Sorting by COP I–coated Vesicles under Interphase and Mitotic Conditions

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Abstract. COP I–coated vesicles were analyzed for their content of resident Golgi enzymes (N-acetylgalactosaminyltransferase; N-acetylglucosaminyltransferase I; mannosidase II; galactosyltransferase), cargo (rat serum albumin; polyimmunoglobulin receptor), and recycling proteins (-KDEL receptor; ERGIC-53/p58) using biochemical and morphological techniques. The levels of these proteins were similar when the vesicles were prepared under interphase or mitotic conditions showing that sorting was unaffected. The average density relative to starting membranes for resident enzymes (14–30%), cargo (16–23%), and recycling proteins (81–125%) provides clues to the function of COP I vesicles in transport through the Golgi apparatus.

THE Golgi apparatus in mammalian cells has a unique architecture comprising a stack of flattened cisternae bounded on one side by the *cis*-Golgi network (CGN)[1] and on the other by the *trans*-Golgi network (TGN) (Rambourg and Clermont, 1990). The CGN is the entry point for the entire output of proteins synthesized and assembled in the ER (Huttner and Tooze, 1989). Cargo proteins may complete their folding in the CGN, and once assembled (Hammond and Helenius, 1994; Tatu et al., 1995), are transported through the stack in a discontinuous process (Rothman, 1994). Movement from cisterna to cisterna is often accompanied by sequential modifications to bound oligosaccharides, mediated by resident Golgi enzymes (Kornfeld and Kornfeld, 1985; Roth, 1987). Once they reach the TGN, cargo molecules are separated, packaged, and sent to their correct destination (Griffiths and Simons, 1986).

Resident Golgi enzymes are thought to share the same pathway as cargo proteins up to the point where they reside and are then prevented from further forward movement. This reflects either their incorporation into heterooligomeric complexes too large for further transport (Nilsson et al., 1994) or a gradient of bilayer thickness that halts further transport once the length of the enzyme's spanning domain matches that of the bilayer (Munro, 1995*a,b*). These models are not mutually exclusive and both may operate to ensure residence in particular cisternae (Pelham and Munro, 1993).

Resident proteins of the ER are retained by less well-characterized mechanisms, but many are equipped with a signal that returns them to the ER should they inadvertently leave (Pelham, 1995). This retrograde pathway recognizes two types of retrieval signal. The first is -KDEL found at the COOH terminus of many soluble ER proteins (Munro and Pelham, 1987), and a putative receptor for this signal has been identified (Semenza et al., 1990; Hsu et al., 1992; Lewis and Pelham, 1992; Tang et al., 1993). The second is a dibasic signal found in the cytoplasmic tails of membrane proteins. Type I membrane proteins have -K(X)KXX at the COOH terminus (Nilsson et al., 1989; Jackson et al., 1990); type II membrane proteins have -RR- at the NH_2 terminus (Schutze et al., 1994). Retrograde pathways have also been implicated in the retrieval of resident Golgi enzymes that leave their place of residence (Hoe et al., 1995; Harris and Waters, 1996). They may also be needed for recycling those proteins thought to be involved in the forward transport of cargo. Candidates for these putative cargo receptors include yeast Emp24p (Schimmöller et al., 1995) and the mannose-binding lectin ERGIC-53/p58 (Lahtinen et al., 1992; Schindler et al., 1993; Arar et al., 1995). Last, retrograde transport is needed to recycle those components of the vesicle transport machinery used in forward transport (Rothman and Warren, 1994).

COP I vesicles have been implicated in both forward and retrograde transport (Pepperkok et al., 1993; Cosson and Letourneur, 1994; Kreis and Pepperkok, 1994; Bednarek et al., 1995; Pelham, 1995). The G protein of

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1. *Abbreviations used in this paper:* CGN, *cis*-Golgi network; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; GalNAcT, N-acetylgalactosaminyltransferase; GalT, β1,4-galactosyltransferase; Mann II, α1,3-α1,6-mannosidase II; NAGT I, β-1,2-N-acetylglucosaminyltransferase I; pIgR, polymeric immunoglobulin receptor; RSA, rat serum albumin.

VSV has long been a paradigm for the forward transport pathway. Immunogold labeling showed that the G protein was present in budding COP I vesicles at the same concentration as the adjacent cisternae, giving rise to the idea that this cargo protein moved by bulk flow through the Golgi stack (Orci et al., 1986). Purified COP I vesicles were also shown to contain G protein and to fuse with acceptor Golgi membranes (Ostermann et al., 1993). The involvement of COP I vesicles in retrograde transport comes from biochemical, genetic, and morphological experiments. One of the signals for retrograde transport, -K(X)KXX, was shown to bind specifically to coatomer, one of the two subunits of the COP I coat (the other is the ADP ribosylation factor [ARF]) (Cosson and Letourneur, 1994). Genetic studies in yeast showed that mutants defective in the return of a fusion protein containing this retrieval signal were defective in one or other of the coatomer polypeptides (Letourneur et al., 1994). Last, immunogold labeling studies have shown that some of the KDEL receptor is present in COP I–coated buds and vesicles (Griffiths et al., 1994b).

Transport through the Golgi apparatus is a constitutive process during interphase but it ceases at the onset of mitosis in animal cells. This happens both in vivo (Collins and Warren, 1992) and in vitro (Mackay et al., 1993; Stuart et al., 1993) and is accompanied by conversion of the stacked cisternae into hundreds of vesicles and small fragments (Lucocq et al., 1987; Lucocq and Warren, 1987). Our original model, put forward in 1985 to explain this phenomenon, suggested that these two processes were linked (Warren, 1985). The model postulated continued budding of transport vesicles (later identified as COP I–coated vesicles) in the absence of fusion with their target membrane. Since membrane lost through budding is normally replenished by the fusion of incoming vesicles, an inhibition of fusion would lead to consumption of cisternae by vesicle budding. This model also postulated a mechanism to unstack the cisternae and a relaxation in the sorting mechanism that normally prevents resident Golgi enzymes from entering the transport vesicles. These two additional postulates ensured complete consumption of the cisternae by continued budding.

Recent work, however, has shown that two pathways are needed for fragmentation of the Golgi apparatus (Misteli and Warren, 1995). Using a cell-free system, COP I vesicles were shown to consume up to 55% of Golgi membranes but the remaining 45% was consumed by a COP I–independent pathway. Sedimentation analyses suggested that resident Golgi enzymes were present in those membranes consumed by the latter pathway raising the possibility that relaxation of sorting need not occur for complete mitotic fragmentation.

Here we have tested this possibility by analyzing the content of COP I vesicles from both interphase and mitotic incubations using biochemical and morphological techniques. A number of Golgi enzymes was analyzed as well as cargo molecules and recycling proteins. The results strongly suggest that COP I vesicles sort proteins in a similar way under both interphase and mitotic conditions. The relative levels of these proteins, however, raise questions as to the precise role played by COP I vesicles in forward and retrograde transport.

Materials and Methods

Materials

The following antibodies were used for Western blotting and/or immunogold labeling: peptide antibodies against all coatomer components of the COP I coat with the exception of ζ-COP and ε-COP (Dr. C. Harter, Institute for Biochemistry I, University of Heidelberg, FRG); polyclonal antibodies against ε-COP (Dr. S. Hara-Kuge, National Institute of Health, Tokyo); monoclonal antibodies M3A5 against β-COP (Allan and Kreis, 1986; Duden, et al., 1991); peptide antibodies against the COOH-terminal tail of the KDEL receptor (erd2 protein)(Dr. P. Nguyen Van, Clinical Biochemistry, University of Goettingen, FRG); polyclonal rabbit antibodies against rat α1,3–α1,6–mannosidase II (Mann II) (Moremen et al., 1991); and polyclonal antibodies against the polymeric immunoglobulin A receptor (pIgR)(Dr. A. Hubbard, Department of Cell Biology and Anatomy, Johns Hopkins University, School of Medicine, Baltimore, MD). Sheep anti-RSA antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Mitotic and Interphase Incubations

Highly purified rat liver Golgi stacks (100–150 μg of protein; prepared as described by Slusarewicz et al. [1994]) were incubated with interphase or mitotic cytosol (8 mg) from spinner HeLa cells (prepared as described by Stuart et al. [1993], except that no phosphatase inhibitors were added, and the cells were swollen in twofold diluted homogenization buffer instead of TEA-KCl buffer) in a final vol of 1.0 ml. Incubations were carried out for 60 min at 37°C in the presence of an ATP-regenerating system (Misteli and Warren, 1994) and 20 μM GTPγS to prevent uncoating of the COP I vesicles that form (Malhotra et al., 1989).

Fractionation of Mitotic and Interphase Golgi Membranes

1-ml incubation mixtures were adjusted to 250 mM KCl by addition of 3.0 M KCl, to release COP I–coated vesicles from other membranes (Malhotra et al., 1989), and layered on top of a step gradient comprising 0.75 ml 30%, 3.0 ml 35%, 4.0 ml 40%, 3.0 ml 45%, and 1.0 ml 50% (wt/wt) sucrose in gradient buffer (25 mM Tris-HCl, pH 7.3, 2.5 mM MgCl₂, 250 mM KCl). Samples were centrifuged to equilibrium at 65,000 rpm, 4°C for 2.5 h in a vertical rotor (VTi 65.1; Beckman Instruments, Inc., Fullerton, CA), and 1.0-ml fractions were collected from the top. COP I vesicles peaked in fractions 6 to 9 (corresponding to 41–45% [wt/wt] sucrose) and were further purified by combining these fractions from two gradients, diluting them to 30% (wt/wt) sucrose with gradient buffer, and applying them to a second step gradient comprising 2.0 ml 35% and 2.0 ml 45% (wt/wt) sucrose. After centrifugation at 65,000 rpm, 4°C for 2.5 h in a VTi 65.1 rotor, 1-ml fractions were collected from the top.

Western Blotting and Quantitation

Fractions from both gradients were diluted threefold with water and membranes recovered by centrifugation at 100,000 rpm for 30 min at 4°C in a rotor (TL 100.3; Beckman Instruments, Inc.). Membrane pellets were dissolved in SDS-PAGE sample buffer, and proteins separated on 6.5–12.5% polyacrylamide gradient gels. After transfer to nitrocellulose (Hybond C; Amersham Life Science, Little Chalfont, UK) blots were blocked using PBS containing 10% (wt/vol) milk, and antibodies were diluted in the same mixture. HRP-conjugated, goat anti–rabbit or anti–mouse antibodies (Tago, Buckingham, UK) were used to detect primary antibodies. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Life Science).

Films were scanned by a video camera under the control of a Screen Machine card (Fast GmbH, Munich, Germany) to produce digital images. Pixel densities were determined using NIH Image 1.51. Standard curves were constructed using serially diluted total incubations that were run on the same gels. For Mann II, pIgR, and the KDEL receptor the amounts in each fraction were expressed as a percentage of the total in the gradient. The amount of β-COP was expressed as arbitrary units corresponding to the pixel densities measured. Recovery of membranes in the first gradient was reproducibly 90% and recovery of the COP I vesicles from the second gradient was 85%.

Enzyme Assays for Golgi Markers

β1,2-N-acetylglucosaminyltransferase I (NAGT I) was assayed as described by Vischer and Hughes (1981) using ovalbumin as an acceptor and an incubation time of 2.5 h. Precipitates were washed once with 1% (wt/vol) phosphotungstic acid/0.5 M HCl and once with ice-cold 95% ethanol before resuspension in unbuffered 0.4 M Tris/4% (wt/vol) SDS for scintillation counting.

β1,4-galactosyltransferase (GalT) was assayed essentially as described by Bretz and Staubli (1977) using ovomucoid as an acceptor and a 30-min incubation time. Precipitates were processed as for the NAGT I Assay.

GalNAc transferase was assayed as described by Schweizer et al. (1994). Aliquots of gradient fractions were diluted 1:1 with 20 mM Tris/HCl (pH 6.5), 1% Triton X-100, 100 mM NaCl, 10 mM $MnCl_2$, and sonicated for 30 s. In all assays the final reaction mixture was 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 5 mM $MnCl_2$, 250 μM UDP-[^{14}C]GalNAc (4,000 cpm/nmol), 0.5% Triton X-100, and 10–50 μg of acceptor peptide. An identical reaction mixture without peptide substrate was used to obtain the background value for each sample. The amino acid sequence of the two peptides used was as follows: Muc2 (human intestinal mucin): NH$_2$-PTTTPISTTTMVTPTPTPTC-COOH; Carl2 (submaxillary mucin): NH$_2$-LSESTTQLPGGGPGCA-COOH.

Phospholipid Assay

Membranes from interphase or mitotic incubations were collected by centrifugation through a 1-ml layer of 10% (wt/wt) buffered sucrose (25 mM Tris-HCl, pH 7.3, 2.5 mM $MgCl_2$, 50 mM KCl) onto a 5-μl cushion of 50% (wt/wt) sucrose in a Beckman TL 100.2 rotor at 100,000 rpm for 15 min at 4°C. Purified COP I vesicles (fractions 10–12 of the second gradient; 39–45% [wt/wt] sucrose) were diluted and recovered by centrifugation at 100,000 rpm for 30 min at 4°C in a Beckman TL 100.3 rotor.

Membrane pellets were resuspended in 500 μl of water, and lipid extractions were performed according to Bligh and Dyer (1959). Aliquots of the extracts were analyzed for phosphate as described by Chalvardjian and Rudnicki (1970). The amounts of phospholipids in the sample were estimated from a standard curve using 0–12.5 nmol of phosphatidyl ethanolamine, and expressed as a percentage of the total phospholipid in the original membranes after correcting (where appropriate) for losses during the purification procedure (see fractionation section).

Cryoelectron Microscopy and Immunogold Labeling

Membranes from interphase or mitotic incubations and purified COP I vesicles were collected as for the phospholipid assay except that the COP I vesicles were sedimented onto a 5-μl cushion of 50% (wt/wt) sucrose to prevent membrane distortion. Uncoated Golgi remnants from the first gradient (fractions 1–4, corresponding to 28–37% [wt/wt] sucrose) were pooled and diluted to 12% (wt/wt) sucrose with gradient buffer. Contaminating cytosol was removed by sedimentation through a 1-ml layer of 15% (wt/wt) sucrose onto a 10-μl cushion of 50% (wt/wt) sucrose at 50,000 rpm for 3 h at 4°C in a Beckman SW 55 rotor.

Membranes were fixed for 2 h at RT in 0.25% (vol/vol) glutaraldehyde, 2% (wt/vol) paraformaldehyde in PBS containing 8% (wt/wt) sucrose. Samples were embedded in 10% (wt/vol) gelatine in H_2O, cut into small blocks, and infiltrated with 2.1 M sucrose in PBS overnight at 4°C. Blocks were cut on an ultramicrotome 2E (Reichert Jung, Vienna, Austria) at −95°C and sections collected on collodion/carbon–coated copper grids.

Grids were incubated at RT on a drop of 50 mM NH$_4$Cl in PBS, and nonspecific binding was blocked with 0.5% (wt/vol) fish skin gelatin in PBS for 10 min. Sections were labeled for 30–60 min depending on the antibody, washed on five drops of PBS with fish skin gelatin over 20 min, and incubated with goat anti–rabbit antibodies coupled to 10 nm gold (Biocell, Cardiff, UK) for 25 min. The grids were washed for 20 min on sequential drops of PBS, and for 30 min with water before staining with 2% uranyl acetate for 6 min at RT followed by incubation on 2% methylcellulose containing 0.2% uranyl acetate for 10 min on ice. Excess methylcellulose was removed using filter paper and the grids air-dried. Sections were observed in an electron microscope (CM10; Phillips Science, Mahwah, NJ).

Stereology

The relative proportion of membranes in COP I–coated vesicles was determined as described by Misteli and Warren (1994), except that contaminating membranes (<10% of the total) were included in the counting. COP I–coated vesicles were defined as circular profiles, 50–70 nm in di-

ameter, with a coat that was visualized in Epon 812–embedded samples using tannic acid (Simionescu and Simionescu, 1976). Comparable results were obtained using cryosections.

The distribution of Mann II, pIgR, and the KDEL receptor in Golgi membranes before and after incubation with either interphase or mitotic cytosols was estimated by assigning gold particles either to coated vesicles and buds or to uncoated profiles (cisternae, tubules, and other fragments). Gold particles were assigned to coated vesicles and buds if they fell within 15 nm of a coated membrane. Approximately 5% of gold particles was unattributable representing either background labeling or labeling of membranes out of the plane of section.

To determine the linear labeling density for marker proteins, the length of membranes was measured by the intersection method (Weibel, 1979). High magnification photographs (×100,000) were overlaid with a square lattice grid of 5 mm, and the number of intersections of the grid with the membrane profiles was counted. Linear density was expressed as gold particles/μm of membrane. All quantitations were performed on at least five photographs from at least two different experiments.

Results

Rat liver Golgi membranes, typically purified 120-fold over homogenate, were incubated with an 80-fold protein excess of either interphase or mitotic cytosol (derived from HeLa cells) to ensure sufficient cytosolic components for the formation of COP I–coated vesicles (Misteli and Warren, 1995). Incubations were carried out for 60 min at 37°C in the presence of an ATP-regenerating system and 20 μM GTPγS to prevent uncoating of the COP I vesicles that formed. The content of the COP I vesicles under both conditions was measured using two independent methods, one biochemical, the other morphological, starting with the resident Golgi enzyme, Mann II.

Mann II Content of Isolated COP I Vesicles

Isolation of COP I Vesicles. COP I vesicles were released from other membranes by raising the concentration of KCl in the samples to 0.25 M (Malhotra et al., 1989). Stepped sucrose gradients (top loaded) were centrifuged to equilibrium in a vertical rotor, and fractionated from the top. Fractions were diluted with water, and the membranes were recovered by high speed centrifugation. Pellets were solubilized in sample buffer, fractionated by SDS-PAGE, and blots probed using antibodies to the β subunit of the coatomer complex. The signals were visualized by ECL and quantitated by digitizing the film image.

As shown in Fig. 1, there were two distinct peaks containing coatomer. The smaller, peaking between 35 and 37% (wt/wt) sucrose, was the only one present when cytosol alone was fractionated (Fig. 1 F) and when the incubation with Golgi membranes was carried out on ice (Fig. 1 E). It likely represents the soluble, cytoplasmic pool of coatomer. The major peak, centered on 42% (wt/wt) sucrose after both interphase and mitotic incubations (Fig. 1, A and C), satisfied all the criteria for COP I–coated vesicles. The equilibrium density was the same as that for the COP I vesicles first isolated and characterized by Malhotra et al. (41.5%; Malhotra et al., 1989). It blotted for other components of the coatomer complex namely, α-, β'-, γ-, δ-, and ε-COP (data not shown). When gradient fractions were processed for cryoelectron microscopy, coated vesicles with a diameter ranging from 50 to 70 nm were found exclusively in fractions containing 40–45% (wt/wt) sucrose, and >85% of these coated vesicles could be labeled with antibodies to ε-COP (data not shown).

Last, comparison of Fig. 1, A and C shows that there is 2.2-fold more coatomer in mitotic than in interphase incubations in these fractions. This agrees well with the analyses described below showing that 2.1-fold more phospholipid and 2.2-fold more membrane surface area was present in this peak after mitotic when compared with interphase incubations.

Mann II in COP I Vesicles. The percentage of total Mann II in COP I vesicles was assessed by probing the same blots with polyclonal antibodies to Mann II. As shown in Fig. 1, A, C, and E, the major peak of Mann II by Western blotting was found at 32% (wt/wt) sucrose, the density of Golgi membranes. This was the only peak found when samples were incubated on ice (Fig. 1 E), but an additional, minor peak appeared after 60 min incubation in either interphase (Fig. 1 A) or mitotic (Fig. 1 C) cytosol. This minor peak cofractionated with the coatomer peak after interphase incubation but was skewed towards lighter membranes after mitotic incubation suggesting the presence of Mann II in membranes other than COP I–coated ones. This was confirmed by EM and is consistent with earlier observations showing that mitotic fragmentation by the COP I–independent pathway generates a heterogeneous population of small, uncoated fragments (Misteli and Warren, 1995). These contaminating membranes were removed by pooling the coatomer fractions from the first gradient (Fig. 1 C, pool) and recentrifuging them on a slightly shallower gradient. As shown in Fig. 1 D, about half the Mann II was removed, the rest cofractionating with COP I vesicles. As expected, this contaminating population of membranes was not present after interphase incubations and all the Mann II cofractionated with COP I vesicles when the coatomer fractions from the first gradient (Fig. 1 A) were recentrifuged (Fig. 1 B). To prevent interference by these contaminating membranes, all subsequent analyses of COP I vesicles (from both interphase and mitotic incubations) were carried out on fractions isolated from the second gradient.

The percentage of Mann II in the starting Golgi stacks that was incorporated into COP I vesicles is shown in Table I. The initial calculation compared the amount of Mann II in the vesicles isolated from the second gradient with the Mann II present in the incubation applied to the first gradient. All samples were fractionated on the same gel and serial dilutions of the gradient load were used to provide an internal standard. The initial percentage values were corrected for the recovery on the two gradients. Typically, 90% of Mann II was recovered from the first gradient and 85% of applied COP I vesicles from the second. The corrected results, presented in Table I, show that the percentage of total Mann II found in COP I vesicles was 5.6 ± 2.3% for interphase and 8.4 ± 1.0% for mitotic incubations.

Phospholipid in COP I Vesicles. These percentage amounts of Mann II had to be corrected since more COP I vesicles were produced under mitotic compared with interphase conditions (Misteli and Warren, 1994, and see below). This correction factor was calculated by measuring the proportion of total phospholipids in COP I vesicles under both conditions. Phospholipids constitute >85% of total lipid in purified rat liver Golgi preparations (Graham, 1992), so they provide a convenient measure of membrane surface area.

Interphase and mitotic COP I vesicles from the second gradient were pelleted, the lipids were extracted with chloroform/methanol, and the dried pellets were assayed for inorganic phosphate as a measure of phospholipid content. The values were corrected for losses on the two gradients. Total membranes from interphase and mitotic incubations were recovered by high speed centrifugation and assayed in the same manner. These values were corrected for the presence of lipoprotein particles that were not present in COP I vesicles. Lipoprotein particles are enclosed by a monolayer of phospholipid (Gordon et al., 1995) that would artificially inflate the phospholipid values for the total membranes if not corrected. Stereological analysis showed that these particles constituted 10% of the surface area of Golgi membranes so the phospholipid values were corrected using half this figure. This correction assumes that the phospholipid content of the lipoprotein monolayer is the same as that of bulk Golgi membranes. This may not be the case since the composition can vary with diet (Wilcox et al., 1975). However, given the small correction factor, even quite large differences in phospholipid content would not significantly affect the result.

The results, presented in Table I, show that there was 2.1 times more phospholipid in mitotic compared with interphase COP I vesicles (51 ± 3.3% vs 24 ± 0.3%). The comparable ratio for Mann II was 1.5 (Table I; 8.4 ± 1.0% vs 5.6 ± 2.3%) suggesting that the increased percentage of Mann II was simply the consequence of increased production of COP I vesicles under mitotic conditions.

Mann II Content of COP I Vesicles Using Immunogold Microscopy

Immunogold Labeling. Starting Golgi membranes and samples from interphase and mitotic incubations were fixed, frozen, and thin sections prepared. Labeling with polyclonal antibodies to Mann II was followed by secondary antibodies coupled to 10 nm gold. Stacked Golgi membranes typically had 2–4 cisternae in the stack and all but one of these were usually labeled (Fig. 2 A).

After incubation with interphase cytosol, stacked structures were still present, but there was a marked increase in the number of COP I–coated vesicles and buds that were easily distinguished morphologically from uncoated membranes (Fig. 2 B). Mann II was still mostly restricted to stacked cisternae and uncoated membranes but 4.5 ± 1.3% of gold label was present over the COP I–coated membranes (Table II).

After incubation with mitotic cytosol, few, if any, stacks were present. Small, uncoated structures as well as COP I vesicles and buds were more prevalent (Fig. 2 C). The percentage of gold particles over COP I–coated membranes was 8.1 ± 3.2% (Table II).

These values are valid only if there is a constant display of Mann II epitopes. The similarity in the labeling density for Golgi membranes before (8 ± 3 gold particles/10 μm membrane) and after interphase (9 ± 3 gold particles/10 μm membrane) or mitotic incubations (7 ± 2 gold particles/10 μm membrane) shows that Mann II epitopes neither appeared nor disappeared during the course of either incubation.
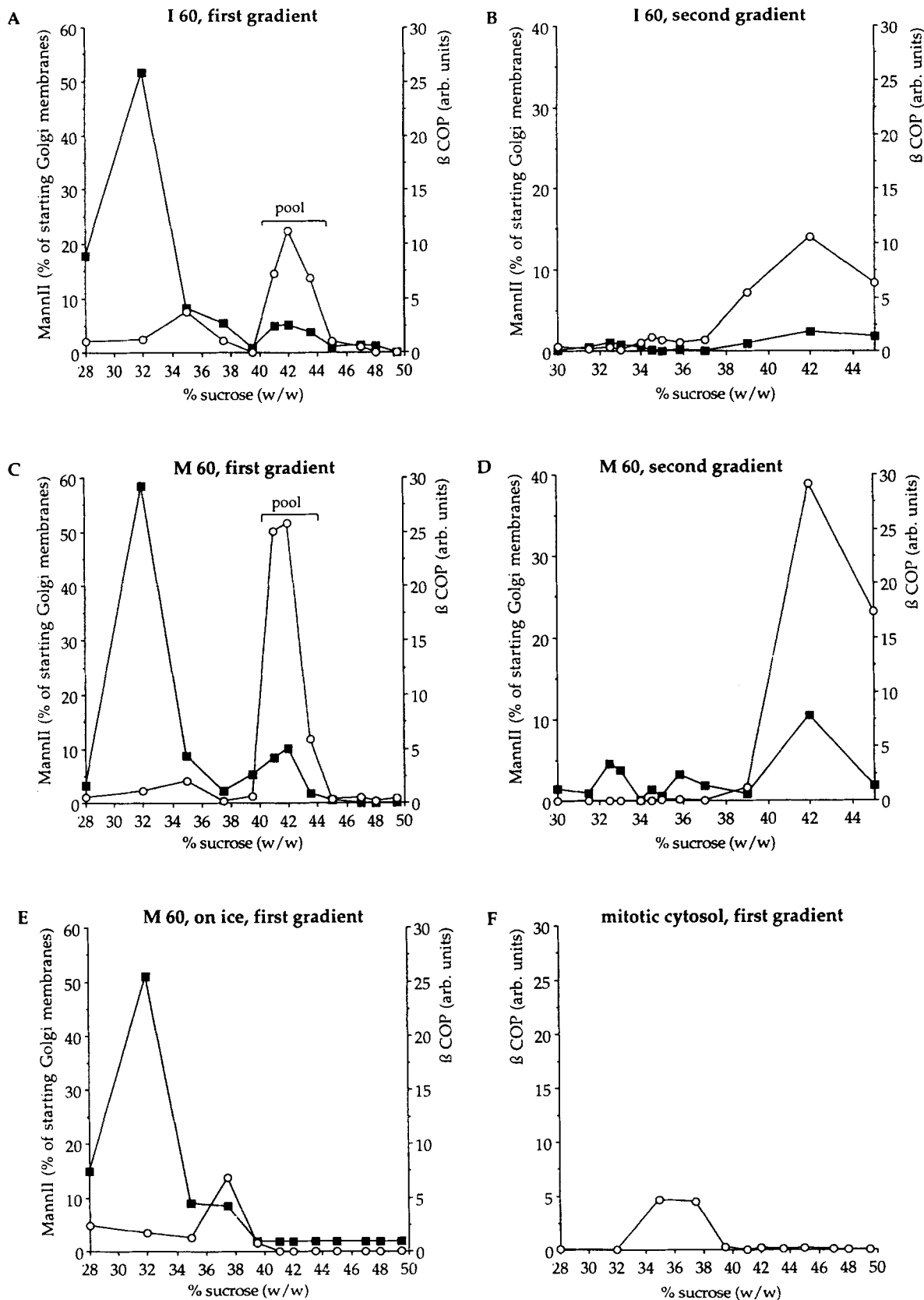
Purified COP I vesicles were also labeled for Mann II

Figure 1. Distribution of Mann II in fractionated interphase and mitotic Golgi membranes. (*A–D*) Purified Golgi membranes were incubated under interphase (*I60*) or mitotic (*M60*) conditions at 37°C for 60 min in the presence of GTPγS. Samples were centrifuged to equilibrium on a 30–50% (wt/wt) sucrose gradient (*first gradient*) and the COP I vesicles (*pool*) further purified on a 30–45% (wt/wt) sucrose gradient (*second gradient*). Pelleted membranes were fractionated by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against Mann II and β-COP. (*E*) Golgi membranes were incubated with mitotic cytosol on ice for 60 min before fractionation on the first gradient. (*F*) Mitotic cytosol alone was fractionated on the first gradient. —■—, Mann II; —O—, β-COP.

Table I. Content of Golgi Proteins in COP I–coated Vesicles under Mitotic and Interphase Conditions: Biochemical Analysis

		Amount in COP I vesicles		Average density in COP I vesicles	
		Interphase	Mitotic	Interphase	Mitotic
		percentage of starting membranes		percentage of starting membranes	
Golgi enzymes	Mann II	5.6 ± 2.3	8.4 ± 1.0	24 ± 9.7	16 ± 2.0
	GalT	3.2 ± 0.6	9.1 ± 1.9	14 ± 2.5	18 ± 3.7
	NAGT I	3.3 ± 0.5	9.8 ± 3.1	14 ± 2.1	19 ± 6.0
	GalNAcT	7.0 ± 1.7	9.7 ± 3.0	30 ± 7.2	19 ± 5.9
Anterograde cargo	pIgR	5.5 ± 1.0	12 ± 2.7	23 ± 4.2	23 ± 5.3
	RSA	4.3 ± 0.6	9.2 ± 3.2	18 ± 2.5	18 ± 6.2
Recycling proteins	KDEL receptor	30 ± 1.5	60 ± 6.3	125 ± 6.3	115 ± 12
	p58/ERGIC 53	23 ± 2.9	42 ± 3.6	93 ± 12	81 ± 7.0
Phospholipids		24 ± 0.3	51 ± 3.3	100	100

COP I–coated vesicles from interphase or mitotic incubations were purified on two sucrose gradients and analyzed either by Western blotting (Mann II, RSA, pIgR, KDEL receptor, p58) or enzyme activity (NAGT I, GalT, GalNAcT). Phospholipid content was assessed by measuring inorganic phosphate. Results are expressed as the percentage of the protein in COP I vesicles over the starting Golgi membranes ± SD (n ranged from 3 to 8). All data were corrected for losses during the purification. Yields were typically in excess of 75%. The average density of the marker proteins in COP I vesicles was estimated by dividing the percentage of the protein by the percentage of phospholipid in the vesicles. Results are expressed as percentage average density of starting membranes.

(Fig. 2 D) and the same percentage was labeled as in unfractionated membranes (11.5% vs 12.5% for mitotic incubations). This eliminates the possibility that Mann II was restricted to cisternal membranes adjacent to budding profiles. Buds were not released by the increased KCl used to separate the COP I vesicles. The few that were could be clearly identified and in those rare instances when they were labeled (double arrows, Fig. 2 D), the gold particles were associated with the coated bud (small arrow, Fig. 2 D), not the associated cisternal remnant.

Stereology. The fraction of Golgi membrane coated by COP I coats was estimated using standard stereological procedures. For interphase incubations, 25 ± 3.2% of Golgi membrane was present in COP I–coated vesicles and buds (Table II), a value somewhat lower than that determined previously in similar incubations (36 ± 7.0%; Misteli and Warren, 1995). The reasons are unclear, but may reflect variability in our interphase cytosol preparations. The comparable figure for mitotic incubations was 56 ± 6.7% (Table II) which compares very well with that determined previously (55 ± 11%; Misteli and Warren, 1995). The ratio of these two figures shows that 2.2 times more coated vesicles were produced under mitotic compared with interphase conditions. The corresponding ratio for Mann II was 1.8 times. These results agree well with those obtained using the biochemical approach (Table I) and together suggest that Mann II enters COP I vesicles to a similar extent under interphase and mitotic conditions. If anything, the ratio showed slightly lower levels of Mann II in COP I vesicles under mitotic conditions though this was of only borderline significance using the t test.

Though sampling of Mann II by COP I vesicles was similar under both conditions, the density of Mann II in these vesicles was higher than expected for a resident enzyme. Density was calculated by dividing the percentage of total Mann II in the COP I vesicles by the amount of membrane in these vesicles. The result, expressed as a percentage of the density in starting Golgi membranes, is an average since Mann II is not present in every Golgi compartment so would not be expected to be in every COP I vesicle that forms.

The average densities calculated for both the biochemi-

cal and the morphological approach (Tables I and II) emphasized the similarity between COP I vesicles generated under interphase or mitotic conditions. For both conditions the average density for Mann II was high, ranging from 15 to 24% of the density in starting Golgi membranes.

Content of Other Resident Golgi Proteins in COP I Vesicles

Since the levels of Mann II in COP I vesicles were unexpectedly high for a resident enzyme, it was important to see whether this was a general feature of Golgi enzymes. COP I vesicles were, therefore, examined for their content of other enzymes from the same and from different parts of the Golgi apparatus.

NAGT I was chosen as another marker of medial/trans–cisternae. It immediately precedes Mann II in the construction of complex oligosaccharides (Kornfeld and Kornfeld, 1985; Roth, 1987), forms hetero-oligomers with it (Nilsson et al., 1994), and has the same distribution in the Golgi apparatus, at least in HeLa cells (Nilsson et al., 1993). GalT is involved in the later stages of construction of complex, N-linked oligosaccharides. It is present in the trans-cisterna and the TGN in HeLa cells, overlapping the distribution of both NAGT I and Mann II (Rabouille et al., 1995). Galactosaminyl transferase (GalNAcT) was chosen as a cis-Golgi marker (Schweizer et al., 1994). It adds the first sugar in the construction of O-linked oligosaccharides.

A lack of suitable antibodies precluded a morphological analysis and a biochemical analysis based on Western blotting. The activity of each enzyme was, therefore, used to determine the distribution between COP I vesicles and other membranes. The results were corrected for recovery of the enzyme on the first gradient and the yield of COP I vesicles on the second. The results in Table I show that all three enzymes had a very similar density to Mann II under mitotic conditions showing no significant differences at the 0.01 < P < 0.05 level. There was more variation in density under interphase conditions but these were again not significant at 0.01 < P < 0.05 level, with the exception of Gal-
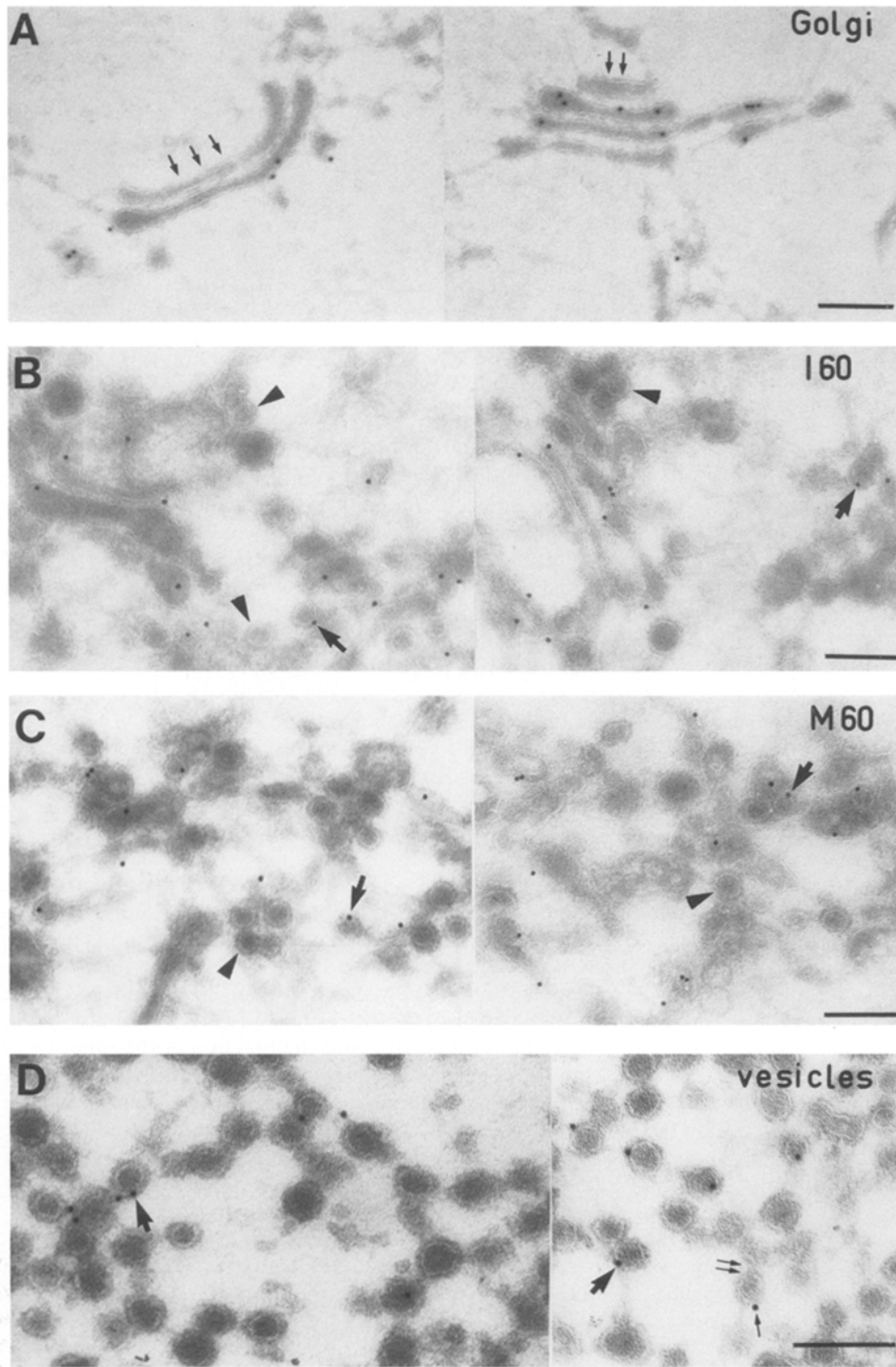
Figure 2. Immunogold labeling for Mann II. Rat liver Golgi stacks (*A*) were incubated with interphase (*I 60, B*) or mitotic (*M 60, C*) cytosol for 60 min at 37°C in the presence of GTPγS. (*D*) COP I vesicles from the mitotic incubation were purified on two sucrose gradients. Samples were fixed and processed for cryo-electron microscopy. Sections were labeled with polyclonal antibodies against Mann II followed by secondary antibodies coupled to 10 nm gold. Two representative fields are shown for each condition. Note the unlabeled cisterna in *A* (*small arrows*), and examples of COP I vesicles that label (*large arrows*) or do not label (*arrowheads*) for Mann II in *B, C,* and *D*. Note in *D* that Mann II labeling is still restricted to COP I vesicle profiles (*thin arrow*) even when they are attached to cisternal remnants (*double arrows*). Magnification, 71,000 (*A–C*); 97,000 (*D*). Bar, 0.2 μm.

NAcT, which was significantly higher than the rest. This might reflect the higher levels of coatomer in the CGN and on the cis-side of the Golgi stack (Oprins et al., 1993) which could lead to a greater production of COP I vesicles. Since the calculations of average density assume that all Golgi compartments are consumed to the same extent by COP I vesicles, selective budding from the cis-most compartments would lead to underestimates in the average density of enzymes from other compartments. In other words, the average densities for NAGT I, Mann II, and

GalT might be higher than the values in Table I (see discussion).

Anterograde Cargo

Given the high levels of resident Golgi enzymes in COP I vesicles, the distribution of two representative cargo proteins was assessed for purposes of comparison. Rat serum albumin (RSA) was chosen as the secretory protein and the pIgR as the membrane protein. Both are rapidly se-

Table II. Content of Golgi Proteins in COP I-coated Vesicles under Mitotic and Interphase Conditions: Stereological Analysis

		Amount in COP I vesicles		Average density in COP I vesicles	
		Interphase	Mitotic	Interphase	Mitotic
		percentage of starting membranes		percentage of starting membranes	
Golgi enzyme	Mann II	4.5 ± 1.3	8.1 ± 3.2	18 ± 5.2	15 ± 1.8
Anterograde cargo	pIgR	4.8 ± 3.7	8.8 ± 5.6	19 ± 15	16 ± 10
Recycling protein	KDEL receptor	16 ± 2.0	34 ± 10	66 ± 8.0	62 ± 18
Golgi membrane surface area		25 ± 3.2	56 ± 6.7	100	100

Mitotic and interphase incubations in the presence of GTPγS were fixed, processed for cryoelectron microscopy, and sections labeled for either Mann II, pIgR, or the KDEL receptor. The percentage labeling for these proteins in COP I vesicles was estimated as was the proportion of total membrane ± SD ($n = 2$). The average density of the marker in COP I vesicles was estimated by dividing the percentage labeling for the protein by the percentage of total membrane in the vesicles.

creted by hepatocytes (Lodish et al., 1983; Fries et al., 1984; Barr and Hubbard, 1993) and appear to be present at about the same concentration throughout the Golgi apparatus (Yokota and Fahimi, 1981; Solari et al., 1986; Dahan et al., 1994). The abundance of these two proteins coupled with the availability of high affinity antibodies meant that both could be analyzed by Western blotting, the same biochemical approach used to determine the distribution of Mann II.

Interphase and mitotic incubations were fractionated on two sucrose gradients and the COP I vesicles analyzed for their content of pIgR and RSA. The results for pIgR after fractionation on the first sucrose gradient are shown in Fig. 3. Most of the pIgR fractionated with Golgi membranes, but a small proportion cofractionated with coatomer after both interphase (Fig. 3 A) and mitotic (Fig. 3 B) incubations. Table I shows that this fraction, when corrected for losses on both gradients and for the phospholipid content, yielded an average density of 23% for both interphase and mitotic COP I vesicles.

A similar set of experiments was carried out and the fractions analyzed for their content of RSA. Since RSA is a soluble protein it is necessary to compare the average concentration in the vesicles with that in the starting Golgi membranes. However, since the ratio of the membrane surface area to volume (surface density per unit volume; Nilsson et al., 1993) did not change significantly during the incubations, the average concentration is directly proportional to the average density. RSA had a slightly lower average density than pIgR, being 18% for both interphase and mitotic conditions (Table I).

These results were confirmed for the pIgR using the morphological approach. Interphase and mitotic incubations were fixed, processed for cryoelectron microscopy and sections labeled with polyclonal antibodies to the pIgR followed by secondary antibodies coupled to gold. Quantitation showed that 4.8 ± 3.7% of the pIgR was present over coated vesicles and buds after interphase incubation compared with 8.8 ± 5.6% for mitotic incubations (Table II). The overall gold labeling was similar for both incubations and the starting material showing that new epitopes for pIgR were not revealed during the incubation nor existing epitopes hidden. COP I vesicles were also purified from a mitotic incubation on two gradients and cryosections labeled for pIgR (Fig. 4 A). The percentage of coated profiles labeled for pIgR (10%) was the same as in unfractionated mitotic incubations (10%), showing

that the purification procedure selects a representative population of COP I vesicles for biochemical analysis.

After correcting the amount of pIgR in COP I vesicles for the different amounts of membrane in the two vesicle populations, the average densities were similar to each other (interphase: 19 ± 15%; mitotic: 16 ± 10%; Table II) and to the densities calculated using the biochemical approach (interphase: 23 ± 4.2%; mitotic: 23 ± 5.3%; Table I). These results, taken together with those for RSA, show that these two cargo proteins are present in COP I vesicles at average densities of only one quarter to one fifth of those in starting Golgi membranes. Sampling of these proteins by COP I vesicles was also unaffected under mitotic conditions.

Recycling Proteins

To complete the analysis, two recycling proteins were studied. The KDEL receptor at steady state is concentrated in the CGN though a significant fraction is present in the Golgi stack and the TGN. It recycles to the ER once ligand is bound (Lewis and Pelham, 1992), most likely in COP I vesicles (Griffiths et al., 1994b; Connolly et al., 1994). Less is known about the function of the other recycling protein, p58 (Lahtinen et al., 1992; Lahtinen et al., 1996). This was originally identified as a marker for the CGN (or ERGIC; Schweizer et al., 1988; Schindler et al., 1993) and is now known to be a mannose-binding protein (Arar et al., 1995). Given the recent evidence implicating lectins in exocytic transport (Fiedler and Simons, 1994, 1996), it is possible that this protein is a cargo receptor, transporting certain proteins from the ER to the entry face of the Golgi stack.

Fractions from the first gradient were immunoblotted with antibodies to the KDEL receptor and the results are presented in Fig. 5. After interphase incubation most of the receptor cofractionated with Golgi membranes having the same density as those containing Mann II (cf. Fig. 1 A and Fig. 5 A) and heavier membranes most likely corresponding to the CGN. The rest cofractionated with coatomer on this (Fig. 5 A) and the subsequent, second gradient (data not shown). After mitotic incubation, most of the receptor cofractionated with coatomer, the rest peaking in membranes lighter than Golgi membranes (Fig. 5 B). The identity of these lighter membranes is presently unclear. Quantitation of Western blots (and appropriate corrections for losses on the gradients) confirmed the extent of incorpora-
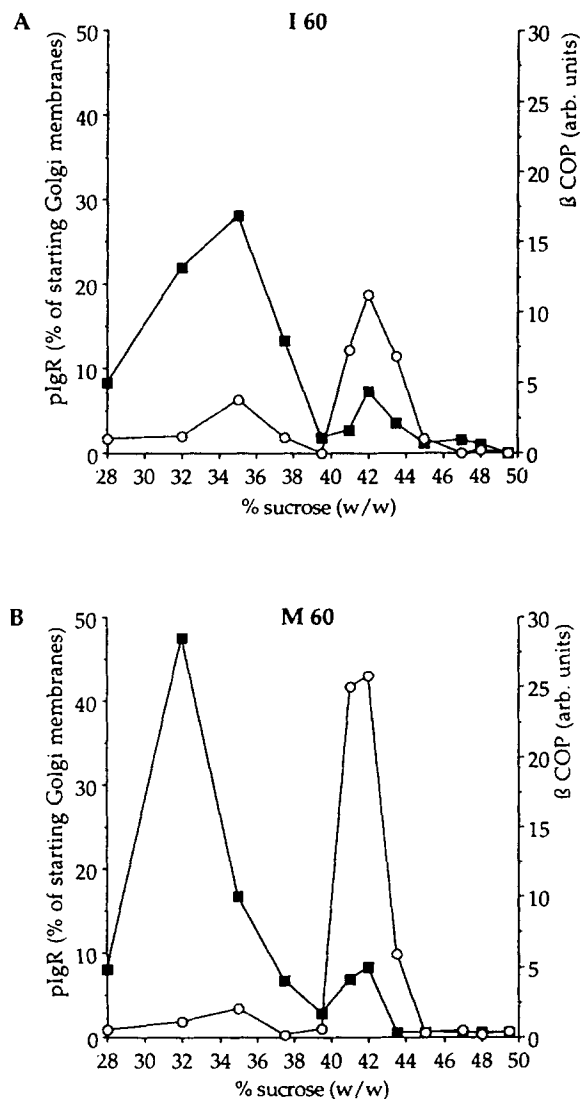
A I 60

B M 60

Figure 3. Distribution of pIgR in fractionated interphase and mitotic Golgi membranes. Samples from (A) interphase or (B) mitotic incubations were centrifuged to equilibrium on 30–50% (wt/wt) sucrose gradients and pelleted membranes fractionated by SDS-PAGE, followed by Western blotting with antibodies to pIgR and β-COP. —■—, pIgR; —O—, β-COP.

tion of the KDEL receptor into COP I vesicles. The average density in COP I vesicles (relative to starting membranes) was 125 ± 6.3% for interphase and 115 ± 12% for mitotic incubations (Table I). For the mannose-binding protein, p58, the average density was 93 ± 12% for interphase and 81 ± 7.0% for mitotic incubations (Table I).

The corresponding morphological analysis was carried out for the KDEL receptor. The average density of the receptor in COP I vesicles (after correction for amount of membrane) was very similar for interphase (66 ± 8.0%) and mitotic (62 ± 18%) incubations, confirming the biochemical data that showed no change in the sorting capacity under mitotic conditions. These densities were, however, significantly lower than those obtained by biochemical analysis. One explanation is that the presence of the COP I coat might have prevented the antibody from gaining ac-

cess to the epitope(s) in the cytoplasmic tail. Similar effects have been reported for the transferrin receptor in endosomal, clathrin-coated buds (Stoorvogel et al., 1996).

The percentage of coated membranes labeled for the KDEL receptor was very similar in unfractionated interphase (35%) and mitotic (37%) incubations as well as in purified COP I vesicles from mitotic incubations (35%) (Fig. 4 B). This again shows that the purified vesicles were a representative population of the unfractionated incubations.

Golgi Compartments Consumed by COP I Vesicles

The two pathways of mitotic fragmentation have been assumed to act at the level of individual cisternae. Peripheral rims would be consumed by continued budding of COP I vesicles, whereas cisternal cores would be fragmented by the COP I–independent pathway (Warren et al., 1995). Alternatively, each pathway could operate on different sides of the Golgi stack. The COP I–dependent pathway could operate on the cis-side of the Golgi stack since this is where most of the coatomer is found (Oprins et al., 1993); the COP I–independent pathway could operate on the rest of the Golgi apparatus since models have been put forward to suggest that vesicles are not needed to transport cargo through the stack itself (Mellman and Simons, 1992).

The first approach to find out whether budding of vesicles occurred equally from all layers of the stack was to incubate Golgi stacks with mitotic cytosol for short time periods. 2–5 min was found to be the optimum time to initiate maximal budding of COP I vesicles from Golgi membranes. Samples were then fixed and processed for immunogold microscopy.

Sections were labeled with antibodies to ε-COP and the result of a typical 5-min incubation is shown in Fig. 6. More than 40% of membranes that met the criteria of cisternae (see Materials and Methods) were labeled for ε-COP and careful examination of many stacks showed that labeling could be seen at all levels.

A second approach was to examine uncoated membranes and compare the percentage labeled for Mann II before and after mitotic incubations. If parts of the Golgi not containing Mann II (such as the CGN in our starting material) were preferentially consumed by COP I vesicles, the percentage of uncoated membranes containing this enzyme should increase during the course of an incubation. As shown in Fig. 7 A, no increase was observed suggesting that budding occurs equally from all Golgi membranes.

The third and final approach was to measure the linear density of Mann II in starting Golgi membranes and uncoated mitotic remnants, isolated from the first sucrose gradient after a mitotic incubation. Since the average density of Mann II in COP I vesicles is much lower than in starting membranes, the formation of COP I vesicles should lead to an increase in the linear density of Mann II in the uncoated remnants that are left. As shown in Fig. 7 B, there was a 2.9-fold increase in linear density after 60 min of mitotic incubation consistent with consumption of more than half the cisternal membrane (Tables I and II). The linear density of pIgR was measured for comparison. The average density in COP I vesicles relative to starting
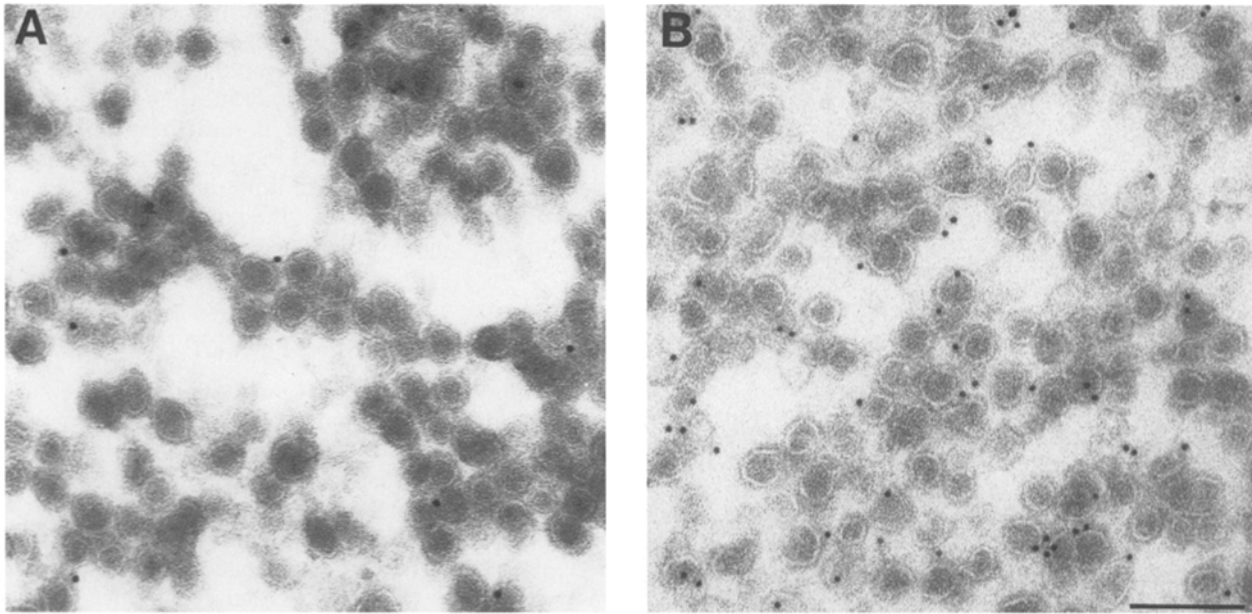
Figure 4. Immunogold labeling for pIgR and the KDEL receptor in purified COP I vesicles. A mitotic incubation was fractionated on two sucrose gradients to purify COP I vesicles that were prepared for cryoelectron microscopy. Sections were labeled with antibodies to pIgR (*A*) and the KDEL receptor (*B*) followed by secondary antibodies coupled to 10 nm gold. Magnification, 97,000; Bar, 0.2 μm.

membranes was similar to Mann II (Table I). After mitotic incubation a 2.4-fold increase in linear density was observed in uncoated remnants (Fig. 7 *B*). Since pIgR is present in all Golgi compartments while Mann II has a more restricted distribution, the similarity in the increase of linear density suggests that budding is occurring more or less equally from all Golgi compartments.

Discussion

Our original model for mitotic Golgi fragmentation postulated complete consumption of Golgi membranes by continued budding of transport vesicles (Warren et al., 1995). This was revised after more detailed analysis using a cell-free system mimicking the fragmentation process which showed that 45% of Golgi membrane could not be consumed by COP I vesicles but was fragmented by another process (Misteli and Warren, 1995). The time course of fragmentation suggested that resident Golgi enzymes were present in this residual membrane, suggesting that a relaxation of the sorting mechanism need not occur. This prompted the present analysis of COP I vesicles.

Initial experiments focused on Mann II and two independent methods were used since each had potential weaknesses. The biochemical approach provided an accurate value for the percentage of total Mann II present in the COP I vesicles but the measurement of membrane surface area was indirect, relying on analysis of membrane lipids. Phospholipids were chosen since they constitute >85% of total lipid in purified rat liver Golgi preparations (Graham, 1992) and this same percentage was found after analysis of purified COP I vesicles (data not shown). Such a high percentage meant that any variation in other lipids within the COP I vesicle population would have minimal effect on the analysis. Cholesterol, for example, is thought

to be present as a gradient through the Golgi apparatus (Orci et al., 1981) but it comprises less than 5% of total Golgi lipid, so even major changes should have no significant effect.

Lipoprotein particles are another potential source of error since they are surrounded by a monolayer of phospholipid. They are present in the starting membranes but not the COP I vesicles, leading to an underestimate of the percentage phospholipid in these vesicles. Stereological analysis, however, showed that these particles constituted no more than 10% of the Golgi membrane surface area, and the correction factor (5%) was sufficiently small as to pose no problems should the composition of the lipoprotein monolayer not match that of Golgi membranes.

A potentially more serious problem for the surface area calculations is the density of membrane proteins in COP I vesicles, which has not yet been determined. This density is relatively constant for ER, Golgi, and plasma membranes at ∼30,000 proteins/μm² (Quinn et al., 1984). Spanning membrane proteins occupy between 25 and 30% of membrane surface area (Branton and Deamer, 1972), so changes in protein density could affect surface area values based entirely on phospholipid content. That said, it is unlikely that the densities are radically different since this should show up as a change in the equilibrium density which depends on the protein to lipid ratio. The density of COP I vesicles after they have lost their coat is very similar to that of Golgi membranes (data not shown).

In contrast to the biochemical approach, the morphological approach suffers more from the opposite problem. The percentage of membrane in COP I vesicles and buds can be accurately estimated using standard stereological procedures since they are homogeneous in size and have a morphologically distinct coat (Malhotra et al., 1989; Orci et al., 1989). The levels of Mann II that can be detected,
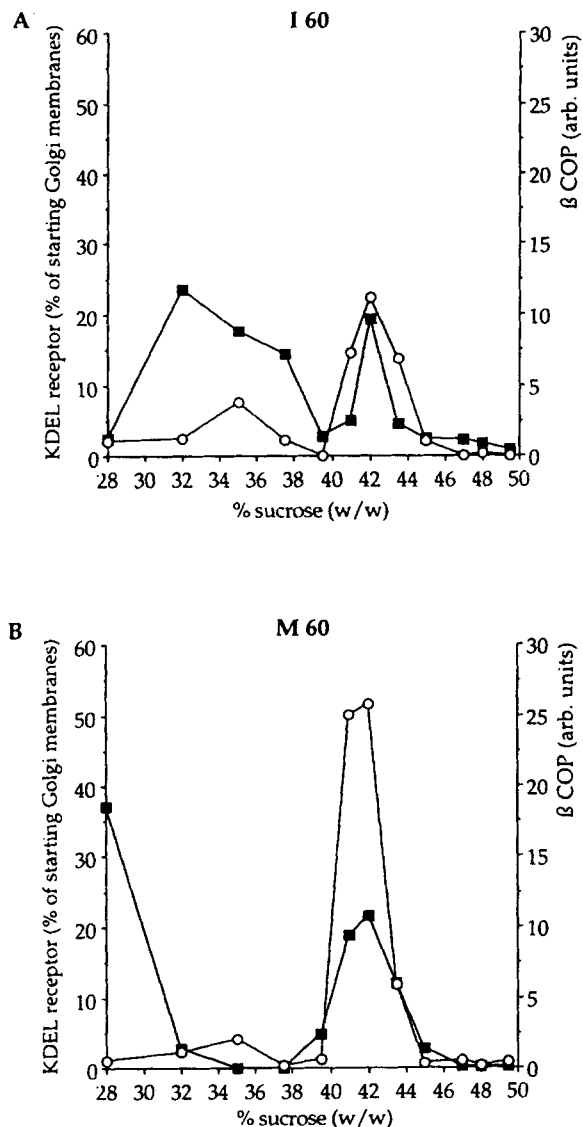
A   **I 60**

B   **M 60**

Figure 5. Distribution of the KDEL receptor in fractionated interphase and mitotic Golgi membranes. Samples from (*A*) interphase or (*B*) mitotic incubations were centrifuged to equilibrium on 30–50% (wt/wt) sucrose gradients and pelleted membranes fractionated by SDS-PAGE, followed by Western blotting with antibodies to the KDEL receptor and β-COP. —■—, KDEL receptor; —O—, β-COP.

Figure 6. Consumption of Golgi cisternae by COP I–coated vesicles. Golgi membranes from a 5-min mitotic incubation in the presence of GTPγS were fixed and processed for cryoelectron microscopy. Sections were labeled with antibodies against ε-COP followed by anti–rabbit antibodies coupled to 10 nm gold. Note the budding of COP I vesicles from several cisternae of the stack (*arrows*). Magnification, 97,000. Bar, 0.2 μm.

however, depend on the number of epitopes accessible to the antibody and this can often be a small proportion of the total (Griffiths et al., 1994*a*). Mann II was chosen because it is a reasonably abundant protein and high affinity antibodies were available. The number of accessible epitopes could also have been affected by the presence or absence of COP I coats. This possibility was eliminated by comparing the labeling density for Mann II in starting membranes and after mitotic incubation. Despite more than half the membrane being covered by COP I coats, the labeling density for Mann II was almost the same. The similarity between these values and that obtained after interphase incubation also argues that Mann II epitopes neither appeared nor disappeared during the course of the incubation.
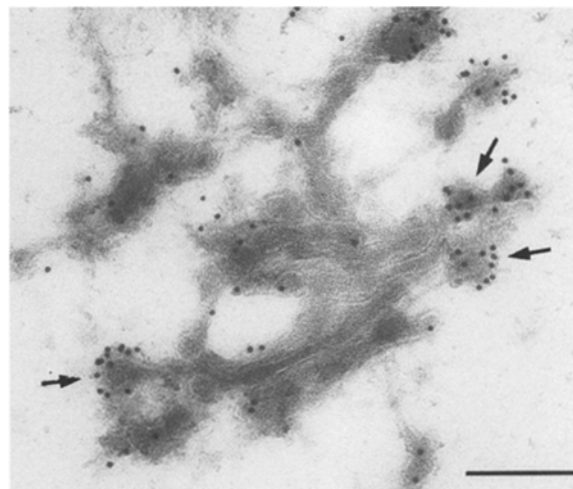
These two independent approaches were validated by the similarity of the results obtained under each condition. Under interphase conditions the average density of Mann II in COP I vesicles was 24% for the biochemical approach and 18% for the morphological approach. The corresponding results for mitotic conditions were 16% (biochemical approach) and 15% (morphological approach). These results also showed that the average density was similar for the interphase and mitotic conditions, a result not limited to Mann II. The biochemical approach was used to determine the average density of three other Golgi enzymes from the same and different parts of the Golgi apparatus (Table I). Using the *t* test, no significant differences between interphase and mitotic vesicles ($0.01 < P < 0.05$) were found for Mann II, NAGT I, and GalT. GalNAcT was the exception and even here the average density was lower, not higher, in mitotic than in interphase COP I vesicles. In other words, there was no relaxation of the sorting mechanism for this or any of the other resident Golgi enzymes.

The two cargo proteins and the two recycling enzymes were also present at very similar densities in COP I vesicles under interphase and mitotic conditions (Tables I and II). There was no significant difference at the $0.01 < P < 0.05$ level using the *t* test. When combined with the data for the resident Golgi enzymes, the results strongly suggest that COP I vesicles carry out the same functions under mitotic conditions the only difference being that more membrane is consumed, perhaps the consequence of cisternal unstacking.

For cargo molecules, the average density in COP I vesicles is an accurate reflection of their density in starting Golgi membranes because they are present in all Golgi compartments at apparently the same concentration. For resident Golgi enzymes this is only true if each compart-
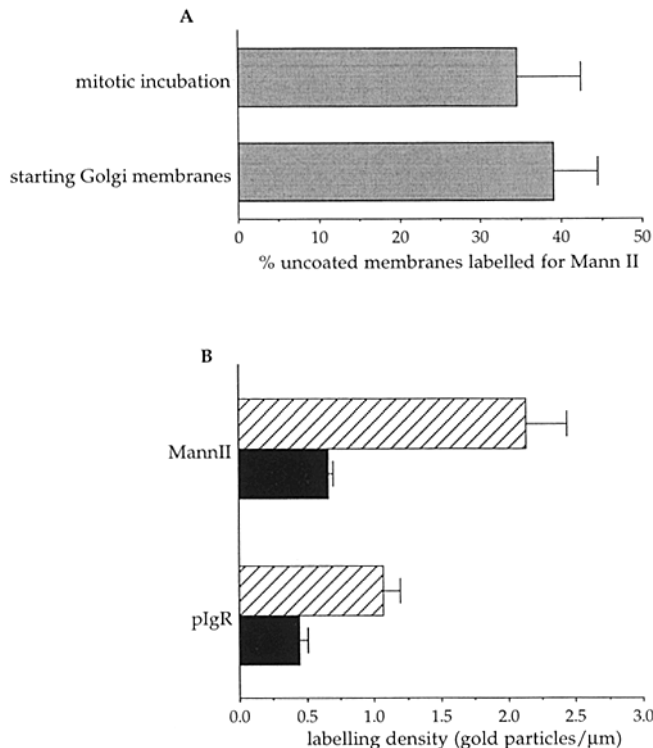
Figure 7. Mann II and pIgR in Golgi membranes and uncoated mitotic Golgi remnants. Golgi membranes and mitotic incubations as well as isolated uncoated remnants from the first gradient were fixed and processed for cryoelectron microscopy. (*A*) Sections were labeled with an antibody to Mann II followed by secondary antibodies coupled to 10 nm gold. Uncoated membranes in Golgi stacks and mitotic incubations were identified and the percentage labeled for Mann II determined. (*B*) Sections were labeled with antibodies to either pIgR or Mann II followed by secondary antibodies coupled to 10 nm gold. The linear density of labeling for Mann II and pIgR was compared for starting membranes and isolated, uncoated remnants. ▨, mitotic uncoated remnants; ■, starting Golgi membranes.

ment is consumed to the same extent by COP I vesicle budding. This is important because each of the resident enzymes has a more restricted distribution in the Golgi apparatus than cargo molecules so not all the vesicles that bud will contain the enzyme. If vesicles bud predominantly from the compartment containing the enzyme, it will be over represented in the COP I vesicle population. If vesicles bud mostly from other compartments it will be under represented. Only if the compartment is consumed to the same extent as other compartments will the average density be an accurate reflection of the average density in the starting Golgi membranes. It is not possible to correct for unequal consumption by estimating the percentage of vesicles that label for a particular resident enzyme. As an example, 11% of the COP I vesicles were labeled for Mann II but this does not mean that only these labeled vesicles contain this enzyme. The immunogold methods are not sufficiently sensitive to ensure that each vesicle containing Mann II will be labeled with the antibody.

The possibility of unequal consumption by COP I vesicles arises because COP I coats are mostly found on the cis-side of the Golgi stack, on the CGN, with much less on the stack and the TGN (Oprins et al., 1993). This indicates that more of the CGN might be consumed than other compartments, a suggestion supported by the higher percentage of GalNAcT in COP I vesicles than the Golgi enzymes. This higher percentage, however, was only found under interphase conditions. Under mitotic conditions, the average density for all the resident Golgi enzymes was remarkably similar, between 16 and 19% of that in starting membranes, suggesting that budding was occurring from all parts of the Golgi apparatus. Several other pieces of evidence support this interpretation. First, short mitotic incubations showed that COP I vesicles could bud from any cisterna in the stack. Second, uncoated membranes containing Mann II were as abundant after mitotic incubations as before. If the cis membranes had been selectively consumed then the percentage of Mann II–containing membranes should have risen. Last, the linear density of Mann II in uncoated membranes at the end of a mitotic incubation was higher than in starting membranes. Since the density of Mann II in COP I vesicles was lower than that in starting membranes this result is consistent with removal of membrane depleted in Mann II by the COP I budding mechanism.

The levels of resident Golgi enzymes and cargo proteins in COP I vesicles were unexpected and it could be argued that they are the consequence of the cell-free assay conditions. Identification and isolation of the COP I vesicles required the addition of GTPγS and this could have altered the content either by changing the sorting capacity or by permitting inappropriate budding from regions other than the cisternal rims. A change in the sorting capacity seems unlikely since Rothman and colleagues have shown that the percentage of VSV G protein cargo that enters COP I vesicles is the same in the presence or absence of GTPγS (Ostermann et al., 1993). Inappropriate budding is also unlikely since the amount of Golgi membrane consumed under mitotic conditions is very similar in the presence (51–56%) or absence (52%) of GTPγS (this paper; Misteli and Warren, 1994, 1995). In the absence of GTPγS, COP I vesicles uncoat but they can be scored under mitotic conditions because a downstream fusion event is inhibited (Levine et al., 1996).

Conversely, it might be argued that the cell-free system does not reflect what happens in vivo. Two lines of evidence argue against this. The first is measurements of the surface labeling density of GalT in HeLa cells treated with okadaic acid, a specific phosphatase inhibitor that mimics the mitotic fragmentation of the Golgi apparatus (Lucocq et al., 1995). The labeling density over uncoated clusters (both tubules and cisternae) rose 2.5-fold, similar to the increase in linear density observed for Mann II in the cell-free system (2.9-fold; Fig. 7). The second line of evidence comes from experiments in which cryosections of rat liver were labeled with antibodies to RSA and ApoE (Dahan et al., 1994). Both of these cargo molecules were detected throughout Golgi cisternae but not in COP I vesicles, consistent with the much lower density of RSA in isolated vesicles compared with starting membranes (Table I).

Golgi enzymes and cargo proteins are major constituents of Golgi membranes, yet all examples tested were found to be depleted in COP I vesicles. Taken together,

their average density ranged from 14 to 30% of that in starting Golgi membranes. This raises the question as to the identity of the proteins which make up the rest of the COP I vesicle membrane. They are probably major proteins because up to 56% of Golgi membrane can be converted into COP I vesicles and because there is no apparent change in the lipid to protein ratio of the vesicle membrane when compared with starting Golgi membranes. A preliminary answer comes from analysis of the COP I vesicle membrane after washing with sodium carbonate. After SDS-PAGE and silver staining, at least 20 proteins found in COP I membranes are either not present in the uncoated remnants or are depleted (data not shown). These are presently being analyzed.

In contrast to cargo and resident enzymes, the KDEL receptor was present in COP I vesicles at levels compatible with its known function. The average density under interphase conditions was 66% for the morphological approach and 125% for the biochemical one. The difference probably reflects inaccessibility of epitopes using the morphological approach since the antibody was raised to the cytoplasmic tail which might well be masked by the coat proteins in the COP I vesicles. This suggests that the average density using the biochemical approach is the more accurate indicating that the receptor is enriched in COP I vesicles relative to Golgi membranes. Such a result is compatible with the suggestion that cycling of the receptor from the Golgi to the ER is triggered by KDEL-bearing ER proteins that have left the ER (Lewis and Pelham, 1992). If ligand binding somehow triggered aggregation of receptor–ligand complexes which then became incorporated into COP I vesicles, this would explain the observed enrichment. Since 35% of the COP I vesicles were labeled for the KDEL receptor using immunogold labeling techniques, this suggests that recycling this receptor is a major function of these vesicles.

ERGIC-53/p58 was present in interphase COP I vesicles at an average density that was 93% of that in starting membranes. The real density is likely to be much higher since this protein is restricted to the CGN so should only be present in vesicles that bud from this compartment. Such an enrichment would also be compatible with its function as a putative cargo receptor. ERGIC-53/p58 is now known to be a mannose-binding protein so it could ferry glycosylated cargo in the forward direction and then return empty along a retrograde pathway.

There are several models for transport within the Golgi apparatus and the present results provide support for some but not all of them. The cisternal maturation model suggests that retrograde transport is the only vesicle-mediated step. It was originally put forward to explain the movement of algal scales too large to enter forward transport vesicles (Brown et al., 1970; Melkonian et al., 1991). Instead, resident Golgi enzymes had to move back to the previous cisterna as each cisterna matured. Such a model predicts that Golgi enzymes, not cargo, will be found in the retrograde vesicles. The levels of cargo were indeed lower than expected and Golgi enzymes were found in COP I vesicles. However, the average densities of Golgi enzymes in the vesicles were lower than in the starting membrane so that budding actually resulted in an increase in the average density of the Golgi enzymes that remained

behind. Such a result is incompatible with the cisternal maturation model unless another, yet-to-be-identified retrograde vesicle is involved.

A second model argues that COP I vesicles are involved in retrograde transport but other, yet-to-be-identified vesicles are involved in forward transport. This model certainly explains the low densities of cargo molecules which would mostly be present in the forward moving vesicles. The levels of Golgi enzymes could be explained either as a salvage pathway for recycling Golgi enzymes that have left their normal location or as a constitutive pathway that ferries Golgi enzymes between adjacent cisternae. The latter would explain the overlapping distribution of many Golgi enzymes in HeLa cells (Nilsson et al., 1993; Rabouille et al., 1995).

The third model argues that COP I vesicles are involved in forward as well as retrograde transport (Rothman and Wieland, 1996). There is considerable biochemical evidence that COP I vesicles are involved in forward transport through the Golgi apparatus, perhaps the most compelling of which is the demonstration that isolated COP I vesicles carrying VSV G proteins will deliver this cargo to acceptor Golgi membranes (Ostermann et al., 1993). Immuno-EM studies showed that ~40% of the COP I–coated vesicles and buds contained the G protein (Orci et al., 1986), suggesting that more or less equal numbers of COP I vesicles are involved in the forward and retrograde pathways. Quantitation showed that the linear density of the viral G protein was similar in the cisternal membranes and the vesicles budding from it, an observation which led to the idea of bulk flow, the G protein being sampled at the density prevailing in the cisternal membrane (Orci et al., 1986). When combined with the percentage labeling this would yield an apparent average density of the G protein in COP I vesicles and buds of 40%, a figure that should be compared with the 18% for RSA and the 16–23% for pIgR. These lower values either suggest that the G protein has access to more of the membrane in COP I vesicles for reasons that are presently unclear, or that the physiological cargo is predominantly carried in other forward moving vesicles. It is, therefore, interesting to note that lipoprotein particles have not been detected in COP I vesicles (Dahan et al., 1994; this paper), perhaps because their size precludes their entry. The same might also be true for other cargo particles, most notably viral particles, that are transported through the Golgi apparatus. Further understanding of the role of COP I vesicles must now await a population analysis, determining which proteins share the same vesicle and which do not. This is now the focus of our research efforts.

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