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Golgi Phosphoprotein 3 Triggers Signal-mediated Incorporation of Glycosyltransferases into Coatomer-coated (COPI) Vesicles

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Background: How Golgi phosphoprotein 3 (GOLPH3) regulates Golgi localization of glycosyltransferases in mammalian cells is poorly understood.
Results: GOLPH3 mediates incorporation of glycosyltransferases into coatomer-coated vesicles.
Conclusion: GOLPH3 regulates localization of glycolsyltransferases by working as a coatomer adaptor.
Significance: This study provides further insight into the molecular mechanism underlying regulation of glycosyltransferases localization by GOLPH3.

Newly synthesized membrane and secreted proteins undergo a series of posttranslational modifications in the Golgi apparatus, including attachment of carbohydrate moieties. The final structure of so-formed glycans is determined by the order of execution of the different glycosylation steps, which seems intimately related to the spatial distribution of glycosyltransferases and glycosyl hydrolases within the Golgi apparatus. How cells achieve an accurate localization of these enzymes is not completely understood but might involve dynamic processes such as coatomer-coated (COPI) vesicle-mediated trafficking. In yeast, this transport is likely to be regulated by vacuolar protein sorting 74 (Vps74p), a peripheral Golgi protein able to interact with COPI coat as well as with a binding motif present in the cytosolic tails of some mannosyltransferases. Recently, Golgi phosphoprotein 3 (GOLPH3), the mammalian homolog of Vps74, has been shown to control the Golgi localization of core 2 *N*-acetylglucosamine-transferase 1. Here, we highlight a role of GOLPH3 in the spatial localization of α-2,6-sialyltransferase 1. We show, for the first time, that GOLPH3 supports incorporation of both core 2 *N*-acetylglucosamine-transferase 1 and α-2,6-sialyltransferase 1 into COPI vesicles. Depletion of GOLPH3 altered the subcellular localization of these enzymes. In contrast, galactosyltransferase, an enzyme that does not interact with GOLPH3, was neither incorporated into COPI vesicles nor was dependent on GOLPH3 for proper localization.

Membrane and secreted proteins are synthesized in the endoplasmic reticulum (ER),[2] transported to the Golgi apparatus for maturation and then sorted at the level of the *trans*-Golgi network (TGN) toward their final destination. Along this pathway, proteins undergo a series of posttranslational modifications, including covalent addition of carbohydrate moieties to their polypeptide backbone. This process, known as glycosylation, is involved in many cellular functions such as protein folding, cell polarization, cell communication, development, or cell differentiation. It is initiated in the ER and then further processed in the Golgi apparatus. The Golgi is a polarized organelle composed of a stack of cisternae. Along transport from the *cis*-cisternae (facing the nucleus) to the *trans*-cisternae (facing the plasma membrane), protein-attached glycans get trimmed and elongated by the successive action of glycosyl hydrolases and glycosyltransferases. The precise sequence of the different glycosylation steps determines the final structure of the glycans and seems intimately related to the accurate localization of the carbohydrate-modifying enzymes within the Golgi. How such a spatial distribution is achieved is still not totally understood.

Most glycosyltransferases are type II integral proteins composed of a short cytoplasmic tail, a single transmembrane domain, a luminal stem region, and a catalytic domain (1). Targeting and retention of these enzymes into specific Golgi compartments seems to rely exclusively on features of the CTS region. According to lipid-based sorting models, the physicochemical properties of the transmembrane domain (length, presence of amino acids with uncharged polar side chains) would trigger enrichment of glycosyltransferases in particular lipid environments. As the lipid composition is not homogeneous within the Golgi, this would result in preferential localization of the various enzymes in different cisternae (2–4). Additionally, glycosyltransferases are able to oligomerize through their transmembrane and/or stem domains. Such interactions can result in the formation of big enzyme aggregates, favoring retention of the enzymes in their respective cisternae (1, 5).

Steady-state localization of many Golgi resident integral proteins depends on dynamic processes involving iterative cycles

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[2] The abbreviations used are: ER, endoplasmic reticulum; GOLPH3, Golgi phosphoprotein 3; COPI, coat protein complex I; C2GnT, core 2 *N*-acetylglucosamine-transferase 1; SiaTI, α-2,6-sialyltransferase 1; GalT, galactosyltransferase; TGN, *trans*-Golgi network.

of transport, either within the Golgi or between the Golgi and the ER (6), through coat protein complex I (COPI)-coated vesicles (7) and COPII vesicles (8). In this context, Vps74p has been implicated in the retention of glycosylation enzymes in the Golgi of yeast (9, 10) Vps74p is a peripheral membrane protein that recognizes a (F/L)(L/I/V)*XX*(R/K) motif, present in the cytosolic tail of numerous mannosyltransferases (9), and interacts with the COPI coat (9, 11). These findings raise the possibility that Vps74p represents an adaptor for incorporation of glycosyltransferases into COPI vesicles, necessary for proper enzyme localization (9).

Two mammalian homologs of Vps74p have been identified: GOLPH3 (MIDAS, GMx33α, GPP34) and GOLPH3L (GMx33β, GPP34R), with the latter being expressed only at low levels (12) and in cell type-dependent manner (13). GOLPH3 dynamically associates with the TGN through binding to phosphatidylinositol 4-phosphate (14–16). This protein has been implicated in diverse biological functions, including mitochondrial biosynthesis (12), regulation of mTOR signaling (17), Golgi morphology (15), and exit from the TGN (15). Overexpression of GOLPH3 is linked to tumor aggressiveness and poor prognosis in several human cancers (18–25). Recent studies highlighted an interaction between GOLPH3 and coatomer (11), as well as with the cytosolic tail of the core 2 *N*-acetylglucosaminyltransferase 1 (C2GnT) (26) and protein *O*-linked mannose-1,2-*N*-acetylglucosaminyltransferase 1 (27). However, evidence for a function of GOLPH3 in COPI-mediated trafficking is still missing.

In this study, we highlight a role of GOLPH3 in the spatial regulation of α-2,6-sialyltransferase 1 (SiaTI). We show for the first time, that GOLPH3 triggers incorporation of both C2GnT and SiaTI into COPI vesicles. Depletion of GOLPH3 led to an altered subcellular localization of these enzymes. In contrast, galactosyltransferase (GalT), an enzyme that does not interact with GOLPH3, was not incorporated into COPI vesicles and was not dependent on GOLPH3 for proper localization.

EXPERIMENTAL PROCEDURES

Stable Cell Lines—Stable cell lines CHO/C2GnT-GFP and CHO/SiaTI-GFP (28) were grown in Hams F12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml G418. CHO/GalT-GFP stable cell line was generated by transfecting CHO-K1 cells with pEGFP/GalT (gift from Frank Perez) using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. After 2–3 weeks in presence of 1 mg/ml G418, single clones were collected and selected for GalT-GFP expression. CHO/GalT-GFP were grown in Hams F12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418.

Antibodies—The following antibodies were used for Western blotting: the rabbit polyclonal anti-δCOP (877) (29), anti-αCOP (29), anti-Arf1(C1) (30), anti-GOLPH3 (15), anti-p24 (31), and anti-GFP (Abcam, ab290).

For immunofluorescence staining, goat anti-calnexin (C-20, Santa Cruz Biotechnology, sc-6465), mouse anti-GM130 (BD Transduction Laboratories, 610823), and an antibody raised against peptides from full-length hGOLPH3 (Peptide Specialty Laboratories) were used. Rabbit serum was negatively purified against hGOLPH3L, the homolog of hGOLPH3.

Recombinant Proteins—*N*-Myristoylated human wild type Arf1 (32), GST (11), GOLPH3-GST (11), and recombinant coatomer isoform $\gamma 1\zeta 1$ (33) were expressed and purified as described.

Preparation of Cell Lysates—Fully confluent 15-cm diameter dishes were washed once with PBS and put on ice. Cells were lysed in 1 ml of lysis buffer (PBS, 1% Triton X-100, 1× proteinase inhibitor mixture, Roche Applied Science), harvested gently with a cell scraper, and incubated in a reaction tube at 4 °C on a turning wheel for 20 h. Residual cell debris was pelleted at 16,000 × *g* for 5 min. Supernatant was snap frozen in liquid nitrogen and stored at −80 °C until used.

Pulldown Assay—50 μg of recombinant GST or GST-GOLPH3, diluted in 500 μl of pulldown buffer (PD buffer: 25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40), was incubated 1h at 4 °C under agitation with 20 μl of glutathione-Sepharose beads. After three washes with PD buffer, beads were mixed with 1 mg of CHO cell lysates and incubated for 1 h at 4 °C under agitation. After three additional washes with PD buffer, bound proteins were analyzed by Western blotting.

RNA Interference—Control siRNA (5′-GAC AGA ACC AGA ACG CCA dTdT-3′) and siRNA to down-regulate hGOLPH3 (siRNA 1: 1, 5′-GGA CCG CGA GGG UUA CAC AdTdT; siRNA 2: 2, 5′-GUU CUU GAC AAA UGG GUA A dTdT-3′) were obtained from Sigma. Cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocols. Experiments were performed 3 days after single transfections.

Preparation of Golgi-enriched Membranes—CHO Golgi membrane fractions were prepared by a modification of the method of Balch *et al.* (34). All procedures were carried out at 4 °C. In a standard preparation, 6 liters of CHO cells (density of 5.10^5 cells/ml) were harvested by centrifugation (10 min at 500 × *g*), washed twice with PBS (10 min at 500 × *g*), twice with homogenization buffer (250 mM sucrose in 10 mM Tris-HCl, pH 7.4, 4 °C) (10 min at 1,500 × *g*), and finally resuspended in four volumes of homogenization buffer. Cells were homogenized by passing 15 times through a ball-bearing homogenizer. The homogenate was brought to a sucrose concentration of 37% (WT/WT) by addition of 62% (WT/WT) sucrose in 10 mM Tris-HCl, pH 7.4. 10 ml of this solution was placed in one SW 32 tube and overlaid with 14 ml 35% (WT/WT) sucrose in 10 mM Tris-HCl, pH 7.4, and 9 ml 25% (WT/WT) sucrose in 10 mM Tris-HCl, pH 7.4. Gradients were centrifuged for 2.5 h at 90,000 × *g*, 4 °C. Typically, 2 ml of a Golgi-enriched membrane fraction was recovered at the 25–35% sucrose interphase. For further purification, collected membranes were diluted 1:3 with 10 mM Tris-HCl, pH 7.4, and pelleted onto a 50% (WT/WT) sucrose cushion in a SW 41 tube (60 min at 90,000 × *g*). Concentrated Golgi-enriched fractions were collected at the cushion interphase and snap frozen in aliquots in liquid nitrogen.

Vesicle Formation Assays from Golgi-enriched Membranes—Prior to each experiment, protein preparations were clarified by centrifugation (10 min, 16,000 × *g*) to remove aggregates. Golgi from CHO/C2GnT-GFP (240 μg) was incubated for 10 min at

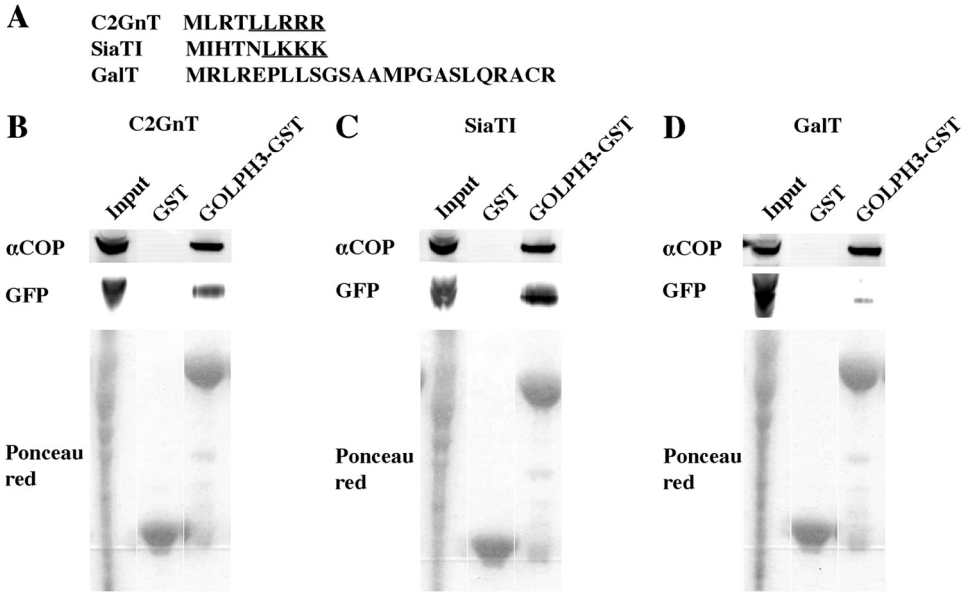

FIGURE 1. **GOLPH3 binds to C2GnT and SiaTI but not GalT.** *A*, amino acid sequences of the cytosolic tails of the Golgi-localized glycosyltransferases C2GnT, SiaTI, and GalT. Putative GOLPH3 binding sites are *underlined*. *B–D*, pulldown from lysate of CHO/C2GnT-GFP (*B*), CHO/SiaTI-GFP (*C*), or CHO/GalT-GFP (*D*) with GST or GST-GOLPH3. *Top panel*: immunoblots; *bottom panel*: Ponceau S-stained PVDF membranes.

37 °C together with 25 μg of recombinant coatomer and 5 μg recombinant Arf1 in the presence of 100 μM GTPγS. Reactions were performed in a total volume of 250 μl in assay buffer (25 mM HEPES-KOH, pH 7.2, 2.5 mM MgAc, 20 mM KCl, 200 mM sucrose, 0.25 mM dithiothreitol (DTT)), and 1% of the input was taken for Western blotting. Samples were then subjected to 250 mM KCl to dissociate tethered COPI vesicles from the donor Golgi membranes, which were then pelleted by centrifugation twice for 5 min each, at 16,000 × *g* and 4 °C. The supernatant, containing COPI vesicles, was laid on top of sucrose cushions of 37.5% (50 μl) and 50% (5 μl) sucrose (% by weight, in assay buffer) in a Herolab thickwall SW60-mini tube. After centrifugation for 50 min at 100,000 × *g*, the vesicles were collected at the 37.5/50% sucrose interphase and analyzed by Western blotting and negative staining electron microscopy.

Semi-intact Cell Assay—Preparation of semi-intact CHO cells was essentially carried out as described previously (35). 200 μg of semi-intact cells were incubated for 15 min at 37 °C in 200 μl of assay buffer (25 mM HEPES/KOH, pH 7.2, 150 mM KOAc, 2 mM MgOAc) in the presence of 0.1 mM GTPγS (Sigma), 2 μg of myristoylated Arf1, and 10 μg of recombinant coatomer. Vesicles were separated from donor semi-intact cells by medium speed centrifugation at 10,000 × *g* for 10 min at 4 °C. Vesicles in the supernatant were subsequently purified and harvested using the same sucrose gradient and centrifugation as described above for the vesicle generation from purified Golgi membranes. Vesicle pellets and semi-intact cells (input) were analyzed by Western blotting and negative staining electron microscopy. Western blot signal quantification was conducted using Image Studio software by comparing background corrected median band intensities.

Electron Microscopy—A Pioloform-coated grid was put on top of a droplet of purified COPI vesicles. After 30 min of adsorption at room temperature, proteins were fixed by putting the grid onto a 20-μl droplet of 1% glutaraldehyde in assay buffer, washed three times with assay buffer each, and incubated for 5 min in 0.05% tannic acid. Afterward, the sample was washed four times with 20 μl of water. All these steps were performed at room temperature. Then, staining was performed with 0.4% uranyl acetate in 1.8% methylcellulose for 10 min on ice.

Flow Cytometry Analysis—The surface-staining experiment was in essence conducted according to the protocol by Engling *et al.* (36). 75,000 CHO cells per condition were transfected with siRNA for 3 days before measurement in 12-well culture plates. For surface staining the cells were washed twice with 1 ml of PBS and then stained with anti-GFP antibody (acidic fraction (36)) in 300 μl of normal growth medium. Cells were incubated agitating at 4 °C for 1 h, washed again twice with cold PBS, and then incubated for further 30 min with allophycocyanin-conjugated goat anti-rabbit IgG (Invitrogen, A10931, 1:1,000) in normal growth medium under the same conditions. After two washing steps with cold PBS, 200 μl of cell dissociation buffer (Invitrogen) was added for 10–15 min until cells were round and detached from the surfaces. Next, the suspension of cells was transferred into 1.5-ml reaction tubes containing 250 μl of growth medium without serum (4 °C). For the adjustment of the photomultiplier values, untransfected CHO cells were treated in the described staining procedure and GFP, and allophycocyanin signals were set to a median fluorescence intensity of 2–5.

Immunofluorescence Microcopy—For immunofluorescence analysis, 35,000 CHO cells per well were seeded into 24-well plates containing autoclaved glass coverslips. On the next day, cells were washed twice with PBS and fixed for 5 min at −20 °C in ice-cold methanol. After two washes with PBS, cells were blocked with 3% (w/v) BSA in PBS for 15 min at room temperature. Coverslips were afterward placed onto droplets of 30 μl 3% (w/v) BSA in PBS containing the indicated primary antibodies for 30 min at room temperature. Before and after the next

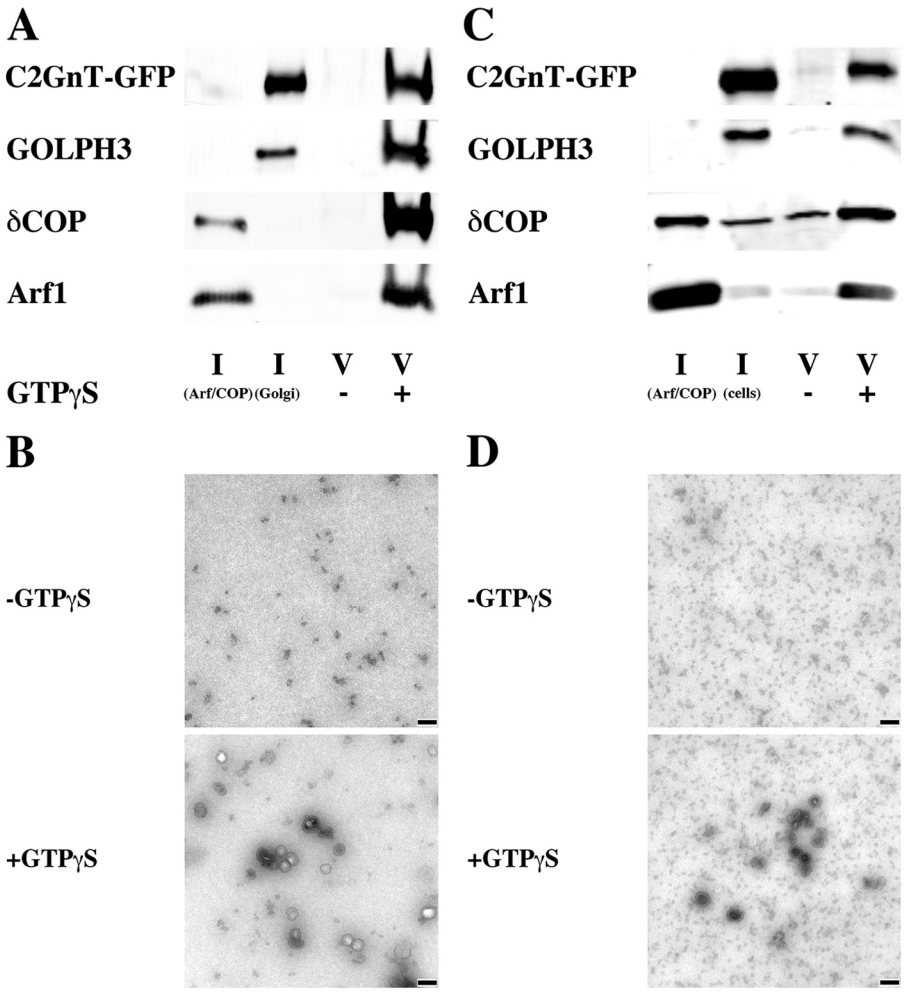

FIGURE 2. **GOLPH3 and C2GnT are incorporated into COPI vesicles.** *A*, COPI vesicles were generated *in vitro* from Golgi-enriched membranes, prepared out of CHO/C2GnT-GFP cells, incubated with recombinant coatomer and myristoylated Arf1 in the presence of GTPγS, and purified by centrifugation through two sucrose gradients (see "Experimental Procedures"). 1% of initial Arf1/coatomer mix (*I* (Arf/COP)), 1% of initial Golgi membrane (*I* (Golgi)), and 50% of the vesicle fraction (*V*) were subjected to SDS-PAGE and Western blot analysis. *B*, samples of the vesicle fractions from *A* were analyzed by negative stain electron microscopy. *Scale bar*, 100 nm. *C*, COPI vesicles were generated from semi-intact CHO/C2GnT-GFP cells incubated with recombinant coatomer and myristoylated Arf1 in the presence of GTPγS, and purified by differential centrifugation. 1% of initial Arf1/coatomer mix (*I* (Arf/COP)), 1% of initial semi-intact cells (*I* (cells)), and 50% of the vesicle fraction (*V*) were subjected to SDS-PAGE and Western blot analysis. *D*, samples of the vesicle fractions from *A* were analyzed by negative stain electron microscopy. *Scale bar*, 100 nm.

antibody incubation (30 min at room temperature, Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 633 secondary antibodies, Invitrogen) coverslips were washed three times for at least 2 min with 3% (w/v) BSA in PBS. After a quick rinse in H_2O, the coverslips were mounted onto glass slides using ProLong Gold (Invitrogen). Fluorescence microscopy was conducted using an Axiovert 200 (Zeiss) microscope. Line analysis was performed using ImageJ software.

RESULTS

GOLPH3 Interacts with C2GnT and SiaTI but Not GalT—Vps74p, the yeast homolog of GOLPH3, binds simultaneously to the molecular coat coatomer and to the consensus sequence ((F/L)(L/I/V)*XX*(R/K)), present in the cytosolic tails of some Golgi-localized mannosyltransferases (9). Similar motifs with juxtaposition of a hydrophobic patch to a positive one could be identified, *in silico*, in many mammalian glycosyltransferases (37). In line with these predictions, a recent study reports an interaction between GOLPH3 and the tail of C2GnT through an LLRRR motif (26). As a similar sequence could be observed at the N terminus of SiaTI (Fig. 1*A*), we analyzed its possible interaction with GOLPH3. To this end, lysates of CHO cells stably expressing C2GnT-GFP or SiaTI-GFP were incubated with recombinant GOLPH3-GST in the presence of glutathione-Sepharose beads. As a result, GOLPH3-GST, but not GST alone, could pull down SiaTI-GFP (Fig. 1*C*), and, with similar efficiency, C2GnT-GFP (Fig. 1*B*). In contrast, no binding could be observed between GOLPH3 and GalT-GFP (Fig. 1*D*), a glycosyltransferase that does not harbor any obvious interacting motif in its cytosolic tail (Fig. 1*A*). In all cases, GOLPH3-GST was efficiently pulling down coatomer (Fig. 1, *B–D*), which is in agreement with Tu and co-workers (11). These data support an interaction between GOLPH3 and both coatomer and mammalian glycosyltransferases through a specific motif present at their N-terminal domains.

GOLPH3, C2GnT, SiaTI, and Not GalT Are Incorporated into COPI Vesicles—Proper localization of many Golgi resident proteins is viewed as a dynamic process involving iterative traffick-

ing steps, including transport via COPI-coated vesicles (6, 7). We thus analyzed whether the glycosyltransferases of interest were present in such transport carriers. In a first approach, we generated COPI vesicles by *in vitro* reconstitution (38). Recombinant coatomer and recombinant Arf1, a small GTPase that triggers vesicle formation, were incubated with Golgi membranes purified from CHO/C2GnT-GFP, in the presence of GTPγS, a slowly hydrolyzing analog of GTP. COPI vesicles formed were then isolated by isopycnic centrifugation through sucrose gradients and analyzed by Western blotting and electron microscopy (Fig. 2, *A* and *B*). 80–100-nm spherical structures, characteristic of COPI vesicles, were formed in a nucleotide-dependent manner (Fig. 2*B*). In addition to Arf1 and the COPI-subunit δCOP, bands specific for GOLPH3 and C2GnT-GFP were observed in the vesicle fraction (Fig. 2*A*).

In a complementary approach, we used a semi-intact cell system recently set-up in the laboratory (39). In this assay (adapted from an assay originally used to study COPII-coated vesicles (40)), the plasma membrane of CHO/C2GnT-GFP cells is permeabilized with digitonin, allowing removal of endogenous cytosol without affecting internal organelles. After several washes, semi-intact cells are provided with coatomer, Arf1, and GTPγS to allow formation of COPI vesicles, which are then separated from the semi-intact cells by centrifugation (Fig. 2, *C* and *D*). These vesicles were similar in shape and composition to those obtained with the *in vitro* reconstitution assay (Fig. 2, *A* and *B*). Once again, GOLPH3 and C2GnT-GFP were found in the vesicle fraction in a nucleotide-dependent manner (Fig. 2*C*). The semi-intact cell system turned out to be a robust and simple tool and was therefore used in the experiments below.

We then generated COPI vesicles from semi-intact CHO/SiaTI-GFP and CHO/GalT-GFP. Vesicle fractions were positive for GOLPH3 and SiaTI-GFP, but devoid of GalT-GFP (Fig. 3). Altogether, these data show that GOLPH3 and its two interacting glycosyltransferases C2GnT and SiaTI are efficiently incorporated into COPI-coated vesicles. In contrast, GalT, which does not bind to GOLPH3, was absent from the vesicle fraction.

GOLPH3 Is Required for Incorporation of C2GnT and SiaTI into COPI Vesicles—GOLPH3 interacts with the cytosolic tails of C2GnT and SiaTI (Fig. 1) (26) and can also bind directly to the COPI coat (Fig. 1) (11). It is thus tempting to assume that GOLPH3 could participate in the incorporation of these enzymes into COPI vesicles. To challenge this hypothesis, we depleted cells of GOLPH3 by siRNA transfection and analyzed them with semi-intact cell assay for vesicle formation. COPI vesicles were efficiently formed even in the absence of GOLPH3 (Figs. 4 and 5). They were similar in shape and quantity compared with those from control cells. Additionally, p24, an integral Golgi protein and component of COPI vesicle machinery was efficiently incorporated into COPI vesicle irrespective of the presence of GOLPH3 (Figs. 4 and 5), suggesting that GOLPH3 does not play a role in vesicle formation *per se*. However, the cargo content of COPI vesicles generated from GOLPH3-depleted cells was strikingly modified. The amount of C2GnT-GFP (Fig. 4*A*) and SiaTI-GFP (Fig. 5*A*) in vesicle fractions was significantly decreased in the absence of GOLPH3. Similar effects were obtained using two different

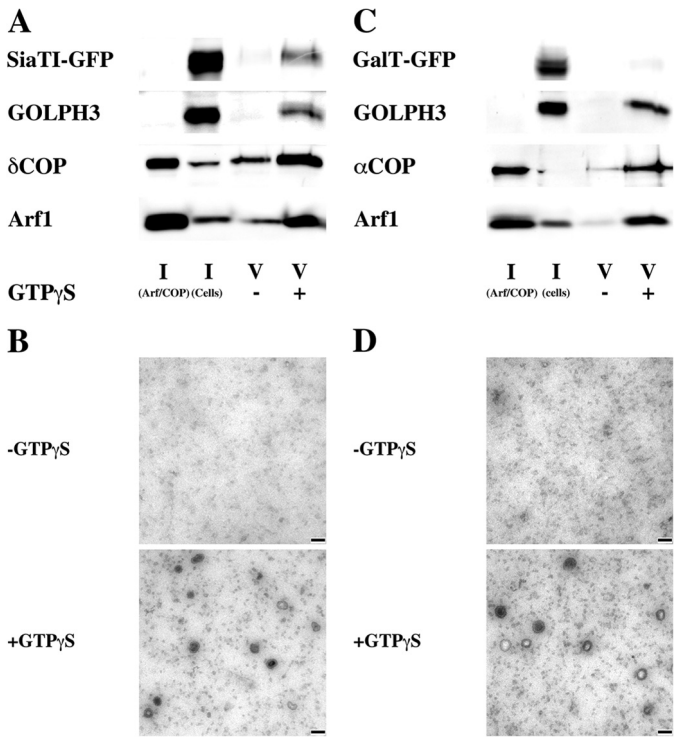

FIGURE 3. **GOLPH3 and SiaTI but not GalT are incorporated into COPI vesicles.** COPI vesicles were generated from semi-intact CHO/SiaTI-GFP cells (*A*) or CHO/GalT-GFP cells (*C*) incubated with recombinant coatomer and myristoylated Arf1 in the presence of GTPγS and purified by differential centrifugation. 1% of initial Arf1/coatomer mix (*I* (Arf/COP)), 5% of initial semi-intact cells (*I* (cells)), and 50% of the vesicle fraction (*V*) were subjected to SDS-PAGE and Western blot analysis. Samples of the vesicle fractions from *A* (*B*) or *C* (*D*) were analyzed by negative stain electron microscopy. *Scale bar*, 100 nm.

siRNAs against GOLPH3. These data suggest that GOLPH3 is required for proper incorporation of C2GnT and SiaTI into COPI vesicles.

Relocalization of C2GnT, SiaTI but Not GalT in GOLPH3-depleted Cells—COPI vesicles have been mainly involved in the retrieval of proteins from the Golgi back to the ER (7). It is thus expected that a default of incorporation of C2GnT and SiaTI into these carriers would result in a redistribution of these enzymes within the secretory pathway. In control cells, C2GnT-GFP and SiaTI-GFP are present mainly in the Golgi but also, to a lower extent, in the ER (Figs. 6 and 7). In contrast, upon GOLPH3 depletion, no colocalization could be observed between these glycosyltransferases and the ER marker calnexin (Fig. 6 and 7). This phenotype is in agreement with a defect of recycling of C2GnT and SiaTI from Golgi to ER by COPI vesicles. However, GOLPH3 depletion had no effect on the subcellular distribution of GalT-GFP (Fig. 8). This is in accordance with an absence of interaction between GalT and GOLPH3.

Leakage of C2GnT, SiaTI but Not GalT from the Golgi to the Plasma Membrane in the Absence of GOLPH3—According to the maturation and progression model for biosynthethic protein transport, cisternae form at the *cis*-Golgi, progress along the Golgi and disassemble at the *trans* side. Proteins would thus be transported by a bulk flow along the *cis-trans* axis before exiting the Golgi and being directed toward the plasma membrane (41). As a consequence, a defect in COPI-mediated recy-

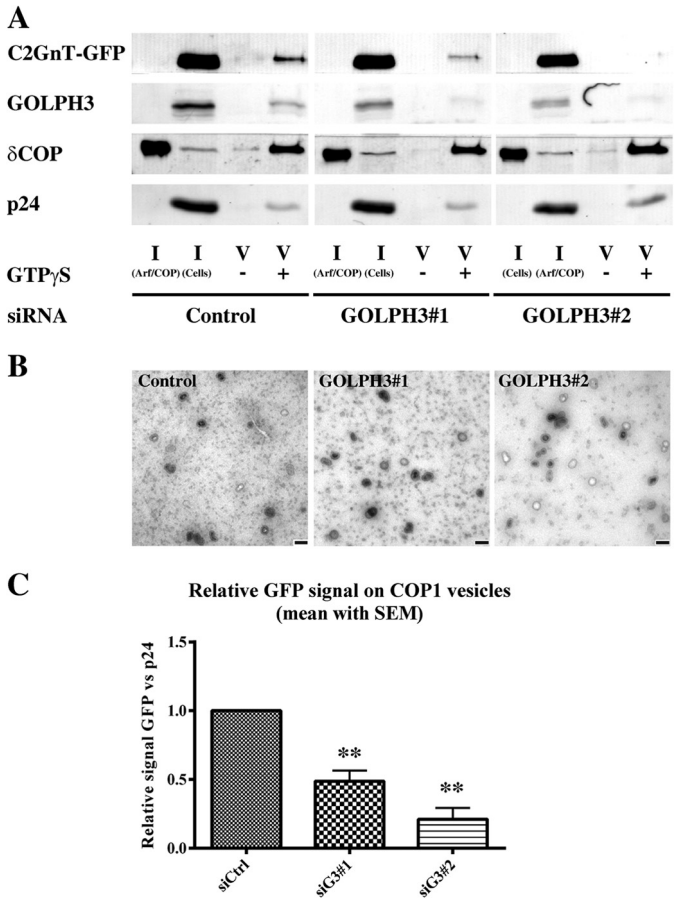

FIGURE 4. **In the absence of GOLPH3, C2GnT is no longer incorporated into COPI vesicles.** *A*, COPI vesicles were generated from semi-intact CHO/C2GnT-GFP cells treated with siRNA control (*siCtrl*), GOLPH3-1 or GOLPH3-2, incubated with recombinant coatomer and myristoylated Arf1 in the presence of GTPγS, and purified by differential centrifugation. 1% of initial Arf1/coatomer mix (*I* (Arf/COP)), 1% of initial semi-intact cells (*I* (cells)), and 50% of the vesicle fraction (*V*) were subjected to SDS-PAGE and Western blot analysis. *B*, samples of the vesicle fractions from *A* were analyzed by negative stain electron microscopy. *Scale bar*, 100 nm. *C*, statistical evaluation of the relative Western blot signal strength of eGFP *versus* p24 specific bands in the vesicle fraction ($n = 4$) generated from semi-intact cells as described above.

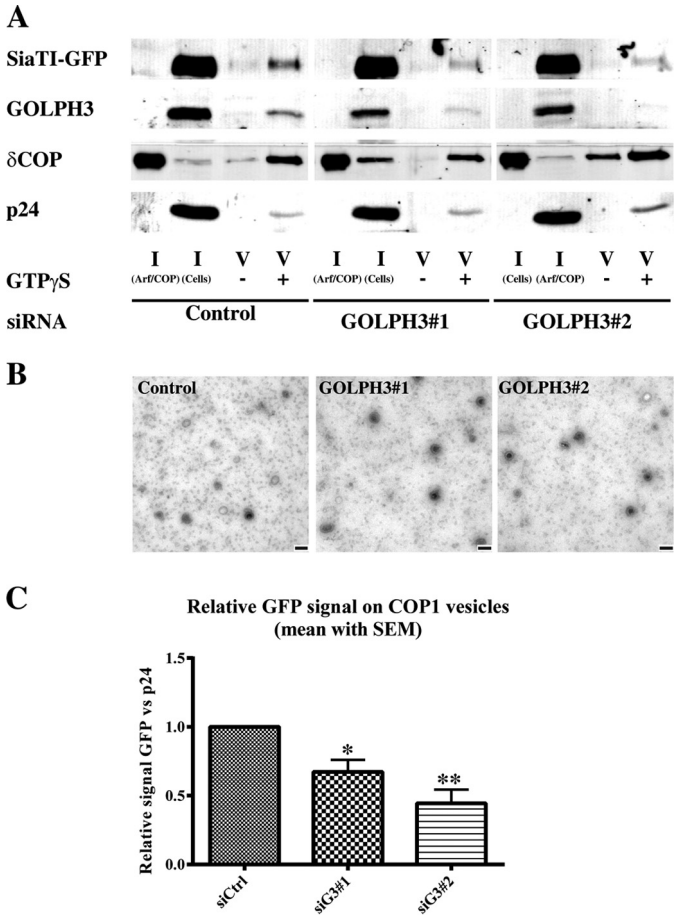

FIGURE 5. **In the absence of GOLPH3, SiaTI is no longer incorporated into COPI vesicles.** *A*, COPI vesicles were generated from semi-intact CHO/SiaTI-GFP cells treated with siRNA control (*siCtrl*), GOLPH3-1 or GOLPH3-2, incubated with recombinant coatomer and myristoylated Arf1 in the presence of GTPγS, and purified by differential centrifugation. 1% of initial Arf1/coatomer mix (*I* (Arf/COP)), 1% of initial semi-intact cells (*I* (cells)), and 50% of the vesicle fraction (*V*) were subjected to SDS-PAGE and Western blot analysis. *B*, samples of the vesicle fractions from *A* were analyzed by negative stain electron microscopy. *Scale bar*, 100 nm. *C*, statistical evaluation of the relative Western blot signal strength of eGFP *versus* p24-specific bands in the vesicle fraction ($n = 4$) generated from semi intact cells as described above.

cling is expected to result in a leakage of Golgi resident proteins toward the plasma membrane.

To quantify the amount of glycosyltransferases present at the cell surface, we used fluorescence activated cell sorting (FACS). C2GnT-GFP, SiaTI-GFP, and GalT-GFP are type II transmembrane proteins fused to GFP at their C terminus. Upon transport to the plasma membrane, their fluorescent tag gets exposed toward the extracellular medium. By using an antibody against GFP, we could thus specifically monitor the population of GFP-labeled glycosyltransferases present at the surface of intact cells. The total amount of GFP proteins expressed in cells was determined by quantifying directly the GFP signal. Results are represented in Fig. 9 as a ratio between antibody and GFP signals. In GOLPH3-depleted cells, the fraction of C2GnT-GFP or SiaTI-GFP at the plasma membrane was significantly increased compared with control cells (Fig. 9, *A* and *B*). In contrast, no significant difference in the distribution of GalT-GFP could be observed upon GOLPH3 depletion (Fig. 9*C*).

Altogether, immunofluorescence and FACS analysis highlight a shift of C2GnT-GFP and SiaTI-GFP localization from the early to the late secretory pathway upon GOLPH3 depletion. In contrast, spatial distribution of GalT-GFP was not affected under similar conditions. These results mirror the differences observed with regard to GOLPH3-dependent incorporation of these enzymes into COPI vesicles (Figs. 4 and 5).

DISCUSSION

Lumenal protein glycosylation seems intimately related to the accurate localization of the different glycosyltransferases and glycosyl hydrolases within the Golgi apparatus. Mechanisms underlying such a spatial regulation are not completely understood. In this study, we show that the peripheral Golgi protein GOLPH3 interacts, in addition to the molecular coat coatomer (9), with the glycosyltransferases C2GnT (26) and SiaTI and mediates their incorporation into COPI-coated vesicles. Under GOLPH3 depletion conditions, these enzymes are no longer included into these recycling carriers and get partially

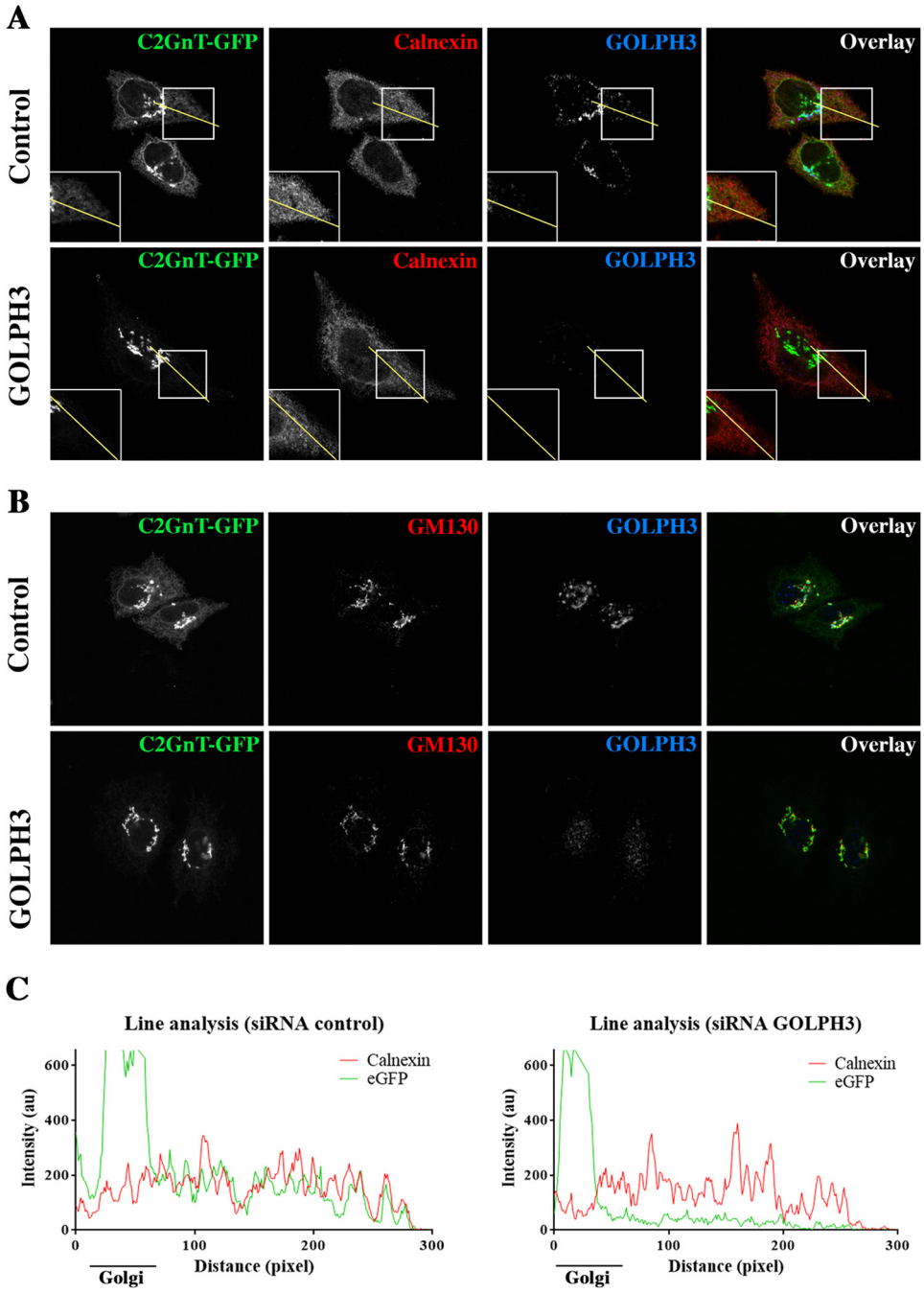

FIGURE 6. **Relocalization of C2GnT-GFP under GOLPH3 depletion.** CHO/C2GnT-GFP were treated with siRNA control (*siCtrl*) or GOLPH3-2. After fixation, cells were stained for GOLPH3 (*blue*), the ER marker calnexin (*red*; *A*), or the Golgi marker GM130 (*red*; *B*). *C*, signal intensity of calnexin (*red*) and eGFP (*green*) fluorescence along a randomly positioned line (indicated in *A* with *yellow lines*) spanning Golgi and ER of cells treated with siRNA control or GOLPH3-2. The part corresponding to Golgi area has been stated on the graph.

relocalized from the early to the late secretory pathway. In contrast, GalT, a glycosyltransferase that does not interact with GOLPH3, was not dependent on GOLPH3 for proper subcellular localization.

GOLPH3-binding Motif—Most glycosyltransferases are type II integral proteins exposing a short N-terminal tail to the cytosol. In yeast, Vps74p, the homolog of GOLPH3, recognizes a (F/L)(L/I/V)*XX*(R/K) sequence present in the cytosolic tails of several of these enzymes (9). Similarly, mammalian GOLPH3 interacts with a LLRRR binding site at the N terminus of C2GnT (26). *In silico* analysis revealed an ~4-fold enrichment of the LL*x*RR motif in the cytosolic tails of Golgi-localized proteins compared with plasma membrane- or endoplasmic reticulum-localized motifs (37). In our experimental study, we found that GOLPH3 efficiently interacts with SiaTI, an enzyme harboring a related motif composed of only one leucine and three lysine residues (LKKK). These results are in agreement with the work of Isaji *et al.* (42), published while our manuscript was under revision. Additionally, a recent article identified L*X*LRR as a putative motif for GOLPH3 recognition (27). Further work will thus be needed to define the precise consensus sequence of the GOLPH3 binding site to get a better estimation

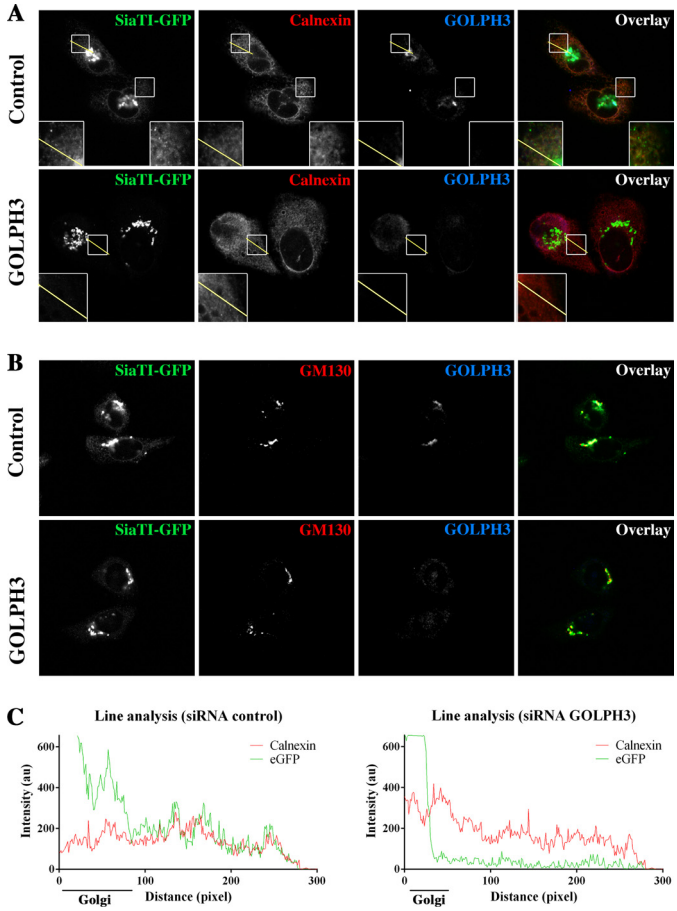

FIGURE 7. **Relocalization of SiaTI-GFP under GOLPH3 depletion.** CHO/SiaTI-GFP were treated with siRNA control (*siCtrl*) or GOLPH3-2. After fixation, cells were stained for GOLPH3 (*blue*), the ER marker calnexin (*red*; *A*), or the Golgi marker GM130 (*red*; *B*). *C*, signal intensity of calnexin (*red*) and eGFP (*green*) fluorescence along a randomly positioned line (indicated in *A* with *yellow lines*) spanning Golgi and ER of cells treated with siRNA control or GOLPH3-2. The part corresponding to Golgi area has been stated on the graph.

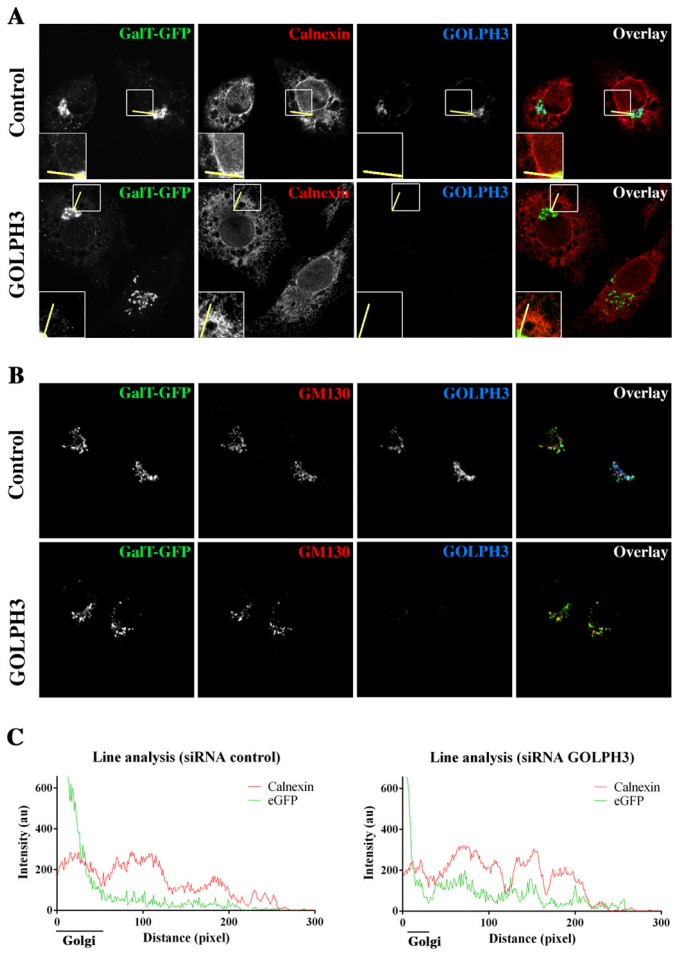

FIGURE 8. **No relocalization of GalT-GFP under GOLPH3 depletion.** CHO/GalT-GFP were treated with siRNA control (*siCtrl*) or GOLPH3-2. After fixation, cells were stained for GOLPH3 (*blue*), the ER marker calnexin (*red*; *A*), or the Golgi marker GM130 (*red*; *B*). *C*, signal intensity of calnexin (*red*) and eGFP (*green*) fluorescence along a randomly positioned line (indicated in *A* with *yellow lines*) spanning Golgi and ER of cells treated with siRNA control or GOLPH3-2. The part corresponding to Golgi area has been stated on the graph.

of the GOLPH3 interactome at the Golgi level. It seems nevertheless clear that the general feature of such a motif is the juxtaposition of a patch of hydrophobic amino acids to a patch of positively charged residues.

Role of GOLPH3 in the COPI System—GOLPH3 interacts with the glycosyltransferases C2GnT (Ref. 26 and this study) and SiaTI (Ref. 42 and this study), as well with coatomer (Ref. 11 and this study), the major component of COPI-coated vesicles that form at the Golgi (7). Using two different biochemical assays, *in vitro* vesicle formation from purified Golgi membranes and a semi-intact cell assay, we found that both GOLPH3, C2GnT and SiaTI are present within COPI vesicles. In absence of GOLPH3, COPI vesicles were still formed efficiently, but with reduced amounts of C2GnT and SiaTI. In contrast, the integral Golgi resident protein p24 was incorporated into the vesicles irrespective of the presence of GOLPH3. These results show that GOLPH3 is not required for COPI carrier biogenesis *per se* and is in agreement with previous work showing that coatomer and the small GTPase Arf1 constitute the minimal cytosolic machinery required to generate COPI vesicles (30, 43). GOLPH3 is, however, necessary for incorporation of C2GnT and SiaTI into COPI vesicles, suggesting an adaptor role for this protein. By binding at the same time to both a glycosyltransferase and to coatomer, GOLPH3 would trigger inclusion of Golgi resident enzymes into COPI transport carriers.

Not all Golgi enzymes exhibit putative GOLPH3 binding sites, suggesting that GOLPH3 action is restricted to a subset of glycosyltransferases (37). Considering that the COPI coat shares mechanistic properties with the well characterized clathrin coat, it is tempting to think that, similar to clathrin, several adaptors could be associated with coatomer. In this context, calsenilin and its close homolog CALP (calsenilin-like protein) have been shown to interact with GalT2 and to be required for its proper Golgi localization (44). Using an approach similar to the one developed in this study would allow deciphering the mechanistic role of calsenilin and CALP.

Role of GOLPH3 in Glycosyltransferase Golgi Localization—According to the maturation and progression model, cisternae are newly formed at the *cis*-Golgi and progress and mature along the Golgi and disassemble at the *trans* side. Proteins would thus be transported along the *cis-trans* axis by bulk flow, whereas maintenance of Golgi resident enzyme localization

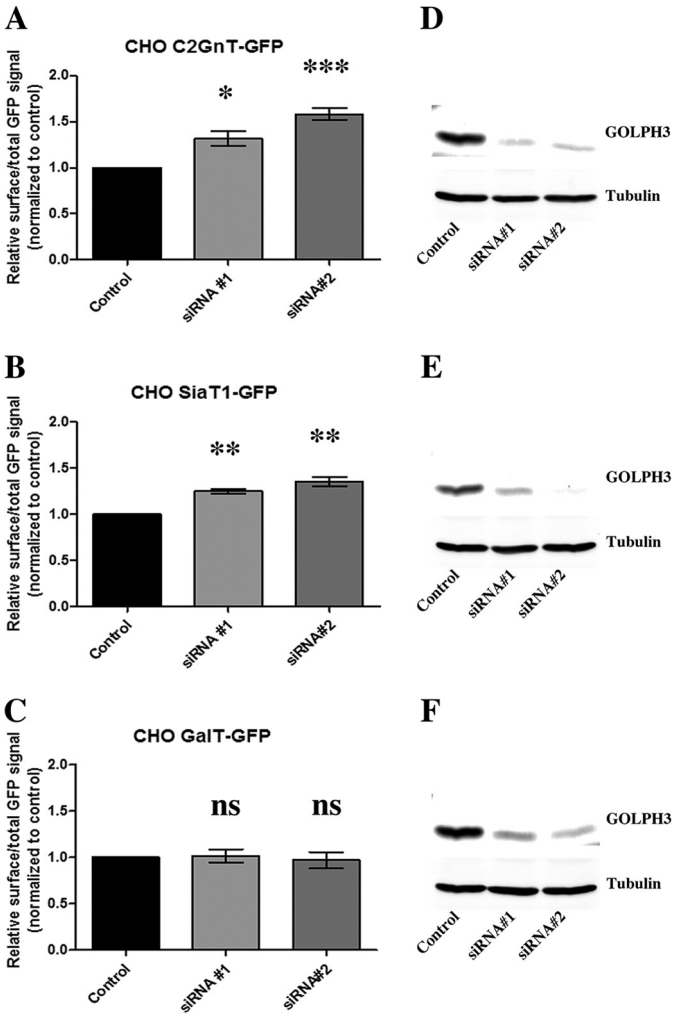

FIGURE 9. **C2GnT and SiaTI but not GalT are relocalized to the plasma membrane in GOLPH3-depleted cells.** *A–C*, CHO/C2GnT-GFP (*A*), CHO/SiaTI-GFP (*C*), and CHO/GalT-eFP (*E*) were treated with siRNA control (*siCtrl*), GOLPH3-1, or GOLPH3-2, stained with anti-GFP antibody and a secondary antibody coupled to allophycocyanin (*APC*) fluorophore, before being analyzed by FACS. Ratio between allophycocyanin signal (corresponding to cell surface-localized GFP proteins) and GFP signal (corresponding to the total amount of GFP proteins expressed in cells) were then calculated. Results are shown as mean of four independent experiments. *D–F*, representative Western blot analysis of cells used in *A–C*. *ns*, nonstatistically significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

would be ensured by retrograde transport in COPI vesicles (41). Thus, by mediating incorporation of C2GnT and SiaTI into these carriers, GOLPH3 would contribute to dynamically maintain the steady-state Golgi localization of these transferases. In agreement with this model, localization of C2GnT and SiaTI was altered upon GOLPH3 depletion.

Upon physiological conditions, C2GnT and SiaTI are localized mainly in the Golgi and, to a lower extent, in the ER. In GOLPH3-depleted cells, however, C2GnT and SiaTI are no longer visible in the ER, and a significant fraction becomes relocalized to the plasma membrane. This early-to-late shift of localization within the secretory pathway is the expected consequence of a default of COPI-mediated Golgi-to-ER recycling. These mislocalization of C2GnT and SiaTI would explain the defect of *O*-glycosylation (26) and sialylation (42) observed in GOLPH3-depleted cells.

GOLPH3 is specifically enriched at the TGN through binding to the lipid phosphatidylinositol 4-phosphate (14–16). It could thus be viewed as a security guard that prevents glycosyltransferases to escape the secretory pathway. When phosphatidylinositol 4-phosphate is redistributed along the whole Golgi, by depletion of the phosphatase Sac1, correct Golgi localization of several enzymes is hampered (45). It might thus be necessary that the action of GOLPH3 is restricted to the TGN for proper spatial organization of the Golgi.

Proper Golgi localization of C2GnT and SiaTI does not rely only in their cytosolic tails but also on their transmembrane domains and flanking regions (3, 46–49). These multiple interactions highlight the cooperation of several mechanisms to maintain the spatial distribution of these transferases and explain why C2GnT and SiaTI are not completely relocalized under GOLPH3 depletion. According to lipid-based sorting models, the physicochemical properties of the transmembrane domain (length, presence of amino acids with uncharged polar side chains) would trigger enrichment of glycosyltransferases into particular lipid environments. As the lipid composition is not homogenous across the Golgi, individual transmembrane segment properties could result in an accumulation of the enzymes in different cisternae (2–4). Additionally, glycosyltransferases are able to oligomerize through their transmembrane and/or stem domains. Such interactions can result in the formation of big enzyme aggregates, favoring retention of the enzymes in their respective cisternae (1, 5).

Three different mechanisms could thus act concomitantly in cells to ensure Golgi retention: lipid-based sorting, kin recognition, and COPI-mediated recycling. Differences in the relative contribution of each of these mechanisms would explain the enzyme- and cell type-dependent localization of each glycosyltransferase. As an illustration of this assumption, we found that C2GnT and SiaTI need to be incorporated into COPI vesicles for proper subcellular distribution, whereas GalT does not. Transport in COPI vesicles might constitute the molecular basis of the trafficking differences observed between SiaTI and GalT (50, 51). It has indeed been suggested that trafficking of GalT within the secretory pathway would rather be mediated by tubular retrograde carrier (52).

Physiological Role of GOLPH3—Several GOLPH3-independent mechanisms contribute to Golgi retention (1), and only a subset of glycosyltransferases harbors putative GOLPH3-binding sites (37). What could thus be the physiological role of GOLPH3? In leukocytes, C2GnT is a key enzyme of the synthesis of core 2-associated sialyl Lewis x (C2-O-sLe^x) antigen, a ligand involved in selectin-mediated adhesion (53, 54). Following tissue injury and infection, an increased production of C2-O-sLe^x on the cell surface leads leukocytes to switch from a circulating to an adhesive state (55). This relies on proper Golgi localization of C2GnT, which depends on GOLPH3 (26). By controlling the localization of specific glycosyltransferases, GOLPH3 might regulate glycosylation patterns of cells, thus modulating their behavior in response to their environment. In line with this hypothesis, GOLPH3, as well as C2GnT, have been involved in cancer progression (18–25, 56–58).

In vertebrates, two isoforms of GOLPH3 have been identified: GOLPH3 and GOLPH3L (14). They both interact differ-

ently with the COPI coat (11) and have antagonistic action on Golgi morphology (13). Although GOLPH3 is ubiquitously expressed, GOLPH3L is present only in some cell lines and tissues (13). Modulating the relative concentration of GOLPH3 and GOLPH3L could constitute another level of regulation to fine-tune spatial localization of glycosyltransferases in a cell type-specific manner. This would explain why, in CHO cells, C2GnT is partially relocalized to plasma membrane upon GOLPH3 depletion (this work), whereas it gets completely relocalized to the ER under similar conditions in lymphoblasts (26).

In conclusion, in this study, we provide for the first time direct evidences for role of GOLPH3 as a coatomer adaptor. By mediating incorporation of glycosyltransferases into COPI vesicles, it contributes to dynamically maintain the localization of Golgi resident enzymes.

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