Genetic Disorders of Glycosylation

Functional analyses of the UDP-galactose transporter SLC35A2 using the binding of bacterial Shiga toxins as a novel activity assay

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Abstract

SLC35A2 transports UDP-galactose from the cytosol to the lumen of the Golgi apparatus and endoplasmic reticulum for glycosylation. Mutations in *SLC35A2* induce a congenital disorder of glycosylation. Despite the biomedical relevance, mechanisms of transport via SLC35A2 and the impact of disease-associated mutations on activity are unclear. To address these issues, we generated a predicted structure of SLC35A2 and assayed for the effects of a set of structural and disease-associated mutations. Activity assays were performed using a rescue approach in Δ SLC35A2 cells and took advantage of the fact that SLC35A2 is required for expression of the glycosphingolipid globotriaosylceramide (Gb3), the cell surface receptor for Shiga toxin 1 (STx1) and 2 (STx2). The N- and C-terminal cytoplasmic loops of SLC35A2 were dispensable for activity, but two critical glycine (Gly-202 and Gly-214) and lysine (Lys-78 and Lys-297) residues in transmembrane segments were required. Residues corresponding to Gly-202 and Gly-214 in the related transporter SLC35A1 form a substrate-translocating channel, suggesting that a similar mechanism may be involved in SLC35A2. Among the eight disease-associated mutations tested, SLC35A2 function was completely inhibited by two (S213F and G282R) and partially inhibited by three (R55L, G266V, and S304P), providing a straight-forward mechanism of disease. Interestingly, the remaining three (V331I, V258M, and Y267C) did not impact SLC35A2 function, suggesting that complexities beyond loss of transporter activity may underlie disease due to these mutations. Overall, our results provide new insights into the mechanisms of transport of SLC35A2 and improve understanding of the relationship between *SLC35A2* mutations and disease.

Key words: congenital disorder, glycosylation, Golgi, Shiga toxin, trafficking

Introduction

Nucleotide sugar transporters belong to the SLC35 family of proteins (Song 2013). These proteins transport nucleotide sugars from the cytosol to the lumen of the endoplasmic reticulum or the Golgi apparatus where the nucleotide sugars serve as substrates for protein glycosylation or glycosphingolipid biosynthesis (Song 2013).

SLC35A2 transports UDP-galactose into the lumen of the Golgi apparatus and the endoplasmic reticulum (Song 2013). In the Golgi apparatus, UDP-galactose is utilized as a substrate by galactosyl-transferase enzymes for galactosylation of proteins (Amado et al. 1999). Additionally, UDP-galactose is also critical for the formation of complex glycosphingolipids in the Golgi. Briefly, within the Golgi

lumen, UDP-galactose is required for conversion of glucosylceramide to lactosylceramide by β 4-galactosyltransferases V and VI (D'Angelo et al. 2013). Lactosylceramide, in turn, serves as a common precursor for the formation of numerous complex glycosphingolipids of the asialo, ganglio, globo/iso-globo, and lacto-neolacto series (D'Angelo et al. 2013). Separately, in the endoplasmic reticulum, UDP-galactose is required for the formation of galactosylceramide, which can then be transported to the Golgi apparatus for further processing to the ganglioside GM4 and to sulfogalactolipids (D'Angelo et al. 2013). SLC35A2 primarily localizes to the Golgi apparatus, but a pool of the transporter is also detected in the endoplasmic reticulum (Sprong et al. 2003; Kabuss et al. 2005). The intracellular localization provides a mechanism for SLC35A2 to transport UDP-galactose into the lumen of both the endoplasmic reticulum and the Golgi apparatus.

Mutations in *SLC35A2* induce a rare congenital disorder of glycosylation in humans (Ng et al. 2013; Westenfield et al. 2018). While multiple organs are affected, developmental delay and neurological deficits (seizures, cerebral atrophy, and delayed myelination) are prominent (Ng et al. 2013; Westenfield et al. 2018). Interestingly, the severity of clinical presentation is variable between patients (Kodera et al. 2013; Ng et al. 2013; Scott et al. 2014; Dorre et al. 2015; Westenfield et al. 2018). A possible reason may be that different mutations have different effects on SLC35A2 function. Additionally, *SLC35A2* is located on the X-chromosome. Random X-inactivation in females and mosaicism observed in both genders may impact the cell-type specific expression of the mutant allele and

thereby also impact disease phenotype (Ng et al. 2013; Scott et al. 2014; Westenfield et al. 2018).

Despite the importance of SLC35A2 in glycosylation and human disease, the mechanisms of UDP-galactose transport via SLC35A2 remain unclear. Additionally, the mechanisms by which disease-associated mutations impact SLC35A2 function are also not properly understood. The crystal structure of SLC35A2 has not been resolved, and the lack of structural information has hindered progress.

Our interest in studying SLC35A2 emerged from a separate project on the intracellular trafficking of bacterial Shiga toxin 1 (STx1) and Shiga toxin 2 (STx2) (Mukhopadhyay and Linstedt 2012; Mukhopadhyay et al. 2013; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). These toxins invade human cells by the retrograde trafficking pathway to reach the cytosol where they block ribosomal protein synthesis and induce cell death (Mukhopadhyay and Linstedt 2013). We recently performed a genome-wide siRNA screen to identify host factors required for toxicosis induced by STx1 or STx2 (Selyunin et al. 2017). SLC35A2 emerged as a hit on the primary screen for both toxins (Selyunin et al. 2017). Additionally, SLC35A2 was also a hit on a related screen performed using CRISPR/Cas9 by another group (Tian et al. 2018). A plausible hypothesis for the requirement of SLC35A2 for STx1- or STx2-toxicity is that, within the lumen of the Golgi, lactosylceramide is a precursor for the synthesis of globotriaosylceramide (Gb3) (D'Angelo et al. 2013), which acts as the cell surface receptor necessary for the binding of STx1 and STx2 (Beddoe et al. 2010; Mukhopadhyay and

Fig. 1. Knockdown of SLC35A2 reduces Gb3 expression and inhibits the binding and toxicity of STx1/STx2. (A) WT HeLa cells were transfected with control siRNA or siRNA against SLC35A2. Three days after transfection, qRT-PCR analyses were performed to quantify the relative levels of SLC35A2 mRNA with levels in control transfected cells expressed as 1 (mean $\pm$ SE; $n = 3$; * $P < 0.05$ by t -test). (B) Cells were transfected as described in A. Three days after transfection, cultures were treated with indicated amounts of STx2 holotoxin for 16 h. Cell viability was then assessed. Viability without toxin exposure was normalized to 100% in each transfection condition (mean $\pm$ SE; $n = 3$). (C–H) Cells were transfected with control siRNA or siRNA targeting SLC35A2. After three days, cultures were stained for cell surface Gb3 (C), or incubated with fluorescently labeled STx2 or STx1 (E and H) and analyzed by flow cytometry. Unstained samples were used for gating (shown as vertical line in respective panels). Cell percentages positive for Gb3 from C (D), for STx2 binding from E (F), or for STx1 binding from G (H) were quantified (mean $\pm$ SE; $n = 3$; * $P < 0.05$ by t -test).

Linstedt 2013; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). As described previously, biosynthesis of lactosylceramide depends on the SLC35A2-mediated transport of UDP-galactose into the Golgi lumen (D'Angelo et al. 2013; Song 2013). Here, we leveraged the requirement of SLC35A2 for STx1/STx2 toxicosis to establish a novel functional assay for SLC35A2 activity. We utilized this assay to analyze the impact of a set of structural and disease-associated mutations on SLC35A2 function; mutations were guided by a predicted structure of SLC35A2, which we generated. We identified four critical residues in SLC35A2 that were required for its transport activity. Additionally, we discovered that disease-associated mutations had differential effects on SLC35A2 activity. Our results provide fundamental new insights into the mechanism of action of SLC35A2 and suggest that there are previously unappreciated complexities in the relationship between SLC35A2 mutations and human disease.

Results

SLC35A2 is required for Gb3 expression and the binding and toxicity of STx1 and STx2

As the first step in this study, we validated the results of our siRNA screen and tested the hypothesis that depletion of SLC35A2 protected against STx1/STx2-toxicosis by reducing Gb3 levels and the cell surface binding of both toxins. For this, we depleted SLC35A2 in HeLa cells using siRNA (Figure 1A). Depletion of SLC35A2 robustly protected against STx2-induced cell death (Figure 1B). Furthermore, loss of SLC35A2 reduced total Gb3 levels and blocked the binding of STx1 or STx2 to the cell surface (Figure 1C–H). Note that these analyses were performed using flow cytometry and, for quantification and statistical analyses, we considered cells with a signal above a set threshold to be positive. Additionally, there is wide variability in the expression of Gb3 and Gb3 synthase, the enzyme necessary for Gb3 production (Keusch et al. 2000), in different HeLa cell clones (Shin et al. 2009), and Gb3 levels correlate with Gb3 synthase expression (Shin et al. 2009). Therefore, similar to our previous work, studies here utilized a HeLa cell clone virally infected with Gb3 synthase to induce overexpression of Gb3 (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). This clone is referred to as wild-type (WT) throughout this study. Importantly, compared with this clone, the Gb3 antibody failed to detect a signal in HeLa cells that did not overexpress Gb3 synthase (Figure S1A–C), implying that the antibody was specific. Finally, we replicated results of Figure 1 using immunofluorescence (Figure S2A and B), validating the flow cytometry approach.

For further confirmation, we used a lentiviral-based CRISPR/Cas9 system to generate Δ SLC35A2 HeLa cells in which SLC35A2 expression was inhibited through mutations introduced in genomic DNA (WT HeLa cells that overexpressed Gb3 synthase were used for the infection). SLC35A2 has 10 transmembrane domains (Aoki et al. 2001; Song 2013). Sequencing of genomic DNA revealed that in Δ SLC35A2 cells, a stop codon was introduced in the third transmembrane domain (Figure 2A). Compared with WT cells, SLC35A2 mRNA levels were lower in Δ SLC35A2 cells (Figure 2B and C). The detection of residual SLC35A2 mRNA in Δ SLC35A2 cells, including with primers designed to amplify a gene product downstream of the stop codon (primer pair 2 in Figure 2B), may be due to skipping of the targeted exon during transcription. Despite this residual mRNA, Δ SLC35A2 cells were robustly protected against STx1- or STx2-induced cell death (Figure 2D and E), and exhibited profound

reductions in Gb3 expression and STx1 or STx2 binding (Figure 2F–K). To ensure that the phenotype of Δ SLC35A2 cells was not due to an off-target effect of the CRISPR/Cas9, we performed rescue assays by expressing SLC35A2_{WT}-GFP in Δ SLC35A2 cells. Note that there are two splice isoforms of SLC35A2 that differ from one another only in eight amino acids at the C-terminal end: SLC35A2 isoform c or UDP galactose transporter 1 (UGT1) and SLC35A2 isoform a or UGT2 (Ishida et al. 1996; Miura et al. 1996; Song 2013). Our construct coded for SLC35A2 isoform c or UGT1. The C-terminus of isoform a/UGT2 has a weak endoplasmic reticulum retention signal not present in isoform c/UGT1 (Kabuss et al. 2005). Since our experiments were designed to assay for the effects of UDP-galactose transport into the lumen of the Golgi apparatus, use of a plasmid coding for SLC35A2 isoform c/UGT1, which lacked the endoplasmic reticulum retention signal, was justified. In this manuscript, SLC35A2_{WT} refers to full-length SLC35A2 isoform c/UGT1. SLC35A2_{WT}-GFP localized to the Golgi apparatus in Δ SLC35A2 cells (Figure 3A). Importantly, expression of SLC35A2_{WT}-GFP rescued Gb3 expression as well as STx1/STx2 binding in Δ SLC35A2 cells (Figure 3B–E). To rule out the possibility that results were reflective of flow cytometry or the GFP-tagged construct used, we repeated the rescue experiment using immunofluorescence and GFP- or myc-tagged SLC35A2_{WT} and obtained similar results (Figure S3A–C). Put together, the above data indicate that SLC35A2 is required for Gb3 synthesis as well as the binding and toxicity of STx1 and STx2. Additionally, these results imply that rescue experiments in Δ SLC35A2 cells could be utilized as a novel strategy to study the function of mutated versions of SLC35A2.

N- and C-terminal cytoplasmic loops are dispensable for SLC35A2 activity

Topology predictions suggest that SLC35A2 has 10 transmembrane domains (Aoki et al. 2001; Song 2013) (Figure 4A). To gain insights into the mechanisms of transport, we generated a predicted structure of SLC35A2 using the algorithms of the PHYRE 2.0 server (Kelley and Sternberg 2009) (Figure 4B). Analyses of the predicted structure revealed that SLC35A2 has six cytoplasmic loops (Figure 4B). The N-terminal cytoplasmic loop preceding the first transmembrane domain (amino acids 1–31), the loop connecting transmembrane domains 2 and 3 (amino acids 88–110), and the C-terminal cytoplasmic loop extending from the end of the 10th transmembrane domain (amino acids 343–393) are relatively large (Figure 4B) and could modulate transport activity. To test the role of these loops, we deleted them individually in SLC35A2 and assayed for the localization and activity of generated mutants in Δ SLC35A2 cells (Table 1). We used Pearson's coefficient to quantitatively assess overlap of each SLC35A2 construct with a marker of the Golgi apparatus or the endoplasmic reticulum. Values for the Pearson's coefficient for colocalization between two fluorophores in two channels may range from –1 to +1 (Leyva-Illades et al. 2014; Zogzas et al. 2016; Zogzas and Mukhopadhyay 2018). Values close to +1 indicate a high degree of overlap. Values close to 0 and negative values imply lack of overlap. The Pearson's coefficient for colocalization between SLC35A2_{WT}-GFP and the Golgi apparatus was 0.76 ± 0.02 (Figure 5A). We considered constructs with a Pearson's co-efficient of <0.5 for overlap with the Golgi apparatus as having failed to localize to the Golgi. Using this criteria, we observed that SLC35A2 Δ _{1–31} and SLC35A2 Δ _{343–393} localized to the Golgi (Figure 5A and B). In contrast, the Δ 88–110 mutant was trapped in the endoplasmic reticulum (Figure 5A and B), suggesting that this

Fig. 2. Generation and phenotypic characterization of Δ SLC35A2 HeLa cells. **(A)** Amino acid sequence of SLC35A2 in WT and Δ SLC35A2 cells. Area with asterisks shows alignment between WT and Δ SLC35A2 cells. Seq., sequence; TM, transmembrane domain. **(B)** RT-PCR analyses were performed to detect expression of SLC35A2 mRNA in WT or Δ SLC35A2 cells. Primer pairs 1 and 2 were used to amplify sequences upstream or downstream, respectively, of the locus targeted by the CRISPR system. TBP was an internal loading control. **(C)** Quantitative RT-PCR analyses were performed to quantify the relative levels of SLC35A2 mRNA in WT or Δ SLC35A2 cells. Levels in WT cells were normalized to 1 (mean $\pm$ SE; $n = 3$; * $P < 0.05$ by t -test). **(D and E)** WT or Δ SLC35A2 cells were treated with indicated amounts of STx2 **(D)** or STx1 **(E)** holotoxin for 16 h. Cell viability was then assessed. Viability without toxin exposure was normalized to 100% for each cell line. For Δ SLC35A2 cells, prominent cell death was not observed even with the highest toxin concentration tested; therefore the LD₅₀ was expressed as $>$ the highest concentration (mean $\pm$ SE; $n = 3$). **(F–K)** WT or Δ SLC35A2 cells were stained for cell surface Gb3 **(F)**, or incubated with fluorescently labeled STx2 or STx1 **(H and J)** and analyzed by flow cytometry. Unstained samples were used for gating (shown as vertical line in respective panels). Cell percentages positive for Gb3 from **F** **(G)**, STx2 binding from **H** **(I)**, or STx1 binding from **J** **(K)** were quantified (mean $\pm$ SE; $n = 3$; * $P < 0.05$ by t -test).

segment of the protein may be required for folding (this mutant was not used in subsequent assays). In rescue assays, both SLC35A2 Δ 1–31 and SLC35A2 Δ 343–393 restored Gb3 expression and STx1/STx2-binding in Δ SLC35A2 cells (Figure 6A–F; Table I). Note that protein

levels of the Δ 1–31 mutant were lesser than SLC35A2_{WT}, while levels of the Δ 343–393 mutant were comparable (Figure 6G). Overall, the N- and C-terminal cytoplasmic loops are dispensable for the trafficking and activity of SLC35A2.

Fig. 3. Expression of SLC35A2-GFP rescues the phenotype of Δ SLC35A2 cells. **(A)** Δ SLC35A2 cells were transfected with SLC35A2_{WT}-GFP. Twenty-four hours after transfection, cultures were processed to detect GFP and the Golgi apparatus, using an antibody against giantin. Scale bar, 20 μ m. **(B–E)** Δ SLC35A2 cells were mock-transfected or transfected with a control GFP construct or SLC35A2_{WT}-GFP. Twenty-four hours after transfection, cells were processed for analyses of Gb3 expression or STx1/STx2 binding. In **B**, numbers in each quadrant represent cell percentages. In **GFP**-expressing cells from **B**, cell percentages positive for Gb3 (**C**), STx2 binding (**D**), or STx1 binding (**E**) were quantified (mean $\pm$ SE; $n = 3$; * $P < 0.05$ by t -test).

Gly-202 and Gly-214 are required for the transport activity of SLC35A2

In SLC35A1, a set of 10 glycine residues form a putative hydrophilic substrate-translocating channel (Eckhardt et al. 1998; Lim et al. 2008; Hadley et al. 2014). Substitution of these glycine residues with amino acids with bulky side chains, such as isoleucine, glutamate or glutamine, inactivates SLC35A1 likely due to steric hindrance (Eckhardt et al. 1998; Lim et al. 2008; Hadley et al. 2014). To test whether a similar mechanism played a role in SLC35A2, we mutated three of the corresponding glycine residues, Gly-178, Gly-202, and Gly-214, individually to isoleucine (Table I). We selected these three residues because they were conserved across species, increasing the possibility that they were important for activity. Quantitative colocalization analyses revealed that SLC35A2_{G202I} predominantly localized to the Golgi (Figure 5A and B). A pool of SLC35A2_{G214I} was trapped in the endoplasmic reticulum, but the Pearson's coefficient for colocalization of SLC35A2_{G214I} and the marker of the Golgi apparatus was 0.56 ± 0.02 (Figure 5A and B), implying that substantial amounts of

the protein still trafficked to the Golgi. In contrast, SLC35A2_{G178I} predominantly localized to the endoplasmic reticulum (Figure 5A and B), and consequently, was not analyzed further. Importantly, in rescue assays, both SLC35A2_{G214I} and SLC35A2_{G202I} failed to restore Gb3 expression or STx1/STx2-binding, indicating that they were required for SLC35A2 activity (Figure 6A–F; Table I). Note that, in these rescue assays, expression of SLC35A2_{G202I} was comparable to SLC35A2_{WT} while that of SLC35A2_{G214I} was lesser (Figure 6G). Overall, Gly-214 and Gly-202 are both required for SLC35A2 activity.

Solvent-exposed Lys-78 and Lys-297 are also required for activity

Analyses of the predicted structure also identified two solvent-accessible lysine residues within the transmembrane domains of SLC35A2, Lys-78 and Lys-297, which could potentially play a role in transport activity (Figure 4B). To test the role of these residues, we generated SLC35A2_{K78A} and SLC35A2_{K297A} constructs

Fig. 4. Predicted topology and structure of SLC35A2. (A) Schematic depicting the topology of SLC35A2. Cytoplasmic aspects and residues of SLC35A2 that were mutated in Figs 5 and 6 are depicted in red; “aa” denotes amino acid. (B) Cartoon depiction of the predicted structure of SLC35A2. Cytoplasmic loops and glycine and lysine residues targeted for mutational analyses in Figs 5 and 6 are depicted in red.

Table I. Summary of SLC35A2 mutations analyzed

Mutation	Location	Localization	Activity
Structural mutations			
△1-31	Cytoplasmic loop 1	Golgi	Fully active
△88-110	Cytoplasmic loop 2	Endoplasmic reticulum	Not tested
△343-393	Cytoplasmic loop 6	Golgi	Fully active
G178I	Transmembrane domain 5	Endoplasmic reticulum	Not tested
G202I	Transmembrane domain 6	Golgi	Inactive
G214I	Transmembrane domain 6	Golgi	Inactive
K78A	Transmembrane domain 2	Golgi	Inactive
K297A	Transmembrane domain 9	Golgi	Inactive
Disease-associated mutations			
S213F	Transmembrane domain 6	Golgi	Inactive
G266V	Transmembrane domain 8	Golgi	Partially active
V331I	Transmembrane domain 10	Golgi	Fully active
R55L	Transmembrane domain 1	Golgi	Partially active
V258M	Luminal loop 4	Golgi	Fully active
Y267C	Transmembrane domain 8	Golgi	Fully active
G282R	Transmembrane domain 8	Golgi	Inactive
S304P	Transmembrane domain 9	Golgi	Partially active

(Table I). Both mutants partially localized to the Golgi (Figure 5A and B) and, importantly, failed to rescue Gb3 expression and STx1 or STx2 binding in Δ SLC35A2 cells (Figure 6A–F; Table I). Thus, both Lys-78 and Lys-297 are required for SLC35A2 activity. Notably, while this manuscript was in preparation, a disease-causing mutation in Lys-297 (mutated to glutamate) was identified in a human patient (Yates et al. 2018).

Effects of disease-associated mutations on SLC35A2 activity

We analyzed eight point mutations in SLC35A2 that are associated with human disease (Tables I & II) (Kodera et al. 2013; Ng et al. 2013; Dorre et al. 2015; Hino-Fukuyo et al. 2015; Bosch et al.

2016; Lopes et al. 2016; Winawer et al. 2018). Except V258, other disease-associated residues resided in the transmembrane domains of SLC35A2 (Figure 7A and B). All mutants tested localized to the Golgi apparatus (Figure 8A and B). Notably, all mutants could be easily detected in cells, but expression levels were lower than SLC35A2_{WT} (Figure 9G). Importantly, in rescue experiments, two mutants, S213F and G282R, failed to restore Gb3 expression and binding of STx1/STx2 (Figure 9A–F; Table I). We verified that the lack of rescue was not an effect of the GFP-tagged constructs used in Figure 9 because similar results were obtained with myc-tagged constructs (Figure S4A–C). Thus, mutations in S213 and G282 strongly impact SLC35A2 function, providing a straight-forward mechanism of disease with these mutations. Importantly, in cells expressing the R55L, G266V, or S304P mutant, Gb3 expression was comparable

Fig. 5. Localization of SLC35A2 structural mutants in Δ SLC35A2 cells. (A and B) Δ SLC35A2 cells were transfected with indicated SLC35A2-GFP constructs. Twenty-four hours after transfection, cultures were imaged to detect GFP and giantin (to demarcate the Golgi apparatus) in A or calnexin (to demarcate the endoplasmic reticulum) in B. "P" denotes values for the Pearson's coefficient for colocalization between channels (mean $\pm$ SEM, $n = 20$ cells per SLC35A2 construct). Scale bar, 20 μ m.

to those expressing SLC35A2_{WT}, but binding of STx1 and/or STx2 was lower (Figure 9A–F; Table I). The partial rescue of toxin binding may be reflective of a subtler defect in SLC35A2 activity that impacts Gb3 levels on a per-cell basis. Such a defect in SLC35A2 activity may be sufficient to induce disease. Expression of the remaining three mutants V331I, V258M, or Y267C fully rescued Gb3 expression and toxin binding (Figure 9A–F; Table I), indicating that these mutations did not impact SLC35A2 function. The means by which these mutations induce disease cannot be explained by a simple loss-of-function of the transport activity of SLC35A2, suggesting more complex mechanisms may be involved.

Discussion

Depletion of SLC35A2 in Δ SLC35A2 HeLa cells reduced Gb3 levels and, consequently, blocked the binding and toxicity of STx1 and STx2.

These effects were rescued by expression of SLC35A2_{WT} in Δ SLC35A2 cells. The above results allowed us to use rescue assays in Δ SLC35A2 cells as a means to investigate SLC35A2 function. Notably, SLC35A2 is also required for toxicity induced by a related bacterial toxin, cholera toxin (Guimaraes et al. 2011). The cell surface receptor for cholera toxin is GM1, which also requires Golgi luminal UDP-galactose for synthesis (Fujinaga et al. 2003; Guimaraes et al. 2011).

Our results showed that the N- and C-terminal cytoplasmic domains were dispensable for SLC35A2 activity. This is consistent with a prior report on murine SLC35A2 (Ishida et al. 1999). Additionally, we discovered that Lys-78, Lys-297, Gly-202 and Gly-214 were required for SLC35A2 activity. Similarities with SLC35A1 raise the possibility that Gly-202 and Gly-214 may be components of a substrate-translocating channel in SLC35A2. Lys-78 and Lys-297 are solvent accessible residues that may be involved in interactions with the substrate or play important roles in the conformational

Fig. 6. Functional analyses of SLC35A2 structural mutants. (A–C) Δ SLC35A2 cells were transfected with indicated SLC35A2-GFP constructs. Twenty-four hours after transfection, cultures were processed to assay for Gb3 expression or STx1/STx2 binding. Numbers in each quadrant represent cell percentages. (D–G). In GFP-expressing cells from A–C, cell percentages positive for Gb3 (D), STx2 binding (E) or STx1 binding (F) were quantified. Separately, GFP levels were also quantified for each construct as median fluorescence intensity (G) (mean $\pm$ SE; $n = 3$; * $P < 0.05$ for the comparison between SLC35A2_{WT}-GFP and all other constructs by one-way ANOVA and Dunnett's *post hoc* test).

change that occurs during the substrate transport cycle. Our results with Lys-297 are consistent with a prior report that additionally demonstrated that the corresponding residue in SLC35A1 is also

critical for activity (Chan et al. 2010). Importantly, mutation in Lys-297 is now associated with human disease (Yates et al. 2018). Characterization of Gly-202, Gly-214, and Lys-78 as critical for

Table II. Disease-associated SLC35A2 point mutations

Point mutation	Identified by	Confirmed by	Disease	Ref.
S213F	WES ^a	Sanger sequencing	Early-onset epileptic encephalopathy	Kodera et al. (2013)
G266V	WES	Sanger sequencing	Congenital disorders of glycosylation	Dorre et al. (2015)
V331I	WES	Sanger sequencing	Congenital disorders of glycosylation	Ng et al. (2013)
R55L	WES	ddPCR ^c	Malformations of cortical development	Winawer et al. (2018)
V258M	aCGH ^b & WES	Sanger sequencing	Rett syndrome	Lopes et al. (2016)
Y267C	WES	Sanger sequencing	Cerebral visual impairment	Bosch et al. (2016)
G282R	aCGH & WES	Sanger sequencing	West syndrome	Hino-Fukuyo et al. (2015)
S304P	WES	ddPCR	Nonlesional focal epilepsy	Winawer et al. (2018)

^aWES: whole exome sequencing; ^baCGH: array comparative genomic hybridization; ^cddPCR: droplet digital PCR.

Fig. 7. Depiction of disease-associated mutations on the predicted structure of SLC35A2. (A and B) All the disease-associated SLC35A2 mutations analyzed are depicted in green on the topology model (A) or predicted structure (B) of SLC35A2.

activity may enhance the ability of clinicians to rapidly identify inactivating mutations in these residues in new patients suspected of suffering from glycosylation deficits due to loss-of-function of SLC35A2.

Since glycine-178 was also expected to be a part of the putative substrate translocating channel of SLC35A2, we assayed for its role in activity. However, SLC35A2_{G178I} was trapped in the endoplasmic reticulum. We did not analyze this mutant further because the altered subcellular localization implied that it will likely lack activity. A prior study reported that substitution of the corresponding glycine residue with aspartate inactivated murine SLC35A2, but substitution with alanine did not impact Golgi localization or activity (Ishida et al. 1999). Presence of a bulky amino acid at this residue may impact SLC35A2-folding and subsequent endoplasmic reticulum exit.

Prior studies provide some insights into the effects of three disease-associated SLC35A2 mutations. Hamster SLC35A2 carrying the Δ S213 mutation, which corresponds to serine-213 in humans, failed to rescue SLC35A2-deficient CHO lec8 cells (Oelmann et al. 2001). SLC35A2_{G266V} also failed to rescue SLC35A2-deficient CHO lec8 and MDCK-RCA^r cells (Dorre et al. 2015). Fibroblasts derived from a patient harboring the V331I mutation were unable to translocate UDP-galactose into the Golgi lumen (Ng et al. 2013).

The loss-of-function effect of the Δ S213 mutation described previously is consistent with our results with the disease-associated S213F mutation. The requirement of the serine-213 residue, as well as of another disease-associated residue, glycine-282 (mutated to arginine in disease), for activity may also be related to the existence of the above described putative substrate-translocating channel. Comparison with residues that form the substrate-translocating channel of SLC35A1 reveals that Gly-214 and Gly-282 may be components of the putative channel in SLC35A2. Serine-213 is adjacent to glycine-214. Substitution of serine-213 and glycine-282 with bulky amino acids, such as phenylalanine or arginine, may induce steric hindrance and thereby block substrate translocation, as proposed for SLC35A1. Further support for this idea comes from the fact that substitution of another putative channel-forming glycine residue with a bulky amino acid (G281D) was identified in a SLC35A2-deficient CHO lec8 clone (Oelmann et al. 2001).

Interestingly, the G266V mutation rescued Gb3 expression and STx1 binding, but did not fully rescue STx2 binding. As described in the Results section, the ability of G266V to rescue Gb3, but not STx2 binding may be due to a subtle inhibition of SLC35A2 activity that reduces total cellular Gb3 levels. Further, the B-subunits of STx1 and

Fig. 8. Localization of SLC35A2 disease-associated mutants in Δ SLC35A2 cells. (A and B) Δ SLC35A2 cells were transfected with indicated SLC35A2-GFP constructs. Twenty-four hours after transfection, cultures were imaged to detect GFP and giantin (to demarcate the Golgi apparatus) in A or calnexin (to demarcate the endoplasmic reticulum) in B. Values for the Pearson's coefficient for colocalization are also indicated (mean $\pm$ SEM, $n = 20$ cells per SLC35A2 construct). Scale bar, 20 μ m.

STx2 mediate membrane binding. The B-subunit of STx1 has a higher binding affinity for glycolipids than that of STx2 (Karve and Weiss 2014), which may provide an explanation for the capability of the G266V mutant to rescue STx1, but not STx2, binding. The R55L and S304P mutations also fully rescued Gb3 expression, but only partially rescued toxin binding. Notably, unlike G266V, the R55L and S304P mutations failed to rescue binding of both STx1 and STx2. It is likely that different mutations inhibit SLC35A2 function to different extents, causing a variation in downstream consequences. From a clinical perspective, the inhibitory effects of the G266V, R55L, or S304P mutation on SLC35A2 function is likely sufficient to induce deleterious effects that lead to disease manifestation.

Three disease-associated mutations, V331I, V258M, and Y267C, did not affect SLC35A2 activity in our assays. In addition to different mutations affecting SLC35A2 function to different extents, the impact

of mutations on activity may be cell-type specific and may depend on the expression of other proteins that interact with SLC35A2. Indeed, as described earlier, fibroblasts isolated from a human patient harboring the V331I mutation were defective in transport of UDP-galactose into the Golgi apparatus (Ng et al. 2013). Overall, an important implication of our findings and this discussion is that the relationship between SLC35A2 function and disease phenotype is complex. These complexities are enhanced by the fact that SLC35A2 is located on the X-chromosome and patient presentation will be influenced by cell-type specific expression of the mutant allele, based on X-inactivation and mosaicism (Ng et al. 2013; Scott et al. 2014; Westenfield et al. 2018).

In conclusion, using a novel activity assay and a predicted structure, we performed functional analyses of SLC35A2. We identified critical residues required for SLC35A2 activity and elucidated the consequences of several mutations associated with human disease on

Fig. 9. Functional analyses of SLC35A2 disease-associated mutants. (A–C) Δ SLC35A2 cells were transfected with indicated SLC35A2-GFP constructs and analyzed for Gb3 expression or toxin binding twenty-four hours post-transfection. Numbers in each quadrant represent cell percentages. (D–G) In GFP-expressing cells from A–C, cell percentage positive for Gb3 (D), STx2 binding (E) or STx1 binding (F) were quantified. Additionally, GFP levels were also quantified as median fluorescence intensity (G) (mean $\pm$ SE; $n = 3$; * $P < 0.05$ for the comparison between SLC35A2_{WT}-GFP and all other constructs by one-way ANOVA and Dunnett's *post hoc* test).

transporter function. These results substantially improve understanding of the transport mechanism of SLC35A2 and of the pathobiology of human disease due to SLC35A2 mutations.

Materials and Methods

Cell culture. All experiments were performed using HeLa cells. WT cells were a HeLa cell clone that overexpresses Gb3 synthase, which

induces overexpression of Gb3 (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). The use of this clone was designed to ensure that we could robustly detect Gb3 expression and STx1/STx2 binding in WT cells, and that a large effect was obtained when these parameters were analyzed after depletion of genes required for Gb3 synthesis. We have extensively utilized this clone to study the trafficking and toxicity of STx1 and STx2 (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). Cells were grown in minimum essential media containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Generation of Δ SLC35A2 cells. Δ SLC35A2 cells were generated by targeting SLC35A2 in WT HeLa cells that overexpressed Gb3 synthase using a lentivirus-based CRISPR/Cas9 system as described previously (Selyunin et al. 2017). The guide RNA sequence was 5'-GGUGCAGUAUGUG GACACGC-3'. It targeted the third exon of the SLC35A2 gene, which codes for amino acids 106–112 (full-length SLC35A2 isoform c or UGT1 has 393 amino acids). Other procedures (lentivirus production and infection, single cell clone selection and propagation, and genomic DNA sequencing) were exactly as described by us previously (Selyunin et al. 2017).

Plasmids and DNA transfections. To generate the SLC35A2-GFP plasmid, we obtained a template plasmid from DNASU (clone HsCD00785042). This template codes for SLC35A2 isoform h, which is expected to be 421 amino acids long. However, the cDNA for this isoform of SLC35A2 has not been isolated from human cells, and it is unclear whether it is expressed. This isoform has an extra 28 amino acids not present in SLC35A2 isoform c or UGT1. We deleted bases 91–174 from clone HsCD00785042 to obtain a plasmid that had the open reading frame of SLC35A2 isoform c or UGT1. This open reading frame is referred to as SLC35A2_{WT}. Subsequently, we moved the SLC35A2_{WT} insert into pEGFP-N3 plasmid by performing a PCR reaction using 5'-GAGCTCAAGCTT ATGGCAGCGTTGGGG-3' and 5'-GGCGATGGATCCCTTCA CCAGACT GACTTTGG-3' primers and digesting the product with *HindIII* and *BamHI* (NEB) enzymes. Myc-tagged SLC35A2_{WT} was generated by looping in a single myc-tag at the N-terminus of SLC35A2 and introducing a stop codon before the C-terminal GFP-tag using QuikChange. Point mutations and deletions were introduced into the above plasmids using QuikChange. We have described all of these cloning techniques previously (Leyva-Illades et al. 2014; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). All plasmids were verified by sequencing. The control plasmid coded for Rab5-GFP, which we have previously described (Leyva-Illades et al. 2014; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). Plasmids were transfected using JetPEI reagent as described previously (Leyva-Illades et al. 2014; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017).

Transfections with siRNA. Transfections with siRNA were performed using Oligofectamine reagent exactly as described by us previously (Leyva-Illades et al. 2014; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). The control siRNA did not target any human gene (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). The SLC35A2 sequence targeted was: 5'-AGAATAACCTCC AGTATGT-3'.

RT-PCR and qRT-PCR. These were performed as described by us previously (Leyva-Illades et al. 2014; Selyunin and Mukhopadhyay 2015; Hutchens et al. 2017; Liu et al. 2017; Selyunin et al. 2017). For RT-PCR in Δ SLC35A2 cells, two pairs of primers were used to amplify SLC35A2. Primer pairs 1 and 2, respectively, amplified fragments upstream or downstream of the region targeted by the CRISPR/Cas9 (primer pair 1: forward, 5'-GAATGCCTCCCTCATC CTCAG-3' and

reverse, 5'-CCTTTGAGCAC TTCCGCCAT-3'; primer pair 2: forward, 5'-GTGCTCATGCTGAATCGCAG-3' and reverse, 5'-CTTGC TGTGCCTGGACAATG-3'). For qRT-PCR, only primer pair 1 was used to amplify SLC35A2. Primers to amplify TBP were 5' CGA ACC ACG GCA CTG ATT TTC 3' (forward) and 5' TTT CTT GCT GCC AGT CTG GAC 3' (reverse). Transcript levels were quantified using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Viability assays. Cells were exposed to indicated concentrations of STx2 (BEI Resources) or STx1 (ListLabs) for 16 h. Viability was assessed using methylthiazolylidiphenyltetrazolium bromide (MTT) assay as described previously (Leyva-Illades et al. 2014; Zogzas et al. 2016; Selyunin et al. 2017; Zogzas and Mukhopadhyay 2018).

Fluorescence activated cell sorting (FACS) analyses. Cells were trypsinized and washed once with ice-cold phosphate buffered saline. To quantify Gb3 expression, cells were incubated with an antibody against Gb3 (Biorad) in phosphate buffered saline containing 1% bovine serum albumin on ice for 30 min. Cells were then washed with ice-cold phosphate buffered saline and stained with Alexa Fluor 555 conjugated secondary antibody (Thermo Fisher, 1:500 dilution in phosphate buffered saline containing 1% bovine serum albumin) on ice for another 30 min. After this, cells were washed in ice-cold phosphate buffered saline, fixed with 1% paraformaldehyde, and utilized for flow cytometry.

To determine binding of STx1 or STx2, the B-subunits of both toxins were used. The B-subunit of STx1 was purified from bacteria and labeled with Alexa Fluor 555 as described by us previously (Mukhopadhyay and Linstedt 2012; Mukhopadhyay et al. 2013; Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017). His-tagged B-subunit of STx2 was obtained from BEI Resources and labeled as above. Labeled toxins were incubated with cells (10 µg/ml for STx1 or 4 µg/ml for STx2) on ice for 30 min in minimum essential media containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were then washed with ice-cold phosphate buffered saline, fixed in 1% paraformaldehyde, and utilized for flow cytometry. This protocol is essentially similar to that described by us previously for analyzing toxin binding and transport using microscopy (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017).

Analyses were performed using a BD Accuri C6 cytometer. The Flowjo software (Tree Star) was used for data analyses.

Generation of a predicted SLC35A2 structure. Predicted structure of SLC35A2 was generated using the PHYRE 2.0 server (Kelley and Sternberg 2009) based on a recently solved crystal structure of Vrg4, a GDP mannose transporter from *Saccharomyces cerevisiae* belonging to the SLC35D family (Parker and Newstead 2017). All protein structure images were generated using the open source software PyMOL (Schrödinger, LLC, New York, NY, 2018).

Antibodies. Monoclonal antibody against Gb3 was from Bio-Rad (Cat# MCA579). We have previously described the monoclonal antibody against the myc-tag and polyclonal antibodies against calnexin and giantin (Selyunin et al. 2017).

Immunofluorescence microscopy and image analyses. Cells were grown on cover-slips. For immunofluorescence without binding of STx1 or STx2, cells were fixed with 1% formaldehyde and processed for immunofluorescence using appropriate primary and secondary antibodies exactly as described previously (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017).

For toxin binding assays, cells were incubated with fluorescently-labeled B-subunits of STx1 or STx2 (5 µg/ml for STx1 and 2 µg/ml for STx2) on ice in minimum essential media containing 10% fetal

bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin for 30 min. Cells were then washed with ice-cold phosphate buffered saline, fixed in 1% formaldehyde, and processed as described above. This protocol is similar to that described by us previously for studying toxin transport (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017).

All images were captured using a Nikon swept-field confocal microscope equipped with a four-line high-power laser launch and a 100 × 1.45 NA oil immersion objective. An iXon3 X3 DU897 electron-multiplying charge-coupled device camera (Andor Technology) was used for image capture. All images were captured as z stacks with 0.2-µm spacing between individual frames. Images depicted in the figures are maximum-intensity projections of the stacks. All analyses were performed using ImageJ (National Institutes of Health) as described previously (Selyunin and Mukhopadhyay 2015; Selyunin et al. 2017).

Statistical analyses. Prism 6 software (GraphPad Software) was used for all statistical analyses. All experiments were repeated at least three times independently. Comparisons between two groups were performed using *t*-test. Comparisons between multiple groups were performed using one-way ANOVA followed by appropriate *post hoc* tests. Non-linear regression was used to calculate the LD₅₀ of STx1 or STx2. For all analyses, *P* < 0.05 was considered to be statistically significant. Asterisks in graphs, where present, denote statistically significant differences.

Supplementary data

Supplementary data is available at *Glycobiology* online.

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Conflict of interest statement

None declared.

Author contributions

S.M. and D.L. designed the study. D.L. performed experiments, analyzed data, and prepared data panels. S.M. wrote the paper with help from D.L.

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