

Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport

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A cisternal progression mode of intra-Golgi transport requires that Golgi resident proteins recycle by peri-Golgi vesicles, whereas the alternative model of vesicular transport predicts anterograde cargo proteins to be present in such vesicles. We have used quantitative immuno-EM on NRK cells to distinguish peri-Golgi vesicles from other vesicles in the Golgi region. We found significant levels of the Golgi resident enzyme mannosidase II and the

transport machinery proteins giantin, KDEL-receptor, and rBet1 in coatamer protein I-coated cisternal rims and peri-Golgi vesicles. By contrast, when cells expressed vesicular stomatitis virus protein G this anterograde marker was largely absent from the peri-Golgi vesicles. These data suggest a role of peri-Golgi vesicles in recycling of Golgi residents, rather than an important role in anterograde transport.

Introduction

The Golgi complex has a pivotal role in the secretory pathway by acting as the central organelle through which newly synthesized proteins pass en route to their final destinations. However, a century after the discovery of the Golgi (Golgi, 1898) and 50 years since the first electron microscopical visualization (Dalton and Felix, 1954) fundamental questions concerning Golgi traffic remain unsolved. Two, not mutually exclusive, models have been launched to explain the movement of newly synthesized proteins through the Golgi stack. The vesicle shuttle model implies that small vesicles formed at the Golgi cisternal rims mediate the forward transport of cargo proteins to the next cisterna. The most likely candidates to mediate such transport are the numerous vesicles at the rims of the Golgi stack, which bud from the Golgi cisternae

in a coatamer protein (COP)*-I-dependent manner (Orci et al., 1986; Malhotra et al., 1989). In this model, each of the more or less stationary Golgi cisternae contains a specific set of resident enzymes, which explains the polarized distribution of these proteins over the stack (Farquhar and Palade, 1981; Rothman, 1994; Rothman and Wieland, 1996; Schekman and Orci, 1996; Orci et al., 1997; Nickel and Wieland, 1998). By contrast, the cisternal maturation or progression maturation model proposes that import of endoplasmic reticulum (ER)-derived membranes at the cis-Golgi side together with the formation of transport vesicles at the trans side of the Golgi results in the progression of entire cisterna from cis to trans (Beams and Kessel, 1968; Glick et al., 1997; Mironov et al., 1997; Allan and Balch, 1999). This model has proven an elegant explanation for the anterograde intra-Golgi transport of large macromolecular complexes and particles, which do not fit in the small sized peri-Golgi vesicles (Melkonian et al., 1991; Becker et al., 1995; Bonfanti et al., 1998).

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*Abbreviations used in this paper: COP, coatamer protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; IEM, immuno-EM; KDELr, KDEL receptor; Man, mannosidase; VSV-G, vesicular stomatitis virus protein G; VTC, vesicular tubular cluster.

The pros and cons of the two models have been discussed in many recent reviews (Bannykh and Balch, 1997; Farquhar and Hauri, 1997; Mironov et al., 1997; Füllekrug and Nilsson, 1998; Nichols and Pelham, 1998; Nickel and Wieland, 1998; Pelham, 1998; Allan and Balch, 1999; Glick, 2000; Pelham and Rothman, 2000). A key issue remains the identity of the peri-Golgi vesicles. An implication of the cisternal maturation model is that to maintain a polarized distribution of and withhold Golgi enzymes from moving along with the anterograde membrane flow, these enzymes must be transported from a trans-moving cisterna backward to a more cis-positioned cisterna. Thus, the vesicle shuttle model predicts that COPI-coated peri-Golgi vesicles contain anterograde transported cargo, whereas the cisternal progression model proposes that they contain Golgi resident proteins and move retrograde. Therefore, it is of fundamental importance to determine the contents of COPI-coated peri-Golgi vesicles and investigate whether they might exist in distinct classes.

So far, studies aimed at elucidating the cargo of peri-Golgi vesicles have resulted in conflicting data. In an early study, a subpopulation of COP-coated vesicles derived from isolated Golgi fractions were found to contain vesicular stomatitis virus protein G (VSV-G), which is considered as a marker for anterograde transported membrane-bound cargo protein (Orci et al., 1986). Indeed, COPI vesicles purified from VSV-infected cells proved to be functional transport intermediates, which could deliver VSV-G protein to acceptor membranes *in vitro* (Ostermann et al., 1993). In a more recent immuno-EM (IEM) study on pancreatic β cells, the precursor secretory protein proinsulin was found in a subpopulation of the COPI-coated peri-Golgi vesicles, albeit with lower concentrations than in the Golgi cisternae (Orci et al., 1997). A different subpopulation of COPI vesicles contained high levels of the KDEL receptor (KDELr), which cycles soluble proteins bearing a KDEL retrieval sequence backward to the ER. Based on these differences in content, the authors sug-

gested the existence of two types of peri-Golgi vesicles involved in a bidirectional traffic at the Golgi level (Orci et al., 1997). Recently, this notion was strengthened by the finding that COPI vesicles with VSV-G but not those with KDELr contain the soluble NSF attachment protein receptor (SNARE) protein GOS28, suggesting that these two types of vesicles have distinct fusion specificities (Orci et al., 2000a). Finally, these same authors found that large engineered protein complexes can be included in big containers at the rims of Golgi cisternae, which were supposed to mediate anterograde intra-Golgi transport (Volchuk et al., 2000). However, in contrast to these studies others have failed to find significant amounts of anterograde-directed cargo in peri-Golgi vesicles (Becker et al., 1995; Sönnichsen et al., 1996; Bonfanti et al., 1998). Notably, this was also the case in systems with a high level of secretory protein synthesis such as liver (Dahan et al., 1994). Thus, despite the fact that several cargo proteins have been studied, the role of peri-Golgi vesicles in anterograde transport has remained ambiguous.

Even more uncertain is a possible involvement of COPI vesicles in the transport of Golgi resident enzymes. Ostermann et al. (1993) found that purified COPI vesicles contained Golgi resident proteins. Sönnichsen et al. (1996) confirmed this finding, but quantitative assays showed that anterograde cargo and Golgi enzymes levels in COPI vesicles were low in comparison to the Golgi stacks from which they were derived. By contrast, retrograde recycling proteins such as KDELr and p58/ERGIC 53 were relatively enriched. These data are consistent with immunocytochemical data, which failed to detect significant labeling of Golgi resident enzymes in peri-Golgi vesicles (Orci et al., 2000b). However, in more recent *in vitro* assays it was shown that Golgi resident enzymes are concentrated actively in COPI vesicles (Love et al., 1998; Lanoix et al., 1999). The discrepancy with the study of Sönnichsen et al. (1996) is explained by the finding that the concentration of proteins in COPI vesi-

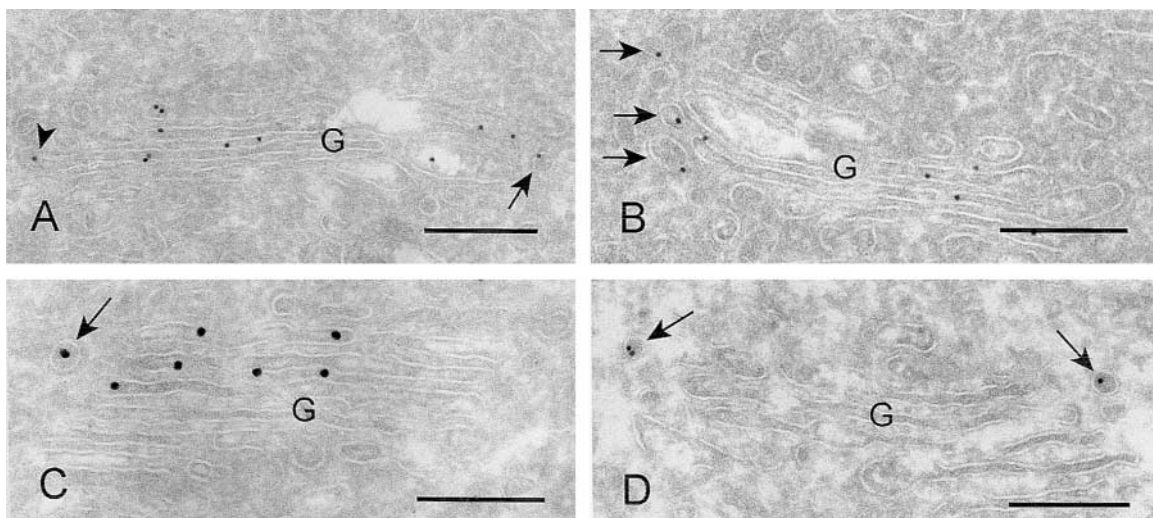


Figure 1. Immunogold labeling of Man II (A, B, and D, 10 nm gold; C, 15 nm gold) on ultrathin cryosections of NRK cells, showing the presence of Man II in the cis-medial Golgi cisternae (G) and in lateral rims of the cisternae and associated vesicular profiles (arrows). Note that the relative distribution of Man II between cisternae and associated vesicles varies considerably between Golgi's. The arrowhead in A points to a Man II-positive Golgi lateral rim with a clearly visible COP coat. The arrow in C points to a Man II-positive vesicle with a visible COP coat. Bars, 200 nm.

Table I. Relative cis-trans distribution of Golgi proteins and VSV-G within the Golgi complex

	G1	G2	G3	G4	G5
Man II	16.3 ± 5.0	42.8 ± 6.4	20.8 ± 3.9	18.3 ± 4.9	1.8 ± 1.3
Giantin	4.7 ± 2.6	21.2 ± 3.8	46.2 ± 4.0	23.4 ± 4.8	4.5 ± 1.9
KDELr	65.3 ± 8.4	24.7 ± 7.6	10.0 ± 5.1		
rBet1	80.1 ± 6.8	13.3 ± 5.8	4.7 ± 4.0	2.0 ± 2.0	
VSV-G ¹⁵⁰⁴⁵ -GFP (20 min, 32°C)					
<i>anti-GFP</i>	16.9 ± 2.8	19.1 ± 2.8	26.1 ± 3.5	20.3 ± 2.7	17.7 ± 3.3

Numbers represent the percentages (mean ± SEM) of total labeling and were obtained by analyzing 25 Golgi complexes for each protein.

cles critically depends on GTP hydrolysis by ARF1 (Love et al., 1998; Nickel et al., 1998; Lanoix et al., 1999; Malsam et al., 1999; Pepperkok et al., 2000). ARF1 hydrolysis is inhibited by GTPγS, which was used in the study by Sönnichsen et al. (1996) to stabilize isolated COPI vesicles. Therefore, no conclusive evidence has been provided for the incorporation of resident Golgi enzymes into peri-Golgi vesicles, leaving a critical implication of the progression maturation model for intra-Golgi traffic unproven.

In this paper, we studied the in situ distributions of Golgi cargo, resident, and machinery proteins by using quantitative IEM and an improved cryosectioning technique (Liou et al., 1996; Martínez-Menárguez et al., 1999). This approach allowed us to directly compare, in the same Golgi's, the in situ distribution of the different proteins under study. Our data show an enrichment of the prototypical Golgi resident enzyme mannosidase (Man) II over VSV-G protein in COPI-coated peri-Golgi vesicles and fit best in the cisternal maturation model for transport through the Golgi complex.

Results

Mannosidase II is present in peri-Golgi vesicles

Man II is a prototypical Golgi resident enzyme with a short transmembrane domain and a luminal catalytic domain involved in N-linked glycosylation (Moremen et al., 1991). To select a cell type yielding high Man II labeling in IEM, we performed immunogold labeling for Man II in various tissues (rat pancreatic islets, exocrine pancreas, and liver) and cell-lines (PC12, CHO, HepG2, and NRK). A high overall labeling density combined with a clear cis-to-trans polarity of the Golgi stacks was found in NRK cells, which we therefore chose for further study. The Golgi complex of NRK cells typically consisted of five cisternae (G1–G5 from cis to trans), although occasionally a larger number of cisternae were observed. In a previous study on NRK cells using the enzymatic immunoperoxidase labeling procedure, Man II was localized to one to three medial cisternae (Velasco et al., 1993). By our immunogold labeling approach, we found the majority of Man II label in G2, lower amounts in G1, G3, and G4, whereas G5 was almost devoid of label (Fig. 1; Table I).

Lateral of the Golgi cisternae, clusters of 50–60 nm diameter vesicles were found, 41% of which bore a visible cytoplasmic COP coat (an example of a COP coat is found in Fig. 1 C). COP coats are clearly distinguishable in cryosections and differ from clathrin coats by a thinner and more regular appearance (for an example of a clathrin coat see Fig. 4 C). A distinction between COPII and COPI coats can only be made by molecular criteria. In agreement with stud-

ies in other cells (Hay et al., 1998; Martínez-Menárguez et al., 1999), we found that COPII labeling was confined to restricted areas, often on ER buds or on vesicles in close vicinity to an ER cisterna (Fig. 2), whereas COPI label was more widely dispersed and also present on the peri-Golgi vesicles and COP-coated rims at the Golgi cisternae (Fig. 3). Notably, significant label of Man II was present in peri-Golgi vesicles (Figs. 1–3, arrows) where it colocalized with COPI (Fig. 3) but not COPII (Fig. 2). Quantitative analysis of double labelings for Man II and either COPI or COPII showed that ~20% of the Man II-positive peri-Golgi vesicles (vesicles within a distance of 200 nm lateral from the stack) was labeled for COPI, and only 2% was labeled for COPII (out of 100 Man II-positive vesicles for each). These data defined the COP-coated cisternal buds and almost all coated peri-Golgi vesicles as the COPI type.

The relative distribution of Man II over distinct Golgi cisternae and associated COPI-coated vesicles was highly variable, ranging from most label in the cisternae to the extreme of all Man II being present in peri-Golgi vesicles (Fig. 1, A compared with D). On average, ~18% of Man II in the Golgi complex (stack plus all peri-Golgi vesicles within a distance of 200 nm lateral from the stack) was present in COPI-coated cisternal buds and COPI-coated peri-Golgi vesicles (Table II). Another 14% was found in lateral rims of cisternae (defined as the last

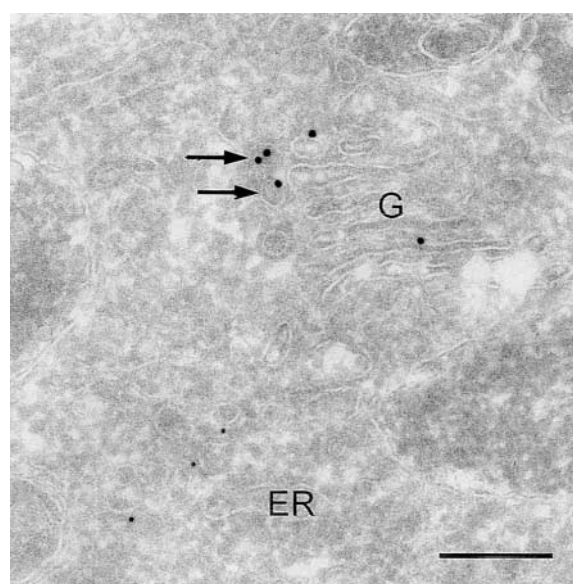


Figure 2. Man II (15 nm gold)-positive peri-Golgi (G) vesicles (arrows) lack COPII (10 nm gold), which is restricted to vesicles close to the ER. Bar, 200 nm.

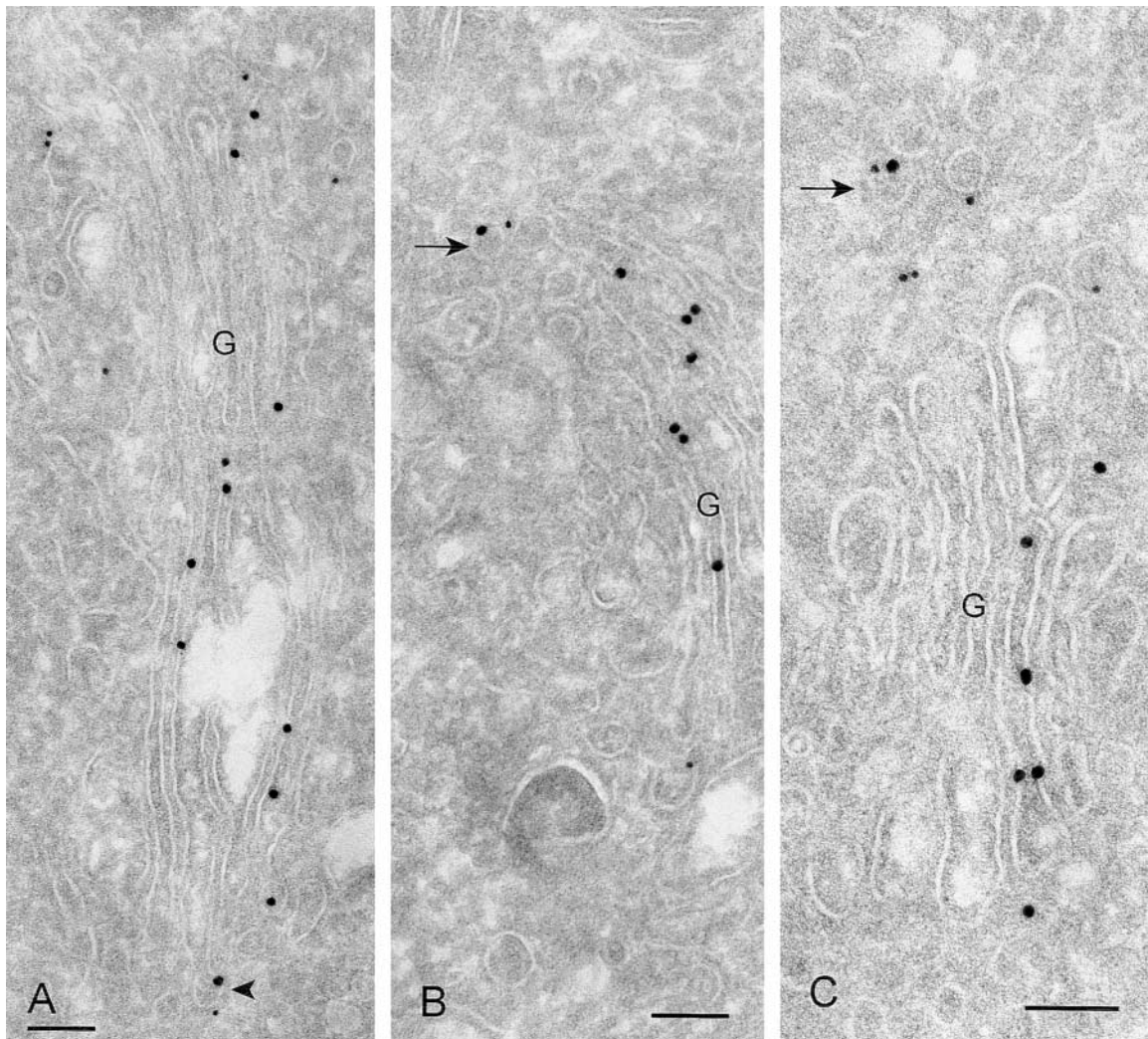


Figure 3. COPI (10 nm gold) colocalizes with Man II (15 nm gold) in lateral rims (arrowhead) of Golgi (G) cisternae and peri-Golgi vesicles (arrows). Bars, 100 nm.

50 nm of a cisterna) on which no COPI coat was visible but which are frequent sites of COPI vesicle formation.

Man II is highly enriched over VSV-G protein in COPI-coated peri-Golgi vesicles

In previous studies, it was reported that the anterograde-directed cargo proteins VSV-G and proinsulin are present in COPI-coated peri-Golgi vesicles (Orci et al., 1986, 1997, 2000a). To directly compare the Golgi distribution of Man II

with that of VSV-G, we transfected NRK cells with the temperature-sensitive mutant of VSV-G with a green fluorescent protein (GFP) tag fused to its cytosolic terminus (VSV-G^{ts045}-GFP). The construct was detected by immunolabeling of ultrathin cryosections with an antibody against GFP. After 2.5 min at the permissive temperature, VSV-G colocalized with KDELr in vesicular tubular clusters (VTCs) but was absent from the Golgi stack (Fig. 4 A). After 5 min release, gold particles could be found in the Golgi complex (unpublished data)

Table II. Relative lateral distribution of Golgi proteins and VSV-G within the Golgi complex

	Golgi stack (cisternae)	Lateral rims (uncoated)	Coated buds and vesicles
Man II	68.9 ± 3.9	13.6 ± 2.7	17.5 ± 2.8
Giantin	58.2 ± 2.6	18.3 ± 2.8	23.5 ± 2.7
KDELr	35.8 ± 4.3	8.9 ± 2.0	55.3 ± 4.5
rBet1	25.6 ± 4.9	15.7 ± 4.0	58.7 ± 5.6
VSV-G ^{ts045} -GFP (20 min, 32°C)			
<i>anti-GFP</i>	93.1 ± 1.4	4.3 ± 1.3	2.6 ± 0.6
<i>anti-VSV-G-lum</i>	94.1 ± 0.8	3.4 ± 0.6	2.5 ± 0.5

Numbers represent the percentages (mean ± SEM) of the total labeling over the distinct membrane categories and were obtained by analyzing 50 Golgi complexes for each antibody.

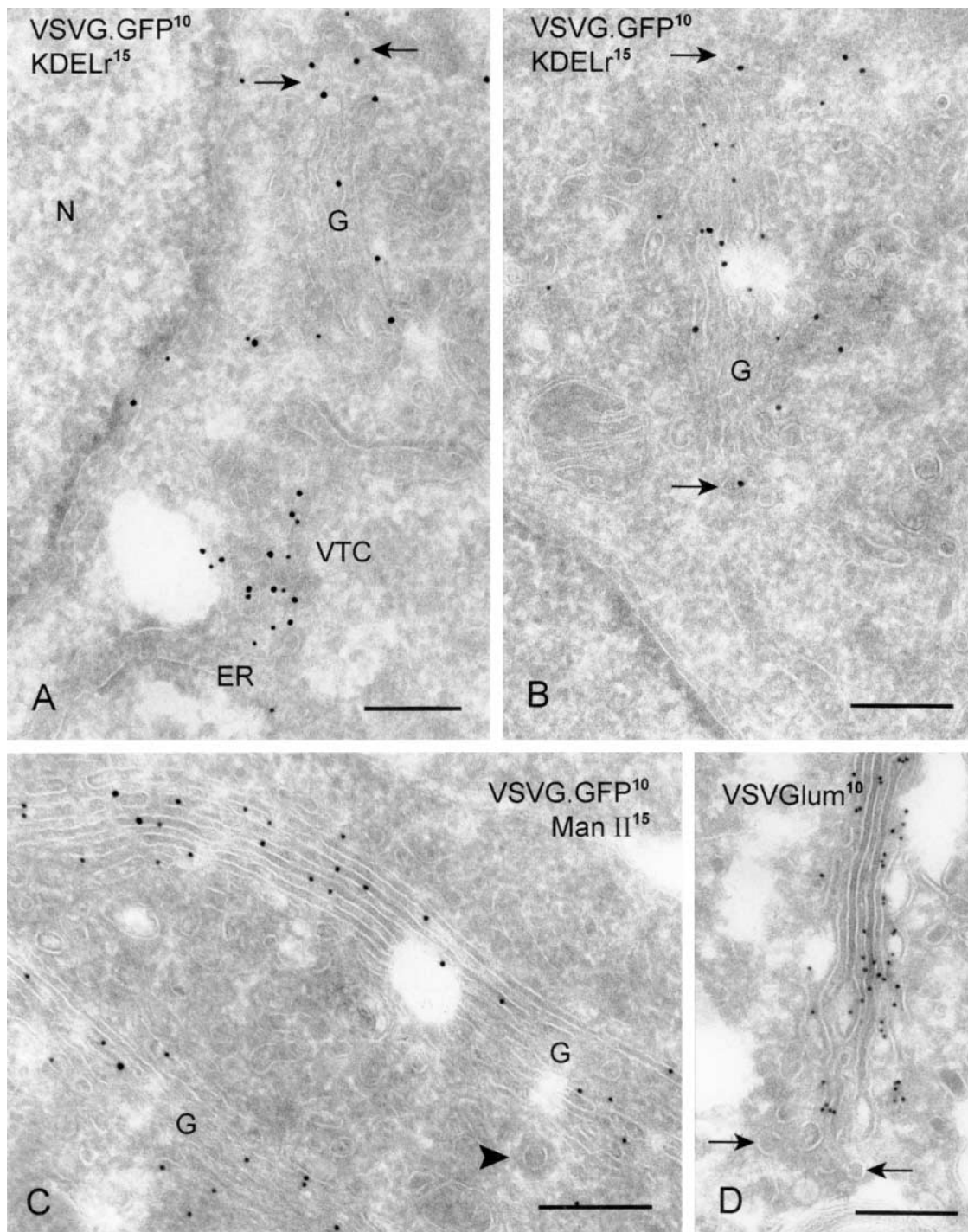


Figure 4. **VSV-G^{ts045}-GFP (10 nm gold) is present in Golgi cisternae of transfected NRK cells but devoid from lateral peri-Golgi vesicles.** In A–C, the protein construct was visualized by using an antibody against the cytosolically exposed GFP tag. In D, an antibody against the luminal domain of VSV-G was used. (A) After 2.5 min at the permissive temperature, VSV-G^{ts045}-GFP colocalizes with KDELr (15 nm gold) in VTCs but is absent from the Golgi complex (G). KDELr is present in Golgi cisternae and in surrounding vesicles (arrows). (B) 20 min after release of the temperature block, VSV-G^{ts045}-GFP is readily detectable in all cisternae of the Golgi complex. Note that KDELr-positive peri-Golgi vesicles do not contain VSV-G^{ts045}-GFP (arrows). (C) Example of a cell with a high expression level of VSV-G^{ts045}-GFP. Man II (15 nm gold) distribution over the Golgi complex is not affected by VSV-G^{ts045}-GFP. The large arrowhead points to a clathrin-coated vesicle. (D) The antibody against the luminal part of VSV-G gives an identical staining pattern to anti-GFP with high levels in the cisternae but no label in the peri-Golgi vesicles (arrows). Bars, 200 nm.

but only after 20 min did the bulk of VSV-G^{ts045}-GFP reach the Golgi complex (Fig. 4 B; Table I), which is why we choose this condition to study the lateral distribution of VSV-G over the Golgi complex. VSV-G^{ts045}-GFP labeling densities dif-

fered considerably between cells, reflecting variation in expression levels (Figs. 4, B compared with C). The distribution of Man II over the Golgi complex was not influenced by VSV-G^{ts045}-GFP expression (unpublished data).

After 20 min release, we found almost similar labeling levels of VSV-G^{ts045}-GFP in G1–G5 (Table I; Fig. 4, B and C). At marked difference with Man II, only minor amounts of VSV-G^{ts045}-GFP were found in peri-Golgi vesicles and at the lateral rims of the Golgi cisternae (Fig. 4 B; Table II). On average, Man II was approximately seven times enriched over VSV-G in COPI-coated Golgi buds and vesicles (Table II). In lateral rims not bearing a visible COPI coat, Man II was three times enriched over VSV-G. To rule out that the scarce VSV-G labeling in COPI vesicles was due to masking of the cytosolic GFP epitope by the COPI coat, we performed a similar analysis of the VSV-G distribution with an antibody directed against the luminal part of VSV-G (Fig. 4 D). As shown in Table II, this resulted in very similar data as with the GFP antibody, leaving peri-Golgi vesicles largely unlabeled.

The relative distribution of Man II over Golgi cisternae and associated vesicles resembles that of recycling proteins but not VSV-G

To compare the labeling patterns of Man II and VSV-G with that of other Golgi-associated proteins, the same approach as used for Man II and VSV-G was performed to establish the cis-trans and lateral Golgi distributions of giantin, KDELr, and rBet1 (Tables I and II).

The membrane protein giantin is present in high concentrations in Golgi-derived COPI vesicles (Linstedt and Hauri, 1993; Sönnichsen et al., 1998) and has been implicated in intra-Golgi traffic. In NRK cells, giantin showed a consistent labeling pattern with the vast majority of label in the Golgi complex and associated vesicles (Fig. 5 and Fig. 6 A). In the Golgi complex itself, giantin was found over all cisternae with highest concentrations in G2–G4 (Table I). In agreement with its proposed function, 24% of Golgi-associated giantin was found in COPI-coated membranes (peri-Golgi vesicles and rims) and 18% in lateral rims on which no visible COPI coat was seen (Table II). Note that these values are in the same order as those for Man II.

The KDELr binds to soluble ER resident proteins bearing KDEL or related sequences and mediates their COPI-dependent retrograde transport from VTCs and Golgi to the ER (Semenza et al., 1990; Majoul et al., 1998). The overall labeling pattern of KDELr varied considerably between cells with the bulk of labeling being present in either VTCs or

Golgi complex or divided over both compartments. KDELr label present in the Golgi complex of NRK cells was largely restricted to the cis-medial cisternae (Table I). When the lateral distribution of KDELr over Golgi membranes was measured, 55% of total Golgi labeling was localized to COPI-coated membranes (Table II).

Finally, we analyzed the lateral distribution of the SNARE protein rBet1, which has been implicated in membrane fusion events early after the formation of ER transport vesicles (Hay et al., 1997, 1998; Zhang et al., 1997; Chao et al., 1999). Consistent with previous studies, the bulk of rBet1 labeling was found in VTCs (Figs. 5 and 7). When double labeled with giantin, rBet1 clearly labeled a distinct population of membranes (Fig. 5). In the Golgi stack, rBet1 was present predominantly in G1 and G2 (Table I). Assessment of its lateral distribution within the Golgi complex showed that almost 60% of rBet1 located to COPI-coated buds and peri-Golgi vesicles (Table II).

Taken together, the data in Tables I and II show that high percentages of the machinery proteins giantin, KDELr, and rBet1 are present in Golgi-associated COPI-coated membranes and lateral rims. The lateral distribution of the Golgi resident enzyme Man II resembles that of the machinery proteins, albeit with a higher steady-state distribution in the Golgi cisternae. Notably, the anterograde-directed cargo protein VSV-G shows an entirely different distribution, being relatively excluded from COPI-coated buds and vesicles.

Man II and giantin concentrations in peri-Golgi vesicles are at least the same as in the Golgi stack, whereas VSV-G concentration is significantly lower

To compare the concentration of Man II in the Golgi cisternae with that in the peri-Golgi vesicles, we counted the number of gold particles per μm membrane in 25 Golgi complexes with a clear cis-trans orientation in the section (Table III). In G2, the cisterna with the most Man II, the average labeling density was 1.37 gold/ μm compared with an average of 0.72 gold/ μm for the whole Golgi stack (G1–G5). In COPI-coated peri-Golgi vesicles, an average labeling of 1.13 gold/ μm was found, which is 1.6 times the average Golgi labeling. Since Man II will be present in only a subpopulation of peri-Golgi vesicles (whereas for technical reasons all COPI-coated peri-Golgi vesicles were taken into account; see Discussion), this figure likely represents the lower limit of Man II concentrations in peri-Golgi vesicles.

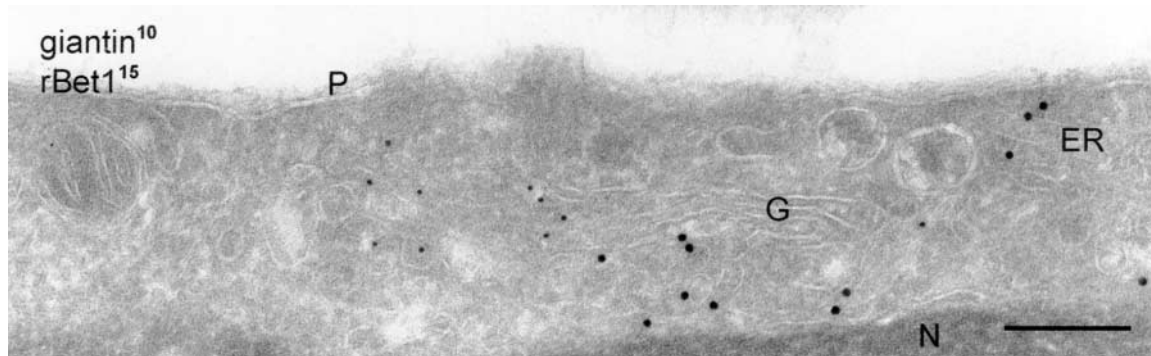


Figure 5. **Giantin (10 nm gold) and rBet1 (15 nm gold) show different distribution patterns.** Most of the cell's giantin is present in the Golgi complex (G) and associated vesicles. rBet1 labels VTC membranes and the cis-Golgi cisterna. Bar, 200 nm.

Table III. Labeling densities (gold/ μm membrane) of Man II, VSV-G, and giantin in Golgi cisternae and COPI-coated peri-Golgi vesicles within 200 nm lateral from the stack

	Man II	VSV-G ¹⁵⁰⁴⁵ -GFP (20 min, 32°C) <i>anti-VSV-G-lum</i>	Giantin
Cisternae			
G1	0.60 \pm 0.16	1.67 \pm 0.39	0.72 \pm 0.31
G2	1.37 \pm 0.17	3.06 \pm 0.75	1.58 \pm 0.28
G3	0.81 \pm 0.16	2.87 \pm 0.46	1.89 \pm 0.41
G4	0.70 \pm 0.19	2.91 \pm 0.56	0.86 \pm 0.26
G5	0.10 \pm 0.08	2.74 \pm 0.44	0.35 \pm 0.20
COPI-coated peri-Golgi vesicles	1.13 \pm 0.38	0.21 \pm 0.12	1.25 \pm 0.32

Numbers represent average labeling densities \pm SEM and were established by analyzing 25 Golgi complexes. Cisternae are numbered from cis (G1) to trans (G5).

Nevertheless, these data clearly show that peri-Golgi vesicles contain substantial concentrations of Man II at least in the same order of magnitude as in the cisternae.

We next performed a similar study for VSV-G (Table III). A considerable labeling density was found in G1, whereas G2–G5 showed higher and equivalent labeling densities. On average, the labeling density of the Golgi cisterna amounted to 2.65 gold/ μm , whereas the labeling density of COPI-coated peri-Golgi vesicles was 0.21 gold/ μm . These data indicate that the concentration of VSV-G in peri-Golgi vesicles is \sim 13 times lower than in the Golgi cisternae and

are strongly in support with an exclusion of VSV-G from COPI-coated peri-Golgi vesicles.

To compare these Man II and VSV-G data to a machinery protein involved in intra-Golgi transport, we finally established the labeling densities of giantin (Table III). Giantin displayed the highest concentrations in G2 and G3, whereas concentrations in G1, G4, and G5 were somewhat lower. The average labeling of the Golgi amounted 1.08 gold/ μm , whereas peri-Golgi vesicles contained 1.25 gold/ μm , indicating a 1.16 times enrichment in peri-Golgi vesicles, which is in the same order as found for Man II.

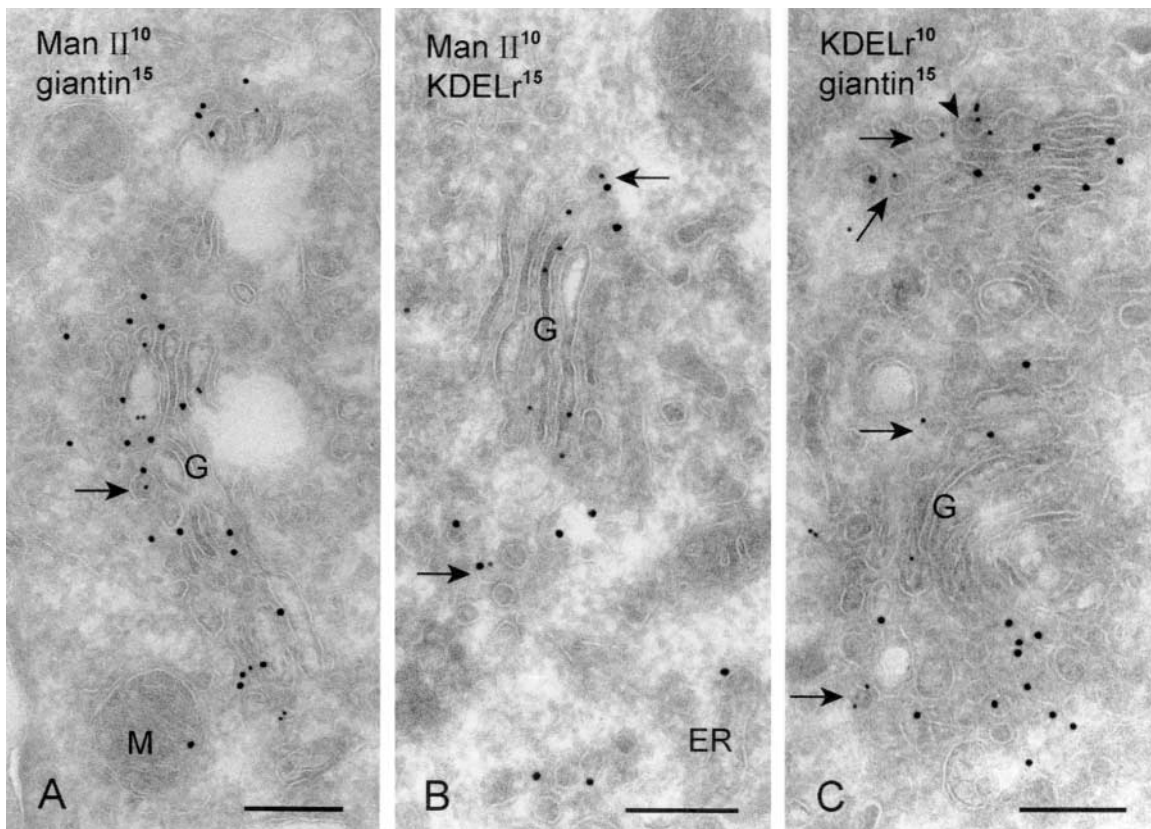


Figure 6. **Man II colocalizes with giantin and KDELr in different populations of peri-Golgi vesicles.** (A) Man II (10 nm gold) colocalizes with giantin (15 nm gold) in peri-Golgi vesicles (arrow). (B) Likewise, Man II (10 nm gold) is regularly found together with KDELr (15 nm gold) in peri-Golgi vesicles (arrows). (C) Despite the high labeling densities of both KDELr (10 nm gold) and giantin (15 nm gold), they are found mostly in different populations of peri-Golgi vesicles. Arrows point to KDELr-positive and giantin-negative vesicles. The arrowhead points to a Golgi cisternal rim that is strongly labeled KDELr and lacks giantin. G, Golgi complex. Bars, 200 nm.

Man II–positive peri-Golgi vesicles contain either giantin or KDELr

COPI vesicles arise from all cisternae, G1–G5 with different marker profiles, which predicts that there will be a heterogeneity in their contents. To test this, we performed a series of double labeling experiments. First, we analyzed Man II–positive vesicles for the presence of giantin, KDELr, and rBet1 (Table IV). For quantitation, all vesicles within 200 nm from the lateral side of a Golgi stack were analyzed. Of all Man II–positive vesicles, >30% also contained giantin (Fig. 6 A; Table IV). A similar high level of colocalization was obtained for Man II and KDELr (Fig. 6 B; Table IV). Colocalization with rBet1 was clearly less pronounced: only 8% of the Man II–positive vesicles also labeled for this SNARE protein (Table IV). It should be noted that the numbers of peri-Golgi vesicles labeled for KDELr or giantin often exceeded that of Man II. This is reflected in the reciprocal experiments in which KDELr and giantin–positive peri-Golgi vesicles were analyzed for the presence of Man II (Table IV), resulting in a lower percentage of vesicles positive for two markers.

When double labelings for giantin and KDELr were performed, despite the high labeling densities of both proteins, only a very limited level of colocalization, ~5%, was found (Fig. 6 C; Table IV). Since both antibodies recognize cytosolic epitopes, we had to exclude whether this low level of colocalization was caused by sterical hindrance. Using different combinations of gold and sequences of labeling, we found that the average number of Golgi complex–associated gold particles did not change when the two antibodies were used in double labeling with each other or with Man II (on average 7.1 ± 0.7 for giantin and 2.6 ± 0.3 for KDELr on 2% formaldehyde plus 0.2% glutaraldehyde–fixed sections, per condition; 50 Golgi counted). These observations show that the presence of

Table IV. Analysis of the codistribution of marker proteins in peri-Golgi vesicles

I	II			
	Man II	Giantin	KDELr	rBet1
Man II		32.0 ± 2.3	30.8 ± 4.1	8.0 ± 1.6
Giantin	13.6 ± 1.3		5.0 ± 2.5	ND
KDELr	17.1 ± 2.6	4.6 ± 2.1		9 ± 3.4
rBet1	6.3 ± 2.6	ND	ND	

Countings comprised 100 Man II-, giantin-, KDELr-, or rBet1-positive vesicles from three different grids. Numbers indicate the percentage (mean \pm SEM) of vesicles positive for the protein indicated under I that also labeled for the protein indicated under II. ND, not determined.

antibody/protein A gold–tagged KDELr did not hamper labeling for giantin, and vice versa, and led us to conclude that KDELr and giantin are indeed present in distinct populations of peri-Golgi vesicles. Since Man II colocalized with both giantin and KDELr, these data also indicate the existence of at least two types of Man II–positive peri-Golgi vesicles, one population containing giantin and another with KDELr.

KDELr–positive peri-Golgi vesicles differ from VTC–derived vesicles by their rBet1 content

Transport vesicle formation from the ER occurs in the Golgi area and at numerous ER exit sites that face so-called peripheral VTCs (Klumperman, 2000). Peripheral VTCs provide an easy identifiable population of VTC membranes that are especially suited to study ER exit. In a previous study (Martínez-Menárguez et al., 1999), we found high levels of KDELr and rBet1 in COPI-coated membranes of VTCs. We now address the question whether rBet1 and KDELr reside in the same COPI-coated VTC membranes. Double labeling of NRK cells showed that in peripheral VTCs $27.0 \pm$

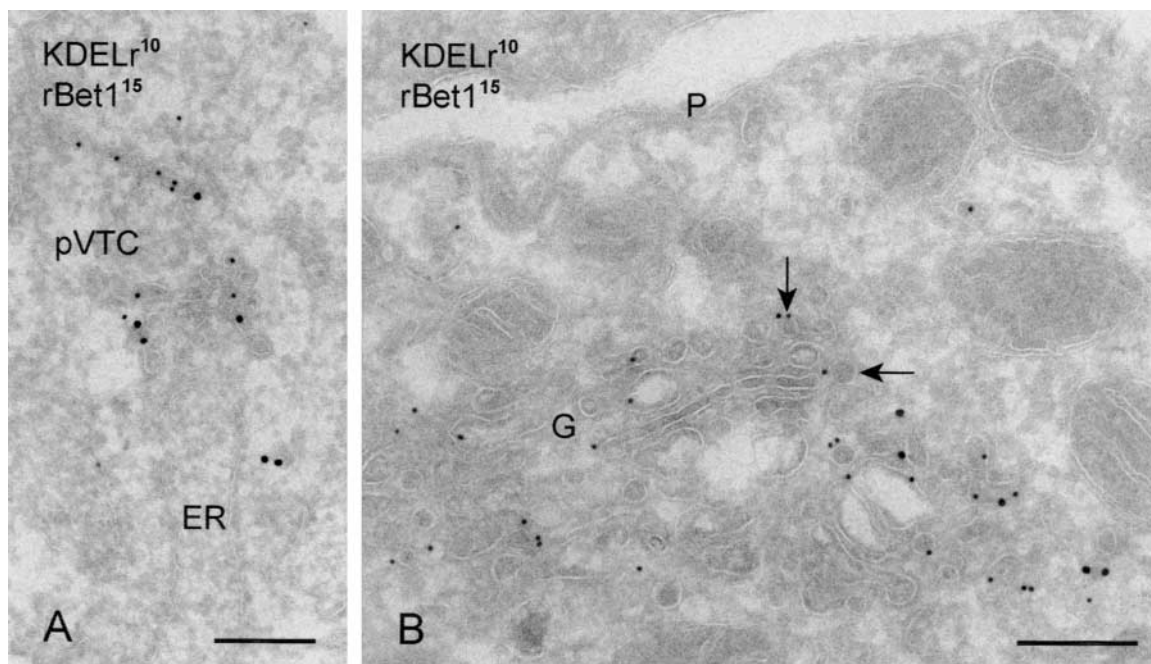


Figure 7. KDELr (10 nm gold) and rBet1 (15 nm gold) colocalize in peripheral VTCs (pVTC) but not peri-Golgi vesicles. (A) High levels of KDELr and rBet1 are found in membranes of a peripheral VTC. (B) In the Golgi (G) region, rBet1 is present in irregularly shaped VTC membranes but absent from peri-Golgi vesicles (arrows). P, plasma membrane. Bars, 200 nm.

1.0% (average \pm SEM) of the KDELr vesicles also contained rBet1 (Fig. 7 A). Since rBet1 is restricted mainly to the cis-Golgi and showed only low colocalization levels with Man II, we next analyzed these percentages for peri-Golgi vesicles (Fig. 7 B). Here, only $9 \pm 3.4\%$ of the KDELr-positive vesicles contained rBet1. These data provide additional evidence that the population of peri-Golgi vesicles analyzed in this study are different from VTC-derived vesicles and indicate a differential SNARE composition of peri-Golgi and VTC-derived COPI-coated vesicles.

Discussion

According to the cisternal progression model of intra-Golgi traffic, the supply of membranes to the cis-side of the Golgi stack in combination with the efflux of membranes at the trans side results in the movement of entire cisternae across the Golgi stack. This model predicts that to withhold resident Golgi enzymes from moving along with the anterograde membrane flow they must be recycled from trans to cis cisternae, supposedly by the vesicles at the lateral rims of the Golgi cisternae. The model also anticipates that anterograde moving cargo is transported by means of Golgi cisternae and is excluded from the peri-Golgi vesicles. Here, we demonstrate in NRK cells that at steady-state significant amounts of the Golgi resident enzyme Man II localize to COPI-coated peri-Golgi vesicles. This observation satisfies a critical requirement for the cisternal progression mode of intra-Golgi transport. In addition, when we evaluated in the same cells, the passage of a cohort of VSV-G protein, a marker for anterograde transport, peri-Golgi vesicles were largely depleted of VSV-G despite the clear presence of VSV-G in neighboring Golgi cisternae. Equivalent results were obtained in low, intermediate, and high VSV-G expressors and with two different antibodies. Hence, both our analyses of a Golgi resident protein and a marker cargo protein are in agreement with a cisternal progression mode of intra-Golgi transport.

Former studies on VSV-G in Golgi fractions (Orci et al., 1986) and proinsulin in β cells (Orci et al., 1997) revealed the presence of these proteins in Golgi-associated buds and vesicles, albeit at lower concentrations than in the Golgi cisternae. We also find VSV-G in peri-Golgi vesicles, but the percentage of total Golgi-bound VSV-G in these vesicles is far less (approximately seven times) than that of the Golgi resident Man II. In addition, labeling density measurements indicated an ~ 13 times reduction of VSV-G concentration in peri-Golgi vesicles when compared with the average labeling density of the Golgi stack. By contrast, Man II and giantin concentrations were enriched slightly in peri-Golgi vesicles. Importantly, all of these measurements were done in the same cells, ruling out that these differences reflect differential steady-state distributions induced by variations in experimental conditions. Together, these findings argue against the idea that peri-Golgi vesicles are important mediators of anterograde transport and rather illustrate their involvement in recycling of Golgi resident proteins.

The exclusion of cargo protein from COPI-coated membranes emerging from the Golgi complex was shown previously for amylase in exocrine pancreatic cells (Martínez-

Menárguez et al., 1999; Fig. 5 C). In this same study, we found that in VTCs secretory proteins are excluded from recycling COPI-coated membranes compared with recycling proteins such as KDELr and rBet1 (Martínez-Menárguez et al., 1999). This led us to propose the “concentration by exclusion” model, stating that anterograde cargo is concentrated in VTC tubules because COPI-coated vesicles without cargo detach from them (Klumperman, 2000). Such a process is likely to continue in the Golgi cisternae that also generate COPI-coated vesicles. Indeed, we established recently in exocrine pancreas cells that the concentration of secretory enzymes increases from cis to trans Golgi (Oprins et al., 2001). The absence of VSV-G and the presence of recycling proteins in the peri-Golgi vesicles (this work) are in line with these findings. Transport of VSV-G through the Golgi and of many secretory proteins is rapid, in the order of 20 min. However, the cisternal progression of newly synthesized aggregates of procollagen through the Golgi complex seems to occur with slower kinetics (Bonfanti et al., 1998). These kinetic differences have led to the theory that cisternal progression and vesicle shuttle systems may coexist, providing for a slow and a fast mode of transport, respectively (Pelham and Rothman, 2000; Volchuk et al., 2000). However, so far such studies were not performed conclusively in the same cells. In this issue, the paper of Mironov et al. (2001) addresses this question and shows that procollagen and VSV-G do travel with similar kinetics. These and our data do not exclude that a population of peri-Golgi vesicles may be involved in anterograde transport, but the contribution of such a pathway is likely to be a minor one. The converging lines of evidence suggest that cisternal maturation provides the general and principal mode of cargo transport through the Golgi stack.

In a study on COPI vesicles, it is of pivotal importance to distinguish COPI vesicles formed at the Golgi cisternae from COPI-coated VTC membranes, which are morphologically very similar. VTCs often occur cis-lateral from the Golgi stacks and may constitute a large portion of the cell's COPI-coated membranes (up to 70% in secretory cells) (Martínez-Menárguez et al., 1999). Because VTC-COPI membranes contain low levels of Golgi resident enzymes, mixing of VTC and peri-Golgi vesicles will falsely decrease the overall concentration of Golgi residents. In our present study, we defined peri-Golgi vesicles as vesicular profiles within 200 nm lateral from a Golgi cisterna. By evaluating several marker proteins, we put this definition to the test. First, VTCs but not peri-Golgi vesicles were found to contain VSV-G 2.5 min after release of the temperature block. Second, the distributions of giantin, KDELr, and rBet1 clearly set VTCs and peri-Golgi vesicles apart. Finally, peri-Golgi vesicles lacked COPII. Therefore, our definitions allowed us to discriminate in NRK cells peri-Golgi vesicles from COPII and COPI-coated VTC vesicles, which provided an appropriate model system to study the occurrence of Man II in these vesicles. However, our definitions cannot simply be extrapolated to all other cells. For instance, in pancreatic β cells clusters of COPII-coated 50–60 nm vesicles frequently occur lateral from the Golgi stack, whereas the contribution of COPI-coated vesicles to these lateral clusters is relatively low (Orci et al., 1994; unpublished

data). The major portion of COPI label is found in coatomer-rich ER, which is present prominently in β cells. In contrast to our data, it was concluded that in β cells Man II is excluded from peri-Golgi vesicles, although some positive vesicles were observed (Orci et al., 2000b). However, double labeling of Man II with appropriate marker proteins or anterograde cargo was not performed in these cells. Such experiments should be performed to unequivocally identify VTCs, COPII vesicles, and coatomer-rich ER, which are all compartments with low amounts of Man II. An erroneous incorporation of membranes poor in Man II in the category peri-Golgi vesicles might explain the differences between our studies.

The rather broad cis-to-trans distributions of Man II and giantin in the Golgi stack differed from those of KDELr and rBet1, which were found mainly at the cis side of the stacks. Interestingly, Golgi-bound Man II and giantin occurred in the order of 20% in peri-Golgi vesicles, whereas both KDELr and rBet1 scored here >55%. These distributions suggest a correlation between the cis-to-trans Golgi distribution profile of a protein and its occurrence in peri-Golgi vesicles. Indeed, the cisternal maturation model predicts that resident proteins that are transported further toward the trans side of the Golgi relatively prevail in the cisternae compared with cis-located proteins because they behave as cargo and travel via the cisternae until they have reached their destination. Although Golgi cisternae progress from cis to trans, retrieval of a given resident protein likely begins from the cisterna after the peak value, which due to the different labeling profiles over the Golgi predicts a heterogeneous population of peri-Golgi vesicles. Indeed, the combined data in Tables I and IV show this heterogeneity and suggest a relation between the site of sorting and the level of colocalization between proteins. Of the Golgi KDELr, 65% was in G1, which flanks the main site of Man II (G2), whereas giantin peaked in G3. KDELr (G1) and Man II (G2) showed a factor of colocalization similar to that of Man II (G2) and giantin (G3). By contrast, giantin (G3) and KDELr (G1) peaked in more distant cisternae and showed only little colocalization in the peri-Golgi vesicles. Thus, markers with predominance in neighboring Golgi cisternae shared presence in peri-Golgi vesicles.

With respect to machinery proteins present in Man II-positive vesicles, our quantitations revealed at least two populations, one also containing KDELr and the other with giantin. These two types of Man II vesicles might mediate different transport pathways of Man II. Although the precise mode of functioning of giantin has not been established conclusively, the prevailing evidence shows that this protein is involved in intra-Golgi traffic, perhaps by interactions with p115 and GM130 (Sönnichsen et al., 1998; Linstedt et al., 2000; Seemann et al., 2000; Alvarez et al., 2001). The Man II/giantin vesicles are therefore putative candidates for intra-Golgi recycling of Golgi resident enzymes to maintain a polarized distribution over the stack during cisternal progression. On the other hand, the Man II/KDELr vesicles may recycle Man II to the ER. Several studies have suggested a continuous recycling of Golgi proteins to the ER, possibly allowing repetitive restoration in the folding environment of the ER (Storrie et al., 1998; for review see Roth, 1999; Zaal

et al., 1999). The low levels of rBet1 in peri-Golgi vesicles together with the restricted localization of rBet1 to the cis-Golgi argue against a role for rBet1 in intra-Golgi transport. It remains to be established which SNARE and other fusion machinery proteins are involved in the transport of the two putative populations of Man II-positive peri-Golgi vesicles.

In an *in vitro* reconstitution study on vesicle formation from Golgi donor membranes, it was found that Golgi enzymes were 10 times concentrated in the vesicles (Lanoix et al., 1999). Our quantitative analysis showed that 30% of the Man II in the Golgi occurred in lateral rims of the cisternae and in peri-Golgi vesicles. We studied the concentrations of Man II in Golgi cisternae and peri-Golgi vesicles by establishing labeling densities. In both models of intra-Golgi transport (vesicles shuttle and cisternal maturation), it is predicted that Man II is present in a subset of vesicles only. However, the Man II labeling of approximately one to two gold particles per vesicle was insufficient to unequivocally identify the subset of Man II-positive vesicles. Therefore, we included all COPI-coated peri-Golgi vesicles in our analysis, which resulted in a labeling density of the peri-Golgi vesicles in the order of those in cis to medial Golgi cisternae. Thus, despite the fact that our data likely represent an underestimation of the labeling density in peri-Golgi vesicles, they clearly show that Man II concentrations in peri-Golgi vesicles are at least in the order of those in the Golgi stack. The same reasoning goes for VSV-G labeling densities in peri-Golgi vesicles. However, according to the vesicle shuttle model VSV-G is expected in a much larger portion of peri-Golgi vesicles than Man II. Nevertheless, we find an ~ 13 times depletion of VSV-G from peri-Golgi vesicles.

Taken together, our study has identified different classes of peri-Golgi vesicles with marker profiles reflecting those of individual Golgi cisternae. The COPI-coated peri-Golgi vesicles are most likely involved in retrograde and not anterograde transport through the Golgi complex because they contain several Golgi markers such as the Golgi resident enzyme Man II and are depleted of the anterograde marker VSV-G.

Materials and methods

Antibodies

Polyclonal antibodies against the catalytic subunit of Man II were obtained from K. Moremen (University of Georgia, Athens, GA) (Velasco et al., 1993). COPI was detected using a polyclonal antibody against the EAGE peptide of β -COP that was supplied by Dr. J. Lippincott-Schwartz (National Institute of Health, Bethesda, MD). COPII was detected with a polyclonal antibody against sec13 provided by W.J. Hong (Institute of Molecular and Cellular Biology, Singapore). The affinity purified rabbit polyclonal antibody against KDELr (ERD2) was a gift from Dr. I. Majoul (University of Göttingen, Göttingen, Germany) (Majoul et al., 1998). A polyclonal antibody against giantin (GCP-372) (Sohda et al., 1994; Toki et al., 1997) recognizes both full-length giantin and its truncated isoform GCP-372 and was provided to us by Dr. Y. Ikehara (Fukuoka University School of Medicine, Fukuoka, Japan). Polyclonal anti-GFP was provided by Dr. G. Warren (University of Yale, New Haven, CT). The mouse monoclonal antibody against rBet1 was described previously (Hay et al., 1998). Mouse monoclonal antibody 8G5F11 (I1) against the luminal part of VSV-G (Lefrancios and Lyles, 1982) was a gift from Dr. D.S. Lyles (Wake Forest University, Winston-Salem, NC).

VSV-G^{ts045}-GFP transfection and trafficking in NRK cells

NRK cells were split and plated in 10-cm plates at 30% confluency. The next day, cells were removed with Trypsin-EDTA (GIBCO BRL) and

washed once with medium and once with PBS by centrifugation (1,000 rpm for 5 min). Cells were resuspended in ice-cold PBS and transferred to a prechilled Gene Pulser Cuvette (0.4-cm gap; Bio-Rad Laboratories). 10 μ g of VSV-G^{iso45}-GFP DNA was added (Chao et al., 1999), and cells were electroporated using the manufacturer's instructions (200 OHMS and 960 μ FD settings were used). Cell pellets were resuspended in 10 ml medium and spun down at 1,000 rpm for 5 min. The cells were plated in T25 flasks at 30% confluency and allowed to recover for 2 h at 37°C after which they were transferred to 40°C and incubated overnight. After incubation at various time points at 32°C, cells were fixed as described.

Ultrathin cryosectioning

NRK cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After washing in buffer, the cells were pelleted by centrifugation, embedded in 10% gelatin, cooled in ice, and cut into 1-mm³ blocks in the cold room. The blocks were infused with 2.3 M sucrose at 4°C for at least 2 h, frozen in liquid nitrogen, and stored until cryo-ultramicrotomy. ~50-nm-thick sections were cut at -120°C using an Ultracut T/FCS (Leica) equipped with an antistatic device (Diatome) and a diamond knife (Drukker). Ultrathin sections were picked up in a mix of 1.8% methylcellulose and 2.3 M sucrose (1:1) according to Liou et al. (1996).

Preparation of immunogold-labeled cryosections

Cryosections were collected on formvar-coated copper grids and incubated with rabbit polyclonal antibodies followed by protein A gold (Slot and Geuze, 1985). A rabbit anti-mouse immunoglobulin antibody was used as a bridging antibody when monoclonal antibodies were used. Double immunolabeling was performed as described before (Slot et al., 1991) with optimal combinations of gold particle sizes and sequences of antibodies. After labeling, the sections were treated with 1% glutaraldehyde, counterstained with uranyl acetate, and embedded in methyl cellulose-uranyl acetate (Slot et al., 1991).

Quantitative IEM

Cis-to-trans and lateral distribution of Golgi-associated proteins. All quantitation was performed on sections from both control cells and VSV-G^{iso45}-GFP-expressing cells (20 min after release of the temperature block). Expression of VSV-G^{iso45}-GFP had no effect on the relative distribution of the endogenous proteins under study.

The cis-to-trans Golgi distributions of Man II, giantin, KDELr, rBet1, and VSV-G were calculated over 25 cross-sectioned Golgi stacks, showing an unambiguous cis-trans polarity using the criteria described previously (Bonfanti et al., 1998). Cisternae were numbered from cis (G1) to trans (G5). The number of gold particles found in each cisterna were counted and expressed as the percentage of the total in the Golgi stacks (Table I). The lateral Golgi distributions of these proteins were calculated over 50 cross-sectioned Golgi stacks. Gold particles were ascribed to the following categories: coated buds and vesicles (whereby the presence of a coat was judged by morphology), lateral rims (the last 50 nm of the lateral tips of cisterna), and Golgi stack (cisterna except for coated buds and lateral rims). The number of gold particles found over each of these compartments was expressed as the percentage of the total (Table II).

Labeling density of mannosidase II, VSV-G, and giantin (gold/ μ m membrane) in peri-Golgi vesicles and Golgi cisterna. To establish the labeling densities in cisternae and peri-Golgi vesicles, 25 micrographs of Golgi areas were taken at a magnification of 30,000 \times and printed at a final magnification of 90,000 \times . Pictures were taken randomly with the only criterion being a well-preserved morphology. Cisternae were numbered from cis (G1) to trans (G5). All COP-coated vesicles within 200 nm of a Golgi cisterna and located at the lateral side of a stack were defined as peri-Golgi vesicles. The surface area of cisterna and vesicles was determined by image analysis using an Imco 10 image computer (Kontron Bildanalyse) and the Microm Image Processing software. Labeling densities in Table III were expressed as the number of gold particles per μ m.

Double immunolabeling analysis of vesicle composition. To analyze the molecular makeup of Man II-positive vesicles, a series of double labelings was performed in which Man II was combined with COPI, COPII, VSV-G, giantin, KDELr, and rBet1, respectively. For each combination, 100 Man II-positive vesicles were analyzed. All Man II-positive vesicles within 200 nm of a Golgi stack were considered. The same approach was performed to study the composition of giantin-, KDELr-, and rBet1-positive vesicles (Table IV).

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COPI, VSV-G-lum, COPII, giantin, and GFP, respectively. We thank R. Scribanek, M. van Peski, and M.T. Castells for excellent photographic and image analysis by computer assistance, respectively.

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