Inhibition of Golgi Apparatus Glycosylation Causes Endoplasmic Reticulum Stress and Decreased Protein Synthesis*

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Nucleotide sugar transporters of the Golgi apparatus play an essential role in the glycosylation of proteins, lipids, and proteoglycans. Down-regulation of expression of the transporters for CMP-sialic acid, GDP-fucose, or both unexpectedly resulted in accumulation of glycoconjugates in the Golgi apparatus rather than in the plasma membrane. Pulse-chase experiments with radiolabeled sugars and amino acids showed decreased synthesis and secretion of both nonglycoproteins and glycoproteins. Further studies revealed that the above silencing induced endoplasmic reticulum stress and inhibited protein translation initiation. Together these results suggest that global inhibition of Golgi apparatus glycosylation may lead to important secondary metabolic changes, unrelated to glycosylation.

The majority of secreted and membrane-bound proteins of eukaryotes undergo post-translational modifications such as glycosylation, sulfation, and phosphorylation in the lumen of the Golgi apparatus. The substrates for these reactions are nucleotide derivatives such as nucleotide sugars, nucleotide sulfate, and ATP. Because none of these compounds are synthesized in the lumen of the Golgi apparatus, they must first be transported into the organelle lumen, by specific transporters, before they can serve as substrates for the corresponding transferases or kinases.

Among the above transporters, those for nucleotide sugars have been the most studied so far and have been described in virtually all eukaryotes (1–3). These polytopic proteins are antiporters that can translocate one or multiple specific nucleotide sugars in exchange with the corresponding nucleoside monophosphate. Therefore Golgi luminal nucleoside diphosphatases are necessary for translocation activity. Although the transport mechanism has not been completely elucidated, both competitive and simultaneous but independent mechanisms have been described for different multisubstrate nucleotide sugar transporters (1, 2).

Mutations of nucleotide sugar transporters have been shown to result in biochemical and developmental phenotypes. Among the former is a significant decrease of the particular sugar covalently bound to glycoproteins, glycolipids, and proteoglycans whose nucleotide sugar transporter is functionally impaired (1, 2).

Diseases caused by mutations in these transporters affect humans (leukocyte adhesion deficiency syndrome II, LAD II (1, 2) and Scheneckenbecken displasia (2, 4, 5)) and cattle (complex vertebral malformation (2, 6)). Developmental phenotypes caused by mutations in nucleotide sugar transporters have been described in Drosophila (2, 7), Caenorhabditis elegans (1, 2), Leishmania (1, 2, 8–10), and yeast (1, 2, 11).

Studies in C. elegans have shown that although expression of nucleotide sugar transporters is tissue-specific, there is functional redundancy among some transporters. Specifically, although the individual silencing of the C. elegans C03H5.2 or srf-3 transporters showed no morphological phenotype when the nematodes are grown on agar with Escherichia coli, the silencing of C03H5.2 in srf-3 mutants caused abnormal gonad migration and impaired oogenesis (12).

We have silenced in this study the Golgi apparatus transporters for CMP-sialic acid and GDP-fucose of HeLa cells either individually or simultaneously. Both sugars are “terminal sugars” of glycoconjugates. We found, surprisingly, that the majority of the nonsialic acid- and nonfucose-containing glycoconjugates were not transported to the cell surface but accumulated in the Golgi apparatus. Furthermore we found decreased synthesis and secretion of nonglycoproteins and glycoproteins, increased ER stress, and inhibition of protein translation initiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Total Cell Lysates—**HeLa, Chinese hamster ovary (CHO) K-1 wild type, and CHO Lec2–4C (CMP-sialic acid transporter mutant (13, 14)) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Total cell lysates were prepared by solubilizing cells in a buffer containing 0.5% Triton X-100, 1% nonidet P-40, 100 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, protease inhibitors, and proteinase inhibitors.

The abbreviations used are: LAD II, leukocyte adhesion deficiency syndrome II; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; siRNA, small interfering RNA; RT, reverse transcription; eLF, eukaryotic initiation factor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; WGA, wheat germ agglutinin; ConA, concanavalin A; CMPST, CMP-sialic acid transporter; GDP, GDP-fucose transporter.

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0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, and protease inhibitor mixture (Sigma).

Antibody against the GDP-Fucose Transporter—Antibodies were raised in rabbits against a synthetic peptide Ac-CEMKK-TPEEPSPKDSKEKSA-amide, corresponding to the C-terminal sequence of the human GDP-fucose transporter (GenBankTM accession number AAK50397.1) plus a N-terminal cysteine for coupling to the carrier protein. The antibodies were further purified by affinity chromatography.

siRNA Transfection and RT-PCR Analysis—Three siRNAs were used in this study, including siRNA for the CMP-sialic acid transporter (5'-CAGCTACCTTGGCCCTTGGG-3'), GDP-fucose transporter (5'-CGTGACTTTGCCAGCTTG-3'), and a control siRNA derived from the luciferase gene. The siRNAs were purchased from Qiagen. HeLa cells (and a control siRNA derived from the luciferase gene. The transporter siRNAs were cultured for 72 h and thereafter—HeLa cells transfected with luciferase control, CMP-sialic acid, and/or GDP-fucose transporter siRNAs were cultured for 72 h. The cells were then equally split into three wells (two for duplicate pulse-chase labeling and one for cell counting). Following 24 h of incubation, the cells were washed three times with glucose-free DMEM and pulse-labeled for 30 min with tritium-labeled galactose (20 μCi, 60 Ci/mmol; Ameri
can Radiolabeled Chemicals (ARC), fucose (40 μCi, 25 Ci/mmol; ARC), and mannose (40 μCi, 44.70 Ci/mmol; New England Nuclear) in glucose-free DMEM, supplemented with 100 μg/ml glucose, and 10% FBS. The cells were then washed with DMEM four times and chased in DMEM with 100 μg/ml glucose plus 10% FBS. After 5 h, the medium was saved, the cells were washed with cold PBS three times, and a cell extract was prepared. Total protein from the extract or medium was precipitated with 10% trichloroacetic acid, and the protein pellet was washed five times and dried with a speed vacuum. The protein pellet was dissolved in 1.0 M NaOH, neutralized with HCl, and counted in a liquid scintillation spectrometer.

Pulse-Chase of Cells with [35S]Methionine and [35S]Cysteine, and Purification of Radiolabeled Glycoproteins—HeLa cells transfected with the luciferase control, CMP-sialic acid, and/or GDP-fucose transporter siRNAs were cultured as described above. After ~96 h, the cells were washed with methionine- and cysteine-free DMEM three times and then pulse-labeled for 30 min with 20 μCi of 35S labeling mix (PerkinElmer Life Sciences) in the same medium plus 10% FBS. The cells were washed with normal DMEM four times and chased with OPTI-MEM medium plus 5% FBS at 37 °C. After 5 h the medium was saved, the cells were washed three times with cold PBS, and total cell lysate was prepared as described above. Glycoproteins were purified by sequential binding to WGA- and ConA-agarose beads according to the manufacturer’s specifications (Pierce). Briefly, 100 μl of medium and one-third of the extracts were diluted with column binding buffer and first incubated with WGA beads for 10 min at room temperature by end-to-top rotation and then passed through the column. The flow-through was saved and subsequently incubated with ConA beads for 10 min with rotation. The flow-through was saved and precipitated with 10% trichloroacetic acid as described above, and the columns with WGA and ConA beads were washed with binding buffer four times, 10 min each with rotation, and then eluted with elution buffer. The precipitated protein from the flow-through, and eluants were dissolved with NaOH, neutralized, and subjected to liquid scintillation spectrometry as described above.

Pulse-Chase of CHO Cells with Radiolabeled Sugars—Equal numbers of CHO-K1 and Lec2 cells were split into three wells for duplicate radiolabeling and cell counting. The cells were washed three times with glucose-free DMEM and then pulse-labeled and chased exactly as described above for HeLa cells.

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RESULTS

Down-regulation of GDP-Fucose and CMP-Sialic Acid Transporter Expression Results in Abnormal Distribution of Glycoproteins—We were interested in investigating whether nucleotide sugar transporters regulate glycoprotein synthesis and the subcellular localization of glycoproteins. For this purpose we used HeLa cells and a similar siRNA approach previously used to down-regulate the expression of the oligosaccharidyltransferase (15) and Pin1 (16), a peptidyl isomerase. Based on the CMP-sialic acid and GDP-fucose transporter DNA sequence analyses, siRNAs were designed, synthesized, and transfected into HeLa cells as described under “Experimental Procedures.” Down-regulation of transporter expression inhibited cell growth by 25–40% of control. The cells were somewhat flatter and larger, and the Golgi apparatus appeared expanded.

Following the down-regulation of the transporters for GDP-fucose and CMP-sialic acid, a fluorescent galactose-binding lectin (*A. precatorius*) was used to visualize glycoproteins within cells. As shown in Fig. 1A (upper left panel), in control (nonsilenced) permeabilized cells, most of the galactose lectin staining was seen, as expected, on the cell surface, with a smaller amount in the Golgi apparatus (as seen by co-localization with the Golgi apparatus marker p58). In cells where either one or both nucleotide sugar transporters were silenced, the majority of galactose lectin staining occurred, unexpectedly, not on the cell surface but in the Golgi apparatus region (Fig. 1A). This was confirmed by co-localization with p58. In most cases an expanded Golgi apparatus can be seen as compared with control cells. Significantly less galactose-binding lectin staining was seen on the cell surface of silenced, nonpermeabilized cells compared with nonsilenced, nonpermeabilized cells (Fig. 1B). Similar results were also obtained when fluorescent wheat germ agglutinin lectin was used (supplemental Fig. S1). Together these results suggest, as a first approximation, that silencing of terminal Golgi glycosylation appears to result in the accumulation of some glycoconjugates in the Golgi apparatus and possibly interference in their movement to the cell surface.

Several lines of evidence were obtained to determine that the silencing was specific (Figs. 2 and 3). As shown in Fig. 2, there was reduced staining when using a lectin that recognizes the particular sugar whose nucleotide sugar transporter had been silenced. As shown in Fig. 2A, when a fluorescent sialic acid-binding lectin was used (*L. polyphemus*), there was considerably less staining of cells where the CMP-sialic acid transporter had been silenced compared with nonsilenced (control) cells or cells where the GDP-fucose transporter had been silenced. Silencing per se did not affect the staining pattern of the Golgi apparatus marker p58 (Fig. 2A).

When a fluorescent fucose-binding lectin was used (*U. europaea*), as shown in Fig. 2B, a similar specific lectin binding pattern as seen for sialic acid was observed. There was reduced staining in GDP-fucose transporter silenced cells compared with control cells or cells where the CMP-sialic acid transporter had been silenced. Again, as seen in Fig. 2A, silencing per se did not affect the labeling of the Golgi apparatus marker p58.

Additional evidence that silencing of the above nucleotide sugar transporters was specific can be seen in Fig. 3. Total RNA from the siRNA-treated cells was obtained and reverse transcribed. RT-PCR was then performed with primers specific for
the transporters of CMP-sialic acid and GDP-fucose as well as for β-actin (as control). Fig. 3A (top panel) shows that the mRNA levels of the CMP-sialic acid and GDP-fucose transporters were specifically decreased as a result of the silencing of the corresponding transporter, whereas that of β-actin was not affected. As shown in Fig. 3A (bottom panel), quantitation showed that silencing was 87–95% effective.

Further proof that silencing was effective and specific can be seen in Fig. 3B (top and bottom panels). An antibody against the C terminus of the GDP-fucose transporter protein showed a decreased amount of protein upon silencing of the corresponding transporter, compared with when the CMP-sialic acid transporter had been silenced (Fig. 3B, top and bottom panels).

In conclusion, two different nucleotide sugar transporters, with different nucleotide sequences, showed upon silencing a very similar phenotype with two sugar-binding lectins, namely increased binding in the Golgi apparatus rather than significant amounts at the cell surface. This suggests that impaired nucleotide sugar transport activity interferes with the traffic of glycoproteins to the cell surface.

**Down-regulation of Expression of the GDP-Fucose and CMP-Sialic Acid Transporters Reduces Glycosylation of Cellular and Secreted Glycoproteins**—In view of the above results, it was important to determine whether upon reduced transport into the Golgi apparatus of nucleotide sugars of terminal sugars such as fucose or sialic acid, incorporation of other sugars into protein was also impaired. To determine whether this indeed had occurred, silenced and control cells were pulse-labeled for 30 min with either radiolabeled galactose, mannose (in medium with low glucose), or fucose. The cells were then washed and incubated with normal growth medium for 5 h. The cell lysates were prepared, total proteins from lysates and growth medium were obtained, and radioactivity in these fractions was determined. As can be seen in Fig. 4, there was a significant decrease in glycosylation of proteins both in the cell lysate and secreted, in cells where the CMP-sialic and GDP-fucose transporters had been silenced separately or simultaneously. However, because the relative decrease in the secreted fraction was less than in the cell lysate fraction, we cannot rule out a relative increase in some secreted proteins.

Chinese hamster ovary Lec2 cells have been previously shown to be mostly (but not completely) defective in CMP-
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FIGURE 3. Down-regulation of GDPFT and CMPST expression with siRNAs. A, RT-PCR analyses of GDPFT and CMPST expression. HeLa cells were transfected with siRNAs specific for luciferase (Luc), CMPST (C), GDPFT (G), or both (C+G). After 96 h, total RNA was isolated and reverse transcribed. cDNA was amplified with the primers for GDPFT (top panel) and quantified (bottom panel). Quantification is based on three independent experiments. B, Western analysis of GDPFT protein expression. A total cell lysate was prepared from siRNA-treated cells as described for A. The proteins were subjected to SDS gel electrophoresis, and the gel was probed with an antibody against GDPFT (top panel) and quantified (bottom panel). Quantification is based on three independent experiments.

Silencing of Transporters of CMP-Sialic Acid and GDP-Fucose Induces ER Stress—The above results showed that silencing of transporters of CMP-sialic acid and GDP-fucose leads to reduced synthesis and secretion of both nonglycosylated and glycosylated proteins. We previously observed that conditions that induce ER stress up-regulate the transcription of the ER/Golgi nucleotide sugar transporter, ude-1, in C. elegans (17). We hypothesized that as a result there is an increase in UMP that can be exchanged with UDP-glucose, relieving ER stress by favoring reglucosylation of unfolded proteins in the ER. Conversely we now hypothesized that inhibition of terminal glycosylation of proteins (sialylation and fucosylation) caused by silencing of either the Golgi apparatus CMP-sialic acid, GDP-fucose transporters, or both may induce ER stress.

To determine whether or not under the above silencing conditions ER stress had occurred, we examined the phosphorylation (at Ser51) of eIF2α, a translation initiation factor that is activated in stressed cells and that, upon being phosphorylated, inhibits protein synthesis (18). Total cell lysates were prepared from control and silenced HeLa cells, and phosphorylation of eIF2α was detected by Western blots. As can be seen in Fig. 7A (top and bottom panels), nucleotide sugar transporter silenced cells showed significant increased phosphorylation of eIF2α over controls. This was not the result of increased eIF2α protein levels as visualized with antibodies against the eIF2α protein (Fig. 7A, top panel). An additional control with antibodies against cdcc2 showed that equal amounts of proteins had been added to each lane.

We next asked whether another stress marker, the shorter spliced form of XPB1, XBP1s (19), was also activated under the glycoprotein fractions that bound to the columns. As can be seen in Fig. 6 (left panels), there was a significant reduction in synthesis and secretion of nonglycoproteins in silenced cells, particularly in the cell lysate fraction. This unexpected result strongly suggests that the reduced synthesis and secretion previously observed with glycoproteins may actually be an even more general effect resulting from reduced synthesis and secretion of total cellular proteins. Reduction of synthesis and secretion of glycosylated proteins (fractions that bound to the above two columns) was also observed (Fig. 6, middle and right panels) in agreement with the above-described results shown with radiolabeled sugars (Fig. 4). However, we cannot rule out that the flow-through fraction in Fig. 6 contains a small amount of glycosylated proteins that do not bind to the above lectin columns.
In this study we have shown that silencing of Golgi apparatus nucleotide sugar transporters, which make terminal sugars such as sialic acid and fucose available in the organelle lumen, results in the following phenotypes: (a) synthesis and secretion of nonsialylated glycoproteins and glycoproteins are inhibited; (b) induction of ER stress and repression of protein translation; and (c) nonsialylated and nonfucosylated glycoproteins accumulate in the Golgi apparatus rather than, as expected, in the plasma membrane.

Previous results with cells in tissue culture (20) and multicellular organisms (21) from this and other laboratories showed that partial inhibition of transport of nucleotide sugars into the Golgi apparatus lumen resulted in a global decrease of the corresponding sugar in glycoconjugates (20). This decrease could be selective, for example, in a mutant MDCK cell line in which we found that even though there was a 80–90% decrease in UDP-galactose transport, synthesis of some but not all galactose-containing glycosaminoglycans was affected (20).

One of the most important implications of this study relates to previously described diseases in humans and cattle resulting from mutations in nucleotide sugar transporters (2). Although in each of these instances the resulting phenotypes were solely attributed to a specific loss of the sugar covalently bound to glycoconjugates whose nucleotide sugar transporter was (partially) inhibited, the results presented here suggest that the overall metabolic defect observed in these previous studies is more complex. For example it had been shown that in LAD II, a decrease in activity of the GDP-fucose transporter resulted in partially (initially) inhibited, the results presented here suggest that the overall metabolic defect observed in these previous studies is more complex. For example it had been shown that in LAD II, a decrease in activity of the GDP-fucose transporter resulted in abnormal lymphoblast rolling. The affected children had growth and mental retardation, abnormal toe development, blood group Bombay (antigen H without fucose) and decreased fucosylation of lymphoblasts (22). The affected children had growth and mental retardation, abnormal toe development, recurrent infections, and abnormal lymphoblast rolling. The results obtained in our current studies with HeLa cells raise the possibility that in addition to the above described biochemical defects in LAD II, nonfucosylated glycoproteins and nonglycoproteins may also be affected and, more importantly, may contribute to some of the phenotypic characteristics of the affected children, such as mental and growth retardation.

In other nucleotide sugar transporter diseases such as in Schneckenbecken dysplasia, a human disease resulting from a defect in a UDP-N-acetylgalactosamine transporter (1, 2, 4, 5), and in complex vertebral malformation (6), a cattle disease...
resulting from a partial loss of function mutation of a UDP-N-acetylglucosamine transporter, the possibility of broader metabolic defects as reported in these studies may also exist. The same conclusions may also be drawn from studies of nucleotide sugar transporters in *C. elegans* (1, 2), *Drosophila melanogaster* (7), and *Leishmania* (8–10), where biological phenotypes were solely attributed to the decrease of the particular sugar in glycoconjugates whose nucleotide sugar transport had been impaired either by mutations or by silencing (2).

It is well known that inhibition of N-glycosylation results in proteins being retained either in the ER or in the Golgi apparatus. Examples of the latter include a chimera of the cytoplasmic and transmembrane regions of the vesicular stomatitis virus G protein and the soluble rat growth hormone. This chimera protein was retained in the Golgi apparatus unless N-glycosylation sites were added to the rat growth hormone sequence and subsequently glycosylated, in which case the chimera reached the cell surface (23). Another example of this was shown by Chen and Colley (24) with a soluble form of a sialyltransferase that was only secreted when glycosylated but was retained in the ER when not glycosylated. In contrast, the membrane-bound sialyltransferase localized to the Golgi apparatus regardless whether or not it was glycosylated. However, we have shown that inhibition of CMP-sialic acid or GDP-fucose transport activity, although only affecting binding of terminal sugars of glycoproteins, is sufficient to inhibit global synthesis and secretion of glycoproteins and nonglycoproteins.

Previous studies have shown that the glucosidase-glucosyltransferase cycle constitutes an important quality control mechanism in the ER that allows correctly folded glycoproteins to proceed through the secretory pathway (25). Inhibition of N-glycosylation results in misfolding of a large number of proteins in the lumen of the ER. This causes
ER stress and triggers the unfolded protein response. As mentioned above, conditions that induce ER stress in *C. elegans* also up-regulate the ER/Golgi nucleoside diphosphatase transcription, thus relieving ER stress by increasing the amount of UMP to exchange with UDP-glucose mediated by the UDP-glucose transporter. Recently, another study with *C. elegans* showed that a nucleotide sugar transporter-like protein is required for maintenance of ER homeostasis (26).

Our results show that impairment of nucleotide sugar transport in the Golgi apparatus also induces ER stress and activates the unfolded protein response.

What is novel in the study herein is that bulk inhibition of either fucosylation or sialylation in the Golgi apparatus is sufficient to induce ER stress and inhibition of protein translation initiation. A recent study with *Trypanosoma brucei* found that blocking synthesis of variant surface glycoprotein resulted in a general arrest of translation initiation. However, in this instance there was no evidence for unfolded protein response (27).

An important open question of the study herein is whether inhibition of other glycosylation events occurring in the Golgi apparatus such as inhibition of transporters for UDP-galactose, UDP-N-acetylgalactosamine, or Golgi apparatus glycosyltransferases lead to inhibition of non-glycoprotein synthesis, induction of ER stress, and inhibition of protein translation. A similar question pertains to inhibition of transport into the Golgi apparatus lumen by transporters for adenosine 3′-phosphate 5′-phosphosulfate as well as ATP. Although such studies will be easier to conduct in mammalian cell culture such as HeLa cells, extension of them to other eukaryotic cell culture systems and to multicellular organisms will be important.

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**FIGURE 7.** Down-regulation of CMPST and GDPFT expression induced ER stress. *A,* down-regulation of CMPST and GDPFT expression induces phosphorylation of initiation factor eIF2α. HeLa cells were transfected with siRNAs as described in Fig. 1. A total cell lysate was obtained and subjected to SDS gel electrophoresis followed by Western blotting. The gels were probed with antibodies to phospho-eIF2α (p-eIF2α). The same blot was also probed with antibodies against eIF2α and cdc2 (*top panel*). Quantification is shown in the *bottom panel*. *B* and *C,* RT-PCR analyses of XBP1 and BiP expression. Total RNA was obtained and reverse transcribed as described for Fig. 3. cDNA was amplified with primers for XBP1 (*B*), BiP (*C*), and β-actin, and the products were analyzed on agarose gels (*top panel*). Quantification is shown in the *bottom panel*.
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