Blood group P1 antigen–bearing glycoproteins are functional but less efficient receptors of Shiga toxin than conventional glycolipid-based receptors

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Kanta Morimoto, Noriko Suzuki, Isei Tanida, Soichiro Kakuta, Yoko Furuta, Yasuo Uchiyama, Kentaro Hanada, Yusuke Suzuki, and Toshiyuki Yamaji

From the 1Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan, the 2Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, Tokyo, Japan, the 3Department of Cellular and Molecular Neuropathology and the 4Laboratory of Morphology and Image Analysis, Biomedical Research Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, and the 5Graduate School of Science and Technology, Niigata University, Niigata, Japan

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Shiga toxin (STx) is a virulence factor produced by enterohemorrhagic Escherichia coli. STx is taken up by mammalian host cells by binding to the glycosphingolipid (GSL) globotriaosylceramide (Gb3; Galα1-4Galβ1-4Glc-ceramide) and causes cell death after its retrograde membrane transport. However, the contribution of the hydrophobic portion of Gb3 (ceramide) to STx transport remains unclear. In pigeons, blood group P1 glycan antigens (Galα1-4Galβ1-4GlcNAc–) are expressed on glycoproteins that are synthesized by α1,4-galactosyltransferase 2 (pA4GalT2). To examine whether these glycoproteins can also function as STx receptors, here we constructed glycan-remodeled HeLa cell variants lacking Gb3 expression but instead expressing pA4GalT2-synthesized P1 glycan antigens on glycoproteins. We compared STx binding and sensitivity of these variants with those of the parental, Gb3-expressing HeLa cells. The glycan-remodeled cells bound STx1 via N-glycans of glycoproteins and were sensitive to STx1 even without Gb3 expression, indicating that P1-containing glycoproteins also function as STx receptors. However, these variants were significantly less sensitive to STx than the parent cells. Fluorescence microscopy and correlative light EM revealed that the STx1 B subunit accumulates to lower levels in the Golgi apparatus after glycoprotein-mediated than after Gb3-mediated uptake but instead accumulates in vacuole-like structures probably derived from early endosomes. Furthermore, coexpression of Galα1-4Gal on both glycoproteins and GSLs reduced the sensitivity of cells to STx1 compared with those expressing Galα1-4Gal only on GSLs, probably because of competition for STx binding or internalization. We conclude that lipid-based receptors are much more effective in STx retrograde transport and mediate greater STx cytotoxicity than protein-based receptors.

Shiga toxin (STx) is a well-studied bacterial exotoxin and is produced by enterohemorrhagic Escherichia coli and Shigella dysenteriae. The toxin causes watery diarrhea, hemorrhagic colitis, and life-threatening hemolytic-uremic syndrome. STx consists of one A subunit (STxA) and five B fragments that constitute the homopentameric B subunit (STxB) (1, 2). Each B fragment possesses receptor-binding sites that bind to specific glycan structures on host cells (3). After binding to the surfaces of host cells, the toxin is internalized using several different pathways, including clathrin-dependent and -independent endocytosis (4, 5). Following entry into the cells, STx is transported into early and recycling endosomes, the trans-Golgi network (TGN), and then the endoplasmic reticulum via the retrograde trafficking route (6, 7). Finally, the cleaved catalytic STxA fragment translocates into the cytosol (8, 9), where it cleaves 28S rRNA, leading to the inhibition of protein biosynthesis and consequent cell death (10). Thus, the binding of STx to cell surface glycans is an important initial step for the entry and intracellular trafficking of this toxin.

STxB binds to a Galα1-4Gal sequence as a minimum essential structure on host cells (11, 12). In mammalian cells, this glycan epitope is found on glycosphingolipids (GSLs) such as globotriaosylceramide (Gb3; Galα1-4Galβ1-4Glc-ceramide). Gb3 is biosynthesized from lactosylceramide (LacCer) by α1,4-galactosyltransferase, encoded by the A4GalT gene in mammals (Fig. 1A) (13, 14). A4GalT-null mutant mice survived with no abnormal behavior even after injection of a lethal level of STx (15). Disruption of Gb3 precursor enzymes also conferred resistance to STx in HeLa cells, including UDP-glucose-ceramide glucosyltransferase (UGCG), which produces glucosylceramide (GlcCer), and β1,4-galactosyltransferase 5 (B4GalT5), which mainly produces LacCer (16). Therefore, Gb3 or GSLs containing the Galα1-4Gal epitope are the functional receptors of STx in mammals.

The Galα1-4Gal glycan epitope is found only on GSLs in mammals, but in some birds, such as pigeons and gulls, it is also found on glycoproteins with the sequence Galα1-4Galβ1-4GlcNAc, called the blood group P1 antigen, at the nonreducing termini of N-glycans (17–20). Unlike in the human genome, which contains only one A4GalT gene (hA4GalT), several A4GalT-like genes are located in tandem in the pigeon genome (21–23). One of these genes, designated pigeon A4GalT (pA4GalT2), is homologous to the gene encoding α1,4-galactosyltransferase, which acts on glycoproteins (24). However, it is
unknown whether the enzyme also acts on GSLs. In addition to pigeon A4GalT1 (pA4GalT1), which acts on GSLs, pigeons and some other birds are likely to express the Galα1-4Gal epitope both on glycolipids and glycoproteins (Fig. 1A).

Several groups have reported that one of the STx variants, STX1, binds to Galα1-4Gal on pigeon egg white glycoproteins (25, 26). STX1 also binds to multivalent O-glycans with P1 determinants on mucin-type fusion proteins in CHO-K1 cells transfected with genes encoding pA4GalT2 and the core 2 β1,6-N-acetylglucosaminyltransferase (C2GnT1) (27). These results suggest that P1-containing glycoproteins potentially function as STx receptors, although this has not been investigated yet. To date, the ceramide moiety of Gb3 is thought to be important for retrograde transport of STx to an appropriate site for exerting cytotoxic activity (28, 29), but there is no conclusive evidence that a ceramide moiety is essential for STx-mediated cytotoxicity. Thus, a comparison of the properties of Gb3 and P1-containing glycoproteins as STx receptors may clarify this issue.

In this study, we constructed a GSL-deficient and P1-expressing HeLa cell clone by disrupting both UGCG and galactosylceramide (GalCer) synthase CGT genes and expressing pA4GalT2 (ΔUGCG/CGT-pA4GalT2 cells). Using the established cells, we demonstrated that STx bound to the cells via P1-containing glycoproteins, and the cells became sensitive to STX1 toxicity even without Gb3 expression. However, compared with that of the parent HeLa cells, the sensitivity of the ΔUGCG/CGT–pA4GalT2 cells was much lower and STx transport into the Golgi was interrupted. Moreover, we also found that the P1 antigens on glycoproteins prevented Gb3-mediated toxin cytotoxicity when both P1-containing glycoproteins and Gb3 were coexpressed.

Results

Pigeon A4GalT2 synthesizes Gb3 with a low activity

One of the pigeon A4GalTs, now termed pA4GalT2, was originally isolated as an enzyme that acts on the Galβ1-4GlcNAc sequence at the nonreducing termini of glycans on glycoproteins to produce a Galα1-4Galβ1-4GlcNAc sequence (i.e. P1 antigen) (24). However, it was not clearly demonstrated whether this enzyme also synthesizes Galα1-4Gal on glycolipids. Thus, to examine whether pA4GalT2 utilizes not only glycoproteins but also GSLs as substrates to synthesize Gb3, pA4GalT2 cDNA was introduced into A4GalT-deficient HeLa cells (ΔA4GalT) that were generated previously (30). Lipids in this transfectant (ΔA4GalT-pA4GalT2) were metabolically labeled with [14C]galactose and analyzed by TLC and radioactive imaging to observe GSL biosynthesis (Fig. 1B). A4GalT cells lost Gb3 biosynthesis, but reintroduction of human A4GalT cDNA completely restored Gb3 expression (ΔA4GalT-hA4GalT). pA4GalT2 also partially recovered Gb3, although the recovery was less than that in ΔA4GalT-hA4GalT cells. This result indicated that pA4GalT2 possesses a low Gb3 synthesis activity. In addition, an unidentified GSL species (marked by an asterisk in Fig. 1B) was also observed in the ΔA4GalT-pA4GalT2 cells, which was not observed in ΔA4GalT-hA4GalT cells, suggesting that pA4GalT2 synthesized an unidentified lipid that may contain the P1 glycan epitope.

Construction of GSL-deficient HeLa cells expressing pA4GalT2

The fact that pA4GalT2 acted not only on glycoproteins but also on GSLs implies that Gb3, as well as other GSLs containing the Galα1-4Gal sequence produced by the action of pA4GalT2, is an obstacle in estimating the function of P1 antigens on glycoproteins as STx receptors. To overcome this problem, we generated GSL-deficient HeLa cells to express P1 antigens on glycoproteins exclusively by pA4GalT2. HeLa cells express both GlcCer and GalCer, which are precursors of Gb3 and galabiosylceramide (Gb2) (Galα1-4Gal-Cer), respectively (31), and GlcCer or GalCer is probably the precursor of the unidentified GSL observed in Fig. 1B when pA4GalT2 was expressed. Therefore, we first constructed double-gene-knockout cells by disrupting both GlcCer synthase (UDP-glucose:ceramide glucosyltransferase; UGCG) and GalCer synthase (UDP-galactose:...
ceramide galactosyltransferase (CGT)). Previously, UGCG-disrupted HeLa cells (ΔUGCG cells) were established by transcription activator-like effector nuclease (16). Therefore, the CGT gene was further disrupted in ΔUGCG cells by the CRISPR/Cas9 system to generate UGCG/CGT double-knockout cells (ΔUGCG/CGT DKO cells). Sequence analyses showed that a coding region within exon 2 of CGT was frameshifted in all alleles in the DKO cell clone (Fig. 2A). To confirm the loss of GSL synthesis in this clone, GSLs were analyzed by the same method as for Fig. 1B. GlcCer-derived GSLs were lost in ΔUGCG cells, and all GSLs including both GlcCer-derived and GalCer-derived lipids were completely lost in ΔUGCG/CGT DKO cells (Fig. S1). The loss of GSLs was also maintained when pA4GalT2, HA-tagged pA4GalT2, or hA4GalT was expressed in UGCG/CGT DKO cells, and the unidentified GSLs observed in Fig. 1B did not appear (Fig. 2B).

Next, surface expression of P1 epitopes and STx receptors on the transfectants were analyzed with a flow cytometer using anti-P1 antibodies and a fluorescent STx1 B subunit (STx1B). STx1B bound to HeLa parent cells, but the binding was completely lost in ΔUGCG/CGT DKO cells (Fig. 2C). However, anti-P1 antibodies that recognize the Galα1-4Galβ1-4GlcNAc epitope did not bind to the parent cells, indicating that the antibodies do not recognize Gb3 and the parent cells do not express P1 glycan antigens (Fig. 2C). Overexpression of pA4GalT2 in ΔUGCG/CGT DKO cells resulted in the binding of anti-P1 antibodies and STx1B, suggesting that pA4GalT2 synthesizes P1 antigens on glycoproteins and that STx1B binds to the epitopes on the glycoproteins. The binding of anti-P1 antibodies was weaker in ΔUGCG/CGT–pA4GalT2 cells than in ΔUGCG/CGT–pA4GalT2 cells. Therefore, the ability of hA4GalT to synthesize P1 antigens was very weak in cells (described below).

Detection of P1 epitopes on N-glycans in pA4GalT2-expressing cells by glycomic analysis

To investigate whether P1 antigens were contained in N-glycans or in O-glycans, MGAT1 (N-acetylglucosaminyl (GlcNAc) transferase I), which is essential for complex- and hybrid-type N-glycan biosynthesis, and C1GalT1 (core 1 β1,3-galactosyltransferase 1), which is essential for elongation of O-glycan biosynthesis, were disrupted by transient transfection of guide RNA (gRNA) in ΔA4Galt–pA4Galt2 cells. Fig. 3A shows that gRNA targeting of MGAT1 reduced the binding of phytohemagglutinin-L (PHA-L), which recognizes specific types of N-glycans.
branched N-glycans, indicating that MGAT1 was disrupted in some cells of the population. In contrast, gRNA targeting of C1GalT1 reduced the binding of peanut agglutinin (PNA) lectin, which binds Galβ1-3GalNAc structures in O-glycans, indicating that C1GalT1 was disrupted in some cells of the population. Under these conditions, only MGAT1 gRNA reduced STx binding, indicating that P1 epitopes were mainly present on N-glycans.

To confirm the presence of P1 epitopes on N-glycans, the N-glycan structures were quantitatively analyzed in ΔUGCG/CYT-pA4GalT2 (or -pA4GalT2-HA) cells (as well as the following cell lines: HeLa parent cells, ΔUGCG/CYT cells, ΔUGCG/CYT-hA4GalT cells, ΔA4GalT cells, and ΔA4GalT-hA4GalT cells) using LC-MS. First, 2-aminopyridine (PA)-derivatized N-glycans from cells were separated by HPLC using an anion-exchange DEAE column into fractions 1–7 (Fig. S2A). Each fraction was analyzed with LC-MS using a reversed-phase column. As shown in Fig. S2B, several peaks in fractions 1 and 3–7 were detected by the fluorescence detector and simultaneously analyzed by both full MS and MS/MS in a data-dependent manner (Supplemental Data S1A and S1B). The profiles of PA-derivatized N-glycans isolated from HeLa parent cells, ΔUGCG/CYT cells, and ΔUGCG/CYT-hA4GalT cells were almost the same, whereas that of ΔUGCG/CYT-pA4GalT2 (or -pA4GalT2-HA) cells was apparently different (Fig. S3A), and P1 epitope-containing glycans were detected by MS and MS/MS analysis (Fig. S4A to C). The quantitative data in Supplemental Data S1A and S1B showed that more than 40% of total N-glycans contained more than one Galα1-4Gal P1 epitope at the nonreducing termini in ΔUGCG/CYT-pA4GalT2 cells (Fig. 3B). The P1 antigens were observed in hybrid-type and complex-type glycans containing bi-, tri-, and tetra-antennae, and 18.7% of complex-type glycans included multiple P1 antigens per glycan (Fig. 3C). Together with the result that ΔUGCG/CYT-pA4GalT2 cells did not express any glycolipids including Gb3 (Fig. 2B), we succeeded in constructing cells that expressed P1 glycan antigens only on the glycoproteins. It should be noted that very small amounts of N-glycans with the Galα1-4Gal epitope were detected on the glycans from ΔUGCG/CYT-hA4GalT and ΔA4GalT-hA4GalT cells (Fig. S3B). This fact was consistent with the result that the P1 epitopes on glycoproteins of ΔUGCG/CYT-hA4GalT cells were detected weakly using anti-P1 mAb (Fig. 2C). These results indicated that hA4GalT had only a very low activity of P1 antigen synthesis on glycoproteins, and P1 antigens were synthesized only when hA4GalT was overexpressed.

P1 epitopes on glycoproteins are partially functional as STx receptors

Next, STx sensitivities were compared among the cells described above (Fig. 4). HeLa parent cells were sensitive to STx toxicity at the lowest concentration tested (0.5 pg/ml), and ΔUGCG/CYT DKO cells were completely resistant to the toxin even at the highest concentration tested (5 ng/ml). When pA4GalT2 was expressed in ΔUGCG/CYT DKO cells (ΔUGCG/CYT-pA4GalT2 and ΔUGCG/CYT-pA4GalT2-HA), the cells became sensitive to 50 pg/ml of STx, indicating that Galα1-4Gal-containing glycoproteins can be functional receptors of STx. However, ΔUGCG/CYT-pA4GalT2 cells were more than 100 times less sensitive to STx than the...
parent cells (a 0.5-pg/ml treatment in the parent cells was comparable to a 50-pg/ml treatment in \( \Delta UGCG/CGT \)-pA4GalT2 cells), although the amount of STx bound to the cell surface was higher than that on the parent cells (Fig. 2C).

These results indicated that the functional activity of P1 antigens on glycoproteins as STx receptors was lower than that of Gb3. A previous study demonstrated that GlcCer is required for the association of STx with detergent-resistant membranes in the endoplasmic reticulum essential for a cytotoxic effect (32). However, the loss of GSLs was not the reason for the low sensitivity of \( \Delta UGCG/CGT \)-pA4GalT2, because \( \Delta B4GalT5 \)-pA4GalT2 cells, which expressed little Gb3 but still expressed GlcCer as well as a high number of STx receptors on glycoproteins, also showed lower sensitivity to STx than the parent cells (Fig. S5).

Differences in intracellular localization of STx mediated by P1-containing glycoproteins and Gb3

Because the P1 antigens on glycoproteins were less functional as STx receptors than Gb3, we speculated that only a small amount of STx reached the cytosol from the cell surface when the P1 antigens on glycoproteins were used as the STx receptors. To examine this assumption, transport of STx1B from the cell surface was compared between \( \Delta UGCG/CGT \)-pA4GalT2 cells and parent cells. STx1B was internalized into the parent cells within 15 min and reached the Golgi within 90 min (Fig. 5A). In \( \Delta UGCG/CGT \)-pA4GalT2 cells, however, some STx1B molecules remained on the cell surface even after 30 min, and no Golgi localization was observed up to at least 3 h. The dot structures of STx1B observed at 1.5 h in \( \Delta UGCG/CGT \)-pA4GalT2 cells were partially colocalized with the early endosome marker EEA1 and only slightly colocalized with the late endosome/lysosome marker LAMP2 (Fig. 5B).

To investigate in detail the membrane structures where the incorporated STx1B was localized, in-resin correlative light and EM (CLEM) were performed to combine the fluorescent imaging of STx1B with the visualization of subcellular structures by field emission scanning electron microscopy. STx1B was mainly localized in vacuole-like structures, which may be derived from endosomes, because these structures were not condensed. However, STx1B was less localized in electron-dense organelles (arrowheads), which indicate lysosomes (Fig. 6). These results indicated that STx was localized mainly in early endosomes when P1 antigens on glycoproteins were used as the receptors.

P1 epitopes on glycoproteins function as decoys in coexpression with Gb3

The fact that P1 antigens on glycoproteins were only partially functional as an STx receptor compared with Gb3 prompted us to investigate the effects of P1 antigens on glycoproteins on Gb3-mediated STx cytotoxicity when coexpressed on the cell surface. To obtain cells that express both P1 antigens on
glycoproteins and Gb3, the pA4GalT2 gene was transfected into the parent cells. Sufficient amounts of STx1B bound to pA4GalT2-expressing cells (Fig. 7A), and the expression level of Gb3 was not significantly different between the two cell lines (Fig. 7B). However, STx sensitivity in pA4GalT2-expressing cells was lower than that in the parent cells as well as in hA4GalT-expressing cells (Fig. 7B). These results suggested that P1 antigens on glycoproteins acted as decoys and inhibited Gb3-mediated STx cytotoxicity when the P1 antigens were coexpressed with Gb3.

**Discussion**

Gb3 is the STx receptor in mammalian cells, and loss of Gb3 confers complete resistance to STx. However, it was unclear to what extent the ceramide moiety of Gb3 contributes to retrograde transport of STx to the appropriate site for exerting a cytotoxic activity and whether membrane proteins could be an alternative receptor. In this study, we established GSL-deficient and pA4GalT2-expressing HeLa cells (ΔUGCG/CGT-pA4GalT2 cells), which expressed the Galα1-4Gal epitope only on glycoproteins, and demonstrated that P1 antigens on glycoproteins were a functional STx receptor. However, the STx sensitivity in ΔUGCG/CGT-pA4GalT2 cells was lower than that in the parent cells, which express Gb3, and when P1 antigens on

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**Figure 6. CLEM images of ΔUGCG/CGT-pA4GalT2 cells.** Alexa Fluor 555–STx1B was incubated on ice for 20 min and then shifted to 37 °C for 1.5 h. Fluorescent images, scanning electron microscopy images, and their overlaid images are shown.

**Figure 7. Effect of P1 antigen on Gb3-mediated STx cytotoxicity.** A, surface binding of STx on parent cells and cells into which cDNA was introduced. The indicated cells were stained with (yellow-green lines) or without (black lines) Alexa Fluor 555-labeled STx1B and analyzed using FACS. B, GSL metabolic analysis of parent cells and cells into which cDNA was introduced. The indicated cells were labeled with [14C]galactose, and the labeled lipids were separated on an HPTLC plate. The amounts of [14C]galactose-labeled Gb3 are expressed as the percentage of band intensity in parent cells (mean percentage ± S.D. from three independent experiments). A paired t test was used for the comparison. n.s., not significant. C, parent cells, pA4GalT2-expressing cells, and hA4GalT cells were treated with STx1 at the indicated concentrations. Viability was estimated as described in the legend to Fig. 4 and expressed as the mean percentage ± S.D. from three independent experiments. The Holm method was used for multiple comparisons (2 comparisons). The asterisk indicates statistical significance.
Differences between lipid- and protein-based STx receptors

Figure 8. Schematic summary of P1 antigen on glycoproteins with positive and negative effects on STx cytotoxicity.

Glycoproteins and GB3 were coexpressed, the P1 antigens on glycoproteins functioned as a decoy to inhibit GB3-mediated cell death (summarized in Fig. 8). Our results clearly indicated that the ceramide moiety of GB3 is important for STx to exert its cytotoxicity efficiently, and only a part of STX1 internalized by glycoproteins can reach the retrograde transport pathway leading to cell death.

P1 antigens on glycoproteins are known to bind STx, and column coupling with P1-containing proteins has been used to purify STx (25, 26, 33). P1 antigens on glycoproteins are frequently observed in mammalian cells, although erythrocytes are likely to express them (34). In this study, hA4GalT synthesized a small amount of P1 antigens on glycoproteins only when overexpressed (Fig. S3). This indicates that hA4GalT uses only GSLs, mainly LacCer, as substrates at normal levels of expression in cells. In contrast, pigeon A4GalT2 synthesized P1 antigens on O-glycans efficiently (Fig. 3B and Figs. S3 and S4), as previously reported (24). Cherian et al. demonstrated that STx could bind to P1 antigens on the core 2 O-glycans of proteins when C2GNT1 (core 2 branching enzyme) and pA4GalT2 were overexpressed in CHO-K1 cells (27). The LacNAC (Galβ1-4GlcNAc) structure is generally present on core 2 O-glycans as well as N-glycans, but core 2 O-glycans are not expressed in HeLa cells (35). Therefore, N-glycans were the main holder of P1 antigens in ΔUGCG/CGT-pA4GalT2 cells (Fig. 3).

In FACS analysis, the amount of STx bound on the cell surface of ΔUGCG/CGT-pA4GalT2 cells was comparable to or greater than that of parent cells (Fig. 2C), and STx actually caused cell death (Fig. 4). These results suggest that STx incorporated via P1 antigens on glycoproteins was also transported to cytosolic ribosomes. However, the STx sensitivity of ΔUGCG/CGT-pA4GalT2 cells was more than 100 times lower (nanograms of STx per milliliter) than that of parent cells (picograms of STx per milliliter) despite its sufficient surface binding. To clarify the reason for the difference in STx sensitivities between ceramide-based and membrane protein-based receptors, STx trafficking was analyzed microscopically, and at least two differences were observed between ΔUGCG/CGT-pA4GalT2 cells and parent cells. First, the internalization of STx was slower in ΔUGCG/CGT-pA4GalT2 cells than in parent cells. After 30 min of permitted incorporation at 37°C, most of the surface-bound STx molecules were internalized into the parent cells, whereas moderate amounts of STx molecules remained on the cell surface in ΔUGCG/CGT-pA4GalT2 cells. The STx pentamer has 15 GB3 binding pockets (36), and recent studies have demonstrated that the STx-Gb3 complex drives the formation of narrow tubular membrane invaginations due to the clustering of several pentamers without the requirement for the clathrin machinery (5, 37). GSLs including GB3 on the outer leaflet of the plasma membrane have less access to cytosolic proteins, but GSLs can form clusters through hydrogen bonding between hydroxyl and amide groups of a ceramide moiety and by hydrophobic interaction between the fatty acid and sphingoid base of ceramides. If the driving force of the toxin clustering depends on the ceramide moiety of GB3, STx may be less clustered when it uses glycoproteins as receptors. There are multiple routes for membrane protein internalization, including clathrin-dependent and -independent ones (38–41), and the physical and physiological properties of membrane proteins are thought to determine the endocytic routes and retention time of proteins on the cell surface. Therefore, STx on some glycoproteins may be incorporated less into the cells or recycled back to the plasma membrane frequently. As another possibility, if STx straddles both a cell surface membrane protein and an extracellular matrix protein secreted from P1-expressing cells, or if it straddles respective membrane proteins on different cells upon contact, STx may be retained on the cell surface.

The second difference in STx trafficking between parent cells and ΔUGCG/CGT-pA4GalT2 cells was that the toxin reached the Golgi/TGN in parent cells, whereas the toxin hardly reached the Golgi and was stuck mainly in early endosomes in ΔUGCG/CGT-pA4GalT2 cells. The STx-Gb3 complex is known to be transported from early endosomes to the TGN (42), and various factors are involved in transport, including the retromer components and GPP130 membrane protein (43–46). The surface-exposed loop (β4–β5 loop) of STx1B and STx2B is required for their endosome-to-Golgi transport. Lack of this region prevents the toxin from entering the TGN, and instead, the toxin is sent to late endosomes/lysosomes (47). The loop of STx1B, but not STx2B, interacts with GPP130, which is involved in the endosome-TGN transport of STx1B (47). Therefore, the amino acid sequence of STx determines its endosome-to-Golgi transport. However, our results indicated that not only the region within STx but also the presence of STx-binding receptors affected the transport. One of the reasons for the difference in the STx transport to the TGN between lipid-based and protein-based receptors may be the distance between STx and the membrane, because GPP130 binds to STx1B at its juxtamembrane region, which is required for transport to the TGN (48). Therefore, if STx1B binds to a transmembrane protein at a distal P1-glycan site, GPP130 may not bind to STx1B, and as a result, STx1B may not be transported to the TGN efficiently.

There are several toxins that recognize glycans on glycoproteins as receptors and require retrograde trafficking, including ricin, a toxin produced in the seeds of the castor oil plant, and subtilase cytotoxin, produced by locus of enterocyte effacement-negative Shiga-toxigenic E. coli strains. Both toxins are also required in nanogram-per-milliliter concentrations to cause cell death in HeLa cells, as for STx in ΔUGCG/CGT-
pA4GalT2 cells (35, 49, 50), which was different from the sensitivity of STx (on the picograms-per-milliliter order) using Gb3 as a receptor. Therefore, only small amounts of toxins may be able to reach the respective appropriate site when proteins are used as receptors.

In conclusion, we constructed GSL-deficient and P1-expressing HeLa cells and compared glycoprotein-mediated STx sensitivity with that of Gb3. Although P1-containing glycoproteins functioned as an STx receptor, the STx sensitivity of P1-expressing cells was lower than that of parent cells. The retrograde transport of STx to the Golgi was suppressed in P1-expressing cells, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx. In contrast, P1 antigens on glycoproteins functioned as a decoy to inhibit Gb3-mediated cell death when both proteins were coexpressed. Previous studies have shown that pigeons express P1 antigen-bearing glycoproteins in various tissues and in serum (51). If pigeons do possess Shiga toxin-producing E. coli (STEC), P1-glycoproteins may act as a protective agent against Shiga toxin, suggesting that pigeons could be less sensitive to the toxin. Consequently, STEC may grow in pigeons, indicating that the ceramide moiety of Gb3 is critical for effective retrograde transport and subsequent cytotoxicity of STx.
**Retroviral infection and preparation of stable transfectants**

Preparation of retroviruses and infection of HeLa-mCAT#8-based cells were performed using the Plat-E system, as described previously (56). When pMXs-IP-based (hA4GalT and pA4GalT2) and pMXs-IB-based (pA4GalT2-HA) retroviruses were used, the concentrations of puromycin (hA4GalT and pA4GalT2) and pMXs-IB-based (pA4GalT2-HA) retroviruses were used, the concentrations of puromycin and blasticidin-S for selection were 2 μg/ml and 7.5 μg/ml, respectively.

**Metabolic labeling of glycolipids and TLC analysis**

Metabolic labeling experiments using D-[1-14C]galactose, including mild alkaline methanolysis, were performed as described previously (59). Cells (3 × 10⁵/well in a 6-well plate) were cultured overnight at 37 °C and then incubated for 16 h with 7.4 kBq of D-[1-14C]galactose in Opti-MEM with 1% Neutridoma-SP (Roche). Cells were lysed with 0.1% SDS, and lysates containing the same amount of protein were used for lipid extraction following the method of Bligh and Dyer (60). For alkali methanolysis to remove glycerolipids, dried lipids were hydrolyzed with 0.1 N KOH in methanol for 1 h at 40 °C. After neutralization with 0.1 N HCl, the methanol layer was washed twice with n-hexane, and the lipids were extracted using the method of Bligh and Dyer (60). The lower fractions collected were dried by SpeedVac. Lipids were separated by HPTLC using chloroform/methanol/0.25% CaCl₂ (65/35/8) as a developing solvent (31). The radioactive lipids on TLC plates were visualized, and the intensity of each band was quantitated using a Typhoon FLA 7000 (GE Healthcare, Buckinghamshire, UK).

**FACS analysis**

Nonconfluent cells were trypsinized and washed with culture medium and wash buffer (1% BSA) in PBS at 4 °C. Cells were incubated with 10 μg/ml Alexa Fluor 555–Stx1B, FITC–PHA-L, and FITC-PNA for 45 min on ice. For FITC-PNA staining, 1 mM each of CaCl₂ and MgCl₂ was further added. For anti-P1 staining, cells were incubated with mouse anti-P1 mAb at a 100-fold dilution followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (heavy plus light chain) on ice. After one washing with wash buffer, cells were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). To determine whether the P1 antigens were expressed on N-glycans or O-glycans, ΔhUGCG/CGT-pA4GalT2 cells (2.5 × 10⁶ cells/well in a 12-well plate) were transfected with 0.5 μg CRISPR plasmids targeting the MGAT1 gene (for N-glycans) and the C1GalT1 gene (for O-glycans) (35). After puromycin treatment and subculture as described above, the cells were subjected to FACS analysis.

**STx treatment and cell viability assay**

Cells (2.5 × 10⁴ to 5 × 10⁴ cells/ml in 12-well or 24-well plates) were cultured overnight at 37 °C and then treated with STx1 at the concentrations indicated in Figs. 4, 7, and Fig. S5 for 3 days. An MTT assay was then performed as described previously to assess cell viability (31).

**Preparation of PA–N-glycans from cultured cells**

Cells cultured in 10-cm dishes were washed with PBS and harvested with 0.5 mM EDTA in PBS. The cell pellets were washed with PBS and suspended in 300 μl deionized water. Cells were homogenized with a Polytron homogenizer and mixed with 800 μl of methanol and 400 μl chloroform. After centrifugation, the supernatants were removed. The residual pellets were air-dried and resuspended in 800 μl of 6 mM guanidine-HCl in 50 mM Tris-HCl, pH 8.4. Cell suspensions were mixed with 200 μl of 120 mM DTT in 6 mM guanidine-HCl and incubated at 37 °C for 1 h. For alkylation of thiols, the suspensions were mixed with 200 μl of 300 mM iodoacetamide in 6 mM guanidine-HCl, incubated at room temperature for 1 h in the dark, and then dialyzed against deionized water. The reaction mixtures were suspended with 50 mM NH₄HCO₃, pH 8.4, and digested with trypsin at 37 °C overnight. After inactivation of the enzymes at 100 °C for 10 min, the digest was lyophilized. N-Glycans were released by glycoamidase F treatment in 10 mM NH₄HCO₃, pH 7.8, at 37 °C overnight (61). The reaction mixtures were adjusted to around pH 5.0 by addition of acetic acid and incubated at 37 °C for 30 min. The mixtures were loaded onto 1 ml Dowex 50WX2 (H⁺ form, 200–400 mesh) packed in an Econo-column (Bio-Rad). The column was washed with 5 ml of water, and the collected effluents were lyophilized. The released N-glycans were derivatized with PA as described previously (62). Mixtures of PA–N-glycans were separated by HPLC using a TSKgel DEAE-5PW column as described previously (63). PA-glycans were detected using a fluorescence spectrophotometer with an excitation wavelength of 310 nm and an emission wavelength of 380 nm.

**ESI-MS and MS/MS conditions**

MS analysis of PA-glycans was performed by electrospray ionization (ESI)-MS on an LTQ XL linear ion trap mass spectrometer coupled to a Dionex U3000 HPLC system and an ESI probe (H–ESI-II; Thermo Scientific, San Jose, CA) as described previously (63). MS conditions were as follows: spray voltage, 4 kV; auxiliary gas flow rate, 2 arbitrary units; sheath gas flow rate, 30 arbitrary units; heated capillary temperature, 250 °C; heated capillary voltage, 40 V; and tube lens voltage, 75 V. MS data were recorded and analyzed with Xcalibur 2.2 software (Thermo Scientific). For general use, MS and MS/MS data were collected in data-dependent mode.

**On-line LC-MS conditions for PA–N-glycans**

PA–N-glycans were separated by reversed-phase LC using an InertSustain AQ-C18 column at a flow rate of 0.2 ml/min at 30 °C. Elution was performed using Eluent A (0.2% (v/v) formic acid) and Eluent B (0.2% formic acid in 20% acetonitrile). The column was equilibrated with Eluent A, and 3 min after sample injection, the A:B ratio was increased linearly from 100:0 to 80:20 over 80 min and then to 0:100 over 5 min. Eluents were separated evenly (1:1) using an ASI 600-PO-10-06 flow splitter (Analytical Scientific Instruments, Richmond, CA, USA) and directed to the MS and fluorescence detector devices. PA-glycans were detected by MS, as described above, and were simultaneously detected using a fluorescence spectrophotometer.
with an excitation wavelength of 315 nm and an emission wavelength of 400 nm. Glycan structures were deduced based on the results of MS and MS/MS as well as known biosynthetic pathways of vertebrate glycans. The presence of α-galactoside on the reducing end of glycans was confirmed by digestion with α-Gal. The relative amount of each PA–N-glycan was quantified based on the integration of fluorescence signals after LC separation. When the fluorescence intensity peaks included more than two kinds of PA-glycans with different mass values, their ratios were estimated using the ratios of integrated ion intensities for each m/z value detected at the corresponding time.

Immunofluorescence microscopy of retrograde transport of STx1B

Parent and ΔUGCG/CGT-pA4GalT2 cells were cultured on a cover glass in a 6-well plate for 3 days. The cells were incubated with 10 μg/ml Alexa Fluor 555–STx1B in 1% BSA/PBS for 20 min on ice. After three washings with complete medium on ice, the cells were cultured in toxin-free medium at 37 °C for the times indicated in Fig. 5. The cells were then fixed with Mildform 10N (Wako), and immunostaining was performed as described previously (64). Specimens were visualized with a wide-field fluorescence microscope, BZ-X700 (Keyence, Osaka, Japan) equipped with a Plan Apo VC 60 × 1.40 oil immersion objective. The haze reduction function (condition 2), which applies a no-neighbor deconvolution algorithm to the captured image, was used to eliminate fluorescence blurring caused by scattered light and capture clear images with high contrast.

In-resin CLEM

After incubation with 10 μg/ml Alexa Fluor 555–STx1B and chasing for 1.5 h as described above, the cells were prefixed with 2.5% glutaraldehyde at 4 °C for 1 h. The fixed cells were washed twice with 0.1 M phosphate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide at 4 °C for 15 min. Fixed cells were incubated in TUK solution (FUJIFILM Wako Chemicals, Tokyo, Japan) at 4 °C for 10 min. Cells were dehydrated with a graded series of ethanol and embedded in Epon 812 epoxy resin (Oken Shoji, Tokyo, Japan) at 60 °C for 72 h. Thin sections (100 nm) were cut with a UC6 ultramicrotome (Leica, Wetzlar, Germany) and placed on glass coverslips that were coated with Pt/Au using an E-1010 ion sputter coater (Hitachi, Tokyo, Japan). Sections were observed in TUK solution using a BZ-X710 fluorescence microscope (Keyence). Sections were then stained with uranyl acetate and lead citrate and observed with a Helios NanoLab 660 scanning electron microscope (FEI, Hillsboro, OR, USA). Scanning electron microscopy images were obtained with a backscattered electron detector (CBS detector) at a voltage of 2.0 kV and a current of 0.4 nA.

Statistical analysis

In Fig. 7B, a two-tailed paired t test was used for statistical analysis, with a p value of <0.05 considered statistically significant. For multiple comparisons, Holm’s sequential Bonferroni procedure (the Holm method) was used (65). A two-tailed paired t test was performed, and the p values were ranked from lowest to highest. The Holm-Bonferroni formula (0.05/(number of comparisons – rank + 1)) was used for the first rank to calculate the index, and the first-ranked p value was compared with the calculated index. When the p value was less than the index, the null hypothesis was rejected, and the difference was considered statistically significant. The Holm-Bonferroni formula was repeated for the next rank until the p value was greater than or equal to the calculated index. The numbers of comparisons were as follows: 7 comparisons in Fig. 4 (parent versus DKO, parent versus DKO-pA4GalT2, parent versus DKO-pA4GalT2-HA, parent versus DKO-hA4GalT, DKO versus DKO-pA4GalT2, DKO versus DKO-pA4GalT2-HA, and DKO versus DKO-hA4GalT), 2 comparisons in Fig. 7C (parent versus hA4GalT and parent versus pA4GalT2-HA), and 3 comparisons in Fig. S5B (parent-mock versus ΔB4GalT5-mock, parent-mock versus ΔB4GalT5-pA4GalT2, and ΔB4GalT5-mock versus ΔB4GalT5-pA4GalT2) at each concentration of STx.

Data availability

The raw MS data for glycan structural analysis have been deposited to GlycoPOST. The ID is GPST000074.

Author contributions—K. M., N. S., I. T., S. K., Y. F., and T. Y. investigation; N. S. and T. Y. conceptualization; N. S., K. H., and T. Y. resources; N. S., K. H., and T. Y. funding acquisition; N. S., I. T., Y. U., and T. Y. methodology; N. S. and T. Y. writing-original draft; K. H. writing-review and editing; Y. S. and T. Y. supervision.

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Abbreviations—The abbreviations used are: STx, Shiga toxin; A4GalT1, α,1,4-galactosyltransferase; B4GalT5, β,1,4-galactosyltransferase 5; C1GalT1, core 1 β,1,3-galactosyltransferase 1; CGT, UDP galactose ceramide galactosyl transferase (GalCer synthase); CLEM, correlative light and electron microscopy; DKO, double knockout; ESI, electrospray ionization; GalCer, galactosylceramide; Gb3, globotriaosylceramide; GlcCer, glucosylceramide; gRNA, guide RNA; GSL, glycosphingolipid; HPTLC, high-performance TLC; LacCer, lactosylceramide; MGAT1, GlcCer transferase I; MIT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PA, 2 amino pyridine; PHA-L, phytohaemagglutinin-L; PNA, peanut agglutinin; STx1B, Shiga toxin 1 B subunit; TGN, trans-Golgi network; UGCG, UDP-glucose ceramide glucosyltransferase (GlcCer synthase).

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