GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles

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Upon addition of GTP γ S to *in vitro* budding reactions, COP I vesicles form but retain their coat, making them easy to isolate and analyze. We have developed an *in vitro* budding assay that reconstitutes the formation of COP I-derived vesicles under conditions where GTP hydrolysis can occur. Once formed, vesicles are uncoated and appear functional as they fuse readily with acceptor membranes. Electron microscopy shows a homogeneous population of uncoated vesicles that contain the *medial/trans* Golgi enzyme α 1,2-mannosidase II. Biochemical quantitation of vesicles reveals that resident Golgi enzymes are up to 10-fold more concentrated than in donor membranes, but vesicles formed in the presence of GTP γ S show an average density of resident Golgi enzymes similar to that seen in donor membranes. We show that the sorting process is mediated by the small GTPase arf-1 as addition of a dominant, hydrolysis-deficient arf-1 Q71L mutant produced results similar to that of GTP γ S. Strikingly, the average density of the anterograde cargo protein, polymeric IgA receptor, in COP I-derived vesicles was similar to that found in starting membranes and was independent of GTP hydrolysis. We conclude that hydrolysis of GTP bound to arf-1 promotes selective segregation and concentration of Golgi resident enzymes into COP I vesicles.

Keywords: arf-1/COP I vesicles/Golgi resident enzymes/GTP hydrolysis/sorting

Introduction

Anterograde transport of newly synthesized proteins through the secretory pathway begins in the endoplasmic reticulum (ER) where cytosolic components of the COP II coat are thought to capture newly synthesized transmembrane proteins in the ER by binding to their cytoplasmic domains. This results in selective packaging and concentration of cargo into budding COP II vesicles. Following budding, COP II vesicles uncoat and fuse to create larger membrane structures termed vesicular tubular clusters

(VTCs) or transport clusters (TCs). TCs subsequently move along microtubules towards the central juxta-nuclear Golgi complex situated in close proximity to the microtubule organizing center. Here, newly synthesized proteins are delivered to the Golgi complex for further rounds of anterograde transport (for reviews, see Rothman and Wieland, 1996; Bannykh *et al.*, 1998; Pelham, 1998).

How proteins make their way through the Golgi stack is presently an open question (for a review see Glick and Malhotra, 1998). The discovery by Rothman and co-workers of transport intermediates termed COP I vesicles led to the suggestion that these ferry newly synthesized proteins from cisterna to cisterna in an anterograde direction (Orci *et al.*, 1986; Malhotra *et al.*, 1989). Several lines of evidence support the notion that COP I vesicles indeed mediate forward transport of cargo proteins. First, COP I vesicles purified from vesicular stomatitis virus (VSV)-infected cells could successfully deliver VSV-G protein to acceptor membranes *in vitro* (Ostermann *et al.*, 1993). Secondly, immunogold labeling of infected cells revealed the presence of VSV-G in COP I vesicles as well as in buds (Orci *et al.*, 1986, 1997). Thirdly, microinjection of antibodies directed towards native coatamer inhibited anterograde transport *in vivo* (Pepperkok *et al.*, 1993). Finally, deletion mutants of coatamer subunits are essential in yeast (Hosobuchi *et al.*, 1992) and a temperature-sensitive mutant of β' -COP was found to impair anterograde transport (Duden *et al.*, 1994).

The nature of anterograde transport was further investigated by means of careful morphometric studies which showed that the concentration of anterograde cargo in COP I vesicles was similar to that of cisternal membranes (Orci *et al.*, 1986, 1997). This observation led to the proposal that anterograde cargo is transported through the secretory pathway by default. The bulk flow hypothesis was formulated which stated that unless endowed with sorting signals, newly synthesized proteins move through the secretory pathway unrestricted. As such, there would be no need for concentration of anterograde cargo into COP I vesicles (Pfeffer and Rothman, 1987; Wieland *et al.*, 1987). The concept of bulk flow has been challenged as several studies have now shown that newly synthesized proteins are actively sorted and concentrated into COP II vesicles (Mizuno and Singer, 1993; Balch *et al.*, 1994; Matsuoka *et al.*, 1998). This process is facilitated by motifs present in cytoplasmic domains of the newly synthesized protein, VSV-G (Nishimura and Balch, 1997), as well as in resident proteins operating in the pathway (Kappeler *et al.*, 1997; Dominguez *et al.*, 1998). A direct interaction between motifs in cytoplasmic domains and COP II coat is thought to allow for lateral sampling in the membrane, resulting in selection and concentration of cargo into budding COP II vesicles (Bednarek *et al.*, 1996). Likewise, resident proteins of the ER and early

parts of the biosynthetic pathway display discrete motifs shown to interact specifically with components of the COP I coat (Cosson and Letourneur, 1994). By inference, one would expect that proteins displaying COP I binding motifs would also be concentrated into COP I vesicles.

Systematic analysis of the protein content of purified COP I vesicles formed *in vitro* in the presence of GTP γ S showed that neither resident proteins nor anterograde cargo were enriched (Sönnichsen *et al.*, 1996). Only one protein, ERGIC-53, a protein carrying a COP I binding motif (Schindler *et al.*, 1993), was found to be slightly concentrated. Most studies, including the one by Sönnichsen *et al.* (1996), rely on the use of GTP γ S. This might not only prevent uncoating, but could also affect the incorporation of proteins into COP I vesicles. The recent work by Love *et al.* (1998) revealed the existence of a steady-state population of vesicles containing relatively high amounts of Golgi-associated glycosylation enzymes. Also, the recent study by Nickel *et al.* (1998) suggests that COP I vesicles formed *in vitro* in the absence of GTP γ S contain more anterograde cargo compared with vesicles formed in the presence of GTP γ S. In their study, only coated vesicles were examined and as COP I vesicles are expected to uncoat rapidly following formation, the majority of vesicles formed may not have been included.

To study COP I vesicles more precisely in terms of protein content, as well as the role of COP I coat in cargo selection, one would ideally like to examine both coated as well as uncoated COP I-derived vesicles. Such studies have so far been hampered by difficulties in isolating uncoated vesicles. We have now overcome these difficulties and developed an *in vitro* budding assay which allows for the formation, isolation and characterization of uncoated COP I-derived vesicles. Our data suggest that Golgi resident proteins are preferentially incorporated into COP I-derived vesicles and that GTP hydrolysis plays an integral role in this process. Furthermore, we identify the small GTPase arf-1, a component of the COP I coat, as the major effector of GTP hydrolysis in this sorting process. Our study therefore highlights a new and unexpected role of arf-1 in promoting the sorting and concentration of resident Golgi proteins into COP I vesicles.

Results

The budding assay

To approximate physiological conditions in vesicle formation better, we modified the COP I vesicle budding assay described previously by Warren and co-workers (Sönnichsen *et al.*, 1996) to allow for GTP hydrolysis. Vesicle formation was reconstituted *in vitro* using highly purified rat liver Golgi membranes and rat liver cytosol. As markers to monitor the extent of incorporation of cargo proteins into COP I vesicles, we followed primarily the abundant *medial/trans* resident Golgi enzyme, α 1,2-mannosidase II (Mann II), as well as the anterograde cargo, polymeric IgG receptor A (pIgR), which is found evenly distributed across the Golgi stacks (Solari *et al.*, 1986). Both Mann II and pIgR are present in relatively high amounts in the Golgi membranes, and are incorporated into COP I vesicles generated in the presence of GTP γ S (Sönnichsen *et al.*, 1996).

Fig. 1. Biochemical characterization of the budding assay. (A) Salt-stripping treatment removes coatomer, but not Mann II, from Golgi membranes. Non-treated Golgi membranes (lane 1) or salt-stripped Golgi membranes (lane 2) were analyzed for their content of Mann II and coatomer by immunoblotting using antibodies against Mann II and β -COP, respectively. (B) GTP γ S decreases the amount of Mann II, but not pIgR, detected in vesicle pellets. Salt-washed Golgi membranes were incubated in assay mixtures containing either 0.5 mM GTP (lane 1) or 20 μ M GTP γ S (lane 2). Budded vesicles were recovered and analyzed for their content of Mann II and pIgR by immunoblotting. (C) Effect of ATP depletion and temperature on the amounts of Mann II and pIgR detected in vesicle pellets. Salt-washed Golgi membranes were incubated at 4°C (lane 1) or at 37°C (lanes 2 and 3) in standard assay mixtures (lanes 1 and 2) or in assay mixtures containing an ATP-depleting system (lane 3). Budded fractions were recovered and the content of Mann II and pIgR was evaluated by immunoblotting. (D) Addition of a sucrose cushion allows for an improved detection of the Mann II signal in vesicle pellets. Low-speed supernatants containing COP I-derived vesicles were centrifuged at high speed without (lane 1) or with (lane 2) the presence of a sucrose cushion. The resulting high-speed pellets were resuspended and solubilized in SDS-PAGE sample buffer and the amount of Mann II signal was detected by immunoblotting. In (B), (C) and (D), standard curves were constructed using increasing amounts of Golgi membranes and were expressed as a percentage of membranes used in the standard assay mixture, as indicated. The data are representative of at least three independent experiments.

The final protocol for vesicle formation is detailed in Materials and methods. Briefly, highly purified Golgi membranes were salt washed to remove a small but significant fraction of preformed vesicles (J.Lanoix and T.Nilsson, data not shown) as well as membrane-bound coatomer (Figure 1A, lane 2). The membranes were then pelleted, resuspended and incubated together with rat liver cytosol for 30 min at 37°C. Incubations were terminated by transferring the membranes on ice. The incubation mixtures were adjusted to 250 mM KCl (final concentration) to detach bound vesicles from membranes. Donor membranes were then separated from slowly sedimenting vesicles by a medium-speed centrifugation and the super-

natant containing the vesicles was overlaid over a 30% sucrose cushion. Underneath this cushion, a second cushion composed of 50% sucrose was added. Following high-speed centrifugation, vesicles were found trapped between the two cushions. The supernatant above the interphase of the sucrose cushions was then carefully removed, and pelleted vesicles were resuspended and subjected to morphological and biochemical analysis.

The density of formed vesicles was determined by equilibrium centrifugation using a protocol described by Sönnichsen *et al.* (1996). This yielded a broad distribution across the gradient extending from 35 to 45% sucrose, in marked contrast to vesicles generated under GTP γ S condition where they distributed in a discrete peak at the expected density of 40% sucrose (J.Lanoix and T.Nilsson, data not shown). A partial or complete uncoating of the vesicles after the budding event might explain the broader distribution seen under GTP condition. Alternatively, vesicles formed under GTP condition could be heterogeneous in terms of protein content, resulting in variations in densities. Nevertheless, this observation ruled out the use of equilibrium centrifugation for efficient vesicle recovery. The addition of the two-step sucrose cushion in this budding assay resolved the problem of a heterogeneous vesicle population since most, if not all, vesicles released from the KCl wash were recovered at the 30/50% interphase. No material could be detected in the remaining supernatant as determined by Western blotting against Mann II and pIgR (data not shown). In parallel experiments, we tested the recovery of vesicles generated in the presence of GTP γ S using the same procedure. Again, all budded material released from the KCl wash was found in the 30/50% interphase.

Formation of COP I-derived vesicles containing high amounts of Mann II

We next monitored the extent of incorporation of Mann II and pIgR into vesicles generated in the presence of GTP and GTP γ S (Figure 1B). Vesicle fractions were subjected to SDS-PAGE followed by Western blotting using polyclonal antibodies against Mann II or pIgR. The signals were then compared with a standard curve of known amounts of Golgi membranes. When a budding reaction was performed in the presence of GTP, high levels of Mann II were detected in the vesicle fraction (lane 1; for quantitation, see Table IV). In contrast, when vesicles were generated in the presence of GTP γ S, the level of Mann II detected was significantly lower (lane 2). The level of pIgR found in the vesicle pellet was low and was not drastically affected upon addition of GTP γ S (compare lanes 1 and 2), indicating a preferential incorporation of Mann II in the absence of GTP γ S.

We also examined the effect of temperature as well as the energy requirement in the budding assay (Figure 1C). When a reaction was performed at 4°C (lane 1) or in the presence of an ATP-depleting system (lane 3), very little Mann II was detected compared with the levels seen at 37°C (lane 2). This shows that the elevated Mann II levels generated at 37°C could not be the result of mechanical fragmentation of membranes, as they required an energy- and temperature-dependent process. Examining vesicles for their content of pIgR revealed only modest levels at either condition (compare lanes 1, 2 and 3). We observed

no apparent difference at 37°C with or without ATP, which was unexpected. The significance of this finding remains unclear and will be discussed further (see Discussion).

The preferential incorporation of relatively high amounts of Mann II into COP I vesicles had not been observed in the past. We therefore examined parameters which, besides omitting GTP γ S, could have permitted such an extent of Mann II incorporation. Two technical parameters were found to be important. Firstly, the use of the sucrose layers was critical, as pelleting in the absence of 30/50% sucrose drastically reduced the amount of Mann II recovered in the vesicle fraction (Figure 1D, compare lanes 1 and 2). Presumably, in the absence of sucrose, sedimented vesicles were packed too densely at the bottom of the tube to be resuspended properly. Secondly, the preparation and source of the cytosol were found to be crucial. Figure 2A (lane 2) shows that subjecting the cytosol to a desalting step caused a dramatic reduction in the amount of Mann II recovered in the vesicle pellet. Preliminary data suggest that the observed reduction is due to a significant loss of coatamer as well as additional factors required for vesicle formation as well as sorting (J.Lanoix and T.Nilsson, data not shown).

Upon testing cytosols from other sources (Figure 2B), we found that compared with rat liver cytosol (lane 1), bovine brain cytosol (lane 2) and HeLa cytosol (lane 3) performed less well in the budding assay (Table I). It is conceivable that these differences are not caused by differences in the amount of vesicles formed, but rather reflect differences in the steady-state concentration of vesicles, which is determined by the rates of both budding and fusion. If vesicles formed in the presence of bovine brain or HeLa cytosols indeed fused more rapidly than those generated in the presence of rat liver cytosol, one would expect that in the presence of GTP γ S, the level of Mann II detected should be comparable between the three cytosols. Figure 2C shows that this is not the case (compare lane 2 with lanes 4 and 6). This supports the conclusion that differences between cytosols reflect differences in vesicle formation.

To rule out that budded vesicles undergo multiple rounds of budding and fusion in our *in vitro* budding assay, we specifically blocked fusion. Components required for COP I vesicle docking and fusion have been identified and characterized in great detail (for a review, see Rothman and Wieland, 1996). The cytosolic ATPase NSF (NEM-sensitive factor) and its cofactor α -SNAP (soluble NSF attachment factor) are key components in vesicle fusion. Their precise roles remain to be determined, although data exist to suggest that they either act to prime the fusion event (Mayer *et al.*, 1996; Sato and Wickner, 1998; Xu *et al.*, 1999) or mediate the actual fusion event (Otter-Nilsson *et al.*, 1999). A mutant of α -SNAP, α -SNAP (L294A), has been shown to block fusion effectively in several cases (Barnard *et al.*, 1997; Christoforidis *et al.*, 1999). As shown in Figure 2D (lane 2), addition of α -SNAP (L294A) to the budding reaction did not result in any detectable differences in Mann II levels (compare lanes 1 and 2), arguing that formed vesicles are not subjected to multiple rounds of budding and fusion.

We then tested whether *in vitro* generated vesicles could fuse with isolated Golgi membranes, and determined whether this step was inhibited by α -SNAP (L294A). We

Fig. 2. Rat liver cytosol supports budding and allows accumulation of Mann II-containing vesicles. **(A)** Desalted rat liver cytosol reduces the amount of Mann II signal detected in vesicle pellets. Salt-washed Golgi membranes were incubated in assay mixtures containing 5 mg/ml of either non-desalted cytosol (lane 1) or desalted cytosol (lane 2). Budded vesicles were recovered and their content of Mann II was analyzed by immunoblotting. **(B)** Effect of various cytosols on the amount of Mann II signal detected in vesicle pellets. Salt-washed Golgi membranes were incubated in assay mixtures containing 5 mg/ml of cytosols from either rat liver (lane 1), bovine brain (lane 2) or HeLa cells (lane 3). Budded vesicles were recovered and their content of Mann II was analyzed by immunoblotting. **(C)** Addition of GTP γ S in bovine brain and HeLa cytosols does not increase the Mann II signal detected in vesicle pellets. Salt-washed Golgi membranes were incubated in assay mixtures containing either 0.5 mM GTP (lanes 1, 3 and 5) or 20 μ M GTP γ S (lanes 2, 4 and 6) and cytosols from rat liver (lanes 1 and 2), bovine brain (lanes 3 and 4) and HeLa (lanes 5 and 6). Budded vesicles were recovered and analyzed for their content of Mann II by immunoblotting. **(D)** Addition of α -SNAP (L294A) mutant does not modify the amount of Mann II detected in vesicle pellets. Salt-washed Golgi membranes were incubated in assay mixtures containing rat liver cytosol without (lane 1) or with 20 μ M α -SNAP (L294) mutant (lane 2). Budded vesicles were recovered and analyzed for their content of Mann II by immunoblotting. **(E)** The α -SNAP (L294) mutant inhibits vesicle fusion with acceptor membranes. Budded vesicles were generated from a standard budding assay. The vesicle pellets were resuspended and the vesicles were used as donor membranes in the *in vitro* Golgi transport assay (Love *et al.*, 1998) either in the absence (filled circles) or in the presence of 20 μ M α -SNAP (L294) mutant (open circles). **(F)** Rat liver cytosol does not support vesicle fusion with acceptor membranes. Budded vesicles were generated from standard budding assay. The vesicle pellets were resuspended and the vesicles were used as donor membranes in the *in vitro* Golgi transport assay in the presence of either CHO cytosol or rat liver cytosol, as indicated. The data are representative of at least three independent experiments.

employed a modified version of the *in vitro* intra-Golgi transport assay originally developed by Balch *et al.* (1984). Instead of using CHO wild-type (wt) membranes, COP I-derived vesicles from rat liver Golgi membranes were incubated together with Golgi membranes purified from VSV-infected CHO 15B cells. As CHO 15B cells are deficient in the *medial/trans* Golgi enzyme *N*-acetylglucosaminyltransferase I (NAGT I), delivery of

NAGT I activity from donor vesicles into the 15B acceptor membranes can be measured by monitoring addition of *N*-acetylglucosamine (GlcNAc) onto the N-linked oligosaccharides of the VSV-G protein. As shown in Figure 2E, the COP I-derived vesicles fused readily with acceptor membranes in the absence of the α -SNAP mutant, but failed to do so in the presence of the mutant. This experiment shows that α -SNAP (L294A) efficiently

Table I. Phospholipid analysis of vesicle pellets generated in the presence of various sources of cytosol (% of starting membranes)

Rat liver	Bovine brain	HeLa
2.5 ± 0.6	0.4 ± 0.1	0.8 ± 0.3

Vesicles were produced at 4°C or at 37°C in assay mixtures containing various cytosols. Budded vesicles were recovered and analyzed for their phospholipid contents as described in Materials and methods. Standard curves of known amounts of starting membranes were included in all experiments and used to evaluate the amounts of phospholipids in vesicle fractions. The amounts of phospholipids recovered in vesicles generated at 4°C were subtracted as non-specific background from that generated at 37°C. Results are expressed as the percentage of the phospholipids in vesicles over the starting membranes ± SD (*n* ranged from 3 to 4).

blocks fusion of COP I-derived vesicles. Furthermore, as COP I-derived vesicles fused efficiently with acceptor membranes in the absence of the α -SNAP (L294A), it shows that they can serve as functional transport intermediates (see also Figure 5).

Further examination of the rat liver cytosol revealed a surprising feature when tested in the *in vitro* intra-Golgi transport assay. To allow for the use of cytosol that was neither desalted nor dialyzed to remove nucleotides, addition of GlcNAc was monitored through the acquired resistance to deglycosylation by Endo H (Fries and Rothman, 1980; Love *et al.*, 1998). When compared with CHO cytosol, rat liver cytosol failed to support fusion. Very little, if any, conversion was observed compared with CHO cytosol which routinely yielded an ~50% conversion of the VSV-G protein into the Endo H-resistant form when used with salt-washed Golgi membranes (Figure 2F). While further work is needed to determine why rat liver cytosol fails to support fusion of COP I-derived vesicles *in vitro* (see Discussion), this finding suggests that once formed, vesicles are not subjected to multiple rounds of budding and fusion. Therefore, this allows us to compare directly the protein contents of vesicles formed under GTP and GTP γ S conditions.

We next examined vesicle pellets by negative-stain electron microscopy. Figure 3 shows typical fields of vesicles generated at 37°C (A), at 4°C (B) and in the presence of an ATP-depleting system (C). Quantitation (Table II) showed that incubations at 37°C generated 5- and 10-fold more vesicles (arrowheads) when compared with incubations performed at 4°C or in the presence of an ATP-depleting system, respectively. Occasionally, we observed other membrane structures. These were mainly vesicular profiles larger than 200 nm (see the arrows in Figure 3A, B and C). However, under all three conditions, the concentration of other membrane structures was identical. As expected, protein aggregates were seen after ATP depleting the system (Traub *et al.*, 1993). We also performed the budding reaction in the absence of Golgi membranes (Figure 3D). No vesicular profiles were seen in this case, which eliminates the possibility that the assay was detecting vesicles present in the cytosol.

Formation of Mann II-containing vesicles is a coatamer-dependent process

To test for the requirement of coatamer, the main cytosolic constituent of the COP I coat, in vesicle formation, rat

liver cytosol was immunodepleted using the monoclonal antibody CM1A10 specific for native coatamer (Palmer *et al.*, 1993). Figure 4A shows that the depletion of coatamer was complete (lane 2) as antibodies to the α , β and γ subunits of coatamer failed to detect any of these components. An additional minor and slower migrating protein was detected by the monoclonal antibody to β -COP. The nature of this protein is at present unclear (most likely, this represents one of the microtubule-associated proteins against which the antibody was originally raised). It does not appear to be depleted by the CM1A10 antibody (asterisk, Figure 4A). As a control, cytosol was mock depleted using an irrelevant antibody (lane 1). To show that immunodepletion was specific for coatamer, we monitored the cytosolic arf-1 protein. Comparison between mock-depleted and CM1A10-depleted cytosols indicated no apparent decrease in the level of arf-1 (compare lanes 1 with 2). As shown in Figure 4B, removal of coatamer from the cytosol resulted in a 5.5 (± 1.6)-fold reduction in the amount of Mann II detected in the vesicle fraction (lane 1). This was in contrast to mock-depleted cytosol which showed no apparent decrease of Mann II (lane 2). Purified coatamer, when added to the coatamer-depleted cytosol, restored Mann II levels to control levels (lane 3). We conclude that our budding assay measures a COP I-dependent process.

Formation of functional transport intermediates is a coatamer-dependent process

We next determined the amount of coatamer present on vesicles formed in the absence of GTP γ S. Figure 5A shows that very little coatamer can be detected on these vesicles (lane 1). In contrast, vesicles formed in the presence of GTP γ S contained coatamer, as expected (lane 2). This suggests that vesicles uncoat rapidly once formed (Elazar *et al.*, 1994). We then tested whether functional transport vesicles in this fraction were formed in a coatamer-dependent process. As shown in Figure 5B, the formation of vesicles in the presence of a coatamer-depleted cytosol (lane 3) resulted in a 4-fold decrease in delivered NAGT I compared with untreated cytosol (lane 2) or mock-depleted cytosol (lane 4). This reduction was comparable to levels obtained upon performing the budding reaction at 4°C (lane 1). Re-addition of purified coatamer to coatamer-depleted cytosol restored its ability to produce functional transport vesicles (lane 5). Taken together, these data demonstrate that coatamer is required for the production of functional transport intermediates.

Inhibition of GTP hydrolysis impairs incorporation of Golgi resident proteins

We next examined vesicles by immunoelectron microscopy. Thin frozen sections were prepared from vesicle pellets generated in the absence or presence of GTP γ S and immunolabeled for the resident Golgi enzyme Mann II. As shown in Figure 6A, clusters of vesicular/tubular profiles of vesicles formed in the absence of GTP γ S were devoid of any detectable coat structure (arrowheads), in agreement with above biochemical data (Figure 5A). Immunogold labeling confirmed that a subpopulation of vesicles contained Mann II. Negligible labeling was seen outside vesicular profiles. When produced in the presence of GTP γ S, similar vesicular/tubular profiles were observed,

Fig. 3. Morphological characterization of the vesicle pellets by electron microscopy. Vesicles were generated in standard assay mixtures after incubation at 37°C (A), at 4°C (B), in the presence of an ATP-depleting system (C) or in the absence of Golgi membranes (D). After high-speed centrifugation, the pellets were resuspended and further processed for negative staining as described in Materials and methods. The arrowheads indicate budded vesicles (defined as vesicular profiles having a diameter between 40 and 120 nm) found in each fraction. Arrows show vesicular profiles larger than 150 nm which are considered to be membrane contaminants. Bar, 200 nm. In (C), big arrowheads indicate large protein aggregates. In (A), the upper left corner shows a higher magnification of three vesicular profiles. Note the apparent connections between the three vesicles. This could either represent tubular connections or filamentous structures (Orci *et al.*, 1998; Sönnichsen *et al.*, 1998). Bar, 100 nm.

Table II. Amount of vesicles generated after various incubation conditions (number of vesicles/ μm^2)

37°C	4.66 ± 1.16
4°C	0.94 ± 0.13
(-)ATP	0.43 ± 0.09

Vesicle pellets were generated after incubations at 37°C or 4°C or in the presence of an ATP-depleting system [(-)ATP]. The pellets were resuspended, processed for negative staining and the amount of vesicles formed was quantified as described in Materials and methods.

but these were clearly coated (arrows, Figure 6B) and contained significantly less Mann II. The latter was confirmed by stereological quantitation which showed a 4-fold reduction in the linear labeling density for Mann II (Table III), in agreement with the biochemical data presented in Figure 1B.

To determine whether inhibition of GTP hydrolysis had also affected the incorporation of other cargo proteins, we extended the range of marker proteins to include two other glycosylation enzymes: NAGT I and the *trans* Golgi/TGN enzyme, β 1,4-galactosyltransferase (GalT).

Biochemical analysis of Mann II, NAGT I and GalT showed that all three were incorporated to a relative high extent, NAGT I being the highest ($25.8 \pm 2.7\%$ of starting material) (Table IV). In marked contrast, vesicles formed in the presence of GTP γ S contained as much as 8-fold less glycosylation enzymes. For the anterograde pIgR, incorporation was relatively low ($\sim 4\%$ of starting material) and no significant decrease was observed upon addition of GTP γ S. To exclude the possibility that vesicle formation was dramatically altered upon addition of GTP γ S, we determined the membrane surface area by measuring amounts of phospholipids recovered in isolated vesicles (Sönnichsen *et al.*, 1996). Table IV shows that the extent of vesicle formation was similar under GTP and GTP γ S conditions, ruling out the possibility that the observed decrease in incorporation of resident Golgi enzymes was a consequence of a GTP γ S-mediated inhibition or stimulation of vesicle budding. From these data, we conclude that GTP hydrolysis selectively promotes incorporation of resident glycosylation enzymes into COP I-derived vesicles.

Fig. 4. Coatomer-dependent formation of Mann II-containing vesicles. (A) Immunodepletion of cytosolic coatomer. Rat liver cytosol was incubated with the anti-coatomer antibody CM1A10 coupled to protein A-beads (lane 2) or with a mock antibody coupled to protein A-beads (lane 1) and analyzed by immunoblotting for the α , γ and β coatomer subunits and for cytosolic arf-1. The asterisk indicates a slower migrating protein that cross-reacts with β -COP antibody but is not depleted by the CM1A10 antibody. (B) Requirement of coatomer for budding of Mann II-containing vesicles. Golgi membranes were incubated in assay mixtures containing a coatomer-depleted cytosol (lane 1), a mock-depleted cytosol (lane 2) or a coatomer-depleted cytosol supplemented with purified coatomer (lane 3). Budded vesicles were recovered and analyzed by immunoblotting for their content of Mann II.

arf-1 promotes selective concentration of resident Golgi enzymes into vesicles

We then set out to identify the GTP binding protein that promoted the selective incorporation of resident Golgi enzymes. We focused on the small GTPase ADP-ribosylation factor 1 (arf-1). This protein has been shown to be involved in the formation of COP I vesicles (Donaldson *et al.*, 1992; Palmer *et al.*, 1993). To test the role of arf-1, we used the recombinant hydrolysis-deficient arf-1 Q71L mutant (Tanigawa *et al.*, 1993; Dascher and Balch, 1994). As shown in Figure 7, addition of the arf-1 Q71L mutant to the budding reaction (lane 3) resulted in a dramatic reduction in the incorporation of Mann II into vesicles. This was not observed with the wild-type arf-1 protein (lane 2). It was noted that the degree of inhibition and the extent of vesicle formation in the presence of the arf-1 mutant were very similar to those obtained upon addition of GTP γ S (Table IV), suggesting a direct role for arf-1 in protein sorting through GTP hydrolysis.

We next calculated the relative concentration of each marker in the vesicles relative to donor membranes by dividing the percentage of the marker by the percentage of phospholipids found in the vesicle pellets (according to values shown in Table IV). As summarized in Figure 8, resident Golgi enzymes were found to be concentrated between 4- and 10-fold in the presence of GTP. In marked contrast, addition of GTP γ S or the arf-1 Q71L mutant dramatically reduced this concentration to levels similar to that seen in the cisternal membranes. In all conditions tested, the anterograde cargo, pIgR, was not significantly concentrated showing that GTP hydrolysis by arf-1 protein preferentially drives packaging

Fig. 5. Budded vesicles are functional intermediates. (A) Vesicles formed in the presence of GTP are free of coatomer. Vesicles were generated in assay mixtures containing either 0.5 mM GTP (lane 1) or 20 μ M GTP γ S (lane 2) and analyzed for the coatomer content by immunoblotting using a monoclonal anti- β -COP antibody. (B) Budded vesicles bind to and fuse with acceptor membranes. Vesicles were generated at 4°C (lane 1) or at 37°C (lanes 2–5) in assay mixtures containing either a non-treated cytosol (lanes 1 and 2), a coatomer-depleted cytosol (lane 3), a mock-depleted cytosol (lane 4) or a coatomer-depleted cytosol supplemented with purified coatomer (lane 5). Budded vesicles were recovered and used as donor membranes in the *in vitro* Golgi transport assay. The data are normalized to total amounts of [3 H]GlcNAc incorporated into VSV-G under standard incubations (lane 2, 37°C). The data (mean $\pm$ SD) are the average of four independent experiments.

of resident glycosylation enzymes into COP I-derived vesicles through a process of selection and concentration. This raises the possibility of a link between cargo selection and the direct recognition of specific cytoplasmic motifs by coatomer. It was therefore of interest to test whether preferential incorporation also applied to other resident Golgi proteins, in particular those for which a direct interaction between coatomer and the cytoplasmic domains had been demonstrated. We monitored incorporation of members of the p24 family of small transmembrane proteins. Two members, p24 α_2 (p25) and p24 δ_1 (p23), display typical K(X)KXX-like motifs in their cytoplasmic domains and can bind coatomer readily *in vitro* (Fiedler *et al.*, 1996; Dominguez *et al.*, 1998). As shown in Figure 9, relatively high amounts (30%) of both p24 α_2 and p24 δ_1 proteins were recovered in the vesicle pellet under GTP conditions (lane 1). In contrast, addition of GTP γ S or the arf-1 Q71L mutant reduced incorporation significantly (lanes 2 and 3). This extends our observations to include those proteins of the p24 family which are known to bind coatomer (p24 α_2 and p24 δ_1). It remains to be seen whether other Golgi resident proteins such as glycosylation enzymes also contain COP I binding motifs. This would support the notion of a link between the recognition of cytoplasmic motifs and preferential sorting.

Fig. 6. Immunogold labeling of budded vesicles for Mann II. Vesicles were generated in assay mixtures containing 0.5 mM GTP (A) or 20 μ M GTP γ S (B). Budded vesicles were sedimented at high speed, and the pellets were fixed and processed for cryoelectron microscopy. Sections were labeled with a polyclonal anti-Mann II antibody followed by protein A coupled to 10 nm gold particles. In (A), arrowheads indicate vesicles devoid of any detectable coat structure. In (B), arrows show a fuzzy coat surrounding the vesicles. Note that vesicles in (A) appear more densely packed than those in (B). However, this does not reflect differences in the amounts of generated vesicles, as shown by phospholipid analysis (see Table IV). Bars, 100 nm.

Table III. Linear labeling density of Mann II in vesicles (gold particles/ μ m of membrane)

GTP	GTP γ S
1.2 ± 0.5	0.29 ± 0.13

Vesicles were generated either in the presence of GTP or GTP γ S. After high-speed centrifugation, the pellets were processed for cryoelectron microscopy and immunogold labeling for Mann II. The linear labeling density was determined as described in Materials and methods.

Table IV. Biochemical analysis of the amount of Golgi proteins and phospholipids in vesicle pellets generated in the presence of GTP, GTP γ S or *N*-myristoylated arf-1 ^{Q71L} mutant

Markers	Amount of vesicles (% of starting membranes)		
	GTP	GTP γ S	arf-1 ^{Q71L}
Golgi enzymes			
Mann II	13.2 ± 1.3	6.1 ± 1.4	4.2 ± 1.1
NAGT I	25.8 ± 2.7	3.1 ± 1.0	3.0 ± 0.8
GalT	11.0 ± 2.0	1.7 ± 0.4	1.8 ± 0.4
Anterograde cargo			
pIgR	4.6 ± 1.0	3.6 ± 1.4	3.3 ± 1.1
Phospholipids	2.7 ± 0.7	3.1 ± 0.7	2.5 ± 0.8

Vesicles were produced at 4°C or at 37°C in assay mixtures containing either 0.5 mM GTP, 20 μ M GTP γ S or 50 μ M *N*-myristoylated arf-1 ^{Q71L} mutant. Budded vesicles were recovered and analyzed for their content of Mann II and pIgR by quantitative Western blotting or by enzyme activity for NAGT I and GalT. The phospholipid contents were determined as described in Materials and methods. Standard curves of known amounts of starting membranes were included in all experiments and used to evaluate the amounts of both protein and phospholipid in vesicle fractions. The amounts of proteins or phospholipids recovered in vesicles generated at 4°C were subtracted as non-specific background from that generated at 37°C. Results are expressed as the percentage of the protein or phospholipid in vesicles over the starting membranes $\pm$ SD (*n* ranged from 3 to 6).

Discussion

GTP hydrolysis mediates preferential incorporation of resident proteins into functional COP I-derived vesicles

We have shown that functional COP I-derived vesicles formed *in vitro* contain high amounts of Golgi resident proteins and that preferential incorporation of these proteins requires GTP hydrolysis by arf-1. While others (see e.g. Sönnichsen *et al.*, 1996) have not previously observed such an enrichment, the difference between these studies is readily explained by the effect of GTP and GTP γ S on cargo incorporation during budding. Our study further revealed that the anterograde cargo was neither depleted nor enriched in COP I-derived vesicles generated in the presence of GTP, GTP γ S or the arf-1 ^{Q71L} mutant. This indicates that the anterograde cargo uptake in vesicles is independent of GTP hydrolysis. This finding is consistent with several morphological and biochemical studies *in situ* showing that the anterograde cargo is present in COP I vesicles or buds at a concentration similar to that found in the cisisternal membranes (Orci *et al.*, 1986, 1997; Ostermann *et al.*, 1993; Griffiths *et al.*, 1995). However, the uptake of pIgR did not appear to require ATP. This could imply that the small fraction of pIgR in the vesicle might represent a contamination of Golgi fragments, rather than transport vesicles.

Our findings contrast those of Nickel *et al.* (1998) who compared cargo uptake in COP I-coated vesicles generated in the presence of GTP or GTP γ S. They showed that, when omitting GTP γ S, incorporation of anterograde cargo increased whereas p24 protein incorporation decreased. However, in the study by Nickel *et al.*, they exclusively analyzed COP I-coated vesicles, omitting uncoated vesicles. As shown in this study, the vast majority of COP I-derived vesicles produced under GTP conditions were uncoated. The lack of complete sampling might, therefore, have contributed to observed differences.

Fig. 7. Addition of arf-1 ^{Q71L} mutant, but not arf-1 wt, inhibits incorporation of Mann II into vesicles. Vesicles were generated in assay mixtures containing 0.5 mM GTP and cytosol (lane 1) supplemented with 50 μM of either arf-1 wt (lane 2) or arf-1 ^{Q71L} mutant (lane 3). Budded vesicles were recovered and analyzed by immunoblotting for their Mann II content.

Fig. 8. Hydrolysis of GTP by arf-1 mediates concentration of resident Golgi enzymes, but not anterograde cargo, in vesicles. The average densities of resident Golgi enzymes Mann II (▨), NAGT 1 (■) and GalT (▤), as well as the anterograde cargo pIgR (■), in vesicles generated under GTP, GTPγS and arf-1 ^{Q71L} conditions were determined by dividing the percentage of the protein by the percentage of phospholipid in the vesicles according to values presented in Table IV. Results are expressed as the percentage average density of starting membranes. The horizontal line represents the average density of Golgi proteins in the cisternal membranes.

Cargo selection and concentration: a new role for arf-1 protein

The observation that the arf-1 ^{Q71L} mutant affected protein sorting to the same extent as GTPγS is intriguing. It has been suggested that GTP hydrolysis by arf-1 triggers uncoating of the COP I coat from the vesicle membrane, rather than cargo sorting. This conclusion is based on the observation that COP I vesicles formed in the presence of the hydrolysis-deficient arf-1 ^{Q71L} mutant remain coated (Tanigawa *et al.*, 1993). As arf-1 has a poor intrinsic GTPase activity, an additional factor, arf-1 GAP, is needed for arf-1 hydrolysis and uncoating (Elazar *et al.*, 1994). In addition, coatamer is known greatly to enhance the GTPase activity by arf-1 bound to arf-1 GAP, suggesting a strong functional link between these three components (Goldberg, 1999). In the light of our findings, we favor the existence of additional GAP activities besides those implicated in uncoating. A possible role for arf-GAP in sorting has recently been suggested by others (Aoe *et al.*, 1997; Poon *et al.*, 1999; Zhu *et al.*, 1999). Further work will be needed to determine whether arf-GAP proteins required for sorting and uncoating are identical.

Fig. 9. GTPγS and arf-1 ^{Q71L} mutant inhibit incorporation of the p24 family members into vesicles. Non-salt-washed Golgi membranes (40 μg) were incubated in assay mixtures containing either 0.5 mM GTP (lane 1), 20 μM GTPγS (lane 2) or 50 μM arf-1 ^{Q71L} mutant (lane 3). Budded vesicles were recovered and analyzed by immunoblotting for the γ₃, α₂ and δ₁ members of the p24 family as well as for Mann II.

Reconstitution of a single round of budding

Vesicle formation was reconstituted from highly purified rat liver Golgi membranes in the presence of rat liver cytosol. The use of heterologous cytosols such as bovine brain or HeLa cytosol proved unsuccessful in the budding reaction and appeared to be due to a reduced ability to support vesicle formation. Further characterization of the assay revealed that once formed, COP I-derived vesicles were sufficiently stable to permit isolation. As we compared cargo of vesicles formed under various conditions, it was crucial that vesicles did not undergo multiple rounds of budding and fusion. Our results strongly suggest that only budding is reconstituted. Addition of the α-SNAP mutant (L294A) did not change the amount of Mann II detected in the vesicle pellet, even though this mutant is an effective inhibitor of fusion (Barnard *et al.*, 1997; Christoforidis *et al.*, 1999; Figure 2E). This suggests that once formed, COP I-derived vesicles do not undergo multiple rounds of transport when formed in this assay. One attractive possibility is that budded vesicles remain tethered to membranes by components designed to ascertain that vesicles are delivered to their target membrane in a controlled and vectorial manner (Weidman *et al.*, 1993; Sönnichsen *et al.*, 1998). In our assay, these events would not be reconstituted.

The observation that rat liver cytosol performed poorly in supporting fusion was unexpected as previous studies have shown that rat liver cytosol provides a rich source for fusion components. Reconstitution of Golgi stack reassembly involves both homo- and heterotypic fusion events, and these are readily supported by rat liver cytosol (Rabouille *et al.*, 1998). Furthermore, rat liver cytosol efficiently supports fusion of liposomes in a minimal fusion system (Otter-Nilsson *et al.*, 1999). However, the fusion of COP I-derived vesicles was inhibited by an α-SNAP mutant (L294A). α-SNAP is known to interact with NSF, and membrane fusion in the assay system used by us requires NSF (for a review, see Rothman and Wieland, 1996). Another ATPase, p97, capable of promoting homotypic fusion, is highly abundant in rat liver cytosol and it is feasible that together with its co-factor, p47, this ATPase effectively competes with NSF/α-SNAP for binding to receptors present on target membranes (Rabouille *et al.*, 1998) and therefore inhibits NSF-dependent fusion.

Vesicles do not arise from mechanical fragmentation

We can clearly rule out that the high amounts of resident proteins recovered in the vesicle fraction were the consequence of a non-specific membrane fragmentation of the Golgi caused by mechanical shearing and/or salt wash prior to the budding reaction. First, electron microscopic analysis showed that most of the profiles were vesicular and displayed the expected size of transport vesicles. Rarely did we observe other types of membrane structures, such as elongated tubules. If broken off as a consequence of membrane fragmentation, one would not expect to see profiles of consistent diameter. It is unlikely that one always cuts a long tubule or membrane fragment perpendicular to the length of the tubule. It is formally possible, though, that COP I drives the formation of small tubules and that these would be mechanically forced off the membrane during the procedure. After release into the supernatant, they would relax and assume the shape of round vesicles with uniform size. The present study does not attempt to resolve this issue or to prove formally the existence of COP I-derived vesicles versus COP I-derived tubules. The key observation is that transport intermediates are selectively enriched in resident proteins and that they form in a coatamer-dependent manner. Secondly, if elevated levels of resident proteins were due to mechanical fragmentation, we would expect to observe similar levels irrespective of the incubation conditions. Thirdly, the level of Mann II detected in vesicles produced with GTP was 3-fold higher than the level of pIgR. Such a difference between two markers found in the same vesicle fraction strongly suggests a selective process, rather than fragmentation. Fourthly, incorporation of Mann II into vesicles is dependent on coatamer. Fifthly, the salt-wash treatment of membranes prior to budding did not modify the extent of incorporation of Mann II into vesicles. The same level of Mann II incorporation was found in vesicles generated from membranes that had not been salt washed (compare Figure 1B with Figure 9). Finally, we exclude the possibility that the high amounts of resident Golgi proteins incorporated in COP I-derived vesicles formed with GTP were caused by increased vesicularization. Phospholipid analysis confirmed that similar amounts of vesicles were formed under all three conditions (see Table IV). We conclude that we have indeed observed the formation of COP I-derived vesicles rather than mechanical shearing or some other non-specific activity.

Consequences for present models of vesicular transport

The ability of COP I-derived vesicles to transport resident proteins between Golgi compartments has consequences on how one views transport through the secretory pathway. That COP I-derived vesicles are preferentially concentrated in glycosylation enzymes as well as other residents argues against a primary role for COP I vesicles in the transport of anterograde cargo. The observed concentration of resident proteins in COP I-derived vesicles suggests that, over time, a given Golgi cisterna is depleted faster of its resident proteins than of its anterograde cargo. Our findings are consistent with the cisternal maturation model which postulates that anterograde cargo remains in the cisternae and that these mature by acquiring the content

Fig. 10. (A) Model depicting the role of coatamer in the selection and concentration of resident proteins in COP I vesicles. Cytosolic coatamer binds selectively to cytoplasmic domains of resident proteins through a process that required the hydrolysis of GTP-bound arf-1. This direct interaction would permit lateral sampling in the membrane, resulting in selective packaging and concentration of resident proteins into COP I vesicles. (B) and (C) Two alternative models illustrating the effect of GTP hydrolysis by arf-1 in cargo selection. In model I (B), GTP-bound arf-1 recruits cytosolic coatamer onto the Golgi membranes. Hydrolysis of GTP by arf-1 induces a conformational change in coatamer which would then allow for a direct interaction with the cytoplasmic tails of resident proteins. In model II (C), GTP-bound arf-1 would interact directly or indirectly with cytoplasmic tails of resident proteins. Hydrolysis of GTP by arf-1 causes a conformational change in the cytoplasmic tails which would then allow for a direct interaction with coatamer.

and shape of previous cisternae (e.g. a *cis* cisterna becomes a *medial* one). This process is thought to be driven by the budding and fusion of retrograde COP I vesicles containing the resident proteins of the pathway (Glick *et al.*, 1997; Mironov *et al.*, 1997; Glick and Malhotra, 1998).

Mechanisms

The finding that resident proteins known to bind directly to coatamer were concentrated into COP I vesicles offers clues to the underlying mechanism of cargo selection and concentration. As with newly synthesized proteins being sampled in the ER membrane through direct interaction with COP II coat, we envisage that COP I coat can bind directly to cytoplasmic domains of resident proteins and that this interaction promotes sorting and concentration into vesicles (see Figure 10A). What is then known about COP I binding motifs to support such a scenario? Recent studies show that COP I-coated vesicles can be generated from synthetic lipid bilayers and that this requires coatamer, arf-1, GTP and cytoplasmic domains containing KKXX or KKXX-like motifs (Bremser *et al.*, 1999). For the p24 family members tested in this study, two members, p24 α_2 (p25) and p24 δ_1 (p23), possess in their cytoplasmic tails the K(X)KKX-like retrieval motif known to bind directly to coatamer (Dominguez *et al.*, 1998). The third member, p24 γ_3 , lacks this motif but appears to behave in a similar manner to p24 α_2 and p24 δ_1 . Since the p24 family members can form large oligomeric complexes (Dominguez *et al.*, 1998), these would ensure that all members, even those lacking the K(X)KKX-like motif, are taken up into the vesicle efficiently. Alternatively, other motifs or variations of the K(X)KKX motif could exist in residents throughout the pathway. It will be

interesting to determine whether cytoplasmic domains of glycosylation enzymes are also equipped with COP I binding motifs, enabling them to interact directly with COP I coat. In support of this, we recently showed that NAGT I binds coatamer *in vitro*, albeit to a lower extent than that observed for p24 α_2 and p23 δ_1 harboring K(X)KXX-like motifs (Dominguez *et al.*, 1998).

Different models can be developed to explain the arf-1-dependent segregation and concentration of resident Golgi proteins into COP I vesicles. The hypothesis outlined in Figure 10B proposes that GTP-bound arf-1 recruits cytosolic coatamer onto Golgi membranes, perhaps via a direct interaction of arf-1 with coatamer (Zhao *et al.*, 1997, 1999). Hydrolysis of GTP by arf-1 (promoted by arf-GAP) then induces a conformational change in coatamer, and this might reveal a high-affinity binding site for cytoplasmic domains of resident proteins. Alternatively (Figure 10C), GTP-bound arf-1 could interact directly or indirectly with cytoplasmic domains of resident proteins. Hydrolysis of GTP by arf-1 could cause a conformational change in the cytoplasmic domains, generating high-affinity binding sites for coatamer. It can also be envisaged that cytoplasmic domains serve as nucleation sites for coat assembly. A continuous cycle of GTP hydrolysis by arf-1 would ensure that polymerization of the coat does not precede incorporation of cargo. This hypothesis would be consistent with the observed effects of GTP γ S and arf-1^{Q71L} which, upon addition, abolished sorting but not vesicle formation. In all these models, a direct interaction of coatamer with resident proteins is predicted to ensure a lateral segregation and subsequent enrichment into COP I vesicles. The unexpected new function of arf-1 in protein sorting revealed in this study opens an exciting novel way to study cargo sorting during vesicle formation.

Materials and methods

Reagents

All reagents were of analytical grade. Aprotinin, leupeptin, pepstatin, antipain, benzamidine, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, GTP, GTP γ S, myristic acid, Triton X-100, lysosyme, apyrase, 2-[N-morpholino]ethanesulfonic acid (MES), trypsin/EDTA, ascorbic acid and ammonium molybdate-4H₂O were from Sigma Chemical Co. (St Louis, MO). Perchloric acid was from Fluka (Neu-Ulm, Switzerland). Five percent fetal calf serum was from PAA Laboratories (Linz, Austria). L-Glutamine and penicillin/streptomycin antibiotics were from Gibco-BRL (Eggenstein, Germany). Non-essential amino acids were purchased from Seromed (Berlin, Germany); ultrafiltration cell (Model 8010) and ultrafiltration membranes YM10 were from Amicon GmbH (Witten, Germany). ATP, creatine phosphate and creatine kinase were from Boehringer Mannheim (Mannheim, Germany). Isopropyl- β -D-thiogalactopyranoside and 1,4 dithiothreitol (DTT) were from Biomol GmbH (Hamburg, Germany). Mono S HR 10/10, superose HR 10/30 and PD-10 columns were from Pharmacia (Uppsala, Sweden). DEAE-cellulose was from Whatman International Ltd (Maidstone, UK). Thirty percent (w/v) acrylamide/0.8% (w/v) bis-acrylamide solution was from National Diagnostics (Atlanta, GA). Protran nitrocellulose membranes (0.45 μ m) were from Schleicher and Schuell (Dassel, Germany). Sucrose and the ECL detection kit were from Amersham Life Science (Amersham, UK). X-Omat XAR5 films were from Eastman Kodak Co. (Rochester, NY). Coatamer was purified from rat liver cytosol as described (Waters *et al.*, 1992).

Antibodies

Polyclonal antibodies against the α_2 , γ_3 and δ_1 members of the p24 family, and against the α and γ coatamer subunits as well as the monoclonal anti- β -COP E5A3, have been described (Dominguez *et al.*, 1998). The monoclonal antibody anti-coatamer CM1A10 has been

described (Love *et al.*, 1998). Polyclonal antibodies against Mann II were a gift from K. Moremen (University of Georgia, Athens). Polyclonal antibody against pIgR and arf-1 were kind gifts from A. Hubbard (The Johns Hopkins University, Baltimore) and Roland LeBorgne (Institut Pasteur, Lille), respectively. Polyclonal anti-IgG mouse antibody was purchased from Sigma.

Expression vectors

The human pET11d/wild-type arf-1 and *Saccharomyces cerevisiae* N-myristoyltransferase pBB131 plasmids were kindly provided by P. Chardin (UCSF Cancer Center, San Francisco, CA). For the mutant arf-1^{Q71L}, the Q71L point mutation in the pET11d-arf-1(Q71L) construct was introduced by PCR-based site-directed mutagenesis using the wild-type arf-1 as a template and synthetic oligonucleotides containing the desired mutation within their sequence to produce two overlapping fragments. These fragments were reamplified in a second PCR round using external primers to generate the whole arf-1 open reading frame bearing the desired mutation.

Production and purification of N-myristoylated wild-type, Q71L mutant arf-1 and His₆-tagged α -SNAP (L294A) mutant

BL21 (Petlyse) bacteria were co-transformed with either the arf-1 wt or the arf-1^{Q71L} mutant and yeast N-myristoyltransferase plasmids, and selected for ampicillin and kanamycin resistance. Two liters of transformed bacteria were grown at 37°C to an OD of 0.6. Myristic acid (50 μ M) was then added as a 100-fold concentrated solution prepared by dissolving myristic acid in 100% ethanol at 42°C. After 10 min, the co-expression of arf-1 and N-myristoyltransferase was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside and the temperature was reduced at 27°C to increase the efficiency of myristoylation (Franco *et al.*, 1995). After 3 h of incubation, the bacteria were harvested and the pellet was homogenized in 80 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 30 mM EDTA, 30 mM MgCl₂, 18% w/v sucrose, 0.05% v/v Triton X-100, 1 mM PMSF, 1 μ g/ml pepstatin, 2.5 mM benzamidine, 5 μ g/ml leupeptin, 0.3 mg/ml lysosyme). Following 30 min of incubation at 4°C, the pellet was re-homogenized, sonicated and centrifuged at 10 000 r.p.m. for 20 min at 4°C in a Sorvall SS-34 rotor. The crude supernatant was applied to a DEAE-cellulose column equilibrated with 20 mM Tris-HCl pH 8.0 and the flow-through containing arf-1 proteins was dialyzed overnight against MES buffer (10 mM MES pH 5.7, 1 mM DTT, 2 mM MgCl₂) containing 1 mM PMSF. The flow-through was centrifuged at 41 000 r.p.m. for 60 min at 4°C using a Ti45 rotor and the supernatant was filtered on Nalgene 0.45 μ m before being applied to a Mono S column equilibrated in MES buffer. The bound proteins were eluted using a salt gradient (0–500 mM NaCl in MES buffer) and the fractions containing arf-1 protein (eluted between 130 and 170 mM salt) were pooled (~6 ml) and concentrated using an Amicon ultrafiltration cell and YM10 ultrafiltration membrane to 1.5 ml. The solution was clarified by centrifugation at 13 000 r.p.m. for 10 min at 4°C using an Eppendorf centrifuge and applied to a superose 200 HR 10/30 equilibrated in MES buffer. Fractions containing arf-1 protein were pooled (2 ml), dialyzed overnight at 4°C against 1 l of budding buffer (25 mM HEPES pH 7.0, 115 mM potassium acetate, 2.5 mM MgCl₂, 1 mM DTT) and stored at –80°C. Two liters of cell culture typically yield 2 mg of $\geq$ 95% pure arf-1.

Recombinant His₆-tagged α -SNAP (L294A) mutant was purified on Ni-NTA-agarose as described in Barnard *et al.* (1997). The recombinant protein was dialyzed overnight at 4°C against 2 l of budding buffer, aliquoted, snap frozen and stored at –80°C.

Subcellular fractionations

Highly purified rat liver Golgi membranes and rat liver cytosol were prepared as described (Slusarewicz *et al.*, 1994). Typically, the purification over homogenate was 60-fold for GalT and 160-fold for NAGT I. The membranes and cytosol were snap frozen in liquid nitrogen and stored at –80°C. The bovine brain cytosol was prepared according to LeBorgne *et al.* (1996), except that the homogenization buffer consisted of 100 mM potassium phosphate pH 6.7 and 0.5 M sucrose. For the production of HeLa cytosol, HeLa cells were cultured in S-MEM media containing 5% fetal calf serum, non-essential amino acids, L-glutamine and penicillin/streptomycin. Five 175 cm² flasks with 80–100% confluency were trypsinized, diluted to 600 ml with media and transferred to 1 l spinner flask. After 2 days at 37°C with stirring, they were expanded to 4 l and left at 37°C with stirring for an additional 4 days. The cells were harvested by centrifugation at 3000 r.p.m. for 10 min at 4°C using a GS-3 rotor. The resulting pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged at 3000 r.p.m. for 10 min at 4°C using a

GS-3 rotor. The pellet was resuspended in PBS, transferred to a Falcon tube and centrifuged at 1500 r.p.m. for 10 min at 4°C using a RT-6000 Sorvall Centrifuge. The cell pellet was then resuspended to a final volume of 15 ml in budding buffer containing 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 1 mM benzamide and 40 µg/ml PMSF. The cell resuspension was homogenized with eight passes in a ball-bearing homogenizer with a 16 µm clearance. The homogenate was centrifuged at 80 000 r.p.m. at 4°C using a TLA 100.4 rotor. The supernatant was aliquoted, snap frozen and stored at -80°C.

For the budding assay, 500 µl of frozen HeLa, rat liver or bovine brain cytosols were thawed. The rat liver and bovine brain cytosols were further diluted with 1 ml and 500 µl of budding buffer, respectively. The cytosols were centrifuged at 60 000 r.p.m. for 60 min at 4°C using a TLA 100.2 rotor. The resulting supernatants were further cleared by centrifugation at 13 000 r.p.m. for 15 min at 4°C. To desalt rat liver cytosol, 2.5 ml of cytosol were applied to a PD-10 column according to the manufacturer's protocol.

Salt treatment of Golgi membranes

Golgi membranes were salt washed to remove endogenous coatamer by diluting the membranes to 200 µg/ml in salt-stripping buffer (0.5 M Tris-HCl pH 7.5, 2 mM EDTA and 1 mM DTT, final concentration) containing a protease inhibitor cocktail (0.5 mM benzamide, 5 µg/ml leupeptin and 2 µg/ml soybean trypsin inhibitor). The solution was incubated for 15 min at 4°C and then for 5 min at 37°C, the latter to remove preformed vesicles. The mixture was centrifuged at 13 000 r.p.m. in an Eppendorf centrifuge for 15 min at 4°C and the resulting pellet was resuspended to 2 mg/ml in budding buffer containing 100 mM sucrose. It is important to note that the level of incorporation of Mann II into vesicles is similar when using either salt-washed or unwashed membranes (compare Figure 1B with Figure 9).

Vesicle budding assay

Incubations were performed in a final volume of 250 µl. Unless otherwise indicated, the standard assay mixture contained 100 µg of salt-washed Golgi membranes, 5 mg/ml of rat liver cytosol, an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 10 U/ml creatine kinase) or an ATP-depletion system (see Otter-Nilsson *et al.*, 1999, for details), 1 mM DTT, a protease inhibitor cocktail and 0.5 mM GTP. The mixture was incubated for 30 min at 37°C and put at 4°C. The salt concentration was then raised to 250 mM to release bound vesicles from membranes and the mixtures were centrifuged at 13 000 r.p.m. for 15 min at 4°C. The medium-speed supernatants containing vesicles were loaded onto a two-step sucrose cushion made up of 50 µl of 30% w/w sucrose and 5 µl of 50% w/w sucrose, and centrifuged at 45 000 r.p.m. for 45 min at 4°C using a TLA 45 rotor. The vesicle pellets were found at the 30/50% sucrose interface and were resuspended. Protein content was analyzed either by SDS-PAGE followed by semiquantitative Western blotting using specific antibodies, or by enzymatic activity using assays specific for NAGT I (Bretz and Staubli, 1977) and GalT (Vischer and Hughes, 1981).

Phospholipid determination

Phospholipid extraction was carried out essentially as described by Bligh and Dyer (1959). Briefly, the vesicle pellets were resuspended in 200 µl of water and shaken at 4°C for 20 min using a thermomixer. The vesicle pellets were sonicated for 5 min in a water bath sonicator at 25°C. The material was then transferred to 1.5 ml Eppendorf tubes and 440 µl of methanol and 200 µl of chloroform were added. The mixtures were vortexed for 1 min and then 200 µl of water and 200 µl of chloroform were added. The mixtures were vortexed for 5 min and centrifuged for 5 min at 13 000 r.p.m. at room temperature. The lower (chloroform) phases were transferred to new 1.5 ml Eppendorf tubes. To the remaining upper phases were added 200 µl of chloroform. The mixtures were vortexed for 1 min and then centrifuged at 13 000 r.p.m. for 5 min at room temperature. The chloroform phases were collected, pooled with the previous ones and centrifuged for 5 min at 13 000 r.p.m. The chloroform phases were carefully collected to avoid any contamination by water and then transferred to glass tubes. The glass tubes were left overnight in the fume hood to evaporate the chloroform. To each glass tube, 50 µl of 70% perchloric acid were added and incubated at 180°C for 60 min. The phospholipid content was then assessed by measuring inorganic phosphate as described by Ames and Dubin (1960). For each independent experiment, a standard curve corresponding to 0.62, 1.25, 2.5 and 5% of starting material was constructed, and used to determine the amount of phospholipids generated in vesicle pellets.

Western blotting and quantitation

After SDS-PAGE, the proteins were electroblotted to nitrocellulose sheets overnight at 30 V at 4°C by the method of Towbin *et al.* (1979). The blot was then blocked using PBS containing 5% defatted milk and 0.05% Tween 20 for 30 min at room temperature, and with primary antibodies diluted in blocking buffer for 90 min at room temperature. After washing, secondary antibodies were incubated for 20 min at room temperature in blocking buffer. Horseradish peroxidase-labeled secondary antibodies were revealed by the ECL system. For quantitation, undersaturating exposures of the autoradiograms were scanned using a Microtek scanmaker III scanner at a resolution of 300 d.p.i. (Microtek Electronics Europe, GmbH, Düsseldorf, Germany). The intensities of the signals were quantitated using the NIH image1.59/ppc software.

Immunodepletion of cytosolic coatamer

Protein A-Sepharose beads were incubated in 500 µl of budding buffer containing 100 mM NaCl and 15 µg of rabbit anti-mouse IgG for 30 min at 4°C. Beads were washed three times and incubated in 500 µl of buffer supplemented either with 10 µl of monoclonal anti-coatamer CM1A10 or 10 µl of monoclonal mock antibody for 45 min at 4°C. Beads were washed three times in budding buffer without NaCl and incubated with 1.5 mg of rat liver cytosol and protease inhibitor cocktail for 45 min at 4°C. The mixture was centrifuged, the supernatant collected and analyzed by Western blotting or used immediately in the vesicle budding assay.

Negative-staining procedure

Vesicle pellets were carefully resuspended in 20 µl (final volume) of budding buffer containing 30% w/w sucrose. One microliter of each vesicle resuspension was then incubated on a formvar and carbon-coated copper grid for 5 min at room temperature. The samples were fixed in 4% paraformaldehyde in PBS for 5 min, rinsed in PBS and incubated for 5–10 min on a drop of incubation buffer [0.1% bovine serum albumin (BSA), 0.1% fish skin gelatin, 0.15% glycine in PBS]. They were washed in distilled water and incubated on methylcellulose/uranyl acetate drops (1.8%/0.3%, respectively) for 5 min on ice. The excess fluid was removed and the grids were air dried. Samples were then observed using a Zeiss electron microscope. For the quantitation, at least five negatives of each vesicle preparation were taken at a magnification of 25 000 and overlaid with a square box filling a surface area of 9 µm². The vesicles (defined as circular profiles with a diameter ranging from 40 to 120 nm) contained in the box were counted. The amount of vesicles produced under various conditions was then expressed as the number of vesicles/µm².

Cryo-immunogold labeling

To obtain sufficient material for cryoelectron microscopy, vesicles were first generated from 24 incubation mixtures. Following high-speed centrifugation, the vesicle pellets were resuspended, pooled, diluted to 8% (w/w) sucrose with budding buffer and repelleted onto a 20 µl cushion of 50% (w/w) at 45 000 r.p.m. for 120 min at 4°C using a TLA45 rotor. The resulting vesicle pellets were fixed for 120 min in 0.2% glutaraldehyde/2% paraformaldehyde in 0.2 M phosphate buffer pH 7.4. After fixation and washing in PBS, the pellets were embedded in 10% gelatin in PBS and fixed overnight in 1% paraformaldehyde in PBS. The gelatin-embedded pellets were infiltrated in 2.4 M sucrose in PBS for 60 min and cut into small cubes (~0.5 mm³). The cubes were quickly frozen in liquid nitrogen and sectioned at -95°C. Sections were collected onto carbon-coated copper grids. The grids were then incubated in blocking buffer (0.1% BSA, 0.1% fish skin gelatin and 0.15% glycine in PBS) at room temperature. After 15 min, the grids were further incubated with a polyclonal antibody against Mann II (Moremen *et al.*, 1991) for 60 min. The grids were washed with blocking buffer and incubated with protein A coupled to 10 nm gold particles (Utrecht School of Medicine, Cell Biology Laboratory). After washing with PBS and distilled water, the grids were incubated in 0.3% uranylacetate/1.8% methylcellulose. Grids were collected and air dried. Sections were visualized using a Zeiss electron microscope. For the quantitation, the linear labeling density was determined by measuring the length of membranes using the intersection method (Weibel, 1979) and expressed as gold particles/µm of membrane.

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