Defective mucin-type glycosylation on α-dystroglycan in COG-deficient cells increases its susceptibility to bacterial proteases

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Deficiency in subunits of the conserved oligomeric Golgi (COG) complex results in pleiotropic defects in glycosylation and causes congenital disorders in humans. Insight regarding the functional consequences of this defective glycosylation and the identity of specific glycoproteins affected is lacking. A chemical glycobiology strategy was adopted to identify the surface glycoproteins most sensitive to altered glycosylation in COG-deficient Chinese hamster ovary (CHO) cells. Following metabolic labeling, an unexpected increase in GalNAz incorporation into several glycoproteins, including α-dystroglycan (α-DG), was noted in cog1-deficient ldlB cells. Western blotting analysis showed a significantly lower molecular weight for α-DG in ldlB cells compared with WT CHO cells. The underglycosylated α-DG molecules on ldlB cells are highly vulnerable to bacterial proteases that co-purify with V. cholerae neuraminidase, leading to rapid removal of the protein from the cell surface. The purified bacterial mucinase StcE can cleave both WT and ldlB α-DG but did not cause rapid degradation of the fragments, implicating other V. cholerae proteases in the final proteolysis of the fragments. Extending terminal glycosylation on the existing mucin-type glycans of ldlB α-DG stabilized the resulting fragments, indicating that fragment stability, but not the initial fragmentation of the protein, is influenced by the glycosylation status of the cell. This discovery highlights a functional importance for mucin-type O-glycans found on α-DG and reinforces a growing role for these glycans as regulators of extracellular proteolysis and protein stability.

The congenital disorders of glycosylation are inherited disorders that are caused by defects in the glycosylation machinery of the cell (1–3). The number of CDG2 subtypes identified is rapidly expanding. These disorders are associated with a wide range of clinical manifestations, including neurological and muscle deficits, gastrointestinal complications, and dysmorphia (4–9). The underlying basis for CDGs includes proteins not directly involved in glycosylation such as the subunits of the conserved oligomeric Golgi (COG) complex (1, 10, 11). The eight-subunit COG complex acts as a vesicle tether for COPI-coated vesicles during intra-Golgi retrograde transport (12, 13). Loss of COG integrity impairs the retrograde transport of glycosylation enzymes, resulting in their mislocalization and/or altered activity (14, 15). COG-deficient cells exhibit defects in both protein and lipid glycosylation, with pronounced alterations in N- and O-glycan galactosylation and sialylation (11, 16–19).

Although glycan profiles in CDG-deficient cells, patient samples, and animal models are described (16, 18–20), there is little information regarding which glycoproteins are most sensitive to COG-associated glycosylation defects, with the notable exception of the LDL receptor. The sensitivity of this receptor to COG-driven glycosylation defects served as the basis for identifying the CHO cell mutants, ldlB and ldlC, and highlighted the functional importance of proper glycosylation on cell-surface receptor stability (21–23). Identifying additional glycoproteins impacted by the altered glycosylation associated with COG deficiency and other different CDG subtypes will better inform our understanding of how global glycosylation defects influence glycoprotein function and may also yield new insight into the tissue-specific pathogenesis of these disorders.

Here we took advantage of metabolic engineering of COG-deficient ldlB cells and identified α-dystroglycan as a protein profoundly affected by the glycosylation defects found in these cells. A glycoprotein normally associated with muscle cells, α-dystroglycan is also expressed in follicular epithelial cells where it localizes to the basal surface and plays a role in establishing polarity during oogenesis (24, 26). Our data show that the mucin-type glycosylation on α-DG is strongly affected by COG deficiency, leading to its increased susceptibility to proteases that co-purify with V. cholerae neuraminidase. Although WT and ldlB α-DG are equally susceptible to fragmentation by the bacterial mucinase, the altered glycosylation on ldlB α-DG enhances the turnover of these fragments by other proteases.

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2 The abbreviations used are: CDG, congenital disorder of glycosylation; COG, conserved oligomeric Golgi; α-DG, α-dystroglycan; LDL, low density lipo-protein; CHO, Chinese hamster ovary; VC, V. cholera; AU, A. ureafaciens; SEEL, selective exo-enzymatic labeling; DANA, N-acetyl-2,3-dehydro-2-deoxyneuraminic acid; HRP, horseradish peroxidase; FBS, fetal bovine serum; PNGase, peptide/N-glycosidase.
that co-purify with *V. cholerae* neuraminidase (Fig. 1). Restoring the terminal glycosylation of existing *O*-glycans on *ldlB*/*H9251*-DG stabilizes the fragments. This work demonstrates for the first time that *H9251*-DG is a substrate for bacterial mucinases and highlights a stabilizing role for the mucin-type *O*-glycans found on this glycoprotein.

**Results**

**Increased labeling of specific glycoproteins with GalNAz in *ldlB* CHO cells**

We envisioned that metabolic labeling of cells with Ac₄ManNAz or Ac₄GalNAz could be an effective way to identify changes in the glycosylation status and abundance of cell-surface glycoproteins in the glycosylation-deficient cells. By analyzing labeled glycoproteins by SDS-PAGE and Western blotting, glycoprotein profiles can be assessed to determine whether the underlying glycosylation defect alters the mobility or intensity of individual glycoproteins. Lec2 and *ldlB* CHO cells, deficient in the CMP-sialic acid transporter and COG complex subunit 1, respectively, were chosen as representative cell lines to determine the impact of impaired sialylation alone (Lec2) or broader glycan alterations (*ldlB*) on labeled glycoproteins. Although ManNAz-treated cells largely display azide-modified sialic acids on their cell surface, GalNAz-treated cells incorporate azido sugars into both *N*- and *O*-glycans, as well as nucleo-cyttoplasmic *O*-GlcnAc-bearing proteins. Thus, the biorthogonal step was performed using S-DIBO-biotin, which is membrane-impermeable, to selectively add biotin to the cell-surface glycoproteins. WT CHO cells showed substantial incorporation of SiaNAz after Ac₄ManNAz and S-DIBO-biotin labeling (which increased after 48 h), but there was no detectable incorporation of this label in either Lec2 or *ldlB* cells. The lack of ManNAz labeling in Lec2 and *ldlB* was anticipated based on the underlying glycosylation defects in these cell lines. WT CHO cells incorporated GalNAz into multiple cell-surface glycoproteins. This labeling was decreased in the Lec2 cells. Overall GalNAz incorporation increased following 48 h of labeling in both WT and Lec2 cells. GalNAz labeling was unexpectedly higher in the *ldlB* cells. These cells showed a strongly labeled band at 60 kDa following 24 h of GalNAz treatment. The intensity of this band decreased after longer GalNAz treatment, possibly reflecting higher turnover of this glycoprotein in *ldlB* cells.

The major GalNAz-labeled 60-kDa band in *ldlB* cells is an *O*-glycosylated protein susceptible to *V. cholerae* proteases that co-purify with its neuraminidase

To determine the nature of the major GalNAz-labeled glycoproteins in *ldlB* cells, GalNAz-treated WT CHO and *ldlB* cells were further treated with or without neuraminidase from *Vibrio cholerae* (VC) and analyzed by SDS-PAGE and Western blotting. Cell lysates from each condition were further treated with PNGase F to assess whether *ldlB* cells incorporated GalNAz into *N*- or *O*-linked glycans of the major labeled glycoproteins including the prominent 60-kDa band. Neuraminidase treatment of WT CHO cells resulted in a shift of the major

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**Figure 1. Domain structure of α-dystroglycan and proposed role of mucin-type glycans in protein stability.** The mucin region of α-dystroglycan bears both GalNAc- and mannose-initiated glycans flanked by *N*- and C-terminal domains that contain *N*-glycans. ManNAz is incorporated into sialic acid on both *N*- and *O*-glycans, whereas GalNAz is mainly incorporated into GalNAc-containing *O*-glycans. The cleavage of α-dystroglycan by bacterial mucinases occurs in both WT and *ldlB* CHO cells despite major effects on the mucin-type glycans of this glycoprotein. The susceptibility of the resulting fragments toward other bacterial proteases, however, is influenced by the extent of mucin-type glycosylation on the fragments.

**Figure 2. Labeling of cell-surface glycoproteins with GalNAz is increased in *ldlB* cells.** WT CHO, Lec2, and *ldlB* cells were labeled with Ac₄ManNAz or Ac₄GalNAz for 24 or 48 h followed by reaction with S-DIBO-biotin and analysis by SDS-PAGE and Western blotting. A representative blot from three independent experiments is shown. IB, immunoblot.
labeled proteins to a lower molecular weight, consistent with the loss of sialic acid. Surprisingly, neuraminidase treatment of GalNAz-labeled ldlB cells resulted in complete loss of the 60-kDa major labeled band (Fig. 3A). This glycoprotein contains both N- and O-glycans but bears the majority of the GalNAz label within O-glycans because PNGase F reduces the apparent molecular weight but did not result in obvious loss of any biotin label.

*V. cholerae* contains a hydrolytic complex of enzymes including a neuraminidase and several proteases (28–31). This complex is highly active toward mucins typically found in the airways and intestines. Commercial preparations of *V. cholerae* neuraminidase contain residual protease activity, because the enzyme is purified from *V. cholerae* extracts. *Arthrobacter ureafaciens* (AU) neuraminidase, however, is prepared as a recombinant protein and thus devoid of the protease contaminants found in *V. cholerae* extracts. We compared VC neuraminidase and AU neuraminidase to address the possibility that the loss of the 60-kDa band in ldlB cells is specific to a certain neuraminidase and/or its substituents. Although both neuraminidases were able to remove azido-sialic acid from ManNAz-treated CHO cells, only the VC neuraminidase removes the 60-kDa band. The relative intensity of this band was quantified (average ± standard error of the mean; n = 2). ManNAz-labeled WT CHO and GalNAz-labeled ldlB cells were treated with *V. cholerae* neuraminidase in the presence of increasing amounts of the general neuraminidase inhibitor DANA. Following S-DIBO-biotin reaction on whole cells, resulting lysates were subjected to SDS-PAGE and Western blotting using anti-biotin–HRP. A representative blot from two separate experiments is shown. Removal of the 60-kDa band is likely a function of its proteolytic degradation by the VC neuraminidase contaminants, so
we tested whether a broad-spectrum matrix metalloproteinase inhibitor, GM6001 (galadin or ilomastat) could impair the removal of this band following treatment of VC neuraminidase. An increase in the level of the 60-kDa band was observed in the presence of GM6001 following neuraminidase treatment of ldIB cells, although this was not sufficient to fully restore the steady-state level seen in non-neuraminidase-treated cells. The abundance of the 60-kDa band was also increased in non-neuraminidase-treated ldIB cells in the presence of GM6001, suggestive of some inherent susceptibility to a metalloprotease in this cell line (Fig. 3).

The protease-sensitive 60-kDa glycoprotein in ldIB cells is identified as α-dystroglycan

The removal of the 60-kDa band in the ldIB cells following VC neuraminidase treatment indicated that the surface stability of this glycoprotein is sensitive to the proteolytic components that co-purify with VC neuraminidase. We next sought to identify this 60-kDa glycoprotein using a proteomics-based strategy that takes advantage of this sensitivity. Metabolic labeling of ldIB cells with GalNAz was followed by treatment with or without neuraminidase at 4 °C or 37 °C. Note that ldIB cells were treated with neuraminidase at 4 °C or 37 °C. Note that the major labeled 60-kDa glycoprotein is partially spared from neuraminidase-induced loss at the lower temperature. Indeed, the abundance of the 60-kDa band was also increased in non-neuraminidase-treated ldIB cells in the presence of GM6001, suggestive of some inherent susceptibility to a metalloprotease in this cell line (Fig. 3D).

Figure 4. Identification of α-dystroglycan as the neuraminidase-sensitive O-glycoprotein in ldIB cells.

A, Western blotting analysis of Ac4GalNAz-labeled WT CHO and ldIB cells with or without post-treatment of V. cholerae neuraminidase (n = 2). Note that ldIB cells were treated with neuraminidase at 4 °C or 37 °C. Note that the major labeled 60-kDa glycoprotein is partially spared from neuraminidase-induced loss at the lower temperature. B, normalized spectral counts of a subset of assigned proteins in ldIB cells under the conditions shown. Both DAG1 and CD44 demonstrated decreased abundance following neuraminidase treatment at 37 °C but partial recovery when treated at 4 °C. C, Western blotting analysis of input and eluted fractions with anti-biotin–HRP following immunoprecipitation of GalNAz and S-DIBO-biotin–labeled cell lysates using an anti-CD44 antibody. The cells were treated with or without V. cholerae neuraminidase prior to lysis. D, Western blotting analysis of input, flowthrough, and eluted fractions with anti-DG (core) antisera following immunoprecipitation of lysates from GalNAz and S-DIBO-biotin labeled cells using an anti-biotin antibody. The cells were treated with or without V. cholerae neuraminidase prior to lysis. The asterisk denotes a nonspecific band detected using the anti-DG (core) antibody. The blots shown in C and D are representative of three independent experiments. IB, immunoblot.

We isolated the protease-sensitive 60-kDa glycoprotein from ldIB cells and attempted to identify it using a proteomics-based strategy that takes advantage of this sensitivity. Metabolic labeling of ldIB cells with GalNAz was followed by treatment with or without neuraminidase at 4 °C or 37 °C (Fig. 4). The 4 °C condition was used to ask whether lowering the temperature of the VC neuraminidase step would slow the loss or turnover of this glycoprotein. Indeed, the abundance of the 60-kDa labeled band was reduced but not absent at 4 °C following neuraminidase treatment, whereas it was removed completely at 37 °C, indicating temperature sensitivity (Fig. 4A). Biotinylated proteins were enriched by immunoprecipitation, resolved by SDS-PAGE, and subjected to proteomic analysis as described under "Experimental procedures." The relative spectral counts of identified proteins among three different conditions (4 °C with and without VC neuraminidase and 37 °C with VC neuraminidase) were compared (Fig. 4B). Among the most abundant protein hits, α-dystroglycan (α-DG; DAG) and CD44 showed significantly decreased spectral counts in a temperature-dependent manner upon neuraminidase treatment. To verify the candidates, ldIB cells were labeled with GalNAz and S-DIBO-biotin, and immunoprecipitation performed with either an anti-CD44 antibody (followed by immunoblotting with anti-biotin–HRP) or an anti-biotin antibody (followed by immunoblotting with anti-DG (core) antibody). The GalNAz labeling of CD44 in ldIB cells was reduced upon neuraminidase treatment, but the molecular weight of the labeled CD44 did not match with the 60-kDa band (Fig. 4C). In contrast, the major GalNAz-labeled 60-kDa band clearly corre-
sponded to the glycoprotein detected by the α-DG core antibody following anti-biotin immunoprecipitation, both with regard to its molecular weight and sensitivity to VC neuraminidase (Fig. 4D).

**Altered mobility of α-DG in COG-deficient CHO cells is a function of COG-dependent defects in O-glycosylation**

The electrophoretic mobility of α-DG in WT CHO cells is much different from that observed in ldlB cells. In WT CHO, the protein exists in multiple bands ranging in size between 110 and 150 kDa, whereas α-DG in ldlB cells shows a single band at 60 kDa. The basis for this large molecular mass shift in α-DG in the ldlB cells was investigated. PNGase F treatment of the lysate showed only a slight decrease of molecular weight (5–10 kDa), suggesting that the major portion of molecular weight difference is not due to defects in N-glycosylation, but rather a defect in its O-glycosylation (Fig. 5A). The large molecular weight difference could arise if N-terminal region of α-DG is retained in WT CHO but not in ldlB cells (32, 33). Thus, WT CHO and ldlB cells were treated with an inhibitor of the proprotein convertase furin (one of the convertases responsible for α-DG maturation) to see whether altered processing of the N-terminal region occurred in either cell line. Inhibitor treatment resulted in a similar, dose-dependent shift in the mobility of α-DG in both cell types (Fig. 5B), suggesting that the molecular weight difference of α-DG is not due to a block in its proteolytic maturation by furin-like convertases. Overall, these results strongly suggest that the molecular weight difference of α-DG in between WT CHO and ldlB cells is due to the defect in its O-glycosylation. The abundance and mobility of α-DG was examined in other glycosylation-deficient CHO cell lines (Fig. 5C). Of note, the steady-state level of α-DG was significantly higher in untreated ldlB and ldlC cells compared with WT CHO. Lec2 cells showed similar but nonsialylated forms of α-DG, and its expression level is even lower than WT CHO cells. The cog2-deficient ldlC cells showed the same behavior following VC neuraminidase/protease treatment as ldlB cells (Fig. 5C). Finally, analysis of the α-DG protein in cog1-corrected ldlB cells showed near complete rescue of the decreased molecular weight of α-DG, suggesting that the altered mobility of α-DG is a direct consequence of COG-related alterations in glycosylation (Fig. 5D).

**StcE mucinase digestion fragments both WT and ldlB α-DG**

In light of the fact that multiple hydrolytic activities are known to co-purify with VC neuraminidase, we asked whether bacterial mucinase activity or another protease was responsible for the fragmentation and degradation of α-DG from the surface of ldlB cells. The effects of VC neuraminidase treatment on cell lysates containing a protease inhibitor mixture was first tested, revealing intact neuraminidase activity but no signifi-
significant degradation of α-DG (Fig. 6A). This experiment confirms the prior observation that the degradation of α-DG in ldlB cells is due to proteolysis and not triggered by desialylation. To ask whether bacterial mucinase activity is specifically responsible for the fragmentation and degradation of the protein, we expressed and purified the mucinase enzyme StcE, a sequenceologue of the TagA mucinase found in V. cholerae (34), and assessed its ability to remove cell-surface α-DG in both WT and ldlB cells. The purified mucinase was equally capable of cleaving both WT and ldlB α-DG into distinct fragments (at 45 and 35 kDa for WT CHO and at 35 and 25 kDa for ldlB cells (Fig. 6, B and C). Notably, the resulting fragments were stable even at the highest concentrations of mucinase, consistent with the more selective action of this protease. These data indirectly suggest that the mucinase in the VC neuraminidase preparations is likely responsible for the fragmentation of α-DG but is not the enzyme that completes the degradation of the α-DG fragments. Comparing the purified mucinase and neuraminidase preparations was not possible because of the unknown concentration of contaminating proteases in the neuraminidase preparations. Using ranges for each treatment, we were able to at least qualitatively compare the fragmentation patterns produced by StcE and VC neuraminidase on the same blot (Fig. 6D). Both treatments caused fragmentation of α-DG in ldlB cells, but the pattern of these fragments was distinct, which reinforces the notion that there are likely multiple proteases that co-purify with VC neuraminidase that are capable to cleaving α-DG and ultimately degrading the fragments produced.

Terminal extension of existing O-glycans partially inhibits α-DG fragmentation and prolongs fragment half-life

The large difference in molecular weight in ldlB α-DG indicates a loss of O-glycan occupancy or failure to extend existing structures with more complex termini. We tested whether exogenously adding sugars back onto cell-surface α-DG in ldlB cells would result in stabilization of the protein and protection from turnover and/or proteolysis. Enzymatic labeling using ST3Gal1 and its natural substrate CMP-Neu5Ac to extend existing galactose-terminated glycans did not show any molecular weight shift of α-DG (Fig. 7A). However, labeling using Drosophila C1GalT1 and UDP-galactose shifted α-DG to a higher molecular weight and substantially reduced the diversity of different MW species. These data suggest the presence of some O-glycans on ldlB-derived α-DG that were initiated but not extended. This is consistent with the earlier demonstration of weak or absent ManNAz incorporation in these cells. When labeling was performed using C1GalT1, ST3Gal1, UDP-galactose, and CMP-Neu5Ac together, the MW shift of the α-DG band increased further (Fig. 7A). This result indicates that α-DG in ldlB cells bears Tn antigen (Ser/Thr–O-GalNAc), which cannot accept sialic acid directly by ST3Gal1 without prior galactosylation. Nonetheless, these shifts noted are relatively small compared with the large difference in molecular weight between ldlB and WT derived α-DG, which can be interpreted as either the substantial loss of O-glycan occupancy on this glycoprotein or the inefficiency of the enzymatic labeling at the cell surface. The exogenous modification of α-DG with C1GalT1 and ST3Gal1 weakly inhibited the fragmentation
of this protein following VC neuraminidase treatment but resulted in significantly greater stability of the 45-kDa α-DG fragment (Fig. 7, B and C). These findings point to a role for the mucin-type glycans in stabilizing the fragments produced by mucinase digestion but not in preventing the initial fragmentation by this enzyme.

Discussion

We took advantage of a metabolic engineering strategy to identify α-DG as a glycoprotein that is strongly sensitive to the glycosylation defects associated with COG deficiency. The profound loss of O-glycan glycosylation on α-DG in the cogI-deficient ldlB CHO mutant results in a form of the protein that is highly susceptible to degradation by bacterial proteases that co-purify with V. cholerae neuraminidase. These findings highlight another possible route whereby altered glycosylation can influence the critical function of this cell-surface glycoprotein. The implications of these findings with regard to the function of mucin-type glycans and disease pathogenesis are considered below.

The rapid loss of α-DG from the surface of ldlB cells upon treatment with commercial VC neuraminidase is a function of the proteases that co-purify with the neuraminidase. V. cholerae is known to contain a complex of hydrolases including the neuraminidase, a hexosaminidase, and several proteases including a mucinase that allow the bacteria to invade epithelial layers in the lung and intestines (28, 30, 31). The specific role of a bacterial mucinase in the degradation of α-DG was tested in both WT and ldlB cells using purified StcE, an enzyme with high similarity to the V. cholerae mucinase TagA. We found that both WT and ldlB α-DG is efficiently fragmented by StcE, suggesting that other VC proteases are likely responsible for the rapid degradation of α-DG in ldlB cells compared with WT CHO cells. Augmenting the existing mucin-type O-glycans on ldlB cells stabilized the α-DG fragments (Fig. 7, B and C), supporting the conclusion that fragment stability is influenced by the glycosylation state of the glycoprotein.

Krieger and co-workers (21–23) identified cogI-deficient ldlB cells as one of four complementation groups that exhibited greatly decreased LDL receptor on the cell surface. Subsequent work lead to the conclusion that altered O-glycosylation of this protein following VC neuraminidase treatment but resulted in significantly greater stability of the 45-kDa α-DG fragment (Fig. 7, B and C). These findings point to a role for the mucin-type glycans in stabilizing the fragments produced by mucinase digestion but not in preventing the initial fragmentation by this enzyme.

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Krieger and co-workers (21–23) identified cogI-deficient ldlB cells as one of four complementation groups that exhibited
limited number of O-mannose–initiated glycans would not account for the dramatic shift in molecular weight in the ldlB cells. Moreover, O-mannose glycans are initiated in the ER, whereas mucin-type glycosylation begins in the Golgi. Effects of COG deficiency may be restricted to glycan initiation and processing events in the Golgi, because the primary action of this complex is maintaining the fidelity of retrograde transport of glycosyltransferase-bearing COPI vesicles in Golgi and post-Golgi compartments. Future studies will be aimed at defining how COG complex deficiency alters the biosynthesis of the M3 glycan in muscle cells and whether the early steps in this process are affected.

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Cell lines and culture
WT CHO cells (clone K1, ATCC), mutant CHO cells (Lec2, ldlB, or ldlC) and a Cog1-transfected ldlB cells, ldlB[ldlB] were cultured in minimum essential medium α 1X (Cellgro) with Earle’s salts, without ribonucleosides, deoxyribonucleosides, and l-glutamine with 10% fetal bovine serum (FBS, Bench-Mark) and penicillin (100 IU/ml)/streptomycin (100 μg/ml, MediaTech). Cells were cultured in a 5% CO2 atmosphere, 37 °C humid incubator.

Metabolic labeling and enzymatic treatment of cells
Metabolic labeling of cell surface was typically done by incubation of cells with 50 – 60% confluency in 12-well, 6-well, or 10-cm dishes with 30 μM of Ac4ManNAz or 100 μM of Ac4GalNAz for 24 h in the same cell culture medium at 37 °C. In case of 48 h of labeling, 25–30% confluent cells were used to obtain confluent cells at the end of labeling. After metabolic labeling, the cells were washed with Dulbecco’s PBS, and then cells were further incubated with 30 μM of S-DIBO-biotin in Dulbecco’s PBS containing 2% FBS for 1 h at room temperature. Treatment of (±) sialidase, in general, was made right after metabolic labeling and before S-DIBO-biotin reaction. Typically, 50 milliunits/ml of V. cholerae neuraminidase or 67 units/ml of A. ureafaciens neuraminidase (determined empirically as equivalent concentration to 50 milliunits/ml of V. cholerae neuraminidase for similar removal of azide-containing sialic acid from metabolically labeled cells with Ac4ManNAz) was treated in serum-free cell culture medium for 2 h at 37 °C. The labeled cells were directly lysed on plate with radioimmunoprecipitation assay buffer (1.0% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, and 0.5% sodium deoxycholate), and the resulting lysate was analyzed by immunoblot using anti-biotin–HRP.

Immunoprecipitation and LC-MS/MS proteomics
For identification of the 60-kDa protein(s), ldlB cells were labeled by Ac4GalNAz, and then the labeled cells were further treated with (±) V. cholerae neuraminidase at 37 °C or 4 °C. When V. cholerae neuraminidase was treated at 4 °C, 460 mM sucrose was used. The resulting cells were then treated with S-DIBO-biotin at room temperature for 1 h. The cells were then lysed with radioimmune precipitation assay buffer, and each 2.8 mg of lysate from was used to pull down the biotinylated proteins. Briefly, each lysate was precleared for 1 h with protein G beads (20 μl), and then the precleared lysate was further incubated with protein G beads (20 μl) preconjugated with antibiotin antibody (13.3 μl) overnight at 4 °C. Elution of biotinylated proteins from beads, SDS-PAGE, silver staining, in gel digestion of gel pieces between 50 and 75 kDa, and LC-MS-MS analysis were done as previously reported (44). The gel bands were destained, denatured by incubating with 10 mM of DTT at 56 °C for an hour, and alkylated by 55 mM of iodoacetamide for 45 min in dark prior to digestion with trypsin overnight. The resulting peptides were separated on a 75-μm (inner diameter) × 15-cm C18 capillary column (packed in house, YMC GEL ODS-AQ120AS-5, Waters) and eluted into the nano-electrospray ion source of an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) with a 180-min linear gra-
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dient consisting of 0.5–100% solvent B over 150 min at a flow rate of 200 nl/min. The spray voltage was set to 2.2 kV, and the temperature of the heated capillary was set to 280 °C. Full MS scans were acquired from m/z 300 to 2000 at 120k resolution, and MS2 scans following collision-induced fragmentation were collected in the ion trap for the most intense ions in the Top-Speed mode within a 3-s cycle using Fusion instrument software (v1.1, Thermo Fisher Scientific). The raw spectra were searched against the human protein database (UniProt, Oct. 2014) using SEQUEST (Proteome Discoverer 1.4, Thermo Fisher Scientific) with full MS peptide tolerance of 20 ppm and MS2 peptide fragment tolerance of 0.5 Da, and filtered using ProteoIQ (v2.7, Premier Biosoft) at the protein level to generate a 1% false discovery rate for protein assignments. UniProt was used to determine cellular localization of the identified proteins. Quantification was performed by normalizing the spectral counts generated in ProteoIQ (v2.7, Premier Biosoft) to the protein level to generate protein expression.

Recombinant StcE was expressed using ER2566 cells (IMPACT system, New England Biolabs) containing StcE expression vector (pTEG11) by following the New England Biolabs manual (New England Biolabs catalog no. E69015 V. 3.1). The ER2566 cells expressing StcE was obtained as a generous gift from Dr. Rodney A. Welch's laboratory. Briefly, the ER2566 cells were grown in 100 ml LB + Amp medium until A600 reached ~0.43 at 37 °C, and then 0.4 mM isopropyl β-D-thiogalactopyranoside was added followed by overnight incubation at room temperature. The resulting cells were harvested and lysed by sonication in 10 ml of column buffer containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100 on ice. The lysed cells were centrifuged at 15,000 × g for 30 min at 4 °C. The resulting supernatant was collected and loaded on the chitin column (New England Biolabs, catalog no. S6651S, 2 ml of bed volume) for purification. After the loading and washing steps, the resulting chitin column was quickly flushed with column buffer containing 50 mM DTT and then incubated overnight at room temperature. Next, StcE was eluted with column buffer (400 μl for each fraction), and StcE-containing fractions (1~5) were determined by Coomassie Blue staining. The resulting StcE-containing fractions were dialyzed and concentrated via spin column (molecular mass cutoff, 30 kDa) to give final volume of ~1.0 ml in 20 mM Tris-HCl, 50 mM NaCl, and 5% glycerol. Protein concentration of StcE (3.1 mg/ml) was determined by using Bio-Rad protein assay dye reagent (Bio-Rad, catalog no. 5000006).

Treatment of StcE on CHO and ldlB cells

Confluent CHO or ldlB cells cultured in 12-well dishes were incubated with various amount of StcE (0, 0.03, 0.06, 0.13, 0.26, 0.53, 1.05, and 2.10 μg/ml) in serum-free cell culture medium for 1 h at 37 °C. The resulting cells were lysed and then analyzed by immunoblot with anti–α-DG (core) antibody. The comparison of V. cholerae neuraminidase and StcE treatment on ldlB cells was made by treating confluent ldlB cells with various amount of StcE (0, 0.26, 0.53, 1.05, and 2.10 μg/ml) or V. cholerae neuraminidase (18, 35, 70, and 140 milliunits/ml) in serum cell culture medium for 2 h at 37 °C. The resulting cells were lysed and then analyzed by immunoblot with anti–α-DG (core) antibody.

Corrective SEEL

Corrective SEEL reaction of ldlB cells were typically done in 6-well dishes with 600 μl of final volume. For SEEL with ST3Gal1, the cells were incubated with ST3Gal1 (25 μg), FastAP (4U), BSA (4 μl of 2 mg/ml), and CMP-Neu5Ac (220 μM) as final concentration in serum-free culture medium for 2 h at 37 °C. For SEEL with C1GalT1, the cells were incubated with C1GalT1 (30 μg), FastAP (8U), BSA (4 μl of 2 mg/ml), MnCl2 (2 mM), and UDP-galactose (2 mM) in serum-free cell culture medium for 2h at 37 °C. For SEEL with both enzymes, the cells were incubated with ST3Gal1 (25 μg), C1GalT1 (30 μg), FastAP (8U), BSA (4 μl of 2 mg/ml), MnCl2 (2 mM), and UDP-galactose (2 mM) in serum-free cell culture medium for 2h at 37 °C. For control, the cells were incubated with serum-free medium with BSA only or treated with the same SEEL reaction mixture without UDP-galactose. After the SEEL reaction was done, optionally, the cells were further treated with V. cholerae neuraminidase for 2h at 37 °C before lysis. After lysis, the cells were analyzed by immunoblot with anti–α-DG (core) antibody. Recombinant forms of ST3Gal1 and C1GalT1 were generated as previously described (25) except that a truncated form of Drosophila melanogaster C1GalT1 (residues 43~388, UniProt Q7K237) was generated in
the pGEn2 mammalian expression vector as a GFP fusion protein. Both fusion proteins were purified from the conditioned HEK293 cell conditioned medium by Ni\(^{2+}\)–nitrilotriacetic acid chromatography and gel filtration prior to use.

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