ER/Golgi Intermediates Acquire Golgi Enzymes by Brefeldin A–Sensitive Retrograde Transport In Vitro

Chung-Chih Lin,* Harold D. Love,* Jennifer N. Gushue,‡ John J.M. Bergeron,‡ and Joachim Ostermann*

*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146; and ‡Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, H3A 2B2 Canada

Abstract. Secretory proteins exit the ER in transport vesicles that fuse to form vesicular tubular clusters (VTCs) which move along microtubule tracks to the Golgi apparatus. Using the well-characterized in vitro approach to study the properties of Golgi membranes, we determined whether the Golgi enzyme NAGT I is transported to ER/Golgi intermediates. Secretory cargo was arrested at distinct steps of the secretory pathway of a glycosylation mutant cell line, and in vitro complementation of the glycosylation defect was determined. Complementation yield increased after ER exit of secretory cargo and was optimal when transport was blocked at an ER/Golgi intermediate step. The rapid drop of the complementation yield as secretory cargo progresses into the stack suggests that Golgi enzymes are preferentially targeted to ER/Golgi intermediates and not to membranes of the Golgi stack. Two mechanisms for in vitro complementation could be distinguished due to their different sensitivities to brefeldin A (BFA). Transport occurred either by direct fusion of preexisting transport intermediates with ER/Golgi intermediates, or it occurred as a BFA-sensitive and most likely COP I–mediated step. Direct fusion of ER/Golgi intermediates with cisternal membranes of the Golgi stack was not observed under these conditions.

Key words: Golgi apparatus • in vitro transport • secretion • transport vesicles • ER

THE intermediate compartment is the compartment through which secretory proteins pass before they reach the Golgi apparatus. It consists of vesicle clusters and tubular networks (or vesicular tubular clusters, VTCs[1]; for review, see Bannykh and Balch, 1997), and it represents a compartment in which long-lived resident proteins of the early secretory pathway are separated from the secretory flow and recycled to the ER (Pelham, 1996). Assembly of the intermediate compartment is usually studied in the context of protein exit from the ER. The goal of this study is to shed light on the next step, transport between the intermediate compartment and the Golgi apparatus.

Important progress in our understanding of protein transport from the ER to the Golgi apparatus was made by visualizing transport in living cells. When a secretory protein is fused to green fluorescent protein, its transport can be directly observed by fluorescence microscopy (Presley et al., 1997; Scales et al., 1997). These studies demonstrated that ER-derived transport vesicles rapidly assemble into larger structures, the previously described VTCs, which form close to ER exit sites. These VTCs appear at the light microscopy level as punctate objects that travel along microtubules towards the Golgi apparatus. Proteins that must be retrieved to the ER are removed from VTCs in a coat protein I (COP I)-dependent process (Letourneur et al., 1994; Lewis and Pelham, 1996). After VTCs have reached the Golgi apparatus, they appear to merge with it. However, visualization of protein transport with green fluorescent protein has done little to elucidate the role of transport vesicles in transport to and through the Golgi apparatus.

Different models have been proposed to explain protein transport through the Golgi apparatus (Farquhar and Palade, 1998). Secretory cargo passes through the Golgi apparatus from cis to trans. Transport in the retrograde direction retrieves Golgi-localized proteins and separates them from the secretory flow. The vesicular transport hypothesis predicts that transport between neighboring compartments occurs by vesicular transport in both directions

(Farquhar, 1985; Orci et al., 1997). The cisternal maturation hypothesis proposes that the cisternae of the Golgi stack are anterograde transport intermediates that are in different stages of maturation (Beams and Kessel, 1968; Becker and Melkonian, 1996; Bonfanti et al., 1999). An important prediction of the cisternal maturation hypothesis is that Golgi cisternae form constantly anew on the cis-side of the organelle, possibly by fusion of ER-derived transport intermediates with Golgi-derived transport vesicles. The retrograde transport of Golgi enzymes between cisternae in the trans-to-cis direction could drive the maturation of cisternae while maintaining a stationary enzyme distribution across the stack. It has been proposed that the competition of different Golgi enzymes for retrograde transport could explain the observed distribution of enzymes in the Golgi stack (Glick et al., 1997).

A less well-developed alternative to these two currently prevailing models is that the cisternae of Golgi apparatus are continuous. Such a continuity would require that the anterograde flow of secretory cargo is coupled to a separate retrograde transport of resident Golgi enzymes. The interactions of Golgi enzymes with their substrates, as well as interactions between the Golgi enzymes themselves, might explain why not all Golgi enzymes are evenly distributed over the Golgi stack (Lippincott-Schwartz et al., 1998). While cisternae often appear to be distinct from each other when observed by electron microscopy, transient connections might rapidly form and break. They could be highly unstable and would be difficult to observe in fixed specimens; nevertheless, there are well-documented examples of continuity between Golgi cisternae (Rambourg et al., 1993; Clermont et al., 1994).

Together with morphology and genetics, in vitro transport assays are used to study intra-Golgi protein transport. Rothman and coworkers characterized an in vitro complementation assay in which Golgi membranes are isolated from wild-type (wt) cells and from vesicular stomatitis virus (VSV)-infected glycosylation-defective mutant cells (Fries and Rothman, 1980). When these membranes are incubated together under the appropriate conditions, VSV-glycoprotein (VSV-G) that is enclosed in mutant cell membranes is glycosylated by Golgi enzymes enclosed in the wt membranes. A morphological analysis of a related assay had provided evidence that this assay might reconstitute the anterograde transport of secretory cargo within the Golgi stack (Braell et al., 1984). It was further suggested that such anterograde transport occurs in COP I–coated vesicles that form on Golgi membranes when incubated in vitro (Orci et al., 1986). Indeed, it was shown that VSV-G could be transferred in this system in a COP I–dependent reaction (Ostermann et al., 1993).

However, the difficulty of interpreting the many complex and sometimes contradictory results from in vitro experiments has resulted in criticism of this approach, validating the assay only as a tool to identify components (Mellman and Simons, 1992). Some of the results are not readily reconciled with the anterograde vesicular transport model that had originally been applied to interpret the experiments. For example, Golgi enzymes are not excluded from COP I vesicles (Ostermann et al., 1993; Sönnichsen et al., 1996; Lanoix et al., 1999), and indeed, transport of Golgi enzymes in the retrograde direction may be suffi-cient to account quantitatively for the glycosylation that is measured in this in vitro assay (Love et al., 1998). The situation is further complicated by the possibility that in vitro complementation assays might measure a combination of several different reactions. Direct fusion of Golgi membranes, transport through continuities of Golgi subcompartments, and vesicular transport in both the anterograde and retrograde direction would all contribute to glycosylation of VSV-G by wt Golgi enzymes in this assay.

That transport occurs even when COP I assembly was inhibited has questioned even the relevance of the COP I pathway in Golgi transport (Orci et al., 1991; Elazar et al., 1994; Taylor et al., 1994; Happe and Weidman, 1998; Dominguez et al., 1999). However, we recently discovered that cellular homogenates contain small membranous structures that are highly active in the complementation assay, and we found these membranes even when cells were homogenized under gentle conditions (Love et al., 1998). They were of much smaller size than the bulk of the Golgi membranes in homogenates. Relative to the amount of Golgi enzyme activity contained in these membranes, they were found to be much more active than routinely prepared Golgi membranes. At that time, we proposed the hypothesis that these active membranes are intermediates in the retrograde transport of Golgi enzymes, possibly generated in a COP I–dependent reaction in cells before homogenization, and possibly identical with the abundant vesicles and small pleiomorphic structures seen in EM reconstitution of the Golgi apparatus (Ladinsky et al., 1999).

Two goals were pursued in the study that is summarized here. First, we assessed critically the target compartment in which the VSV-G protein must reside to become glycosylated with the highest efficiency. We found that glycosylation occurred with the highest yield not when VSV-G resided in the Golgi apparatus or in the ER but when it was arrested in transit between them. Second, we determined what type of transport was reconstituted when transport was studied after removal of preexisting transport vesicles. We found that Golgi enzymes gained access to VSV-G not by direct fusion of ER/Golgi intermediates with each other or the Golgi stack, but by COP I–dependent transport.

Materials and Methods

Cell Growth

CHO wt and 15B cells were grown on tissue culture plates in MEMα supplemented with 10% fetal calf serum plus penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C and split 1 to 10 every 2 d. For large scale production of cells for isolation of membrane fractions or cytosol, cells were grown in spinner flasks until confluent.

VSV Infection

15B cells from a confluent spinner culture were pelleted, resuspended in 50 ml of infection media (serum free media, 1 ml VSV, 0.1 mg/ml actino-mycin D, 25 mM Hepes, pH 7.2), and incubated for 45′ at 37°C in a small spinner flask. 200 ml of media with serum were added, and the infection was continued for an additional 2 h 15 min.

Pulse–Chase Labeling

Cells were pelleted by centrifugation, washed once, and resuspended in 10

ml of methionine-free media per 1 ml of cell pellet. After 5 min at 37°C, 0.1 to 0.5 mCi per ml of ^{35}S-methionine/cysteine were added, and cells were incubated for an additional 5 min at 37°C (pulse). The labeling mixture was then diluted into a spinner flask containing a large volume of methionine-containing media in a water bath set to the desired temperature to be incubated for the indicated times (chase). For an additional chase at 37°C, aliquots were withdrawn and incubated for the indicated times in a water bath at 37°C.

Gradient Fractionation of Cellular Homogenates

Cells were collected, washed once in PBS, and resuspended in 4 ml per ml cell pellet of ST buffer (200 mM sucrose, 10 mM Tris, pH 7.2). This suspension was homogenized in a ball bearing homogenizer. For fractionation by sucrose density gradient centrifugation, homogenate was loaded on a 5-ml linear sucrose gradient (20–50% weight per weight) in KHM (150 mM KCl, 10 mM Hepes, pH 7.2, 2.5 mM MgOAc) buffer. Gradients were centrifuged for 16 h in an SW55 rotor at 50,000 rpm at 4°C. 10 fractions were collected from the top. Velocity sedimentation was done by loading homogenate on a 15 to 35% sucrose/KHM gradient and centrifuging it in an SW55 centrifuge tube for 20 min at 50,000 rpm. 10 fractions were collected from the top.

Preparation of Cisternal wt Golgi Membranes

To prepare Golgi-enriched membranes, homogenate was centrifuged for 5 min at 1,000 g. The supernatant was loaded on a 1-ml cushion of 40% sucrose in 10 mM Tris, pH 7.2, and Golgi membranes were pelleted on this cushion by a 10-min centrifugation at 17,500 g. The bottom 2 ml were collected, mixed in an SW40 tube with 62% sucrose to ~40% final, and overlaid with 4 ml of 35% and 3 ml of 29% sucrose. After 90 min centrifugation at 40,000 rpm, Golgi membranes were harvested at the interface between 29% and 35% sucrose. For small scale preparations of Golgi membranes from cells after pulse–chase incubations, homogenates were mixed with 62% sucrose to 37.5% sucrose final and were overlaid with 2 ml 35% and 1 ml 29% sucrose in an SW55 centrifuge tube. After 90 min centrifugation at 50,000 rpm, Golgi membranes were harvested at the interface between 29% and 35% sucrose. To prepare salt-washed Golgi membranes, Golgi membranes harvested after flotation were diluted with 4 vol of 10 mM Tris, pH 7.2, and KCl was added to 250 mM final concentration. After 20 min incubation on ice, the mixture was loaded on 1 ml 40% and 3 ml 20% sucrose in an SW40 centrifuge tube. Golgi membranes were pelleted through the 20% layer on the 40% cushion by 20 min centrifugation at 15,000 rpm and were harvested at this interface.

Isolation of Membranes from Supernatant of Permeabilized Cells

To prepare fractions enriched in active transport intermediates, cells resuspended in ST were frozen in liquid nitrogen and kept at −80°C until use. Cells were thawed in a water bath at room temperature. Permeabilized cells and other debris were removed by centrifugation at 1,000 g and 17,500 g. In some experiments, vesicles were separated from cytosol by loading 400 μl of the supernatant on 400 μl 20% sucrose and 200 μl 40% sucrose. After 15 min centrifugation at 100,000 rpm in the TLA120.2 rotor, the top 600 μl was discarded and the bottom 400 μl was collected. For large scale preparations, membranes in the supernatant of permeabilized cells were pelleted by centrifugation for 20 min at 75,000 rpm in the TLA100.4 rotor, resuspended in KHM buffer and fractionated by velocity sedimentation on a 30-ml linear 15–35% sucrose/KHM gradient in a SW28 tube (25′ at 28,000 rpm). Vesicle containing fractions were identified by measuring GalT activity and were pooled and layered on top of a 0.75-ml cushion of 50% iodixanol in HM. Vesicles were pelleted on this cushion by 3 h centrifugation at 40,000 rpm in a SW40 rotor. 1.5 ml were collected from the bottom and mixed with 0.5 ml of 50% iodixanol in HM. A step gradient of 2 ml of 25% and 1 ml of 10% iodixanol in KHM was layered on top of this sample, and the gradient was centrifuged for 3 h at 55,000 rpm in a SW55 rotor. Vesicles were harvested at the interface between the 10 and 25% layers.

Electron Microscopy

Membrane fractions were prepared for routine electron microscopy using a filtration apparatus for random sampling exactly as described previously by Dominguez et al. (1999). Membranes associated with magnetic beads were processed identically to the conditions described in Dominguez et al. (1999), except that the material was retained by magnets during throughout the washing and fixation procedures.

Preparation of Cytosol

wt or 15B cells were gently broken by a cycle of freezing and thawing as described above or were homogenized in a ball bearing homogenizer. Cellular debris and membranes were pelleted by subsequent centrifugations at 1,000, 17,500, and 250,000 g. Repeated centrifugations at 250,000 g were done when active vesicles needed to be completely removed from wt cytosol. To remove low molecular mass components in cytosol, it was either passed over a PD10 gel filtration column, or it was dialyzed against 25 mM Tris, pH 7.2, 50 mM KCl using dialysis tubing with a molecular weight cutoff of 12–14 kD. To deplete coatomer from cytosol, 100 μl protein A–Sepharose slurry was incubated with 100 μl CM1A10 ascites fluid. The Sepharose beads were reisolated and washed. Half of the beads were incubated with 150 μl cytosol for 20 min at 4°C. Cytosol was recovered and incubated with the other half of the beads for 60 min at 4°C.

Immunoisolation of Membranes

M450 (for gel electrophoresis and enzyme assays) or M500 (for electron micrographs) magnetic beads were cross-linked with anti-rabbit IgG according to the manufacturer's instructions. Typically, 10 and 40 μl of the bead suspension was used per immunoisolation. Beads were preincubated with affinity-purified p23 cytoplasmic tail antibodies and reisolated with a magnet. Antibody coated magnetic beads were incubated with membranes in immunoisolation buffer (KHM buffer plus 0.2 M sucrose and 0.5 mg/ml milk powder) for ~2 h in the cold room with gentle agitation. Beads were reisolated with a magnet and washed repeatedly in buffer (last wash without milk powder). Beads were either extracted with electrophoresis buffer (for gel electrophoresis and Western blotting) or in NAGT, GalT, or mannosidase assay buffers. Membranes that remained in the supernatant after immunoisolation were pelleted by ultracentrifugation. For EM analysis, samples were frozen for shipment and storage.

Enzyme Assays

Mannosidase activity was measured in 100-μl samples. Each contained 58 μl 0.2 M Na-phosphate, pH 6.0, 20 μl 20 mM 4-methylumbelliferyl-α-D-mannopyranoside in DMSO, 2 μl 10% Triton X-100, and 20 μl sample (or water). After 60 min at 37°C, samples were diluted in 1 ml 0.25 M sodium carbonate. Fluorescence was measured at 448 nm for emission and 364 nm for excitation light. NAGT activity was measured in 100 μl samples. Each contained 5 μl 1 M Tris, pH 6.8, 0.33 μl 3 M KCl, 1 μl 1 M MnCl$_2$, 1 μl 1 M MgCl$_2$, 10 μl 20 mg/ml ovalbumin, 1 μl 10% Triton X-100, 1 μl ^{3}H-UDP-GlcNAc (0.1 mCi/ml), and 20 μl sample (or water). After 2 h at 37°C, 1 ml of ice-cold 1% phosphotungstic acid in 0.5 M HCl was added to each sample. After 10 min on ice, the precipitate was pelleted by a short centrifugation (~5″ in a microcentrifuge). The pellet was resuspended in 1 ml of phosphotungstic acid in 0.5 M HCl, centrifuged again, resuspended in 1 ml ice-cold 95% ethanol, and pelleted again. Pellets were completely dissolved in 200 μl of 1% SDS and 50 μl of 2 M Tris base and mixed with 15 ml scintillation cocktail to determine the amount of ^{3}H bound to protein. GalT activity was measured in 50-μl samples. Each contained 2.5 μl 1 M Tris, pH 6.8, 1 μl 10% Triton X-100, 2 μl 1 M MnCl$_2$, 5 μl 10 mg/ml ovalbumin, 2.5 μl 40 mM ATP, 1 μl ^{3}H-UDP-Gal (0.1 mCi/ml), and 10 μl sample (or water). After 1 h at 37°C, samples were spotted in the middle of 2.5-cm-wide squares drawn on filter paper. Filters were incubated in 10% trichloroacetic acid for 10 min at room temperature, rinsed quickly with water and washed three times for 5 min with water. Filters were dried, each square was cut out, and filter-bound radioactivity was measured by scintillation counting.

In Vitro Complementation Assay

Unless indicated otherwise, 50 μl incubation would include 5 μl wt Golgi membranes, 5 μl 15B Golgi membranes isolated from VSV-infected cells, 15 μl CHO cytosol, 75 mM ATP, 3 mM creatine phosphate, and 12 IU/ml creatine kinase. The final buffer conditions contained 25 mM Hepes, pH 7.2, 2.5 mM MgOAc, 50 mM KCl, and 200 mM sucrose. When salt-washed Golgi membranes were used, 200 ng/ml N-ethylmaleimide sensitive factor was also added. Incubations were done for 1 h at 37°C. At the end of the incubation, Golgi membranes were pelleted by centrifugation (10 min 17,500 g), resuspended in 20 μl 50 mM citrate, pH 5.5, 1 mM DTT, and

0.4% SDS, and denatured for 3 min at 95°C. 20 μl of 50 mM citrate were added, and 1.7 mIU endo H was added when indicated. After at least 1 h incubation at 37°C, an aliquot of the digest was mixed with electrophoresis sample buffer and separated by gel electrophoresis. To observe late Golgi modifications, 4 mM each of UDP-galactose and CMP-sialic acid were added during the initial incubation. When indicated, 0.2 IU/ml of neuraminidase was added together with endo H.

Determination of BFA Sensitivity

To assure that cytosol would not contain any wt Golgi-derived membranes, cytosol prepared from 15B cells was used for most BFA inhibition experiments. A 10 mg/ml solution of BFA (purchased from Calbiochem) in ethanol was prepared and kept at −20°C until use. To add BFA to the transport assay, the BFA stock solution was diluted 1 to 100 in water, and 10 μl of this dilution was included in each incubation mixture.

Gel Electrophoresis, Phosphorimaging, and Western Blotting

Proteins were separated by denaturing protein gel electrophoresis using a Biorad Miniprotean® 2 or 3 gel system. For optimal resolution of the differently glycosylated forms of VSV-G, proteins were separated on a 3.5-ml 10% acrylamide gel (acrylamide to bisacrylamide, 37.5:1) with a 1-ml 5% stacking gel. After 85 min electrophoresis at 200 V, gels were fixed in 40% methanol and 10% acetic acid and dried on filter paper. Visualization of radioactive VSV-G was done by phosphorimaging. Typically, exposures were chosen with a maximum intensity (100% black) between 10^2 and 10^3. The minimum intensity (0% black) was set to 1/100 of the maximum, and intensities in between were linearly assigned gray scale values from 0 to 100%. For detection of p25 by Western blotting, the acrylamide concentration was increased to 14%, and electrophoresis was done for 40 min. Proteins were electrophoretically transferred on PVDF filter, and the filter was incubated with a polyclonal antiserum raised against p25 (Dominguez et al., 1998). Bound antibody was detected by chemiluminescence.

Results

The goals of this study were to determine the cellular compartment in which VSV-G resides when it is glycosylated by in vitro complementation with wt Golgi membranes, and to evaluate how Golgi enzymes and VSV-G in different compartments gained access to each other. Towards this goal, we performed pulse–chase experiments at different temperatures to introduce labeled VSV-G into different compartments of the secretory pathway. Coimmunoisolation was affected with Golgi and ER/Golgi marker proteins to confirm the fidelity of the temperature block. Golgi-enriched membranes isolated from cells containing VSV-G in various secretory compartments were assessed to determine the yield with which labeled VSV-G was glycosylated by in vitro complementation. Finally, experiments were designed to determine whether glycosylation occurred by transport vehicle shuttling between Golgi subcompartments or by direct fusion of Golgi membranes.

Accumulation of Secretory Cargo at 15 and 20°C

In vitro complementation assays to measure intra-Golgi transport are most frequently performed by measuring incorporation of radiolabeled sugar into VSV-G. As we had done previously (Love et al., 1998), we used this assay in a modified form by directly labeling the VSV-G protein in the glycosylation-deficient cells. To accumulate radiolabeled VSV-G in different parts of the secretory pathway, we labeled newly synthesized VSV-G (pulse) and then incubated cells at different temperatures for different times to allow forward transport of VSV-G (chase). When cells
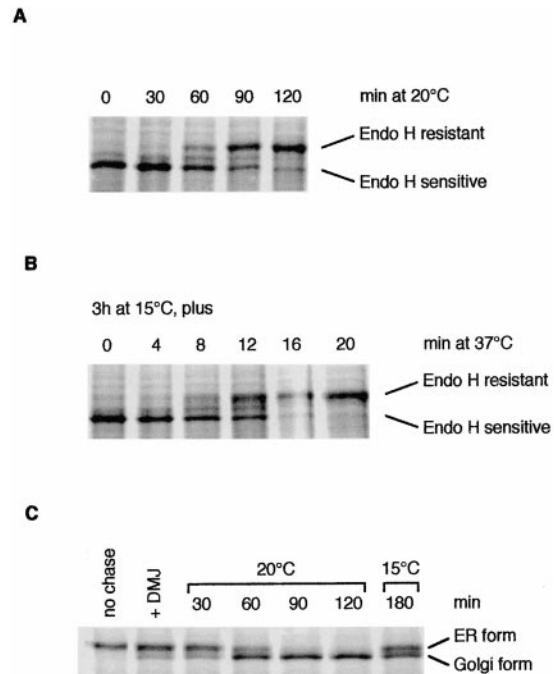

Figure 1. Glycosylation kinetics of VSV-G at 15°C, 20°C, and 37°C. (A) CHO wt cells were infected with VSV and newly synthesized VSV-G was metabolically labeled for 5 min at 37°C. Cells were then incubated at the indicated temperature in the presence of an excess of unlabeled amino acids. At the indicated times, aliquots were withdrawn, and half of each aliquot was digested with endo H. Proteins were separated by gel electrophoresis and visualized by phosphorimaging. The VSV-G band is shown. (B) As in A, except that 15B cells were used instead of wt cells and that the endo H digestion was omitted. When indicated, 10 μM deoxymannojirimycin was added. ER form and Golgi form of VSV-G refer to VSV-G that was not or was processed by Man I, respectively.

are incubated at 15°C, secretory cargo leaves the ER, but instead of being transported to the Golgi apparatus it accumulates in ER/Golgi intermediates that remain in the cellular periphery (Saraste and Kuismanen, 1984). When cells are incubated at 20°C, secretory proteins accumulate in late Golgi compartments (Matlin and Simons, 1983). Golgi processing of VSV-G was detected by measuring its sensitivity to endoglycosidase H (endo H) that distinguishes between the ER and Golgi forms of N-linked glycans. As expected, at 20°C VSV-G was slowly converted from an endo H–sensitive to an endo H–resistant form (Fig. 1 A). When cells were incubated for up to 3 h at 15°C, VSV-G did not become endo H resistant. When these cells were subsequently incubated at 37°C, glycosylation resumed, and after 20 min VSV-G had mostly been converted to the endo H–resistant form (Fig. 1 B). This gives us a lower limit for the time VSV-G resides in Golgi membranes, but it is very likely that VSV-G remains in the stack for longer than these kinetics suggest. VSV-G transport can be more directly followed by fluorescence microscopy, and in such experiments VSV-G can still be found in the Golgi apparatus for as long as 1 h after release of the 15°C block (for example, see Rojo et al., 1997). Further-

more, Golgi enzyme distributions overlap, and even late-acting enzymes can be found in early Golgi compartments (Nilsson et al., 1993; Velasco et al., 1993; Lovelock and Lucocq, 1998).

In all subsequent experiments, we used CHO clone 15B cells so that we could measure the transport of Golgi enzymes to membranes isolated from these cells by in vitro complementation of the glycosylation defect of this cell line (Gottlieb et al., 1975; Fries and Rothman, 1980). Only one Golgi glycosylation step can occur in these cells, the trimming of the high mannose glycan that is present on proteins that leave the ER by the Golgi enzyme Mann I. We found that at 20°C, VSV-G was converted to a form with slightly higher mobility (Fig. 1 C). This conversion was prevented by addition of the Mann I inhibitor deoxymannojirimycin, and therefore we conclude that this increase in mobility is caused by Mann I trimming. At 15°C, Mann I trimming was reduced, but not abolished; indeed approximately half of the total VSV-G was processed.

Sucrose Gradient Fractionation of ER/Golgi Intermediates

We studied the fractionation of VSV-G–containing membranes after the 15°C chase by velocity sedimentation and density gradient centrifugation to confirm that the bulk of VSV-G had exited the ER during the 15°C chase. After pulse labeling of newly synthesized VSV-G, half of the cells were kept on ice, and the other half was incubated in the presence of an excess of unlabeled amino acids for 3 h at 15°C. We prepared homogenates from both and fractionated them by sucrose gradient centrifugation. After equilibrium density gradient centrifugation, we found that approximately half of the radiolabeled VSV-G in membranes of cells that had been incubated at 15°C was redistributed to a lighter membrane fraction (Fig. 2 A, fractions 4–6). When the 15°C chase was omitted, much of the VSV-G protein was found at higher densities in fractions 7–10.

We also fractionated homogenates by velocity sedimentation to determine the sedimentation speed of the 15°C compartment, following the same technique that we had used before to distinguish between large cisternal Golgi membranes and small transport intermediates (Love et al., 1998; Fig. 2 B). We found that after the chase at 15°C, the peak of the VSV-G distribution was found in fractions 6 and 7 of the gradient (or in aggregated material at the bot-

tom of the gradient). In our earlier work, we had found a similar fractionation behavior for Golgi membranes, and we had found that small transport vesicles did not sediment this far during the short centrifugation time and were found in fractions 2 and 3 (Love et al., 1998). When the 15°C chase was omitted, VSV-G was evenly distributed over most gradient fractions, most likely due to the breakup of the ER into variably sized microsomes during homogenization of cells.

In summary, at 15°C VSV-G redistributed into lighter membranes that were larger and less heterogeneous in size, and we conclude that the bulk of VSV-G had exited the ER during the 15°C chase.

Immunoisolation of ER/Golgi Intermediates with p23 Antibodies

Our results (Fig. 1 C), as well as earlier results (Balch et al., 1986), indicated that Mann I processing is only partially inhibited at 15°C, and this suggests that VSV-G had entered the Golgi apparatus. Equally possible is that a fraction of Mann I might be present in ER/Golgi intermediates.

In intact cells, ER/Golgi intermediates are readily distinguishable from Golgi membranes due to their peripheral localization, but they are not readily separable from Golgi membranes in cellular homogenates. A clear separation of the two may well be impossible. However, ER/Golgi intermediates are enriched in proteins of the p24 family (Schimmoller et al., 1995; Stamnes et al., 1995; Belden and Barlowe, 1996; Rojo et al., 1997; Dominguez et al., 1998). We determined whether it was possible to make use of an antibody that recognized the cytoplasmic tail of the p23 protein, one member of this family, to distinguish between membranes of the Golgi stack on the one hand and ER/Golgi intermediates and the cis-Golgi network on the other (Rojo et al., 1997). As p24 proteins could be present in smaller amounts in the Golgi stack (Dominguez et al., 1998), we did not expect that immunoisolation with p23 antibodies would allow us to obtain a true separation of ER/Golgi intermediates and Golgi membranes. However, we did hope to find a depletion of Golgi enzymes from the immunoisolated membranes as Golgi enzymes are most concentrated in the membranes of the stack where the concentration of p24 proteins is the lowest.
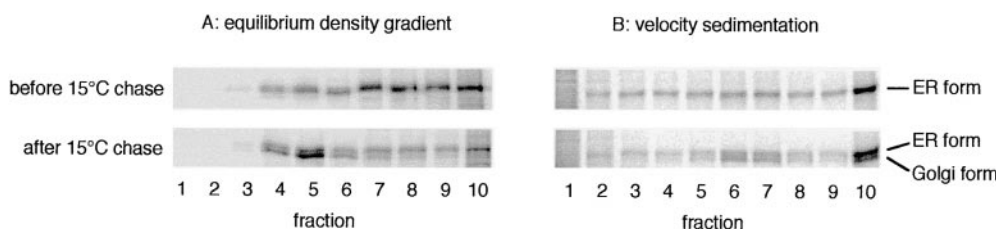
Electron microscopy allowed us to visualize the mem-

Figure 2. Fractionation of pulse-labeled VSV-G in homogenate before and after the 15°C chase. 15B cells were infected with VSV and newly synthesized VSV-G was pulse labeled for 5 min with ^{35}S-methionine. One-half of the cells was transferred on ice and the other half was incubated for 3 h at 15°C in the presence of an excess of unlabeled methionine. Cellular homogenates were prepared from both. (A) An aliquot of the homogenate was fractionated by flotation on a sucrose density gradient (equilibrium density gradient centrifugation). (B) An aliquot of the homogenates was loaded on a 15–35% sucrose gradient and membranes were fractionated by velocity sedimentation. 10 fractions were collected from the top, and aliquots of each fraction were fractionated by gel electrophoresis. VSV-G was visualized by phosphorimaging.
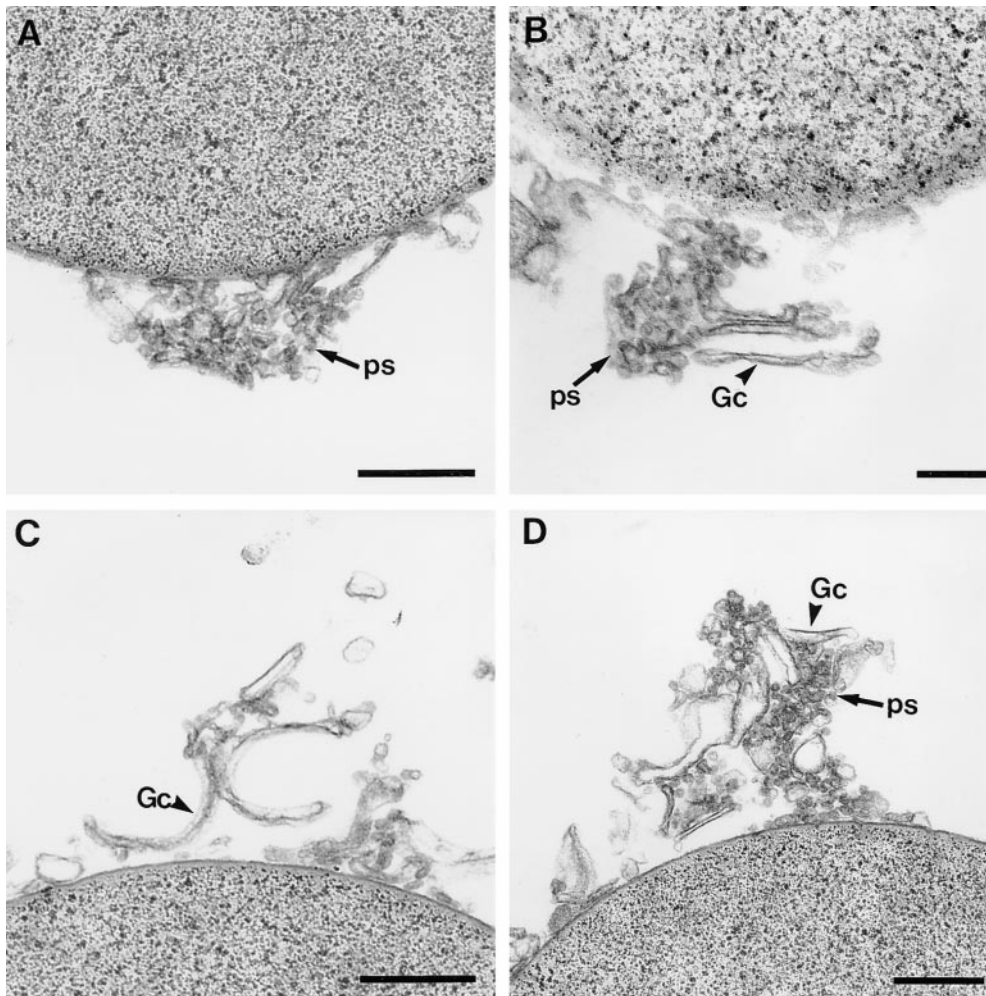
Figure 3 (continues on facing page).

branes that are immunoisolated with the p23 antibody. We coated magnetic beads with anti-rabbit IgG antibody together with or without affinity-purified p23 antibody and incubated these with Golgi-enriched membranes. After incubation, we recovered the beads with a magnet and washed them to remove any material that was not tightly bound. The beads were then processed for electron microscopy (Fig. 3). In the absence of this antibody, no membranes were found bound to the beads. However, when p23 antibodies were present, beads were coated with membranes that were enriched in the anastomosing sectioned tubules characteristic of ER/Golgi intermediates and the cis-Golgi network (Dominguez et al., 1998). However, in addition to these, we also observed in some images clear evidence for cisternal membranes that appeared to be derived from the Golgi stack in continuity with these cis-Golgi tubules. The variability in recovery of stacked Golgi membranes and the abundance of tubular elements in these images suggests that, as we had expected, the p23 antibody allows us to prepare a membrane fraction that is enriched in cis-Golgi and pre-Golgi intermediates, but also contains a variable amount of associated Golgi membranes.

Coimmunoisolation of VSV-G and Golgi Enzymes with p23

We prepared Golgi-enriched membranes from cells that contained radiolabeled VSV-G in the 15°C compartment, and, for comparison, we prepared Golgi-enriched membranes from cells that had been incubated for an additional 20 min at 37°C. This would allow the labeled VSV-G to enter the Golgi stack. We then incubated these membranes with magnetic beads coated with or without p23 antibody as indicated. Beads were collected with a magnet, and membranes that remained in the supernatant were recovered by centrifugation. We determined the yield with which the ER/Golgi intermediates were immunoisolated by measuring the recovery of p25, another member of the p24 protein family (Dominguez et al., 1998), and we found that p25 was completely removed from the supernatant and efficiently bound to p23 antibody-coated beads (Fig. 4). VSV-G that had been accumulated in the 15°C compartment was almost as well precipitated. When the p23 antibody was omitted, both VSV-G and p25 remained in the supernatant, and very little binding to the beads was detectable. When cells were incubated after the 15°C chase for an additional 20 min at 37°C before isolation of
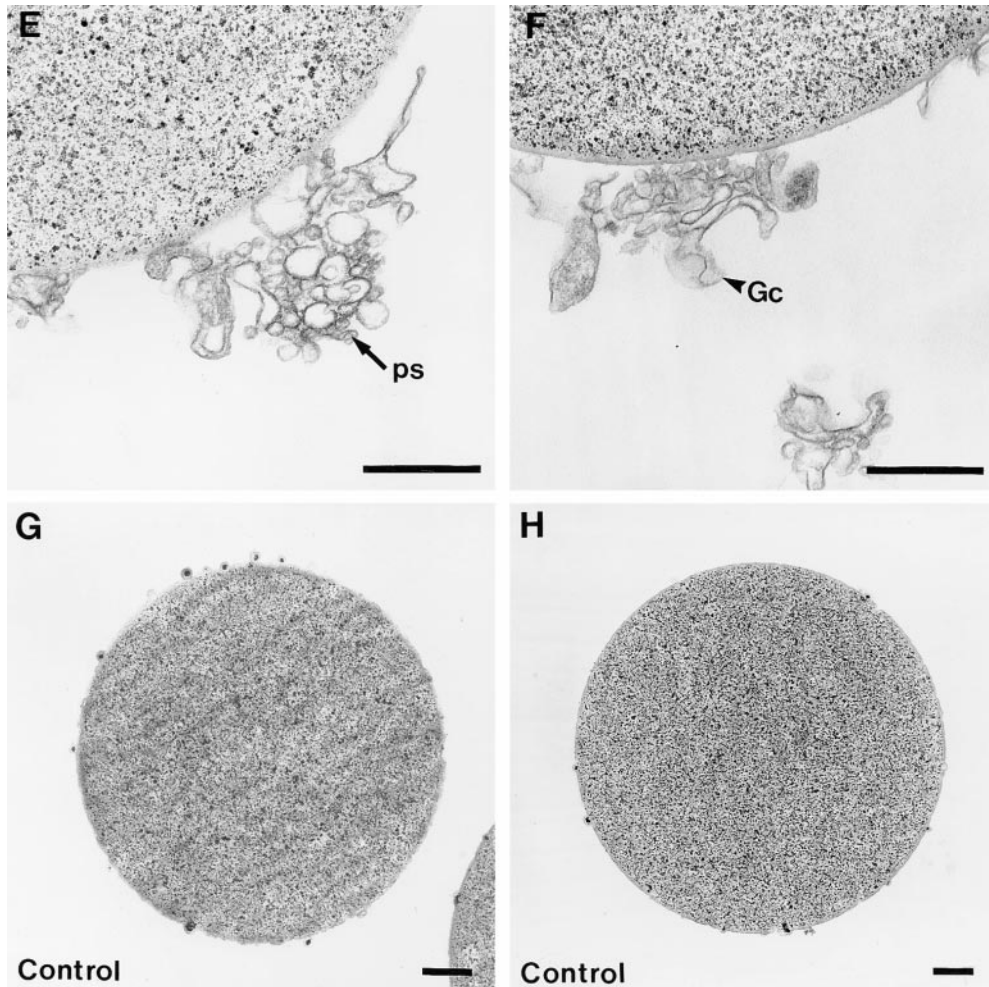
Figure 3. Immunoisolation of the p23 compartment. Elements of the cis-Golgi network are prominent. Partial stacks of Golgi cisternal also observed (Gc) as well as pleiomorphic tubular structures (ps). The magnification bars (500 nm) are indicated as are the sectioned magnetic beads themselves. Control beads (G and H) are indicated. The variation in two separate experiments are shown (A, B, C, D, and G are from one experiment; E, F, and H are from a separate experiment).

membranes, then only approximately one-third of VSV-G was recovered with p23 antibodies, even though immunoisolation of p25 was unaffected.

We also measured which percentage of different Golgi enzyme activities was precipitated with p23 antibodies. We first measured mannosidase activity as it is easily measured using a fluorescent substrate that allows this activity to be measured without interference of the radiolabeled VSV-G. We found that about 30% of the total mannosidase activity could be immunoisolated with p23 antibodies, which is a yield similar to the recovery of VSV-G after release of the 15°C block. Next, we measured two Golgi-specific marker enzymes, N-acetylglucosamine (GlcNAc) transferase (NAGT) and galactosyl transferase (GalT). These measurements were done in separate experiments for two reasons: the metabolic labeling of VSV-G would interfere with the activity assays for NAGT and GalT as incorporation of 3H–labeled sugars is measured, and 15B cells contain only low levels of NAGT activity, as they lack NAGT I. When we immunoisolated p23-containing membranes from wt cells, we found that on average 38% of NAGT and 23% of GalT activities were immunoprecipitated as well. Mannosidase activity and p25 were immunoisolated with p23 antibodies from wt membranes with the same yield as from 15B membranes (data not shown).

As we had expected based on the morphological analysis of magnetic beads after immunoisolation, p23 immunoisolation recovers quantitatively ER/Golgi intermediates but only partially Golgi membranes. This makes this technique useful to distinguish between a localization in ER/Golgi intermediates and Golgi membranes. We conclude that by the end of the 15°C incubation, VSV-G was in ER/Golgi intermediate and cis-Golgi network membranes, but after a 20-min chase at 37°C, it entered the Golgi stack.

In Vitro Glycosylation of VSV-G in ER/Golgi Intermediates

We employed the pulse–chase conditions described above to introduce labeled VSV-G into the ER, ER/Golgi intermediates, or the Golgi stack. This allows us to determine where in the secretory pathway VSV-G must reside to be efficiently glycosylated by in vitro complementation. When we incubated 15B membranes containing labeled VSV-G in the 15°C compartment with wt Golgi membranes, we observed that VSV-G was converted to the endo H–resistant form with very high efficiency (Fig. 5 A). As a control, wt membranes were omitted, and under those conditions VSV-G remained endo H sensitive. VSV-G also remained endo H sensitive when incubations were
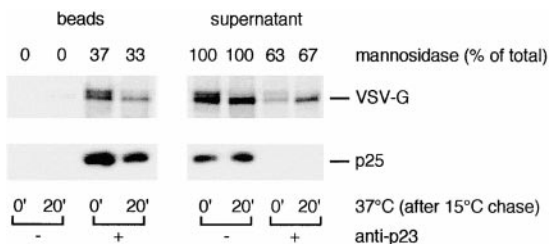
Figure 4. Immunoisolation of VSV-G and Golgi marker enzymes with p23 cytoplasmic tail antibodies. Magnetic beads were coated with p23 antibodies and incubated with membranes isolated from VSV-infected 15B cells containing labeled VSV-G in the 15°C compartment. Beads were recovered with a magnet and membranes that remained in the supernatant were pelleted by ultracentrifugation. When indicated, cells were incubated for 20 min at 37°C before homogenization of cells and isolation of membranes. A fraction of membranes bound to beads and membranes that were pelleted by ultracentrifugation were dissolved in electrophoresis sample buffer, and another fraction was dissolved in mannosidase assay buffer. VSV-G was visualized by phosphorimaging, p25 by Western blotting, and mannosidase activity was determined using a fluorescent substrate.

done on ice, or without addition of cytosol, ATP, or the activated sugar UDP-GlcNAc (data not shown). We carefully quantified the glycosylation yield in this reaction to assure that we could reproducibly obtain high glycosylation yields in vitro after accumulation of VSV-G in the 15°C compartment (Table I). We added saturating amounts of wt membranes so that we could be sure that glycosylation of VSV-G had reached the maximal level that could be obtained under these conditions. In the series of experiments that were quantified for this table, we used six different batches of membranes and tested them under experimental conditions that were typical for the experiments shown here. We found that 68 ± 6% of VSV-G became endo H resistant at the end of the incubation indicating reproducibility in comparing different batches of membranes.

In Vitro Complementation after Exit from ER/Golgi Intermediates

The batch-to-batch reproducibility of this assay allowed us to compare membranes isolated from cells after different

Table I. Endo H Resistance of VSV-G after In Vitro Complementation (Labeled VSV-G in 15°C Compartment of 15B Cells)

% Endo H resistant	Average ± SD
66.7	68.1 ± 6.1
70.9	
74.0	
63.0	
57.9	
75.0	

Each data point represents one individually prepared batch of membranes. The first four points represent averages of several experiments with a given batch of membranes. The last two data points represent individual incubations with different batches of membranes in different experiments.
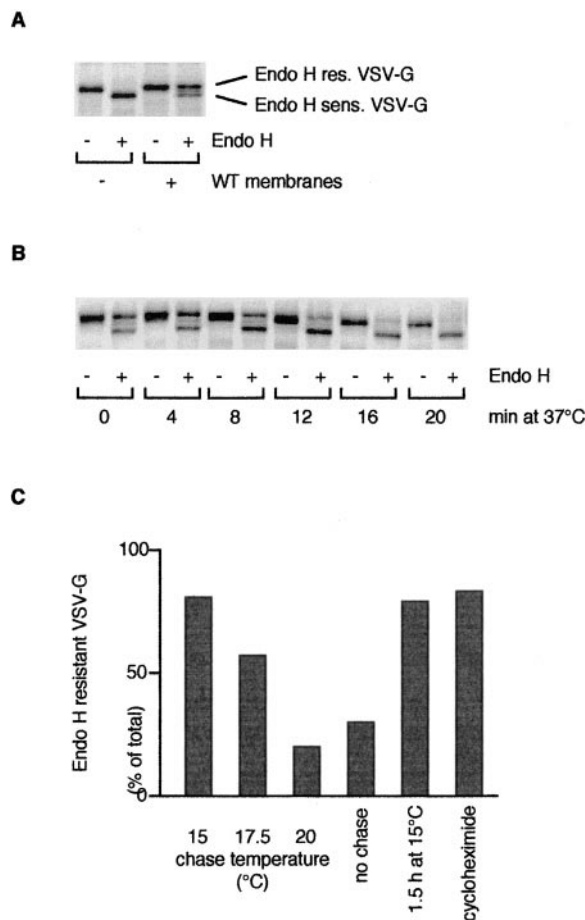

Figure 5. Glycosylation of VSV-G in 15B membranes after fusion with wt Golgi-derived membranes. (A) VSV-G in the 15°C compartment is efficiently processed by wt Golgi-derived NAGT I. Membranes isolated from VSV-infected 15B cells after pulse labeling and 15°C chase were incubated with vesicles isolated from wt cells in the presence of cytosol, ATP, and UDP-GlcNAc. At the end of the incubation, samples were digested with endo H. (B) Biochemical complementation is rapidly lost as VSV-G enters the Golgi stack. After the 15°C chase, cells were incubated at 37°C for the indicated times before membranes were isolated. These membranes were then incubated with both wt Golgi-derived vesicles and cisternal membranes. (C) Quantitation of endo H resistance of VSV-G in complementation assay. After pulse labeling, VSV-infected 15B cells were incubated for 3 h at 15°C, 17.5°C, 20°C, or the chase was omitted, or cells were incubated for 1.5 h at 15°C. Golgi enriched membranes were isolated and incubated with wt membranes as described above. When indicated, wt membranes from cycloheximide treated cells were added in the complementation assay. The standard deviations between independent experiments were between 5 and 10%.

chase conditions in the complementation assay. After 5 min pulse labeling at 37°C and accumulation of VSV-G in ER/Golgi intermediates by a 3-h incubation at 15°C, we incubated cells for up to 20 min at 37°C. This allowed labeled VSV-G to exit the 15°C compartment and enter the Golgi stack. We found that for up to 4 min after transfer to 37°C, the majority of VSV-G in our membrane preparation could be processed (Fig. 5 B; temperature shift from 15 to 37°C was performed by transferring the culture flask

from one water bath to the other, and only after 4 min did the temperature inside the flask reach 37°C). However, after 20 min, when, according to the glycosylation kinetics and the immunoisolation experiments most of VSV-G is in the Golgi stack, the in vitro complementation yield was much reduced. Membranes containing labeled VSV-G in the 15°C compartment or after up to 20 min chase at 37°C were routinely prepared at different times throughout the course of this study. Only when the chase time was kept very low (<4 min at 37°C) did we observe glycosylation yields that remained constant (within 5%), irrespective of the chase time (data not shown). In all experiments in which longer chase times were included, a marked drop similar to the one shown in Fig. 5 B was observed.

To determine whether the in vitro complementation might be the result of fusion of ER/Golgi intermediates containing newly synthesized Golgi enzymes in transit from the ER to the Golgi, we isolated membranes from wt cells that had been treated with cycloheximide. We found that these were equally active in the complementation assay as the membranes isolated from control cells (Fig. 5 C). We also tested how the yield of the complementation reaction changed when we either omitted the 15°C chase, shortened the time of the 15°C chase, or increased the chase temperature to 17.5 or 20°C (Fig. 5 C). We found that the yield of the complementation reaction dropped when the chase was done for the same time but at an even slightly higher temperature. When the chase time at 15°C was shortened to 1.5 h instead of 3 h, less of the total VSV-G was recovered in the Golgi-enriched fraction, but the relative yield of the complementation reaction was unaffected. Even when the chase after pulse labeling was entirely omitted, a fraction of VSV-G became endo H resistant. This could indicate transport of Golgi enzymes to the ER, as has been suggested by some, but the evidence remains contradictory (Cole et al., 1998; Shima et al., 1998; Storrie et al., 1998). More likely is that a small fraction of VSV-G exited the ER during the labeling step, and this fraction of VSV-G is enriched during preparations of membranes used in the in vitro transport assay (see Fig. 2 A; fractions 4–6 are the membranes used in the in vitro assay).

In summary, we conclude that in vitro complementation becomes efficient as VSV-G exits the ER and reaches ER/Golgi intermediates. As VSV-G progresses into the Golgi stack, in vitro complementation again becomes inefficient. We conclude that ER/Golgi intermediates are the optimal target compartment for the in vitro transport of wt Golgi enzymes.

Completion of VSV-G Glycosylation in ER/Golgi Intermediates

The in vitro complementation assay that we used in our experiments relies on processing by NAGT I, and this is followed by Mann II processing so that VSV-G will become resistant to endo H. We were interested in determining whether later Golgi modifications could also occur in vitro. This would suggest that ER/Golgi intermediates contained at least small amounts of the enzymes that are required to complete Golgi processing of N-linked glycans. Such late-acting Golgi enzymes might be present in ER/Golgi intermediates of 15B cells, or they might be acquired by retrograde transport during the in vitro incubation. Indeed, the observation that antibodies against p23, a protein highly enriched in ER/Golgi intermediates and the cis-Golgi network, immunoisolated a significant fraction of GalT activity suggests physical continuity among these compartments.

We determined the time course of glycosylation after complementation of the 15B defect. In previous experiments, we had included UDP-N-acetyl glucosamine (UDP-GlcNAc), the activated sugar that is transferred by NAGT. Now, we also added UDP-galactose and CMP-sialic acid, the substrates for GalT and sialyl transferase, respectively (Fig. 6 A). We found that at intermediate time points during the incubation, a form of VSV-G appeared that was resistant to endo H and migrated with the same mobility as the endo H–resistant VSV-G that was seen when only UDP-GlcNAc was added (Fig. 6 A, endo H resistant). After longer time points, the mobility of endo H–resistant VSV-G decreased further, indicating the addition of more sugar molecules. After 2 h, most endo H–resistant VSV-G no longer migrated as a sharp band
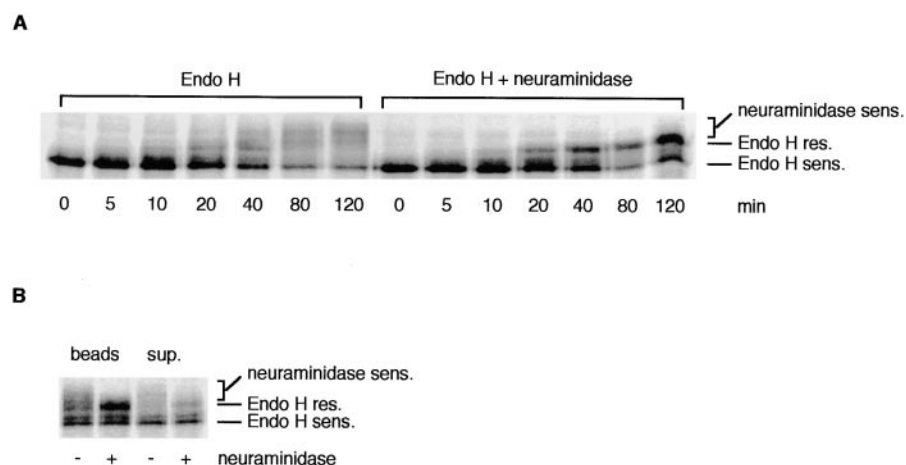

Figure 6. Complementation of the 15B defect allows processing of VSV-G by late-acting Golgi enzymes. (A) Time course of glycosylation. 15B membranes isolated from cells containing radiolabeled VSV-G in the 15°C compartment were incubated with wt Golgi-derived vesicles for the indicated times as described above, except that not only UDP-GlcNAc but also UDP-galactose and CMP-sialic acid were added. After the incubation, samples were digested with either endo H alone or with endo H and neuraminidase. (B) Immunoisolation of membranes after incubation. At the end of the incubation, magnetic bead coated with p23 cytoplasmic tail antibodies were used to immunoisolate membranes as described in Fig. 4.

but was spread out over a wider area (Fig. 6 A, neuraminidase sensitive). To confirm that this increase in mobility was caused by addition of sialic acid, we digested VSV-G with neuraminidase. We found that after neuraminidase treatment, VSV-G migrated as a sharp band that was very similar in mobility to the transiently appearing endo H–resistant band.

We used immunoisolation to confirm that sialylated VSV-G still resided in the p23 compartment at the end of the incubation (Fig. 6 B). When we reisolated p23-containing membranes from the reaction mixture using magnetic beads with bound p23 cytoplasmic tail antibodies, the majority of VSV-G bound to the beads. Most of the endo H–resistant and sialylated material bound to magnetic beads, and only membranes containing VSV-G that was not processed by Golgi enzymes were incompletely recovered by immunoisolation at the end of the incubation. These membranes might be an ER contamination in this membrane preparation, or the result of inactivation or aggregation of membranes during the incubation. We conclude that VSV-G remains in a p23-containing compartment throughout the incubation.

ER/Golgi Intermediates and Golgi Membranes Remain Distinct during Incubation

Having established in which compartment VSV-G must reside to be glycosylated by in vitro complementation, our attention turned towards studying the mechanism of this process. Specifically, we wished to determine whether in vitro complementation occurred as a consequence of dissociative vesicular transport, or by direct fusion of Golgi compartments.

So far, we provided evidence that VSV-G must reside in a pre-Golgi compartment at the beginning of the incubation. As VSV-G was still recovered with p23 antibodies at the end of the incubation, most of it was not separated from proteins of ER/Golgi intermediates. However, as membrane fusion occurs in this assay, ER/Golgi intermediates might directly fuse with wt Golgi membranes (Happe and Weidman, 1998). If this had occurred, then VSV-G might still colocalize with p23, but the compartmental distinction between ER/Golgi intermediates and membranes of the Golgi stack might have been lost. We therefore determined whether the immunoisolation yield of Golgi marker enzymes and VSV-G changed after the in vitro incubation (Fig. 7). As expected, we found only a slight increase in the amount of Golgi enzyme activity that was recovered after immunoisolation, suggesting transport of Golgi enzymes to ER/Golgi intermediates. We also observed a similarly small decrease of VSV-G recovery, which may be caused by a separation of forward moving VSV-G from retrograde moving p23.

While small changes in recovery were found after incubation, it is primarily established that the recovery did not drastically change. This suggests that ER/Golgi intermediates and Golgi membranes did not undergo extensive fusion and remained at least partially distinct from each other.

Characterization of the wt Golgi Activity
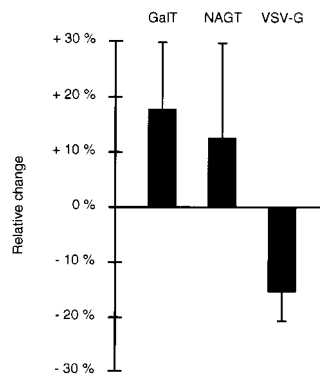
Two results now suggest that cisternal Golgi membranes

Figure 7. Relative change of the immunoisolation yield before and after in vitro incubation. wt Golgi (NAGT, GalT) or 15B Golgi membranes (VSV-G) containing radiolabeled VSV-G in the 15°C compartment were incubated in vitro. Before and after the incubation, p23-containing membranes were immunoisolated. The increase or decrease in the recovery of GalT, NAGT, and VSV-G after incubation was determined and is expressed as a percentage of the amount that is immunoisolated at the beginning of the incubation (mean ± SD, $n = 4$).

do not fuse with each other in this assay. First, complementation efficiency is maximal after the 15°C incubation and decreases thereafter. If all Golgi membranes were capable of fusion, then complementation should remain constant for as long as VSV-G resides in Golgi membranes. Second, as discussed in the preceding paragraph, we observed only small changes in the p23 immunoisolation after incubation.

We showed in past work that wt Golgi membranes can form functional Golgi enzyme-containing transport vesicles, and we showed that enough of them are formed to account for the observed glycosylation yield (Love et al., 1998). In a recently completed study, cargo selection into COP I vesicles was studied, and it was found that NAGT I is efficiently packaged into functional transport vesicles (Lanoix et al., 1999). However, an important and seemingly contradictory observation is that BFA, an inhibitor of COP I assembly, had no effect on transport (Orci et al., 1991; Elazar et al., 1994; Taylor et al., 1994; Happe and Weidman, 1998; Dominguez et al., 1999).

We hypothesized that the BFA-resistant transport described earlier is fusion of preexisting transport intermediates. To determine whether this hypothesis was reasonable, we first quantified the activity of different sources of wt Golgi activity in this assay. The most active wt Golgi fraction that we could obtain were the small vesicles that remained in the supernatant of permeabilized cells (Love et al., 1998). We therefore prepared such a supernatant and compared its activity with the activity of large, cisternal wt Golgi membranes that were isolated in parallel (Fig. 8 A). To assure that transport was measured under identical conditions, the reaction in which cisternal Golgi membranes were used was supplemented with a supernatant fraction from which all vesicles were removed by ultracentrifugation. This assured that both incubations contained equal amounts of cytosol so that any differences detected between the different fractions were due to differences in activity of the membranes, rather than cytosolic factors. We found that based on volume, both the vesicle-containing supernatant fraction and the cisternal Golgi membrane fraction contained approximately equal activity. However, when we measured the amount of NAGT activity in each
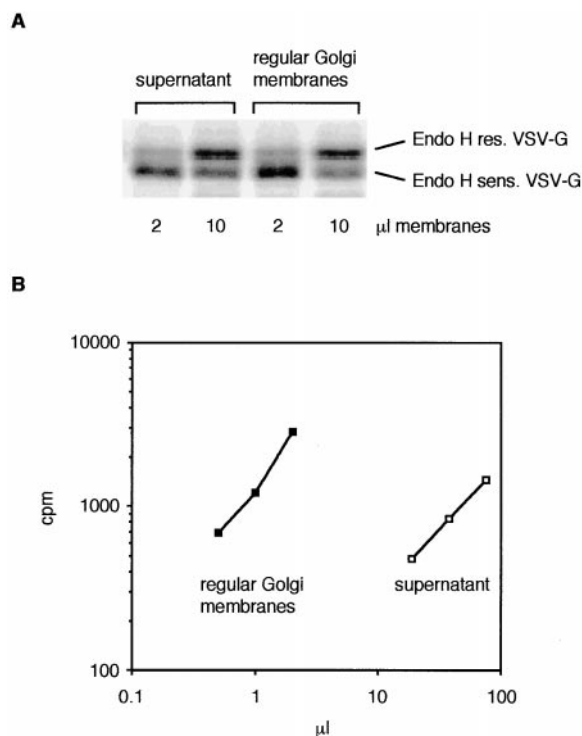
Figure 8. Comparison of different sources for wt activity. (A) 2 and 10 μl of either a vesicle-containing supernatant from permeabilized cells (supernatant) or regularly prepared cisternal Golgi membranes (regular Golgi membranes) were incubated in the in vitro assay with VSV-G–containing 15B membranes. (B) Total NAGT activity contained in each of the two sources for wt Golgi activity was determined at three different concentrations. Numbers are plotted on a logarithmic scale.

fraction, we found that the Golgi fraction contained 1 to 2 orders of magnitude more NAGT activity (Fig. 8 B).

While the focus of this study is a functional characterization of the different sources of wt activity in this transport assay, we wished to include at least an initial morphological characterization of the membranes contained in this supernatant. In Fig. 9, we show results from an electron micrograph of membranes that were isolated from the freeze/thaw supernatant. To enrich the Golgi-derived membranes in this supernatant, we isolated small vesicles from this fraction by a combination of velocity sedimentation and equilibrium density gradient centrifugations, as we had done previously (Love et al., 1998). Electron micrographs show an abundance of small pleiomorphic vesicular structures as well as small tubular elements and occasional larger contaminants. Although a mixed fraction, the element are in majority very small (54 ± 33 nm diameter [mean ± SD, *n* = 100]).

The most significant property of the supernatant fraction is that it contains extraordinarily active membranes. When the findings of in vitro experiments were compared with morphological observations, it was assumed that the wt Golgi membranes that were observed morphologically were the same membranes that generated the in vitro assay signal. If, however, the very slowly sedimenting membranes observed by us are present even as a minor contamination of any Golgi membrane preparation, then their contribution to the assay signal might still be higher than that of the morphologically observed Golgi elements.

Transport in the Absence of Preexisting Transport Intermediates Is Inhibited by BFA

Before we could detect transport that originated from cisternal Golgi membranes, it was necessary to add a salt wash step to the isolation procedure to remove loosely bound transport intermediates. To confirm that this was necessary, we incubated Golgi membranes with 250 mM KCl and then pelleted the Golgi membranes by a medium speed centrifugation. Even though the bulk of the Golgi membranes were pelleted, the supernatant was approximately as active when incubated with 15B Golgi membranes as the total membranes from which the supernatant was derived (Fig. 10). No active membranes remained in the supernatant when salt was omitted. From this we conclude that active transport intermediates remain bound to Golgi membranes during their isolation. Unless removed, they are likely to be an important if not exclusive contributor to the assay signal.

While salt-washed Golgi membranes had been used by others, it is noteworthy that they recovered membranes by ultracentrifugation (Clary et al., 1990). In this study, however, we used a medium speed centrifugation to reisolate Golgi membranes, and this change assured that we would not recover any small vesicles. A serendipitous observation allowed us to reconstitute transport with these membranes: only cytosol that was prepared as a crude supernatant, without desalting or dialysis, was found to be active in the assay with salt-washed Golgi membranes (data not shown). Crude, dialyzed, and desalted cytosols were equally active when fusion of preexisting transport intermediates was measured. This observation suggests that some cytosol preparations lack components needed to reconstitute vesicle formation. As all these cytosols contained coatomer and ADP ribosylation factor, it is likely that additional factors are needed that remain to be identified.

We found that when salt-washed wt-Golgi membranes were used in the in vitro complementation assay, the reaction was efficiently inhibited by 20 μg/ml BFA (Fig. 11 A). While lower concentrations are sufficient for the in vivo effects of BFA, the concentration used here is identical to the one used previously to inhibit COP I assembly in vitro (Donaldson et al., 1991). We also observed that the transport reaction with salt washed membranes was very sensitive to addition of ethanol, a known inhibitor of vesicle formation (Pfanner et al., 1989; Ktistakis et al., 1996). BFA was added as a solution in ethanol, but at the low concentration used here, the solvent had only a small inhibitory effect (Fig. 11 A, compare lanes 3 and 6 or 5 and 8).

As the fusion of preexisting transport intermediates does not require coat assembly, it should be resistant to BFA. As expected, we found that BFA did not inhibit transport when small transport-active vesicles were used (Fig. 11 B). Instead, we observed an approximately twofold stimulation by the addition of BFA, possibly due to a
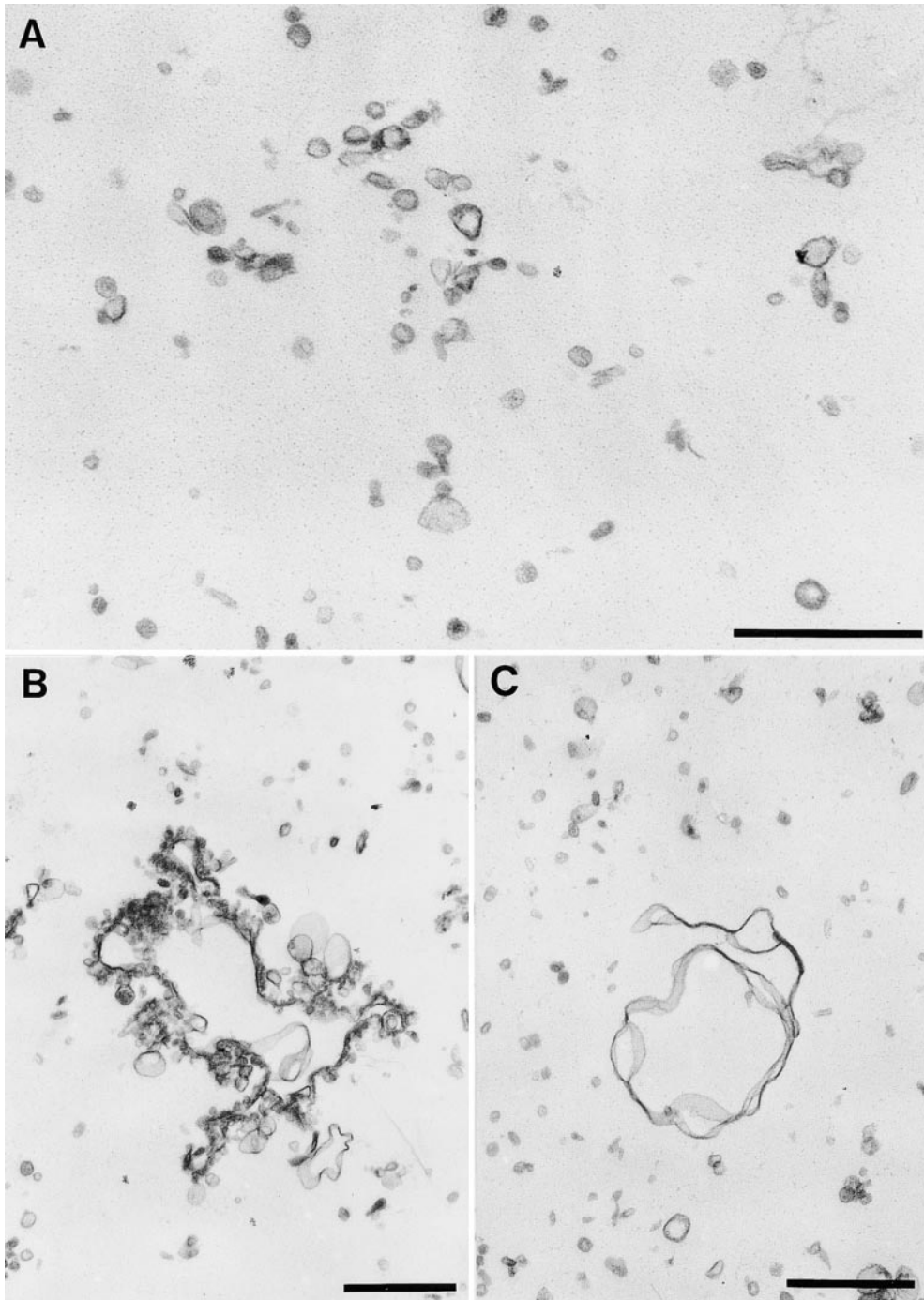
Figure 9. Transport active enzyme-containing fraction. Velocity and density gradient centrifugation was used to isolate the transport active fraction containing Golgi resident enzymes as described in Materials and Methods. These compartments are mainly small but markedly heterogeneous in shape and size (54 ± 33 nm diameter; mean ± SD, *n* = 100). Occasional contaminating larger structures are seen in B and C. The bars represent 500 nm and the preparation is from a filtered pellicle ensuring a random view of the preparation as described in Dominguez et al. (1999).

reduction in the amount of COP I coat associated with the 15B membranes which might facilitate membrane fusion. Addition of ethanol was less inhibitory when vesicles were used, as was to be expected as ethanol inhibits more strongly vesicle budding then membrane fusion.

As BFA is not inhibitory but stimulatory when vesicles are added, it is established that BFA inhibition in the transport assay with salt-washed wt Golgi membranes is not caused by an inhibition of the fusion reaction. It also demonstrates that, as is predicted by our hypothesis of vesicular transport from wt to 15B Golgi, BFA inhibits by acting on wt membranes, and not on the 15B membranes.

If, for example, BFA inhibited by preventing maturation of the 15B membranes, then we should observe this inhibition irrespective of the source of wt Golgi activity.

Transport Is Inhibited by Antibodies against Coatomer and Coatomer Depletion

The BFA inhibition suggests that COP I assembly is required for transport in this system, and a direct test of this hypothesis is to deplete cytosol of COP I components and show that it is inactivated. We found that even though cytosol was not completely inactivated by immunodepletion,
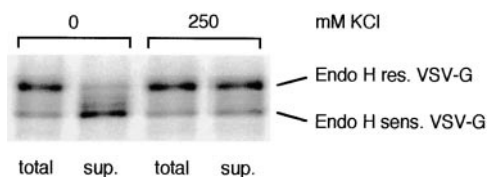
Figure 10. Reconstitution of transport with salt-washed membranes. Regular wt Golgi membranes were incubated with or without 250 mM KCl as indicated. Subsequently, a supernatant was prepared from each and incubated with VSV-G containing 15B membranes in vitro (sup.). For comparison, the VSV-G containing 15B membranes were incubated with the preincubation mixture without removal of cisternal Golgi membranes by centrifugation (total).

its activity was significantly reduced (Fig. 12 A). We also determined whether addition of purified CM1A10 antibody inhibited transport. We found that as little as 5 ng antibody was partially inhibitory, and maximal inhibition was observed with 25 ng (Fig. 12 B). When wt activity was added as vesicles instead of cisternal membranes, no antibody inhibition was observed at any of the concentrations tested (Fig. 12 C). This allows us to conclude that just as BFA, the coatomer antibody inhibits transport at the stage of coat assembly on wt membranes. Membrane fusion with and glycosylation in 15B membranes are not inhibited.

Surprising and contradictory to our hypothesis is, however, that the activity of cytosol could not be restored by adding back purified coatomer. Indeed, addition of purified coatomer inhibited the residual activity after coatomer depletion (data not shown). Similar to what is found here is the observation by Peter et al. (1993) that isolated coatomer did not overcome the inhibition of ER export by coatomer antibodies. While we recently used purified coatomer to reconstitute coat assembly after coatomer depletion (Lanoix et al., 1999), the vesicles were then released from Golgi membranes by a high salt wash, purified and assayed for activity in the presence of complete cyto-

sol. As was originally suggested by Peter et al. (1993), it seems that purified coatomer differs from the active coatomer in cytosol. A full understanding of the coat protein requirement in this step will therefore require a characterization of all the factors required for the formation of functional transport intermediates. While the currently available data are fully explained by the hypothesis that BFA inhibition is mediated by an inhibition of COPI assembly, the possibilities of other types of coat remains open.

Discussion

While the experimental approach of our work is not without complexity, the hypothesis that emerges is surprisingly simple and has been anticipated by many. In vitro, Golgi enzymes are transported in COP I–coated vesicles by retrograde transport from the Golgi stack to ER/Golgi intermediates. Our work establishes ER/Golgi intermediates as the site in which VSV-G must reside to become efficiently glycosylated by in vitro complementation, demonstrating this compartment's ability to receive Golgi enzymes from the Golgi stack. As the information that one can gain from pulse–chase experiments is limited, it seems prudent to include ER exit sites and the earliest membranes of the Golgi stack in our definition of ER/Golgi intermediates. Most likely, the yield of in vitro complementation gradually increases as ER exit sites form ER-derived transport intermediates and ER/Golgi intermediates, and decreases as these assemble into the Golgi stack.

Only one membrane fusion reaction appears to occur in vitro under our experimental conditions: the fusion of Golgi enzyme-containing transport vesicles with ER/Golgi intermediates. Two sources for Golgi enzyme-containing transport intermediates have been identified: preexisting transport intermediates, some of which are released when cells are permeabilized or when Golgi membranes are incubated with salt, and newly generated transport intermediates that are formed by Golgi membranes in a BFA-sen-
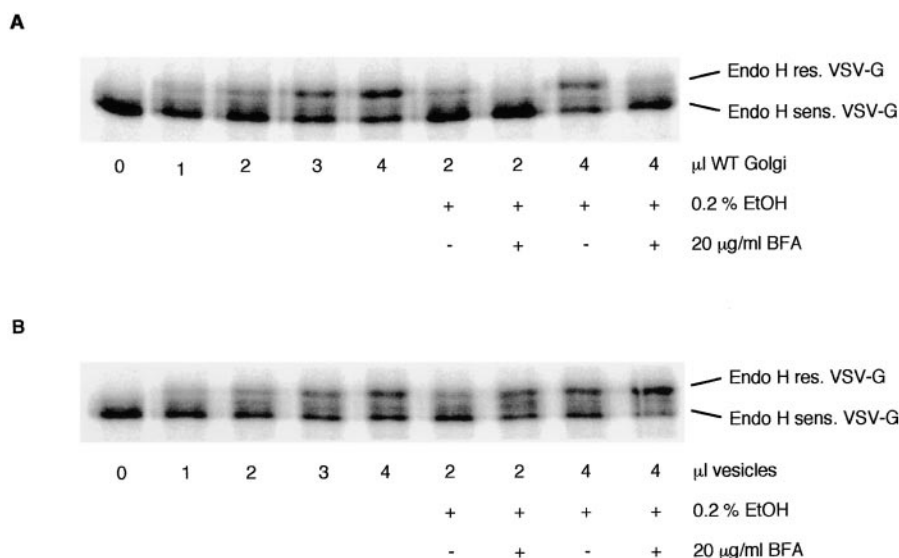

Figure 11. Transport is BFA sensitive. (A) The indicated amounts of salt-washed wt Golgi membranes were incubated with VSV-G–containing 15B Golgi. Ethanol and BFA were added as indicated. (B) Same experiment as A, except that transport active membranes isolated from permeabilized cell supernatant were added as source of wt Golgi activity.
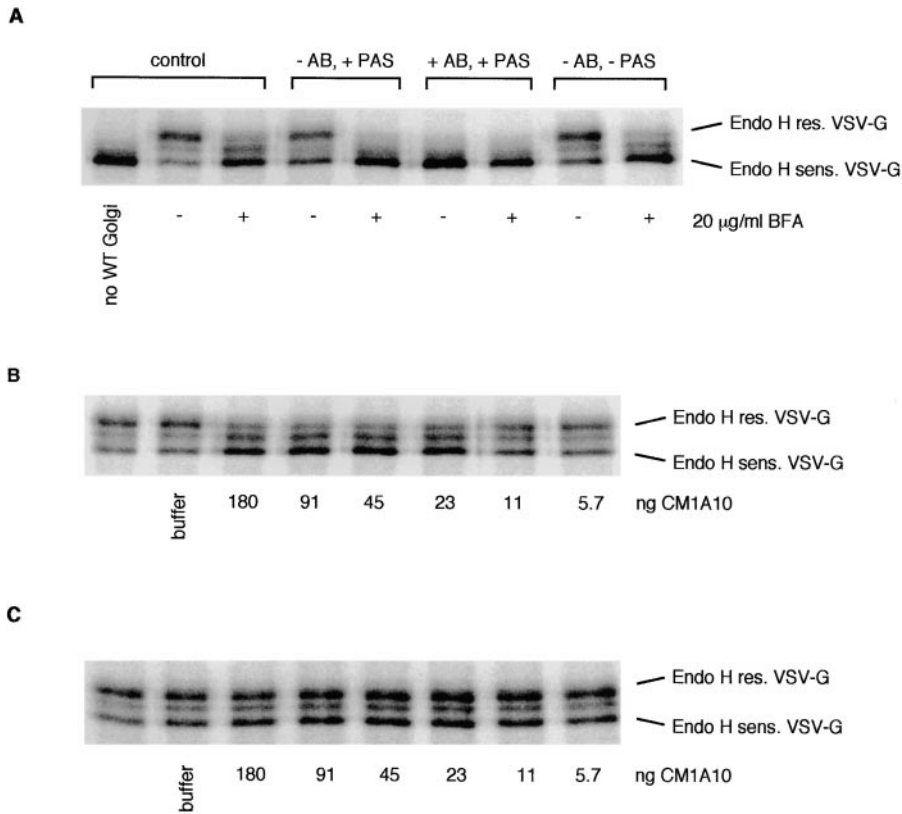
A

control   - AB, + PAS   + AB, + PAS   - AB, - PAS

— Endo H res. VSV-G

— Endo H sens. VSV-G

no WT Golgi   -   +   -   +   -   +   -   +   20 µg/ml BFA

B

— Endo H res. VSV-G

— Endo H sens. VSV-G

buffer   180   91   45   23   11   5.7   ng CM1A10

C

— Endo H res. VSV-G

— Endo H sens. VSV-G

buffer   180   91   45   23   11   5.7   ng CM1A10

Figure 12. Transport is inhibited by coatomer antibodies. (A) Cytosol was incubated with or without protein A–Sepharose (PAS) and CM1A10 antibody (AB) to deplete coatomer before its use in the in vitro complementation assay. Salt-washed wt Golgi membranes were used as the source for wt Golgi activity. (B and C) The indicated amounts of purified CM1A10 IgG (or buffer, when indicated) were added to transport incubations with salt-washed wt Golgi membranes (B) or vesicles (C).

sitive reaction. In earlier work, we had established that Golgi enzymes could be transported in COP I vesicles (Love et al., 1998; Lanoix et al., 1999). However, only with the reconstitution of a BFA-sensitive mode of transport have we eliminated the possibility that cisternal Golgi compartments might also fuse directly with each other, or that pre-Golgi intermediates might fuse directly with the Golgi stack (Happe and Weidman, 1998).

The impact of this study on our understanding of the Golgi apparatus is straightforward. That ER/Golgi intermediates acquire Golgi enzymes is an important prediction of the cisternal maturation model, and it has now been confirmed. Future work can now focus on the mechanism by which the contents of ER/Golgi intermediates is exchanged during their conversion from an ER-derived to a Golgi-derived structure. This will provide important insights that are likely to be also applicable to other transport steps, such as transport through the Golgi stack or endocytosis. However, other than this promise, little has been accomplished here to understand the next transport step, progression through the Golgi stack. We propose that this transport is not reconstituted in our system. Possibly, transport through the Golgi stack requires components or specific conditions that have yet to be identified. For example, it may require tethering of vesicles to cisternae (Orci et al., 1998; Sönnichsen et al., 1998), or it may involve other modes of transport that are impossible to detect by an in vitro complementation assay that can only detect freely diffusible transport intermediates.

Additional work is needed to understand the nature of the active intermediates. At this time, we can say little

more of them other than that they exist, that they are highly active, and that they must be removed if one wants to study a full round of transport. An unexpectedly large fraction of Golgi enzymes were found to cofractionate with these structures, and preliminary data from our group suggest a functional and structural heterogeneity in this membrane fraction. The activity described here, fusion with ER/Golgi intermediates, is likely to represent only one of several intermediates present in this fraction. Further morphological and biochemical characterization of these membranes is likely to provide insights into the mechanism of later transport steps. The exceptionally high activity of these membranes is the principal reason why we expect them to be of significance, rather than an artifact of cell breakage. However, the validity of this study's conclusions is not put in question by this uncertainty. Even if the membranes we think of as retrograde transport intermediates were caused by an artifact, they would still have to be removed before transport between Golgi membranes could be studied.

The in vitro assay used here has a long history of controversy. However, only two findings, both entirely plausible and readily observable by independent approaches, were required to resolve this puzzle. The first is that COP I vesicles contain Golgi enzymes, and, due to the catalytic effect, this means that transport of the enzyme will dominate the assay signal. The second finding is that cellular homogenates are produced from growing and secreting cells and contain steady state levels of transport intermediates. Such active intermediates have not only been observed by us here and elsewhere (Love et al., 1998), but vesicles and

budding profiles on cisternae have been observed by electron microscopy. When these two observations are accounted for, interpretation of the results of the in vitro assay becomes straightforward, and contradictions are removed without the need to invoke hypothetical modes of transport.

References

Balch, W.E., M.M. Elliott, and D.S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein between the endoplasmic reticulum and the Golgi. *J. Biol. Chem.* 261:14681–14689.

Bannykh, S.I., and W.E. Balch. 1997. Membrane dynamics at the endoplasmic reticulum Golgi interface. *J. Cell Biol.* 138:1–4.

Beams, H.W., and R.G. Kessel. 1968. The Golgi apparatus: structure and function. *Int. Rev. Cytol.* 23:209–276.

Becker, B., and M. Melkonian. 1996. The secretory pathway of protists: spatial and functional organization and evolution. *Microbiol. Rev.* 60:697–721.

Belden, W.J., and C. Barlowe. 1996. Erv25p, a component of COP II-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. *J. Biol. Chem.* 271:26939–26946.

Bonfanti, L., A.A. Mironov, Jr., J.A. Martinez-Menarguez, O. Martella, A. Fusella, M. Baldassarre, R. Buccione, H.J. Geuze, A.A. Mironov, and A. Luini. 1999. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell.* 95:993–1003.

Braell, W.A., W.E. Balch, D.C. Dobbertin, and J.E. Rothman. 1984. The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of cisternae. *Cell.* 39:511–524.

Clary, D.O., I.C. Griff, and J.E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell.* 61:709–721.

Clermont, Y., A. Rambourg, and L. Hermo. 1994. Connections between the various elements of the cis- and mid-compartments of the Golgi apparatus of early rat spermatids. *Anat. Rec.* 240:469–480.

Cole, N.B., J. Ellenberg, J. Song, D. DiEuliis, and J. Lippincott-Schwartz. 1998. Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* 140:1–15.

Dominguez, M., K. Dejgaard, J. Fullekrug, S. Dahan, A. Fazel, J.P. Paccaud, D.Y. Thomas, J.J.M. Bergeron, and T. Nilsson. 1998. gp25L/emp24/p24 protein family members of the cis-Golgi network bind COP I and II coatomer. *J. Cell Biol.* 140:751–765.

Dominguez, M., A. Fazel, S. Dahan, J. Lovell, L. Hermo, A. Claude, P. Melançon, and J.J.M. Bergeron. 1999. Fusogenic domains of Golgi membranes are sequestered into specialized regions of the stack that can be released by mechanical fragmentation. *J. Cell Biol.* 145:673–688.

Donaldson, J.G., R.A. Kahn, J. Lippincott-Schwartz, and R.D. Klausner. 1991. Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science.* 254:1197–1199.

Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa, and J.E. Rothman. 1994. ADP-ribosylation factor and coatomer couple fusion to vesicle budding. *J. Cell Biol.* 124:415–424.

Farquhar, M.G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.

Farquhar, M.G., and G.E. Palade. 1998. The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol.* 8:2–10.

Fries, E., and J.E. Rothman. 1980. Transport of vesicular stomatitis virus glycoprotein in a cell-free extract. *Proc. Natl. Acad. Sci. USA.* 77:3870–3874.

Glick, B.S., T. Elston, and G. Oster. 1997. A cisternal maturation mechanism can explain the asymmetry of the Golgi stack. *FEBS Lett.* 414:177–181.

Gottlieb, C., J. Baenziger, and S. Kornfeld. 1975. Deficient uridine diphosphate-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyltransferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. *J. Biol. Chem.* 250:3303–3309.

Happe, S., and P. Weidman. 1998. Cell-free transport to distinct Golgi cisternae is compartment specific and ARF-independent. *J. Cell Biol.* 140:511–523.

Ktistakis, N.T., H.A. Brown, M.G. Waters, P.C. Sternweis, and M.G. Roth. 1996. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi-coated vesicles. *J. Cell Biol.* 134:295–306.

Ladinsky, M.S., D.N. Mastronarde, J.R. McIntosh, K.E. Howell, and L.A. Staehelin. 1999. Golgi structure in three dimensions: functional insights from the normal rat kidney cell. *J. Cell Biol.* 144:1135–1149.

Lanoix, J., J. Ouwendijk, C.-C. Lin, A. Stark, H.D. Love, J. Ostermann, and T. Nilsson. 1999. GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:4935–4948.

Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, and S.D. Emr. 1994. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207.

Lewis, M.J., and H.R. Pelham. 1996. SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. *Cell.* 85:205–215.

Lippincott-Schwartz, J., N.B. Cole, and J.G. Donaldson. 1998. Building a secretory apparatus: role of ARF1/COPI in Golgi biogenesis and maintenance. *Histochem. Cell Biol.* 109:449–462.

Love, H.D., C.C. Lin, C.S. Short, and J. Ostermann. 1998. Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport. *J. Cell Biol.* 140:541–551.

Lovelock, C., and J. Lucocq. 1998. Quantitative immuno electron microscopy reveals a2,6 sialyltransferase is concentrated in the central cisternae of rat hepatocyte Golgi apparatus. *Eur. J. Cell Biol.* 76:18–24.

Mellman, I., and K. Simons. 1992. The Golgi complex: in vitro veritas? *Cell.* 68:829–840.

Matlin, K., and K. Simons. 1983. Reduced temperature prevents transfer of a glycoprotein to the cell surface but not terminal glycosylation. *Cell.* 34:233–243.

Nilsson, T., M. Pypaert, M.H. Hoe, P. Slusarewicz, E.G. Berger, and G. Warren. 1993. Overlapping distribution of two glycosyltransferases in the Golgi apparatus of HeLa cells. *J. Cell Biol.* 120:5–13.

Orci, L., B.S. Glick, and J.E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell.* 46:171–184.

Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J.G. Donaldson, J. Lippincott-Schwartz, R.D. Klausner, and J.E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.

Orci, L., M. Stamnes, M. Ravazzola, M. Amherdt, A. Perrelet, T.H. Söllner, and J.E. Rothman. 1997. Bidirectional transport by distinct populations of COP I-coated vesicles. *Cell.* 90:335–349.

Orci, L., A. Perrelet, and J.E. Rothman. 1998. Vesicles on strings—morphological evidence for progressive transport within the Golgi stack. *Proc. Natl. Acad. Sci. USA.* 95:2279–2283.

Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elazar, and J.E. Rothman. 1993. Stepwise assembly of functionally active transport vesicles. *Cell.* 75:1015–1025.

Pelham, H.R.B. 1996. The dynamic organisation of the secretory pathway. *Cell Struct. Funct.* 21:413–419.

Peter, F., H. Plutner, H. Zhu, T.E. Kreis, and W.E. Balch. 1993. Beta-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro. *J. Cell Biol.* 122:1155–1167.

Pfanner, N., L. Orci, B.S. Glick, M. Amherdt, S.R. Arden, V. Malhotra, and J.E. Rothman. 1989. Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae. *Cell.* 59:95–102.

Presley, J.F., N.B. Cole, T.A. Schroer, K. Hirschberg, K.J.M. Zaal, and J. Lippincott-Schwartz. 1997. ER-to-Golgi transport visualized in living cells. *Nature.* 389:81–84.

Rambourg, A., Y. Clermont, and F. Képès. 1993. Modulation of the Golgi apparatus in *Saccharomyces cerevisiae* sec7 mutants as seen by three-dimensional electron microscopy. *Anat. Rec.* 237:441–452.

Rojo, M., R. Pepperkok, G. Emery, R. Kellner, E. Stang, R.G. Parton, and J. Gruenberg. 1997. Involvement of the transmembrane protein p23 in biosynthetic protein transport. *J. Cell Biol.* 139:1119–1135.

Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.

Scales, S.J., R. Pepperkok, and T.E. Kreis. 1997. Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COP II and COP I. *Cell.* 90:1137–1148.

Schimmoller, F., B. Singer-Kruger, S. Schroder, U. Kruger, C. Barlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COP II-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1329–1339.

Shima, D.T., N. Cabrera-Poch, R. Pepperkok, and G. Warren. 1998. An ordered inheritance strategy for the Golgi apparatus: visualization of mitotic disassembly reveals a role for the mitotic spindle. *J. Cell Biol.* 141:955–966.

Sönnichsen, B., R. Watson, H. Clausen, T. Misteli, and G. Warren. 1996. Sorting by COP I–coated vesicles under interphase and mitotic conditions. *J. Cell Biol.* 134:1411–1425.

Sönnichsen, B., M. Lowe, T. Levine, E. Jämsä, B. Dirac-Svejstrup, and G. Warren. 1998. A role for giantin in docking COP I vesicles to Golgi membranes. *J. Cell Biol.* 140:1013–1021.

Stamnes, M.A., M.W. Craighead, M.H. Hoe, N. Lampen, S. Geromanos, P.

Tempst, and J.E. Rothman. 1995. An integral membrane component of coatomer-coated transport vesicles defines a family of proteins involved in budding. *Proc. Natl. Acad. Sci. USA.* 92:8011–8015.

Storrie, B., J. White, S. Rottger, E.H. Stelzer, T. Suganuma, and T. Nilsson. 1998. Recycling of Golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. *J. Cell Biol.* 143:1505–1521.

Taylor, T.C., M. Kanstein, P. Weidman, and P. Melançon. 1994. Cytosolic ARFs are required for vesicle formation but not for cell-free intra-Golgi transport: evidence for coated vesicle-independent transport. *Mol. Biol. Cell.* 5:237–252.

Velasco, A., L. Hendricks, K.W. Moremen, D.R. Tulsiani, O. Touster, and M.G. Farquhar. 1993. Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. *J. Cell Biol.* 122:39–51.