since the mechanisms behind the formation of these heterogeneous microdomains are at present completely unknown, it is unclear whether GM4 and GM3 are clustering independently. If GM4 molecules in the plasma membrane localize distinctly from the other gangliosides, including GM3, then the degradation rate of the GM4 cluster during the endocytic pathway from the plasma membrane to the lysosome might be much faster than that of GM3. This hypothesis should be clarified by future studies.

GM3/GM4S knock out mice reportedly exhibit several phenotypes, including enhanced insulin sensitivity in skeletal muscle and liver [2], complete hearing loss [24], and deficient $CD4^+$ T-cell activation [25]. GalCer is not expressed in skeletal muscle, liver, or $CD4^+$ T-cells, so GM4 is also not synthesized. In the mouse inner ear, however, not only a-series gangliosides (GM3, GM1, GD1a, GD1b, and GT1b) but also sulfatides (SM3 and SM4), which are synthesized from LacCer and GalCer, respectively, are highly expressed [24]. Although the expression of GalCer was not confirmed in that report, the data nevertheless indicate that GalCer are abundant in the mouse inner ear. Thus, GM4 may also be synthesized in that structure and may be involved in the functional maturation of the cochlea, which is essential for the acquisition and maintenance of hearing function. Consequently, it will be an important subject for future studies to clarify the biological functions of GM4 at the cellular level, and the GM4-reconsituted cells (GM-95/GalCerS/GM3/GM4S cells) established in this study could be useful tools.

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