The regulation of ER export and Golgi retention of ST3Gal5 (GM3/GM4 synthase) and B4GalNAcT1 (GM2/GD2/GA2 synthase) by arginine/lysine-based motif adjacent to the transmembrane domain

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Abstract

In the Golgi maturation model, the Golgi cisternae dynamically mature along a secretory pathway. In this dynamic process, glycosyltransferases are transported from the endoplasmic reticulum (ER) to the Golgi apparatus where they remain and function. The precise mechanism behind this maturation process remains unclear. We investigated two glycosyltransferases, ST3Gal5 (ST3G5) and B4GalNAcT1 (B4GN1), involved in ganglioside synthesis and examined their signal sequences for ER export and Golgi retention. Reports have suggested that the \([R/K](X)[R/K]\) motif functions as an ER exporting signal; however, this signal sequence is insufficient in stably expressed, full-length ST3G5. Through further analysis, we have clarified that the \([R/K](X)[R/K]\) sequence in ST3G5 is essential for ER export. We have named the sequence the R/K-based motif. On the other hand, for ER export of B4GN1, the homodimer formation in addition to the R/K-based motif is required for ER export suggesting the importance of unidentified luminal side interaction. We found that ST3G5 R2A/R3A and K9A/K13A mutants localized not only in Golgi apparatus but also in endosomes. Furthermore, the amounts of mature type asparagine-linked (N)-glycans in ST3G5 R2A/R3A and K9A/K13A mutants were decreased compared with those in wild-type proteins, and the stability of the mutants was lower. These results suggest that the R/K-based motif is necessary for the Golgi retention of ST3G5 and that the retention is involved in the maturation of N-glycans and in stability. Thus, several basic amino acids located on the cytoplasmic tail of ST3G5 play important roles in both ER export and Golgi retention.

Key words: ER export, glycosphingolipid, glycosyltransferase, Golgi retention
Introduction

Glycosphingolipids, which exist in the outer leaflet of the plasma membrane, regulate cellular processes (Takamiya et al. 1996; Chiavegatto et al. 2000; Inoue et al. 2002; Okada et al. 2002; Yamashita et al. 2003, 2005; Yoshikawa et al. 2009; Nagafuku et al. 2012). Furthermore, changes in the sphingolipid composition of cell membranes are known to be causatively associated with various diseases. For example, levels of the ganglioside GM3, a glycosphingolipid, are increased in the adipose tissue of Zucker fa/fa rats and ob/ob mice, typical rodent models of obesity (Tagami et al. 2002). Insulin resistance in such obesity models is significantly improved after treatment with a glucocerebrosidase synthase inhibitor that decreases the GM3 levels (Aerts et al. 2007; Zhao et al. 2007). Moreover, patients with early-onset refractory epilepsy or salt and pepper syndrome (characterized by severe intellectual disability, epilepsy, scoliosis, choreoathetosis, dysmorphic facial features and altered dermal pigmentation) are known to carry a mutation in the gene encoding ST3Gal5 (Fragaki et al. 2013; Boccuto et al. 2014). Thus, the regulation of glycosphingolipid synthesis is an important area to understand.

The glycosphingolipid synthases are type II integral membrane proteins comprising a short cytoplasmic NH2-terminal tail, a cosphingolipid synthesis is an important area to understand. paratus by retrograde transport between the Golgi cisterna and/or may be possible that glycosyltransferases are retained in the Golgi apparatus, and glycosphingolipid synthesis occurs mainly on the lumenal side (Paulson and Colley 1989). Once folded in the endoplasmic reticulum (ER), the enzymes are transported to the Golgi apparatus by COPII vesicles, which are formed by the small GTPase Sar1 and the coat proteins Sec23, Sec24, Sec13 and Sec31 (Barlowe et al. 1994; Matsuoka et al. 1998).

Type I integral membrane proteins interact with the binding pockets (known as sites A, B and C) of Sec24, via the signal sequence located on their COOH-terminal side. For example, the vesicular stomatitis virus glycoprotein (VSV G) interacts with Sec24 via a di-acidic DxEx motif (where x is any amino acid) (Nishimura and Balch 1997). Likewise, the LXXLE sequence of the v-SNARE Bet1 binds to site B, and the LXXLE and YNNNPF sequences of the t-SNARE Sec5 bind to site B and site A, respectively (Mossessova et al. 2003). The FF sequence of the ER-Golgi intermediate compartment marker ERGIC53 is necessary for the protein to interact with the Sec23–Sec24 complex (Kappeler et al. 1997). Other signal sequences having functions similar to that of the FF sequence are known; these include the sequences FXX, YXX, LLX(X), IIX(X) and VX (Dominguez et al. 1998; Nufer et al. 2002; Otte and Barlowe 2002).

In type II integral membrane proteins such as glycosyltransferases, which include sphingolipid synthases, the NH2-terminal (R[K]) motif as well as the cytoplasmic tail of M2-ST3G5. On the other hand, M3-ST3G5 has a longer half-life than M2-ST3G5, yet both M2- and M3-ST3G5 localize in the Golgi apparatus. This suggests that the cytoplasmic tail of M2-ST3G5 affects the stability of M3-ST3G5.

In the study presented here, we investigated M3-ST3G5 as well as β-1,4-N-acetylglucosaminyltransferase I (B4GnT1) (B4GN1), which catalyzes GM2, GD2 and GA2 synthesis (Nagata et al. 1992) and examined their ER export and Golgi-retention signals. The M3-ST3G5(N)-GFP chimeric protein and the M3-ST3G5 full-length protein were exported from the ER, dependent on its 2R3R[X]5R[K] sequence. However, only B4GnT1(N)-GFP, and not the full-length protein, was exported from the ER, dependent on the 2R3R[X]5R[K] motif. Moreover, we found that the 2R3R[X]5R[K] sequence of M3-ST3G5 is essential for its Golgi retention and is necessary for the maturation of N-glycans.

Results

ER export signals in M3-ST3G5(N)-GFP and B4GN1(N)-GFP chimeric proteins

It has been reported that the [R[K][X][R/K]] motif, which is located just above the transmembrane domain in many glycosyltransferases, functions as an ER exporting signal through its interaction with the small GTPase Sar1 (Giraudo and Maccioni 2003). However, this finding was described on the basis of experiments on cells transiently expressed with chimeric proteins in which the luminal regions of the glycosyltransferases were replaced with GFP, rather than full-length proteins. Accordingly, we investigated the function of the [R[K][X][R/K]] motif in the full-length versions of two glycosyltransferases, M3-ST3G5 and B4GN1, both of which are involved in ganglioside synthesis.

First, to confirm the previous finding, we transiently expressed a chimeric protein for the NH2-terminal of M3-ST3G5 or B4GN1 containing transmembrane region and GFP tag, M3-ST3G5(N)-GFP and B4GN1(N)-GFP, in CHO-K1 cells and observed their subcellular localization by fluorescence microscopy (Figure 1A–C). Both from the Golgi to the ER. To date, the well-defined Golgi-retention signal is the only (F/L)[L/A][X][R/K] sequence in yeast Kre6 known to be involved in the synthesis of beta-1,6 glycans (Tu et al. 2008). This signal sequence is necessary for interaction of the enzyme with the sorting protein Vps74, which binds to the heptameric COPI coat complex. However, this sequence is not conserved among mammalian glycosyltransferases. Furthermore, the expression of mammalian homologs of Vps74, Golph3 and Golph3L cannot be recovered the defect of Golgi retention of Kre6 in the vps74A mutant (Tu et al. 2008). Thus, we must entertain the possibility that other Golgi-retention signals or mechanisms may function in mammalian glycosyltransferases.

ST3Gal5 (ST3G5), which catalyzes GM3 and GM4 synthesis, has three isoforms (M1-, M2- and M3-ST3G5), each having a distinct length in its NH2-terminal cytoplasmic tail and a common transmembrane and luminal domains (Uemura et al. 2009, 2014). M1-ST3G5, in which the NH2-terminus is the longest, stably localizes in the ER through its arginine (R)-based motif (Uemura et al. 2009). The R-based motif is only located in the extended NH2-terminus of M1-ST3G5. The R-based motif has to be positioned distally from a lipid bilayer to function as a retrograde transport signal (Shikano and Li 2003). Since the mutation of R-based motif leads to the Golgi localization of M1-ST3G5, the R-based motif function as only a retrograde transport signal, but not an ER export signal. Thus, we assumed that M1-ST3G5 exports from ER through the [R[K][X][R/K]] motif as well as M2- and M3-ST3G5. On the other hand, M3-ST3G5 has a longer half-life than M2-ST3G5, yet both M2- and M3-ST3G5 localize in the Golgi apparatus. This suggests that the cytoplasmic tail of M2-ST3G5 affects the stability of M3-ST3G5.
M3-ST3G5(N)-GFP and B4GN1(N)-GFP localized to a compact juxtanuclear reticulum. These proteins co-localized with GM130, a Golgi apparatus marker protein (Supplementary data, Figure S1A and B). The [R/K][X][R/K] sequences correspond to R2R and R5R in M3-ST3G5 and B4GN1, respectively (Figure 1A). Accordingly, we generated M3-ST3G5(N)-GFP R2A/R3A and B4GN1(N)-GFP R5A/R6A mutants by site-directed mutagenesis and examined their subcellular localizations. The M3-ST3G5(N)-GFP R2A/R3A mutant mainly exhibited a reticular fluorescence pattern that was apparent in the perinuclear and cytosolic regions and co-localized with ER proteins containing the ER retention signal KDEL (Figure 1B and Supplementary data, Figure S1A). Thus, the mutant localized in both the Golgi apparatus and ER (Golgi/ER) or the ER. This result was similar to the previous report (Giraudo and Maccioni 2003). On the other hand, the B4GN1(N)-GFP R5A/R6A mutant localized in not only the Golgi/ER and the ER but also the Golgi apparatus (Figure 1C and Supplementary data, Figure S1B), suggesting that R5R is not enough for an ER exporting signal in B4GN1. As shown in Figure 1A, the second amino acid of B4GN1 is a basic amino acid (R) in addition to RXXR. To clarify whether R is also involved in the ER export of B4GN1, we constructed B4GN1(N)-GFP R2A and R2A/R5A/R6A mutants and observed their subcellular localizations. The B4GN1(N)-GFP R2A mutant localized in the Golgi apparatus, similar to the wild-type (WT) protein, but the R2A/R5A/R6A mutant mainly localized in the ER (Figure 1C and Supplementary data, Figure S1B). These results indicate that the R2R (ST3G5) and RXXR6R (B4GN1) sequences function as ER exporting signals when ST3G5(N)-GFP and B4GN1(N)-GFP are transiently expressed.

Next, we examined whether ST3G5(N)-GFP R2A/R3A and B4GN1(N)-GFP R2A/R5A/R6A mutants stay in the ER when these proteins were stably expressed in CHO-K1 cells (Figure 2 and Supplementary data, Figure S2). Both the M3-ST3G5(N)-GFP WT protein and the B4GN1(N)-GFP WT protein localized in the Golgi apparatus. Surprisingly, the M3-ST3G5(N)-GFP R2A/R3A mutant localized in the Golgi apparatus or the Golgi/ER, whereas the B4GN1(N)-GFP R2A/R5A/R6A mutant mainly localized in the ER. These results indicate that the R2A/R3A mutations in M3-ST3G5(N)-GFP are insufficient to prevent ER export of the stable transfectants, although the RXXR6R sequence in B4GN1(N)-GFP functions as an ER exporting signal.

In M3-ST3G5(N)-GFP, we assumed that other amino acid residues in the cytoplasmic region are also necessary for ER export. The cytoplasmic tail carries 12 amino acid residues in addition to R2R (Figure 1A). We constructed five mutants (M3-ST3G5(N)-GFP R2A/R3A/P4A/S5A, R2A/R3A/L6A/L7A, R2A/R3A/I8A/K9A, R2A/R3A/D10A/I11A, R2A/R3A/C12A/K13A) in which the amino acids behind R2R were substituted for by alanine. We stably expressed these in CHO-K1 cells and observed their subcellular localization (Figure 3A). The M3-ST3G5(N)-GFP R2A/R3A/I8A/K9A and R2A/R3A/C12A/K13A mutants were mainly localized in the Golgi/ER or ER, while the other mutants remained localized in the Golgi apparatus. The M3-ST3G5(N)-GFP R2A/R3A/I8A/K9A and R2A/R3A/C12A/K13A mutants carried substitutions of lysine (underlined), a basic amino acid. We investigated K because the positive charge of Alexa 488-conjugated anti-chicken IgY. The cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were stained with anti-GM130 (a cis-Golgi marker) or anti-KDEL (an ER marker) antibodies and Alexa 594-conjugated anti-mouse IgG. The localization patterns (Golgi, Golgi/ER and ER) were quantified with cell counting, and the quantitative data were expressed as mean values with SD from more than three independent cell counting. Bar, 10 μm.

**Fig. 1.** ER exporting signals of M3-ST3G5(N)-GFP and B4GN1(N)-GFP in transient expression. A, the structure of cDNA constructs used in the present experiment. We constructed plasmids, each encoding a fusion protein of the NH2-terminus of M3-ST3G5 or B4GN1 containing the transmembrane region and GFP (M3-ST3G5(N)-GFP and B4GN1(N)-GFP). TM indicates the transmembrane region. An underline indicates an amino acid residue involved in ER export. B and C, CHO-K1 cells were transiently transfected with M3-ST3G5(N)-GFP WT, B4GN1(N)-GFP WT or their indicated mutated proteins. The cells were fixed, permeabilized with 0.5% SDS in PBS and stained for 1 h with anti-GFP antibodies, followed by incubation with M3-ST3G5(N)-GFP and B4GN1(N)-GFP localized to a compact juxtanuclear reticulum. These proteins co-localized with GM130, a Golgi apparatus marker protein (Supplementary data, Figure S1A and B). The [R/K][X][R/K] sequences correspond to R2R and R5R in M3-ST3G5 and B4GN1, respectively (Figure 1A). Accordingly, we generated M3-ST3G5(N)-GFP R2A/R3A and B4GN1(N)-GFP R5A/R6A mutants by site-directed mutagenesis and examined their subcellular localizations. The M3-ST3G5(N)-GFP R2A/R3A mutant mainly exhibited a reticular fluorescence pattern that was apparent in the perinuclear and cytosolic regions and co-localized with ER proteins containing the ER retention signal KDEL (Figure 1B and Supplementary data, Figure S1A). Thus, the mutant localized in both the Golgi apparatus and ER (Golgi/ER) or the ER. This result was similar to the previous report (Giraudo and Maccioni 2003). On the other hand, the B4GN1(N)-GFP R5A/R6A mutant localized in not only the Golgi/ER and the ER but also the Golgi apparatus (Figure 1C and Supplementary data, Figure S1B), suggesting that R5R is not enough for an ER exporting signal in B4GN1. As shown in Figure 1A, the second amino acid of B4GN1 is a basic amino acid (R) in addition to RXXR. To clarify whether R is also involved in the ER export of B4GN1, we constructed B4GN1(N)-GFP R2A and R2A/R5A/R6A mutants and observed their subcellular localizations. The B4GN1(N)-GFP R2A mutant localized in the Golgi apparatus, similar to the wild-type (WT) protein, but the R2A/R5A/R6A mutant mainly localized in the ER (Figure 1C and Supplementary data, Figure S1B). These results indicate that the R2R (ST3G5) and RXXR6R (B4GN1) sequences function as ER exporting signals when ST3G5(N)-GFP and B4GN1(N)-GFP are transiently expressed.

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basic amino acids such as arginine and lysine is necessary for interactions with Sar1. As expected, the M3-ST3G5(N)-GFP R2A/R3A/K9A and R2A/R3A/K13A mutants localized in the Golgi/ER or ER, and the localization of the M3-ST3G5(N)-GFP R2A/R3A/K9A/K13A mutant exhibited a full ER pattern (Figure 3B and Supplementary data, Figure S3).

Since the M3-ST3G5(N)-GFP K9A/K13A mutant mainly localized in the Golgi/ER, the K9A/K13A mutation also partially affected the ER export (Figure 3B).

These results indicate that the \( \text{R/K}[X]\text{R[K]} \) motif functions as an ER exporting signal in M3-ST3G5 and M3-ST3G5(N)-GFP. We would like to propose an "R/K-based motif" as a new ER exporting signal, since \( \text{R}[X]\text{R[K]} \) in M3-ST3G5 and \( \text{R}[X]\text{R[K]} \) in B4GN1 do not conform to the \( \text{R[K][X]} \) motif.

Functions of an R/K-based motif as an ER export or Golgi-retention signal in full-length M3-ST3G5

To examine whether the R/K-based motif functions as an ER exporting signal in full-length M3-ST3G5 and B4GN1, we constructed M3-ST3G5 R2A/R3A/K9A, R2A/R3A/K13A and R2A/R3A/K9A/

K13A mutants and B4GN1 R2A/R3A/R6A mutants and stably expressed them in CHO-K1 cells. The M3-ST3G5 and B4GN1 proteins were stained with antibodies against ST3G5 (Uemura et al. 2006) and...
B4GN1, respectively (Figure 4 and Supplementary data, Figure S5). Since CHO-K1 cells express only GM3 as gangliosides (Uemura et al. 2014), CHO-K1 cells should possess endogenous ST3G5 proteins but not B4GN1 proteins. However, our anti-ST3G5 antibody used in this study cannot recognize the endogenous ST3G5 probably due to its low expression. As the reference of negative control, we showed the image of CHO-K1 cells staining anti-ST3G5 or anti-B4GN1 antibody in Supplementary data, Figure S4. M3-ST3G5 and B4GN1 WT proteins mainly co-localized with GM130, similar to results for M3-ST3G5(N)-GFP and B4GN1(N)-GFP. The M3-ST3G5 R2A/R3A/K9A and R2A/R3A/K13A mutants localized in the ER, indicating that the R/K-based motif functions as an ER export signal (Figure 4A and Supplementary data, Figure S5A). Unexpectedly, the localization of the M3-ST3G5 R2A/R3A/K9A/K13A mutant could not be examined due to its low expression level (data not shown). In contrast, the B4GN1 R2A/R5A/R6A mutant remained in the Golgi apparatus, indicating that the R/K-based motif is not essential for the ER export of full-length B4GN1 (Figure 4B). These localization patterns were similar in transient expression (Supplementary data, Figures S6 and S7). Reportedly, B4GN1 forms a homodimer through two disulfide bonds, and the dimer formation is necessary for ER export (Li et al. 2000). To confirm the result of this report, we constructed a B4GN1 C80S/C82S mutant in which the dimer formation is inhibited and observed its subcellular localization when stably expressed (Figure 4B and Supplementary data, Figure S5B). The localization of the B4GN1 C80S/C82S mutant was mainly consistent with the staining pattern of the anti-KDEL antibody, indicating that the dimer formation is necessary for B4GN1 to be exported from the ER.

Interestingly, staining of the ST3G5 R2A/R3A mutant was observed not only in the Golgi apparatus but also as several dots in the cytosolic region (Figure 5). These dots partially colocalized with Rab5a (an early endosome marker) or Rab7 (a late endosome marker) (Figure 5 and Supplementary data, Figure S8). In the quantitative data, we counted the number of cells possessing vesicles that both M3-ST3G5 and Rab5a are positive. M3-ST3G5 R2A/R3A mutant co-localized with Rab5a in all cells, whereas M3-ST3G5 WT co-localized with Rab5a in about 20% of the cells (Figure 5B). These results indicate that the R2A/R3A mutant is transported from the Golgi apparatus to the early endosome and late endosome. Moreover, the R2K/R3K mutant showed a similar localization pattern to the R2A/R3A mutant. These results suggest that, among basic amino acids, the arginine residue is important for the Golgi retention of M3-ST3G5. The M3-ST3G5 R2A, R3A and K9A/K13A mutants also partially colocalized with Rab5a or Rab7 (Figure 5 and Supplementary data, Figure S8). The rate of co-localization of ST3G5 R2A, R3A or K9A/K13A mutant and Rab5a also increased about two to four times (Figure 5B), indicating that the R/K-based motif functions as a Golgi-retention signal.

**Effects of dysfunction in the R/K-based motif on the structure of N-glycans and enzyme stability**

Mouse ST3G5 is a glycoprotein with three N-glycans in its luminal domain (Uemura et al. 2006). Most N-glycans on M3-ST3G5 are Endoglycosidase H (Endo H)-resistant, whereas N-glycans on M2-ST3G5, which exhibits a lower stability compared with M3-ST3G5, are Endo H-sensitive, indicating that they are high-mannose-type glycans (Uemura et al. 2009). Accordingly, we examined whether the change of localization by the R/K-based motif mutations in M3-ST3G5 affects the structure of N-glycans. The fractions of integral membrane proteins were prepared from CHO-K1 cells expressing WT proteins or R2A/R3A, R2K/R3K, R2A, R3A, K9A/K13A, R2A/R3A/K9A or R2A/R3A/K13A mutants. The fractions were treated with Endo H or peptide N-glycosidase F (PNGase F) and subjected to immunoblotting using anti-ST3G5 antibodies (Figure 6). WT M3-ST3G5 was detected as bands of 43 and 44.5 kDa, which were resistant to Endo H treatment, whereas all bands shifted to 36 kDa after treatment with PNGase F. These results indicated that most M3-ST3G5 carried matured N-glycans modified in the Golgi apparatus. In the ST3G5 R2A/R3A mutant, the band of 43 kDa increased and the band of 44.5-48 kDa remarkably decreased. Most bands of the R2A/R3A mutant shifted to 36 kDa after Endo H treatment.

**Fig. 4.** Function of R/K-based motif in full-length M3-ST3G5 as an ER exporting signal. CHO-K1 cells stably expressing WT ST3G5, WT B4GN1 or the indicated mutant were fixed, permeabilized with 0.5% SDS in PBS, and stained for 1 h with anti-ST3G5 antibodies **A** or anti-B4GN1 antibodies **B**, followed by incubation with Alexa 488-conjugated anti-rabbit IgG. Cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were stained with anti-GM130 (a cis-Golgi marker) or anti-KDEL (ER marker) antibodies and Alexa 594-conjugated anti-mouse IgG. Bar, 10 μm.
Fig. 5. Function of the R/K-based motif in full-length M3-ST3G5 as a Golgi apparatus retention signal. CHO-K1 cells stably expressing a ST3G5 R2A/R3A, R2K/R3K, R2A, R3A or K9A/K13A mutant were fixed, permeabilized with 0.5% SDS in PBS and stained for 1 h with anti-ST3G5 antibodies, followed by incubation with Alexa 488-conjugated or Alexa 594-conjugated anti-rabbit IgG. Cells were visualized by confocal laser-scanning microscopy. For colocalization studies, cells were stained with anti-GM130 (a cis-Golgi marker) and Alexa 594-conjugated anti-mouse IgG. To visualize the early endosome, we used CellLight® Early Endosomes-GFP (Rab5a-GFP). Arrows indicate merged dots. Bar, 10 μm. This is figure available in black and white in print and in color at glycobiology online.
indicating that most of the R2A/R3A mutant proteins carried immature N-glycans carrying the high-mannose type. The amounts of the Endo H-resistant bands in the R2K/R3K, R2A, R3A and K9A/K13A mutants also decreased compared with that of the WT protein (Figure 6A and B). The R2A/R3A/K9A and R2A/R3A/K13A mutants carried only Endo-H sensitive N-glycans, supporting the contention that these mutants cannot be exported from the ER. Among the mutants, which are transported from the ER to the Golgi apparatus, the amount of the Endo H-resistant band of the R2A/R3A mutant decreased most notably. These results suggest that Golgi retention could be associated with the maturation of N-glycans in ST3G5.

Next, we investigated whether the loss of Golgi retention affects enzyme stability (Figure 7). CHO-K1 cells expressing WT M3-ST3G5 or R2A/R3A, R2K/R3K, R2A, R3A, K9A/K13A, R2A/R3A/K9A or R2A/R3A/K13A mutant were cultured for 3 h with or without cycloheximide (CHX), an inhibitor of protein synthesis, and total cell lysates and fractions of integral membrane proteins were prepared. The total cell lysates and membrane protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were subjected to immunoblotting using anti-β-actin and anti-ST3G5 antibodies, respectively. All fractions of integral membrane proteins were treated with PNGase F to compare the protein levels of each sample accurately. After a 3-h treatment with CHX, a band representing WT ST3G5 was detected. This result was similar to that in a previous report of a pulse-chase experiment (Uemura et al. 2009). In contrast, the amount of the R2A/R3A mutant remarkably decreased after treatment with CHX.

Figure 6. The structures of N-glycans on WT M3-ST3G5 and its mutants. A The fractions of integral membrane proteins were prepared from CHO-K1 cells stably expressing WT M3-ST3G5 or R2A/R3A, R2K/R3K, R2A, R3A, K9A/K13A, R2A/R3A/K9A or R2A/R3A/K13A mutant. The proteins were treated with (+) or without (−) Endo H (E) or PNGase F (P) and were analyzed by immunoblotting with anti-ST3G5 antibodies. B The amounts of Endo H resistant bands were quantified with ImageJ software. The quantitative data were expressed as mean values with SD from more than three independent experiments. * P < 0.005 versus WT.
The roles of R/K-based motif in glycosyltransferases

In this study, we attempted to clarify the regulation of glycosphingolipid biosynthesis in the Golgi apparatus by analyzing the intracellular dynamics of ST3G5 and B4G1N. It had been reported that the [R/K](X)[R/K] motif of the cytoplasmic tail functions as an ER export signal in many glycosyltransferases (Giraudo and Maccioni 2003). Certainly, we could confirm that the R2A/R3A mutations in the ST3G5(N)-GFP chimeric protein, which are defective in the [R/K](X)[R/K] motif, caused the relatively strong suppression of ER export in transient expression system (Figure 1B). However, we also found that the motif, caused the relatively strong suppression of ER export in transiently expressed condition. In fact, the R2A/R3A mutations caused the ER accumulation of ST3G5(N)-GFP in only the high expressed condition. Similarly, B4G1N(N)-GFP needs the 2RXX5R6R sequence, but not the 5R6R sequence, for ER export (Figure 2C), indicating that B4G1N(N)-GFP is also transported from the ER, dependent on the R/K-based motif. However, the ER export of full-length B4G1N was slightly decreased by a mutation in the R/K-based motif (Figure 4B). These results suggest that the lumenal domain of B4G1N is involved in ER export. B4G1N forms a homodimer via two disulfide bonds (80Ca and 82C residues) in the luminal domain (Li et al. 2000), and a C80S/C82S mutation suppressed ER export (Figure 4B). This result is consistent with a previous report (Li et al. 2000). Since the dimer formation is necessary for the protein to be folded properly, it is likely that the C80S/C82S mutant was not concentrated in COPII-coated vesicles due to its abnormal structure. Thus, there may be cargo receptors that recognize the proper conformation of B4G1N and concentrate it in COPII-coated vesicles.

It has been proposed that the [R/K](X)[R/K] motif functions by interacting with the small GTPase Sar1. We assume that, similar to the [R/K](X)[R/K] motif, the R/K-based motif interacts with Sar1. However, we wonder if just the interaction between the R/K-based motif and Sar1 is enough for cargo proteins to be concentrated in COPII-coated vesicles. The formation of COPII-coated vesicles begins with the conversion from Sar1-GDP to Sar1-GTP by the guanine nucleotide exchange factor, Sec12 (Barlowe and Schekman 1993). The Sar1-GTP is anchored to the ER membrane and recruits the Sec23–Sec24 complex through an interaction with Sec23 (Yoshihisa et al. 1993). The Sar1–Sec23/Sec24 complex generally recruits many cargo proteins by interacting with Sec23 or Sec24 to generate a pre-budding complex (Aridor et al. 1998; Kuehn et al. 1998; Springer and Schekman 1998; Peng et al. 1999; Votsmeier and Gallwitz 2001). Finally, the pre-budding complexes are polymerized by Sec13–Sec31 complexes, resulting in COPII-coated vesicles (Barlowe et al. 1994; Matsuoka et al. 1998). Importantly, Sec32 associated with Sar1 exhibits GTPase-activating protein activity for Sar1-GTP (Yoshihisa et al. 1993). As soon as Sar1 forms the pre-budding complex, Sar1-GTP is hydrolyzed by Sec23 and is dissociated from the pre-budding complex (Sato and Nakano 2005). The pre-budding complex without Sar1-GTP is unstable, but Sar1 is immediately reactivated by Sec12 and is again incorporated into the pre-budding complex. When the pre-budding complex without Sar1 contains unfolded proteins, the complex breaks before the reassembly of Sar1-GTP due to the weak interaction between the unfolded proteins and Sec24. The GTP/GDP cycle of Sar1 is important for the selective transport of cargo proteins (Sato and Nakano 2005). If the R/K-based motif interacts with only Sar1, the cargo proteins should dissociate from the pre-budding complex when Sar1-GTP is converted to Sar1-GDP, resulting in the cargo proteins not being efficiently concentrated in COPII-coated vesicles. Thus, we have speculated that the R/K-based motif functions as the only guide to the pre-budding complex through interactions with Sar1. Subsequently, the glycosyltransferases may be transferred to other cargo receptors carrying ER export signals in the pre-budding complex.

Most glycosyltransferases carry N-glycans on the large catalytic domains in the luminal side. Mouse ST3G5 carries three N-glycans on 180N, 224N and 334N residues (Uemura et al. 2006). Since all

![Figure 7. Protein stability of WT M3-ST3G5 and its mutants.](https://academic.oup.com/glycob/article-abstract/25/12/1410/2355502)

Discussion

In this study, we attempted to clarify the regulation of glycosphingolipid biosynthesis in the Golgi apparatus by analyzing the intracellular dynamics of ST3G5 and B4G1N. It had been reported that the [R/K](X)[R/K] motif of the cytoplasmic tail functions as an ER export signal in many glycosyltransferases (Giraudo and Maccioni 2003). Certainly, we could confirm that the R2A/R3A mutations in the ST3G5(N)-GFP chimeric protein, which are defective in the [R/K](X)[R/K] motif, caused the relatively strong suppression of ER export in transient expression system (Figure 1B). However, we also found that the ST3G5(N)-GFP R2A/R3A mutant mainly localized in the Golgi apparatus when the mutant was stably expressed (Figure 2A). These results suggest that the [R/K](X)[R/K] motif is insufficient as an ER export signal in ST3G5(N)-GFP. In general, the expression level of transiently expressed condition. In fact, the R2A/R3A mutations caused the ER accumulation of ST3G5(N)-GFP in only the high expressed condition. Similarly, B4G1N(N)-GFP needs the 2RXX5R6R sequence, but not the 5R6R sequence, for ER export (Figure 2C), indicating that B4G1N(N)-GFP is also transported from the ER, dependent on the R/K-based motif. However, the ER export of full-length B4G1N was slightly decreased by a mutation in the R/K-based motif (Figure 4B). These results suggest that the lumenal domain of B4G1N is involved in ER export. B4G1N forms a homodimer via two disulfide bonds (80Ca and 82C residues) in the luminal domain (Li et al. 2000), and a C80S/C82S mutation suppressed ER export (Figure 4B). This result is consistent with a previous report (Li et al. 2000). Since the dimer formation is necessary for the protein to be folded properly, it is likely that the C80S/C82S mutant was not concentrated in COPII-coated vesicles due to its abnormal structure. Thus, there may be cargo receptors that recognize the proper conformation of B4G1N and concentrate it in COPII-coated vesicles.

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N-glycans of ST3G5 are necessary for its activity, the N-glycans are involved in the maintenance of the structure and/or the folding (Uemura et al. 2006). We have found that a specific amino acid can substitute for the function of N-glycans (Uemura et al. 2006). Therefore, H177D/N180S, N224K or T336Q mutants maintain enzyme activity similar to that of WT enzymes, although these mutants are defective at each N-glycan. Interestingly, the amount of secretion to the culture medium of an H177D/N180S mutant was decreased compared with that of WT, N224K and T336Q mutated proteins, suggesting that the ER export of the H177D/N180S mutant is abnormal. Glycosyltransferases exhibit different substrate specificities and the structures of luminal domains are varied. If the cargo receptors specifically recognize each structure in the luminal domain, many kinds of cargo receptors are necessary for transport. However, if the cargo receptors can recognize a common structure like N-glycans, the number is drastically reduced. Our proposed idea is that cargo receptors having lectin activity are involved in the ER export of glycosyltransferases.

The M3-ST3G5 R2A/R3A mutant could not stay in the Golgi apparatus and partly localized in late and early endosomes (Figure 5 and Supplementary data, Figure S8). The M3-ST3G5 K9A/K13A mutant also localized in not only the Golgi apparatus but also in endosomes (Figure 5). Considering the Golgi maturation model, retrograde transport between Golgi cisternae is necessary for glycosyltransferases to stay in the Golgi apparatus. It has been reported that the conserved oligomeric Golgi (COG) complex, which is a hetero-oligomer consisting of eight subunits (Cog1–Cog8), is involved in retrograde transport (Ungar et al. 2002). Cog2-null mutant CHO cells exhibit defective GM3 and sphingomyelin synthesis (Spessott et al. 2010a, 2010b). In the Cog2-null cells, the localization of ST3G5 and sphingomyelin synthase 2 is abnormal, and endogenous sphingomyelin synthase 2 can be observed in the cytosol as dots (Spessott et al. 2010a). The localization pattern is very similar to that of the M3-ST3G5 R2A/R3A mutant. This result indicates that glycosyltransferases localize in endosomes when retrograde transport is defective. Thus, we speculate that mutations in the R/K-based motif (R2A/R3A and K9A/K13A) also cause defects in retrograde transport. In addition, the function of the R/K-based motif as a Golgi-retention signal is found only in the full-length protein as the M3-ST3G5(N)-GFP R2A/R3A and K9A/K13A mutants localized in the Golgi apparatus similar to the WT (Figures 2 and 3). This result implies that the R/K-based motif does not have a major function such as being a binding partner of COPI-coated proteins in retrograde transport. This raises the possibility that the luminal domain of ST3G5 accelerates the export from the Golgi apparatus, while the R/K-based motif suppresses the transport. Consequently, ST3G5 can stay in the Golgi apparatus by the retrograde transport system. Moreover, we surmise that the long cytoplasmic tail of the M2-ST3G5 isoform inhibits such functions of the R/K-based motif and shortens its stability (Uemura et al. 2009).

The ST3G5 R2A/R3A mutant carries immature (Endo H sensitive) N-glycans, although the WT carries mature (Endo H resistant) N-glycans, suggesting that retrograde transport is required for the maturation of N-glycans (Figure 6). This result implies that the

![Fig. 8. Intracellular dynamics of ST3G5 and B4GN1.](https://academic.oup.com/glycob/article-abstract/25/12/1410/2355502/fig8)
maturation of N-glycans progresses gradually while repeating the retrograde transport between Golgi cisterna. B4GN1 exists in not only the intracellular membrane but also the culture medium. The NH2-terminal region of B4GN1 containing the transmembrane domain is cleaved by cathepsin D or another unknown cathepsin D-like protease, and the B4GN1 is secreted to culture medium (Jaskiewicz et al. 1996). The most secreted form carries mature N-glycans, while the intracellular form carries both mature and immature N-glycans (Jaskiewicz et al. 1996). It is likely that only the B4GN1 repeating the retrograde transport cycle is secreted to culture medium, selectively and rapidly. If the activity of the cleavage enzyme depends on the degree of maturation of N-glycans of its substrates, the mechanism of the selective transport can be explained. Since many plasma membrane proteins carry N-glycans, these proteins may also pass through the maturation cycle of N-glycans in the Golgi apparatus. Thus, it is important to clarify these checkpoints to judge the maturation of N-glycans of glycoproteins and export them selectively from trans-Golgi. Moreover, it is apparent from the COG complex study that the retrograde transport of glycosyltransferases is necessary to synthesize N-glycans efficiently. The involvement of the R/K-based motif in retrograde transport presents a new insight into the potential regulatory mechanism of GM3 and GM4 synthesis (Figure 8).

Material and methods

Plasmids

The pSU157 plasmid encoding fusion proteins of the NH2-terminus of mouse M3-ST3G5 and GFP (M3-ST3G5(N)-GFP) was constructed using mouse M3-ST3G5/pcDNA3.1(+) (Uemura et al. 2006) and primers 5′-CCCTATTGACGTCAATGACGG-3′ and 5′-CCCGGGCAGGGTCCGACATGTCATTTCG-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pSU151. The 0.6-kb EcoRI-SmaI fragment of pSU151 was then cloned into the EcoRI-SmaI site of a pEGFP-N1 vector (BD Bioscience Clontech Laboratories, Palo Alto, CA) to generate pSU157.

The pKM9 (B4GN1(N)-GFP) plasmid was constructed using mouse brain cDNA and primers 5′-ATGCGCCGTAGACGGGCCGCGCTCTC-3′ and 5′-TACAGTGCATGACGGGGGCTGTC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM1. To insert the kozak sequence (GCCACC) of start codon (ATG), B4GN1 was amplified using pKM1 and primers 5′-GGATCCGCCACCATGGCCGCTAGACGCCGGGCGCCCTC-3′ and 5′-TGGATCCTCGGGCTGTCGATGACTGCATTAGCC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM3. The 1.6-kb BamHI fragment of pKM3 was then cloned into the BamHI site of pEGFP-N1 vector to generate pKM5. Moreover, NH2-terminus of B4GN1 was amplified using pKM5 and primers 5′-GAGATCTGGCAAGGTCAGTCTCAATGACGTCAATGACGG-3′ and 5′-CCCGGGGCAAGGTCTAGCAGATCGAGTCTC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM7. The 0.7-kb EcoRI-SmaI fragment of pKM7 was then cloned into the EcoRI-SmaI site of a pEGFP-N1 vector to generate pKM9.

To produce a lentiviral, the pKM150 (M3-ST3G5(N)-GFP/CSII-CMV-RI) plasmid was constructed using pSU157 and primers 5′-CACCATGAGAAGACCCAGCTTGTTAATAAAAGA-3′ and 5′-GCTAGACCGCCGGGCGCCCTC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM1. To insert the kozak sequence (GCCACC) of start codon (ATG), B4GN1 was amplified using pKM1 and primers 5′-GGATCCGCCACCATGGCCGCTAGACGCCGGGCGCCCTC-3′ and 5′-TGGATCCTCGGGCTGTCGATGACTGCATTAGCC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM3. The 1.6-kb BamHI fragment of pKM3 was then cloned into the BamHI site of pEGFP-N1 vector to generate pKM5. Moreover, NH2-terminus of B4GN1 was amplified using pKM5 and primers 5′-GAGATCTGGCAAGGTCAGTCTCAATGACGTCAATGACGG-3′ and 5′-CCCGGGGCAAGGTCTAGCAGATCGAGTCTC-3′. The resulting fragments were cloned into a pGEM-T Easy vector to generate pKM7. The 0.7-kb EcoRI-SmaI fragment of pKM7 was then cloned into the EcoRI-SmaI site of a pEGFP-N1 vector to generate pKM9.

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Table I. Plasmids used in this study

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CA to generate pKM144. The CSII-CMV-MCS plasmid was converted to a Gateway® destination vector (CSII-CMV-RfA) using the Gateway® vector conversion system (Life Technologies). The M3-ST3G5(N)-GFP of pKM144 was inserted into the lentiviral expression vector, CSII-CMV-RfA, using the LR clonase reaction to generate pKM150. The pKM155 (B4GN1(N)-GFP), pFS311 (M3-ST3G5) (Uemura et al. 2014) and pKM91 (B4GN1) plasmids were created by similar procedures.

Point mutants of M3-ST3G5(N)-GFP, B4GN1(N)-GFP, M3-ST3G5 and B4GN1 in Table I were created by site-directed mutagenesis using PrimeSTAR® Mutagenesis Basil Kit (Takara, Shiga, Japan) according to the manufacturer’s instructions.

Cell culture
Chinese hamster ovary (CHO)-K1 cells were cultured in the nutrient mixture F-12 HAM (N6558; Sigma, St Louis, MO), which was supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, respectively. CHO-K1 cells were transiently transfected using Lipofectamine® 2000 (Life Technologies) according to the manufacturer’s instructions. The stable transfectants were established using lentiviral vectors.

Preparation of lentiviral vectors
Twenty-four hours before transfection, 293T cells (0.5 × 10⁶) were seeded in a poly-lysine coated 60-mm dish. The 293T cells were transfected with 3 μg of CSII-CMV-RfA plasmid cloning M3-ST3G5 (N)-GFP, B4GN1(N)-GFP, M3-ST3G5 or B4GN1 (WT or mutants) in Table I, 1.5 μg of pCAG-HIVgp plasmid and 1.5 μg of pCMV-VSV-G-RSV-Rev plasmid using Lipofectamine 2000. The cells were incubated for 24 h at 37°C, then the medium was replaced with 4 mL of high-glucose DMEM containing 10 μM Forskolin. Approximately 32 h after transfection, the culture temperature was shifted from 37 to 32°C to maintain virus stability. The lentivirus-containing supernatant was harvested 48 h after transfection and centrifuged at 200 × g for 3 min to remove living cells. The lentivirus supernatant was used immediately for experiments or snap-frozen in liquid nitrogen and stored at −80°C for later applications. For the lentiviral transduction of CHO-K1 cells, 4 mL of the lentiviral supernatant was added to 0.25 × 10⁶ 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.

Immunofluorescent microscopy
Transfected cells were cultured on cover glass then fixed for 15 min with 3.7% formaldehyde in phosphate-buffered saline (PBS) at room temperature. After being rinsed with PBS, the cells were permeabilized, if necessary, by 0.5% SDS or 0.5% TritonX-100 in PBS, treated with a blocking solution (10 mg/mL bovine serum albumin in PBS) for 30 min, then incubated for 1 h with anti-ST3G5 (Uemura et al. 2006), anti-B4GN1 that had been raised against the lumenal domain (28–533 aa) (rabbit polyclonal antibodies), anti-KDEL (StressGen Bioreagents Corp., Victoria, BC, Canada) or anti-GM130 antibodies (BD Bioscience, Franklin Lakes, NJ) diluted 1:1000 with a blocking solution. After three washes in PBS, the cells were incubated for 30 min with Alexa 488-conjugated anti-rabbit IgG antibodies (Life Technologies) or Alexa 594-conjugated anti-mouse IgG antibodies (Life Technologies) diluted in the blocking solution to 5 μg/mL. Coverslips were washed in PBS three times and mounted onto glass slides using ProLong™ Gold antifade reagent (Life Technologies). Slides were analyzed by fluorescence microscopy FV1000 (Olympus, Tokyo, Japan).

To visualize early endosomes, we used CellLight® Early Endosomes-GFP BacMam2.0 reagent (Life Technologies). The transfected cells treated with the reagent were cultured on cover glass for 16 h, fixed for 15 min with 3.7% formaldehyde in PBS at room temperature, and permeabilized by 0.5% SDS in PBS. The early endosomes-GFP (Rab5a-GFP) was detected using anti-GFP antibodies (IgY fraction; diluted 1:100 with the blocking solution; Aves Labs Inc., Tigard, OR) and Alexa 488-conjugated anti-chicken IgY antibodies (diluted 1:400 with the blocking buffer; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) by the same manner.

Preparation of cell lysates
Cells were washed twice with ice-cold 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× Complete™ protease inhibitor mixture (ethylenediaminetetraacetic acid-free; Roche Diagnostics)], and lysed by sonication. After removal of cell debris by centrifugation at 1000 × g for 3 min at 4°C, the supernatant (total cell lysates) was centrifuged at 100,000 × g for 1 h at 4°C. The precipitates (integral membrane proteins) 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 10% 2-mercaptoethanol and incubated for 5 min at 37°C. Endo H or PNGase F (New England Biolab, Beverly, MA) digestion was performed on the integral membrane proteins at 37°C for 1 h according to the manufacturer’s recommended procedure.

Immunoblotting
Immunoblots were performed on the total cell lysates and the fractions of 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-ST3G5 antibodies or anti-β-actin antibodies (Sigma). After a wash, the membrane was incubated for 1 h with a 1:20,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (ab®/ fragment (GE Healthcare Bio-Sciences, Piscataway, NJ). Labeling was detected using an ECL select™ kit (GE Healthcare Bio-Sciences).

CHX chase
CHO-K1 cells stably expressing with GM3S WT, R2A/R3A, R2K/R3K, R2A and R3A mutants were cultured in the nutrient mixture F-12 HAM with or without 50 μg/mL CHX for 3 h at 37°C. The total lysates and fractions of integral membrane proteins were prepared as described above.

Supplementary data
Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement
None declared.
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Abbreviations
B4GN1, B4GalNT1/β-1,4-N-acetylglactosaminyltransferase 1; CHX, cycloheximide; COG, conserved oligomeric Golgi; Endo H, endoglycosidase H; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PNGase F, peptide:N-glycosidase F; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ST3Gn, ST3GAl3/ST3 β-galactoside α-2,3-sialyltransferase 5; WT, wild type.

References


