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Zebrafish and Mouse α2,3-Sialyltransferases Responsible for Synthesizing GM4 Ganglioside*[S]

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We have previously reported that fish pathogens causing vibriosis specifically adhere to GM4 on the epithelial cells of fish intestinal tracts (Chisada, S., Horibata, Y., Hama, Y., Inagaki, M., Furuya, N., Okino, N., and Ito, M. (2005) *Biochem. Biophys. Res. Commun.* 333, 367–373). To identify the gene encoding the enzyme for GM4 synthesis in the fish intestinal tract, a phylogenetic tree of vertebrate ST3GalVs, including *Danio rerio* and *Oryzias latipes,* was generated in which two putative subfamilies of fish ST3GalVs were found. Two putative ST3GalVs of zebrafish (zST3GalV-1 and -2), each belonging to different subfamilies, were cloned from the zebrafish cDNA library. Interestingly, zST3GalV-1 synthesized GM3 (NeuAcα2–3Galβ1–4Glcβ1-1′Cer) but not GM4, whereas zSTGalV-2 synthesized both gangliosides *in vitro* when expressed in CHO-K1 and RPMI1846 cells. Flow cytometric analysis using anti-GM4 antibody revealed that the transformation of RPMI1846 cells with zST3GalV-2 but not zST3GalV-1 cDNA increased the cell-surface expression of GM4. Whole mount *in situ* hybridization showed that the zST3GalV-2 transcript was strongly expressed in the gastrointestinal tract, whereas zST3GalV-1 was expressed in the brain and esophagus but not gastrointestinal tract in 3-day post-fertilization embryos. It has long been a matter of controversy which enzyme is responsible for the synthesis of GM4 in mammals. We found that three isoforms of mouse ST3GalV (mST3GalV) having different N-terminal sequences can synthesize GM4 as well as GM3 when expressed in RPMI1846 and CHO-K1 cells. Furthermore, mST3GalV knock-out mice were found to lack GM4 synthase activity and GM4 in contrast to wild-type mice. These results clearly indicate that zST3GalV-2 and mST3GalV are the enzymes responsible for the synthesis of GM4 in zebrafish and mice, respectively.

Glycosphingolipids (GSLs)[2] form microdomains in the outer leaflet of plasma membranes with and without cholesterol, and GSLs could be involved in many cellular events basically by interacting with cell-surface proteins and in some cases with glycan chains of glycoconjugates (1). During the past decades accumulating evidence has indicated that pathogens such as bacteria and virus invade mammalian host cells after adhering to cell-surface GSL receptors (2). In contrast to mammals, there have been very few reports on the interaction between fish pathogens and GSL receptors of host cells, although damage to fish cultures and probably natural stocks by specific pathogens is widely recognized as a serious problem (3, 4). We have reported that *Vibrio harveyi* causing fish vibriosis, a terminal hemorrhagic septicemia and serious disease for fish culture, specifically adhere to the ganglioside GM4 (NeuAcα2–3Galβ1-1′Cer) on the epithelial cells of the intestinal tract of red sea bream, *Pagrus major* (5). However, the enzyme responsible for synthesizing GM4 in fish has not yet been identified.

GM4 was first characterized as a minor ganglioside of white matter of human brain (6) and then was isolated from myelin (7) and astrocytes of human brain (8). This less polar ganglioside is distributed in a wide variety of vertebrates such as the mouse (9), rat (10), chicken (11), frog (12), and fish (13). Although the biological relevance of GM4 in vertebrates, including humans, is not yet fully understood, this GSL exhibits immunosuppressive activity *in vitro* and an ability to prevent experimental encephalomyelitis in guinea pigs (14).

Several sialyltransferases (STs) responsible for the sialylation of GSLs have been cloned (15). Among them, ST3GalV/SAT-1 was found to catalyze the transfer of *N*-acetylneuraminic acid (NeuAc) from CMP-NeuAc to lactosylceramide (LacCer, Galβ1–4Glcβ1-1′Cer) generating GM3 ganglioside (NeuAcα2–3Galβ1–4Glcβ1-1′Cer). However, cloned

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[2] The abbreviations used are: GSL, glycosphingolipid; EGCase, endoglycoceramidase; hpf, hours post-fertilization; dpf, days post-fertilization; HPTLC, high performance thin layer chromatography; PBS, phosphate-buffered saline; SCDase, sphingolipid ceramide *N*-deacylase; ST, sialyltransferases; hST3GalV, human α2,3-sialyltransferase; mST3GalV, mouse α2,3-sialyltransferase; zST3GalV, zebrafish α2,3-sialyltransferase; RT, reverse transcription; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; MRM, multiple reaction monitoring.

mouse (16, 17) and human GM3 synthases (18) were not able to utilize GalCer as a substrate. On the other hand, GM4 synthase activity was reported to be present in mouse brain (19) and in rat brain and kidney (20). In contrast to previous reports, Berselli *et al.* (21) found that a variant of human GM3 synthase possessing a long N-terminal region utilized not only LacCer but also GalCer as an acceptor substrate. Thus, it is still necessary to clarify which ST or which isoform of ST is responsible for synthesizing GM4 in mammals.

Here we report the cDNA cloning of two α2,3-STs (zST3GalV-1 and -V-2) of zebrafish. Interestingly, zST3GalV-1 synthesized only GM3, whereas zSTGalV-2 synthesized GM4 as well as GM3. Furthermore, it was revealed that the zST-GalV-2 but not zST3GalV-1 transcript was strongly expressed in the gastrointestinal tract in 3- and 6-dpf embryos. Furthermore, we clearly show for the first time that three isoforms of mouse ST3GalV (mST3GalTV) differing in N-terminal length are able to synthesize not only GM3 but also GM4 when expressed in hamster RPMI1846 and Chinese hamster CHO-K1 cells. It is worth noting that mST3GalV knock-out mice lacked the GM4 synthase activity of the brain, and GM4 in the brain and erythrocytes, in contrast to wild-type mice. These results clearly indicate that zST3GalV-2 and mST3GalV are enzymes responsible for the synthesis of GM4 in zebrafish and mice, respectively.

EXPERIMENTAL PROCEDURES

Materials—Chinese hamster ovary-derived CHO-K1 cells and GM3 synthase (mST3GalV) knock-out mice were kindly provided by Dr. Y. Igarashi (Hokkaido University, Sapporo, Japan) and Dr. M. Saito (Meiji Pharmaceutical University, Tokyo, Japan) (22), respectively. Hamster melanoma-derived RPMI1846 cells (CCL-49^{TM}) were obtained from the ATCC. Anti-GalCer monoclonal antibody (AMR-20) and anti-GM4 monoclonal antibody (AMR-10) were generated as described (23). Anti-GM4 polyclonal antibody (rabbit IgG) was purchased from Matreya. Rhodamine-labeled anti-mouse IgM antibody and horseradish peroxidase-conjugated anti-rabbit IgG Fab fragment were purchased from Sigma and GE Healthcare, respectively. GM1a was isolated from bovine crude gangliosides as detailed previously (24). GM4 and GalCer were prepared from the red sea bream, *P. major*, by a method described previously (5). GM4 (d18:1-C18:0) was also purchased from Wako. LacCer was purchased from Sigma, and the other GSLs used were obtained from Matreya. HPTLC plates were purchased from Merck. Endoglycoceramidase (EGCase) II was prepared from *Rhodococcus* sp. M-750 as described (25) or purchased from Takara Bio Inc. Sialidase from *Vibrio cholerae* was purchased from Calbiochem. [14C]Stearic acid and CMP-[^{14}C]NeuAc were purchased from PerkinElmer Life Sciences. SCDase was prepared by the method described previously (26) or purchased from Takara Bio Inc. Lyso-GalCer (psychosine, Galβ1-1′Sph) was obtained from Sigma, and lyso-LacCer (Galβ1–4Glcβ1-1′Sph) was prepared by digestion of LacCer with SCDase as described (27). All other reagents used in this study were of the highest quality available.

Preparation of [^{14}C]GSLs Using SCDase—[^{14}C]GalCer and [^{14}C]LacCer were prepared using SCDase by the method described previously (28). Briefly, 250 nmol of psychosine or lyso-LacCer was incubated at 37 °C overnight with 250 nmol of [^{14}C]stearic acid in 1 ml of 25 mM phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$ and 0.1% Triton X-100 in the presence of 5 milliunits of SCDase. The reaction mixture was subjected to Folch's partition, and then the lower phase containing [^{14}C]GSLs and free [^{14}C]stearic acids was evaporated to dryness. The pellet was dissolved in 1 ml of hexane/diethyl ether/acetic acid (50:50:1, v/v) and applied to a Sep-Pak Plus silica cartridge previously equilibrated with the same solvent. [^{14}C]GSLs were adsorbed on the cartridge, whereas [^{14}C]stearic acids were eluted with the solvent. Then [^{14}C]GSLs were eluted from the cartridge with 10 ml of chloroform/methanol (2:1, v/v).

Phylogenic Tree—A phylogenic tree of vertebrate ST3GalVs was generated as described (15).

cDNA Cloning of zST3GalV-1 and zST3GalV-2—To obtain DNA fragments encoding zST3GalV-1 and zST3GalV-2, the PCR was performed using sense and antisense oligonucleotide primers based on the cDNA sequence in the data base NCBI; the sense primer (5′-ATGAGGAGAGTCATGAAACA-3′) and the antisense primer (5′-GAAGCTGCAGTGTATCCC-TCC-3′) for zST3GalV-1 and the sense primer (5′-ATGGTG-ACTCTGAAACACTTGAGTG-3′) and the antisense primer (5′-TCACAGTGCCCCAGTGAGGT-3′) for zST3GalV-2 were used for PCRs with the cDNA library of adult zebrafish as a template in a GeneAmp PCR System 9700 (Applied Biosystems) using Advantage^{TM} 2 (Clontech). The cycling parameters for PCR were 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1.5 min, and the cycle number was 35. Amplified 1092- and 1149-bp PCR products containing the putative zST3GalV-1 sequence and the putative zST3GalV-2 sequence, respectively, were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and then sequenced. To obtain cDNAs containing the open reading frames of putative zST3GalV-1 and zST3GalV-2, PCRs were performed using a sense primer with an EcoRI site (5′-GGAATTCATGAGGAGAGTCATGAAACA-3′) and an antisense primer with an XhoI site and a disrupted stop codon (5′-ACTCGAGGAAGCTGCAGTGTATCCCTCC-3′) for zST3GalV-1 and a sense primer with an EcoRI site (5′-GGA-ATTCATGGTGACTCTGAAACAC-3′) and an antisense primer with an XhoI site and a disrupted stop codon (5′-GCT-CGAGCAGTGCCCCAGTGAGGTC-3′) for zST3GalV-2. The PCR products were subcloned into pcDNA3.1/Myc-His(+) (Invitrogen) to generate pcDNA/zST3GalV-1 and pcDNA/zST3GalV-2. The nucleotide and deduced amino acid sequences were evaluated using GENETYX version 8 software (GENETYX, Tokyo, Japan). The homology search of deduced amino acid sequences was performed with BLAST (29). The alignment of amino acid sequences was performed with ClustalW (30).

cDNA Cloning and Point Mutation of mST3GalV—To construct the plasmid encoding a short form of mST3GalV (described simply as mST3GalV in this study, M3-ST3GalV), we cloned the 1.1-kb BstXI-NotI fragment of pBluescript KS(+) encoding mST3GalV (a generous gift from Dr. M. Saito, Meiji Pharmaceutical University, Tokyo, Japan) into the BstXI-NotI site of pcDNA3.1 Zeo(+). Long form (M1-ST3GalV) and middle form (M2-ST3GalV) of mST3GalV were cloned from B16 melanoma cells using cDNAs prepared from the cells and

primers 5′-GGCGGCTTGCCAGCGCTCCCTC-3′ and 5′-GGTTTGCCGTGTTCCGAGTTC-3′. The resulting fragments were cloned into pGEM-T Easy vector (Promega) to generate the pSY03. The 1.3-kb NotI fragment of pSY03 was then cloned into the NotI site of pcDNA3.1 Zeo(+) vector (Invitrogen) to generate pSY08. pSY14 and pSY15 encoding M1-ST3GalV and M2-ST3GalV, which is inserted to the Kozak sequence (GCCACC) prior to ATG (M1 and M2), were constructed using pSY08 and primers 5′-GGATCCGCCACCATGCACACAGAGGCGGTGGG-3′ and 5′-GGTTTGCCGTGTTCCGAGTTC-3′, and primers 5′-GGATCCGCCACCATGCCAAGTGAGTTCACCTCTG-3′ and 5′-GGTTTGCCGTGTTCCGAGTTC-3′, respectively. The resulting fragments were cloned into pGEM-T Easy vector to generate the pSY12 and pSY13. The 1.3-kb NotI fragments of pSY12 and pSY13 were then cloned into the NotI site of pcDNA3.1 Zeo(+) vector to generate pSY14 and pSY15. Point mutations of mST3GalV generating M1-ST3GalV and M2-ST3GalV were performed by site-directed mutagenesis using the QuikChange™ kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions.

Cell Culture, Transfection, and Preparation of Cell Lysates—Chinese hamster CHO-K1 cells and hamster melanoma RPMI1846 cells were grown at 37 °C in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified incubator containing 5% CO_2. cDNA transfection was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. ST3GalV cDNA- or mock (empty vector)-transfected cells were detached from culture dishes by treatment with PBS containing 0.2 M EDTA. The cells were collected by centrifugation (1,800 rpm for 5 min) and suspended in lysis buffer (5 mM Tris-HCl, pH 7.5, containing 1 μg/ml pepstatin A and leupeptin). Cell suspension was then subjected to pipetting with a 27-gauge needle (0.4 × 19 mm)-equipped syringe to prepare cell lysate which was used as crude ST.

Preparation of Crude ST from Mouse Brains—All steps were performed at 0–4 °C. Brains from 12-week-old female wild-type mice and 12-week-old female mST3GalV knock-out mice (about 0.35 g each) were homogenized with 3 ml of 3 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.1 mM EDTA. The homogenates were subjected to centrifugation at 100 × *g* for 4 min. The same volume of 3 mM Tris-HCl buffer, pH 7.4, containing 0.34 M sucrose and 0.1 mM EDTA was added to the supernatant which was then centrifuged at 7,000 × *g* for 10 min. The supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 h. Next, 150 μl of 15 mM sodium cacodylate buffer, pH 6.5, containing 5% glycerol, 0.1% Lubrol PX, and protease inhibitors (pepstatin A and leupeptin, each 1 μg/ml) was added to the precipitate, which was then kept for 10 s in a sonic bath. The suspension was subjected to centrifugation at 20,000 × *g* for 15 min. The supernatants obtained were used as crude ST (microsomal fraction).

ST Assay—Enzymatic activity was determined with the radioactive donor substrate CMP-[^{14}C]NeuAc (assay I) or radioactive acceptor substrate [^{14}C]GSLs (assay II). For assay I, the reaction mixture (20 μl) contained 10 mM $MnCl_2$, 0.15% Triton X-100, 0.4 nmol of CMP-[^{14}C]NeuAc (60 nCi), 2 nmol of each acceptor GSL (LacCer, GalCer, or other GSLs), 40 μM NeuAc (if necessary), and cell lysate (60 μg of protein). For assay II, the reaction mixture (20 μl) contained 10 mM $MnCl_2$, 0.15% Triton X-100, 1 nmol of CMP-NeuAc, 0.2 nmol of [^{14}C]GSLs (10.8 nCi), and 1 nmol of nonradioactive GSLs, 40 μM NeuAc (if necessary), and cell lysate (60 μg of protein). The specific activity of the lysate of RPMI1846 cells transfected with mST3GalV was determined by assay II. For both assays, 80 μl of chloroform/methanol (2:1, v/v) was added to the reaction mixture and mixed well after incubation at 37 °C for 1 h, and then the lower phase was taken and dried under a stream of N_2 gas. By this partition, almost all radioactive GM4 or GM3 was recovered in the lower phase. The dried sample was dissolved in chloroform/methanol (2:1, v/v) and then subjected to HPTLC. After development with chloroform, methanol, 0.2% $CaCl_2$ (5:4:0.8, v/v), the plate was exposed to an imaging plate, which was quantified with a FLA-5000 imaging analyzer (Fuji Film Co., Kanagawa, Japan).

Protein Determination—The content of protein was determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard.

Flow Cytometric Analysis—Cells were washed with PBS, detached from the dishes by incubation with PBS containing 0.2% trypsin and 1 mM EDTA at 37 °C for 1 min, and then suspended in PBS. After centrifugation, cells were treated with the anti-GM4 antibody AMR-10 or anti-GalCer antibody AMR-20 at 4 °C for 1 h, followed by rhodamine-labeled anti-mouse IgM antibody. Incubation was continued for 30 min at room temperature. After being washed with PBS at 4 °C, the cells were analyzed with a flow cytometer (EPICS XL System-IC, Beckman Coulter).

RNA Extraction and Quantitative Real Time RT-PCR—Total RNA was prepared from the embryos at each developmental stage (0, 6, 9, 12, 24, 36, 48, 72, 84, and 96 hpf) using Sepasol RNA super I (Nacalai Tesque, Japan). First strand cDNA synthesis was performed version. 3.0 (Takara Bio Inc., Japan). After the removal of unincorporated primers using Illustra™ MicroSpin G-25 columns (GE Healthcare), quantitative real time RT-PCR was performed with the FullVelocity™ SYBR® Green QPCR Master Mix (Stratagene) in a Mx3000P™ Real Time PCR system (Stratagene), using the following primers: qST3GalV-2U, CAAACCTACGCAGAACAGCA, and qST3GalV-2L, ATGCGGAGAGAGCCGTAGTA. The amount of gene expression was quantified using a standard curve generated by amplification of a series of standard samples containing a known amount of zSTGalV-2.

Whole Mount in Situ Hybridization—The analysis was carried out as described (31). The sense (as negative control) and antisense RNA probes labeled with digoxigenin-UTP were synthesized using the digoxigenin RNA labeling mix (Roche Applied Science) from zST3GalV-1 or zST3GalV-2 cDNA subcloned into pGEM-T Easy. The hybridization signals were detected with anti-digoxigenin antibody conjugated with alkaline phosphatase and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate.

TLC Analysis—GSLs were subjected to HPTLC or TLC plate that was developed with chloroform, methanol, 0.2% $CaCl_2$ (5:4:1, v/v) for GSLs from zebrafish intestines and mouse brains and chloroform, methanol, 0.2% $CaCl_2$ (55:45:10, v/v) for GSLs from mouse erythrocytes. GSLs were visualized with orcinol-

H_2SO_4 or immunostaining using anti-GM4 antibody as described below. [^{14}C]GSLs were quantified with an imaging analyzer FLA-5000.

TLC Immunostaining—TLC immunostaining was performed as described (32) with slight modifications. Briefly, GM4 was spotted on a TLC plate and developed with chloroform, methanol, 0.2% $CaCl_2$ (5:4:1, v/v). The dry plate was dipped in diethyl ether/*n*-hexane (1:5, v/v) containing 0.5% (w/v) polyisobutylmethacrylate for 30 s to air-dry overnight and then blocked by incubation in PBS containing 2.5% skim milk at 37 °C for 2 h. It was rinsed five times with PBS containing 0.25% skim milk, PBS (wash buffer) and incubated with anti-GM4 monoclonal antibody AMR-10 or polyclonal anti-GM4 antibody (rabbit, Matreya) in PBS at 4 °C overnight. The plate was then washed five times with washing buffer, and the plate was re-incubated with anti-mouse IgM alkaline phosphatase conjugate antibody for AMR-10 or horseradish peroxidase-conjugated anti-rabbit Fab fragment for rabbit polyclonal antibody at 37 °C for 4 h. As a final step, alkaline phosphatase or horseradish peroxidase was detected by adding appropriate substrates according to the manufacturer's instructions. The specificity of AMR-10 and polyclonal antibody was practically the same, and both specifically recognize GM4 but not other GSLs such as GM3 and GalCer.

Extraction of GSLs from Zebrafish Gastrointestinal Tracts—The gastrointestinal tract (about 0.19 g, dry weight) of adult zebrafish was homogenized with 4 ml of chloroform/methanol (2:1, v/v) and left for 30 min at room temperature. The extract was subjected to partition with chloroform/methanol/water (8:4:3, v/v), and the lower phase was drawn and evaporated dry. The residue was dissolved in chloroform/methanol (1:1, v/v) and subjected to mild alkaline hydrolysis (0.2 M KOH). After neutralization with 0.2 M acetic acid, the extract was subjected to partition with chloroform/methanol/water (8:4:3, v/v) and the lower phase was evaporated dry. The residue was dissolved in chloroform/methanol/H_2O (30:60:8, v/v) and applied to a cartridge of Sep-Pak Plus QMA (Waters) equilibrated with chloroform/methanol/H_2O (30:60:8, v/v). Acidic GSLs were eluted from the cartridge with chloroform, methanol, 0.8 M sodium acetate (30:60:8, v/v) and de-salted with a Sep-Pac Plus silica cartridge (Waters). For purification of GM4, the fraction was treated with EGCase II to digest contaminating GSLs. The reaction mixture was subjected to Folch's partition, *i.e.* 4× volume of chloroform/methanol (2:1, v/v) was added to the reaction mixture and mixed well, and the lower phase was taken and dried under a stream of N_2 gas.

Extraction of GSLs from Mouse Brains—The brains (wild-type mice, about 88 mg; mST3GalV KO-mice, about 68 mg, dry weight) were cut to pieces with scissors. GSLs were extracted with chloroform/methanol (2:1, v/v) three times. The extracts were combined and subjected to mild alkaline hydrolysis. After neutralization, the extract was subjected to the partition with chloroform/methanol/water (8:4:3, v/v), and then the lower phase was evaporated to dryness. The residue was dissolved in chloroform/methanol/H_2O (30:60:8, v/v) and applied to a cartridge of Sep-Pak Plus QMA (Waters) to obtain acid GSL fraction as described above.

Immunohistochemistry of GM4—Zebrafish gastrointestinal tracts were fixed with 4% paraformaldehyde in PBS at 4 °C overnight. The materials were embedded in OCT compound (Sakura Finetechnical, Japan), rapidly frozen using liquid nitrogen, and stored at −80 °C. The frozen materials were cut into 10-μm-thick sections using a Cryostat (Leica CM1850, Germany) and mounted on aminopropyl silane-coated slide glasses. After treatment with PBS containing 2.5% skim milk at room temperature for 2 h, the samples were incubated with the polyclonal anti-GM4 antibody (rabbit, diluted by 500 times) at 4 °C overnight, followed by Cy3-labeled anti-rabbit IgG antibody (diluted by 2000 times) at room temperature for 2 h. For staining of actin filament, samples were treated with fluorescein isothiocyanate-conjugated phalloidin (Sigma). Immunostained samples were observed with a confocal laser-scanning microscope (Digital Eclipse C1, Nikon, Japan) (33).

Extraction of GSLs from Mouse Erythrocytes—Blood collected from the hearts of mice was kept at room temperature for 1 h and then left at 4 °C overnight. The GSLs were successively extracted with chloroform/methanol (1:1, 1:2, then 1:2, v/v). The extracts were combined and then applied to DEAE-Sephadex A-25 equilibrated with chloroform/methanol/water (30:60:8, v/v/v). After washing the column with the same solvent, the acidic GSL fraction was eluted with chloroform, methanol, 1 M sodium acetate (30:60:8, v/v). The fraction was de-salted with a Sep-Pak silica cartridge.

Digestion of GSLs with EGCase II or Sialidase—To distinguish GM4 from GM3, EGCase II was employed. GM3 was hydrolyzed by the enzyme to generate sialyl lactose and ceramide, but GM4 was completely resistant to hydrolysis by the enzyme (34, 35). On the other hand, *V. cholerae* sialidase hydrolyzes both GSLs. The reaction mixture containing sample and 0.2 milliunit of recombinant EGCase II in 20 μl of 50 mM acetate buffer, pH 5.0, containing 0.1% Triton X-100 was incubated at 37 °C for 6 h. Sialidase treatment of samples was conducted as follows: the reaction mixture containing sample and 20 milliunits of the sialidase in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$ was incubated at 37 °C for 2 h.

Mass Analysis of GM4 from Mouse Erythrocytes—To identify the molecular species of GM4 in red blood cells, reverse phase LC/electrospray ionization (ESI)-MS and multiple reaction monitoring (MRM) were performed using a 4000Q TRAP quadrupole-linear ion trap hybrid MS (Applied Biosystems, Foster City, CA) with an UltiMate 3000 nano/cap/micro LC system (Dionex Corp., Sunnyvale, CA) as described (36). MS^2 analyses of GM4 and MS^3 analyses of individual ceramide molecular species derived from the MS^2 analyses were conducted in the positive ion mode with a scan speed of 1000 atomic mass units/s, trap fill time of 20 ms, extraction time of 200 ms, ion spray voltage of 5500 V, declustering potential of 120 V, and collision-induced dissociation of 40 V.

RESULTS

Detection of GM4 in Zebrafish Gastrointestinal Tract—Prior to identifying the gene encoding the GM4 synthase of zebrafish, we examined whether GM4 is present in zebrafish. GM4 was detected in the acidic GSL fraction from zebrafish gastrointestinal tract using orcinol-H_2SO_4 reagent (Fig. 1*A*, *lanes 1, 4,* and

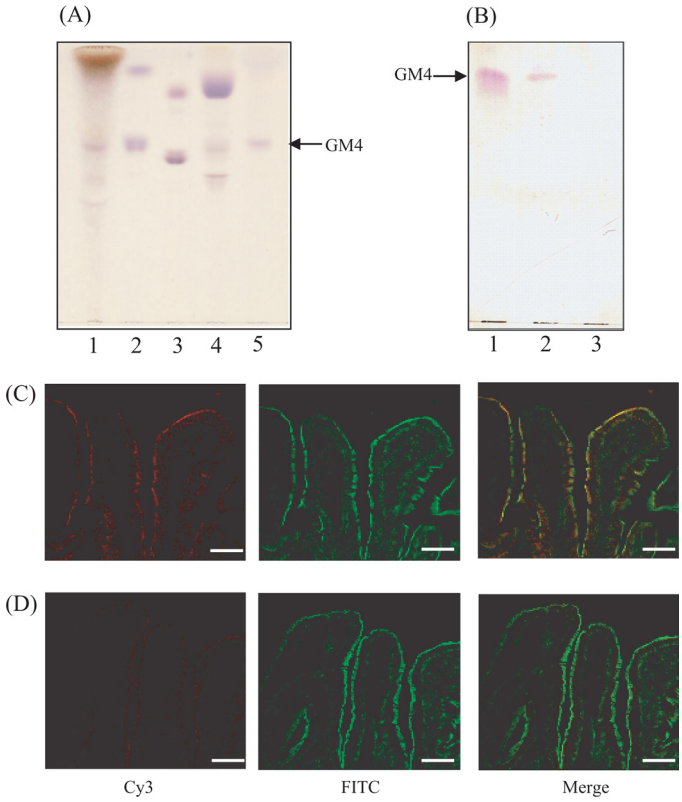

FIGURE 1. **Detection of GM4 of the gastrointestinal tract of zebrafish *Danio rerio*.** GSLs were separated by HPTLC using chloroform, methanol, 0.02% $CaCl_2$ (5:4:1, v/v) and visualized with the orcinol-H_2SO_4 reagent (*A*) and separated by plastic TLC using the same solvent and visualized with anti-GM4 monoclonal antibody AMR-10 (*B*), respectively. *A, lane 1*, crude GSL fraction (lower phase from Folch's partition) of *D. rerio* gastrointestinal tract; *lane 2*, standard GalCer and GM4 from top to bottom; *lane 3*, standard LacCer and GM3 from top to bottom; *lane 4*, acidic GSL fraction from the gastrointestinal tract. *lane 5*, GM4 purified from the gastrointestinal tract. *B, lane 1*, standard fish *P. major* GM4; *lane 2*, purified GM4 from *D. rerio* gastrointestinal tract; *lane 3*, standard GM1a. *C,* immunohistochemistry of zebrafish intestinal tract with anti-GM4 polyclonal antibody and Cy3-conjugated anti-rabbit IgG antibody as a second antibody. *D,* same experiment of *C* without anti-GM4 antibody (negative control). *C* and *D,* GM4 signal (*red, left panel*); actin signal (*green, center panel*); and merge (*yellow, right panel*). For staining of actin filament, samples were treated with fluorescein isothiocyanate (*FITC*)-conjugated phalloidin (Sigma). *Calibration bar*, 15 μm. Details for TLC immunostaining and immunohistochemistry of GM4 are described under "Experimental Procedures."

5) and TLC immunostaining with the anti-GM4 monoclonal antibody AMR-10 (Fig. 1*B*, *lane 2*). To investigate the distribution of GM4 in the intestinal tract of zebrafish, immunostaining using a polyclonal antibody for GM4 and Cy3-labeled anti-rabbit IgG as a secondary antibody was performed with frozen sections of the intestinal tract. As shown in Fig. 1*C*, signals for GM4 (Cy3, *red*) were observed at the luminal surface of epithelial cells in zebrafish intestine and merged with signals for actin filaments (Fig. 1*C*, *FITC*, *green*). Fig. 1*D* shows negative controls with the secondary antibody but not the anti-GM4 antibody.

Phylogenetic Tree of Vertebrate ST3GalVs—Zebrafish ST3GalV (accession number AJ619960 for zST3GalV-1) was registered as a putative GM3 synthase in the data base ZFIN). Using the sequence of zST3GalV-1, a search for homologous sequences was made with BLAST. As shown in supplemental Fig. 1, the ST3GalV family seems to be divided into two subfamilies. One subfamily, which in this phylogenic tree (upper branch in supplemental Fig. 1), contains the GM3 synthases of humans (accession number AB018356), mice (accession number AAF66147), chickens (accession number AAS83519), and zST3GalV-1. The other subfamily (under branch in supplemental Fig. 1) contains putative ST3GalVs from various fish but not other vertebrates. Interestingly, another putative zebrafish ST3GalV (zST3GalV-2) was found in this subfamily. The supplemental Fig. 2 shows the alignment of the deduced amino acid sequences of ST3GalVs of various sources. zST3GalV-1 and zST3GalV-2 show 39.5 and 33.4% identity to human ST3GalV (hST3GalV), 37.6 and 32.6% identity to mouse ST3GalV (mST3GalV), respectively, and 30.3% identity to each other. The N-terminal sequence of zST3GalV-2 was found to be longer than that of other STGal3Vs (supplemental Fig. 2). All sialyl motifs, large, small, motif III, and motif VS, were identified in two zST3GalV sequences.

ST Activities of zST3GalV-1, zST3GalV-2, and mST3GalV—The lysates of RPMI1846 cells transformed with zST3GalV-1 and zST3GalV-2 cDNAs were subjected to assays II as described under "Experimental Procedures" using [^{14}C]GalCer for GM4 synthase and [^{14}C]LacCer for GM3 synthase as an acceptor substrate, respectively. As shown in Fig. 2*A*, a new band corresponding to the R_f value of GM4 was generated from the reaction of [^{14}C]GalCer and CMP-NeuAc with zST3GalV-2 (*lane 2*) but not zST3GalV-1 (*lane 3*). On the other hand, a new band corresponding to the R_f value of GM3 was generated from the reaction of [^{14}C]LacCer and CMP-NeuAc with zST3GalV-1 (Fig. 2*A*, *lane 7*) and zST3GalV-2 (*lane 6*). Fig. 2, *B* and *C,* represents the quantification of the synthesis of GM4 and GM3 by STs as shown in Fig. 2*A*. The synthesis of GM4 by zST3GalV-2 was increased by 3.4-fold as compared with that by mock (Fig. 2, *A* and *B*, *lane 1 versus lane 2*). It is worth noting that zST3GalV-1 was unable to synthesize GM4, but the synthesis of GM3 by zST3GalV-1 was much greater than that by zST3GalV-2 under the conditions used (Fig. 2*A, lane 6 versus lane 7*; Fig. 2*C, lane 2 versus lane 3*). These results indicate that zST3GalV-1 synthesized GM3 but not GM4, whereas zST3GalV-2 synthesized both gangliosides.

Mouse GM4 synthase has yet to be identified, although GM4 is present in mouse tissues as a minor ganglioside (9). Because zST3GalV-2 but not zST3GalV-1 showed GM4 synthase activity, we searched for an orthologous sequence of zST3GalV-2 in mouse gene data bases. However, we did not find the zST3GalV-2 orthologues except mST3GalV, although several reports have indicated that the enzyme did not utilize GalCer as an acceptor substrate (16, 17). Thus, we re-examined carefully whether mST3GalV could synthesize GM4 or not. First, mST3GalV cDNA was introduced into RPMI1846 cells, and ST activity was determined by assay II. Surprisingly, a new radioactive band corresponding to the R_f values of [^{14}C]GM4 (Fig. 2*A*, *lane 4*) was generated from the reactions of [^{14}C]GalCer and CMP-NeuAc with mST3GalV, in addition to the generation of [^{14}C]GM3 from the reactions of [^{14}C]LacCer and CMP-NeuAc with the enzyme. To distinguish GM4 and GM3 generated, we used sialidase and EGCase II, the former hydrolyzed both GSLs but the latter only GM3. As a result, it was found that the radioactive band corresponding to the R_f value of GM3 disappeared on treatment with EGCase II (Fig. 2*A*, *lane*

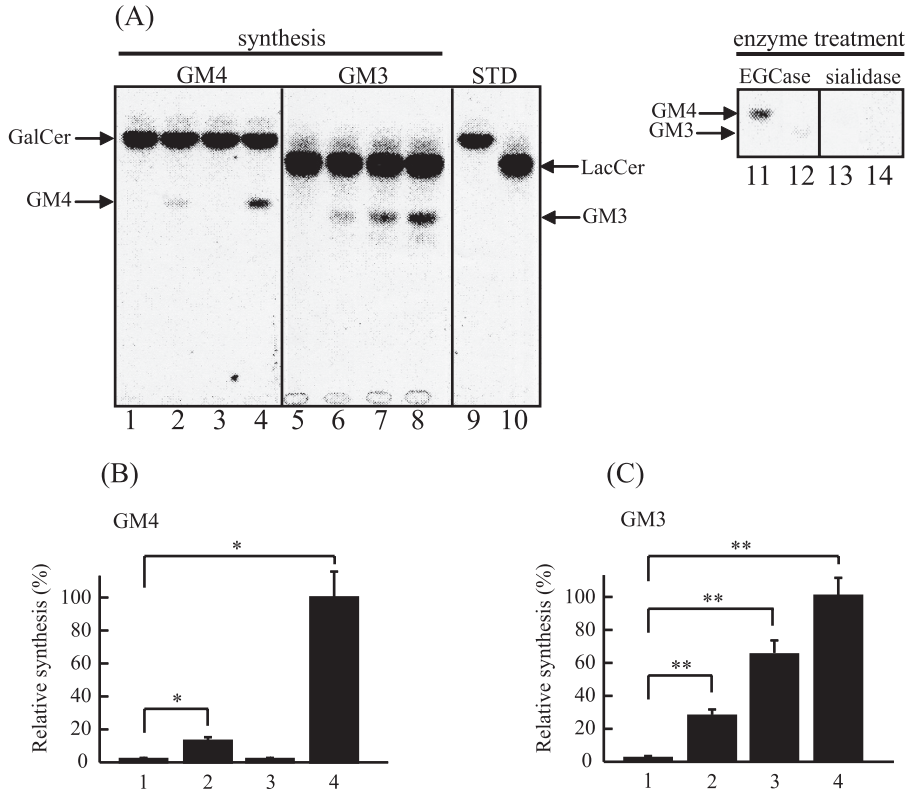

FIGURE 2. **ST activity of RPMI1846 cells transfected with empty vector (mock) or vector containing zST3GalV-1, zST3GalV-2, or mST3GalV cDNA.** GM4 synthase activity was measured by assay II using 60 μg of cell lysates of transfected cells as the enzyme and CMP-NeuAc and [^{14}C]GalCer as substrates. For the measurement of GM3 synthase activity, [^{14}C]LacCer was used as the acceptor substrate instead of [^{14}C]GalCer. Cells transfected with the empty vector pcDNA3.1/Myc-His(+) (mock) were used as a negative control. *A*, autoradiogram of the TLC showing the reaction products from the incubation with lysate of mock transfectants (*lanes 1* and *5*), zST3GalV-2 transfectants (*lanes 2* and *6*), zST3GalV-1 transfectants (*lanes 3* and *7*), and mST3GalV transfectants (*lanes 4* and *8*). *Lanes 1–4* and *5–8* show the activities of GM4 and GM3 synthases, respectively. *Lanes 9* and *10* show the standard [^{14}C]GalCer and [^{14}C]LacCer, respectively. *Lanes 11* and *13* show the reaction products of mST3GalV with [^{14}C]GalCer and CMP-NeuAc after treatment with EGCase II and sialidase, respectively. *Lanes 12* and *14* show the reaction products of mST3GalV with [^{14}C]LacCer and CMP-NeuAc after treatment with EGCase II and sialidase, respectively. *STD*, standard. *B*, quantification of the radioactivity corresponding to GM4 in *A*. Data are presented relative (%) to the radioactivity of [^{14}C]GM4 in *lane 4* of *A*. *Lane 1*, mock (*lane 1* of *A*); *lane 2*, zST3GalV-2 (*lane 2* of *A*); *lane 3*, zST3GalV-1 (*lane 3* of *A*); *lane 4*, mST3Gal (*lane 4* of *A*). *C*, quantification of radioactivity corresponding to GM3 in *A*. Data are presented relative (%) to the radioactivity of [^{14}C]GM3 in *lane 8* of *A*. *Lane 1*, mock (*lane 5* of *A*); *lane 2*, zST3GalV-2 (*lane 6* of *A*); *lane 3*, zST3GalV-1 (*lane 7* of *A*); *lane 4*, mST3Gal (*lane 8* of *A*). Values of *B* and *C* are the average of three independent determinations with S.D. * and ** represent $p < 0.01$ and $p < 0.001$, respectively, compared with mock transfectants.

12) and sialidase (Fig. 2*A*, *lane 14*), whereas that corresponding to [^{14}C]GM4 disappeared on treatment with sialidase (Fig. 2*A*, *lane13*) but not EGCase II (Fig. 2*A*, *lane 11*). The synthesis of GM4 and GM3 by mST3GalV increased 27.5-fold (Fig. 2*B*, *lane 1 versus lane 4*) and 38-fold (Fig. 2*C*, *lane 1 versus lane 4*) as compared with that of mock transfectants. Collectively, zST3GalV-2 and mST3GalV synthesized GM3 as well as GM4 *in vitro*, whereas zST3GalV-1 synthesized GM3 but not GM4.

Enzymatic Properties of GM4 Synthases—Some enzymatic properties for the synthesis of GM4 by zST3GalV-2 and mST3GalV were examined using lysates of RPMI1846 cells transformed with zST3GalV-2 and mST3GalV cDNAs, respectively. Both STs were found to be activated in the presence of Mg^{2+}, Mn^{2+}, and Fe^{2+}, but not Ca^{2+}, at a concentration of 10 mM. On the other hand, Zn^{2+} and Cu^{2+} at the same concentration showed strong inhibitory effects (supplemental Fig. 3, *A* and *B*). The optimum pH of both STs for the synthesis of GM4 was found to be 6.5 when the activity was determined in 25 mM sodium cacodylate buffer (supplemental Fig. 3, *C* and *D*). Triton X-100 and Nonidet P-40 were found to activate the GM4-synthesizing activity of the two STs at a concentration of 0.15%, whereas taurodeoxy cholate showed inhibitory effects at the same concentration (supplemental Fig. 3, *E* and *F*). On the other hand, almost no activity was found in the absence of detergents. These properties seem to be very similar, if not identical, to those for the GM3 synthesizing activity of mST3GalV as reported previously (16, 20, 37). The specific activity of the ST in the lysate of RPMI1846 cells transformed with mST3GalV was estimated to be 77.1 and 689.6 pmol/mg/h using GalCer and LacCer, respectively, indicating the level of GM3 synthesizing activity to be nine times higher than that of GM4 synthesizing activity under the conditions used.

GM4 Synthase Activity of mST3GalV Isoforms—As shown in Fig. 3*A*, several putative initiation codons were found in mST3GalV based on the NCBI data base. To investigate the effects of the length of the N-terminal region on the specificity of mST3GalV, a short form of mST3GalV cDNA (described simply as mST3GalV, M3-ST3GalV) and two mutant forms, in which Met-28 and Met-56 (M1-ST3GalV) or Met-56 (M2-ST3GalV) had been changed to Ala, were generated (Fig. 3*A*). These cDNA constructs were separately transfected into CHO-K1 cells, and ST activity was examined by assay I using transformed cell lysate as the enzyme source. As shown in Fig. 3, *B* and *C*, all transfectants showed GM4 synthase activity, and the ratio of GM3/GM4 synthase activity among the three isoforms was almost the same, indicating the length of the N-terminal region had no effect on the activity and specificity of mST3GalV.

It is worth noting that M1-ST3GalV, M2-ST3GalV, and M3-ST3GalV clearly differed in molecular weight as estimated by Western blotting in which no cross-contamination was detected (Fig. 3*D*), and these isoforms were found to be expressed in mice tissues (47).

Expression of Cell-surface GM4 after Transfection with zST3GalV-2 and mST3GalV—First, RPMI1846 and CHO-K1 cells were subjected to a flow cytometric analysis using the anti-GalCer antibody AMR-20 to examine the expression of GalCer,

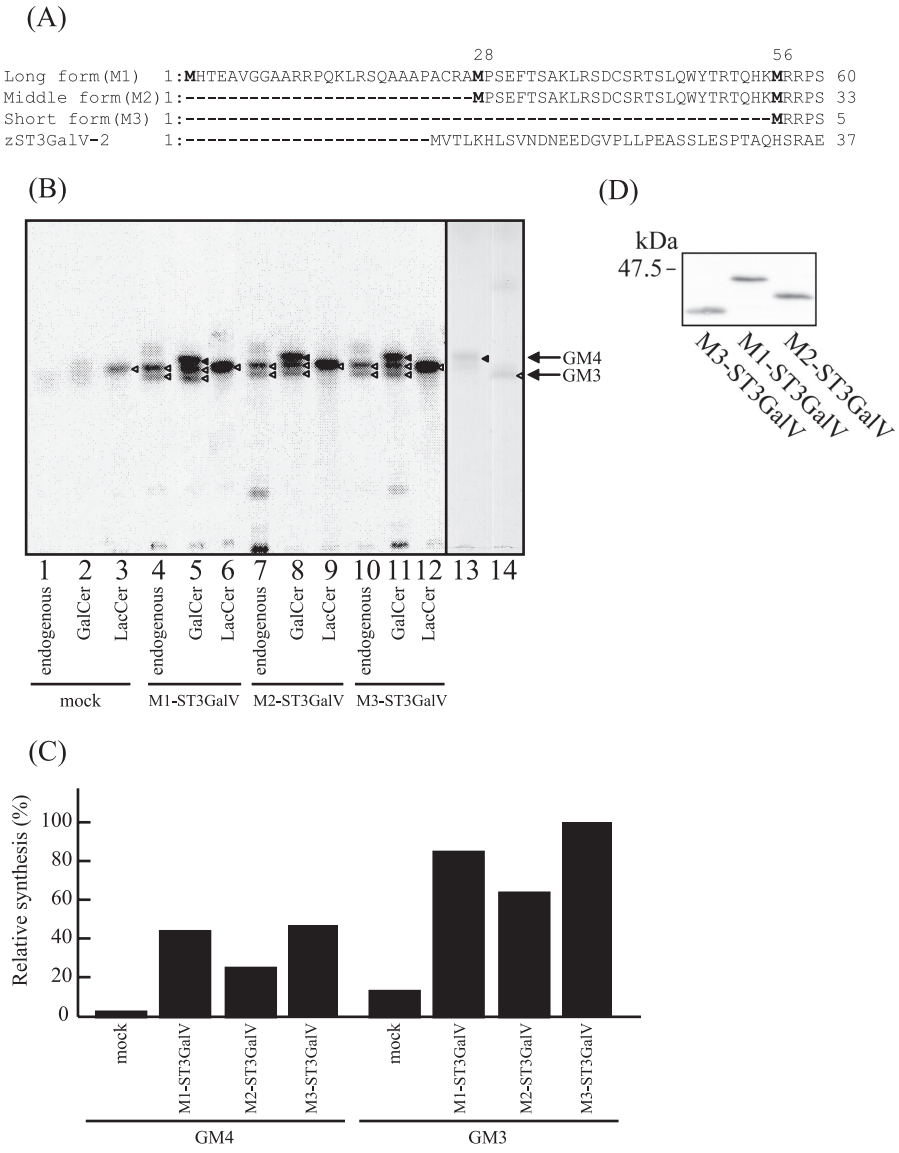

FIGURE 3. **Effects of N-terminal sequence of mST3GalV on ST activity.** *A*, deduced N-terminal sequences of mST3GalV isoforms registered in the NCBI data base. *M1*, *M2*, and *M3* represent M1-ST3GalV (*long form*), M2-ST3GalV (*middle form*), and M3-ST3GalV (*short form*), respectively. *B*, autoradiogram of TLC showing the generation of [^{14}C]GM3 and [^{14}C]GM4 by the actions of three isoforms of mST3GalV expressed separately in CHO-K1 cells. ST activity was measured by assay I using 60 μg of cell lysates of CHO-K1 cells transfected with cDNA encoding each isoform of mST3GalV. The products from the reaction with the lysates of mock transfectants (*lanes 1–3*), M1-ST3GalV transfectants (*lanes 4–6*), M2-ST3GalV transfectants (*lanes 7–9*), and M3-ST3GalV transfectants (*lanes 10–12*). GalCer (*lanes 2, 5, 8,* and *11*), LacCer (*lanes 3, 6, 9,* and *12*), and no substrates (*lanes 1, 4, 7,* and *10*) were used for the reaction as the acceptor substrate. *Lanes 13* and *14* were standard GM4 and GM3 visualized by orcinol-H_2SO_4 reagent. The *closed* and *open arrowheads* show GM4 and GM3, respectively. This was confirmed by the treatment of samples with EGCase II and sialidase by the method described in Fig. 2. *C,* quantification of [^{14}C]GM4 and [^{14}C]GM3 generated from the reaction with mock and M1-ST3GalV, M2-ST3GalV, and M3-ST3GalV. Data are presented relative (%) to the radioactivity of [^{14}C]GM3 synthesized by M3-ST3GalV. Values are the average of two independent determinations. *D,* Western blotting of M1-ST3GalV, M2-ST3GalV, and M3-ST3GalV. Integral membrane fractions were prepared from CHO-K1 cells transfected with cDNA encoding each isoform of mST3GalV, treated with peptide:*N*-glycosidase F, and then analyzed by Western blotting with anti-ST3GalV antibody that recognizes the C-terminal 55 amino acids of mST3GalV (46).

the precursor for the synthesis of GM4 by GM4 synthase. GalCer was expressed on the surface of RPMI1846 cells (Fig. 4*A*) but not CHO-K1 cells (Fig. 4*B*), indicating RPMI1846 cells to be suitable for the validation of GM4 expression. Expression of GM4 was then analyzed by flow cytometry using AMR-10 after transformation of the cells with zST3GalV-2 and mST3GalV cDNAs. No GM4 expression was observed when CHO-K1 cells were transformed with these cDNAs possibly due to the absence of GalCer in the cells (Fig. 4*C*). In contrast, cell-surface expression of GM4 was markedly increased when zST3GalV-2 as well as mST3GalV cDNAs were transfected into RPMI1846 cells and incubated at 37 °C for 24 (Fig. 4*D*) and 36 h (Fig. 4*E*). It was confirmed that the expression of cell-surface GM4 was not increased by transfection of zST3GalV-1 cDNA into RPMI1846 cells (Fig. 4*F*).

mRNA Expression of zST3GalV-2 during Zebrafish Embryogenesis—The expression of mRNA of the putative zebrafish GM4 synthase during embryogenesis was examined using real time RT-PCR. As shown in Fig. 5, the expression of zST3GalV-2 gradually increased after fertilization with peaks at 9 and 24 h, then decreased, and increased again with a maximum peak at 84 h. Whole mount *in situ* hybridization of zebrafish embryos using antisense probe for zST3GalV-2 revealed that the zST3GalV-2 transcript was strongly expressed in the pronephric duct in 24-hpf embryos (Fig. 6*B*) and in the gastrointestinal tract in 3- and 6-dpf embryos (Fig. 6, *D* and *F*). On the other hand, zST3GalV-1 was strongly expressed in the brain in 24-hpf embryos (Fig. 6*H*) and weakly expressed in the esophagus in 3-dpf embryos (Fig. 6*J*). However, zST3GalV-1 was not expressed in the gastrointestinal tract. These results indicated that zST3GalV-2 is an enzyme responsible for the synthesis of gastrointestinal GM4 in zebrafish.

ST Activity in the Brains of Wild-type and mST3GalV Knock-out Mice—Our data strongly suggest that mST3GalV synthesizes GM4 as well as GM3 in mice. To obtain a further conclusive result, levels of ST activity in the wild-type and mST3GalV knock-out mice were measured using the microsomal fraction of the brain as an enzyme source. In wild-type mice, activities of both GM4 synthase and GM3 synthase were detected by assay I (Fig. 7, *A* and *B*, *lanes 1* and *3*). The activity of GM3 synthase was ~5 times greater than that of GM4 synthase (Fig. 7*B*, *lane 1 versus lane 3*). On the other hand, almost no ST activity was detected in the lysate of the brains of mST3GalV knock-out mice using either GalCer or

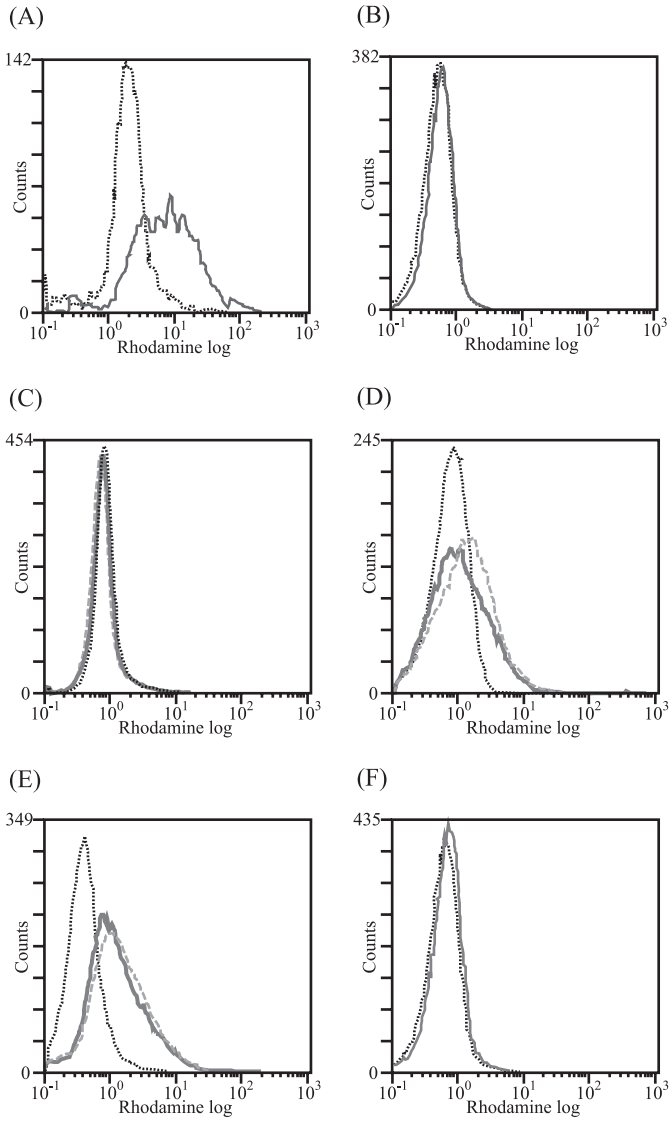

FIGURE 4. **Flow cytometric analysis of cell-surface GSLs of cells transfected with zST3GalV-1, zST3GalV-2, and mST3GalV cDNAs.** Cells transfected with zST3GalV-2 and mST3GalV were incubated with the anti-GalCer monoclonal antibody AMR-20 (*A* and *B*) and anti-GM4 monoclonal antibody AMR-10 (*C–F*), followed by rhodamine-labeled goat anti-mouse IgM. After washing, the cells were analyzed with a flow cytometer (EPICS XL System-IC, Beckman Coulter). *A*, GalCer expression of RPMI1846 cells; *B*, GalCer expression of CHO-K1 cells; *C*, GM4 expression of CHO-K1 cells after transformed with zST3GalV-2 and mST3GalV cDNAs. The GM4 expression of RPMI1846 cells after transforming with zST3GalV-2 and mST3GalV cDNAs at 24 h (*D*) and at 36 h (*E*) is shown. *F*, GM4 expression of CHO-K1 cells after transforming with zST3GalV-1 cDNA. *A* and *B*, *dotted lines* show negative control (secondary antibody treatment without AMR-20) and *solid lines* show the expression of GalCer (AMR-20 and secondary antibody treatment); *C–E*, *dotted lines* show the expression of GM4 in mock transfectants; *solid lines* show that in zST3GalV-2 transfectants, and *broken lines* show that in mST3GalV transfectants. *F*, *dotted line* shows the expression of GM4 in mock transfectants, and *solid line* shows that in zST3GalV-1 transfectants. Details are described under "Experimental Procedures."

LacCer as an acceptor substrate (Fig. 7, *A* and *B lanes 2* and *4*), indicating that not only GM3 but also GM4 synthase activity is absolutely lacking in the mST3GalV knock-out mice.

Lack of GM4 in the Brain and Erythrocytes of mST3GalV Knock-out Mice—The analysis of the acid GSL fraction of the brain and erythrocytes in wild-type and mST3GalV knock-out mice was performed by TLC immunostaining using anti-

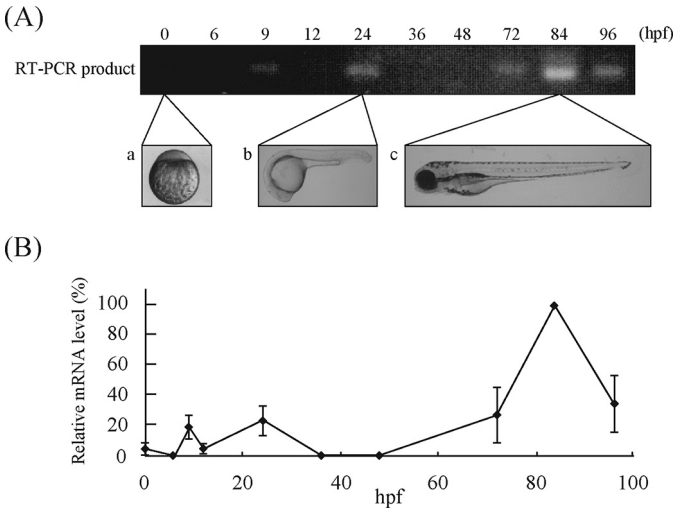

FIGURE 5. **Quantitative real time RT-PCR analysis of zST3GalV-2 during zebrafish embryogenesis.** *A*, total RNA was isolated from the embryos at each developmental stage (0, 6, 9, 12, 24, 36, 48, 72, 84, and 96 hpf), and then quantitative real time RT-PCR was performed as described under "Experimental Procedures." *B*, gene expression was quantified using a standard curve made using PCR products of a known amount of zST3GalV-2 mRNA. Quantitative profile data of real time RT-PCR at each developmental stage are presented relative (%) to the mRNA level at the 84-hpf stage and the average of three independent determinations.

GM4 antibody (Fig. 8). The amount of GM4 in wild-type mice was very small and barely detectable in erythrocytes and the brain by conventional TLC with chemical staining such as orcinol-H_2SO_4 (data not shown). However, GM4 was clearly detected by TLC immunostaining in the brain (Fig. 8*A*) and erythrocytes (Fig. 8*B*) of wild-type mice but not mST3GalV knock-out mice.

To verify the molecular species of the erythrocyte GSL that disappeared in mST3GalV knock-out mice, the GSL was extracted from the TLC plate and subjected to reverse-phase LC/ESI-MS in the negative ion mode. Several peaks corresponding to GM4 possibly with different species of ceramide were detected by the multiple reaction monitoring (MRM) (C24 series, Fig. 9*A*, and C22 series, supplemental Fig. 4*A*). An MS^2 analysis of these peaks conducted in the positive ion mode revealed the carbohydrate of each GSL to be composed of one hexose and one sialic acid (Fig. 9*B* and supplemental Fig. 4*B*). An MS^3 analysis revealed that the sphingoid base of each ceramide was d18:1 (*m/z* 264) or d18:2 (*m/z* 262) (Fig. 9*C* and supplemental Fig. 4*C*). From these results, the GSLs were identified as GM4 containing different molecular species of ceramide (d18:1-C22:0, d18:2-C22:0, d18:1-C24:0, d18:2-C24:0, and d18:2-C24:1). Interestingly, GM4 of mouse erythrocytes contained long chain fatty acids, and thus its R_f value seemed to be somewhat different from that of standard GM4 composed of a d18:1-C18:0 ceramide (Fig. 8*B*). Collectively, GM4 was absent in the brain and erythrocytes of mST3GalV knock-out mice in contrast to wild-type mice, indicating that mST3GalV is the enzyme responsible for the synthesis of GM4 in mice.

DISCUSSION

In vertebrates, almost all of the ganglio series GSLs are synthesized from GM3, and thus GM3 synthase (ST3GalV/SAT-1) is

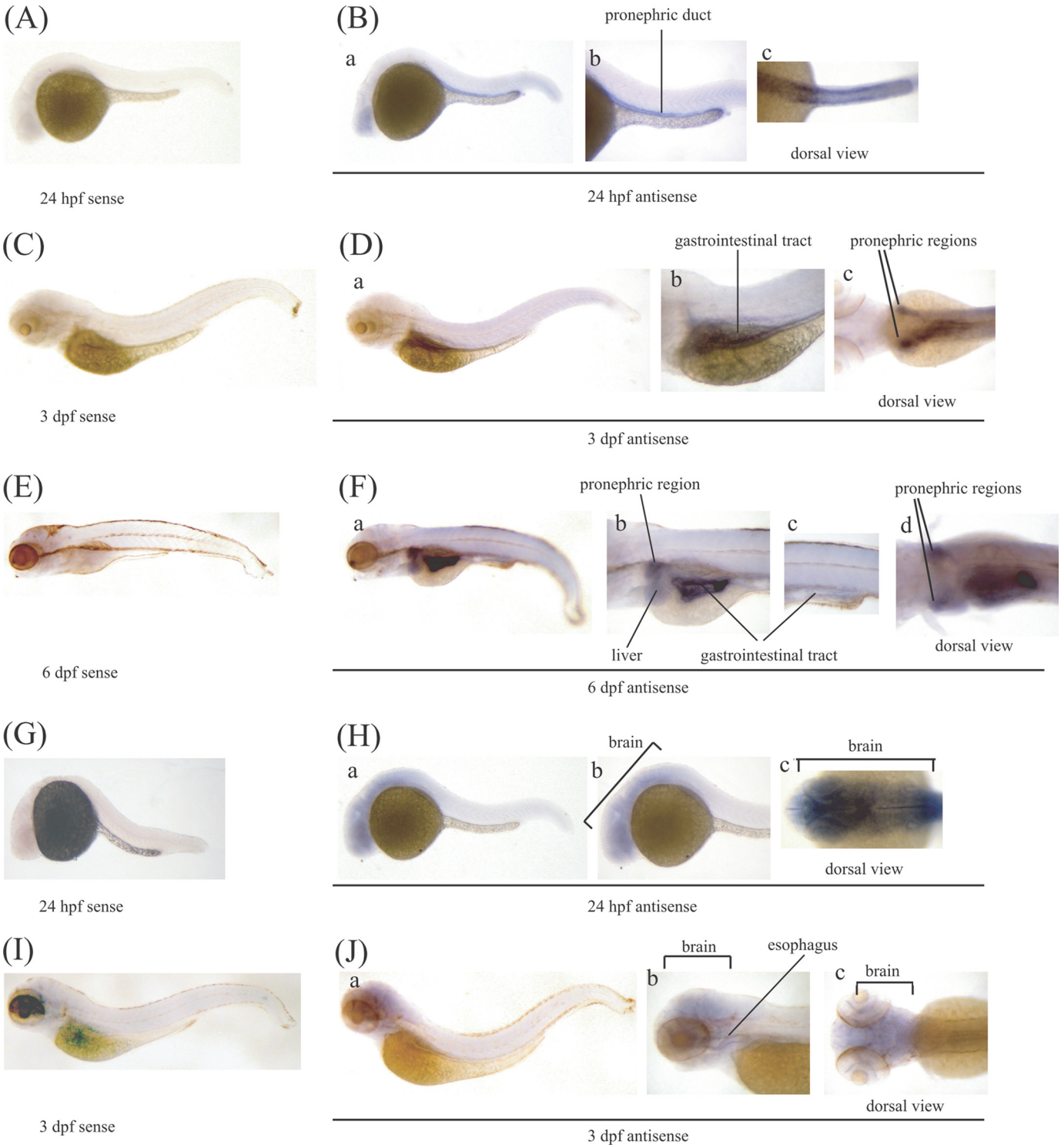

FIGURE 6. **Expression of zST3GalV-1 and zST3GalV-2 transcripts in zebrafish embryos.** *A–F,* whole mount *in situ* hybridization of zST3GalV-2 transcripts; *G–J*, whole mount *in situ* hybridization of zST3GalV-1 transcripts. *A, B, G,* and *H*, embryos at 24-hpf; *C, D, I,* and *J*, embryos at 3-dpf; *E* and *F*, embryos at 6-dpf. *A, C, E, G,* and *I*, whole mount *in situ* hybridization using the sense RNA probes (negative control); *B, D, F, H,* and *J*, that using the antisense RNA probes. *Panel a*, whole body; *panel b*, abdominal region; *panel c*, dorsal region in *B* and *D*; *panel a*, whole body; *panel b*, abdominal region; *panel c*, lateral region; *panel d*, dorsal region in *F*; *panel a*, whole body; *panel b*, head region; *panel c*, dorsal region in *H* and *J*. Details are described under "Experimental Procedures."

considered a key enzyme for ganglioside production. Several lines of evidence indicate GM3 to be involved in insulin signaling (38–40), cell differentiation (41), and neural cell death (42). The first molecular cloning of a human GM3 synthase was achieved by Saito and co-workers (18) and was followed by the cloning of mouse cDNAs encoding ST3GalV (16, 17). It is worth noting that the acceptor specificity of these cloned GM3 synthases seemed to be strict, and LacCer was the only acceptor substrate (16–18). On the other hand, GM4 synthase activity, which utilizes GalCer as an acceptor substrate instead of LacCer, was found in the microsomal

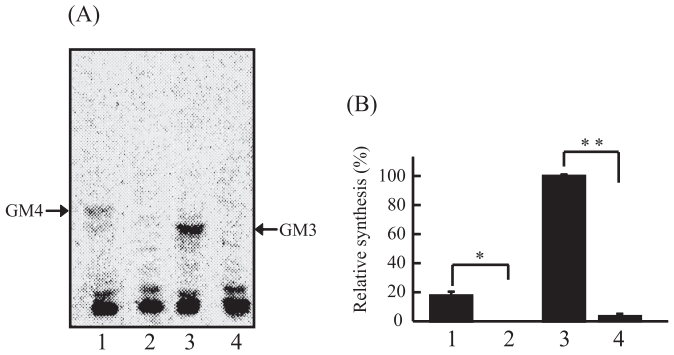

FIGURE 7. **ST activity in the brains of wild-type and mST3GalV knock-out mice.** *A*, autoradiogram of HPTLC showing the generation of [^{14}C]GM4 and [^{14}C]GM3 by the action of brain STs from 12-week-old female wild-type and 12-week-old female mST3GalV knock-out mice. ST activity was measured by assay I using 70 μg of microsomal fraction of the brain. *Lanes 1* and *2*, GM4 synthase activity measured using GalCer and CMP-[^{14}C]NeuAc. *Lanes 3* and *4*, GM3 synthase activity measured using LacCer and CMP-[^{14}C]NeuAc. *Lanes 1* and *3*, wild-type mice; *lanes 2* and *4*, mST3GalV knock-out mice. Details of crude enzyme preparation from mouse brains are given under "Experimental Procedures." *B*, quantification of *A* by a FLA-5000 imaging analyzer. *Lanes 1* and *2*, GM4 synthase activity. *Lanes 3* and *4*, GM3 synthase activity. *Lanes 1* and *3*, wild-type mice; *lanes 2* and *4*, mST3GalV knock-out mice. Autoradiograms were subjected to quantification with a FLA-5000 imaging analyzer. Data are presented as relative activity (%), with the GM3 synthase activity in wild-type mice taken as 100%. Values are averages of three independent determinations with the S.D. * and ** represent $p < 0.01$ and $p < 0.001$, respectively, compared with wild-type mice.

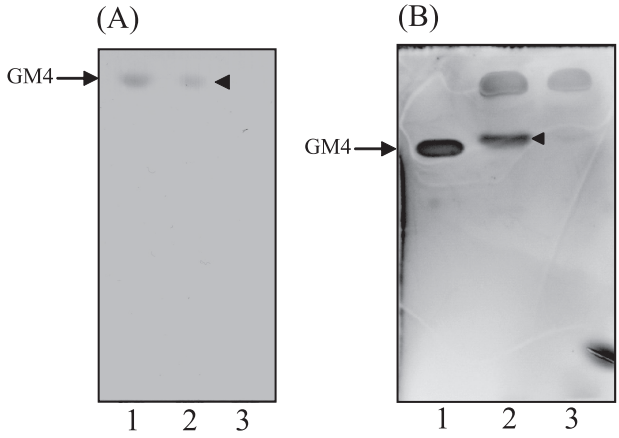

FIGURE 8. **Detection of GM4 of the brain and erythrocytes from wild-type and mST3GalV knock-out mice by TLC immunostaining.** *A*, TLC immunostaining of the brain GSLs from wild-type and mST3GalV knock-out mice using anti-GM4 monoclonal antibody (AMR-10). *Lane 1*, 0.4 nmol of standard fish GM4 (5); *lane 2*, acidic GSL fraction of the wild-type mouse brain; *lane 3*, acidic GSL fraction of the mST3GalV knock-out mouse brain. *B*, TLC immunostaining of the erythrocyte GSLs from wild-type and mST3GalV knock-out mice using anti-GM4 polyclonal antibody. *Lane 1*, standard synthetic GM4 (d18:1-C18:0); *lane 2*, acidic GSL fraction from wild-type mouse erythrocytes; *lane 3*, acidic GSL fraction from mST3GalV knock-out mouse erythrocytes. *Black triangles* show GM4. We confirmed that the specificity of anti-GM4 monoclonal antibody AMR-10 was practically the same as that of polyclonal anti-GM4 antibody (rabbit, Matreya) used in this study. Details for TLC-immunostaining are described under "Experimental Procedures."

fraction of mouse brain (19, 20), but the enzyme responsible for synthesizing GM4 was not identified at the molecular level. Recently, Berselli *et al.* (21) reported a new mRNA variant encoding an N-terminal extended form of human GM3 synthase. Interestingly, they showed that the variant utilized not only LacCer but also GalCer, asialo-GM1 (Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1-1′Cer), and asialo-GM2, although LacCer was found to be the most suitable substrate. Considering these contradictory reports, which enzyme or which isoform of enzyme is responsible for the synthesis of GM4 is still ambiguous.

GM4 is widely distributed in mammals, but its levels are very low compared with GM3. However, GM4 is relatively abundant in some fish, especially in the gastrointestinal tract where it could be utilized by microbes to invade epithelial cells (5). Our attempt to identify the GM4 synthase of zebrafish revealed two putative ST3GalVs (zST3GalV-1 and -2) in the zebrafish gene data base. Biochemical and flow cytometric analyses showed that zST3GalV-1 synthesized only GM3, whereas zST3GalV-2 synthesized GM4 as well as GM3 under the conditions used. Interestingly, the mRNA expression of the two enzymes during zebrafish embryogenesis was completely different, *i.e.* zST3GalV-1 mRNA was strongly expressed in the brain in 24-hpf embryos and in the esophagus in 3-dpf embryos whereas zST3GalV-2 mRNA was strongly expressed in the pronephric duct in 24-hpf embryos and in the gastrointestinal tract in 3- and 6-dpf embryos. We thus concluded that zST3GalV-2 is responsible for the synthesis of zebrafish intestinal GM4.

Next, we searched for mouse orthologue of zST3GalV-2 to identify GM4 synthase in mammals. Unexpectedly, we could not find zST3GalV-2 orthologue except mST3GalV-2. Thus, we carefully re-examined in this study whether mST3GalV could synthesize GM4 using two sets of acceptor and donor substrates as follows: CMP-[^{14}C]NeuAc and nonradioactive GalCer (assay I, see Fig. 3) and [^{14}C]GalCer and nonradioactive CMP-NeuAc (assay II, see Fig. 2). It was clearly shown that mST3GalV is able to synthesize [^{14}C]GM4 in both cases. Furthermore, cell-surface GM4 was found to be significantly increased by transformation of the hamster melanoma cell line RPMI1846 (Fig. 4, *D* and *E*) with a vector containing mST3GalV cDNA but not mock vector. Finally, conclusive evidence was obtained from mST3GalV knock-out mice that were found to lack GM4 as well as GM3 synthase activities in the brain and GM4 in the brain and erythrocytes, in contrast to wild-type mice (43). Thus we concluded that mST3GalV is responsible for the synthesis of mouse GM4 *in vitro* and *in vivo*.

Kapitonov *et al.* (44) reported that three possible initiation Mets were present in the N-terminal region of both hST3GalV and mST3GalV. Recombinant GM3 synthase activity of mST3GalV had been measured using a short form (17) and a protein A-fused N-terminal truncated soluble form of mST3GalV (lacking the first 60 amino acids from the second deduced initiation Met-28 in Fig. 3*A*) (16). Interestingly, a new mRNA variant encodes an N-terminal extended form of hST3GalV that could be expressed in the human placenta and in undifferentiated HL60 cells but not in the monocytic lineage showed broader substrate specificity (21). In this study, we generated three constructs containing cDNA encoding M1-ST3GalV (long form), M2-ST3GalV (middle form), or M3-ST3GalV (short form) of mST3GalV. Met-28 and Met-56, and Met-28 were altered to Ala in the long form and the middle form, respectively, whereas no mutation was conducted in the short form. It was revealed that all isoforms of mST3GalV utilized LacCer as well as GalCer as acceptor substrates, although

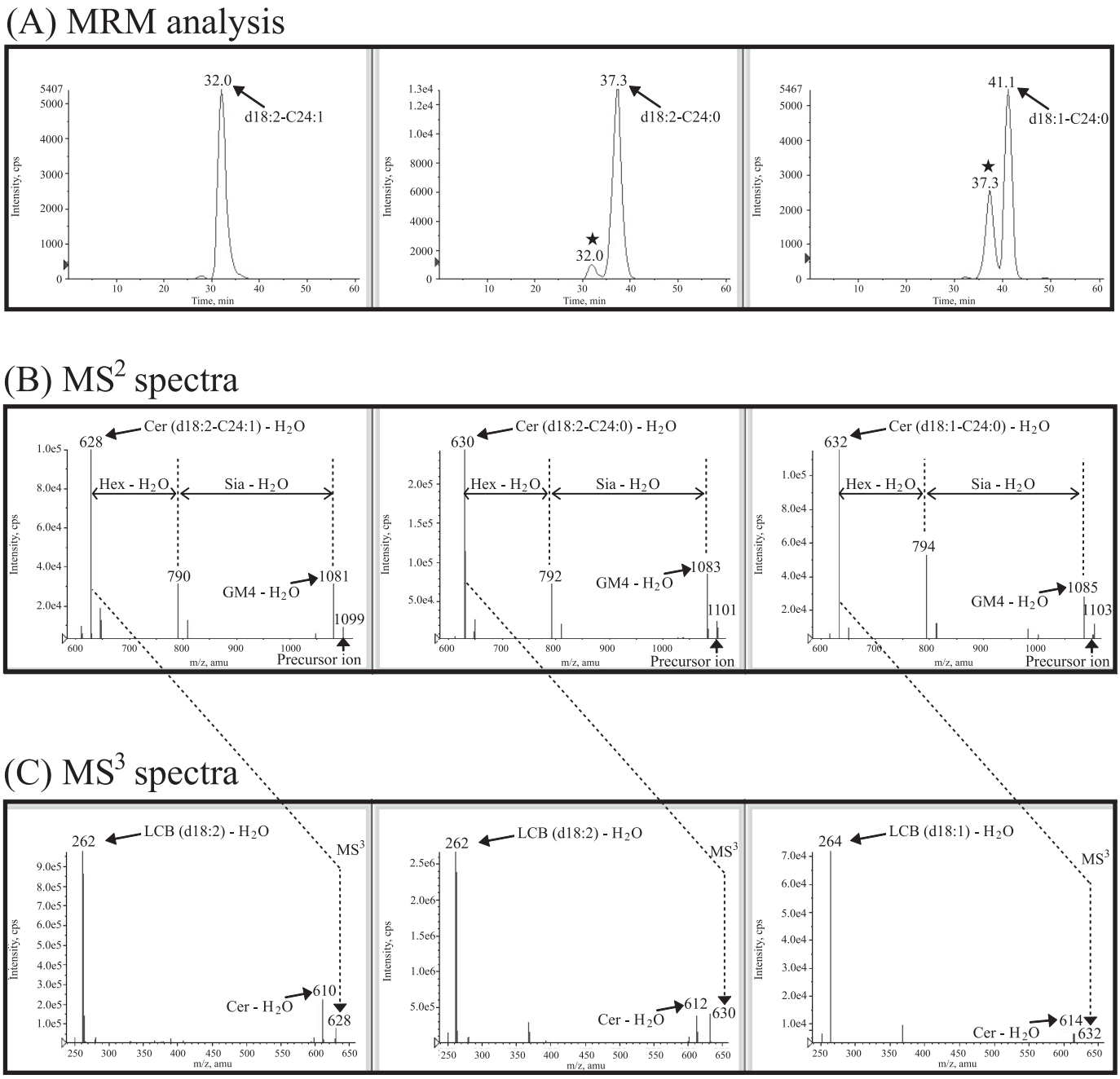

FIGURE 9. **Reverse-phase LC/ESI-MS and MRM of the erythrocyte GM4 present in wild-type mice but not in mST3GalV knock-out mice.** *A*, MRM of mouse erythrocyte GM4 with different ceramide moieties (C24 series). *B*, MS^2 analysis of the GM4 revealing its carbohydrate structure. *C*, MS^3 analysis of the GM4 revealing a long chain base structure. *Sia*, sailic acid; *Hex*, hexose; *LCB*, long chain base (sphingoid base). ⋆ shows isotope peaks of individual GM4 species by MRM. Details for reverse phase LC/ESI-MS and MRM are described under "Experimental Procedures."

LacCer was preferred to GalCer (Fig. 3, *C* and *D*), indicating the length of the N-terminal region had no effect on the substrate specificity of mST3GalV under the conditions used. The N-terminal region of zST3GalV-2 was longer than zST3GalV-1 (supplemental Fig. 2), but this difference was not likely to affect the specificity of these two STs.

mST3GalV utilized a synthesized GalCer whose ceramide consisted of d18:1 and C18:0 (Fig. 2) and a natural fish GalCer whose ceramide mainly consisted of d18:1 and 2-hydroxy-C24:1 (Fig. 3) (5). Thus the specificity of mST3GalV for the ceramide moiety seems to be quite broad.

Why previous reports concluded that cloned mST3GalV did not synthesize GM4 is not clear, but we propose several possibilities. 1) Cultured cells, including various cancer cell lines, highly express sialidases such as NEU3, which cleaves GM3 (46) and possibly GM4, and thus we added 40 μM NeuAc to the reaction mixture to inhibit the sialidase activity in the enzyme preparation if necessary, and the reaction products (GM3 and GM4) eventually increased compared with the experiment without inhibitors. It is not clear whether such sialidase inhibitors were used for the assay of ST3GalVs in previous experiments. 2) For the assay of GM4

synthase in this study, the lower phase of the reaction mixture after Folch's partition was directly applied to a TLC plate to detect the product GM4. It was found that almost all [^{14}C]GM4 was extracted in the lower phase. On the other hand, the reaction products were applied to a TLC plate after purification with a reverse phase column in the previous reports (16–18). GM4, the amount of which would be much smaller than that of GM3, could be lost through the purification procedure. 3) Specific enzymes and monoclonal antibodies were employed to distinguish GM3 and GM4 in the present study. We found that distinguishing GM4 from GM3 was not easy using normal silica-based TLC because GM4 migrates slower than expected possibly due to the high concentration of hydroxy fatty acids in the ceramide moiety, which are derived from GalCer used for the acceptor substrate. We found that EGCase II is very useful for distinguishing GM3 and GM4, because GM3 is hydrolyzed by EGCase II very efficiently, and GM4 is completely resistant to hydrolysis by the enzyme. For example, we found that several reaction products were generated in assay I in which CMP-[^{14}C]NeuAc and GalCer were used as a donor and an acceptor substrate, respectively (Fig. 3*B*, *lanes 5, 8,* and *11*). These products were determined to be [^{14}C]GM4 (*closed arrowheads*) and [^{14}C]GM3 (*open arrowheads*) using EGCase II, *i.e.* the former was completely resistant to hydrolysis by the enzyme but the latter was hydrolyzed. [^{14}C]GM3 was likely to be produced by the ST with endogenous LacCer as a donor substrate (Fig. 3*B*, *lanes 4, 7,* and *10*). Using this method, the synthesis of GM4 by mST3GalV was clearly demonstrated. In addition, it is noteworthy that both anti-GM4 monoclonal and polyclonal antibodies used in this study were specific for GM4 and useful to determine the GM4 of zebrafish gastrointestinal tracts (Fig. 1), mouse melanoma cells transfected with zST3GalV and mST3GalV cDNAs (Fig. 4), and mouse brains and erythrocytes (Fig. 8). 4) Although either [^{14}C]GalCer or CMP-[^{14}C]NeuAc could be used as a radioactive tracer substrate for the assay of GM4 synthase, [^{14}C]GalCer seems more suitable. This is because not only [^{14}C]GM4 but also [^{14}C]GM3 were generated when CMP-[^{14}C]NeuAc, but not [^{14}C]GalCer, was used as a substrate when the lysate of CHO-K1 cells was used as an enzyme source. [^{14}C]GM3 could be generated from CMP-[^{14}C]NeuAc and endogenous LacCer of CHO-K1 cells that contain no GalCer. CMP-[^{14}C]NeuAc has been frequently used as a substrate in previous studies possibly because [^{14}C]GalCer is not commercially available. It is worth noting that [^{14}C]GSLs such as [^{14}C]GalCer, [^{14}C]LacCer, [^{14}C]GM3, and [^{14}C]GM4 were easily prepared by using the condensation reaction of [^{14}C]stearic acid and corresponding lyso-GSLs with SCDase as described under "Experimental Procedures" (28).

In conclusion, GM4 is synthesized in the zebrafish and mouse by zST3GalV-2 and mST3GalV, respectively, both of which are also able to synthesize GM3, indicating that the tissue-specific distribution of GM4 and GM3 depends primary on the supply of their precursors GalCer and LacCer, respectively.

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