The Cytoplasmic Tail of GM3 Synthase Defines Its Subcellular Localization, Stability, and In Vivo Activity

Satoshi Uemura,* Sayaka Yoshida,* Fumi Shishido,* and Jin-ichi Inokuchi**

*Division of Glycopathology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai, Miyagi 981-8558, Japan; and **Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Agency (JST), Saitama, 332-0012, Japan

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GM3 synthase (SAT-I) is the primary glycosyltransferase responsible for the biosynthesis of ganglio-series gangliosides. In this study, we identify three isoforms of mouse SAT-I proteins, named M1-SAT-I, M2-SAT-I, and M3-SAT-I, which possess distinct lengths in their NH₂-terminal cytoplasmic tails. These isoforms are produced by leaky scanning from mRNA variants of mSAT-Ia and mSAT-Ib. M2-SAT-I and M3-SAT-I were found to be localized in the Golgi apparatus, as expected, whereas M1-SAT-I was exclusively found in the endoplasmic reticulum (ER). Specific multiple arginines (R) arranged in an R-based motif, RRXXXXR necessary for ER targeting, were found in the cytoplasmic tail of M1-SAT-I, and in vivo GM3 biosynthesis by M1-SAT-I was very low because of restricted transport to the Golgi apparatus. In addition, M1-SAT-I and M3-SAT-I had a long half-life relative to M2-SAT-I. This is the first report demonstrating the presence of an ER-targeting R-based motif in the cytoplasmic tail of a protein in the mammalian glycosyltransferase family of enzymes. The system, which produces SAT-I isoforms having distinct characteristics, is likely to be of critical importance for the regulation of GM3 biosynthesis under various pathological and physiological conditions.

INTRODUCTION

Ganglioside GM3 is a common precursor for most gangliosides, and the GM3 synthase SAT-I/ST3Gal-V (Ishii *et al.*, 1998; Kono *et al.*, 1998; Fukumoto *et al.*, 1999; Kapitonov *et al.*, 1999) provides an essential function in the biosynthesis of more complex ganglio-series gangliosides. In addition, GM3 itself forms lipid microdomains (rafts) that, with cholesterol and sphingomyelin, function as platforms for effective signal transduction in the plasma membrane (Simons and Toomre, 2000) and participate in the regulation of numerous cellular processes, such as proliferation, differentiation, and adhesion (Hakomori, 2004).

GM3 is also known to be involved in type 2 diabetes. GM3 levels are increased in the adipose tissue of Zucker *fa/fa* rats and *ob/ob* mice, which are typical rodent models of obesity (Tagami *et al.*, 2002), and in SAT-I knockout mice insulin sensitivity is enhanced (Yamashita *et al.*, 2003). In 3T3-L1 adipocytes, exposure to TNF- α increases the expression of GM3 (Tagami *et al.*, 2002), and TNF- α -induced insulin resistance can be prevented by treatment with a glucosylceramide synthase inhibitor, D-PDMP, that decreases GM3 content (Tagami *et al.*, 2002). The accumulation of GM3 in insulin resistance results in the dissociation of the insulin receptor from caveolae (Kabayama *et al.*, 2005) after an electrostatic

interaction between GM3 and a lysine residue (Lys-944) located just above the transmembrane of the insulin receptor (Kabayama *et al.*, 2007). Overall, treatment with glucosylceramide synthase inhibitors significantly improves insulin sensitivity and glucose homeostasis in rodent models of obesity (Aerts *et al.*, 2007; Zhao *et al.*, 2007). Consequently, it is important to understand the regulation of GM3 biosynthesis as it relates to diabetes, as well as other pathological states.

The primary enzyme responsible for GM3 synthesis, SAT-I, transfers a sialic acid to lactosylceramide (LacCer) in the Golgi apparatus. The regulation of SAT-I expression at the transcriptional level has been partially clarified. All promoters of glycosyltransferase genes utilized in ganglioside synthesis are TATA-less and have no CCAAT box, but they do contain GC-rich sequences in their SP1- and AP2-binding sites. Indeed, it has been reported that mouse SAT-I genes are regulated by SP1 and AP2 (Xia *et al.*, 2005), although the transcription factor regulation of human SAT-I, including the basal transcription machinery, is not understood (Kim *et al.*, 2002; Zeng *et al.*, 2003). However, CREB, the cAMP response element-binding protein, is known to be crucial for the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced expression of human SAT-I mRNA in HL-60 and K562 cells (Choi *et al.*, 2003, 2004).

Mouse SAT-I transcripts are classified into two types, mSAT-Ia and -Ib variants, according to the position of transcription initiation, exon 1 or 2 (Figure 1A; Kono *et al.*, 1998; Kapitonov *et al.*, 1999). In contrast, human SAT-I transcripts can be classified into three types, hSAT-Ia, -Ib, and -Ic variants, according to the position of transcription initiation, exon 1, 2, or 4 (see Figure 1B; Ishii *et al.*, 1998; Kapitonov *et al.*, 1999; Kim *et al.*, 2001; Berselli *et al.*, 2006). In addition, the structures of hSAT-Ia and -Ib mRNA are each further classified into two types: hSAT-Ia-1 and -Ia-2 variants and

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Address correspondence to: Jin-ichi Inokuchi (jin@tohoku-pharm.ac.jp).

Abbreviations used: aa, amino acid residues; CHO, Chinese hamster ovary; Cys, cysteine; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; Endo H, endoglycosidase H; ks, kozak sequence; LacCer, lactosylceramide; Met, methionine; PNGase F, peptidase N-glycosidase F; 5'-UTR, 5'-untranslated region.

Figure 1. Transcriptional variants of mouse and human SAT-I. The structures of mouse SAT-I (mSAT-I; A) and human SAT-I (hSAT-I; B) genes and their mRNA variants are illustrated. Black boxes and horizontal lines indicate exons and introns, respectively. M1, M2, and M3 denote initiation codons, and TGA denotes a termination codon.

hSAT-Ib-1 and -Ib-2 variants, respectively, reflecting alternative splicing of exon 3 (Figure 1B; Kim *et al.*, 2001). According to the first-AUG rule, translation is initiated at the AUG codon nearest the 5' end of an mRNA (Kozak, 2002). Thus, in the mSAT-Ib and hSAT-Ic variants, it has been assumed that the first AUG codons, M2 and M2', respectively, are the initiation codons (Figure 1; Kono *et al.*, 1998; Berselli *et al.*, 2006). However, in the mSAT-Ia, hSAT-Ia-1 and -Ia-2, and hSAT-Ib-1 and -Ib-2 variants, a second or third AUG, M3, has been considered as the initiation codon, based on speculation that the context presenting M1 and M2 is insufficient for either to be recognized by the ribosome as an initiation codon (Ishii *et al.*, 1998; Kapitonov *et al.*, 1999; Kim *et al.*, 2001). Although the exact translational sites remain undetermined, the translational product from M3 (M3-SAT-I) has been used in many studies (Giraud *et al.*, 2001; Giraud and Maccioni, 2003a,b; Uemura *et al.*, 2006; Uliana *et al.*, 2006; D'Angelo *et al.*, 2007). Because the catalytic domain of the SAT-I protein exits on the lumen side of Golgi apparatus and M3-SAT-I can produce GM3 *in vivo* (Ishii *et al.*, 1998), little attention has been given to the significance of the position of the initiation codon; however, the position of the initiation codon would determine the length of the cytoplasmic region of a SAT-I protein. For example, in the mouse SAT-I protein, the lengths of the cytoplasmic tails translated from M1, M2, and M3 are 69, 42, and 14 amino acids (aa), respectively (Figure 2). Notably, the length of M1-SAT-I is the greatest among the members of the sialyltransferase family (Figure 2).

In the present study, we have demonstrated that the three isoforms: M1-SAT-I, M2-SAT-I, and M3-SAT-I and the two isoforms M2-SAT-I and M3-SAT-I are translated from mSAT-Ia and -Ib, respectively, by leaky scanning, which suggests that various isoforms coexist in cells. To clarify the role of the cytoplasmic tail of the SAT-I protein in intracel-

Figure 2. Comparison of the lengths of the NH₂-terminal cytoplasmic region of mouse sialyltransferases. The sequences are from mouse ST3Gal-I (Lee *et al.*, 1993), ST3Gal-II (Lee *et al.*, 1994), ST3Gal-III (Kono *et al.*, 1997), ST3Gal-IV (Kono *et al.*, 1997), ST3Gal-VI (Okajima *et al.*, 1999), ST6Gal-I (Hamamoto *et al.*, 1993), ST6Gal-II (Takashima *et al.*, 2003), ST6GalNAc-I (Kurosawa *et al.*, 2000), ST6GalNAc-II (Kurosawa *et al.*, 1996), ST6GalNAc-III (Lee *et al.*, 1999), ST6GalNAc-IV (Lee *et al.*, 1999), ST6GalNAc-V (Ikehara *et al.*, 1999), ST6GalNAc-VI (Okajima *et al.*, 2000), ST8Sia-I (Yamamoto *et al.*, 1996), ST8Sia-II (Kojima *et al.*, 1995), ST8Sia-III (Yoshida *et al.*, 1995a), ST8Sia-IV (Yoshida *et al.*, 1995b), ST8Sia-V (Kono *et al.*, 1996), and ST8Sia-VI (Takashima *et al.*, 2002). The number of amino acid residues are shown in white. TM indicates a transmembrane region.

lular dynamics, we examined the subcellular localization, stabilities, and activities of SAT-I isoforms. M1-SAT-I was stably localized in the endoplasmic reticulum (ER), due to retrograde transport signals (arginine [R]-based motifs) and, consequently, its *in vivo* GM3 synthesis activity was very low compared with that of other isoforms. In contrast, both M2-SAT-I and M3-SAT-I were localized in the Golgi apparatus, but their stabilities differed. M2-SAT-I was rapidly degraded in the lysosomes, whereas M3-SAT-I was retained in the Golgi apparatus. Thus, we propose a new regulatory mechanism for the intracellular dynamics of SAT-I proteins.

MATERIALS AND METHODS

Plasmids

The pSY08 encoding mSAT-Ia was constructed using cDNA prepared from B16 melanoma cells and primers 5'-GGCGGCTTGCCAGCGCTCCCTC-3' and 5'-GGTTTCCGCTGTTCCGAGTTC-3'. The resulting fragments were cloned into a pGEM-T Easy vector (Promega, Madison, WI) to generate pSY03. The 1.3-kb NotI fragment of pSY03 was then cloned into the NotI site of a pcDNA3.1 Zeo(+) vector (Invitrogen, Carlsbad, CA) to generate pSY08. To insert the kozak sequence (ks) before ATG (M1) (ks-M1-SAT-I), M1-SAT-I was amplified using pSY08 and primers 5'-GGATCCGCCACCATGCACACAGAGCGGTGGG-3' and 5'-GGTTTCCGCTGTTCCGAGTTC-3'. The resulting fragments were cloned into a pGEM-T Easy vector to generate pSY12. The 1.3-kb NotI fragment of pSY12 was then cloned into the NotI site of pcDNA3.1 Zeo(+) vector to generate pSY14. The plasmids encoding mSAT-Ib, hSAT-Ia-1, hSAT-Ia-2, ks-M2-SAT-I, and ks-M3-SAT-I were created by similar procedures.

The pSU180 [M1-SAT-I(N)-enhanced green fluorescent protein (EGFP)] plasmid encoding fusion proteins of the NH₂-terminus of M1-SAT-I and EGFP was constructed using pSY14 and primers 5'-CCCCTATTGACGT-

CAATGACGG-3' and 5'-CCCGGGCAGGGTCCACATAGTGCATTC-3'. The resulting fragments were cloned into a pGEM-T Easy vector to generate pSU147. The 0.33-kb EcoRI-SmaI fragments of pSU147 were then cloned into the EcoRI-SmaI site of the pEGFP-N1 vector (BD Bioscience Clontech Laboratories, Palo Alto, CA) to generate pSU180. The pSU181 [M2-SAT-I(N)-EGFP] and pSU182 [M3-SAT-I(N)-EGFP] plasmids were created by similar procedures.

Point mutants of mSAT-Ia, mSAT-Ib, ks-M1-SAT-I, ks-M2-SAT-I, and M1-SAT-I(N)-EGFP were created by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

The primers used to amplify the deletion mutants of M1-SAT-I(N)-EGFP ($\Delta 28-55$) were 5'-TGCTCGGCAGGCAGGTGCCGCTGC-3' and 5'-ATGAGAA-GACCCAGCTGTGTAATAAAGACATC-3'. The antisense primers were treated with T4 polynucleotide kinase (Takara, Shiga, Japan). PCR was carried out with KOD plus (Toyobo, Osaka, Japan) in the presence of 0.2 mM dNTPs, 1 mM MgSO₄, and 0.3 μ M primers for 30 cycles. PCR products were ligated using a DNA Ligation Kit Ver.2.1 (Takara). Other deletion mutants were created by similar procedures.

To produce a retrovirus, the pSU213 (ks-M1-SAT-I/pBABE-puro) plasmid was constructed using pSY14. The 1.2-kDa BamHI-EcoRI fragment of pSY14 was cloned into the BamHI-EcoRI site of a pBABE-puro vector (Addgene, Cambridge, MA) to generate pSU213. The pSU214 (ks-M2-SAT-I/pBABE-puro), pSU215 (ks-M3-SAT-I/pBABE-puro), pSU216 (ks-M1-SAT-I M28/56A mutant/pBABE-puro), and pSU217 (ks-M2-SAT-I M29A mutant/pBABE-puro) plasmids were created by similar procedures.

Real-Time PCR

First-strand cDNA synthesis was performed using a First-Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Diagnostics, Penzberg, Germany) and total RNA isolated from mouse tissues or a First Choice Human Total RNA Survey Panel (Applied Bioscience, Foster City, CA). Primers and probes (Taq-man probe) specific for mSAT-Ia (primers, 5'-TGCGAAGCCAAG-CAGCG-3' and 5'-AGCAATCACTTCTCAGCTTGCA-3'; probe, 5'-TGC-CGAGCAATGCCAA-3'), mSAT-Ib (Mm00488232-m1), mSAT-I all (primers, 5'-GGCATCTCTGACGGACTAGA-3' and 5'-CGCACCTCTGGGTAAGTCA-3'; probe CGATGTGGTAAATAAGGTGTGAACAGTGGCG-3'), hSAT-Ia-1 (primers, 5'-GGCCGAGGCACTGTGAAG-3' and 5'-TGCAGATTCCATGCAAGCA-3'; probe, 5'-AGTGGTGTGCAAAAGC-3'), hSAT-Ia-2 (primers, 5'-CCGGCCGAG-CAATGC-3' and 5'-GGCCTCGAGCAATCACTTCT-3'; probe, 5'-TGAGTA-CACCTATGTGAAAC-3'), hSAT-Ib-1 (primers, 5'-CATTCTCTGCTAGGC-GAGAAA-3' and 5'-TGCAGATTCCATGCAAGCA-3'; probe, 5'-CAGG-CACCTGTGAAGAC-3'), hSAT-Ib-2 (primers, 5'-CATTCTCTGCTAGGC-GAGAAA-3' and 5'-AGGCCTCGAGCAATCACTTC; probe, 5'-ACAGCAAT-GCCAACTGAG-3'), rodent GAPDH (4308313), and 18S rRNA (Hs99999901-s1) were purchased from Applied Biosystems (Foster City, CA). A 2 $\times$ PCR universal master mix (Applied Biosystems), containing a PCR buffer, MgCl₂, dNTPs, and thermally stable AmpliTaq Gold DNA polymerase, was used in PCR reactions. In addition, the PCR reaction mixture contained forward and reverse primers at 0.9 μ M, a 0.25 μ M Taq-man probe, and 0.05 μ g first-strand cDNA. RNase- and DNase-free water was added to a final volume of 10 μ L. The PCR reaction mixture was incubated at 50°C for 2 min and at 95°C for 10 min and then run for 40 cycles at 95°C for 15 s and 60°C for 60 s on an Applied Biosystems 7500 Real-Time PCR System.

Cell Culture

Chinese hamster ovary (CHO) cells and the amphotopic retroviral packaging cell line Phoenix_{eco} (American Type Culture Collection, Manassas, VA) were cultured in the nutrient mixture F-12 HAM (N6558, Sigma, St. Louis, MO) and high-glucose DMEM (Invitrogen), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, respectively. CHO cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected in the same medium used for culture in the presence of zeocin (Invitrogen).

Primary Cell Isolation and Immobilization

Two breeding pairs of SAT-I knockout mice were generous gifts from M. Saito (Pharmacodynamics, Meiji Pharmaceutical University, Tokyo, Japan; Tsukamoto *et al.*, 2005). Mouse embryonic fibroblasts (MEFs) were derived and cultured from GM3 synthase knockout (−/−) embryos obtained by mating GM3 synthase (+/−) $\times$ GM3 synthase (+/−). Embryos were separated from the maternal tissues and yolk sack and then minced and incubated in 0.1% trypsin/0.02% EDTA (Sigma) at room temperature for 35 min. The supernatant was centrifuged at 300 $\times$ g for 5 min at 4°C, and the precipitations were resuspended in DMEM (D6046, Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin and seeded onto plastic plates for culture. To establish an immortalized MEF cell line, MEFs were transfected using Lipofectamine 2000 with SV40 Large T antigen/pcDNA3.1Zeo(+) (a gift from Dr. J. Gu, Division of Regulatory Glycobiology, Tohoku Pharmaceutical University, Sendai, Japan). SV40 Large T antigen-transfected cells were selected in the same medium used for culture in the presence of zeocin.

Preparation of Cell Lysates

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), suspended in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 4 M urea, 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ Complete protease inhibitor mixture [EDTA-free]; Roche Diagnostics), and lysed by sonication. After removal of cell debris by centrifugation at 1000 $\times$ g for 3 min at 4°C, the supernatant (total cell lysates) was centrifuged at 100,000 $\times$ g for 1 h at 4°C. The precipitates (integral membrane proteins) were suspended in a 2 $\times$ SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and a trace amount of bromophenol blue) containing 5% 2-mercaptoethanol and incubated for 5 min at 37°C. Endoglycosidase H (Endo H) or Peptide: N-glycosidase F (PNGase F; New England Biolabs, Beverly, MA) digestion was performed on integral membrane proteins at 37°C for 1 h according to the manufacturer's recommended procedure.

Immunoblotting

Immunoblots were performed on integral membrane proteins prepared as described above. Proteins were separated by SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was then incubated for 1 h with a 1:1000 dilution of anti-SAT-I antibodies that had been raised against GST or polyhistidine fusion proteins encompassing the COOH-terminal 55 aa (C-term; Uemura *et al.*, 2006) or 267 aa (9129). After washing, the membrane was incubated for 1 h with a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab')₂ fragment (GE Healthcare Bio-Sciences, Piscataway, NJ). Labeling was detected using an ECL plus kit (GE Healthcare Bio-Sciences).

Detection of Endogenous Mouse SAT-I Isoforms

Mice (SAT-I^{+/+} and SAT-I^{−/−}) were kept under deep anesthesia and briefly perfused with physiological saline. Brain tissues were isolated, minced, and homogenized in 0.25 M sucrose containing 1 $\times$ Complete using Teflon homogenizer (10 strokes). After removal of nuclear and cell debris by centrifugation at 1000 $\times$ g for 10 min at 4°C, the supernatant was centrifuged at 8000 $\times$ g for 10 min at 4°C to remove mitochondria. Finally, the supernatant was centrifuged at 100,000 $\times$ g for 1 h at 4°C. The precipitates (microsome) were suspended with immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 $\times$ Complete) and incubated with protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. After centrifugation, the supernatant was incubated with anti-SAT-I antibodies (9129) and protein A/G plus-agarose for 1 h at 4°C. The agarose beads were then washed four times with washing buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.05% Tween20), and bound proteins were eluted with 2 $\times$ SDS sample buffer containing 5% 2-mercaptoethanol and incubated for 5 min at 37°C. Immunoprecipitates were treated with PNGase F and analyzed by immunoblotting using anti-SAT-I antibodies (C-term) and Rabbit IgG TrueBlot (eBioscience, San Diego, CA).

Immunofluorescence Microscopy

Transfected 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, if necessary, by 0.5% SDS or 0.5% Triton X-100 in PBS, treated with Image-iT FX Signal Enhancer (Invitrogen) for 30 min, and then incubated for 1 h with anti-SAT-I (C-term), anti-KDEL (StressGen Bioreagents, Victoria, BC, Canada), anti-GM130 (BD Bioscience, Franklin Lakes, NJ), or anti-hemagglutinin (HA) antibodies (InvivoGen, San Diego, CA; diluted 1:100 with blocking solution). After washing three times in PBS, the cells were incubated for 30 min with Alexa 488-conjugated anti-rabbit IgG antibodies (Invitrogen) or Alexa 594-conjugated anti-mouse IgG antibodies (Invitrogen) diluted in blocking solution to 5 μ g/ml. Coverslips were washed in PBS three times, mounted on glass slides using ProLong Gold antifade reagent (Invitrogen), and analyzed by fluorescence microscopy FV1000 (Olympus, Tokyo, Japan).

Pulse Chase and IP

CHO cells stably expressing ks-M1-SAT-I, ks-M2-SAT-I, ks-M3-SAT-I, and ks-M1-SAT-I R11/125 were cultured in DMEM Nutrient Mixture F-12 HAM (D8437, Sigma) with or without 200 μ M chloroquine. The culture medium was then changed to DMEM without methionine (Met)/cysteine (Cys) (21013-024, Invitrogen) with or without 200 μ M chloroquine and incubated at 37°C for 1 h. Cells were pulse-labeled with [³⁵S]Met/[³⁵S]Cys (27.5 mCi/ml EXPRESS protein labeling mix; Perkin Elmer Life Science, Waltham, MA) for 30 min and chased with unlabeled Met (final concentration, 0.5 mg/ml) and Cys (final concentration, 0.1 mg/ml) in DMEM containing 10% fetal bovine serum with or without 200 μ M chloroquine. At predetermined times, cells were suspended with RIPA buffer (50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ Complete) and kept on ice. Cells were disrupted by bath sonication, and debris was removed by centrifugation at 20,000 $\times$ g for 3 min at 4°C. Cell lysates with equal radioactivity were incubated with anti-SAT-I antibodies (C-term) and protein A/G plus-agarose

overnight at 4°C. After two washes with RIPA buffer and one with 10 mM Tris-HCl, pH 7.5, agarose beads were suspended in a 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and a trace amount of bromophenol blue) containing 5% 2-mercaptoethanol and incubated for 5 min at 37°C. Precipitates were treated with PNGase F and separated by SDS-PAGE. Radioactivities associated with SAT-I were quantified using a Bio-Imaging Analyzer BAS5000 (Fuji Photo Film, Tokyo, Japan).

Phoenix Retroviral Expression System

At 24 h before transfection, Phoenix_{eco} cells were seeded at 0.5×10^6 cells per 60-mm dish. Phoenix_{eco} cells were transfected with ks-M1-SAT-I, ks-M2-SAT-I, ks-M3-SAT-I, ks-M1-SAT-I M28/56A, and ksM2-SAT-I M29A/pBABE-puro plasmids using Lipofectamine 2000. Approximately 32 h after transfection, the culture temperature was shifted from 37 to 32°C to maintain virus stability. The retrovirus-containing supernatant was harvested 48 and 72 h after transfection and centrifuged at $200 \times g$ for 3 min to remove living cells. The retroviral supernatant was either used immediately for experiments or snap-frozen in liquid nitrogen and stored at −80°C for later applications. For the retroviral transduction of immortalized SAT-I knockout cells, 4 ml of the retroviral supernatant containing 10 µg/ml polybrene (Sigma) 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 to 37°C, and the cells were incubated for 48 h.

Assay of Enzymatic Activity In Vitro

SAT-I activity was performed as described previously (Uemura et al., 2006).

Lipid Analysis

Cells were washed twice with PBS, and total lipids were extracted from the cells with chloroform/methanol (1/1 and 1/2 vol/vol, successively). Samples were equalized for protein concentration, which was determined using a BCA kit (Pierce Chemical, Rockford, IL). The total lipid extract was dissolved in 6 ml of diisopropyl ether/butanol (3/2) and added to 3 ml of 50 mM NaCl. The mixture was vortexed for 1 min and centrifuged at $700 \times g$ for 5 min. The upper layer was removed, and the ganglioside-containing lower layer was washed with 6 ml diisopropyl ether/butanol (3/2) again. The lower layer was diluted with 5 ml of 50 mM NaCl solution and then applied to a Sep-Pak C18 reverse-phase cartridge (Waters Associates, Milford, MA). The cartridge was washed with 40 ml water, and lipids were eluted with 10 ml methanol and chloroform/methanol (1/1, vol/vol), successively. Gangliosides were separated by thin-layer chromatography (TLC) using chloroform/methanol/0.2% CaCl₂ (55/45/10, vol/vol/vol) and detected with an orcinol-sulfuric acid reagent. Standard compounds were purchased from Matreya (Pleasant Gap, PA).

RESULTS

Tissue Distribution of mSAT-I and hSAT-I Transcriptional Variants

The structures of gene and mRNA variants for mouse and human SAT-I are shown in Figure 1. To examine the tissue distributions of mouse and human SAT-I transcripts, we performed real-time PCR analyses. In mouse tissues, the expression pattern of the mSAT-Ia transcript was quite similar to that of the mSAT-Ib transcript, and both transcripts were highly expressed in brain, heart, and testis (Figure 3A). SAT-I mRNA levels have been found by others to be high in the liver and spleen of mice (Kono et al., 1998), so transcriptional variants other than mSAT-Ia and -Ib may be present. In human tissues, expression levels of hSAT-Ia-1 and -Ia-2 were high in brain, thymus, lung, spleen, testis, skeletal muscle, thyroid, ovary, and cervix (Figure 3B). In contrast, the expression levels of the hSAT-Ib-1 and -Ib-2 variants were very low in all tissues. We could not examine the tissue distribution of the hSAT-Ic variant because of difficulties in the design of specific primers and probes for real-time PCR measurements. The observed tissue distribution of the human SAT-I transcripts was consistent with a previous report of a Northern blot analysis of human SAT-I (Ishii et al., 1998). Moreover, our analyses revealed that in all tissues the hSAT-Ia-2 variant exhibited the highest relative levels. These results suggest that the transcription of human SAT-I is initiated mainly from exon 1 and not from exon 2, and that exon 3 is efficiently spliced. Thus, coexpression of the SAT-I vari-

Figure 3. Tissue distributions of mSAT-I and hSAT-I transcripts. Quantitative analyses of mSAT-I (A) and hSAT-I (B) transcripts in various mouse and human tissues, as determined by real-time PCR, are presented. Expression levels of mSAT-I and hSAT-I mRNA were normalized to 18S rRNA transcript.

ants that utilize M1 and M2 as their first AUGs was found in both mouse and human tissues (Figures 1 and 3).

Translation of SAT-I Isoforms by Leaky Scanning

To investigate the position of initiation codons in the mSAT-I variants, CHO cells were transiently transfected with plasmids encoding mSAT-Ia or -Ib, which contains the 5'-untranslated region (UTR) of each variant (Figure 4A). Integral membrane fractions were prepared from the mSAT-Ia- or mSAT-Ib-transfected cells and subjected to immunoblotting with anti-SAT-I antibodies. Each SAT-I protein was detected as a broad band of 44.5–48 kDa (Figure 4B). Because mouse SAT-I carries three N-glycans (Uemura et al., 2006), samples of integral membrane fractions were also treated with PNGase F before immunoblotting. Interestingly, the broad bands in mSAT-Ia-transfected cells shifted to 46, 40, and 36 kDa, whereas the broad band from mSAT-Ib-transfected cells resolved into bands at 40 and 36 kDa (Figure 4B). These results suggest that the band of 46 kDa in mSAT-Ia-transfected cells is a SAT-I protein translated from M1 (M1-SAT-I), and the 40- and 36-kDa bands in both mSAT-Ia- and mSAT-Ib-transfected cells are SAT-I proteins translated from M2 and M3 (M2-SAT-I and M3-SAT-I), respectively. Accordingly, site-directed mutagenesis of Met (M2 and M3 in mSAT-Ia and -Ib, respectively) to Ala mutants (mSAT-Ia M28/56A and mSAT-Ib M29A) was performed. Integral membrane fractions were prepared from these mutant-transfected cells and subjected to immunoblotting with anti-

Figure 4. Translation of SAT-I isoforms from mRNA variants by leaky scanning. (A and D) The structure of cDNA constructs used in these experiments. Horizontal lines indicate a 5'-UTR of (A) mSAT-Ia or -Ib and (D) hSAT-Ia-1 or -Ia-2. M1, M2, and M3 denote initiation codons, and TGA denotes a termination codon. TM and CS indicate a transmembrane region and common sequence, respectively. (B, C, and E) CHO cells were transiently transfected with a pcDNA3 Zeo(+) vector (vector) or a plasmid encoding mSAT-Ia or -Ib, the mSAT-Ia M28/56A mutant, the mSAT-Ib M29A mutant, or hSAT-Ia-1 or -Ia-2, as indicated. Integral membrane fractions were prepared, treated with (+) or without (-) PNGase F, and then analyzed by immunoblotting with anti-SAT-I antibodies (B and C, C-term; E, 9129). An asterisk indicates a nonspecific band. (F) Microsome fractions prepared from brain tissue of SAT-I^{+/+} and SAT-I^{-/-} mice were subjected to immunoprecipitation with anti-SAT-I antibodies (9129). Immunoprecipitates were treated with PNGase F and then analyzed by immunoblotting with anti-SAT-I antibodies (C-term) and Rabbit IgG TrueBlot.

SAT-I antibodies. As shown in Figure 4C, the bands of 40 and 36 kDa in the mSAT-Ia M28/58A-transfected cells,

and the band of 36 kDa in the mSAT-Ib M29A-transfected cells disappeared, indicating that mSAT-Ia and mSAT-Ib produce three and two independently initiated proteins, respectively. This supports the finding from the tissue distribution analysis above (Figure 3A) that M1-SAT-I, M2-SAT-I, and M3-SAT-I isoforms may coexist in mouse cells and tissues.

To examine whether several SAT-I isoforms are also translated from each human mRNA variant, CHO cells were transfected with hSAT-Ia-1 or -Ia-2 (including the 5'-UTR), which are the major variants in human tissues (Figure 3B). Membrane fractions were again prepared and subjected to immunoblotting with anti-SAT-I antibodies. Two bands, a major band from M1-SAT-I and a weak band from M3-SAT-I, were detected in the hSAT-Ia-2-transfected cells, whereas only a single band, from M3-SAT-I, was detected in the hSAT-Ia-1-transfected cells (Figure 4E). This suggests that not only M1, but also M3 functions as an initiation codon for hSAT-Ia-2, but only M3 is weakly recognized as an initiation codon for hSAT-Ia-1.

Next, to confirm the expression pattern of endogenous SAT-I isoforms, we purified SAT-I proteins from brain tissue of SAT-I^{+/+} and SAT-I^{-/-} mice by IP using anti-SAT-I antibodies. Because the expression levels of mSAT-Ia and mSAT-Ib were high in brain tissue (Figure 3A), we expected that three SAT-I isoforms would be detected. Three bands equivalent to the three isoforms were detected only in the SAT-I^{+/+} mice (Figure 4F). The expression level of M3-SAT-I was slightly higher than the other isoforms. These results firmly support the contention that the three SAT-I isoforms endogenously coexist in tissues after leaky scanning from mRNA variants of mSAT-Ia and -Ib.

In general, the first AUG codon is recognized as an initiation codon by the ribosome, a phenomenon known as the first-AUG rule (Kozak, 2002). However, the first AUG codon is sometimes skipped, and a downstream AUG codon is then recognized as an initiation codon; this is known as a leaky scanning system (Kozak, 2002). In mammals, the optimal context for recognition of the AUG start codon is GCCRCCAUGG. Within this motif, the purine nucleotide (R = A or G) in position -3 is the most highly conserved and the most functionally important. The G in position +4 is also highly conserved and, especially in the absence of A in position -3, makes a strong contribution to recognition. When the first AUG residue is presented in a weak context, especially lacking both R in position -3 and G in position +4, some ribosomes will initiate at that point, but most continue scanning and then initiate transcription further downstream. Such leaky scanning enables the production of two distinct proteins from one mRNA. Positions -3 and +4 in mSAT-Ia are occupied by A and C, respectively (Figure 5A, mSAT-Ia WT). To identify the optimal context for recognition of the M1 site in mSAT-Ia, CHO cells were transfected with mutants that replaced the A in position -3 with G, C, or U (Figure 5A, mSAT-Ia m1, m2, and m3, respectively), and immunoblots were performed. In cells carrying mSAT-Ia m2 or m3, the expression of M1-SAT-I decreased and the expression of M2-SAT-I increased, whereas the band pattern for the mSAT-Ia m1 cells was similar to that for mSAT-Ia wild-type (WT) cells (Figure 5B). These results suggest that for initiation, A or G is the optimum residue for position -3 in mSAT-Ia (Kozak, 2002). Next, CHO cells were transfected with mutants that replaced the C in position +4 with G, A, or U (Figure 5A, mSAT-Ia m4, m5, and m6, respectively) and examined the cells by immunoblotting. Only in cells transfected with mSAT-Ia m4 did the expression levels of M2-SAT-I and M3-SAT-I decrease slightly,

Figure 5. Leaky scanning of mSAT-Ia and mSAT-Ib depends on the upstream sequence of AUG codon. (A) Nucleotide sequences of mutants of mSAT-Ia and -Ib used in the presented experiments. Underlining indicates a mutated nucleotide. (B and C) CHO cells were transiently transfected with wild type (WT) or mutants (m1–m8) of each variant. Integral membrane fractions were prepared, treated with PNGase F, and then analyzed by immunoblotting with anti-SAT-I antibodies (C-term).

indicating that G at position +4 is optimum for efficient initiation (Figure 5B, top panel). Nevertheless, leaky scanning of the mSAT-Ia m4 mutant, which would be expected to represent the optimal condition, was not completely eliminated. We next created a mutant (Figure 5A, mSAT-Ia m7) in which the nucleotide sequence upstream of M1 was replaced with GCCACC (ks), and expressed it in CHO cells. In immunoblots of cells carrying mSAT-Ia m7, the M2-SAT-I band disappeared, and the amount of M3-SAT-I decreased slightly (Figure 5B, bottom panel). In addition, the band pattern of the mSAT-Ia m8 mutant, in which the C in position +4 of mSAT-Ia m7 was replaced with G, was similar to that of the mSAT-Ia m7 mutant. These results suggest that the nucleotide at position +4 is not restricted if the nucleotide sequence before M1 is GCCACC. On the other hand, the expression level of M3-SAT-I also decreased remarkably in cells carrying the mSAT-Ib m1 in which the nucleotide sequence from position –6 to –1 at the M2 site was switched for GCCACC (Figure 5C). These results suggest that the entire sequence from position –6 to –1 is important for recognition of the initiation codon in mSAT-I variants. Subsequently, to investigate posttranslational regulation of each SAT-I isoform, we used constructs in which GCCACC was inserted before M1, M2, or M3 to minimize leaky scanning.

Distinct Differences in Subcellular Localization of M1-SAT-I, M2-SAT-I, and M3-SAT-I

As shown in Figure 2, the cytoplasmic region of M1-SAT-I is much longer than that found in other sialyltransferases. To examine the significance of the long cytoplasmic tail, CHO cells were stably transfected with ks-M1-SAT-I, ks-M2-SAT-I, or ks-M3-SAT-I, in which GCCACC (ks) was inserted before M1, M2, or M3 (Figure 6A). Integral membrane fractions were prepared, treated with or without endoglycosidase (Endo H or PNGase F), and analyzed by immunoblotting with anti-SAT-I antibodies. In ks-M1-SAT-I-transfected cells, M1-SAT-I carried Endo H-sensitive N-glycans, whereas M3-SAT-I carried Endo H-resistant N-glycans (Figure 6B, top panel). In ks-M2-SAT-I-transfected cells, some M2-SAT-I and most M3-SAT-I proteins carried Endo H-resistant N-glycans (Figure 6B, middle panel). M3-SAT-I in ks-M3-SAT-I-transfected cells also carried Endo H-resistant N-glycans (Figure 6B, bottom panel). These results indicate that the cytoplasmic regions of SAT-I isoforms influence the structures of their N-glycans. In addition, M3-SAT-I generated by the transfection of ks-M1-SAT-I and ks-M2-SAT-I is the result of leaky scanning.

On the basis of the different structures of the N-glycans on M1-, M2-, and M3-SAT-I, we speculated as to whether there may be distinct subcellular localizations for these isoforms. Accordingly, we performed indirect immunofluorescence microscopic analysis to determine the subcellular localization of these SAT-I isoforms in the transfected CHO cells. M1-SAT-I exhibited a reticular immunofluorescence staining pattern that was apparent in perinuclear and cytosolic regions (Figure 6C). This staining pattern was attributable to the ER localization of M1-SAT-I, because it could be superimposed on the ER pattern produced by an antibody against the ER retention signal KDEL. In contrast, M2- and M3-SAT-I localized to a compact juxtanuclear reticulum (Figure 6, D and E). Both M2- and M3-SAT-I colocalized with SAT-II-HA, which transfers sialic acid to GM3 to generate GD3 at the *trans*-Golgi (Daniotti *et al.*, 2000), but they partially colocalized with GM130, a *cis*-Golgi marker protein (Figure 6, D and E). Thus, M1-SAT-I is localized in the ER, whereas M2- and M3-SAT-I exhibit a distinct localization, mainly in the *trans*-Golgi.

Selective Retrograde Transport of M1-SAT-I from the Golgi Apparatus to the ER

To clarify whether the cytoplasmic tails of the SAT-I proteins influenced their subcellular localization, we stably expressed in CHO cells a fusion protein for the NH₂-terminus of M1-, M2-, or M3-SAT-I containing the transmembrane region and EGFP (M1-SAT-I(N)-EGFP, M2-SAT-I(N)-EGFP, and M3-SAT-I(N)-EGFP; Figure 7, A and B). M1-SAT-I(N)-EGFP was localized in the ER, whereas M2-/M3-SAT-I(N)-EGFP was localized in the Golgi apparatus (Figure 7B), indicating that the cytoplasmic tail of each of the SAT-I isoforms defines its subcellular localization.

To investigate the presence of the ER-targeting signal of M1-SAT-I in its cytoplasmic tail, we constructed a series of deletion mutants for M1-SAT-I(N)-EGFP (Figure 7C). CHO cells were transiently transfected with each M1-SAT-I(N)-EGFP mutant, fixed, and observed by confocal laser-scanning microscopy. M1-SAT-I(N)-EGFP Δ 12–26 and Δ 12–21 deletion mutants localized in the Golgi apparatus, although other mutants remained in the ER, suggesting the presence of ER-targeting signals in the amino acid residues between 12 and 21 (Figure 7D).

As shown in Figure 8A, the NH₂-terminal amino acid sequence of SAT-I is highly conserved between mice and

Figure 6. Differences in subcellular localization among M1-SAT-I, M2-SAT-I, and M3-SAT-I. (A) The structure of cDNA constructs used in the presented experiment. M1, M2, and M3 denote initiation codons, and TGA denotes a termination codon. TM and ks indicate a transmembrane region and a kozak sequence (GCCACC), respectively. (B) CHO cells were stably transfected with ks-M1-SAT-I, ks-M2-SAT-I, or ks-M3-SAT-I. Integral membrane fractions were prepared, treated with (+) or without (–) Endo H (E) or PNGase F (P), and then analyzed by immunoblotting with anti-SAT-I antibodies (C-term). (C–E) CHO cells stably expressing ks-M1-SAT-I, ks-M2-SAT-I, or ks-M3-SAT-I were fixed, permeabilized with 0.5% SDS in PBS, and stained with anti-SAT-I antibodies (C-term) and Alexa 488–conjugated anti-rabbit IgG. Cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were incubated with antibodies against KDEL (an ER marker) or GM130 (a *cis*-Golgi marker) for 1 h, followed by incubation with Alexa 594–conjugated anti-mouse IgG for 30 min. To visualize the *trans*-Golgi, CHO cells were transiently transfected with SAT-II-HA and stained with anti-HA antibodies and Alexa 594–conjugated anti-mouse IgG. Merged images are shown in the right panels. Bar, 5 μ m.

humans. Multiple arginine residues, especially Arg-11, Arg-12, and Arg-17, are conserved in both species, corresponding to the region essential for ER targeting. R-based motifs located on the cytoplasmic side of a transmembrane protein reportedly function as retrograde transport signals (Michelsen *et al.*, 2005). We generated R11S, R12S, and R11/12S M1-SAT-I(N)-EGFP mutants by site-directed mutagenesis and examined their subcellular localizations. R11/12S double-substitution mutant resulted in a shift of localization from the ER to the Golgi apparatus, although R11S and R12S single mutants were localized in the ER (Figure 8B). The RR motif is well known as an R-based motif (Schutze *et al.*, 1994), yet if only the RR motif functioned as an ER-targeting signal in M1-SAT-I, then the R11S and R12S single mutants would shift from the ER to the Golgi apparatus (Schutze *et al.*, 1994). However, as demonstrated in Figure 8B, these single mutants remained in the ER. We also tried to determine the role of Arg-17 in the ER targeting of M1-SAT-I, by generating and expressing R17S, R11/17S, or R12/17S M1-SAT-I(N)-EGFP mutants. As expected, R11/17S and R12/17S mutants shifted their localization to the Golgi apparatus, whereas the R17S mutant stayed in the ER (Figure 8B). These results indicate that not only the RR motif but also RXXXXR and RXXXXXR motifs function as ER-targeting signals of M1-SAT-I.

It has been reported that R-based motifs have to be positioned distally from a lipid bilayer to function (Shikano and Li, 2003). To confirm this observation, we created various deletion mutants of the M1-SAT-I(N)-EGFP WT sequence and examined their subcellular localization (Table 1). Only the Δ 28–55 mutant (distance: 25 aa) changed its localization from the ER to the Golgi apparatus. This result indicates that the RXXXXXR motif requires a distance of at least 30 aa from the membrane. Here, we use the term “functional distance” to describe the space essential for R-based motifs to function as a retrograde transport signal. We next examined the localization of the single deletion mutants M1-SAT-I(N)-EGFP R11S, R12S, and R17S to determine whether the functional distances among RR, RXXXXR, and RXXXXXR were

different. As summarized in Table 1, the functional distances of M1-SAT-I(N)-EGFP R11S (SRXXXXR), R12S (RSXXXXR), and R17S (RRXXXXS) were 40, 45, and 25 aa, respectively. These results indicate that the functional distances of the RXXXXR and RXXXXXR motifs are longer than that of the RR motif. In addition, the functional distance of the R17S (RR motif) single mutant is similar to that of WT M1-SAT-I. Thus, the sequences of the R-based motifs greatly affect the functional distance.

To confirm that full-length M1-SAT-I is localized in the ER by its RXXXXXR motif, CHO cells were stably transfected with the M1-SAT-I R11/12S mutant. An integral membrane fraction was prepared and analyzed by immunoblotting with anti-SAT-I antibodies. The mutant protein was detected as bands of 47.5 and 49 kDa, and part of the mutant carried Endo H-resistant N-glycans similar to M2-SAT-I (Figure 9A). To determine the subcellular localization of the M1-SAT-I R11/12S mutant in CHO cells, we performed indirect immunofluorescence microscopic analysis. As expected, the M1-SAT-I R11/12S mutant also exhibited colocalization with SAT-II-HA and partial colocalization with GM130, but this mutant did not colocalize with KDEL-containing proteins (Figure 9B and data not shown).

Differences in Intracellular Stability among SAT-I Isoforms

We next examined whether the differences in their cytoplasmic tails affect the stability of M1-SAT-I, M2-SAT-I, and M3-SAT-I. CHO cells stably expressing the ks-M1-SAT-I, ks-M2-SAT-I, ks-M3-SAT-I, or ks-M1-SAT-I R11/12S mutant were labeled with [35 S]Met/Cys for 30 min and then incubated with excessive amounts of unlabeled Met and Cys for 1, 2, and 3 h. SAT-I proteins were immunoprecipitated from cell lysates using anti-SAT-I antibodies. Before analysis by SDS-PAGE, the immunoprecipitated proteins were treated with PNGase F to remove N-glycans. As shown in Figure 10A, M1- and M3-SAT-I were not significantly degraded over the time of the experiment. In contrast, the half-life of the M2-SAT-I or the M1-SAT-I R11/12S mutant was consid-

Figure 7. Identification of the cytoplasmic region of M1-SAT-I essential for ER targeting. (A and C) The structure of cDNA constructs used in the presented experiment. We constructed plasmids, each encoding a fusion protein of the NH₂-terminus of M1-SAT-I, M2-SAT-I, or M3-SAT-I containing the transmembrane region, and EGFP (M1-SAT-I(N)-EGFP, M2-SAT-I(N)-EGFP, and M3-SAT-I(N)-EGFP). TM indicates the transmembrane region. M1, M2, and M3 denote initiation codons, and TGA denotes a termination codon. (B) CHO cells were stably transfected with M1-SAT-I(N)-EGFP, M2-SAT-I(N)-EGFP, and M3-SAT-I(N)-EGFP. Expression of the EGFP fusion proteins was observed in fixed cells by confocal laser-scanning microscopy. (D) CHO cells were transiently transfected with M1-SAT-I(N)-EGFP WT or the indicated deletion mutant. Expression of the EGFP fusion proteins was observed in fixed cells by confocal laser-scanning microscopy. Bar, 10 μ m.

erably shorter, and significant degradation was observed for each after a 3-h chase.

We previously reported that the lipid-mannosyltransferases responsible for sphingolipid synthesis in yeast (i.e., Csg1 and Csh1) are sorted to the vacuoles, which correspond to lysosomes in mammals, for degradation (Uemura *et al.*, 2007). Accordingly, we examined whether M2-SAT-I and the M1-SAT-I R11/12S mutants were also sorted to lysosomes for degradation. SAT-I-expressing cells were treated with or without 0.2 mM chloroquine, a lysosomal protein degradation inhibitor, for 24 h, and then the cells were labeled with [³⁵S]Met/Cys for 30 min and incubated for 3 h with excessive amounts of unlabeled Met and Cys.

Figure 8. ER targeting of M1-SAT-I (N)-EGFP by multiple arginine residues. (A) Comparison of the amino acid sequence of mouse SAT-I (mSAT-I) and human SAT-I (hSAT-I). Alignment was generated using the Clustal W and BOXSHADE (Institute for Animal Health, Surrey, United Kingdom) programs. Black boxes indicate identical residues, and gray boxes amino acid similarities. Asterisks indicate mutated amino acids. Arrow head (RR) indicates the ER exporting signal (Giraud and Maccioni, 2003a). TM indicates a transmembrane region. M1, M2, and M3 denote initiation codons. (B) CHO cells were transiently transfected with M1-SAT-I(N)-EGFP WT or the indicated mutant. Expression of the EGFP fusion proteins was observed in fixed cells by confocal laser-scanning microscopy. Bar, 10 μ m.

SAT-I proteins were immunoprecipitated from cell lysates using anti-SAT-I antibodies. Before analysis by SDS-PAGE, immunoprecipitated proteins were treated with PNGase F to remove N-glycans. As expected, the disappearance of M2-SAT-I and its M1-SAT-I R11/12S mutant was inhibited by treatment with chloroquine (Figure 10B). These results suggest that M2-SAT-I is sorted to lysosomes for degradation, whereas M1-SAT-I and M3-SAT-I escape this degradation pathway.

Reduction of the In Vivo GM3 Synthesis of M1-SAT-I by ER Targeting

To clarify whether differences in intracellular dynamics among SAT-I isoforms affect GM3 synthesis activity, we examined their activities in vitro and in vivo. The introduction of ks-M1-SAT-I or ks-M2-SAT-I into CHO cells led to the production of M3-SAT-I by leaky scanning (Figure 6B). To completely suppress leaky scanning, we constructed ks-M1-SAT-I M28/56A and ks-M2-SAT-I M29A mutants. The subcellular localization and stability of these mutants were similar to those of the WT sequences (data not shown). To compare the activities of SAT-I isoforms and their mutants, we determined the activities in vitro using lysates from CHO-K1 cells transiently expressing the protein or a mutant as enzyme sources. The activity of ks-M3-SAT-I was about

Table 1. Functional distance of arginine-based motifs

RRXXXXR (wild type)		SRXXXXR (R11S)	RSXXXXR (R12S)	RRXXXXS (R17S)	Distance ^a (aa)
Wild type	ER	ER	ER	ER	53
Δ48–55	ER	ER	ER>Golgi	ER	45
Δ43–55	ER	ER>Golgi	ER<Golgi	ER	40
Δ38–55	ER	ER<Golgi	ER<Golgi	ER	35
Δ33–55	ER	ER<Golgi	ER<Golgi	ER	30
Δ28–55	ER<Golgi	ER<Golgi	ER<Golgi	ER<Golgi	25
Δ28–32	ER	—	—	—	48
Δ28–37	ER	—	—	—	43
Δ28–42	ER	—	—	—	38

—, no information; aa, amino acid residues.
^a Distance is the number of amino acid residues from R/S to the lipid bilayer.

three fold higher than that of either ks-M1-SAT-I or ks-M2-SAT-I (Figure 11A). The activity of the ks-M1-SAT-I M28/56A mutant was similar to that of ks-M1-SAT-I, whereas the activity of the ks-M2-SAT-I M29 mutant was decreased, to ~50%, relative to the activity of ks-M2-SAT-I (Figure 11A). To examine the GM3 synthesis activities of SAT-I isoforms and their mutants *in vivo*, we used a retroviral transfection system to introduce the constructs into immortalized SAT-I knockout MEFs, which mainly express GM1b (o-series ganglioside). Using real-time PCR, we confirmed that the expression levels of SAT-I were almost the same in the cells expressing the SAT-I isoforms and their mutants (Figure 11B) and then analyzed their ganglioside compositions. The introduction of SAT-I led to a reduction in GM1b and an

increase in GM3, GM1, and GD1a levels (Figure 11, C and D). However, the differences in the activities of the SAT-I isoforms and mutants observed *in vitro* did not correlate with ganglioside synthesis *in vivo*. Because the immortalized SAT-I knockout MEF were overexpressing SAT-I proteins after retroviral transfection, GM3 synthesis should be saturated in these cells. Remarkably, the amounts of gangliosides in the ks-M1-SAT-I M28/56A mutant-transfected cells were greatly reduced compared with those of cells transfected with the other isoforms or mutants (Figure 11D). The ganglioside levels in ks-M1-SAT-I-transfected cells were similar to those in ks-M2-SAT-I- or ks-M3-SAT-I-transfected cells, suggesting that the amount of M3-SAT-I produced by the transduction of ks-M1-SAT-I is enough to saturate GM3 synthesis. In conclusion, these results indicate that the sorting of M1-SAT-I to the *trans*-Golgi is restricted

Figure 9. The deletion of sequential double arginines in M1-SAT-I caused loss of its ER-targeting ability. (A) CHO cells were stably transfected with M1-SAT-I R11/12S. Integral membrane fractions were prepared, treated with (+) or without (–) Endo H (E) or PNGase F (P), and then analyzed by immunoblotting with anti-SAT-I antibodies (C-term). (B) CHO cells stably expressing the M1-SAT-I R11/12S mutant were fixed, permeabilized with 0.5% SDS in PBS, and stained with anti-SAT-I antibodies (C-term) and Alexa 488–conjugated anti-rabbit IgG. Cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were incubated with antibodies against GM130 (a *cis*-Golgi marker) for 1 h, followed by incubation with Alexa 594–conjugated anti-mouse or anti-goat IgG for 30 min. To visualize the *trans*-Golgi, CHO cells were transiently transfected with SAT-II-HA and stained with anti-HA antibodies and Alexa 594–conjugated anti-mouse IgG. Merged images are shown in the right panels. Bar, 5 μ m.

Figure 10. Distinct differences of the lysosomal turnover of SAT-I isoforms. (A) CHO cells stably expressing ks-M1-SAT-I, ks-M2-SAT-I, ks-M3-SAT-I, or the ks-M1-SAT-I R11/12S mutant were pulse-labeled with [³⁵S]Met/[³⁵S]Cys for 30 min and incubated with excessively cold Met/Cys for 1, 2, and 3 h. Total cell lysates were prepared, subjected to immunoprecipitation with anti-SAT-I antibodies (C-term), treated with PNGase F, and separated by SDS-PAGE. Gels were fixed and radiolabeled proteins were detected by autoradiography. (B) CHO cells stably expressing ks-M1-SAT-I, ks-M2-SAT-I, ks-M3-SAT-I, or the ks-M1-SAT-I R11/12S mutant were treated for 24 h with (+) or without (–) 0.2 mM chloroquine (C), a lysosomal protein degradation inhibitor. During treatment with chloroquine, cells were pulse-labeled with [³⁵S]Met/[³⁵S]Cys for 30 min and incubated for 3 h with excess amounts of unlabeled Met/Cys. Total cell lysates were prepared, subjected to immunoprecipitation with anti-SAT-I antibodies (C-term), treated with PNGase F, and separated by SDS-PAGE. Gels were fixed, and radiolabeled proteins were detected by autoradiography.

Figure 11. Comparison of in vitro and in vivo GM3 synthesis activities among SAT-I isoforms and their mutants. SAT-I knockout MEF cells were transfected with (+) the indicated SAT-I isoform or mutant using a retrovirus expression system. A minus sign indicates no transfection. (A) Whole cell extracts were examined for enzyme activity using LacCer and cytidine-5'-monophosphate- $[^{14}\text{C}]$ -N-acetyl-neuraminic acid as substrates. Products were separated by TLC, and the radioactivities associated with GM3 were quantified using a Bio-Imaging analyzer BAS5000. The relative enzyme activities were calculated using the radioactivities associated with GM3, and the corresponding protein levels were determined by immunoblotting with anti-SAT-I antibodies (C-term) after PN-Gase F treatment of the sample. (B) Quantitative analyses of SAT-I isoforms and mutants in transfected cells were performed by real-time PCR. Expression levels of the SAT-I transcripts were each

by the R-based motif, but M1-SAT-I does not completely remain in the ER. Therefore, some M1-SAT-I is sorted to the *trans*-Golgi by escaping retrograde transport, and then it participates in GM3 synthesis.

DISCUSSION

Generally, only SAT-I proteins with a short cytoplasmic tail (14 aa) are chosen as subjects for ganglioside biosynthesis studies. However, we have presented evidence here that three mouse SAT-I isoforms with cytoplasmic tail lengths of 69 aa (M1-SAT-I), 42 aa (M2-SAT-I), and 14 aa (M3-SAT-I) are translated by leaky scanning and are produced as several proteins separately initiated from a single mRNA. Furthermore, we have identified distinct intracellular dynamics for each. Remarkably, M1-SAT-I was found to be localized in the ER, mainly due to a retrograde transport signal (RRXXXXR) located on its cytoplasmic tail. Because the consensus sequences of R-based motifs were previously reported to be RR and RXR (Schutze *et al.*, 1994), the RXXXXR and RXXXXXR sequences identified in this study are clearly new consensus sequences. ER targeting restricts M1-SAT-I transport to the Golgi apparatus, leading to the suppression of its GM3 synthesis activities in vivo. On the other hand, although both M2-SAT-I and M3-SAT-I were found to be localized in the Golgi apparatus, we observed distinct differences in their protein stabilities. Further analysis revealed that M2-SAT-I is sorted to lysosomes via the Golgi for degradation, whereas M3-SAT-I remains relatively stable in the Golgi apparatus. Observations regarding the tissue distributions of mouse and human mRNA variants also determined that SAT-I isoforms with different properties coexist in most tissues. Thus, we speculate that changes in the relative composition and/or intracellular trafficking of these isoforms are important for the regulation of GM3 synthesis.

There are many reports indicating that a single mRNA can produce several independently initiated proteins via leaky scanning (Kozak, 2002). This phenomenon often results from a suboptimal context of the first AUG_{start} codon. There are, however, rare instances of leaky scanning occurring despite the first AUG being presented in a strong context (R^{-3} and G^{+4} ; Kozak, 2002). Such a result can occur when the first AUG codon is too close to the 5'-end to be efficiently recognized. Most leaky scanning of mouse SAT-I can be explained by a suboptimal context. However, the production of M3-SAT-I from mSAT-Ia and mSAT-Ib was not completely abolished when the first AUG was replaced with an optimal sequence by site-directed mutagenesis. Because mSAT-Ia and mSAT-Ib were inserted into an expression vector for mammalian cells [pcDNA3.1 Zeo(+)], the possibility that the first AUG is too close to the 5'-end to be recognized as the initiation codon could not be confirmed. Thus, M3-SAT-I may be produced by context-independent leaky scanning, although the mechanism remains unclear.

R-based motifs function as retrograde transport signals when positioned distally from a lipid bilayer. Several reports suggest that the minimum distance would be between 16

normalized to GAPDH transcripts. (C) The biosynthesis pathway of o- and a-series gangliosides. Cer, ceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide. (D) Gangliosides (corresponding to 0.75 mg total protein) were extracted from SAT-I-knockout MEF cells expressing the indicated SAT-I isoform or mutant and were separated on TLC plates and stained with an orcinol-sulfuric acid reagent. The asterisk indicates bands of globosides.

highly matured N-glycans, retrograde transport in the Golgi apparatus may be necessary for the maturation of N-glycans.

and 46 Å (Shikano and Li, 2003). Our results also indicate that a distance of between 24 and 47 aa from the lipid bilayer is necessary for the functioning of R-based motifs, a distance we refer to as the “functional distance.” For example, the ER-exporting signal R/K(X)R/K, located just above the transmembrane domain in many glycosyltransferases, cannot function as a retrograde transport signal (Giraud and Maccioni, 2003a). R-based motifs need to interact with COP I subunits, β -COP and δ -COP, to function as a signaling sequence, and binding affinities to β -COP and δ -COP depend on the sequences of R-based motifs (Michelsen *et al.*, 2007). We also confirmed that M1-SAT-I WT, but not R11/12S mutant, interacts with β -COP and/or δ -COP (data not shown). Thus, we speculate that a functional distance is essential for R-based motifs to interact with β -COP and δ -COP. On the basis of this idea, we also assume that for a variety of R-based motifs, the distinct binding affinities to β -COP and δ -COP influence the functional distance. In other words, the functional distance is longer if the R-based motif has weak binding affinity. As shown in Table 1, RXXXXR and RXXXXR motifs require a long range to function, relative to the RR motif. These results suggest that the binding affinities of RXXXXR and RXXXXR motifs to β -COP and δ -COP are weaker than that of the RR motif. In conclusion, we propose that the functional distance should be the criterion that defines the affinity of R-based motifs to β -COP and δ -COP.

M2-SAT-I and the M1-SAT-I R11/12S mutant are both sorted to lysosomes via the Golgi and rapidly degraded (Figure 10A). M3-SAT-I is more stable than M2-SAT-I or the M1-SAT-I R11/12S mutant, indicating that the N-terminus of M2-SAT-I (1–28 aa) may be involved in trafficking to the lysosomes. Many membrane proteins are known to be sorted to the lysosomes when carrying multiple monoubiquitins or short polyubiquitin chains (Pelham, 2004; Piper and Luzio, 2007). It might be possible that the lysine residues present in the M2-SAT-I confer the lower stability to this protein when compared with M3-SAT-I. In contrast, M3-SAT-I remains stable in the *trans*-Golgi by escaping the degradation pathway (Figure 10A). Based on the Golgi maturation model (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006), this result suggests that M3-SAT-I undergoes retrograde transport between the Golgi cisternae. Most N-glycans on M3-SAT-I are of the Endo H-resistant, mature type, whereas most N-glycans on M2-SAT-I are of the

Figure 12. Intracellular dynamics of SAT-I isoforms. M1-SAT-I, containing R-based motifs in its cytoplasmic tail, is targeted for ER by retrograde transport. However, a small amount of M1-SAT-I is sorted to the *trans*-Golgi by escaping retrograde transport, and is involved in GM3 synthesis. The significance of the ER targeting of M1-SAT-I remains unclear, but M1-SAT-I might have chaperone activity in addition to GM3-synthesis activity. In contrast, the M1-SAT-I R11/12S mutant, which is not targeted to the ER, and M2-SAT-I are localized in the *trans*-Golgi and rapidly sorted to lysosomes for degradation. M3-SAT-I is also localized in the *trans*-Golgi, but the half-life is more stable relative to that of M2-SAT-I or the M1-SAT-I R11/12S mutant, so the protein is more stably localized. Perhaps, based on the Golgi maturation model, retrograde transport within the Golgi apparatus is involved in the stabilization of M3-SAT-I. Because only M3-SAT-I carries

Endo H-sensitive, high-mannose type. This suggests further that the retrograde transport cycle between the Golgi cisternae is involved in the maturation of N-glycans on SAT-I proteins.

Most M1-SAT-I is targeted to the ER by R-based motifs, but a small population of M1-SAT-I is sorted to the Golgi apparatus, which participates in GM3 synthesis (Figure 11D). To escape ER targeting, it would be necessary for the R-based motif containing M1-SAT-I to be masked. Such masking has been proposed as a mechanism utilized by ATP-binding cassette proteins. ATP-sensitive K^+ channels are octameric complexes that consist of four pore-forming, inwardly rectifying α subunits (Kir6.1/2) and four sulfonylurea-binding β subunits (SUR1/2A/2B) belonging to the ATP-binding cassette family. Each subunit contains an R-based motif, and unassembled subunits or incompletely assembled complexes stay in the ER. During the process of heteromultimeric assembly, R-based motifs are masked, which permits transport of the complex to the cell surface (Zerangue *et al.*, 1999). The R-based motif in Kir6.2 is sterically masked by the SUR1 subunit. In contrast, the R-based motif in SUR1 can be inactivated by the recruitment of 14-3-3 (Heusser *et al.*, 2006). For SAT-I, proteins involved in masking R-based motifs have not been identified. However, the affinity of the NH_2 -terminus of M1-SAT-I for masking proteins is expected to be lower than that for β - and/or δ -COP. Consequently, M1-SAT-I is predominantly remains in the ER and then sorted slowly to the Golgi. The significance of the slow sorting of M1-SAT-I must be a subject for future research. Currently, we assume that there are two possibilities, either M1-SAT-I functions as a means to stably supply GM3, or it has a distinct function in the ER. In regards to the latter function, Protein O-fucosyltransferase 1 (OFUT1), an enzyme that glycosylates the epidermal growth factor-like domains of Notch, is localized in the ER by the C-terminal KDEL-like sequence and displays a Notch chaperone activity (Okajima *et al.*, 2005).

In the presented study, we were able to demonstrate distinct intracellular dynamics for several SAT-I isoforms and possible regulatory mechanisms for *in vivo* GM3 synthesis (Figure 12). Further work will be required to confirm whether various pathological conditions alter the composition and/or intracellular trafficking of endogenous SAT-I isoforms, and thereby affect GM3 synthesis.

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REFERENCES

- Aerts, J. M., *et al.* (2007). Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes* 56, 1341–1349.
- Berselli, P., Zava, S., Sottocornola, E., Milani, S., Berra, B., and Colombo, I. (2006). Human GM3 synthase: a new mRNA variant encodes an NH₂-terminal extended form of the protein. *Biochim. Biophys. Acta* 1759, 348–358.
- Choi, H. J., Chung, T. W., Kang, N. Y., Kim, K. S., Lee, Y. C., and Kim, C. H. (2003). Transcriptional regulation of the human GM3 synthase (hST3Gal V) gene during monocytic differentiation of HL-60 cells. *FEBS Lett.* 555, 204–208.
- Choi, H. J., Chung, T. W., Kang, N. Y., Kim, K. S., Lee, Y. C., and Kim, C. H. (2004). Involvement of CREB in the transcriptional regulation of the human GM3 synthase (hST3Gal V) gene during megakaryocytoid differentiation of human leukemia K562 cells. *Biochem. Biophys. Res. Commun.* 313, 142–147.
- D'Angelo, G., *et al.* (2007). Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature* 449, 62–67.
- Daniotti, J. L., Martina, J. A., Giraudo, C. G., Zurita, A. R., and Maccioni, H. J. (2000). GM3 alpha2,8-sialyltransferase (GD3 synthase): protein characterization and sub-Golgi location in CHO-K1 cells. *J. Neurochem.* 74, 1711–1720.
- Fukumoto, S., Miyazaki, H., Goto, G., Urano, T., and Furukawa, K. (1999). Expression cloning of mouse cDNA of CMP-NeuAc:lactosylceramide alpha2,3-sialyltransferase, an enzyme that initiates the synthesis of gangliosides. *J. Biol. Chem.* 274, 9271–9276.
- Giraudo, C. G., Daniotti, J. L., and Maccioni, H. J. (2001). Physical and functional association of glycolipid N-acetyl-galactosaminyl and galactosyl transferases in the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* 98, 1625–1630.
- Giraudo, C. G., and Maccioni, H. J. (2003a). Endoplasmic reticulum export of glycosyltransferases depends on interaction of a cytoplasmic dibasic motif with Sar1. *Mol. Biol. Cell* 14, 3753–3766.
- Giraudo, C. G., and Maccioni, H. J. (2003b). Ganglioside glycosyltransferases organize in distinct multienzyme complexes in CHO-K1 cells. *J. Biol. Chem.* 278, 40262–40271.
- Hakomori, S. (2004). Glycosynapses: microdomains controlling carbohydrate-dependent cell adhesion and signaling. *Ann. Acad. Bras. Cienc.* 76, 553–572.
- Hamamoto, T., Kawasaki, M., Kurosawa, N., Nakaoka, T., Lee, Y. C., and Tsuji, S. (1993). Two step single primer mediated polymerase chain reaction. Application to cloning of putative mouse, beta-galactoside alpha 2,6-sialyltransferase cDNA. *Bioorg. Med. Chem.* 1, 141–145.
- Heusser, K., Yuan, H., Neagoe, I., Tarasov, A. I., Ashcroft, F. M., and Schwappach, B. (2006). Scavenging of 14-3-3 proteins reveals their involvement in the cell-surface transport of ATP-sensitive K⁺ channels. *J. Cell Sci.* 119, 4353–4363.
- Ikehara, Y., Shimizu, N., Kono, M., Nishihara, S., Nakanishi, H., Kitamura, T., Narimatsu, H., Tsuji, S., and Tatematsu, M. (1999). A novel glycosyltransferase with a polyglutamine repeat; a new candidate for GD1alpha synthase (ST6GalNAc V)(1). *FEBS Lett.* 463, 92–96.
- Ishii, A., Ohta, M., Watanabe, Y., Matsuda, K., Ishiyama, K., Sakoe, K., Nakamura, M., Inokuchi, J., Sanai, Y., and Saito, M. (1998). Expression cloning and functional characterization of human cDNA for ganglioside GM3 synthase. *J. Biol. Chem.* 273, 31652–31655.
- Kabayama, K., Sato, T., Kitamura, F., Uemura, S., Kang, B. W., Igarashi, Y., and Inokuchi, J. (2005). TNFalpha-induced insulin resistance in adipocytes as a membrane microdomain disorder: Involvement of ganglioside GM3. *Glycobiology* 15, 21–29.
- Kabayama, K., Sato, T., Saito, K., Loberto, N., Prinetti, A., Sonnino, S., Kinjo, M., Igarashi, Y., and Inokuchi, J. (2007). Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. *Proc. Natl. Acad. Sci. USA* 104, 13678–13683.
- Kapitonov, D., Bieberich, E., and Yu, R. K. (1999). Combinatorial PCR approach to homology-based cloning: cloning and expression of mouse and human GM3-synthase. *Glycoconj. J.* 16, 337–350.
- Kim, K. W., Kim, S. W., Min, K. S., Kim, C. H., and Lee, Y. C. (2001). Genomic structure of human GM3 synthase gene (hST3Gal V) and identification of mRNA isoforms in the 5'-untranslated region. *Gene* 273, 163–171.
- Kim, S. W., Lee, S. H., Kim, K. S., Kim, C. H., Choo, Y. K., and Lee, Y. C. (2002). Isolation and characterization of the promoter region of the human GM3 synthase gene. *Biochim. Biophys. Acta* 1578, 84–89.
- Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y. C., and Tsuji, S. (1995). Enzymatic activity of a developmentally regulated member of the sialyltransferase family (STX): evidence for alpha 2,8-sialyltransferase activity toward N-linked oligosaccharides. *FEBS Lett.* 360, 1–4.
- Kono, M., Ohya, Y., Lee, Y. C., Hamamoto, T., Kojima, N., and Tsuji, S. (1997). Mouse beta-galactoside alpha 2,3-sialyltransferases: comparison of in vitro substrate specificities and tissue-specific expression. *Glycobiology* 7, 469–479.
- Kono, M., Takashima, S., Liu, H., Inoue, M., Kojima, N., Lee, Y. C., Hamamoto, T., and Tsuji, S. (1998). Molecular cloning and functional expression of a fifth-type alpha 2,3-sialyltransferase (mST3Gal V:GM3 synthase). *Biochem. Biophys. Res. Commun.* 253, 170–175.
- Kono, M., Yoshida, Y., Kojima, N., and Tsuji, S. (1996). Molecular cloning and expression of a fifth type of alpha2,8-sialyltransferase (ST8Sia V). Its substrate specificity is similar to that of SAT-V/III, which synthesizes GD1c, GT1a, GQ1b and GT3. *J. Biol. Chem.* 271, 29366–29371.
- Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. *Gene* 299, 1–34.
- Kurosawa, N., Inoue, M., Yoshida, Y., and Tsuji, S. (1996). Molecular cloning and genomic analysis of mouse Galbeta1,3GalNAc-specific GalNAc alpha2,6-sialyltransferase. *J. Biol. Chem.* 271, 15109–15116.
- Kurosawa, N., Takashima, S., Kono, M., Ikehara, Y., Inoue, M., Tachida, Y., Narimatsu, H., and Tsuji, S. (2000). Molecular cloning and genomic analysis of mouse GalNAc alpha2, 6-sialyltransferase (ST6GalNAc I). *J. Biochem.* 127, 845–854.
- Lee, Y. C., Kaufmann, M., Kitazume-Kawaguchi, S., Kono, M., Takashima, S., Kurosawa, N., Liu, H., Pircher, H., and Tsuji, S. (1999). Molecular cloning and functional expression of two members of mouse NeuAcalpha2,3Galbeta1,3GalNAc GalNAcalpha2,6-sialyltransferase family, ST6GalNAc III and IV. *J. Biol. Chem.* 274, 11958–11967.
- Lee, Y. C., Kojima, N., Wada, E., Kurosawa, N., Nakaoka, T., Hamamoto, T., and Tsuji, S. (1994). Cloning and expression of cDNA for a new type of Gal beta 1,3GalNAc alpha 2,3-sialyltransferase. *J. Biol. Chem.* 269, 10028–10033.
- Lee, Y. C., Kurosawa, N., Hamamoto, T., Nakaoka, T., and Tsuji, S. (1993). Molecular cloning and expression of Gal beta 1,3GalNAc alpha 2,3-sialyltransferase from mouse brain. *Eur. J. Biochem.* 216, 377–385.
- Losev, E., Reinke, C. A., Jellen, J., Strongin, D. E., Bevis, B. J., and Glick, B. S. (2006). Golgi maturation visualized in living yeast. *Nature* 441, 1002–1006.
- Matsuura-Tokita, K., Takeuchi, M., Ichihara, A., Mikuriya, K., and Nakano, A. (2006). Live imaging of yeast Golgi cisternal maturation. *Nature* 441, 1007–1010.
- Michelsen, K., Schmid, V., Metz, J., Heusser, K., Liebel, U., Schwede, T., Spang, A., and Schwappach, B. (2007). Novel cargo-binding site in the beta and delta subunits of coatamer. *J. Cell Biol.* 179, 209–217.
- Michelsen, K., Yuan, H., and Schwappach, B. (2005). Hide and run. Arginine-based endoplasmic-reticulum-sorting motifs in the assembly of heteromultimeric membrane proteins. *EMBO Rep.* 6, 717–722.
- Okajima, T., Chen, H. H., Ito, H., Kiso, M., Tai, T., Furukawa, K., and Urano, T. (2000). Molecular cloning and expression of mouse GD1alpha/GT1aalpha/GQ1balpha synthase (ST6GalNAc VI) gene. *J. Biol. Chem.* 275, 6717–6723.
- Okajima, T., Fukumoto, S., Miyazaki, H., Ishida, H., Kiso, M., Furukawa, K., and Urano, T. (1999). Molecular cloning of a novel alpha2,3-sialyltransferase (ST3Gal VI) that sialylates type II lactosamine structures on glycoproteins and glycolipids. *J. Biol. Chem.* 274, 11479–11486.
- Okajima, T., Xu, A., Lei, L., and Irvine, K. D. (2005). Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. *Science* 307, 1599–1603.
- Pelham, H. R. (2004). Membrane traffic: GGAs sort ubiquitin. *Curr. Biol.* 14, R357–R359.
- Piper, R. C., and Luzzio, J. P. (2007). Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes. *Curr. Opin. Cell Biol.* 19, 459–465.
- Schutze, M. P., Peterson, P. A., and Jackson, M. R. (1994). An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J.* 13, 1696–1705.
- Shikano, S., and Li, M. (2003). Membrane receptor trafficking: Evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localization signals. *Proc. Natl. Acad. Sci. USA* 100, 5783–5788.
- Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1, 31–39.
- Tagami, S., *et al.* (2002). Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J. Biol. Chem.* 277, 3085–3092.

- Takashima, S., Ishida, H. K., Inazu, T., Ando, T., Ishida, H., Kiso, M., Tsuji, S., and Tsujimoto, M. (2002). Molecular cloning and expression of a sixth type of alpha 2,8-sialyltransferase (ST8Sia VI) that sialylates O-glycans. *J. Biol. Chem.* 277, 24030–24038.
- Takashima, S., Tsuji, S., and Tsujimoto, M. (2003). Comparison of the enzymatic properties of mouse beta-galactoside alpha2,6-sialyltransferases, ST6Gal I and II. *J. Biochem.* 134, 287–296.
- Tsukamoto, K., Kohda, T., Mukamoto, M., Takeuchi, K., Ihara, H., Saito, M., and Kozaki, S. (2005). Binding of *Clostridium botulinum* type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *J. Biol. Chem.* 280, 35164–35171.
- Uemura, S., Kihara, A., Iwaki, S., Inokuchi, J., and Igarashi, Y. (2007). Regulation of the transport and protein levels of the inositol phosphorylceramide mannosyltransferases Csg1 and Csh1 by the Ca²⁺-binding protein Csg2. *J. Biol. Chem.* 282, 8613–8621.
- Uemura, S., Kurose, T., Suzuki, T., Yoshida, S., Ito, M., Saito, M., Horiuchi, M., Inagaki, F., Igarashi, Y., and Inokuchi, J. (2006). Substitution of the N-glycan function in glycosyltransferases by specific amino acids: ST3Gal-V as a model enzyme. *Glycobiology* 16, 258–270.
- Uliana, A. S., Crespo, P. M., Martina, J. A., Daniotti, J. L., and Maccioni, H. J. (2006). Modulation of GalT1 and SialT1 sub-Golgi localization by SialT2 expression reveals an organellar level of glycolipid synthesis control. *J. Biol. Chem.* 281, 32852–32860.
- Xia, T., Zeng, G., Gao, L., and Yu, R. K. (2005). Sp1 and AP2 enhance promoter activity of the mouse GM3-synthase gene. *Gene* 351, 109–118.
- Yamamoto, A., Haraguchi, M., Yamashiro, S., Fukumoto, S., Furukawa, K., Takamiya, K., Atsuta, M., and Shiku, H. (1996). Heterogeneity in the expression pattern of two ganglioside synthase genes during mouse brain development. *J. Neurochem.* 66, 26–34.
- Yamashita, T., *et al.* (2003). Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc. Natl. Acad. Sci. USA* 100, 3445–3449.
- Yoshida, Y., Kojima, N., Kurosawa, N., Hamamoto, T., and Tsuji, S. (1995a). Molecular cloning of Sia alpha 2,3Gal beta 1,4GlcNAc alpha 2,8-sialyltransferase from mouse brain. *J. Biol. Chem.* 270, 14628–14633.
- Yoshida, Y., Kojima, N., and Tsuji, S. (1995b). Molecular cloning and characterization of a third type of N-glycan alpha 2,8-sialyltransferase from mouse lung. *J. Biochem.* 118, 658–664.
- Zeng, G., Gao, L., Xia, T., Tencomnao, T., and Yu, R. K. (2003). Characterization of the 5'-flanking fragment of the human GM3-synthase gene. *Biochim. Biophys. Acta* 1625, 30–35.
- Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* 22, 537–548.
- Zhao, H., Przybylska, M., Wu, I. H., Zhang, J., Siegel, C., Komarnitsky, S., Yew, N. S., and Cheng, S. H. (2007). Inhibiting glycosphingolipid synthesis improves glycemic control and insulin sensitivity in animal models of type 2 diabetes. *Diabetes* 56, 1210–1218.