Exclusion of Golgi Residents from Transport Vesicles Budding from Golgi Cisternae in Intact Cells

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Abstract. A central feature of cisternal progression/maturation models for anterograde transport across the Golgi stack is the requirement that the entire population of steady-state residents of this organelle be continuously transported backward to earlier cisternae to avoid loss of these residents as the membrane of the oldest (trans-most) cisterna departs the stack. For this to occur, resident proteins must be packaged into retrograde-directed transport vesicles, and to occur at the rate of anterograde transport, resident proteins must be present in vesicles at a higher concentration than in cisternal membranes. We have tested this prediction by localizing two steady-state residents of medial Golgi cisternae (mannosidase II and *N*-acetylglucosaminyl transferase I) at the electron microscopic level in intact cells. In both cases, these abundant cisternal constituents were strongly excluded from buds and vesicles. This result suggests that cisternal progression takes place substantially more slowly than most protein transport and therefore is unlikely to be the predominant mechanism of anterograde movement.

Key words: secretion • cisternal maturation • coatomer • cargo • glycosyltransferase

Introduction

What is the role of the numerous COPI (coatomer)-coated transport vesicles (Orci et al., 1986) that bud from the cisternae at all levels of the Golgi stack? Two principal hypotheses have been proposed. In one view, these vesicles transport cargo across a stack of static cisternae in the cis-to-trans (anterograde) direction (Orci et al., 1986, 1989; Ostermann et al., 1993), among other roles they may play (Letourneur et al., 1994). Consistent with this, about half of these Golgi-derived vesicles contain such anterograde-directed cargo; the other half are retrograde-directed, containing receptors for retrieval of escaped ER residents from the Golgi stack (Orci et al., 1997).

An alternative view that recently has become prominent, cisternal maturation/progression (Glick et al., 1997; Glick and Malhotra, 1998; Pelham, 1998; Allan and Balch, 1999), proposes that anterograde transport results from movement of the cisternae themselves; transport vesicles would have no role in anterograde transport, but rather would function exclusively in retrograde transport. Recent observations (Bonfanti et al., 1998) concerning the anterograde transport of procollagen aggregates (which are much larger than can be accommodated by standard size COPI vesicles) suggest that cisternal progression may occur, though on a time scale that seems too slow to account for the transport of most proteins (Bonfanti, L., O. Martella, A. Miranov, and A. Luini. 1999. *Mol. Biol. Cell.* 10: S114a; Volchuk et al., 2000).

To create a cisternal progression, new cisternae would have to be continuously added at the entry (cis) face of the stack, whereas at the exit (trans) face of the stack, the oldest cisterna would be shed as it is converted into post-Golgi and other vesicles. As a result, in the absence of a special mechanism for preventing this, the steady-state residents of the Golgi stack (including glycosyltransferases and other processing enzymes) would be rapidly lost from the trans face. Therefore, all current cisternal progression models (Glick et al., 1997; Glick and Malhotra, 1998; Pelham, 1998; Allan and Balch, 1999) postulate that Golgi residents are continuously retrieved from later to earlier Golgi cisternae. Indeed, it is this essential feature that is connoted by the term maturation. Steady-state Golgi resident proteins are known to cycle through the ER on a time scale of several hours (Storrie et al., 1998), whereas anterograde transport across the stack typically occurs within 10–20 min (Green et al., 1981; Quinn et al., 1984). This means that if cisternal progression is to keep pace with this rapid anterograde transport, the proposed retrograde-directed recycling of Golgi residents would have to be much faster than return to the ER and be directed to earlier cisternae within the Golgi stack (Glick and Malhotra, 1998).

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Because they are the major, if not exclusive, class of vesicle budding throughout the stack, this retrieval is proposed to be carried out by Golgi-derived COPI vesicles moving in the retrograde direction (Glick et al., 1997; Glick and Malhotra, 1998; Pelham, 1998; Allan and Balch, 1999). Furthermore, if transport vesicles have no role in anterograde transport, then most, if not all, of the COPI vesicles budding from the stack are predicted to carry retrograde-moving Golgi residents.

If cisternal progression were the exclusive mechanism of anterograde transport, then Golgi resident proteins must be present in the vesicles at a higher concentration than in the cisternal membranes from which these vesicles bud (Glick et al., 1997). This is the case because when the trans-most cisterna departs, the resident population must be effectively removed together with only a fraction of the membrane surface to leave the anterograde-directed cargo behind in maturing secretory vesicles. On the other hand, if cisternal progression were to occur at a slower rate than most protein transport (i.e., operating in parallel with a faster vesicle transport pathway), then this strict requirement is relaxed. Now, many of the vesicles should still have Golgi residents, but at lower concentrations than in cisternae; how low depending on how slow the progression is. The anterograde vesicle transport model is indifferent to this issue.

These basic predictions of cisternal progression/maturation models have not yet been adequately or convincingly tested. Certainly, it is known that steady-state residents of the cis-Golgi cisternae encounter trans-Golgi processing enzymes during their lifetime, implying that retrograde transport of residents occurs within the stack at some rate in vivo (Hoe et al., 1995; Harris and Waters, 1996; Linstedt et al., 1997; Wooding and Pelham, 1998). However, in the absence of more quantitative data, this fact does not speak to the level of resident proteins in anterograde- or retrograde-directed vesicles.

Measurements have been made of the concentration of certain resident proteins (glycosyltransferases and saccharide-processing enzymes) in COPI-coated vesicles produced in cell-free systems (Sönnichsen et al., 1996; Lanoix et al., 1999). In one study, COPI-coated vesicle fractions were isolated from cell-free incubations of Golgi membranes, and the concentration of resident and other proteins in the vesicles was compared with that in parental Golgi membranes using both electron microscope immunocytochemistry and biochemical determinations, with excellent agreement between these methods (Sönnichsen et al., 1996). The concentrations in the vesicles of the four resident proteins examined ranged from 14 to 30% of that present in cisternae, a result that is inconsistent with the concentration requirement for exclusive anterograde transport by cisternal progression.

However, questions have been raised about this study (Lanoix et al., 1999), because the COPI vesicles were formed in the presence of GTPγS, needed to keep the COPI coats attached. Lanoix et al. (1999) report that vesicles produced with GTP instead of GTPγS have a much higher concentration of residents than starting Golgi membranes. However, since Lanoix et al. (1999) studied an uncoated membrane fraction, it is difficult to prove conclusively that COPI vesicles were obtained, despite the many excellent controls that were reported.

Given the special importance of this measurement for evaluating the significance of cisternal progression for anterograde transport, it is important that such data now be obtained in intact cells, and we do so here. As matters stand, cell-free incubations used to produce COPI vesicles may not, for one or another reason (not limited to use of GTPγS), effectively reconstitute the recycling process for resident proteins; or there might be some systematic contamination artifact in subcellular fractionation used to obtain either coated or uncoated COPI vesicles. All of these uncertainties are avoided by obtaining data from intact cells.

Here, we provide measurements of the concentrations of mannosidase II (Man II)[1] (Burke et al., 1982; Novikoff et al., 1983; Baron and Garoff, 1990; Velasco et al., 1993), and *N*-acetylglucosaminyltransferase I (NAGT I; Kornfeld and Kornfeld, 1985), both residents of medial Golgi cisternae (Nilsson et al., 1993; Rabouille et al., 1995).

Materials and Methods

Cells were fixed in phosphate-buffered glutaraldehyde and processed directly for cryoultramicrotomy after sucrose infiltration, following the protocol of Tokuyasu, (1986). The cryosections were incubated with the respective antibodies, revealed by the protein A gold method using 10-nm gold particles (Roth et al., 1978). Golgi profiles showing stacked cisternae and associated vesicles were evaluated. The following antibodies were used: anti-Man II antibody supplied by Dr. K.W. Moremen (University of Georgia, Athens, Georgia; Moremen and Robbins, 1991; Moremen et al., 1991), dilution 1:50; NAGT I tagged with *myc* was detected using the 9E10 mAb (Evan et al., 1985), dilution 1:2,000, followed by rabbit anti–mouse IgG, diluted 1:400. COPI was labeled with the anti-p36 (ε-COP) antibody (Hara-Kuge et al., 1994), dilution 1:400. Double-labeling was carried out by exposing the sections to a mixture of rabbit anti–Man II and mouse anti–β-COP (M3A5 from the late Dr. T. Kreis, University of Geneva, Geneva, Switzerland), followed by washing with PBS and labeling with goat anti–rabbit and goat anti–mouse IgG coupled to gold of 15 and 10 nm, respectively. Cryosections were examined and photographed in a Philips LS420 electron microscope with calibrated magnifications. Quantitative evaluation followed the procedures of Orci et al. (1997).

Results

To quantitatively establish the concentration of Golgi resident proteins in vesicles relative to cisternae in intact cells, we used immunogold particles directed against two well-characterized resident Golgi proteins, Man II and NAGT I.

As expected, Man II immunogold labeling in intact pancreatic β cells was essentially restricted to the Golgi stack (Fig. 1 and Table I). We limited the quantification of the density of gold particles ($n/\mu m^2$) to those Golgi cisternae that were positive (as distinct from the Golgi area as a whole) and to those 70–90-nm vesicles that were immediately adjacent (lateral) to the labeled cisternae. The lateral vesicles do not migrate significantly in the cis-trans direction away from their budding sites (Orci et al., 1997, 1998) due to tethering (Orci et al., 1998; Sönnichsen et al., 1998), and based on earlier studies are almost exclusively COP I vesicles, many of which have already uncoated (Orci et al., 1997). The density of gold particles over vesicles lateral to Man II-staining cisternae (7 ± 3 gold particles/μm^2) was not significantly above the background level of mitochon-

[1]*Abbreviations used in this paper:* Man II, mannosidase II; NAGT I, *N*-acetylglucosaminyltransferase I.

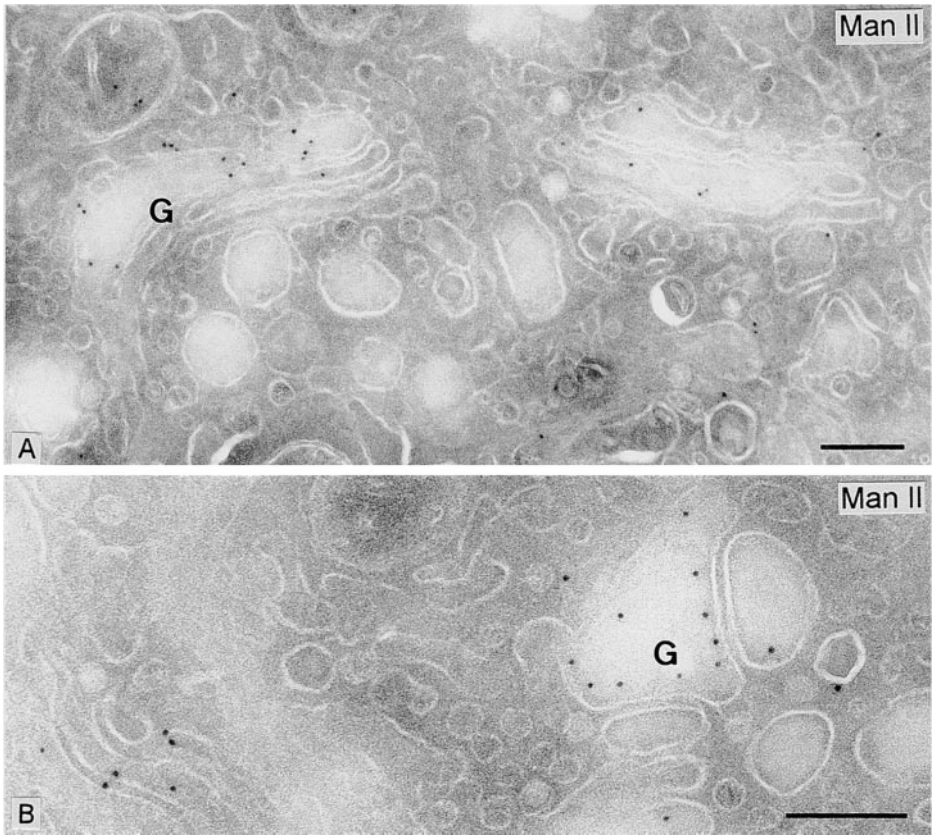

Figure 1. Immunogold labeling of Man II in insulin cells. A, The immunogold particles are predominantly associated with the cisternal profiles of the Golgi complex. Two neighboring Golgi complexes (G) are indicated. Surrounding cisternae-associated Golgi vesicles are virtually unlabeled. B, Detail of one dilated Golgi cisterna showing immunogold associated with the inner aspect of the limiting membrane; the surrounding vesicles are free of labeling. For quantitation, see Table I. Bars, 0.2 μm.

drial staining (8 ± 1 gold particles/μm^2; see Table I), and only 3% of these lateral vesicles had a gold particle (including background labeling), setting an upper limit. By contrast, Man II was present at 91± 6 gold particles/μm^2 in positive cisternae. Therefore, the concentration of Man II in lateral vesicles cannot be greater than (7/91) = 7.7% of that in parental cisternae.

The presence of Man II in cisternae and its virtual absence in lateral COPI vesicles is emphasized in Fig. 2, A–C. A and B show Golgi stacks in a comparable orientation stained either for Man II or for ϵ-COP, respectively. The segregation of the overall labeling is evident. Double-labeling experiments (Fig. 2 C) confirmed that COPI vesicles have low levels of Man II, as compared with cisternae. Quantitative evaluation revealed that 42 ± 4% of the lateral vesicles are COPI positive and another 4 ±1% are Man II positive, but no vesicles labeled for both antigens (145 vesicles counted on 17 different Golgi stacks).

The virtual absence of staining for Man II in the lateral COPI vesicles is unlikely to be due to a systematic inability to detect antigens in these vesicles (versus in the cisternae) because of their much smaller size, since a number of antigens have previously been detected and quantified in COPI buds and vesicles (Sönnichsen et al., 1996; Orci et al., 1997), and because the labeling density for Man II in whole versus mitotically fragmented Golgi complex (the fragments frequently being in the size range of COP I vesicles) are similar (Sönnichsen et al., 1996).

A similar analysis was performed on sections of HeLa cells expressing an epitope-tagged NAGT I (Nilsson et al., 1993). Most of the labeling was restricted to the medial Golgi cisternae (Fig. 3 and Table I), and was present in these Man II-positive cisternae at a density of 214 ± 11 gold particles/μm^2. Only 6 ± 2% of the lateral vesicles were labeled, and much of this labeling (17 ± 4 gold particles/μm^2) was due to background (10 ± 2 gold particles/

Table I. Localization of Man II and NAGT I in Golgi Cisternae and Adjacent 70–90-nm Diameter Vesicles

	Density of labeling	
Enzymatic/antigenic marker	Man II (insulin cells)	NAGT I (HeLa cells)
	Gold particles/μm^2 ± SEM	*Gold particles/μm^2 ± SEM*
Labeled Golgi cisternae	91 ± 6	214 ± 11
Vesicles lateral to labeled cisternae	7 ± 3	17 ± 4
Buds on labeled cisternae	17 ± 10	24 ± 14
Mitochondria (e.g., background)	8 ± 1	10 ± 2

20 Golgi areas were evaluated in each case. A total of 3 ± 1% and 6 ± 2% of the vesicles lateral to the Golgi cisternae are labeled for Man II and NAGT I, respectively. For buds, the corresponding numbers were 7 ± 4% and 9 ± 6%.

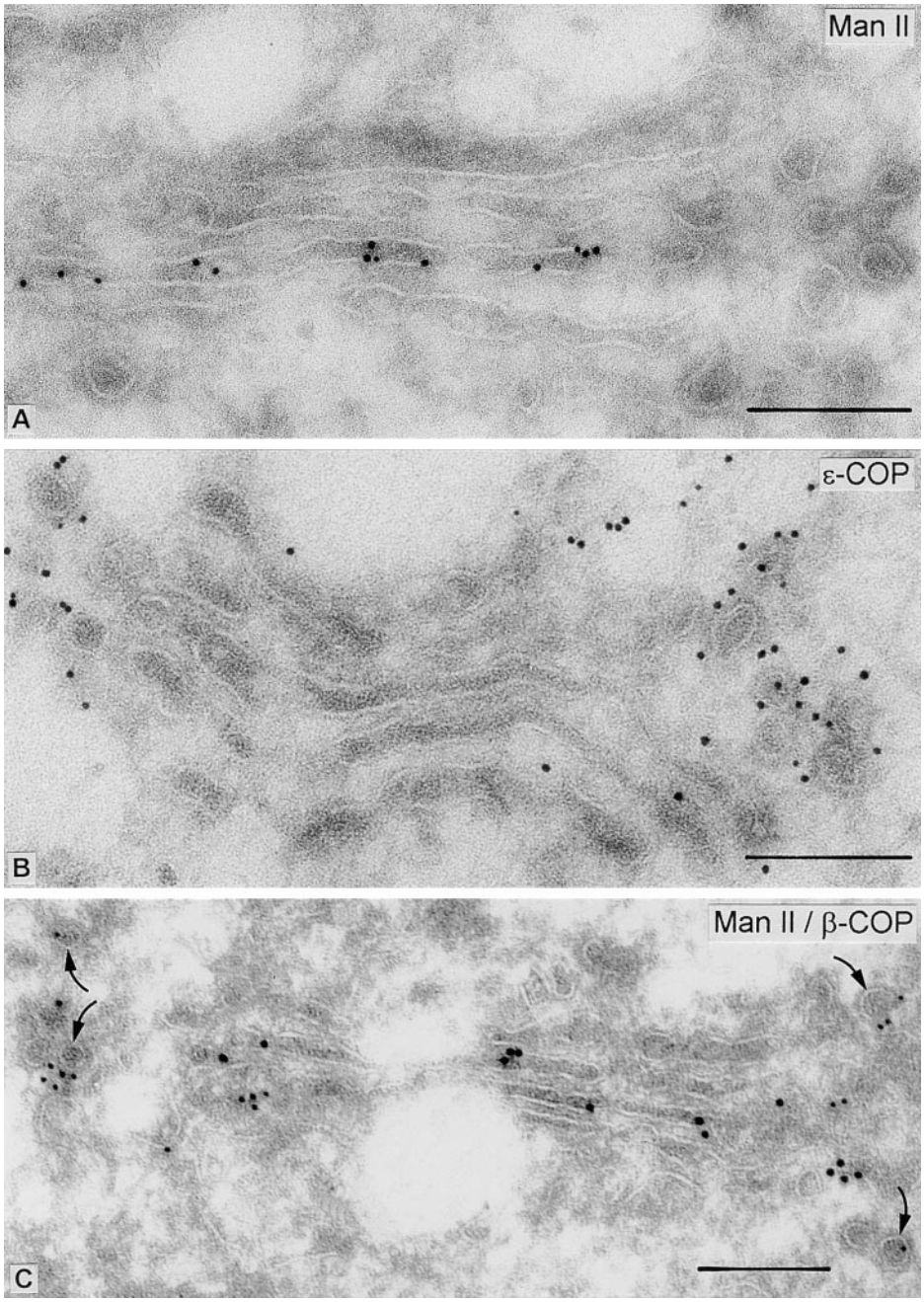

Figure 2. Immunogold-labeling of Man II and COPI (ε-COP) in fibroblasts. The comparable orientation of the Golgi stacks in cryosections A and B shows that Man II staining is present on medial cisternae, but is absent from vesicles lateral to the cisternal tips (A), whereas COPI-labeling has the reverse distribution, being localized to the vesicles lateral to the Golgi stack (B). C shows double-labeling with Man II and β-COP antibodies. The large (15 nm) gold particles labeling Man II are confined to cisternae, whereas the surrounding vesicles (arrows) stain for β-COP (10-nm gold). Quantitative evaluation reveals that 42 ± 4% of vesicles are COPI positive, 4 ± 1% are Man II positive, and no colocalization was observed (145 vesicles counted on 17 different Golgi stacks). Bars, 0.2 μm.

μm^2 over mitochondria). The concentration of NAGT I in lateral vesicles is therefore not greater than (17/214) = 8.0% of that in parental cisternae.

To be certain that these resident proteins are excluded from Golgi-derived COPI vesicles, we separately quantified immunostaining of buds forming from (but still continuous with) the Golgi cisternae (Table I). As would be predicted, the buds had dramatically less Man II and NAGT I than the cisternae that they are in the process of budding from. Earlier studies have established that all of these budding profiles are COPI-coated (Orci et al., 1997).

Discussion

Although we are unaware of previous studies using EM of sections of intact cells that quantify or specifically call attention to the presence or absence of steady-state resident proteins of the Golgi stack in lateral vesicles and buds, micrographs consistent with our result, a striking exclusion of residents from vesicles surrounding positive cisternae, are to be found in some earlier work. For example see: Figure 4 in Novikoff et al. (1977) and Figure 4 in Griffiths et al. (1989), both concerning cytochemical staining of TPPase; Figure 5 in Burke et al. (1982), concerning immunogold labeling of a then-unidentified 135-kD protein subsequently

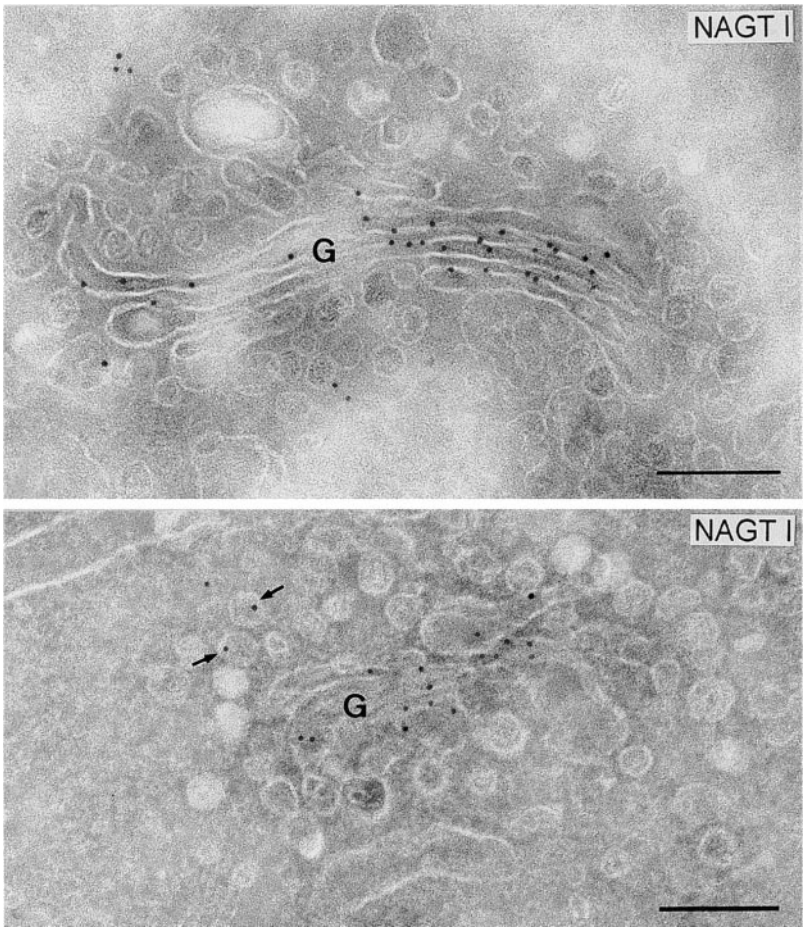

Figure 3. Gold labeling for *myc*-tagged NAGT I expressed in HeLa cells (Nilsson et al., 1993) is largely restricted to the cisternal profiles of the Golgi complex (top and bottom panel). The arrows indicate two labeled vesicular profiles in the bottom panel. For quantitation, see Table I. Bars, 0.2 μm.

identified as ManII (Baron and Garoff, 1990); and Figure 5 in Nilsson et al. (1993), concerning both NAGT I and galactosyltransferase.

Other studies have reported the presence of Man II and additional steady-state Golgi resident proteins in highly purified COPI-coated vesicle-containing fractions obtained from cell-free incubations of Golgi membranes (Ostermann et al., 1993; Sönnichsen et al., 1996) or partially purified from cell homogenates (Love et al., 1998), in the latter case without morphological evidence of purity. The concentration of resident proteins in COPI-coated vesicles relative to cisternae was measured in only one study (Sönnichsen et al., 1996) and did not exceed 30% of that in cisternae. Since COPI-coated vesicle preparations are invariably contaminated with sheared-off COP-coated buds that contain portions of parental cisternae (see Figure 3 D in Ostermann et al. 1993), which contribute resident proteins as an artifact of fractionation, the presence of resident proteins in bona fide COPI vesicles is likely overestimated by analyzing isolated COPI vesicles, whether by biochemical or morphological methods.

Recently, Lanoix et al. (1999) have analyzed the resident protein (glycosyltransferase) content of an uncoated membrane fraction produced from Golgi membranes in vitro (in the presence of GTP) that is thought to be derived from COPI-coated vesicles, and compared this with bona fide COPI-coated vesicles prepared with GTPγS. They report (Table IV in Lanoix et al., 1999) a 9.6-fold higher concentration (protein/phospholipid) of NAGT I and a 4.8-fold higher concentration of Man II, in the uncoated (GTP) vesicles than in the starting Golgi fraction and an exclusion of residents in the GTPγS -prepared coated vesicles. There was no corresponding enrichment in anterograde-directed cargo in the GTP-produced uncoated vesicles (1.7-fold for pIgR) or in bona fide COP I-coated vesicles made with GTPγS (1.2-fold). In contradiction to this, Nickel et al. (1998) analyzed bona fide coated COPI vesicles produced in the presence of GTP versus GTPγS, and report that anterograde-directed cargo is up to 50-fold more concentrated in GTP-prepared as compared with GTPγS-prepared coated vesicles. It is possible that the explanation for this serious discrepancy with regard to the concentration of anterograde cargo is that the GTP-prepared uncoated membrane fraction of Lanoix et al. (1999) is for some reason not derived mainly from once-coated COPI vesicles, despite reasonable arguments made to the contrary; the establishment of purity of morphologically distinct coated vesicles is inherently more reliable than uncoated vesicles that look in the electron microscope like many other types of vesicles. Also, since the steady-state resident protein population of the Golgi accounts for ~95% of the total protein mass (Quinn et al., 1984; Griffiths et al., 1989), it is not evident how further concentration within COPI vesicles, as claimed by Lanoix

et al. (1999), would be possible. This raises the possibility of a systematic error in quantitation in these studies that warrants further investigation.

All of these potential difficulties, including possible differences due to the use of GTP versus GTPγS (Lanoix et al., 1999), that may be inherent to fractionation or cell-free approaches are circumvented by measurements made on sections of whole cells, as we have done. Our results set a conservative upper limit for the concentration of steady-state Golgi residents in Golgi-associated COPI vesicles in vivo at ~8% of their prevailing concentration in parental cisternae. This result is consistent with the previously established upper limit of ~14–30% set by analyses of highly purified and morphologically pure COPI coated vesicle fractions (Sönnichsen et al., 1996).

The low level of resident proteins in COPI vesicles observed in vivo, and by Sönnichsen et al. (1996) in vitro is consistent with a role for these vesicles in bidirectionally redistributing steady-state residents within the stack (Pelham and Munro, 1993; Hoe et al., 1995; Harris and Waters, 1996; Linstedt et al., 1997; Wooding and Pelham, 1998) to achieve their asymmetric steady-state distributions (Rabouille et al., 1995) via a dynamic equilibrium (Rothman and Wieland, 1996), since in principle this equilibrium need not occur on the time scale of transport of cargo in the anterograde direction, except when cisternal progression is the sole means for anterograde transport.

In summary, our results are inconsistent with the view that cisternal progression is the sole or predominant means of anterograde transport, as this model does, as a matter of principle, require that the density of Golgi enzymes be at least as high in COPI vesicles as in the cisternal membranes (Glick et al., 1997). While our results are inconsistent with the possibility that cisternal progression occurs in lock-step with anterograde transport, they do not speak against the possibility that cisternae may progress on a substantially slower time scale than vesicle transport, as is independently indicated by other studies (Bonfanti et al., 1998; Bonfanti, L., O. Martella, A. Miranov, and A. Luini. 1999. *Mol. Biol. Cell.* 10:S114a; Volchuk et al., 2000).

We thank Dr. K.W. Moremen for kindly providing the Man II antibody. and Dr. G.B. Warren for kindly providing the HeLa cell line expressing epitope-tagged NAGT I.

This work was supported by grants from the Swiss National Science Foundation (to L. Orci) and from the National Institutes of Health (to J.E. Rothman).

Submitted: 19 April 2000
Revised: 19 July 2000
Accepted: 21 July 2000

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