In Vitro Transport on Cis and Trans Sides of the Golgi Involves Two Distinct Types of Coatomer and ADP-ribosylation Factor-independent Transport Intermediates*

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The cisternal maturation model proposes that secretory proteins transit the Golgi in cisternae that mature by the continuous retrograde transport of Golgi enzymes in vesicles. We have tested the hypothesis that de novo generation of transport intermediates containing medial, trans, and trans Golgi network (TGN) enzymes is reconstituted in vitro. Our analysis shows that the majority of transport is mediated by a steady state of transport intermediate production and consumption by Golgi cisternae, with only a minor contribution of pre-existing transport intermediates. Transport in the medial and trans regions of the stack involved intermediates containing Golgi enzymes, apparently moving in a retrograde direction. In contrast, transport between the trans Golgi and TGN was exclusively mediated by intermediates containing secretory protein, as expected for anterograde transport. These intermediates may be physiologically relevant, because only these two specific types of intermediates can be detected in cell homogenates. By analogy to the coatomer (COPI)-independent transport of Golgi enzymes to the endoplasmic reticulum, the steady-state production of intra-Golgi transport intermediates was not impaired by inhibition of COPI vesicle formation. These data suggest a model for COPI-independent intra-Golgi transport by cisternal maturation with a shift in mechanism to anterograde transport at the trans Golgi and TGN boundary.

The Golgi complex is a major crossroads for membrane trafficking in eucaryotic cells. Consisting of stacked cisternae with anastomosing membrane networks at the entry and exit faces, the Golgi accepts the entire secretory output of the ER,† removing non-secretory components for recycling while modifying carbohydrates on secretory cargo as it passes through the stack. Upon exiting the stack at the trans Golgi network, a final sorting occurs and the various secretory components are delivered to the appropriate cellular organelles. Although these functions of the Golgi are well known, the mechanism by which secretory cargo moves through the stack has remained elusive. It is generally agreed that small vesicular carriers, particularly COPI-coated vesicles, play an essential role in this process, but the nature of their participation is a subject of intense debate (1–4).

In vivo studies have definitively demonstrated that COPI vesicles play an essential role in the recycling of non-secretory components from the Golgi to the ER (5, 6). It has been proposed that a subpopulation of COPI vesicles also transport secretory protein in anterograde direction through the Golgi stack (7, 8). Consistent with this hypothesis, immunoelectron microscopy has revealed a population of Golgi-associated COPI vesicles that contain secretory protein but lack detectable recycling components or resident Golgi enzymes (9, 10). Engineered protein aggregates that are too large to enter COPI vesicles appear to transit the stack in anterograde-directed megavesicles (11). This hypothesis has been challenged, however, by similar studies that show an apparent enrichment of COPI enzymes but not secretory cargo in peri-Golgi COPI vesicles (12). In addition, large aggregates of procollagen were observed to transit through the Golgi without apparently leaving the cisterna (13). This mode of transport appears to apply to small cargo proteins as well. A simultaneous pulse of a small secretory protein and procollagen entered the Golgi together and remained together as they transited the stack (14). These in vivo observations, along with evidence that COPI vesicles generated in vitro selectively concentrate Golgi-resident enzymes (15), favor a model for intra-Golgi transport by cisternal maturation (reviewed in Refs. 1 and 2). In this model, secretory cargo is transported forward in cisternae that sequentially mature through the systematic retrograde transport of Golgi enzymes from cisterna to cisterna. Although in theory anterograde vesicular transport and transport by cisternal maturation should be distinguishable by direct testing, a clear resolution to the dilemma has not been attained.

The transport processes occurring within the compact Golgi stacks of living cells are not resolvable by the in vivo imaging techniques used so successfully in the analysis of transport to and from the Golgi (16–18). Consequently, most of what is known about intra-Golgi transport has been gleaned from the analysis of this process as reconstituted in vitro. The archetypal in vitro system reconstitutes transport between mutant Golgi (mut-Golgi) lacking the medial Golgi enzyme N-acetylglucosaminyl transferase I (GlcNAc T1) and glycosylation-competent wild type Golgi (wt-Golgi) (19). Transport is detected when the secretory protein, vesicular stomatitis virus glycopro-
tein (VSV G-protein) originating in the mut-Golgi, becomes glycosylated by GlcNAc T1 originating from wt-Golgi. This \textit{in vitro} reconstitution system has been a powerful tool for the identification of protein components involved in transport, including COPI and its regulator ARF (20, 21). Nevertheless, even though COPI vesicles form during an \textit{in vitro} transport incubation (22–24), it has been impossible to unambiguously demonstrate that COPI vesicles are required for intra-Golgi transport \textit{in vitro} (25–28).

Various explanations have been proposed to account for this unexpected discrepancy. The coupling hypothesis proposes that COPI vesicles are obligatory transport intermediates when formed. However, when COPI vesicle formation is prevented, uncoupling leads to a non-physiological fusion between cisternae (25). A few studies have also suggested that the \textit{in vitro} system reconstitutes the fusion between pre-existing transport intermediates that contain GlcNAc T1 and mut-Golgi cisternae containing VSV G-protein (29, 30). These intermediates could be \textit{bona fide} transport intermediates that were formed \textit{in vivo}, or they could be fusogenic fragments of cisternae that were formed by disruption of the cells. Generalizing from these observations, it has been proposed that transport at all levels of the Golgi stack might occur by retrograde vesicular transport of Golgi enzymes, rather than anterograde transport of cargo. A final possibility is suggested by the finding that the retrograde transport of Golgi enzymes to the ER \textit{in vivo} occurs by a completely COPI-independent process (31, 32). It is thus possible that the transport of Golgi enzymes \textit{in vitro} could involve carriers formed by a similar COPI-independent mechanism.

In the current study, we have measured the extent to which each of these potential mechanisms contributes to Golgi transport reconstituted \textit{in vitro} using minimal perturbation of the system from its original formulation. The analysis was extended to \textit{in vivo} transport at different levels of the Golgi stack to determine whether the mechanisms are conserved throughout the Golgi. Our data demonstrate that the majority of \textit{in vitro} Golgi transport arises, not from pre-formed transport intermediates, but from a steady state of transport intermediates that differ in their cargo and destination. The same two specific types of carriers were the only functional transport intermediates detected in broken cells, suggesting that they may be physiologically relevant intermediates in intra-Golgi transport.

### EXPERIMENTAL PROCEDURES

**Reagents**—Radiolabeled nucleotide sugars were purchased from PerkinElmer Life Sciences (Boston, MA). Palmitoyl coenzyme A and unlabeled nucleotide sugars were obtained from Roche Biochemicals. All other chemicals were purchased from either Sigma or VWR Scientific Products. Monoclonal antibody to COPI (CMA110) was generous gift of Dr. J. Ostermann. Monoclonal antibodies against ARF (1D9) and β-COP (M3A5) were purchased from Affinity Bioreagents. Cytoplasmic preparation—CHO (33) and bovine brain (34) cytosols were prepared as previously described. ARF-depleted cytosol was prepared by anion exchange chromatography according to Happe et al. (27). Briefly, cytosolic protein in TD buffer (10 mM Tris, pH 7.4, 1 mM dithiothreitol) supplemented with 25 mM KCl and 10 μM EDTA was loaded onto a Fast-Flow Q anion exchange matrix (Amersham Biosciences). After elution of unbound protein, ARF was eluted with 65 mM KCl and 1 mM magnesium chloride (in TD), and the remaining protein was eluted with 500 mM KCl (in TD). ARF-depleted cytosol was prepared by mixing equal parts of ARF-depleted cytosol and the similarly concentrated ARF-containing fraction. Unfractionated and concentrated cytosols had identical capacity to inhibit transport in the presence of the non-hydrolysable analog of GTP, GDPβS, indicating that ARF function was reconstituted (27). COPI-depleted cytosol was prepared by immunodepletion with the COPI monoclonal antibody, CMA110 (28). Cytosol was incubated twice with either CMA110 or protein G beads to produce COPI-depleted cytosol or with c-Myc monoclonal antibody (9E10) protein G beads to produce mock-depleted cytosol. Depleted cytosols contained less than 2% of the normal content of ARF or COPI, as determined by Western blotting with the anti-ARF antibody 1D9 or the anti-β-COP antibody M3A5. All cytosols were free of transport incompetent vesicles from WT-Golgi.

**Cell-free Transport Assays**—Cell-free assays that measure transport between the cis and medial Golgi (medial assay (19 and 35)), medial and trans Golgi (trans assay (27)), and trans Golgi and TGN (TGN assay (27 and 36)) were as previously described (27). Briefly, transport mixtures contained buffer salts, cytosol, an ATP-regenerating system, palmitoyl coenzyme A, mut-Golgi, and unlabeled nucleotide sugars (27). Each 25-μl assay contained an amount wt-Golgi (−0.5–0.75 μg) that was just sufficient to attain optimal transport with 2.5 μl of mut-Golgi (−1.5 μg). Golgi-enriched membranes were isolated by sucrose density flotation from freshly prepared cell homogenates (19). wt-Golgi isolated from CHO Pro-5 cells were mixed with mut-Golgi containing defects in either medial, trans, or TGN-localized N-linked carbohydrate modifications. The mut-Golgi were prepared from VSV-infected CHO Lec1 (GlcNAc T1-deficient (37)), Lec8 (UDP-galactose transporter-deficient (37, 38)), and Lec2 (CMP-sialic acid transporter-deficient (37, 39)). Transport was measured as the incorporation of [3H]GlcNAc, [3H]galactose, or [3H]-sialic acid into VSV G-protein carbohydrates after a 60-, 90-, or 180-min transport incubation for the medial, trans, or TGN assays, respectively (27).

**Two-stage Transport Assays**—Functional vesicular transport intermediates in the medial, trans, and TGN assays were detected using a two-stage transport assay. In stage I, free transport intermediates were allowed to form by preincubating either wt-Golgi or mut-Golgi in a 25-μl transport mixture without the complementary partner. After 0–60 min of preincubation at 37°C, 12.5 μl of the mixture was removed and centrifuged for 3 min at 16,000 × g at room temperature. Transport was measured in stage II of the reaction by mixing either 12.5 μl of the supernatant or 12.5 μl of the unfraccionated stage I reaction with 12.5 μl of a transport mixture containing the complementary membrane partner (mut-Golgi or wt-Golgi). In stage II experiments, the membrane pellet from the stage I reaction was resuspended in 12.5 μl of transport mixture containing 0.2 μm sucrose and then subjected to a stage II transport incubation. Functional intermediates in the medial, trans, and TGN assays were detected using the transport mixtures and mut-Golgi specific for each assay, as described above (19, 27). A background value from a transport incubation without cytosol was subtracted from the value obtained for each sample.

The cumulative release of intermediates during preincubation was measured using a variation of this two-stage assay where the membrane pellet of the stage I reaction was subjected to repeated cycles of preincubation and re-isolation. After each preincubation step, the membrane pellets were centrifuged for 1 min at 16,000 × g at room temperature. The supernatant was added directly to a stage II transport mixture. The pellet was either gently re-suspended by pipetting up and down, or simply overlaid with a transport reaction mixture containing 0.2 μm sucrose and incubated again.

**Fractionation of Membranes by Velocity Sedimentation**—Sucrose solutions (% w/v) were prepared in 10 mM Hepes-KOH, pH 7.4, 150 mM KCl, and 2.5 mM magnesium acetate. Equal volumes of 10–37.5% sucrose in 2.5% increments were used to prepare linear sucrose gradients. Cell homogenates or Golgi-enriched membranes were layered on top of the gradient and centrifuged at 4°C in either an SW41 rotor (40,000 rpm, 274,355 × g) for 55 min or in an SW60.1 rotor (50,000 rpm, 390,000 × g) for 17 min. 10–20 μl fractions of equal volume were collected from the top of each tube. Each fraction was diluted in two steps with an equal volume of 10 min Tris, pH 7.4 (4°C), and the membranes were collected by ultracentrifugation in a TLSA100.2 rotor at 100,000 rpm (435,680 × g) for 20–30 min at 4°C. Membrane pellets were soaked in 1 M sucrose, diluted to 0.25 μm sucrose with 10 mM Tris, pH 7.4 (4°C), and then gently resuspended in 10–20 μl of equal volume. Pellets were assayed immediately or flash-frozen in liquid nitrogen and stored at −80°C for later analysis.

**Glycosyl Transferase Assay and Nucleotide Sugar Uptake Assays**—Previously described methods were used to measure galactosyl transferase and sialyl transferase (40) activities. Each 50-μl reaction contained 50 mM Hepes-KOH, pH 7.4, 1 mM ATP, 5 mM palmitoyl-CoA, 0.1 mM unlabeled UDP-galactose, 10 μCi/ml UDP-[3H]galactose, and 14 mg/ml ovalbumin as substrate. The sialyl transferase reaction mixture contained 0.5 mM unlabeled CMP-SA, 20
ARF-independent Golgi Transport Intermediates

RESULTS

Steady-state Vesicle Production Occurs in Vitro—Recent studies demonstrated that vesicular intermediates containing the medial Golgi enzyme GlcNAc T1 can be extracted from Golgi or produced by mechanical disruption of rat liver Golgi. These intermediates effectively substituted for wt-Golgi in the cell-free Golgi transport system referred to here as the medial assay (29, 30). It was thus proposed that similar intermediates normally present in isolated CHO Golgi membranes might be responsible for most or all of the transport reconstituted in the in vitro Golgi transport system. We directly tested this hypothesis by determining the contribution of pre-formed intermediates to in vitro transport using the original formulation of the system with Golgi-enriched membranes from CHO cells.

A consideration in this analysis was that pre-existing intermediates might be loosely bound to the Golgi. Because salt extraction of these intermediates might also remove essential components for de novo generation of transport intermediates (30, 42), we used an alternative approach for depleting pre-existing intermediates. Wt-Golgi were first preincubated in a transport reaction in the absence of mut-Golgi to allow any pre-existing intermediates to target and fuse with wt-Golgi cisternae and be consumed. If these intermediates were the primary source of transport activity in the wt-Golgi preparation, preincubated wt-Golgi should exhibit a loss of transport capacity when subjected to a second incubation in the presence of mut-Golgi. In contrast, if other processes contribute to the activity of the wt-Golgi, such a preincubation might be expected to have little effect on the subsequent transport capacity of wt-Golgi. Fig. 1A (triangles) shows the results obtained when a wt-Golgi were preincubated for various lengths of time in a transport reaction without mut-Golgi (stage I) and then incubated again in the presence of mut-Golgi to measure transport (stage II). Preincubation of the wt-Golgi for up to 40 min had little effect on the subsequent transport activity with mut-Golgi in stage II, even though the amount of wt-Golgi was limiting for transport. This suggests that the transport capacity of the wt-Golgi cannot be solely attributed to pre-existing transport intermediates.

The observation that wt-Golgi do not lose their transport activity after prolonged preincubation might be explained if the wt-Golgi cisternae were capable of directly fusing with mut-Golgi. Alternatively, a steady state of transport intermediate production and consumption by wt-Golgi might be maintained during the incubations. To distinguish between these possibilities, a medium speed centrifugation was used to separate wt-Golgi cisternal membranes from any slowly sedimenting transport intermediates produced in the stage I preincubation. Each of these fractions was then incubated with mut-Golgi in stage II to measure transport.

In the absence of a 37 °C preincubation in stage I (Fig. 1A, 0-min time point), about 15–25% of the total transport capacity of the wt-Golgi (triangle) was recovered in the supernatant (square). This presumably represents pre-existing free intermediates that co-purified with the Golgi membranes. After a 10-min preincubation at 37 °C, the transport activity of free intermediates increased ~3-fold (Fig. 1A, squares) and remained constant for up to 40 min. The transport activity of the residual cisternal membranes (circles) also remained nearly constant for up to 40 min of wt-Golgi preincubation. This persistence of transport activity in both fractions is indicative of a steady state of free intermediate production and consumption by the wt-Golgi during stage I. Significantly, the activity of the residual cisternal membranes was not due to liberation of fusogenic membrane fragments during resuspension (29), because similar transport activity was obtained without resuspension (data not shown, but see Figs. 3A and 7). Furthermore, the steady-state level of transport intermediates was found to increase with increasing cytosol concentration in stage I (Fig. 1B). This would not be expected if the intermediates were generated by mechanical disruption of the Golgi. Thus, the in vitro system appears to reconstitute both production and consumption of functional vesicular intermediates containing GlcNAc T1 during a normal in vitro incubation.

Enzyme-containing Intermediates Are Not Universal Carriers—The observation that vesicular intermediates containing GlcNAc T1 are functional in the medial Golgi transport assay suggests the possibility that Golgi enzyme-containing intermediates might mediate transport throughout the stack, as predicted by the cisternal maturation model for intra-Golgi transport (1, 2). We addressed this possibility by employing two additional cell-free assays that measure transport either between the medial and trans Golgi (trans assay (27)) or between the trans Golgi and TGN (TGN assay (27, 36)). By analogy to the medial assay, these cell-free assays employ mut-Golgi that contain VSV G-protein and are defective in producing N-linked carbohydrate modifications associated with either the trans Golgi (galactosylation) or TGN (sialylation). Transport between mut- and wt-Golgi is thus measured by the incorporation of 3H-galactose (trans assay) or 3H-sialic acid (TGN assay) into VSV G-protein carbohydrates during the in vitro incubation. Although the medial, trans, and TGN assays require similar components, the rate of transport, sensitivity to inhibitors, and optimal transport conditions are assay-specific (27). This might indicate that there are some mechanistic differences in transport at different levels of the stack.

Free intermediates were generated in a 10-min preincubation of wt-Golgi and tested for transport activity in stage II medial, trans, and TGN assays using the appropriate mut-Golgi and radiolabeled nucleotide sugars. Unexpectedly, the level of functional intermediates released from wt-Golgi was highest in the medial assay, lower in the trans assay, and negligible in the TGN assay (Fig. 2A). The same profile was
obtained in a comparable unfractionated transport reaction (10,146 respectively. Data are the average of a minimum of two independent experiments and are expressed as the percentage of maximal transport obtained in a comparable unfractionated transport reaction (10,146 ± 169, 7,603 ± 1,554, and 2,656 ± 654 cpm in the medial, trans, and TGN assays, respectively). Data are the average of a minimum of two independent experiments and are expressed as the percentage of maximal transport obtained in a comparable unfractionated transport reaction (10,146 ± 169, 7,603 ± 1,554, and 2,656 ± 654 cpm in the medial, trans, and TGN assays, respectively).

observed with different membrane and cytosol preparations, concentrations of cytosolic protein, and lengths of preincubation (data not shown), indicating that these variables do not contribute to the observed differences. The inability to detect Golgi-enzyme-containing intermediates that are functional in the TGN assay led us to test for the production of functional intermediates containing secretory cargo. When the appropriate wt-Golgi were subjected to a 10-min stage I preincubation and then assayed in stage II with wt-Golgi, no free intermediates were detected that were functional in the medial and trans assays (Fig. 2B). In contrast, intermediates derived from mut-Golgi supported a level of transport in the TGN assay that was more than half that of unfractionated wt-Golgi intermediates (Fig. 2B). This suggests that in vitro transport is mediated by two different types of intermediates on the cis and trans side of the Golgi stack. 

Free Intermediates Fuse with Cisternal Membranes—If the transport intermediates derived from both the wt- and mut-Golgi are physiologically relevant, they should target and fuse with cisternal membranes and not with other vesicular intermediates. This hypothesis was tested by preincubating both wt- and mut-Golgi in separate stage I reactions. For the stage II transport incubation, the free intermediates from a wt-Golgi or mut-Golgi were then mixed with either the free intermediates or residual cisternae from a wt-Golgi or wt-Golgi stage I preincubation, respectively. In the medial and trans assays (Fig. 2C, black and crosshatched bars), free intermediates derived from wt-Golgi fused exclusively with the mutant residual cisternae in the stage II transport reaction. In the TGN assay, there was a corresponding preference for free vesicular intermediates derived from mut-Golgi to fuse with wild type cisternae (Fig. 2D, white bar). Under no condition was transport observed between free intermediates observed. Intermediates containing either Golgi enzymes or secretory cargo thus exhibit the fusion specificity expected for physiological transport intermediates in vectorial transport. 

Intermediates Are Continuously Generated during a Transport Reaction—The continuous production of free intermediates by Golgi membranes was demonstrated by measuring the cumulative release of enzyme-containing intermediates after repeated cycles of wt-Golgi preincubation. In the medial assay (Fig. 3A), seven rounds of wt-Golgi preincubation and re-isolation over a period of 60 min released free intermediates with a cumulative transport activity (closed squares) equivalent to the total transport capacity of the starting membranes (0 min, triangle). The transport activity of the residual cisternae was almost completely exhausted after the final round (60 min, open diamond). Importantly, similar results were obtained when the cisternal membranes were not resuspended between rounds (closed circles), indicating that mechanical disruption of the cisternal membranes during resuspension was not the cause for continued release of free intermediates. 

The cumulative release of free intermediates in the medial assay was half-maximal at ~10 min of cumulative preincubation of wt-Golgi (Fig. 3A, closed squares), and at ~20 min in the trans assay (Fig. 3B, closed squares). These values are identical to the t1/2 for transport in these assays (27), suggesting that the production of intermediates containing medial and trans Golgi enzymes is the rate-limiting step for transport in the medial and trans assays. In contrast, there was almost no detectable cumulative transport activity released from wt-Golgi in the TGN assay (Fig. 3C, closed squares). Thus, functional intermediates containing TGN enzymes do not appear to be produced in the in vitro transport system.

Fig. 2. Distinct vesicular intermediates participate in transport at different levels of the stack. A and B, two distinct types of vesicular intermediates participate in transport on the cis and trans side of the stack. A, wt-Golgi were preincubated for 10 min in stage I, and the isolated free intermediates were assayed for transport in stage II with the assay-specific mut-Golgi for detecting transport of medial, trans, and TGN-localized Golgi enzymes. B, mut-Golgi for the medial, trans, and TGN transport assays were preincubated for 10 min in stage I, and free intermediates were tested for transport activity with wt- Golgi in stage II. C and D, intermediates derived from wt- and mut-Golgi selectively fuse with cisternal membranes. C, free intermediates from a 10-min preincubation of wt-Golgi were mixed with free intermediates or residual cisternae from a 10-min stage I preincubation of the appropriate mut-Golgi to measure transport. D, free intermediates from mut-Golgi were mixed with wt-Golgi-free intermediates or residual cisternae to measure transport. The black, hatched, and white bars indicate transport in the medial, trans, and TGN transport assays, respectively. Data are the average of a minimum of two independent experiments and are expressed as the percentage of maximal transport obtained in a comparable unfractionated transport reaction (10,146 ± 169, 7,603 ± 1,554, and 2,656 ± 654 cpm in the medial, trans, and TGN assays, respectively).

Fig. 3. Vesicular intermediates are continuously generated during an in vitro transport reaction. Preincubated cisternal membranes were re-isolated, resuspended, and subjected to another round of preincubation at the indicated times. The free intermediates released at each time point were directly added to a stage II transport reaction. Cumulative transport was tabulated by adding the transport activity of free intermediates obtained at a given time point to the sum of transport for each of the preceding time points. Data are plotted as the cumulative transport produced by preincubation of either wt-Golgi (closed squares) or mut-Golgi (open squares) in the medial (A), trans (B), and TGN (C) transport assays. Also shown in A is the total transport capacity of unfractionated wt-Golgi (closed triangle, 0 min); the cumulative free intermediates produced by wt-Golgi without resuspension of the cisternal pellet at each step (closed circles); and the transport capacity of residual cisternae after 60 cumulative minutes of preincubation with (open diamond) or without (closed diamond) resuspension at each step. Transport is given as 3H-sugar incorporated into VSV G-protein (cpm × 10⁻⁵). Data are representative of a minimum of two independent experiments.
When the cumulative release of functional intermediates from mut-Golgi was analyzed, virtually all of the transport activity in the TGN assay could be attributed to the production of free intermediates containing VSV G-protein (Fig. 3C, open squares). Only a minor component of such activity was detected in the medial and trans assays (Fig. 3, A and B, open squares). Interestingly, the $t_{1/2}$ for free intermediate generation in the TGN assay (~17 min) was significantly faster than the $t_{1/2}$ for transport (~37 min) (27). This difference suggests that the production of free intermediates from mut-Golgi is not the rate-determining step for in vitro transport to the TGN.

We conclude that there is a uniform mechanism of in vitro transport on the cis side of the stack involving apparent retrograde transport of Golgi enzymes in vesicular intermediates. At the interface with the TGN, however, there is a completely unexpected switch to a mechanism of anterograde transport of cargo in vesicular intermediates. In both cases, the continuous production of these intermediates is sufficient to account for the entire transport activity of cisternal membranes in the in vitro system.

Golgi Enzyme Intermediates Are Abundant in Cells—The hypothesis that the transport intermediates generated in vitro are physiologically relevant would further predict that intermediates with the same specificity for cargo and target destination are also present at steady state in cells. This prediction was tested by using velocity sedimentation of wild type cell homogenates to separate slowly sedimenting intermediates from cisternal membranes and determining whether the slowly sedimenting membranes could substitute for wt-Golgi in the three transport assays.

A typical fractionation profile for wild type membranes is shown in Fig. 4. The cisternal membranes in cell homogenate sedimented in the bottom half of the gradient, as detected by the activity of Golgi galactosyl transferase (Fig. 4A, open circles). Sialyl transferase activity and the uptake of UDP-$^3$H-GlcNAc and CMP-$^3$H-SA were also maximum in these fractions (data not shown). Although the slowly sedimenting membranes in the top half of the gradient (fractions 3–5) contained negligible glycosyl transferase activities, they exhibited the highest transport activity with mut-Golgi in the medial (Fig. 4B, open circles) and trans assays (Fig. 4C, open circles). In contrast, essentially all of the wt-Golgi activity in the TGN assay was restricted to fractions containing cisternal membranes (Fig. 4D, open circles). These data therefore show the specificity predicted by our in vitro analyses.

The abundance of pre-existing functional transport intermediates in cell homogenates was somewhat surprising, given the small amount of pre-existing free intermediates that could be detected with isolated Golgi in the absence of a stage I preincubation (Fig. 1A, 0-min time point). This discrepancy appears to be due to a difference between the density of the vesicle intermediates and Golgi cisternae. Velocity sedimentation of Golgi-enriched membranes (isolated from the same homogenate by equilibrium density centrifugation (19)) revealed only low levels of pre-existing free intermediates (Fig. 4, B–D, closed circles, fractions 2–6). The majority of the wt-Golgi activity in all three in vitro assays reproducibly appeared in the lower half of the gradient with cisternal membranes. This further supports the conclusion that the majority of the free intermediates detected in the in vitro transport system are, in fact, generated de novo. We conclude that there is a strong correlation between the presence of functional transport intermediates containing Golgi enzymes in cell homogenates and the ability of wt-Golgi to generate such functional transport intermediates in vitro.

Cells Contain Functional Intermediates for TGN Transport—The extension of this correlation is that cell homogenates from VSV-infected mutant cells will contain vesicular transport intermediates that are functional in the TGN assay but not in the medial or trans assays. Fractionation of homogenates from VSV-infected CHO Lec 1 and Lec 8 cells, the source for mut-Golgi in the medial and trans assays, respectively, revealed that essentially all of the transport activity coincided with the cisternal membrane fractions (data not shown). This is consistent with the findings of Love et al. (30) in the medial assay. In contrast, functional VSV G-protein-containing intermediates were abundant in fractionated homogenates from VSV-infected CHO Lec 2 cells, the source of mut-Golgi for the TGN assay. The majority of the galactosyl transferase activity was associated with fractions 8–11 (Fig. 5A, open circles), as expected for cisternal membranes. The transport activity of the fractionated homogenate, however, was greatest in fractions 3–8, with only a minor peak of activity associated with cisternal membranes (Fig. 5B, open circles). This broad distribution suggests that the VSV G-protein-containing intermediates may more resemble the pleomorphic tubular carriers operating in post-Golgi trafficking (16) than vesicular carriers. Consistent with this possibility, the VSV G-protein-containing intermediates were extremely labile to sedimentation and reisolation, making further characterization difficult.

For comparison, Golgi-enriched membranes (isolated by equilibrium density centrifugation from the same cell homogenate) were also subjected to velocity sedimentation analysis. Only a minor component of slow sedimenting transport intermediates were detected (Fig. 5B, closed circles). The majority of the transport activity reproducibly coincided with the peak of galactosyl transferase activity (Fig. 5A, closed circles). This suggests that isolation of Golgi cisternal membranes by equi-
Enzyme Intermediates Formed in Vivo and in Vitro Are Indistinguishable—If the transport intermediates formed in vivo and during an in vitro transport incubation are related, their size and density should be the same. To test this hypothesis, a medium speed supernatant of wt CHO cell homogenate, unincubated wt-Golgi, and wt-Golgi that had been subjected to a 20-min stage I preincubation were fractionated by velocity sedimentation, and the fractions were tested for transport activity in the medial assay (Fig. 6A). The transport activity of the unincubated wt-Golgi was found in the lower half of the gradient (Fig. 6A, closed circles), as expected for cisternal membranes. The preincubated wt-Golgi exhibited this same peak of activity, as well as a new peak of activity near the top of the gradient (Fig. 6B, closed squares). This new peak exactly coincided with the activity of transport intermediates in the medium speed supernatant of wt cell homogenate (Fig. 6A, open circles). By this criterion, the functional intermediates formed in vivo and in vitro are indistinguishable.

When similar samples were fractionated by equilibrium density centrifugation, nearly identical fractionation profiles were obtained (data not shown). The Golgi enzyme-containing intermediates produced both in vivo and in vitro equilibrated at a density of ~1.06 gm/ml. This is also the approximate density of the fractions containing the slow sedimenting transport intermediates in the velocity sedimentation gradients of Figs. 4 and 6. Thus, these sedimentation profiles cannot be strictly interpreted in terms of the relative size and shape of the functional transport intermediates. Nevertheless, we can conclude that the Golgi enzyme-containing transport intermediates produced in vivo and in vitro have the same density and that this density is considerably lower than the density of Golgi cisternae (1.16 g/ml) or COPI-coated vesicles (1.19 g/ml) (43).

Golgi Enzyme Intermediates Form without COPI Coats—The steady-state level of free intermediates produced in the in vitro system after a 10-min preincubation is highly dependent on the concentration of cytosol (Fig. 1B, squares). A straightforward explanation for this cytosol requirement might be that the steady-state level of free intermediates is dependent on the amount of Golgi coat proteins available to produce them. COPI is the dominant coat protein associated with Golgi vesicles, and depletion of COPI from cytosol essentially eliminates the production of coated vesicles on cisternae during an in vitro incubation (26). Paradoxically, COPI depletion does not inhibit transport in these in vitro assays (26). It was therefore important to determine whether COPI depletion would suppress the formation of Golgi enzyme-containing intermediates during a stage I incubation.

Wt-Golgi membranes were subjected to a 20-min preincubation with either cytosol depleted of greater than 98% of the endogenous COPI or mock depleted cytosol (Fig. 6D). The transport mixtures were then subjected to velocity sedimentation and each fraction tested for activity in a stage II medial assay. As shown in Fig. 6D, the profiles of wt-Golgi incubated with COPI-depleted cytosol (open circles) and mock depleted cytosol (closed circles) were essentially identical, indicating that COPI coats are not required for the formation of Golgi enzyme-containing transport intermediates.
cargo-containing intermediates form without COPI coats—

The instability of the VSV G-protein-containing intermediates required that an alternative approach be used to analyze the involvement of COPI coats in their formation. We therefore determined whether COPI depletion could suppress the continuous release of transport intermediates during multiple rounds of Golgi preincubation, using the approach illustrated in Fig. 3. In these experiments, the membrane pellets were not resuspended after each preincubation step, because previous studies suggested that incubation of Golgi membranes with COPI-depleted cytosol destabilizes cisternal structure (26). As a control, the cumulative production of Golgi-enzyme containing intermediates in the medial assay was also analyzed under the same conditions (Fig. 7A). As expected from the data in Fig. 6, the cumulative release of intermediates during a preincubation of wt-Golgi with COPI-depleted cytosol (circles) was nearly identical to that observed with mock depleted cytosol (squares) in the medial assay. Significantly, similar results were obtained when the cumulative production of VSV G-protein-containing intermediates from mut-Golgi was analyzed in the TGN assay (Fig. 7B). The production of intermediates was the same in the presence (circles) or absence (squares) of cytosolic COPI for four cycles of incubation over 20 min. After that time, intermediate production occurred at a slightly slower rate in the COPI-depleted reaction. This decline might reasonably be attributed to the greater instability of the mut-Golgi and their intermediates, which could be enhanced by a general membrane destabilizing effect of COPI depletion. In all of these reactions, the continuous harvesting of free intermediates depleted the transport capacity of the cisternal membranes (Fig. 7, open symbols). It thus appears that functional intermediates containing either Golgi enzymes or VSV G-protein are produced by a COPI-independent mechanism in these in vitro systems.

We conclude that there is no correlation between the concentrations of ARFs and COPI, the steady-state level of coated vesicles on the Golgi and the steady-state level of functional transport intermediates produced by the Golgi during an in vitro incubation. The de novo production of free intermediates during these in vitro incubations thus appears to involve a COPI-independent mechanism.

**DISCUSSION**

The mechanism of intra-Golgi transport remains one of the most hotly debated subjects in intracellular organelle trafficking (for reviews see Refs. 1, 2–4, 44). Fluorescence imaging of membrane dynamics in living cells has dramatically expanded our understanding of transport pathways to and from the Golgi but has not resolved the details of membrane trafficking within the complex itself. In vitro reconstitution of intra-Golgi transport has been an extremely useful tool for identifying transport factors. However, the fidelity of reconstituted transport to the in vivo transport process remains a controversial issue, because the unexpected and often paradoxical behavior of the system has frustrated efforts to provide a universally acceptable interpretation of the in vitro transport mechanism. We believe that our findings not only resolve some of these difficulties but reveal novel insights into mechanisms of intra-Golgi transport that have a greater relevance to the in vivo process than previously thought.

**Preformed Transport Intermediates Versus Steady-state Production**—Recent studies have suggested that intra-Golgi transport in modified cell-free systems is mediated by pre-formed transport intermediates, such as Golgi-derived fusogenic membrane fragments (29) or Golgi-associated vesicles that had formed in vivo (42). We believe, however, that the behavior of the cell-free intra-Golgi transport assays described here can only be fully explained by the existence of a steady state of Golgi transport intermediate production and consumption during the in vitro incubation.

Dominguez et al. (29) have proposed that in vitro transport is mediated largely by fusion of Golgi-derived membrane fragments that are produced during cell homogenization. This conclusion was based on their observation that highly intact rat liver Golgi stacks could not substitute for CHO wt-Golgi in the medial transport assay unless subjected to harsh mechanical disruption. This treatment produced slowly sedimenting cisternal fragments that were both enriched in Golgi enzymes and highly fusogenic in the assay. Our data indicate that such fusogenic cisternal fragments make little, if any, contribution to the transport observed between CHO wt- and mut-Golgi in the archetypal in vitro system. After isolation, the bulk of the Golgi enzyme and transport activity of CHO Golgi-enriched membranes is associated with cisternal membranes and not with slowly sedimenting membranes (Figs. 4 and 5, closed circles). Functional transport intermediates are released only after transport incubation (Fig. 1A), and their continual release does not require additional mechanical disruption of the CHO Golgi (Figs. 3A and 7). Lastly, fusogenic membrane fragments generated by mechanical disruption would be expected to contain a random sampling of Golgi enzymes and cargo proteins and be able to fuse with other fragments as well as with cisternal membranes. In contrast, a high degree of selectivity for both content and fusion partner is exhibited by the functional transport intermediates found in cell homogenates and produced by CHO Golgi in vitro (Figs. 2–5). The inability of highly intact rat liver Golgi to function in the in vitro transport assay thus appears to be due to a fundamental difference.
between rat and CHO Golgi that is unrelated to, but circumvented by, fragmenting the membranes.

An alternative proposal suggested that transport intermediates formed in vivo and remaining loosely tethered to isolated Golgi membranes are responsible for transport in the medial assay (42). If this were true in our intra-Golgi transport assays, then the transport activity of preincubated wt-Golgi would be expected to decline during a preincubation, because the pre-existing intermediates would fuse with wt-Golgi cisternae and be consumed. In contrast, we find only a modest decline in transport activity even after a 40-min preincubation of wt-Golgi (Fig. 1A, triangles). Moreover, rather than observing consumption of intermediates during a preincubation, their level initially rises and then remains constant (Fig. 1A, squares). The persistence of these intermediates cannot be attributed to a slow release of attached vesicles from the cisternae, because the transport activity of the residual cisternal membranes is not depleted during the preincubation (Fig. 1A, circles). An excess of intermediates bound to the cisternae can also be ruled out by the fact that transport capacity of the cisternal membranes can be exhausted by repeatedly removing the intermediates during a preincubation (Fig. 3, A–C). We conclude that our data are most consistent with a rapid establishment of a steady state in transport intermediate production and consumption during the preincubation and not the release and fusion of preformed intermediates that would be consumed during the reaction.

It is important to emphasize that there are conditions where pre-existing transport intermediates might account for all of the transport signal in these cell-free intra-Golgi transport assays. The traditional formulation of the medial in vitro assay employs equal volumes of wt- and mut-Golgi, conditions were the transport capacity of wt-Golgi is typically well above saturating for the assay (35). Even though pre-existing intermediates constitute a minor fraction of wt-Golgi transport capacity, their abundance under these conditions of excess capacity might be sufficient to drive optimal transport without de novo generation of intermediates. We have also observed that repeated freezing and thawing of the membranes seems to increase the level of free intermediates while impairing the de novo production of intermediates. It is thus possible that variations in the quality and concentration of wt-Golgi might account for some of the contradictory findings and interpretations that are at the core of the controversy surrounding this cell-free transport system.

Stability of Golgi Membranes during the in Vitro Incubation—A final issue is the possibility that Golgi membranes might spontaneously fragment or disassemble as a consequence of prolonged in vitro incubation. Several considerations indicate that this is an unlikely explanation for the production of functional transport intermediates during these in vitro incubations. First, we and others (45, 46) have demonstrated that Golgi stacks remain intact and exhibit a normal density of buds after 45–60 min of in vitro incubation. Consistent with this observation, the transport activity of Golgi membranes sedimented at moderate g force (1.5 min × 16,000 × g) remains constant for at least 45 min of incubation (Fig. 1A). Second, although depletion of COPI or ARF increases the size and number of cisternal fenestrations, the average number of cisternae per stack and the size of the cisternae is not significantly altered after 20 min of in vitro incubation (26, 27). Third, Golgi breakdown, for example, as occurs upon treatment with mitotic cytosol (47), illimaquione (48), okadaic acid (49), or amphiphatic peptides (41), results in the inhibition, not activation, of intra-Golgi transport. A final concern is that cytosolic proteases might destabilize the membranes during extended incubations. Fragments formed by such a mechanism are unlikely to be active, however, because proteolysis of Golgi membranes eliminates transport competency (35). It thus seems highly improbable that the production of functional transport intermediates in these assays is a byproduct of destabilizing conditions arising from the in vitro incubation per se.

Two Distinct Types of Intra-Golgi Transport Intermediates—In the past, the analysis of vesicular intermediates in reconstituted intra-Golgi transport has been restricted to transport as detected by complementation of a medial Golgi enzyme defect (19), here termed medial transport. Although functional VSV G-protein-containing vesicular intermediates have been demonstrated in this assay (50), their contribution to in vitro transport appears negligible in comparison to that of vesicles containing medial Golgi enzyme (30). Our analysis of three separate reconstitution systems that measure transport by complementation of medial Golgi, trans Golgi, and TGN-localized enzymes reveals that the generality of Golgi-enzyme transport in vesicular intermediates does not extend to the TGN.

As predicted by the cisternal maturation model for transport, the majority of the in vitro transport in the medial and trans assays can be accounted for by the production of Golgi enzyme-containing intermediates from wt-Golgi, with only a modest contribution by VSV-G protein-containing vesicles from mutant Golgi (Figs. 2 and 3). In contrast, essentially no functional carriers were produced by wt-Golgi that could complement the glycosylation defect in the TGN assay (Fig. 3C). The absence of functional Golgi enzyme-containing intermediates in the TGN assay appears to be completely compensated for by the production of functional VSV G-protein-containing intermediates. For example, in the experiments in Fig. 3, the cumulative incorporation of sialic acid into VSV G-protein when mut-Golgi were preincubated was 0.34 pmol and only 0.04 pmol for preincubation of wt-Golgi. In comparison, the amount of GlcNAc incorporated into VSV-G protein during preincubation of wt-Golgi was 0.55 pmol, and preincubation of mut-Golgi was 0.11 pmol. These estimates of transport efficiency are admittedly crude, but are consistent with expectations if the observed mechanistic differences are real. Unlike the small VSV G-protein-containing vesicles operating in the medial assay (50), functional VSV G-protein-containing intermediates operating the TGN assay are both heterogeneous in size and abundant in cell extracts (Fig. 5). Conversely, functional TGN-enzyme-containing intermediates are neither produced in vitro nor detectable in cell extracts. It thus appears that there is a switch in the mechanism of transport at the boundary between the trans Golgi and the TGN. The sharpness of this boundary may arise from the fact that sialylation is restricted almost exclusively to the TGN in CHO cells (51).

This inability to detect transport intermediates containing TGN enzymes in intra-Golgi transport was surprising because of the report that TGN-specific enzymes are transported from the Golgi to the ERGIC in vitro (42). This discrepancy might be due to a fundamental difference between in vitro transport within the Golgi and from the Golgi to the ERGIC. Alternatively, the prolonged 15 °C infection employed by Lin et al. to trap VSV-G protein in the ERGIC might have also resulted in the accumulation of small amounts of recycling Golgi enzymes in the ERGIC during the infection. These enzymes could be inactive during the infection, because the in vitro activity of nucleotide-sugar transporters and Golgi glycosyl transferases is significantly inhibited at 15 °C. A subsequent in vitro incubation at 37 °C with appropriate nucleotide sugar substrates

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* P. Weidman, unpublished observations.
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would, however, unmask their presence. It is thus possible that the TGN-specific modifications detected by Lin et al. (42) were not due to in vitro transport of TGN enzymes to the ERGIC per se but to activation of enzymes already accumulated in the ERGIC during the 15 °C in vitro infection. Although this explanation remains to be verified, it leads us to believe that the difference between our findings and those of Lin et al. (42) are reconcilable.

The apparent switch in the direction of transport between the trans and the TGN assays observed here, although unanticipated, may have a physiological basis. It has long been believed that there is a physical as well as functional boundary between the cisternal portions of the Golgi complex and the trans-most tubular reticulum that defines the TGN (3, 52, 53). The existence of such a boundary was reinforced by the discovery that TGN marker proteins do not relocate to the ER with other Golgi marker proteins upon treatment with the fungal metabolite, brefeldin A (51, 54). Although the precise nature of this boundary remains elusive, recent tomographic reconstructions of the Golgi in several cell types have provided structural evidence that sorting of anterograde cargo proteins into vesicular/tubular carriers may occur from multiple trans cisternae, as well as the TGN (55, 56). This hypothesis was suggested by the numerous membrane tubules extending from trans cisternae into the region trans of the stack. The VSV G-containing intermediates functioning in the TGN transport assay might therefore be related to these postulated sorting structures on trans Golgi cisternae.

Functional Golgi Transport Intermediates Are Not COPI Vesicles—It is generally believed that vesicle formation requires cytoplasmic coat proteins to drive membrane budding and to select vesicle cargo. The analysis of intra-Golgi transport has thus focused on the role of COPI-coated vesicles, the only known coat protein that participates in vesicle formation at all levels of the Golgi stack (1, 9, 10, 30). The core of the dispute over the fidelity of reconstitution in the cell-free transport assays arose from the unexpected finding that COPI vesicle formation is not required for intra-Golgi transport in vitro (25–28).

Explanations for this apparent discrepancy have included the possibility that non-physiological fusion occurs directly between cisternae when COPI vesicle formation is blocked (25), or that the in vitro system reconstitutes only the fusion of pre-existing Golgi transport intermediates with cisternae (29, 30). The alternative explanation suggested by our analysis is that the de novo generation of functional transport intermediates in cell-free Golgi transport occurs by COPI-independent mechanisms (Figs. 6 and 7). The extents of COPI and ARF depletion attained in this study are sufficient to nearly or completely eliminate COPI vesicle budding on Golgi cisternae (26–28). Nevertheless, suppression of COPI vesicle formation has no effect on the steady-state level of either Golgi enzyme-containing or VSV G-containing intermediates after 20 min of preincubation and only slightly impairs production after prolonged and repeated preincubation (Figs. 6 and 7). Because these functional transport intermediates appear to be unrelated to COPI-coated vesicles, it is not necessary to invoke a switch in the mechanism of in vitro transport to explain the lack of a requirement for COPI or ARF in these assays.

These data also indicate that the COPI vesicles that do form during a normal contribution to in vitro transport incubation make no detectable contribution to in vitro transport. Although COPI vesicles formed in vitro might simply be non-functional, it is just as likely that the acceptor compartment for these vesicles lies outside the Golgi stack, and thus the detection capabilities of these assays. The absence of a requirement for cytosolic ARF in these intra-Golgi transport assays further indicates that other ARF-dependent coat proteins, such as AP1 (57) or the trans Golgi/TGN vesicle coat proteins called GGAs (Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding proteins) (58, 59) are not involved. It is important to note that, even though coated vesicles are abundant on Golgi cisternae after an in vitro incubation, a low but constant density of uncoated vesicles, buds, and tubules are present, even when COPI vesicle formation is blocked (27). It is thus possible that these uncoated elements correspond to the functional transport intermediates detected in these in vitro assays.

Alternatives to COPI-dependent Transport—There is now an in vitro precedent for COPI-independent transport of Golgi enzymes. The constitutive recycling of Golgi enzymes to the ER involves a Rab6-dependent pathway that is distinct from the COPI-dependent pathway to the ER (31, 32). The Rab6 transport intermediates appear as dynamic globular and tubular carriers that not only translocate toward the ER but emerge from and re-enter or retract into the Golgi (32). This behavior, which might be expected for intra-Golgi transport intermediates, has also been observed with Golgi enzyme-green fluorescence protein chimeras in vivo (18). It is thus possible the same Rab6-dependent mechanism, or a similar mechanism regulated by related Golgi Rabs, such as Rab6A’ (60) or Rab33b (61), is responsible for the trafficking of Golgi enzymes within the stack itself. The fact that Rab6 antibodies and Rab6 mutants defective in GTP-binding inhibit the medial cell-free transport assay (62) makes this an attractive possibility.

The morphogenesis of post-Golgi tubular carriers is not well understood and there are as yet no other in vitro precedents for coat protein-independent sorting of anterograde cargo. It has, however, been demonstrated in vitro that sorting of cargo into tubules of the transitional ER can occur in the absence of ER coat proteins (63). If the segregation of secretory cargo and membrane deformation does not necessarily require coat proteins, it is reasonable to expect that other physiologically relevant coat protein-independent mechanisms of sorting and transport remain to be discovered.

Obviously, until such time when both types of transport intermediates generated by the Golgi in vitro can be isolated and characterized, their origin and relationship to physiological carriers remains necessarily speculative. Nevertheless, we believe our analysis indicates a need to look beyond the constraints imposed by ARF and coat protein-dependent transport mechanisms (64). The morphogenesis and dynamics of the pleomorphic carriers that figure so prominently in living cells (65, 66) clearly warrant more consideration in conceptualizing mechanisms for intra-Golgi transport.

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