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Intra-Golgi Protein Transport Depends on a Cholesterol Balance in the Lipid Membrane*

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Transport of proteins between intracellular membrane compartments is mediated by a protein machinery that regulates the budding and fusion processes of individual transport steps. Although the core proteins of both processes are defined at great detail, much less is known about the involvement of lipids. Here we report that changing the cellular balance of cholesterol resulted in changes of the morphology of the Golgi apparatus, accompanied by an inhibition of protein transport. By using a well characterized cell-free intra-Golgi transport assay, these observations were further investigated, and it was found that the transport reaction is sensitive to small changes in the cholesterol content of Golgi membranes. Addition as well as removal of cholesterol (10 ± 6%) to Golgi membranes by use of methyl-β-cyclodextrin specifically inhibited the intra-Golgi transport assay. Transport inhibition occurred at the fusion step. Modulation of the cholesterol content changed the lipid raft partitioning of phosphatidylcholine and heterotrimeric G proteins, but not of other (non) lipid raft proteins and lipids. We suggest that the cholesterol balance in Golgi membranes plays an essential role in intra-Golgi protein transport and needs to be carefully regulated to maintain the structural and functional organization of the Golgi apparatus.

Cholesterol is an essential lipid constituent in the membranes of mammalian cells and has a profound effect on the physical properties of these membranes. By interacting with the acyl-chain of other (phospho)lipids, cholesterol increases lipid packing in the membranes, thereby causing thickening of the membrane. Increased packing of the membrane affects the fluidity of the membrane and makes it less permeable for the transbilayer passage of small water-soluble molecules like glucose (1–3). Total cellular cholesterol levels are determined by *de novo* synthesis in the endoplasmic reticulum, by the uptake of extracellular cholesterol from, *e.g.* lipoproteins, and by esterification and subsequent release of cholesterol to the outside circulation or storage in lipid droplets (4). Regulation of these processes keeps total free cholesterol levels in cells precisely controlled. A crucial step in cholesterol homeostasis is action of a cholesterol-sensing machinery at the endoplasmic reticulum (5). Within the cell, the cholesterol distribution between membranes is also actively regulated, resulting in a concentration gradient along the secretory pathway, with low concentrations of cholesterol in the ER[1] and high concentrations in the plasma membrane (6, 7). The Golgi apparatus contains intermediate levels of cholesterol (7), and there are indications of a cholesterol gradient even within the Golgi complex (8–11). Also along the endocytic route a heterogeneous cholesterol distribution has been observed (12).

The tight regulation of cholesterol homeostasis and subcellular distribution suggests a critical role of this lipid in biological processes. Due to the cholesterol gradient along the secretory pathway and its implication on lipid bilayer thickness, cholesterol has been postulated to be involved in the targeting of Golgi resident proteins to this organelle (3).

Cholesterol is also involved in the stabilization and function of lipid-enriched microdomains (lipid rafts) within a membrane. The scaffold of these microdomains is built by sphingolipids and cholesterol (13–16). Cholesterol tightly interacts with sphingolipids that contain predominantly long-chain saturated fatty acids. This results in the segregation of these lipids from other membrane lipids into microdomains. Due to the physical properties of these microdomains, distinct classes of membrane proteins are incorporated, causing their segregation as well (13).

Recent advances suggest that cholesterol exerts many of its actions mainly by maintaining sphingolipid rafts in a functional state (16). Microdomains were postulated to exist at the *trans*-Golgi network, to explain the observed sorting of sphingolipids to the apical surface in polarized cells (17, 18). Functionally, sorting is explained by the transport of microdomain-associated proteins such as glycosylphosphatidylinositol-anchored proteins and influenza virus hemagglutinin to the apical surface (19–22). Likewise, microdomains have been implicated in sorting processes along the endocytic pathway (23, 24). At the plasma membrane, the most important role of microdomains may be their function in

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[1] The abbreviations used are: ER, endoplasmic reticulum; MβCD, methyl-β-cyclodextrin; ESI-MS/MS, nano-electrospray ionization tandem mass spectrometry; DRM, detergent-resistant membrane; GTPγS, guanosine 5′-*O*-(thiotriphosphate); VSV-G, vesicular stomatitis virus G; CHO, Chinese hamster ovary; Endo H, endoglycosidase H; RT, room temperature; Chol, cholesterol; SM, sphingomyelin; GlcCer, glucosylceramide; PC, phosphatidylcholine; COPI, coat protein complex I; NSF, NEM-sensitive protein; SNARE, soluble NSF attachment protein receptor.

signal transduction (14–16, 25, 26). Activation of signaling cascades can cause raft clustering into patches as well as change the partitioning of signaling molecules in lipid rafts. For example, in T-cell antigen receptor signaling, clustering of rafts is an essential feature in the formation of an immunological synapse between T-cells and antigen-presenting cells (27–29).

Here we report that small changes in cholesterol content of Golgi membranes specifically inhibit the fusion reaction of intra-Golgi protein transport. Thus, cholesterol levels at the Golgi complex must be precisely balanced to allow protein transport to occur. We considered the possibility that a change in cholesterol levels might affect the function of microdomains at the Golgi complex. Recently we obtained evidence for the existence of such microdomains at the early Golgi complex (30). We now show that changing the cholesterol content of membranes specifically affects the phase partitioning of heterotrimeric G proteins, suggesting a possible involvement of microdomains in the regulation of protein transport through the Golgi apparatus.

EXPERIMENTAL PROCEDURES

Materials

Methyl-β-cyclodextrin (average degree of substitution of 10.5–14.7), α-cyclodextrin, and brefeldin A were from Sigma. Stock solutions of cyclodextrins were freshly prepared at 50 mM in water. [3,4-$^{13}C_2$]Cholesterol was kindly provided by Dr. Wolfgang D. Lehmann (German Cancer Research Center, Germany). 2,2,3,4,4,6-D_6-cholesterol was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Cholesterol was from Merck (Darmstadt, Germany). Triton X-100 (purchased as a 10% solution) and GTPγS were from Roche Diagnostics GmbH (Mannheim, Germany).

Methods

Intra-Golgi Transport Assay—The standard intra-Golgi transport was performed as described previously (31). A standard intra-Golgi transport assay (25 μl) contained 2.5 mM Hepes buffer, pH 7.0, 15 mM KCl, 2.5 mM $Mg(AcO)_2$, 0.4 μCi of UDP-*N*-[^{3}H]acetylglucosamine, 5 μl of 1:1 mixture of donor and acceptor Golgi membranes (2–3 μg of protein), 10 μM palmitoyl-coenzyme A, ATP- and UTP-regenerating systems, and 16.5–17.5 μg of rat or 16 μg of bovine brain cytosol. The transport reactions were incubated for 2 h at 30 °C. The incorporation of *N*-[^{3}H]acetylglucosamine into the VSV-G protein was determined as described previously (32).

Glycosylation Assay—This assay is based on a method described by Taylor *et al.* (33). Prior to isolation of Golgi membranes, CHO wild type cells were infected with the vesicular stomatitis virus. Isolated membranes were treated with 1 mM *N*-ethylmaleimide for 15 min on ice to inhibit transport. Subsequently, excessive *N*-ethylmaleimide was quenched with 2 mM dithiothreitol. The glycosylation assay was performed under standard transport conditions without the addition of cytosol (for details, see Ref. 32).

Metabolic Labeling and Endoglycosidase H Digestion—CHO cells infected with VSV-G (2×10^6 cells for each time point) were pulse-labeled with [^{35}S]cysteine-methionine and chased as previously described (34). Cells were incubated with serum-free medium in the absence or presence of 10 mM MβCD·Chol (4:1) or 10 mM MβCD during the 1-h period of methionine and cysteine starvation and were present throughout the experiment. In case cells were depleted of cholesterol with 10 mM MβCD, lovastatin (5 μM) was added to the preincubation (1 h) as well as during the subsequent experiment. Cells were collected in 300 μl of ice-cold water and 30 μl of ×10 Endo H buffer (3% SDS, 1 M sodium acetate, pH 5.6, 200 μl/ml 2-mercaptoethanol) were then added to the samples prior to incubation at 95 °C for 2 min. Aliquots (45 μl) were incubated in the presence or absence of 3 milliunits of Endo H (Roche Applied Science) overnight at 37 °C, boiled in Laemmli sample buffer, resolved by 10% SDS-PAGE, and subjected to autoradiography.

Lipid Analysis—Cholesterol was measured with nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS) as described by Sandhoff *et al.* (35). Golgi membranes were diluted 5-fold in assay buffer (25 mM Hepes, pH 7.2, 2.5 mM $Mg(OAc)_2$) and incubated for 2 h at 30 °C with or without methyl-β-cyclodextrin prior to centrifugation at 100,000 × *g* for 1 h (4 °C) through a 15% (w/v) sucrose cushion. The pellet was resuspended in the original volume with 1,4-dioxane. For quantitation, an internal standard was added to the samples, and after sulfatation of the cholesterol, cholesterol was measured with ESI-MS/MS by scanning in the negative mode for the parent ions of m/z = 97 (HSO_4^-) or 80 (SO_3^-), depending on the internal standard ([3,4-$^{13}C_2$]cholesterol and 2,2,3,4,4,6-D_6-cholesterol, respectively). Phosphatidylcholine, sphingomyelin, and glucosylceramide analysis by ESI-MS/MS was performed as described previously (36).

Preparation of Methyl-β-cyclodextrin·Cholesterol Complex—Initially, cyclodextrin was pre-loaded with cholesterol according to Ref. 37. By using ESI-MS/MS, it was determined that the efficiency of loading was 1:12 (cholesterol:MβCD; mol:mol). This method was then modified to allow for maximal loading of MβCD. To this end, 12.5 mg of methyl-β-cyclodextrin (average molecular weight of 1,309) was dissolved in 250 μl of H_2O and heated to 80 °C. To this solution, 150 μl of 10 mg/ml cholesterol in methanol/chloroform (2:1) was added dropwise during continuous vortexing and incubated for 4 h at 80 °C. Non-solubilized cholesterol was removed by centrifugation for 10 min at RT for 14,000 rpm. The clear supernatant was transferred to a new Eppendorf tube, frozen in liquid nitrogen, and lyophilized. The cyclodextrin·cholesterol complex was dissolved in 322.5 μl of water (29.4 mM). Under these conditions, the efficiency of preloading was increased to 1:4 (cholesterol: MβCD; mol:mol). The concentration mentioned for MβCD·cholesterol complexes throughout this report is based on the MβCD concentration (unless mentioned otherwise, the cholesterol concentration is 4-fold lower).

Preparation of DRMs—Isolated Golgi membranes were centrifuged at 100,000 × *g* for 30 min at 4 °C and resuspended in ice-cold PEN (25 mM Pipes, pH 6.5, 2 mM EDTA, 150 mM NaCl) containing 1% Triton X-100. After incubation for 30 min on ice, the detergent-insoluble material (DRM) was separated from the detergent-soluble material by centrifugation at 100,000 × *g* for 1 h at 4 °C.

Transfection with a ts-O45-VSV-G-GFP—CHO WT cells were cultured in α minimal essential medium (Biochrom AG, Berlin) supplemented with 10% fetal calf serum on 11-mm diameter coverslips in 24-multiwell plates (Corning B.V., Life Sciences, The Netherlands) for 12 h. Cells were transfected with a plasmid (0.4 μg) containing a GFP-tagged temperature-sensitive mutant of VSV-G (ts-G-GFP) (38). Transfections were performed using LipofectAMINE Plus™ reagent (Invitrogen) according to the manufacturer's instructions. The cells were then incubated at 40 °C for 4 h to accumulate the VSV-G protein in the ER. Subsequently, the cells were incubated for 1 h at 40 °C in serum-free medium in the absence or presence of 10 mM MβCD·Chol (4:1) or 10 mM MβCD. In case cells were depleted of cholesterol with 10 mM MβCD, lovastatin (5 μM) was added to the preincubation (1 h) as well as during the release (45 min at 32 °C). Secretion of VSV-G protein was induced by replacement of the medium with fresh prewarmed medium (serum-free) at 32 °C for 45 min, cells were fixed in 3.5% paraformaldehyde for 20 min at RT, and images were taken with the use of a Leica confocal laser scan microscope.

RESULTS

Cholesterol Affects Transport within the Golgi Complex—Cholesterol (Chol) can be incorporated into cellular membranes by the addition methyl-β-cyclodextrin·cholesterol complexes (MβCD·Chol) to intact cells. To study the effect of cholesterol on the secretory pathway, CHO cells were incubated with MβCD·Chol. Such treatment resulted in a significant change in the subcellular distribution of the Golgi complex. In these experiments, the Golgi apparatus was visualized by using several Golgi markers, including p115, GM130, and GFP-tagged *N*-acetylglucosamine transferase (39, 40). In control cells, the Golgi complex exhibits a typical perinuclear localization (Fig. 1). However, upon loading of the cells with cholesterol the Golgi complex dissociated into small punctuated structures throughout the cells (Fig. 1*A*). When cells were depleted of cholesterol by incubation with MβCD (confirmed by determination of total cellular cholesterol levels, data not shown), the Golgi localization did not change as significantly, but a more condensed perinuclear localization was consistently observed.

The morphology of the Golgi apparatus is dynamically regulated by membrane flow processes at the *cis*- (incoming) and *trans*-site (outgoing) of the complex. This is dramatically visualized by the addition of Brefeldin A to intact cells. Brefeldin A inhibits anterograde transport to the Golgi complex and causes a complete disruption of the perinuclear Golgi structure (41).

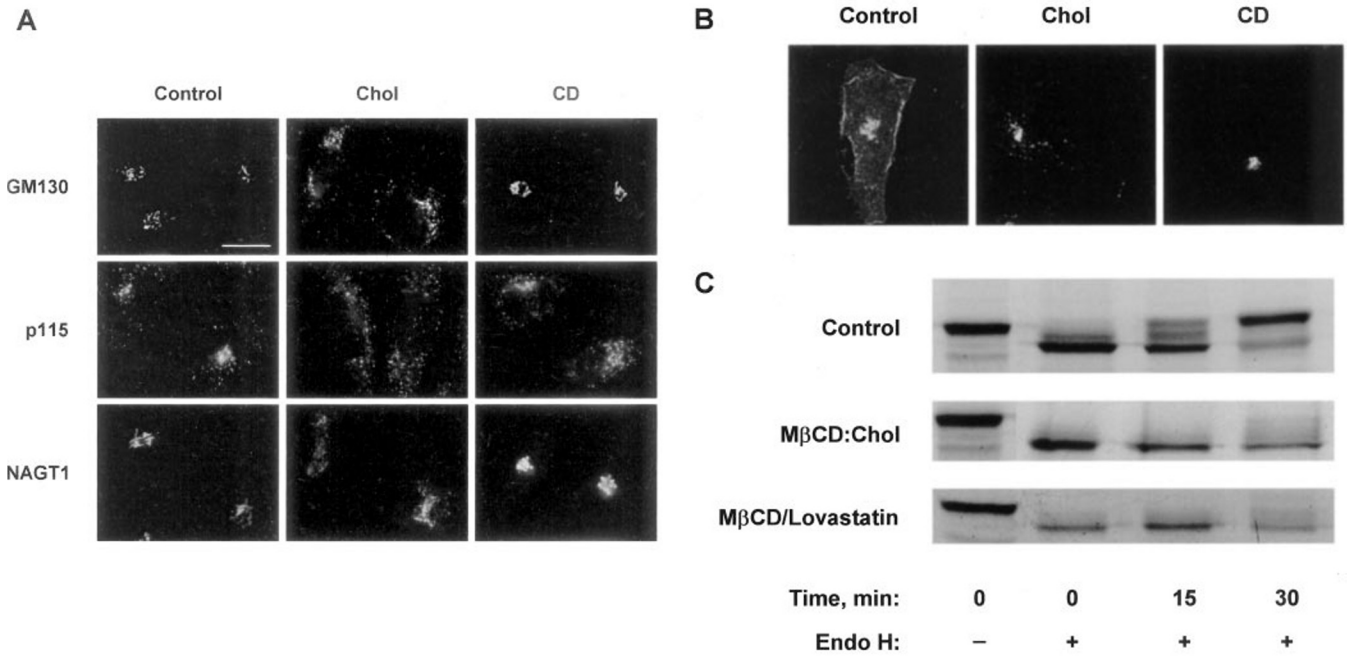

FIG. 1. **Cholesterol affects the morphology and function of the Golgi complex.** *A*, CHO cells were incubated in the absence (control, *left column panels*) or in the presence of 10 mM MβCD·Chol (4:1, mol:mol) (*middle column panels*) or 10 mM MβCD (*right column panels*) for 1 h at 37 °C. The concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration (*i.e.* the cholesterol concentration is 4-fold lower). After fixation, the morphology of the Golgi complex was determined by use of primary antibodies against three different Golgi markers, GM130 (*top row panels*), p115 (*middle row panels*), and NAGT1 (*bottom row panels*). *B*, CHO cells were transfected with a plasmid containing a GFP-tagged temperature-sensitive mutant of VSV-G protein. Prior to release of the protein (45 min at 32 °C), cells were incubated in the absence (control, *left panel*) or presence of 10 mM MβCD·Chol (4:1) (*Chol*, *middle panel*) or 10 mM MβCD (*CD*, *right panel*) for 1 h at 40 °C as described under "Experimental Procedures." After fixation, the subcellular localization of VSV-G-GFP was analyzed by fluorescence microscopy. *C*, CHO cells were subjected to pulse-chase labeling 4 h after VSV infection. Cells were starved for 1 h and then pulse-labeled for 30 min in the presence of [^{35}S]cysteine-methionine and chased in non-radioactive medium for the indicated time periods in the absence (*upper panel*) or presence of 10 mM MβCD·Chol (4:1, mol:mol) (*middle panel*) or 10 mM MβCD (*lower panel*), which were added to the cells during starvation and were present during the pulse and throughout the chase periods (see also "Experimental Procedures" for details). Samples were incubated at 37 °C with no addition (−) or with Endo H (+), resolved by 10% SDS-PAGE, and subjected to autoradiography.

Concomitant with changes in cholesterol content and Golgi morphology, protein transport through was the Golgi apparatus was also inhibited. As shown in Fig. 1*B*, loading of cells with cholesterol resulted in a strong inhibition of transport of GFP-tagged VSV-G protein (a secretory protein), as illustrated by its accumulation at the Golgi complex (Fig. 1*B*, *middle panel*) and sensitivity to Endo H (Fig. 1*C*). Under normal conditions, this protein accumulates at the plasma membrane (Fig. 1*B*, *left panel*) and is processed normally in the Golgi apparatus as shown by its resistance to Endo H (Fig. 1*C*). We could not observe inhibition intra-Golgi protein transport by extraction of cholesterol using MβCD alone. At high concentrations of MβCD, it becomes toxic to the cells resulting in cell death (data not shown). To determine the effect of cholesterol extraction on intra-Golgi protein transport, cells were pretreated with for 1 h with lovastatin, an inhibitor of cholesterol synthesis (42). After treatment of the cells with lovastatin and MβCD, protein transport through the Golgi complex was inhibited (Fig. 1, *B* (*right panel*) and *C*). Upon prolonged incubation with Lovastatin (24 h), addition of MβCD was not required to inhibit protein transport at the Golgi complex, further indicating the specificity of cholesterol extraction with MβCD (data not shown).

To study an effect of cholesterol on transport processes related to the Golgi complex in more detail, we made use of a well characterized cell-free system that reconstitutes intra-Golgi protein transport (31). As shown in Fig. 2*A*, addition of increasing amounts of MβCD·Chol complexes to the transport reaction causes a strong inhibition of intra-Golgi protein transport, with half-maximal inhibition at 0.17 mM MβCD·Chol.

To exclude the possibility that the inhibition of the assay signal by MβCD·Chol resulted from decreased glycosylation activity of GlcNAc transferase rather than from inhibition of transport, we used a glycosylation assay that determines GlcNAc activity under conditions that block fusion (32). MβCD·Chol, however, did not inhibit the glycosylation of VSV-G protein under these assay conditions (Fig. 2*B*), indicating that MβCD·Chol specifically inhibits the transport process. Another criterion for specificity of inhibition is the existence of transport intermediates, resistant to the inhibitor. In the experiment described in Fig. 2*C*, the transport reaction was either terminated by placing the reaction on ice, or at these time points the inhibitor MβCD·Chol was added and the reaction was allowed to proceed for 2 h (see, *e.g.* Refs. 32, 43–45 for similar kinetic determinations). Control samples treated with buffer only were incubated likewise at 30 °C until the end of the 2-h incubation period and served to represent the maximal transport signal. When MβCD·Chol was added 20–30 min after initiation of the reaction, a significant portion of the transport reaction became resistant to MβCD·Chol. Furthermore, 80 min (60–80 min in different experiments) after the beginning of the reaction, the full assay signal was obtained. Taken together, these data indicate that MβCD·Chol acts within the time frame of the transport process and that MβCD·Chol inhibits the transport reaction in a specific manner.

Cholesterol Distribution in Golgi Membranes—The loading of Golgi membranes with cholesterol by the MβCD·CD complex was quantitated by nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS) as described by Sandhoff *et al.* (35). As shown in Fig. 3, isolated CHO Golgi membranes (0.6 μg of

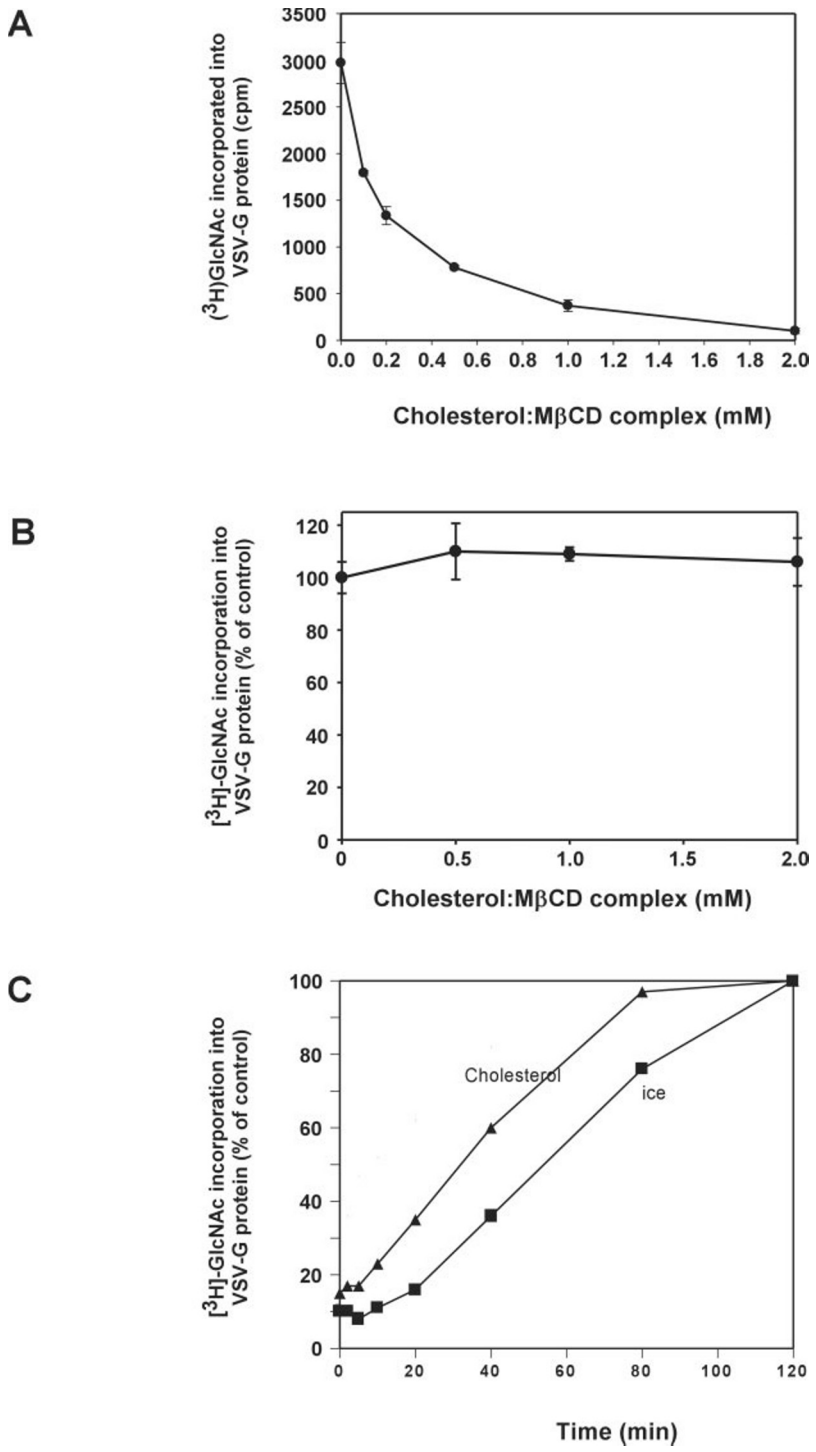

FIG. 2. **Cholesterol inhibits the intra-Golgi protein transport.** *A*, increasing concentrations of methyl-β-cyclodextrin·Chol (12:1, mol:mol) were added to a standard intra-Golgi transport assay, and the incorporation of [^{3}H]GlcNAc into VSV-G protein was measured as described. *B*, methyl-β-cyclodextrin·Chol (4:1, mol:mol) does not inhibit the glycosylation reaction. In the glycosylation assay, the activity of *N*-acetylglucosamine transferase I in Golgi membranes from VSV infected wt-CHO cells was measured as described under "Experimental Procedures" after treatment of the membranes with the indicated amounts of methyl-β-cyclodextrin·Chol complex. *C*, kinetics of pre-loaded methyl-β-cyclodextrin (MβCD·chol) mediated inhibition of intra-Golgi transport. At the indicated time points, MβCD·Chol (*triangles*) was added to the transport assay. The incubation of all samples was then continued for a total time of 2 h. The progression of transport (in the absence of inhibitors) was measured by transferring samples to ice at the indicated time points (*squares*). The incorporation of [^{3}H]GlcNAc was measured as described. The concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration.

protein) contain 320 pmol of cholesterol, in agreement with previously reported values (35). Pre-loaded MβCD dramatically changes the cholesterol content of Golgi membranes. As shown in Fig. 3, with increasing concentrations of pre-loaded MβCD, we found an increased association of cholesterol with Golgi membranes, up to a 4.5-fold increase (at 2 mM MβCD) over the endogenous pool of cholesterol.

Cholesterol is a major lipid in CHO Golgi membranes (0.57 mol of cholesterol/mol of phospholipid) (36) and has been implicated in the formation of microdomains (13–16). Golgi-derived microdomains can be isolated as detergent-resistant membranes, enriched in sphingolipids and cholesterol (30). To determine the amount of cholesterol that partitions in microdomains, Triton X-100 was added to solubilize Golgi membranes. After centrifugation, a detergent-soluble (soluble) and detergent-insoluble (DRM) fraction was obtained and their cholesterol contents quantified. Under these conditions, 62 ± 7% of the cholesterol partitions in DRMs (Fig. 3). This partitioning changes significantly in the presence of exogenously incorporated cholesterol. At 2 mM MβCD·Chol, 89% of the cholesterol is present in the detergent-insoluble phase (resulting in a 5.4-fold increase of this pool). 11% of the cholesterol is present in the detergent-soluble phase (resulting in a 2.5-fold increase of this pool). These results show that most of the exogenous cholesterol is incorporated in the detergent-insoluble phase.

The capacity of Golgi membranes to absorb severalfold its endogenous cholesterol content suggests that under these conditions the general membrane fluidity might also be affected.

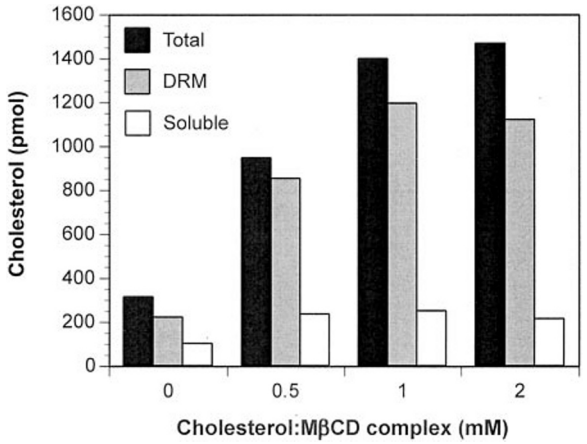

FIG. 3. **Incorporation of cholesterol in Golgi membranes affects the solubility of membranous cholesterol in Triton X-100.** Golgi membranes were incubated with increasing concentrations of MβCD·Chol for 2 h at 30 °C (the concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration). After centrifugation, the membrane pellet was resuspended in PEN buffer, and detergent-soluble and -insoluble phases were prepared as described under "Experimental Procedures." Total cholesterol (*Total*, *black bars*), cholesterol in the detergent-insoluble phase (*DRM*, *gray bars*), and detergent-soluble cholesterol (*Soluble*, *white bars*) were determined by ESI-MS/MS after sulfatation, as described under "Experimental Procedures." Values have been corrected for precipitation of cholesterol in the absence of membranes (under all conditions less than 5% of the data shown).

We therefore determined the partitioning of other known microdomain-enriched lipids such as sphingomyelin (SM) and glucosylceramide (GlcCer) and of a bulk lipid (phosphatidylcholine (PC)) upon addition of exogenous cholesterol. In Golgi membranes, 85% of SM and GlcCer were present in DRMs. In contrast, most of the PC was detergent-soluble and only 28% partitioned to the detergent-insoluble phase (Table I). When Golgi membranes were loaded with cholesterol prior to detergent solubilization, the detergent insolubility of SM and GlcCer did not change significantly. Surprisingly, the pool of PC in the detergent-insoluble fraction was increased 1.5-fold to 42%. Because PC is a major membrane lipid (representing 37% of all phospholipids in Golgi membranes (36)), these data indicate a significant increase of total Golgi surface area of microdomains.

MβCD has also been used to selectively and rapidly extract cholesterol from membranes (46–48). We determined the efficiency of cholesterol extraction by MβCD from Golgi membranes by ESI-MS/MS (35). As shown in Fig. 4*A*, increasing amounts of cholesterol were extracted from isolated Golgi membranes with increasing concentrations of MβCD. The extraction was specific for MβCD, because αCD did not extract cholesterol from the membranes, in accordance with the much higher affinity of MβCD for cholesterol as compared with αCD (46). It was determined whether under these conditions cholesterol is extracted from the fluid- or liquid-ordered phase in the membrane. To this end, increasing amounts of cholesterol were extracted from Golgi membranes with MβCD, and subsequently the partitioning of the remaining membrane-bound cholesterol between the detergent-soluble and detergent-insoluble membranes was measured. As shown in Fig. 4*B*, both pools of cholesterol are similarly affected by MβCD. Because the detergent-insoluble pool represents the larger pool of cholesterol (62%, see above), most of the extracted cholesterol is derived from this pool. Comparison of Fig. 4*A* and Fig. 4*B* shows that at low concentrations of MβCD (*e.g.* 1 mM MβCD), the efficiency of cholesterol extraction from Golgi membranes varied to a significant extent (between 45% (Fig. 4*A*) and 10% (Fig. 4*B*) at 1 mM MβCD). For unknown reasons, these variations were observed between different batches of isolated Golgi membranes (data not shown).

TABLE I
Partitioning of Golgi membrane lipids between detergent soluble phase and DRMs

Golgi membranes (18 μg) were incubated in the absence (*rows 1* and *3*) or presence (*rows 2* and *4, + Chol*) of 2 mM MβCD·Chol (9.5:2.2) for 60 min at 30 °C. Under these conditions, the cholesterol pool of Golgi membranes is increased 4.5-fold (Fig. 3). The membranes were centrifuged, and the pellet was resuspended in ice-cold PEN buffer in the absence (*rows 1* and *2,* Golgi) or presence of 1% Triton X-100 (*rows 3* and *4, Insol. complex*) and incubated for 30 min on ice. Subsequently the incubations were centrifuged for 1 h at 100,000 × *g,* and the pellets were analysed for PC, SM, and GlcCER by ESI-MS/MS as described under "Experimental Procedures." The concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration.

	PC	SM	GlcCer
		nmol / μg	
Golgi	313 ± 27	170 ± 16	4.7 ± 0.5
Golgi + Chol	380 ± 45	173 ± 25	5.7 ± 0.8
Insol. complex	88 ± 16	144 ± 21	4.0 ± 0.2
Insol. complex + Chol	158 ± 36	145 ± 24	4.3 ± 0.1

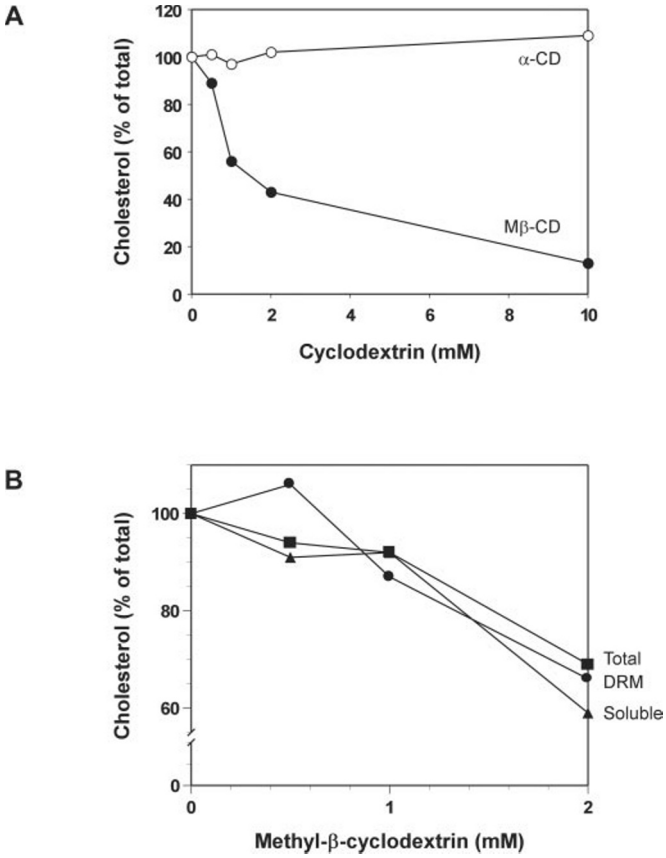

FIG. 4. **Extraction of cholesterol from Golgi membranes by cyclodextrins.** *A*, Golgi membranes were incubated with increasing amounts of methyl-β-cyclodextrin (*closed circles*) or α-cyclodextrin (*open circles*) for 2 h at 30 °C. After 100,000 × *g* centrifugation, the membranes were resuspended in 1,4-dioxane. The pellets were resuspended in 1,4-dioxane, and, after sulfatation, the cholesterol content was determined by ESI-MS/MS. *B*, Golgi membranes were incubated with increasing concentrations of methyl-β-cyclodextrin for 2 h at 30 °C. After centrifugation, the membrane pellet was resuspended in PEN buffer, and detergent-soluble and -insoluble phases were prepared as described under "Experimental Procedures." For cholesterol determination, the membrane pellet (*Total*, *squares*), the detergent-insoluble material (*DRM*, *circles*), and the lyophilized material of the supernatant (*Soluble*, *triangles*) were resuspended in 1,4-dioxane, and after sulfatation the cholesterol content was determined by ESI-MS/MS.

Removal of Cholesterol from Golgi Membranes Blocks Late Stages of Intra-Golgi Transport—Because MβCD and pre-

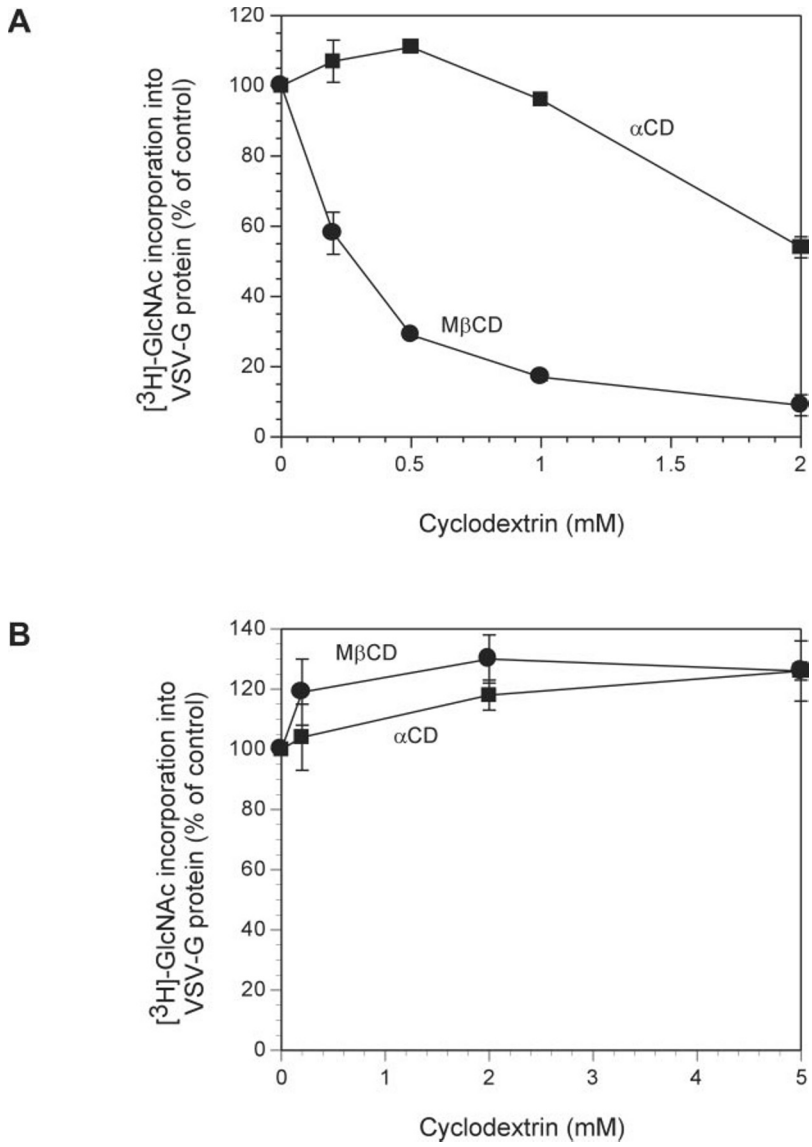

FIG. 5. **Methyl-β-cyclodextrin inhibits intra-Golgi protein transport.** *A*, increasing concentrations of methyl-β-cyclodextrin (*circles*) and α-cyclodextrin (*squares*) were added to a standard intra-Golgi transport assay, and the incorporation of [^{3}H]GlcNAc into VSV-G protein was measured as described. *B*, methyl-β-cyclodextrin does not inhibit the glycosylation reaction. In the glycosylation assay, the activity of *N*-acetylglucosamine transferase I in Golgi membranes from VSV-infected wt-CHO cells was measured as described under "Experimental Procedures" after treatment of the membranes with the indicated amounts of methyl-β-cyclodextrin (*circles*) and α-cyclodextrin (*squares*).

loaded MβCD have opposite effects on the cholesterol content of membranes and have differential effects on the phase partitioning of cholesterol, we tested the effect of MβCD on the cell-free intra-Golgi transport assay. As shown in Fig. 5, MβCD also efficiently inhibited the transport assay with half-maximal inhibition at 0.3 mM and 80–90% inhibition at 1–2 mM MβCD, respectively. The inhibition was specific for methyl-β-cyclodextrin, because α-cyclodextrin inhibited the transport assay only at much higher concentrations (Fig. 5*A*, IC_{50} = 2.2 mM). With this batch of Golgi membranes, 10 ± 6% cholesterol was extracted from the membranes at 1 mM MβCD (data not shown). MβCD did not artificially inhibit the glycosylation of VSV-G protein under the assay conditions (Fig. 5*B*). Other cholesterol-binding agents such as filipin also affected the transport assay. At a concentration of 40 μg/ml, filipin III inhibited transport by 90% (data not shown).

The transport reaction becomes fully resistant to the inhibition by MβCD when added 60 min after onset of the incubation (Fig. 6*A*). Resistance to MβCD inhibition occurred after the GTPγS inhibition, which acts on the budding step, indicating that MβCD inhibits a later stage in the transport process, possibly the fusion process. This is supported by experiments with the cell-free intra-Golgi transport assay is in the presence of brefeldin A. Under these conditions, an assay signal is obtained in the absence of COPI vesicles, and therefore monitors predominantly the docking and fusion processes. The fusion machinery operating under these conditions is the same as the machinery involved in the fusion of COPI-coated vesicles (49, 50). As shown in Fig. 6*B*, MβCD (and MβCD·Chol, data not shown) inhibits the cell-free assay with the same efficiency in the absence and presence of brefeldin A, suggesting that MβCD and MβCD·Chol both act at the same transport step, *i.e.* the fusion machinery of intra-Golgi transport. In accordance with this, we find that MβCD has no significant effect on vesicle formation in the cell-free intra-Golgi transport system as determined by the accumulation of COPI-coated vesicles in the presence of GTPγS (data not shown).

If MβCD inhibits the transport assay by extraction of cholesterol from membranes, then preincubation of the membranes with MβCD and subsequent incubation of these membranes in the transport reaction in the absence of MβCD should also inhibit the transport assay. As shown in Fig. 7*A*, preincubation of the membranes with MβCD inhibited the transport assay with the same efficiency. In contrast, preincubation of cytosol did not affect the transport reaction (Fig. 7*B*). To test directly whether MβCD acts by removing cholesterol from the Golgi membranes, we performed similar two-stage assays in which the Golgi membranes were preincubated under various conditions. When Golgi membranes were preincubated with either MβCD·Chol or MβCD, the transport reaction was effi-

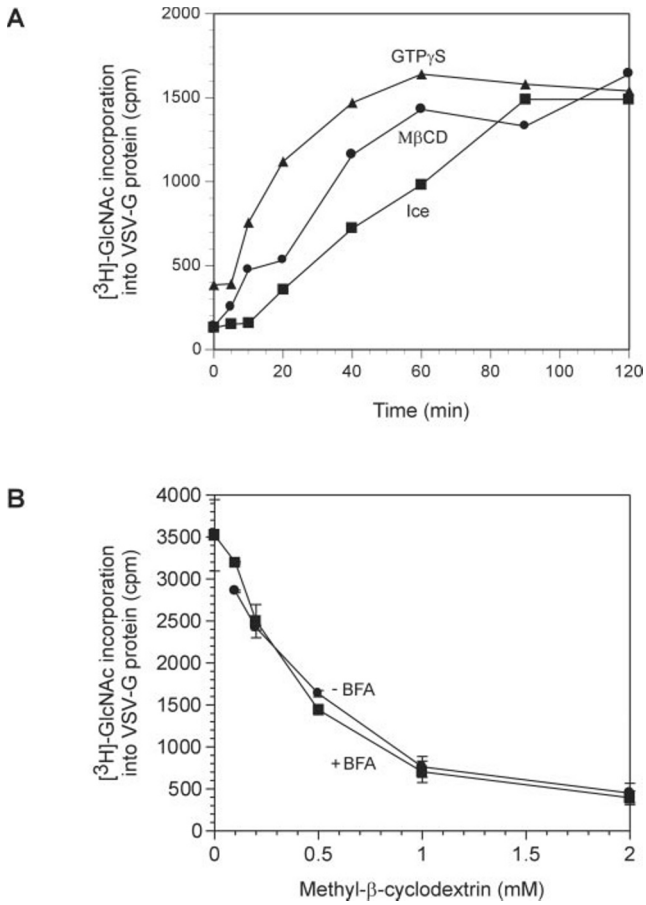

FIG. 6. **Methyl-β-cyclodextrin inhibits a step in the fusion reaction in the intra-Golgi transport assay.** *A*, comparison of the kinetics of inhibition mediated by methyl-β-cyclodextrin and by GTPγS. At the indicated time points, GTPγS (50 μM final concentration) (*triangles*) or methyl-β-cyclodextrin (2 mM final concentration) (*circles*) was added to the transport assay. The incubation of all samples was then continued for a total time of 2 h. The progression of transport (in the absence of inhibitors) was measured by transferring samples to ice at the indicated time points (*squares*). The incorporation of [^{3}H]GlcNAc was measured as described. *B*, increasing concentrations of methyl-β-cyclodextrin were added to the cell-free system in the absence (*circles*) or presence (*squares*) of brefeldin A (150 μM). After incubation for 2 h at 30 °C, the incorporation of [^{3}H]GlcNAc into VSV-G protein was measured as described.

ciently inhibited (Fig. 7*C*). However, addition of both MβCD·cholesterol and MβCD had only a partial effect on transport (Fig. 7*C*). These results clearly indicate that MβCD affects intra-Golgi transport by removing cholesterol from the Golgi membranes, because the inhibition of MβCD can be partially overcome by cholesterol replenishment of the membrane with MβCD·Chol.

Cholesterol Balance in the Golgi Membrane Regulates the Phase Partitioning of Heterotrimeric G Proteins within Membranes—Above, we have presented evidence that modulating the level of cholesterol in Golgi membranes affects the fusion reaction. Recently, SNARE proteins were shown to be concentrated in lipid rafts (51–53). Here we show for the first time that two Golgi SNARE proteins, GOS28 (54) and Syntaxin 5 (55), also partially localize to the Triton X-100-insoluble phase after detergent solubilization of isolated Golgi membranes (Fig. 8). The partitioning between the two different detergent phases of these two SNARE proteins was investigated after the relatively small changes in the cholesterol content of the membrane that caused inhibition of intra-Golgi protein transport. Neither cholesterol depletion by MβCD nor cholesterol binding by MβCD·Chol complexes affected their distribution between the detergent-insoluble and detergent-soluble phases. The partitioning of a non-raft transmembrane protein of the Golgi complex, p23, was also not affected by these treatments and remained in the detergent-soluble fraction. Typical Golgi raft proteins such as Flotillin-1 and GAPR-1 (30, 56) localized predominantly to the detergent-insoluble fraction and were also not affected by the relatively small cholesterol variations in the membrane (note: microdomain partitioning of these proteins is, however, affected at higher cyclodextrin concentrations, see *e.g.* Ref. 30). However, the phase partitioning of heterotrimeric G proteins did change upon cholesterol addition or depletion. Upon cholesterol depletion both the $G\alpha_{i3}$ and the Gβ subunits favored the detergent-soluble phase (Fig. 8). In contrast, both G protein subunits relocalized predominantly to the detergent-insoluble phase upon cholesterol addition. Because localization of proteins to microdomains can affect their activity, these data indicate that alterations in the cholesterol content of membranes might affect the function of heterotrimeric G proteins. These data support previous reports on the involvement of heterotrimeric G proteins in the fusion reaction of COPI vesicles with Golgi membranes (45).

DISCUSSION

Maintenance of a Cholesterol Balance—Here we have shown that cholesterol levels in Golgi membranes play a crucial role in intra-Golgi protein transport. Because both cholesterol extraction from Golgi membranes and cholesterol insertion into Golgi membranes inhibits the transport assay, we suggest that a critical cholesterol concentration needs to be maintained to allow protein transport to occur. It is not known how cholesterol is transported to and from the Golgi complex, nor is it known how maintenance of a balanced concentration is regulated. There is convincing evidence that differences between cholesterol contents of organelles are maintained dynamically, because cholesterol can be rapidly transferred between membranes (4). Vesicular transport processes do not seem to play a major role in intracellular cholesterol transport (57–59). This is in agreement with the recent observation that cholesterol segregates from COPI-coated vesicles during budding from Golgi membranes (36). Current candidates for mediating intracellular cholesterol transport include caveolin (26), possibly in a complex with other proteins (60).

Several other intracellular transport pathways are affected by increased or decreased cholesterol levels. These pathways include clathrin-dependent endocytosis, caveolae endocytosis, biogenesis of synaptic-like microvesicles, plasma membrane to Golgi apparatus, *trans*-Golgi network to apical surface, endosome to Golgi transport, recycling endosome-mediated transport, and yeast vacuole fusion (reviewed in Refs. 61–63). Most of these transport systems are inhibited by modulation of the cholesterol content of the membranes. A remarkable exception is the stimulation of vacuolar fusion by addition of ergosterol, but these membranes have a low ergosterol to phospholipid ratio. Finally, ER to Golgi and the *trans*-Golgi network to basolateral plasma membrane transport pathways are not affected by changes in the cholesterol content of membranes (20, 64, 65). It is not clear why cholesterol might have such a differential effect on various transport events, but there seems to be a tendency that membranes with a high cholesterol content are more sensitive to changes in cholesterol concentrations. This again suggests that the cholesterol content of various membranes in the cells must be carefully balanced to allow intracellular transport to occur.

Inhibition of the Fusion Machinery—Extraction from membranes of cholesterol with MβCD results in a specific inhibition of intra-Golgi transport. Kinetic analysis of the inhibition and the experiments with brefeldin A suggest that MβCD inhibits

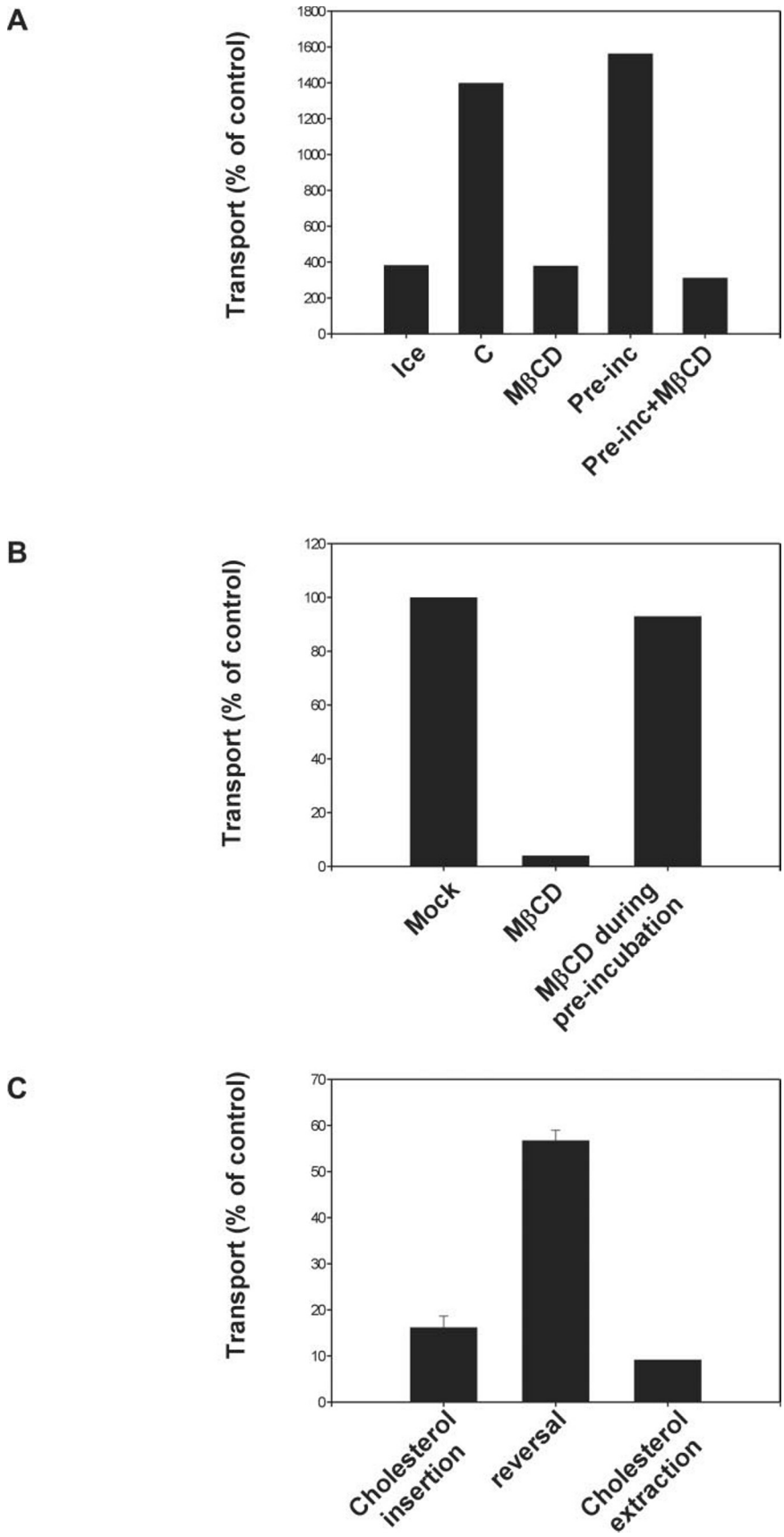

FIG. 7. **MβCD inhibits intra-Golgi protein transport by extraction of cholesterol.** *A*, Golgi membranes (2.4 μg) were preincubated (*lanes 4* and *5*) in the presence of buffer (*lane 4*) or 3 mM MβCD for 10 min at RT. The membranes were then isolated by centrifugation, washed, and tested for their transport activity in the presence of fresh cytosol. As a control, Golgi membranes were not preincubated but added directly to the transport reaction and incubated at 0 °C (*lane 1*), under standard conditions (*lane 2*), or as *lane 2*, but in the presence of 2 mM MβCD. *B*, 6.3 mg/ml bovine brain cytosol in PEN buffer was preincubated with buffer (*lane 1*) or 1 mM MβCD (*lane 3*) for 60 min at 30 °C. After removal of MβCD by gel filtration, the pre-treated cytosol was added to the transport reaction under standard conditions. As a control for the efficiency of inhibition, MβCD was added to a standard transport reaction without preincubation (*lane 2*). *C*, Golgi membranes were preincubated for 20 min at 30 °C with MβCD·Chol complex (ratio 12:1, mol:mol) (*lane 1*), MβCD·Chol complex (ratio 48:1, mol:mol) (*lane 2*) or 3 mM MβCD. Preincubation with buffer served as a control (assay signal of 100%). After incubation the membranes were isolated by centrifugation, washed, and tested for their transport activity in the presence of fresh cytosol. The concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration.

the fusion machinery. Various mechanisms for the observed inhibition can be postulated: (i) Cholesterol is required for the catalytic activity of proteins active in the fusion machinery. So far, however, this has not been reported for any of the fusion proteins involved, including NSF, SNAPs, SNAREs and Rab proteins (for reviews see Refs. 66 and 67). (ii) Cholesterol affects the fusion machinery by affecting membrane fluidity. A general effect of cholesterol on membrane fluidity seems un-

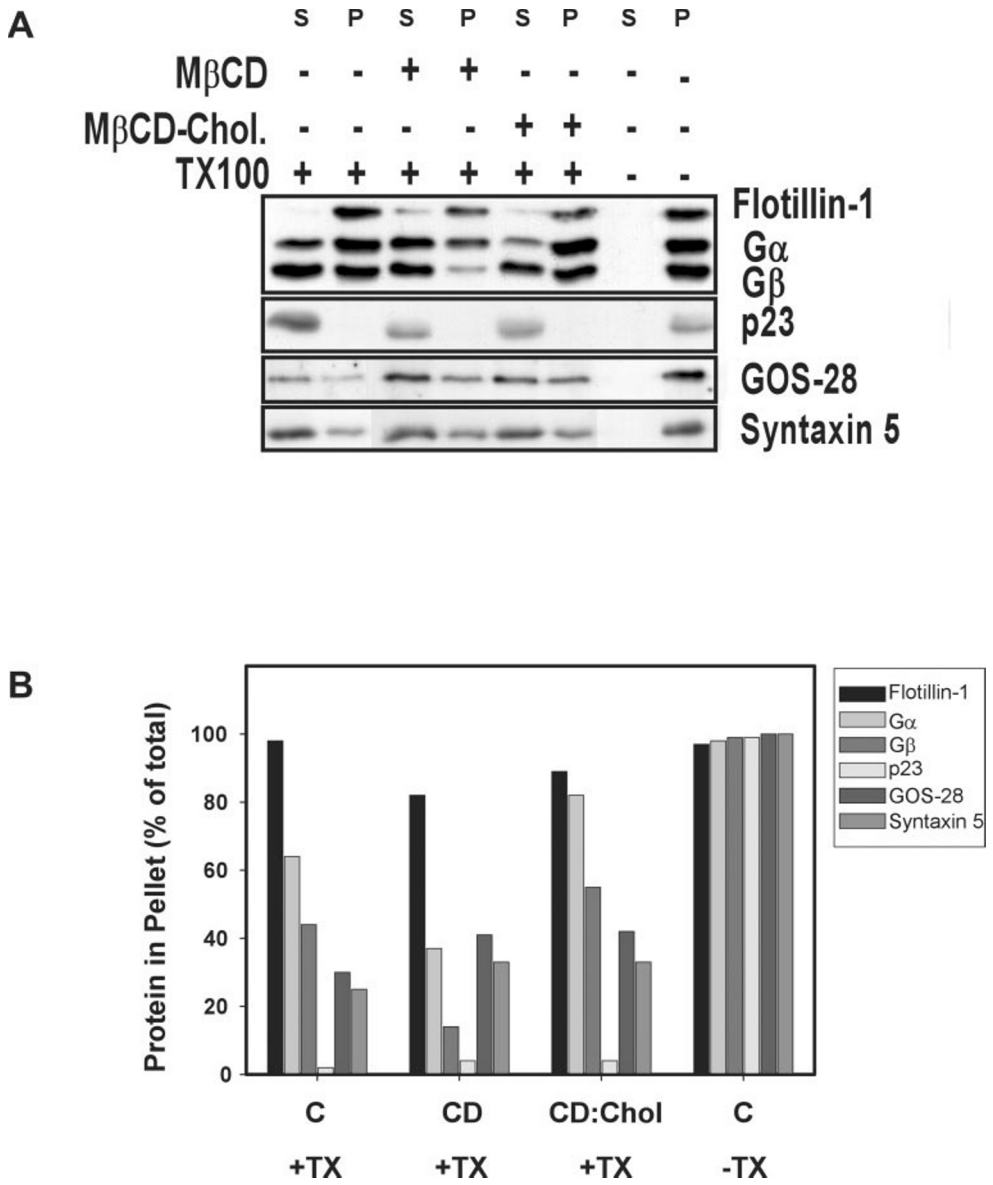

FIG. 8. **Effect of cholesterol on phase-partitioning of proteins.** *A*, CHO Golgi membranes (60 μg) were incubated in the absence (*lanes 1*, *2*, *7*, and *8*) or presence of 2 mM MβCD (*lanes 3* and *4*) or 2 mM MβCD·Chol (4:1) for 2 h at 30 °C. After incubation, the membranes were pelleted and resuspended in PEN-buffer (*lanes 7* and *8*) or PEN buffer containing 1% TX-100 (*lanes 1–6*). After incubation for 30 min on ice, the incubations were centrifuged for 1 h at 100,000 × *g*, and the pellets (*P*, *lanes 2*, *4*, *6*, and *8*) and supernatants (*S*, *lanes 1*, *3*, *5*, and *7*) were analyzed for the presence of the indicated proteins. Proteins from the supernatant were precipitated prior to gel loading by precipitation in chloroform/methanol. The concentration mentioned for MβCD·cholesterol complexes is based on the MβCD concentration. *B*, quantitation of the signal intensity (*panel A*) of the proteins partitioning into the pellet. Signals were scanned and quantified using Gel-Pro Analyzer (Media cybernetics). Data are expressed as percent signal in the pellet, relative to the total signal in pellet and supernatant.

likely, because depletion of cholesterol increases the membrane fluidity, and this should, if anything, facilitate membrane deformations that occur during the fusion reaction (13). In addition, reconstitution of SNARE-mediated fusion of liposomes functions in the absence of cholesterol, and thus, removal of cholesterol should not affect the SNARE-mediated fusion step (68). Finally, such a general mechanism should affect other membrane deformations as well, such as budding of transport vesicles. The formation of COPI-coated vesicles in the cell-free system (data not shown) and a reconstituted assay system (69) does, however, not dependent on cholesterol. This is in contrast to the formation of clathrin-coated vesicles along the endocytic pathway and the formation of synaptic vesicles, which are affected by MβCD treatment (70, 71). In the latter case, a specific cholesterol-binding protein has been implicated in the budding process. (iii) In the presence of other lipids such as sphingomyelin, cholesterol is involved in stabilization of microdomains. The involvement of microdomains in the regulation of intra-Golgi protein transport will be discussed below.

Microdomains and Intra-Golgi Protein Transport—There are several indications for the existence of microdomains at the Golgi complex. Originally, microdomains were postulated to exist at the *trans*-Golgi network, to explain the observed sorting of sphingolipids in polarized cells to the apical surface (17, 18). Subsequently, it was found that glycosylphosphatidylinositol-anchored proteins become associated with DRMs during Golgi passage (72). Microdomains have also been suggested to occur as a result of the cholesterol and sphingomyelin gradient along the secretory pathway to explain protein sorting at the Golgi complex (3). Recently, it was found that cholesterol and sphingomyelin are segregated from Golgi-derived COPI-coated vesicles, implicating a segregation process that might involve microdomains within the Golgi complex (36). By detergent extraction, Golgi-derived detergent-soluble complexes (GIC mi-

crodomains) could be identified and characterized in isolated Golgi membranes. These microdomains were shown to be present in Brefeldin A-sensitive, *i.e.* early Golgi compartments (30, 56), which coincide with Golgi cisternae that produce COPI-coated vesicles. Thus, microdomains could potentially be involved in the regulation of intra-Golgi protein transport.

In agreement with previous data (30) we find that, after solubilization of Golgi membranes in Triton X-100, one pool of cholesterol is detergent-soluble (38%), whereas the other pool is detergent-insoluble (62%). This indicates that, in the Golgi membrane, a significant part of the cholesterol fraction is present in microdomains. Interestingly, only a small reduction (10%, this amount may vary between 10 and 45% depending in the isolated Golgi membranes) of the cholesterol content of Golgi membrane (with 1 mM MβCD) resulted in 80–85% inhibition of *in vitro* protein transport. Thus, minor changes in the cholesterol concentration have a dramatic effect on the function of the cell-free system. This could be explained by an effect of cholesterol on microdomain partitioning of membrane proteins. Several models have been postulated that describe the partitioning of membrane constituents to microdomains and the dynamic behavior of microdomains and its constituents (13). Some of these models have in common that very small local changes in lipid or protein composition might be able to induce large changes in phase separation and thus change the dynamic behavior of microdomains constituents. We tested several microdomain components of the Golgi apparatus for altered microdomain partitioning under conditions that inhibit the transport assay by cholesterol addition or removal. We found that most microdomain proteins are not affected by this treatment suggesting a high microdomain affinity, likely stabilized by protein-protein interactions (30). In contrast, heterotrimeric G proteins are affected by these treatments, suggesting that G proteins only have a relatively week affinity for microdomains. The significance of the 20–30% change in microdomain partitioning of G protein subunits upon cholesterol loading or removal remains to be established. Because G proteins are signaling molecules, the effect of this redistribution between microdomains and the fluid membrane may be enhanced during subsequent signaling cascades. The observed sensitivity to relative small alterations in cholesterol content of Golgi membranes is in agreement with previous work, in which we showed that, upon removal of cholesterol, G protein subunits are not associated with a non-raft protein complex containing several raft proteins (30). According to the lipid shell hypothesis, the affinity of individual raft proteins for raft lipids is also an important determinant that could regulate raft formation (73). According to this hypothesis, a relatively low affinity of G proteins for raft lipids could also explain the observed effects by preferential extraction of raft lipids from the shell surrounding heterotrimeric G proteins, preventing its association with lipid rafts.

G Proteins in the Regulation of Golgi Structure and Function—Our results implicate a role for heterotrimeric G proteins in the fusion reaction of intra-Golgi protein transport. This is in agreement with previous data showing that activation of heterotrimeric G proteins inhibits the fusion of COPI-coated vesicles with Golgi cisternae (45). Because the morphology of the Golgi is changed upon cholesterol treatment of the cells, these data implicate a function for heterotrimeric G proteins in the maintenance of the Golgi structure as well. This is supported by previous findings showing that possibly free G$\beta\gamma$ subunits are involved in maintenance of the Golgi structure (74, 75). Although the mechanism of G protein-mediated signaling cascades at the Golgi complex is not well understood, our data now suggest that these cascades could be regulated by lipid-enriched microdomains, similar to many other signaling events at the plasma membrane (16).

In summary, changes in the cholesterol concentration cause a shift in the phase partitioning of raft proteins and provide a means to affect the activity of these proteins. Thus, the cholesterol level in the Golgi membranes needs to be carefully regulated to balance the equilibrium between the fluid membrane and microdomains, allowing protein transport to occur. It is not known how cholesterol is transported to the Golgi complex, but identification of novel transport pathways will also provide insight in the regulation of a carefully maintained cholesterol balance at the Golgi complex.

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