

Sorting of Golgi resident proteins into different subpopulations of COPI vesicles: a role for ArfGAP1

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We present evidence for two subpopulations of coatamer protein I vesicles, both containing high amounts of Golgi resident proteins but only minor amounts of anterograde cargo. Early Golgi proteins p24 α_2 , β_1 , δ_1 , and γ_3 are shown to be sorted together into vesicles that are distinct from those containing mannosidase II, a glycosidase of the medial Golgi stack, and GS28, a SNARE protein of the Golgi stack. Sorting into each vesicle population is Arf-1 and GTP hydrolysis dependent and is

inhibited by aluminum and beryllium fluoride. Using synthetic peptides, we find that the cytoplasmic domain of p24 β_1 can bind Arf GTPase-activating protein (GAP)1 and cause direct inhibition of ArfGAP1-mediated GTP hydrolysis on Arf-1 bound to liposomes and Golgi membranes. We propose a two-stage reaction to explain how GTP hydrolysis constitutes a prerequisite for sorting of resident proteins, yet becomes inhibited in their presence.

Introduction

The cell recycles most of its constituents of the secretory pathway. This is needed to offset anterograde transport of newly synthesized proteins and lipids and is achieved via two different mechanisms, one coatamer protein (COP)*I independent the other COPI dependent (for review see Storrie et al., 2000). Whereas COPI-independent recycling is so far only poorly understood, COPI-dependent recycling has been investigated extensively, revealing sorting motifs and recycling transport intermediates (e.g., COPI vesicles) operating throughout the pathway. One such sorting motif, the K(X)KXX present in many resident proteins of the early secretory pathway (Nilsson et al., 1989; Jackson et al., 1990), was shown to interact specifically with coatamer, the cytoplasmic coat complex of the COPI coat (Cosson and

Letourneur, 1994; Letourneur et al., 1994). This was demonstrated in vitro and by yeast genetics, thus firmly linking the role of COPI vesicles to recycling. In accordance, we found recently that COPI vesicles formed in vitro contain high amounts of Golgi resident glycosylation enzymes (Lanoix et al., 1999). Upon inhibition of GTP hydrolysis, the level of resident proteins in vesicles was diminished. We also found that regardless of GTP hydrolysis, the level of anterograde cargo recovered in vesicles was low comparable to that observed in the cisternal membranes. This suggests that GTP hydrolysis by Arf-1 selectively favors incorporation of resident proteins into budding COPI vesicles. These findings are in agreement with the postulates of the cisternal maturation process where anterograde cargo is transported through cisternal progression and resident proteins recycle via COPI vesicles (for review see Glick and Malhotra, 1998).

Of the many constituents thought to recycle, the p24 family has recently received considerable attention. These reside at the ER-Golgi interface and are conserved from yeast to mammals. We showed previously that four p24 members, α_2 , β_1 , δ_1 , and γ_3 , exist in a heterooligomeric complex consisting roughly of equimolar amounts of each member (Füllekrug et al., 1999). Similar complex formation of corresponding orthologs has been observed also in yeast, suggesting a conserved property (Marzioch et al., 1999). However, when and where complex formation occurs is unclear. We found that all four p24 proteins are required to

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*Abbreviations used in this paper: CGN, cis-Golgi network; COP, coatamer protein; GAP, GTPase-activating protein; GlcNAc-T1, *N*-acetylglucosaminyltransferase I; GT-1, galactosyltransferase; KDR, KDEL receptor; Mann, mannosidase; RSA, rat serum albumin; TGN, trans-Golgi network.

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leave the ER. This was shown in two ways. First, mutations in residues that are required for export of one member resulted in ER arrest of all four members. Second, systematic expression of one, two, three, or four of the members in different combinations provided further evidence for complex formation as a prerequisite for ER export, most likely for entry into budding COPII vesicles (Dominguez et al., 1998; Füllekrug et al., 1999). As the coprecipitating p24 complex was revealed in Golgi membranes, it is possible that complex formation is also required for entry into COPI vesicles. This notion predicts sorting of all four p24 proteins into the same COPI vesicle. Even though all eight can be deleted in yeast without loss of cell viability or protein transport (Springer et al., 2000), p24 proteins have been ascribed important functions in mammalian cells. For example, p24 δ_1 (p23) has been suggested to serve as a coatomer receptor needed in the formation of retrograde COPI vesicles (Malsam et al., 1999), and likewise p24 β_1 (p24) has been suggested to serve as a coat receptor needed for the formation of anterograde COPI vesicles (Goldberg, 2000). This was based on the observation that cytoplasmic domain of p24 δ_1 and p24 β_1 can interact directly with coatomer (Fiedler et al., 1996; Sohn et al., 1996; Dominguez et al., 1998; Goldberg, 2000). However, several resident proteins with cytoplasmic K(X)KXX motifs bind coatomer equally well (e.g., E19 and p24 α_2) and sometimes even better (for a comparative study see Dominguez et al., 1998). If using binding as a criteria, each coatomer binding protein would then constitute a receptor. What sets p24 proteins apart is that cytoplasmic domains of some members can induce a conformational change in coatomer, causing it to polymerize (Reinhard et al., 1999). This is important, since coatomer is able to stimulate Arf-1-mediated GTP hydrolysis 1,000-fold in an Arf GTPase-activating protein (GAP)1-dependent fashion, at least in solution (Goldberg, 1999). A tripartite complex for optimal GTP hydrolysis was suggested. In a subsequent study, Goldberg showed that a synthetic peptide corresponding to the cytoplasmic domain of p24 β_1 inhibits coatomer and ArfGAP1-dependent GTP hydrolysis. Inhibition of hydrolysis was thought to occur through coatomer via its interaction with the p24 β_1 cytoplasmic domain. Such downmodulation of GTP hydrolysis was proposed to enable formation of priming complexes (Springer et al., 1999) where coatomer remain attached to the membrane via the resident proteins and Arf-1^{GTP}/ArfGAP1 long enough to allow for polymerization of the COPI coat, leading to vesicle formation. However, the role of coatomer in stimulating GTP hydrolysis by Arf-1 has been challenged recently in studies using full-length myristoylated Arf-1 bound to phospholipid vesicles. Under these conditions, coatomer had no effect on ArfGAP1-dependent GTP hydrolysis on Arf-1 (Szafer et al., 2000). Nevertheless, the proposed ability of p24 β_1 (and perhaps other Golgi resident proteins) to downmodulate ArfGAP1 activity and hence Arf-1-mediated GTP hydrolysis as a prerequisite for sorting contrasts our previous findings, showing a clear requirement for GTP hydrolysis by Arf-1 for sorting of Golgi resident glycosylation enzymes into COPI vesicles (Lanoix et al., 1999).

To investigate sorting of p24 proteins and other Golgi resident proteins into COPI vesicles and possible cargo modu-

lation of ArfGAP1 activity, we use a modified in vitro budding assay where consumption of formed vesicles is blocked, thereby overcoming previous technical limitations. We find that p24 α_2 , β_1 , δ_1 , and γ_3 are sorted together in a GTP hydrolysis- and Arf-1-dependent manner into COPI-derived vesicles and that these vesicles are distinct from those containing mannosidase (Mann) II and GS28. We also tested for the effect of the cytoplasmic domain of p24 β_1 on ArfGAP1 activity. We find that on liposomes and Golgi membranes, the cytoplasmic domain peptide of p24 β_1 binds to ArfGAP1 and strongly inhibits Arf-1-mediated GTP hydrolysis directly. Analysis with peptides bearing amino acid substitutions suggests that inhibition is mediated through the previously identified F/YXXXXF/Y motif that is present in some p24 proteins (Dominguez et al., 1998).

Results

GTP-dependent sorting of p24 proteins into COPI-derived vesicles

To ensure a reproducible build-up of COPI-derived vesicles, we had previously subjected membranes to a salt wash before the budding event (Lanoix et al., 1999). A drawback of this was that fenestrated membranes such as the cis-Golgi network (CGN) and trans-Golgi network (TGN) tended to be lost during the washing procedure. However, performing the budding assay using nonwashed membranes often resulted in a low recovery of Mann II signal compared with that obtained when using salt-washed membranes. A likely explanation for this is that by removing peripheral components, such as NSF and α -SNAP, formed vesicles cannot readily fuse back once uncoated. To avoid vesicle fusion, we performed the budding reaction in the presence of a dominant negative mutant of α -SNAP, L294A (α -SNAP^{dn}), which is known to effectively block NSF-dependent fusion (Barnard et al., 1997) by locking the 20S fusion complex on the membrane (McBride et al., 1999). The effect of the α -SNAP^{dn} mutant on salt-washed and nonwashed membranes is shown in Fig. 1 A. Using salt-washed membranes, the level of Mann II in vesicles was high (\sim 20% of that observed in Golgi membranes), whereas the level of p24 proteins was very low in both the Golgi and the vesicle fraction, which suggests that most of the CGN had been lost due to the salt wash. In contrast, when using nonwashed membranes the level of Mann II obtained in vesicles was reduced greatly, whereas the amount of p24 proteins was now high. Upon addition of the α -SNAP^{dn} mutant, the level of Mann II increased significantly, whereas the level of p24 proteins remained unaffected. The idea that the salt wash had removed peripheral NSF and α -SNAP, thereby preventing vesicle consumption, was supported by the lower Mann II signal upon addition of recombinant and complexed NSF/ α -SNAP (Fig. 1 B). Addition of the α -SNAP^{dn} mutant together with NSF/ α -SNAP^{wc} blocked reduction. From a technical standpoint, addition of the α -SNAP^{dn} mutant clearly improved the recovery of resident proteins when using nonwashed membranes, permitting us to use membranes that more accurately represent the entire Golgi apparatus including the CGN and TGN.

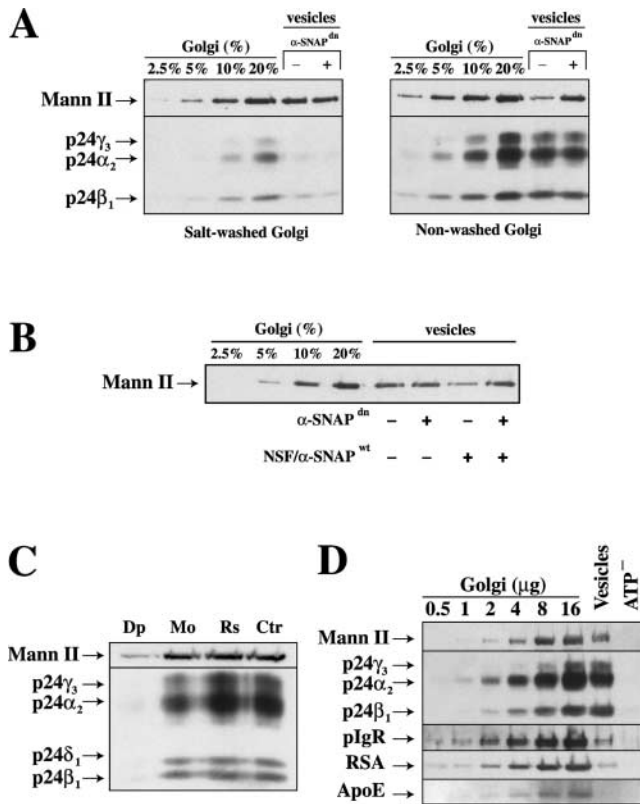


Figure 1. Accumulation of COPI vesicles in the presence of the α -SNAP^{dn} mutant. (A) Vesicles formed using salt-washed Golgi membranes were compared with vesicles formed using nonwashed membranes in the absence or presence of the α -SNAP^{dn} mutant. Proteins from solubilized membranes or vesicles were separated by SDS-PAGE and subjected to Western blot analysis using specific primary antibodies to Mann II, p24 γ_3 , p24 α_2 , and p24 β_1 followed by ECL. Note the lower amount of Mann II in the absence of the α -SNAP^{dn} mutant. Golgi membranes are shown in increasing amounts expressed as the percentage of starting material. (B) Vesicles were formed using salt-washed Golgi membranes in the presence or absence of exogenously added NSF/ α -SNAP^{wt} and/or the α -SNAP^{dn} mutant. (C) Coatomer was depleted (Dp) from cytosol using specific monoclonal antibodies, recognizing native coatomer or mock (Mo) depleted using an irrelevant antibody and used in budding reactions with nonwashed membranes and the α -SNAP^{dn} mutant. Depleted cytosol was rescued (Rs) subsequently by adding back exogenously purified coatomer and compared with vesicles formed using untreated cytosol (Ctr). (D) Vesicle budding was performed using nonwashed membranes in the presence of the α -SNAP^{dn} mutant and tested for anterograde cargo incorporation (pIgR, RSA, and ApoE). Vesicles were also generated in the presence of an ATP depletion system (ATP⁻) for comparison. Proteins from solubilized membranes or vesicles were separated by SDS-PAGE and subjected to Western blot analysis and revealed by the ECL method using antibodies.

Therefore, we added the α -SNAP^{dn} mutant into subsequent budding experiments.

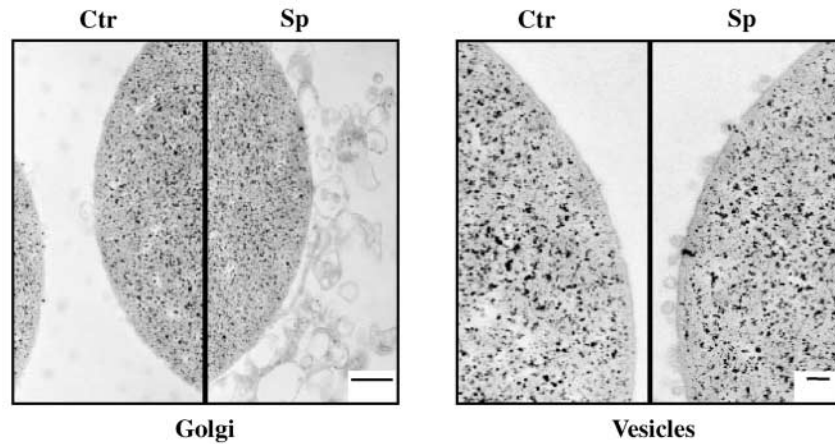
Having modified the budding assay, it was important to verify that vesicles containing p24 proteins and/or Mann II were still formed in a COPI-dependent manner. Coatomer was depleted (Dp) from the cytosol before budding using affinity purified mAb CM1A10. This resulted in a marked decrease in both Mann II and p24 proteins recovered in the vesicle pellet (Fig. 1 C). Adding back purified coatomer (Rs)

or using mock-depleted cytosol (Mo) gave rise to comparable levels to those observed in the control using untreated cytosol (Ctr). Thus, both p24- and Mann-containing vesicles were formed in a COPI-dependent manner. We next examined to what extent anterograde cargo had been incorporated into formed vesicles. Three different cargo proteins were examined: the polymeric immunoglobulin receptor (pIgR), apolipoprotein E (ApoE), and rat serum albumin (RSA). As can be seen in Fig. 1 D, only small amounts of anterograde cargo were detected in the vesicle fraction (Mann II, p24 α_2 , β_1 , and γ_3 are also shown for comparison). This suggests that in the total pool of vesicles, only minor amounts of anterograde cargo had been incorporated. Since budding was performed under conditions where vesicle consumption is blocked, it negated the possibility that putative anterograde vesicles could have been missed if these had fused back at a higher rate than those containing resident proteins. Moreover, since addition of the α -SNAP^{wt} mutant clearly improved the Mann II signal it argues in favor of the budding assay faithfully producing transport intermediates (e.g., vesicles) that are noncontinuous with the membrane before termination of the budding reaction. This is important, since concerns have been raised suggesting that perhaps the in vitro assay mainly registered a fragmentation event caused by subsequent salt treatments, which we use to release formed vesicles from membranes before fractionation and analysis. We conclude that COPI-derived vesicles formed in this improved budding assay carry mainly resident proteins.

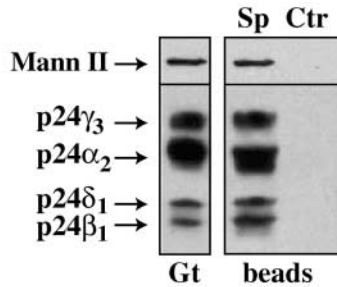
Evidence for a p24 subpopulation of COPI-derived vesicles

Since p24 proteins reside in the early part of the secretory pathway and Mann II in the Golgi stack centered around the medial cisternae (Velasco et al., 1993; Rabouille et al., 1995; Sohn et al., 1996; Rojo et al., 1997; Dominguez et al., 1998; Füllekrug et al., 1999), one would predict only a small overlap between these proteins in recycling COPI vesicles. This is reflected in the budding assay by the differential response to the α -SNAP^{dn} mutant, improving a selective recovery of Mann II but not p24 proteins (Fig. 1 A, right). To examine this more directly, we immunisolated p24-containing vesicles using magnetic beads coated with a cytoplasmic domain antibody to p24 γ_3 (Füllekrug et al., 1999). Affinity purified antibody attached to magnetic beads was first tested for its ability to bind Golgi membranes (Fig. 2 A, left). After incubation, membranes bound to beads were analyzed at the ultrastructural level and for protein content by SDS-PAGE followed by Western blotting. As can be seen, large membrane structures were observed on beads only when using the p24 γ_3 antibody (Sp) but not on beads coated with an irrelevant control antibody (Ctr). Addition of magnetic beads to the vesicle fraction (Fig. 2 A, right) revealed binding of vesicular structures with a diameter of 50–60 nm, the expected size of uncoated COPI vesicles using the p24 γ_3 antibody (Sp). No vesicles were seen binding to beads coated with the control antibody (Ctr). Western blots probed with antibodies to Mann II, p24 α_2 , β_1 , δ_1 , and γ_3 showed that no preferential parts of the Golgi (Gt) had been selected for (Fig. 2 B, Gt compared with Sp). No signal could be detected when using beads coated with the control antibody (Ctr).

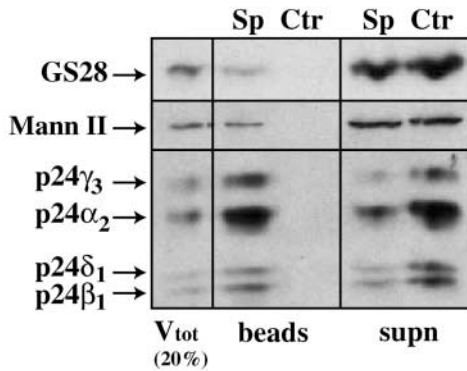
A



B



C



D

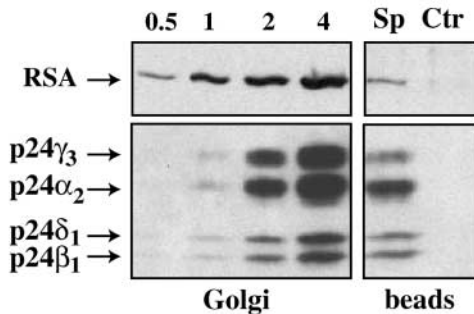


Figure 2. Immunoprecipitation of p24-containing vesicles reveals subpopulations of COPI vesicles. (A–D) Magnetic beads precoated with either an irrelevant control antibody (Ctr) or specific (Sp) antibodies to the cytoplasmic domain of p24 γ_3 were incubated with nonwashed Golgi membranes (A, left, and B) or with COPI vesicles formed from such membranes in the presence of the α -SNAP^{dn} mutant (A, right, and C and D). Isolated membranes and vesicles were pelleted, fixed, embedded, and processed for EM or solubilized and subjected to SDS-PAGE. After transfer to nitrocellulose, Mann II, p24 γ_3 , p24 α_2 , p24 δ_1 , and p24 β_1 were detected using specific antibodies followed by ECL. Vesicles remaining in the supernatant (Supn) after immunoprecipitation were also monitored in terms of their protein content (C, left lanes). (D) Immunoprecipitated vesicles were probed for the anterograde cargo marker, RSA, and compared with p24 γ_3 , p24 α_2 , p24 δ_1 , and p24 β_1 . Lanes containing total Golgi (Gt) and 20% of total vesicles (V_{tot}) used for immunoprecipitation are included for comparison.

In immunoprecipitated vesicles (Fig. 2 C, beads, Sp), the four p24 proteins were detected roughly at the same relative intensities as those seen in the total vesicle population (V_{tot}). Thus, p24 γ_3 did not appear to segregate away from p24 α_2 ,

β_1 , or δ_1 , suggesting that p24 α_2 , β_1 , δ_1 , and γ_3 were present in the same COPI-derived vesicle population. A small amount of Mann II was detected in p24-containing vesicles, but as can be seen the majority remained in vesicle superna-

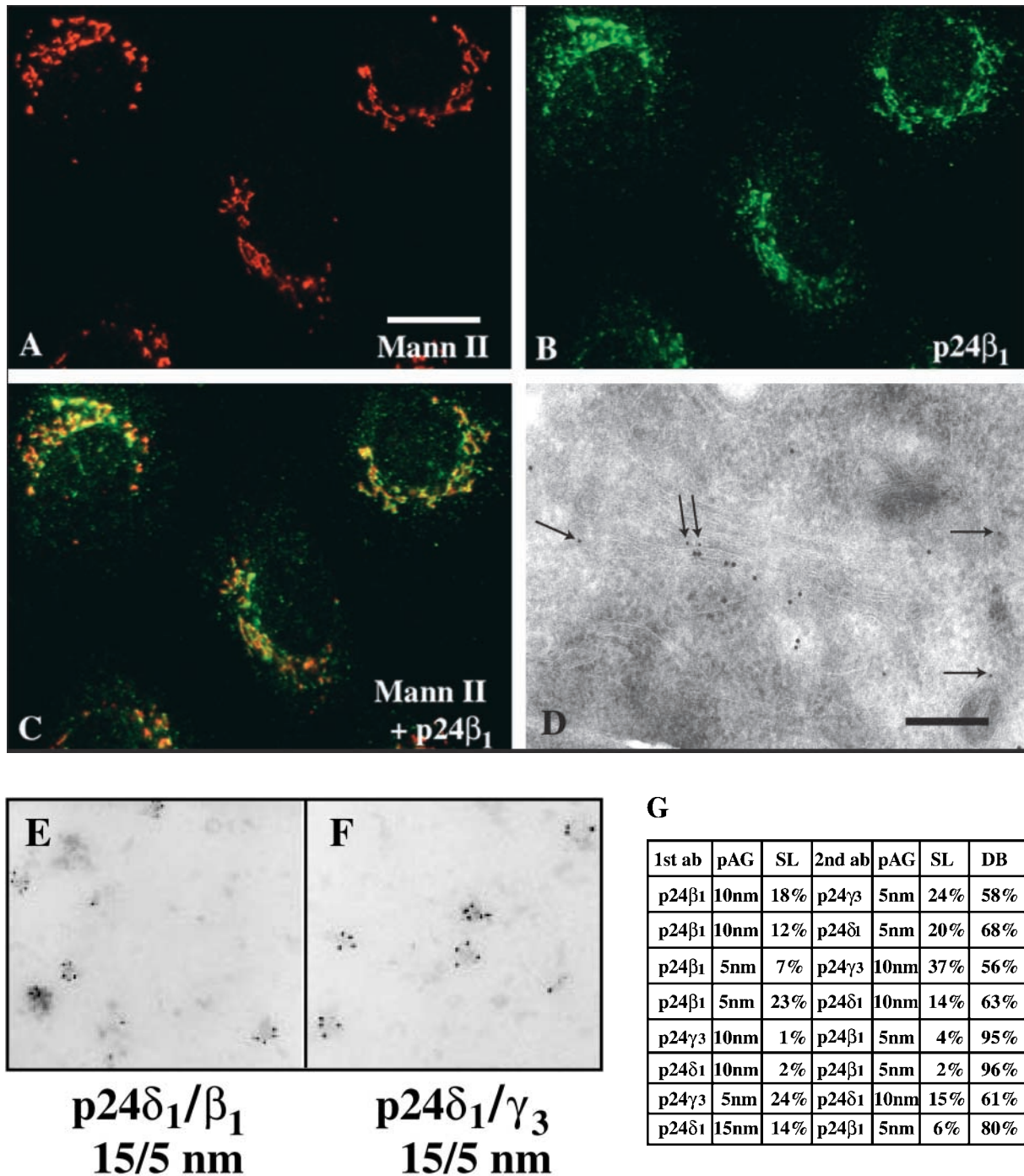


Figure 3. Immunolocalization of p24 proteins in cells and vesicles. (A–C) Subcellular localization of p24β₁ (b, green) as determined by indirect immunofluorescence localization. For comparison, labeling of Mann II (red) is shown in A. The extent of overlap is shown in C. (D) Thin frozen sections of NRK cells were labeled with antibodies to p24β₁ (10 nm gold). Gold particles were found at one side of the Golgi stack, codistributing with antibodies to p24γ₃ (5 nm gold, arrows). (E and F) Vesicles bound to grids were labeled with antibodies against two different p24s and revealed by protein A gold conjugates. The majority of the labeled vesicles contained both p24 proteins of each combination. (G) Quantification of p24 proteins in pair-wise labeling experiments. Different antibodies and protein A gold sizes (pAG) were used. Vesicles labeled with only one type of gold particle (two or more of the same gold particle) are expressed as the percentage of single labeled (SL), and vesicles containing both types of gold particle (two or more different gold particles) are expressed as double labeled (DL).

tant left over after immunoisolation (Fig. 2 C, supn, Sp compared with Ctr). This was also the case for GS28, a vesicle SNARE protein of the Golgi stack (Fig. 2 C, supn, Sp compared with Ctr) (Nagahama et al., 1996; Subramaniam et al., 1996; Hay et al., 1998). Next, we examined to what

extent anterograde cargo was present in p24-containing vesicles. Immunisolated vesicles were probed for RSA and for comparison p24α₂, β₁, δ₁, and γ₃. Only a small amount of RSA was seen in isolated vesicles (Fig. 2 D, beads, Sp) at a comparable level to that observed in the total vesicle popula-

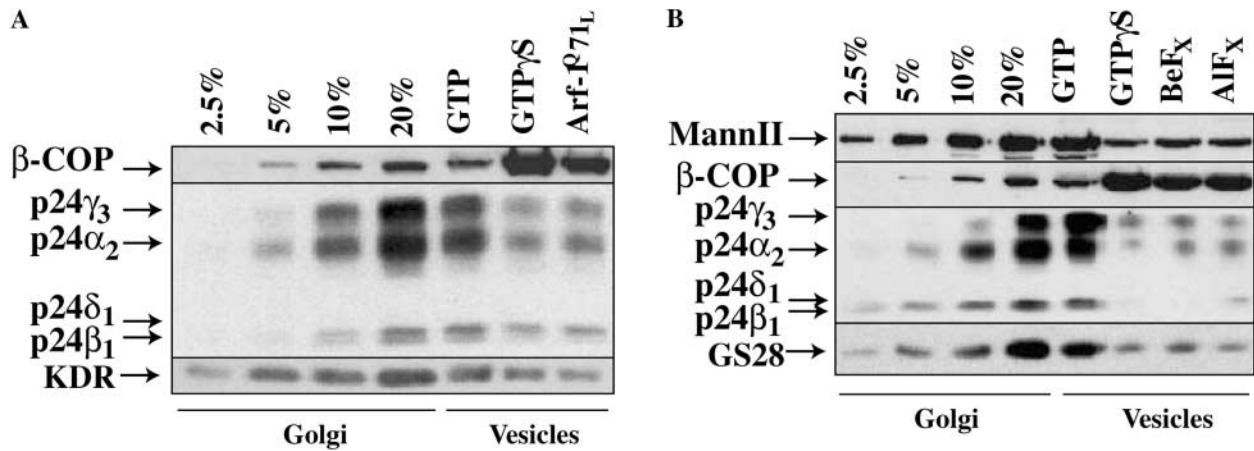


Figure 4. **A requirement for GTP hydrolysis by Arf-1.** (A) Vesicles were formed in the presence of GTP, GTP γ S, or Arf-1^{Q71L}. (B) Vesicles were formed in the presence of GTP, GTP γ S, BeF_x, or AIF_x. Proteins from solubilized membranes or vesicles were separated by SDS-PAGE and subjected to Western blot analysis using specific primary antibodies to Mann II, β -COP, p24 γ_3 , p24 α_2 , p24 δ_1 , p24 β_1 , and the KDR followed by ECL.

tion (compare with Fig. 1 D), making it unlikely that COP-derived p24-containing vesicles transport anterograde cargo.

The immunoisolation data suggests that p24 β_1 is present in vesicles together with p24 α_2 , δ_1 , and γ_3 . Whereas the steady-state localization of p24 α_2 , δ_1 , and γ_3 have been mapped to the cis side of the Golgi stack at the ultrastructural level (Sohn et al., 1996; Rojo et al., 1997; Dominguez et al., 1998; Füllekrug et al., 1999), Golgi localization of p24 β_1 has only been determined at the level of light microscopy (Gommel et al., 1999). Therefore, we determined the steady-state localization of p24 β_1 by immuno-EM. A rabbit peptide antibody was raised against the cytoplasmic domain of p24 β_1 and affinity purified. By indirect immunofluorescence, the antibody gave rise to a juxta nuclear Golgi-like staining pattern (Fig. 3, A–C) with extensive but not complete colocalization with the Golgi stack marker, Mann II.

Peripheral punctate staining was also observed as seen previously when staining for p24 α_2 , δ_1 , and γ_3 . In NRK cells at the ultrastructural level, p24 β_1 (10 nm) showed extensive colocalization with p24 γ_3 (5 nm, arrows) mapped previously to the cis side of the Golgi stack (Füllekrug et al., 1999). This completes the mapping of all four p24 proteins to the cis side of the Golgi stack.

The extent of different p24 proteins in the same COPI-derived vesicle was also examined using affinity purified cytoplasmic domain antibodies. Since cytoplasmic domain antibodies to p24 α_2 failed to produce significant labeling despite the obvious presence of this member in vesicles as determined by Western blotting, we could not monitor this member. Two sets of representative double labeling experiments of COPI-derived vesicles, showing p24 β_1 , δ_1 , and γ_3 in different combinations, are shown in Fig. 3, E and F. A range of different antibody concentrations and gold particle sizes were tested. As summarized in Fig. 3 G, the majority of vesicles revealed double labeling. In summary, we conclude from the above data that at the level of the Golgi stack, p24 proteins enter budding COPI vesicles together, presumably in order to recycle back to the ER. Isolated vesicles were distinct from those containing Mann II and GS28, and neither contained significant amounts of anterograde cargo.

Table I. Outline and summary of peptides

		L	G	V	B
p24 α_2	□	CQMRHLKSFFFAKKLIV	–	–	–
p24 δ_1	■	CQVFLYLRFFKAKKLIE	(+)	(+)	+
p24 β_1	○	CQIYYLKRFFVRRVV	+	+	+
p24 β_1 (YL)	□	CQIYAAKRFFVRRVV	–	–	+
p24 β_1 (LK)	▽	CQIYAAARFFVRRVV	+	+	+
p24 β_1 (FF)	■	CQIYYLKRAAEVRRVV	–	–	–
p24 β_1 (EV)	△	CQIYYLKRFFAARRVV	(+)	(+)	(+)
p24 β_1 (RR)		CQIYYLKRFFVEAAVV			(–)
p24 β_1 (VV)	◆	CQIYYLKRFFVRRAA	+		+
p24 β_1 (+A)	◇	CQIYYLKRFFVRRVVA	+		+
p24 γ_3		CQVLLKSFSDKRTTTRVGS			+
p24 γ_4		CQVLLKSFTEKRPISRAVHS			+
p24 $\gamma_{1/2}$		CQVCTLKRFFQDKRPVPT			(–)
E19		CKYSRRSFIDEKKMP			–
RER1		CKRRYRGKEDAGKAFAS			–

Amino acid residues are written as single amino acid code. Symbols correspond to those used in Fig. 5. The inhibitory effect of each peptides, where assayed for, is summarized on the right. Inhibition of ArfGAP1 activity on liposomes (L) or Golgi membranes (G), inhibitory activity in the vesicle budding assay (V), and ArfGAP1 binding (B) is denoted with a +. No inhibitory activity or no binding is denoted with – and intermediate activity/binding with either (+) or (–).

A need for GTP hydrolysis and a role for GAP in protein sorting

We showed previously that Mann II and two glycosyltransferases, *N*-acetylglucosaminyltransferase I (GlcNAc-T1) and galactosyltransferase (GT-1), are sorted and concentrated into COPI vesicles in an Arf-1 GTP hydrolysis-dependent manner (Lanoix et al., 1999). Yet, resident proteins such as p24 proteins are proposed to inhibit GTP hydrolysis, thereby ensuring that coatomer can remain on the membrane in order to form “priming complexes” required for vesicle formation (Springer et al., 1999). To investigate this, we first tested whether the four p24 proteins require GTP hydrolysis by Arf-1 for incorporation into vesicles. As seen in Fig. 4 A, we observed a clear decrease for p24 α_2 , β_1 , δ_1 , and γ_3 upon addition of GTP γ S, a nonhydrolyzable analogue of GTP or the GTP-

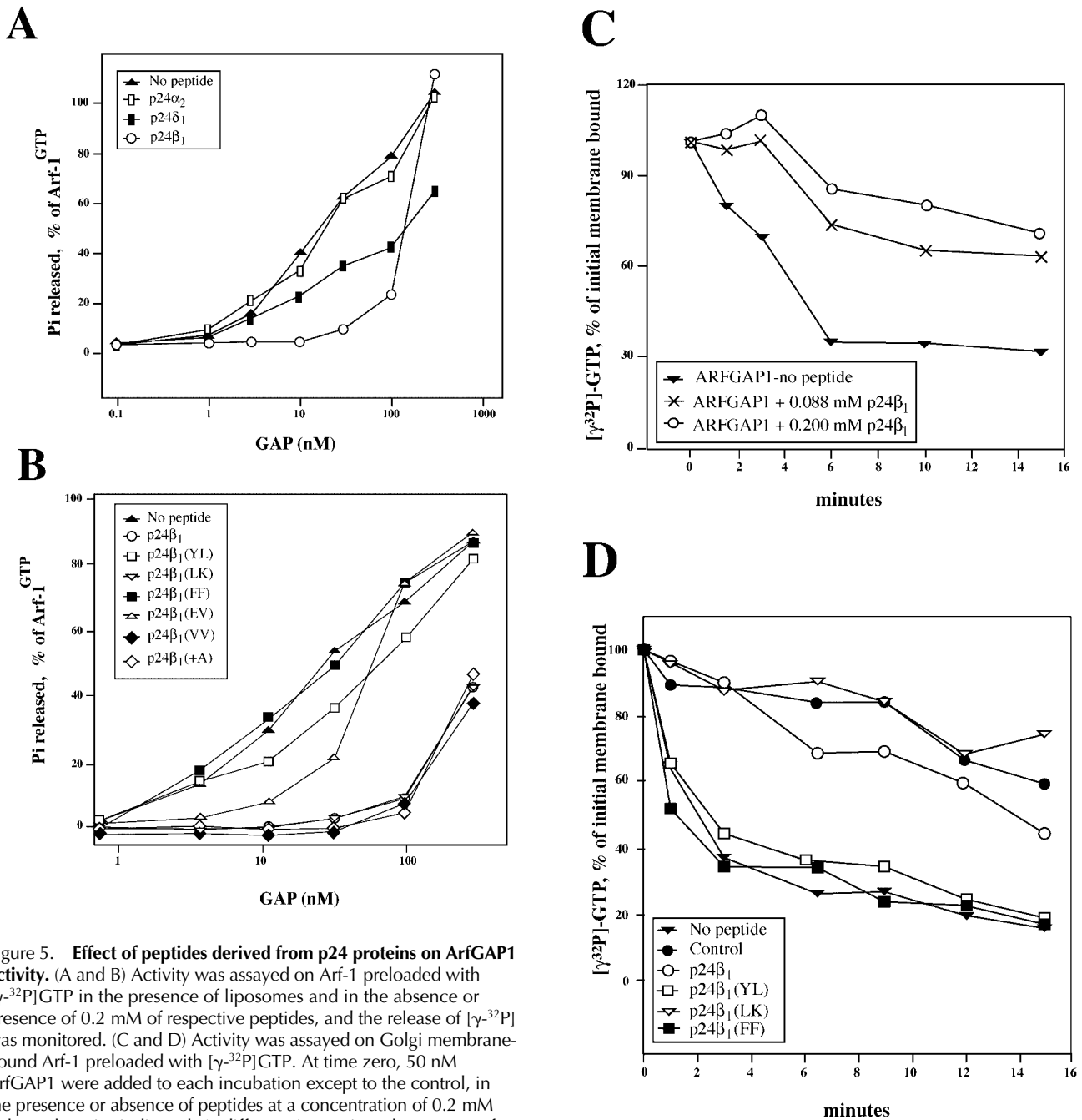


Figure 5. Effect of peptides derived from p24 proteins on ArfGAP1 activity. (A and B) Activity was assayed on Arf-1 preloaded with [γ - 32 P]GTP in the presence of liposomes and in the absence or presence of 0.2 mM of respective peptides, and the release of [γ - 32 P] was monitored. (C and D) Activity was assayed on Golgi membrane-bound Arf-1 preloaded with [γ - 32 P]GTP. At time zero, 50 nM ArfGAP1 were added to each incubation except to the control, in the presence or absence of peptides at a concentration of 0.2 mM unless otherwise indicated. At different time points, the amount of [γ - 32 P]GTP, which remained bound to membranes, was determined.

restricted mutant of Arf-1, Arf^{Q71L}. A similar inhibition, though less striking, was observed upon probing for the KDEL receptor (KDR). As expected, coatomer was most readily detected (monitored using monoclonal antibodies to β -COP) on vesicles generated under conditions where GTP hydrolysis was inhibited. However, a small amount of coatomer persisted on vesicles generated in the presence of GTP. This was observed consistently using our modified assay, suggesting that perhaps uncoating efficiency was somewhat lowered.

The requirement for GTP hydrolysis in sorting was further investigated using phosphate analogs, aluminum, or beryllium fluoride (AlF_x or BeF_x). Previous studies have high-

lighted the dramatic effects of these compounds in both the COPI and COPII systems. A recent study examining the dynamics of COPII coat assembly on liposomes (Antony et al., 2001) demonstrated that BeF_x can mimic the γ -phosphate of GTP on GDP bound to the small GTPase Sar1, resulting in the generation of a stable COPII complex. Other studies have shown that AlF_x induced the assembly of the COPI coat (Donaldson et al., 1991), possibly due to inhibition of GTP hydrolysis (Finazzi et al., 1994). We thus tested if these fluoride complexes would affect sorting in a manner similar to that observed with GTP γ S. As can be seen in Fig. 4 B, vesicles were still formed in the presence of AlF_x or BeF_x as evidenced from the β -COP signal, which was comparable

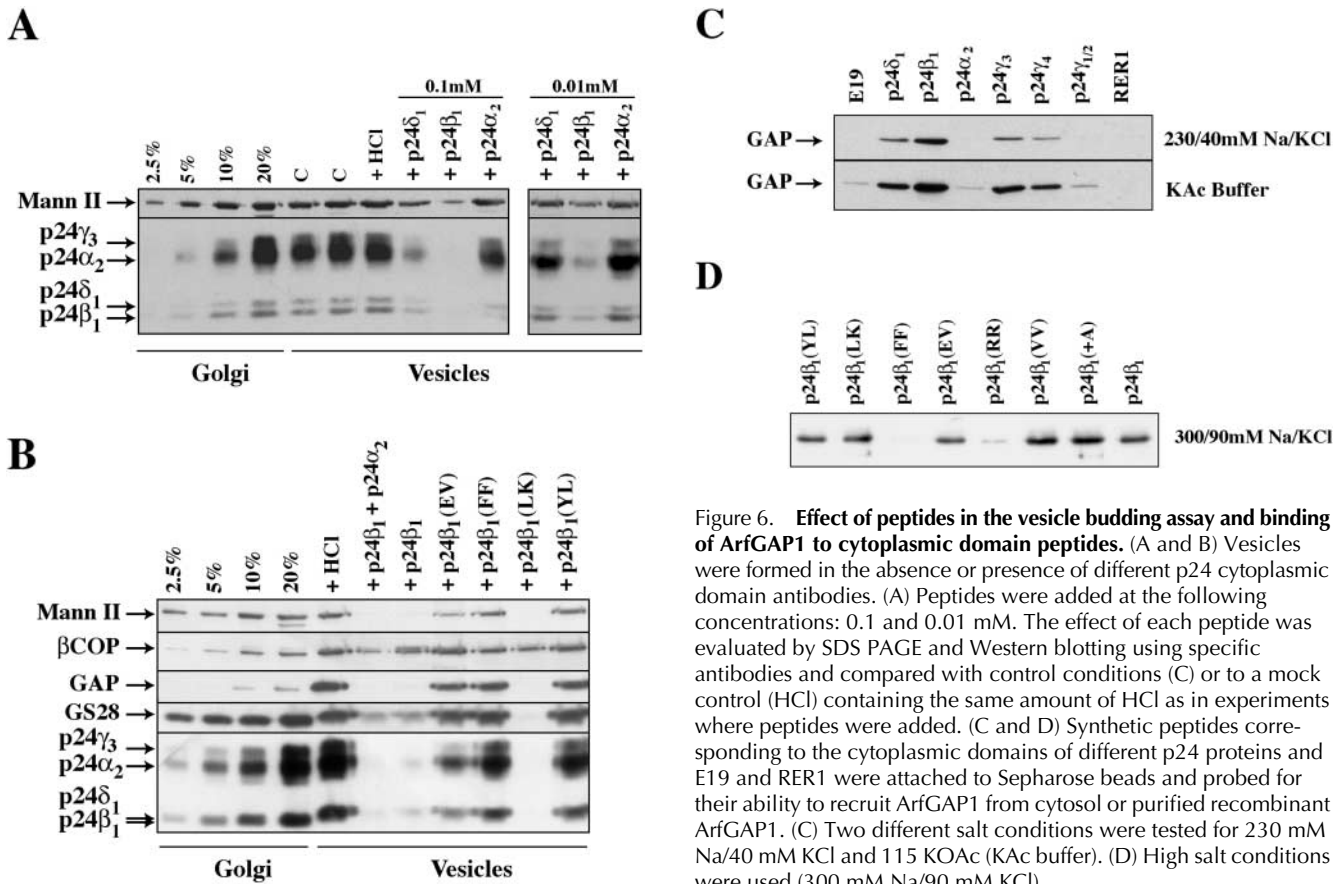


Figure 6. Effect of peptides in the vesicle budding assay and binding of ArfGAP1 to cytoplasmic domain peptides. (A and B) Vesicles were formed in the absence or presence of different p24 cytoplasmic domain antibodies. (A) Peptides were added at the following concentrations: 0.1 and 0.01 mM. The effect of each peptide was evaluated by SDS PAGE and Western blotting using specific antibodies and compared with control conditions (C) or to a mock control (HCl) containing the same amount of HCl as in experiments where peptides were added. (C and D) Synthetic peptides corresponding to the cytoplasmic domains of different p24 proteins and E19 and RER1 were attached to Sepharose beads and probed for their ability to recruit ArfGAP1 from cytosol or purified recombinant ArfGAP1. (C) Two different salt conditions were tested for 230 mM Na/40 mM KCl and 115 KOAc (KAc buffer). (D) High salt conditions were used (300 mM Na/90 mM KCl).

to that seen upon addition of GTP γ S. As with GTP γ S, incorporation of Mann II, GS28, and the four p24 proteins was inhibited, thus providing further evidence for a GTP hydrolysis-dependent sorting event. We conclude that GTP hydrolysis by Arf-1 is a prerequisite for proper sorting of p24 proteins into COPI-derived vesicles.

Since GTP hydrolysis by Arf-1 is required, the observed inhibition by BeF_x and AlF_x suggests a possible role for ArfGAP1, a Golgi-localized Arf GAP, in the sorting process. How is this then reconciled with the proposed need for an inhibition of GAP activity (and hence GTP hydrolysis) to form priming complexes (Springer et al., 1999)? Since evidence exists in favor of cargo modulation of ArfGAP1 activity by the cytoplasmic domain of p24 β ₁, we tested for the ability of p24 cytoplasmic domain peptides to inhibit ArfGAP1 activity. In solution using Δ 17Arf-1 in the presence of coatomer, we observed a strong inhibition in the presence of the p24 β ₁ peptide but not with the p24 δ ₁ peptide, essentially confirming previous observations (Goldberg, 2000; unpublished data). When using full-length myristoylated Arf-1 bound to phospholipid vesicles, coatomer stimulation of GAP activity is not observed (Szafer et al., 2000), whereas on Golgi membranes coatomer approximately doubles ArfGAP1 activity (Szafer et al., 2001). Therefore, it was of interest to examine the effect of p24 peptides under conditions that more closely resemble those prevailing in the cells. The result of an experiment where GAP activity on Arf-1 was monitored on liposomes in the presence of 0.2 mM p24 α ₂, p24 β ₁, or p24 δ ₁ cytoplasmic domain peptides

is shown in Fig. 5 A. As can be seen, addition of the p24 α ₂ peptide had little if any effect on GAP activity, whereas both p24 β ₁ and p24 δ ₁ caused inhibition. Strongest inhibition was obtained with the p24 β ₁ peptide. To examine which amino acid residues in the p24 β ₁ peptide are important for inhibitory effect, we substituted two amino acids at the time throughout the cytoplasmic domain (Table I). The effect of each peptide except for the p24 β ₁ (RR) peptide, which could not be solubilized, is shown in Fig. 5 B. Strikingly, the p24 β ₁ (FF) peptide showed no inhibitory effect. Similarly, the p24 β ₁ (YL) peptide showed only a minor inhibitory effect, whereas p24 β ₁ (EV) revealed partial inhibition. No loss of inhibitory effect was seen with p24 β ₁ LK, VV, and the +A peptide, which contained an additional alanine residue at the COOH terminus. A similar pattern was observed when using Golgi membranes in place of the liposomes (Fig. 5 C). In this experiment, Golgi membranes were preincubated with myristoylated Arf-1 and γ ³²P-GTP to allow for the binding of the nucleotide to Arf-1. Nucleotide exchange was then stopped by the addition of BFA followed by addition of ArfGAP1. The decrease in the amount of membrane-bound GTP was monitored as a measure for GTP hydrolysis on Arf-1 (Szafer et al., 2001). A strong inhibition was obtained using the p24 β ₁ peptide at 0.2 mM. Under these conditions, neither p24 α ₂ nor p24 δ ₁ revealed significant inhibition (unpublished data), suggesting that the inhibition on Golgi membranes was p24 β ₁ specific. We also examined selected p24 β ₁ substitution peptides. As seen with liposomes, substituting YL or FF to AA resulted in a loss of in-

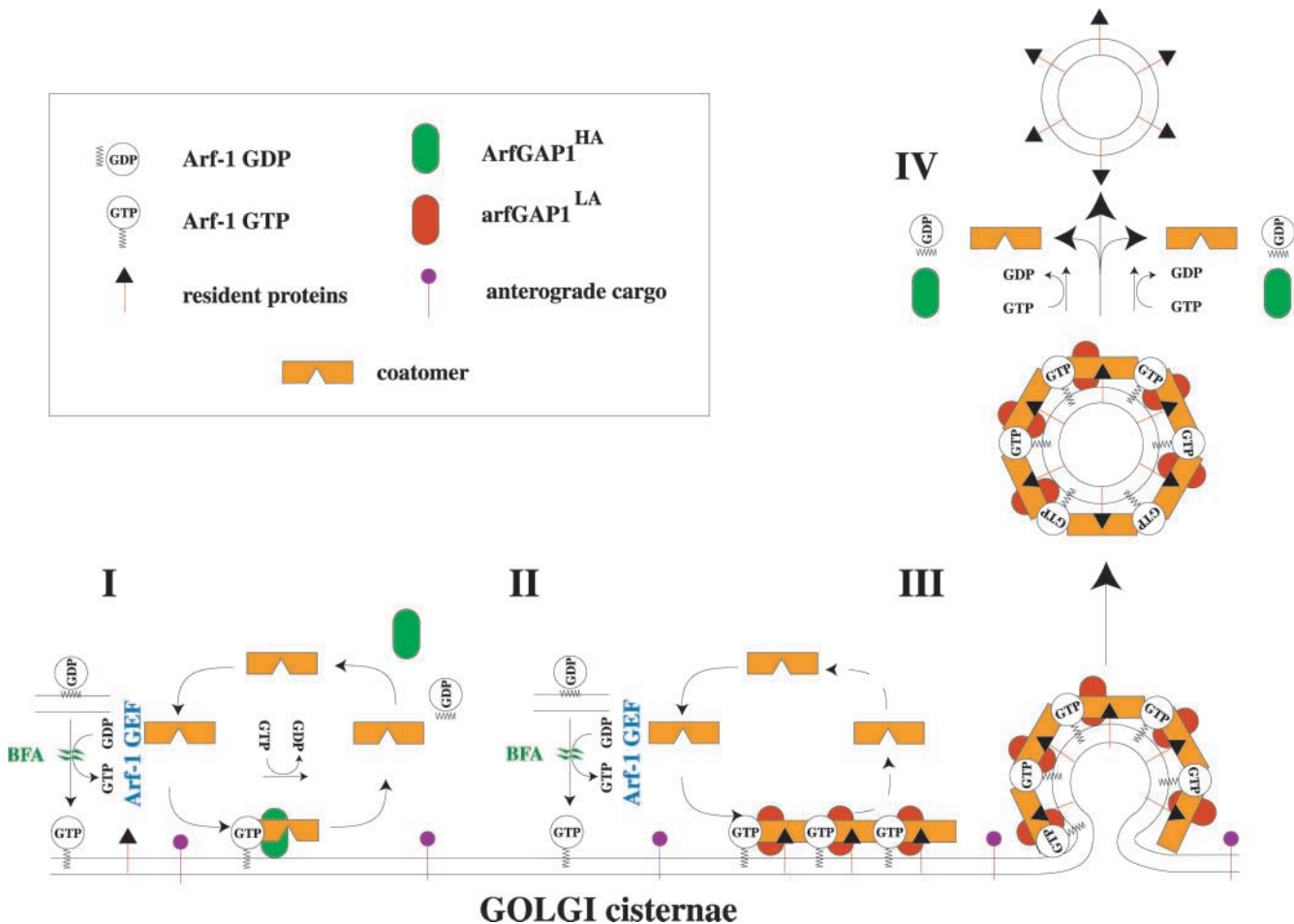


Figure 7. GTP hydrolysis-driven sorting coupled with ArfGAP1 modulation. The model is divided into four steps from left to right and then from bottom to top. (I) GDP bound to Arf-1 attached to the membrane is replaced with GTP by Arf-1 guanine exchange factor. Cytosolic coatmer binds Arf-1^{GTP} but is quickly released upon GTP hydrolysis by Arf-1. This step requires ArfGAP1 and is denoted in green as having a high activity (HA) on Arf-1. Resident cargo proteins bind coatmer directly through cytoplasmic domain motifs (e.g., K[X]KXX). In order for coatmer to capture resident proteins, coatmer first needs to be released from the membrane. This “sorting step” requires GTP hydrolysis by Arf-1. If resident cargo proteins are in addition induced to cluster (Weiss and Nilsson, 2000), coatmer is predicted to bind more strongly, thus favoring these over other resident proteins. (II) The activity of ArfGAP1 is downmodulated by captured resident cargo proteins. The rate of GTP hydrolysis by Arf-1 is as a consequence slowed down, enabling coatmer to remain on the membrane. The presence of resident cargo proteins promotes polymerization of the coat leading up to vesicle formation and budding (III). Though downmodulated in its activity, residual ArfGAP1 activity will permit Arf-1 to hydrolyze its GTP, and coatmer is released back into the cytosol. For simplicity, the release of coatmer, Arf-1, and ArfGAP1 from the vesicle is shown as one event.

hibitory activity (Fig. 5 D). Again, the p24 β_1 (LK) peptide was comparable to the p24 β_1 wild-type peptide and showed strong inhibition.

To test the functional consequence of the inhibition of GAP activity, we tested the effect of p24 peptides on sorting and vesicle formation in the vesicle-budding assay (Fig. 6 A). Vesicle formation was performed as above but in the presence of 0.1 or 0.01 mM p24 δ_1 , β_1 , or α_2 peptides. As controls, vesicle formation was performed in the absence of peptides (two GTP controls are shown) or in the presence of HCl, which was added to the same extent to the residual amounts present in the peptide solutions (of 0.1 mM solubilized peptides). Addition of 0.1 mM p24 β_1 cytoplasmic domain peptide gave rise to a strong decrease in p24 α_2 , β_1 , δ_1 , and γ_3 . Incorporation of Mann II was decreased also, suggesting that the p24 β_1 peptide acted on ArfGAP1 regardless of whether vesicles formed from early or medial-Golgi mem-

branes. A similar but less complete inhibition was seen with the p24 δ_1 peptide, whereas no inhibition was observed upon addition of the p24 α_2 peptide. At 0.01 mM concentration, the p24 β_1 cytoplasmic domain peptide gave rise to a similar decrease in p24 proteins, and Mann II recovered in the vesicle fraction, whereas now the inhibition observed with the p24 δ_1 peptide was less obvious. Selected p24 β_1 substitution peptides were also examined for their ability to affect sorting and vesicle formation (Fig. 6 B). Again, substituting FF or YL to AA removed the inhibitory effect seen with the p24 β_1 wild-type peptide. Furthermore, when using the EV to AA substitution peptide, only a minor inhibitory effect could be seen mirroring the partial activity seen when used in the liposome based assay (compare with Fig. 5 B). It is interesting to note that both the p24 β_1 wild-type peptide and the LK to AA substitution peptide showed strong inhibitory effect in the assay. The amount of coatmer (revealed by a mAb to

the β COP subunit) detected in the vesicle fraction was still comparable to that observed in the control (HCl but no peptide added), suggesting that vesicles still formed but that sorting had been affected. When adding the p24 β_1 and p24 α_2 wild-type peptides together, the amount of β COP was reduced slightly, whereas sorting remained strongly inhibited. Taken together, this indicates that those p24 β_1 peptides that inhibit sorting in the vesicle budding assay and show inhibitory effects in ArfGAP1 activity assays (Fig. 5) do so by interacting with ArfGAP1 directly.

To test for a direct interaction between cytoplasmic domain peptides and ArfGAP1, we monitored ArfGAP1 binding to cytoplasmic domain peptides of six different p24 proteins and the E19 adenovirus protein and RER1, the latter two included as controls. Peptides were attached to Sepharose beads as described previously (Dominguez et al., 1998) and incubated with rat liver cytosol or with purified components. Binding was examined at two different conditions, in the presence of 230 Na mM/ 40 mM KCl or 115 mM KAc (Lanoix et al., 1999). Cytosolic ArfGAP1 could be seen binding strongly to the cytoplasmic domain peptide of p24 β_1 and to a lesser extent to p24 δ_1 (Fig. 6 C) at both high and low salt concentrations. Minor binding was also observed to two cytoplasmic domain peptides of the γ subfamily, γ_3 and γ_4 . The other peptides showed little or no binding. We also monitored binding of Arf-1. No binding of Arf-1 could be revealed to either of the peptides using cytosol or purified Arf-1 (unpublished data). The p24 β_1 substitution peptides were also tested using purified full-length recombinant ArfGAP1 expressed and purified from insect cells. All substitution peptides except for p24 β_1 FF and RR revealed ArfGAP1 binding. This included the YL, LK, and EV peptide, which showed binding but to a lower extent than that seen in the wild-type peptide. Thus, the inability of the p24 β_1 (FF) peptide to bind ArfGAP1 corresponds well with its lack of inhibitory activity in the assays described in Fig. 5 and Fig. 6, A and B. This suggests that the two phenylalanines (FF) possibly in combination with upstream amino acids such as tyrosine (Y) and leucine (L) mediate the observed inhibitory activity of the p24 β_1 cytoplasmic domain peptide. Since we could not assay for the RR peptide in the two ArfGAP1 activity assays or in the vesicle budding assay due to insolubility problems of the peptide, we cannot exclude the possibility that the two arginines might also play a role.

The need for GTP hydrolysis by Arf-1 and a downregulation of ArfGAP1 activity is best explained in a two-stage mechanism (Fig. 7, I and II). First, release of coatamer is required for rebinding to membrane patches occupied by resident proteins. Coatamer units are predicted here to detach and rebind multiple times in order to capture the resident proteins. This process requires GTP hydrolysis by Arf-1 and full activity provided by ArfGAP1 (denoted in green and HA for high activity). Wherever coatamer units successfully capture resident proteins, ArfGAP1 activity is lowered as a consequence of cargo modulation (denoted in red and LA for low activity). We presented evidence in favor of a direct interaction between ArfGAP1 and cytoplasmic domain peptides of p24 β_1 (and to a lesser extent, p24 δ_1) and their ability to downmodulate ArfGAP1 activity on membranes in the absence of coatamer. However, we do not rule out an in-

volvement of coatamer also in this process. Thus, the cytoplasmic domain of the resident protein is shown as interacting both with ArfGAP1 and with coatamer, and the two latter are also shown as interacting. A binding release cycle (Fig. 7 I) coupled with downmodulation of ArfGAP1 by resident cargo (Fig. 7 II) will lead to the enrichment of "priming complexes." Polymerization of the COPI coat (Fig. 7 III) is then promoted by the presence of high concentration of resident proteins (Reinhard et al., 1999): at least four cytoplasmic domains of p24 δ_1 /coatamer unit were required for optimal coat polymerization. The vesicle can then bud and although downmodulated, ArfGAP1 will stimulate GTP hydrolysis by Arf-1, resulting in coat release (Fig. 7 IV).

Discussion

Multiple populations of COPI-derived vesicles

The cisternal maturation model predicts that anterograde cargo remains in cisternal membranes and that cisternae mature in a cis-to-trans direction, whereas resident proteins move in the opposite direction via COPI vesicles. All anterograde cargo is predicted to move at the same rate, whereas resident proteins are sampled at different stages and concentrated into budding COPI vesicles. In the vesicular transport model, each cisterna instead constitutes a stable compartment, and anterograde cargo move via COPI anterograde vesicles from cisterna to cisterna in a cis-to-trans direction. In an attempt to merge the two models, Pelham and Rothman (2000) proposed recently that vesicles containing either anterograde or retrograde cargo would bud and fuse randomly with adjacent cisternae. At the same time, cisternae would progress slowly forward. The prediction of this model is that neither anterograde nor retrograde cargo should be concentrated into budding COPI vesicles. This prediction is clearly not born out by our findings where incorporation of resident proteins is favored over anterograde cargo, a notion in direct support of a cisternal maturation scenario. Selective sorting of resident proteins into more than one population of COPI vesicles is supported by our findings that Mann II/GS28 and the four p24 proteins, α_2 , β_1 , δ_1 , and γ_3 , segregate into distinct subpopulations, neither of which contain significant amounts of anterograde cargo. We monitored three different anterograde cargo markers in the vesicle population, one membrane bound (pIgR) and two soluble (RSA and ApoE), all of which distribute equally across the Golgi stack en route to the cell surface. The two soluble cargo proteins are highly abundant in rat liver, which underscores the selectivity of the observed sorting process in favor of resident proteins. Of interest to note is that GS28 is represented poorly in the vesicle subpopulation containing p24 proteins. This is consistent with the observation that upon shifting cells to 15°C to block ER to Golgi transport (Saraste and Kuismanen, 1984) GS28 does not redistribute to peripheral structures (Hay et al., 1998). Under these conditions, proteins such as p24 α_2 , β_1 , δ_1 , and γ_3 , KDR and ERG153/58 of the CGN/cis cisternae do redistribute to peripheral structures, indicating that GS28 resides in later cisternae (e.g., medial and trans). It will be interesting to see if p24-containing vesicles contain SNARE proteins, such as membrin,

rsec22b, and rbet1, and whether syntaxin 5 is present in both vesicle populations. The latter has been shown to bind GS28 or membrin/rsec22b/rbet1, yielding distinct complexes (Hay et al., 1998). Using immunisolated p24-containing vesicles, it should also be possible to investigate their preferred target membranes (e.g., the ER and/or CGN) (Love et al., 1998).

Vesicle formation and concentration of resident proteins upon sorting

As in our previous study (Lanoix et al., 1999), vesicles generated *in vitro* using rat liver Golgi and cytosol were uncoated. However, some coatomer was detected on vesicles in this study but much less than that would have been observed if these had been formed under conditions where GTP hydrolysis was inhibited (Fig. 4). We term these COPI-derived vesicles, since they form in a COPI-dependent manner, and display the expected size of 50–60 nm but have lost most of their original coat. We rule out that isolated vesicles represent cisternal membrane fragments caused by mechanical fragmentation, since in the presence of an ATP depletion system, in the absence of coatomer, or at +4°C vesicles could not be detected (Lanoix et al., 1999). Likely, GTP hydrolysis by Arf-1 induces formation of domains enriched in resident proteins in a coatomer-dependent manner. We favor this possibility, since such sorting domains would constitute assemblies of priming complexes required for vesicle budding (Fig. 7; Springer et al., 1999). Also, since the Mann II signal increased upon addition of the α -SNAP^{dn} mutant (Fig. 1 B) to inhibit NSF mediated fusion it argues that discontinuous transport intermediates (e.g., vesicles) indeed form. Under these conditions, we can further rule out the possibility that putative anterograde vesicles could have been missed if these had a shorter half-life than retrograde vesicles.

In the previous study, we observed that Mann II was concentrated about fourfold over cisternal membranes as judged by Western blotting (after correcting for phospholipids in vesicles and membranes) or by immuno-EM (comparing the linear density of Mann II) of vesicles formed under conditions where GTP hydrolysis was permitted or inhibited by GTP γ S (Lanoix et al., 1999). A higher concentration effect (up to 10-fold) was estimated by enzymatic activity assays for GlcNAc-T1 and GT-1. Whereas Mann II is very abundant (~1.5% of total Golgi proteins) in the Golgi, both GlcNAc-T1 and GT-1 represent ~0.1% of Golgi resident proteins. The high degree of concentration of GlcNAc-T1 and GT-1 was questioned recently. It was argued that concentration of resident proteins into vesicles would be unlikely, since Golgi cisternae already contain high amounts of resident proteins (Pelham and Rothman, 2000). This is based on the estimate that both ER and Golgi membranes contain 30,000–40,000 protein molecules per square micron (Quinn et al., 1984). If so, a concentration factor of three to four times might seem more feasible than one order of magnitude. The idea that there is room for concentration is supported by recent findings by Luini and coworkers, revealing extensive plasticity in Golgi stacks. These can be seen shrinking or enlarging as a consequence of temperature shifts without losing their resident protein content (Luini, A., personal communication; Griffiths et al., 1989). How-

ever, of further importance is that anterograde cargo appears deselected, thus highlighting a clear sorting preference for resident protein into COPI vesicles (Fig. 1 D and Fig. 2 D; Lanoix et al., 1999). We conclude that the GTP hydrolysis-driven sorting highlighted in this and our previous study favors the concentration of resident proteins into COPI vesicles. As different resident proteins were found in distinct subpopulations, we propose that COPI vesicles mainly recycle constituents from different levels of the pathway.

ArfGAP1-dependent sorting of p24 proteins

The presence of four different p24 proteins in the same subpopulation suggests that these move together into the budding COPI vesicle. Comparing the stoichiometry and the occurrence of the four p24 proteins in Golgi membranes with that in immunisolated vesicles suggests that all four p24 proteins are sorted together. We showed previously that p24 α_2 , β_1 , δ_1 , and γ_3 exist as a heterooligomeric complex in Golgi membranes by coimmunoprecipitation using antibodies to p24 δ_1 or γ_3 (Füllekrug et al., 1999). In that study, a 1:1:1:1 stoichiometry between the four p24 proteins was proposed. Furthermore, two-dimensional gel analysis showed that other p24 proteins such as p24 γ_4 were not present in this complex. The amount of coprecipitating p24 proteins using antibodies to p24 γ_3 was 15% of the total p24 γ_3 . This might reflect a dynamic equilibrium between p24 homodimers and heterooligomers where the latter form as a prelude to sorting (Weiss and Nilsson, 2000). A similar complex formation between p24 α , β , δ , and γ has also been observed in yeast, suggesting that this is a conserved feature (Marzioch et al., 1999). Since p24 α_2 and δ_1 have been shown to distribute to a larger extent to the ER and to the ER-to-Golgi intermediate membranes than p24 β_1 and γ_3 by subcellular fractionation (Dominguez et al., 1998), we do not rule out the possibility that p24 α_2 and δ_1 could also recycle in the absence of p24 β_1 and γ_3 but from earlier membranes not represented in our assay.

Sorting of the four p24 proteins was dependent on GTP hydrolysis by Arf-1 (Fig. 4 A), since GTP γ S and the GTP-restricted mutant of Arf-1 (Q71L) were both found to be inhibitory. A less striking inhibition was seen with the KDR, perhaps suggesting that this receptor can be sorted and concentrated independently of GTP hydrolysis (Sönnichsen et al., 1996). The fact that proper sorting of p24 proteins and Mann II requires GTP hydrolysis by Arf-1 is further demonstrated by experiments using BeF_x and AlF_x. Both compounds act on trimeric G proteins and small GTPases but in the case of the latter only if their respective GAP is present (for review see Chabre, 1990). These fluoride compounds are thought to arrest the GTPase in a conformation, mimicking that observed in an early phase in the transition from the GTP to GDP state. We observed a clear inhibitory effect upon addition of either compounds together with GTP comparable to that observed using GTP γ S and Arf-1^{Q71L}. This makes it likely that Arf-1 was the target of either fluoride compounds rather than trimeric G proteins, although an additional involvement of trimeric G proteins cannot be excluded. The idea that sorting and not vesicle budding is inhibited is supported by the observation that the β -COP signal (used to monitor coatomer) is high comparable to

what was observed when using GTP γ S. Therefore, the inhibitory effects of BeF_x and AlF_x suggested a direct role for ArfGAP1 (and perhaps other GAPs acting on Arf-1) in protein sorting. Experimental evidence in favor of this comes from work by Goldberg (2000) where he monitored Arf-1 activity in the presence of synthetic peptides corresponding to the cytoplasmic domains of p24 proteins. He showed in a coatomer-dependent assay that GTP hydrolysis by Arf-1 together with ArfGAP1 was downregulated in the presence of a cytoplasmic domain peptide of p24 β ₁ in solution. He interpreted this as a conformational change in coatomer caused upon binding to the cytoplasmic domain peptide, which resulted in a reduced ability of coatomer to stimulate GAP activity on Arf-1. However, a coatomer-stimulated GTP hydrolysis through ArfGAP1 was challenged recently, showing that if using full-length Arf-1, coatomer stimulation was undetected (Szafer et al., 2000). In the study presented here, we observed a clear inhibitory role of the p24 β ₁ cytoplasmic domain peptide but independent of coatomer, both on liposomes and Golgi membranes. Therefore, we suggest that this cytoplasmic domain peptide can exert its ability to inhibit GTP hydrolysis through a direct interaction with ArfGAP1 rather than through coatomer. The region mediating ArfGAP1 binding and inhibitory activity corresponds well to the previously identified cytoplasmic domain motif, Y/FXXXXY/F, in p24 δ ₁ for Sec23/24 binding (Dominguez et al., 1998). In that study, p24 δ ₁ was examined, since this peptide binds both to coatomer and Sec23/24. However, p24 β ₁ was shown to bind Sec23/24 stronger than p24 δ ₁, similar to what we observe with ArfGAP1. Since Sec23 is the GAP for the small GTPase Sar1 of the COPII coat (Yoshihisa et al., 1993), it is likely that both p24 β ₁ and p24 δ ₁ have evolved to bind GAPs to ensure incorporation into COPII and COPI vesicles, allowing p24 proteins to operate at the ER-to-Golgi interface. The ability of p24 β ₁ to downregulate ArfGAP1 activity raises the question if resident proteins that recycle from later compartments can also modulate ArfGAP1. Since the p24 β ₁ cytoplasmic domain peptide inhibited both Mann II and the p24 proteins and the fact that the Arf-1^{Q71L} mutant blocks sorting infers that ArfGAP1 is required for sorting into both vesicle populations. Therefore, one would predict that resident proteins other than p24 β ₁ should be able to bind and modulate ArfGAP1 activity for protein sorting. If so, this would offer an attractive mechanism ensuring that vesicles could only form when cargo proteins are present. As proposed by Schekman and coworkers (Springer et al., 1999), downregulation of GAP activity slowing down GTP hydrolysis by Arf-1 (or Sar1 for the COPII coat) will allow coatomer to reside longer on the membrane (Fig. 7). Such a “priming complex” would then be able to interact laterally with other priming complexes to form a lattice, serving as a vesicle-budding site. This model also predicts that vesicle formation is coupled with the presence of cargo, a notion supported by recent findings examining sorting of vesicular stomatitis virus G protein into COPII vesicles (Aridor et al., 1999). The important addition to the scheme proposed by Springer et al. (1999) is that GTP hydrolysis is initially required for the sorting event and a subsequent downmodulation of GAP activity for the formation of the priming complex.

In conclusion, we have presented evidence in favor of two populations of COPI-derived vesicles formed from Golgi membranes. Both contain predominantly Golgi resident proteins and only minor amounts of anterograde cargo. Sorting into each vesicle population requires GTP hydrolysis by Arf-1. Subsequently, the ability of ArfGAP1 to stimulate Arf-1-mediated GTP hydrolysis is decreased by the resident proteins, thereby stabilizing coatomer on the membrane, allowing the COPI coat to polymerize.

Materials and methods

Reagents

Antipain aprotinin, apyrase benzamide, beryllium chloride, GSH, GTP, leupeptin, pepstatin, PMSF, potassium acetate, polyvinylpyrrolidone (PVP-40T), soybean trypsin inhibitor, and Tween 20 were from Sigma-Aldrich. Aluminium chloride was from ICN Biomedicals. ATP, creatine phosphate, and creatine kinase were from Roche. 1,4-dithiothreitol and Hepes were from Biomol GmbH. PD-10 columns, sucrose, and the ECL detection kit were from Amersham Pharmacia Biotech. 30% (wt/vol) acrylamide/0.8% (wt/vol) bis-acrylamide solution was from Bio-Rad Laboratories. Sodium cacodylate was from BDH Chemicals. Glutaraldehyde, glycerol, and potassium fluoride were from Merck. Ethanol was from Kematyl. Osmium tetroxide was from Electron Microscopy Sciences. Uranyl acetate was from Serva. Defatted dry milk was from De-Vau-Ge Gesundheitswerk GmbH. Protran nitrocellulose membranes (0.45 μ m) were from Schleicher and Schuell. X-Omat XAR5 films were from Eastman Kodak Co. Dynabeads M-500 subcellular was purchased from Dynal.

Antibodies

Polyclonal antibodies against α ₂, δ ₁, and γ ₃ have been described previously (Dominguez et al., 1998; Füllekrug et al., 1999). Rabbit polyclonal antibodies to the cytoplasmic domain of p24 β ₁ were raised against a synthetic peptide (CQIYLLKRFEEVRRVV) conjugated to KLH and affinity purified using the same peptide coupled to acrylamide support. Rabbit polyclonal antibodies to ArfGAP1 were raised against truncated recombinant His-tagged ArfGAP1 (1–257) (Δ ArfGAP1^{His}) (Makler et al., 1995) expressed in bacteria. Antibodies were affinity purified over Δ ArfGAP1^{His} attached to nickel beads (QIAGEN). Polyclonal antibody against Mann II was purchased from K. Moremen (University of Georgia, Athens, Greece). Polyclonal antibody against pIgR and Apo E were gifts from A. Hubbard (The Johns Hopkins University, Baltimore, MD) and John Bergeron (McGill University, Montreal, Canada), respectively. Affinity purified mAb CM1A10 was a gift from J. Rothman (Memorial Sloan Kettering Cancer Center, New York, NY), and GS28 was purchased from BD Transduction Laboratories. Polyclonal rabbit and mouse anti-IgG antibodies were purchased from Sigma-Aldrich. Polyclonal sheep antibodies to RSA was from Bidesign International. HRP-labeled polyclonal antibodies against rabbit, mouse, and sheep were purchased from Dianova.

Production and purification of NSF, His₆-tagged α -SNAP^{wt}, α -SNAP^{dn} mutant, and GAP

Recombinant His₆-tagged NSF, α -SNAP^{wt}, and α -SNAP^{dn} mutant were purified on Ni-NTA agarose as described (Barnard et al., 1997). Recombinant proteins were desalted using a PD-10 column preequilibrated in 20 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 5% glycerol, 1 mM GSH, and in the case of NSF, 5 mM ATP. Recombinant His₆-tagged α -SNAP^{dn} mutant was aliquoted and snap frozen directly after elution and diluted in budding buffer at 37°C before use. NSF was complexed with α -SNAP^{wt} before addition as described previously (Otter-Nilsson et al., 1999). Recombinant His₆-tagged Δ ArfGAP1^{His} containing amino acids 1–257 was purified as described (Cukierman et al., 1995). Purified fractions were pooled, and glycerol was added (10% final) before aliquoting, snap freezing, and storing at –80°C. Full-length His₆-tagged ArfGAP1 inserted into baculovirus and infected into Sf9 cells was purified essentially as described (Huber et al., 2001).

Membrane and cytosol preparation

Highly purified rat liver Golgi membranes and rat liver cytosol were prepared and treated as described (Slusarewicz et al., 1994; Lanoix et al., 1999). Typically, the purification was ~100-fold over homogenate. Cytosol was subjected to centrifugation at 41,000 rpm for 60 min at 4°C using a Ti45 rotor. Supernatant was then aliquoted, snap frozen, and stored at

–80°C. For the budding assay, 500 μ l of frozen rat liver cytosol was thawed and diluted with 1 ml of budding buffer (25 mM Hepes, pH 7.0, 115 mM potassium acetate, 2.5 mM MgCl₂). The cytosol was then centrifuged at 60,000 rpm for 60 min at +4°C using a TLA 100.2 rotor. The supernatant was further cleared by centrifugation at 13,000 rpm for 15 min at 4°C. Immunodepletion of cytosolic coatome was performed as described previously (Lanoix et al. 1999).

Vesicle budding assay

The standard assay mixture contained 40 μ g of either salt-washed or non-washed Golgi membranes, 5 mg/ml of rat liver cytosol, an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase), 1 mM DTT, a protease inhibitor cocktail, and 0.5 mM GTP in a final volume of 250 μ l. To inhibit NSF-dependent fusion, 0.5 μ M recombinant His₆-tagged α -SNAP^{dn} mutant was added. 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 rpm for 15 min at 4°C. The medium speed supernatants containing vesicles were loaded on a two-step sucrose cushion made up of 50 μ l of 30% wt/wt sucrose and 5 μ l of 50% wt/wt sucrose and centrifuged at 45,000 rpm for 45 min at 4°C using a TLA 45 rotor. Vesicles were found at the 30%/50% sucrose interface and were resuspended and analyzed for protein content by SDS-PAGE followed by Western blotting and detection using specific antibodies. When vesicle preparations were used for immunoisolation, vesicles were generated from 48 standard assay mixtures. After incubation for 30 min at 37°C and addition of KCl, the mixtures were spun at 13,000 rpm for 15 min at 4°C. The medium speed supernatants were pooled (13 ml), divided in four parts, and overlaid on top of a sucrose step cushion made of 800 μ l of 30% wt/wt sucrose and 150 μ l of 50% wt/wt sucrose. After centrifugation at 55,000 rpm for 60 min at 4°C using a SW60 rotor, all but 350 μ l of each supernatant was discarded. Vesicles were found at the 30%/50% sucrose interface and resuspended, pooled, aliquoted, snap frozen, and stored at –80°C. In cases where AlF_x or Bef_x was used, 10 mM of potassium fluoride and 250 μ M aluminium chloride or 250 μ M beryllium chloride was added to the reactions and to any buffer used at a later stage. Synthetic peptides were dissolved in 4 mM HCl at high concentration. Actual concentration was then determined by measuring OD₂₁₀, OD₂₁₅, and OD₂₂₀ before dilution.

Immunoisolation using magnetic beads

Dynabeads were coupled to goat anti-rabbit polyclonal antibodies according to the manufacturers description. For immunoisolation of Golgi membranes, Eppendorf tubes containing 7.5 μ l Dynabeads precoupled with α -rabbit IgG were incubated with 1 μ g of affinity purified α -p24 γ_3 antibody or with 1 μ g of control antibody for 90 min at 4°C on a rotating wheel in 200 μ l buffer A (budding buffer, 0.5 mg/ml fat-free dry milk, and 0.5 mg/ml PVP-40T). Beads were then washed three times in buffer A to remove unbound antibodies and resuspended in 250 μ l buffer A. Golgi membranes (9.6 μ g) and protease inhibitors were then added to each tube and incubated for 4 h at 4°C. Beads were finally washed three times with 500 μ l of buffer B (budding buffer, 2 mg/ml fat-free dry milk, and 1 mg/ml PVP-40T), and Golgi membranes bound to the beads were analyzed by SDS-PAGE and Western blotting or processed for EM. For immunoisolation of vesicles, beads were prepared as above but 15 μ l of beads/tube was used instead and incubated with 80 μ l of vesicles and protease inhibitors in a final volume of 300 μ l of buffer C (budding buffer, 0.5 mg/ml of nonfat dry milk, 0.5 mg/ml PVP-40T, and 0.5 M KCl) for 4 h at 4°C. Beads were then washed three times with 500 μ l of buffer C and bound material was analyzed by SDS-PAGE and Western blotting or processed for EM as described below.

GAP activity assays

GTP hydrolysis by myristoylated Arf-1 in the presence of phospholipid vesicles (40% phosphatidylcholine, 30% phosphatidylserine, and 30% phosphatidylethanolamine) was assayed as described (Huber et al., 2001) except that KCl was added to give 200 mM. GTP hydrolysis by Golgi membrane-bound ARF1 was assayed as described (Szafer et al., 2001). Briefly, rat liver Golgi membranes were washed with 0.75 M KCl to remove endogenous GAP; this treatment also removed coatome that was present in the membrane preparation. The membranes were incubated at 30°C with 2 μ M bovine myristoylated ARF1 in a GTP-loading mixture consisting of 4 μ M [γ -³²P]GTP (~200,000 cpm), 0.18 M KCl, 0.2 M sucrose, 2.5 mM MgCl₂, 25 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 1 mM ATP plus ATP-regenerating system (5 mM phosphocreatine and 50 μ g/ml creatine phosphokinase) in a final volume of 50 μ l. After 15 min, nucleotide exchange was stopped by the addition of 0.3 mM brefeldin A (from a

30 mM methanolic stock solution) followed by the rapid addition of ARFGAP1 with or without p24 peptides. At different times, aliquots were removed for the determination of membrane-associated [γ -³²P]GTP by the nitrocellulose filtration assay (Weiss et al., 1989).

Peptide binding experiments

Synthetic peptides were coupled to thipropyl sepharose beads as described previously (Dominguez et al., 1998). Briefly, coupled peptides (~3 nmol) were incubated with 1 mg/ml rat liver cytosol in either 230 mM Na/40 mM KCl buffer (30 mM Tris, pH 7.5, 230 mM NaCl, 40 mM KCl, 8.5 mM MgCl₂, 1 mM EDTA, 7% glycerol, 0.1% Triton X-100, and proteinase inhibitors) or KAc buffer (Lanoix et al., 1999) for 1 h at 4°C with rotation. When monitoring binding to full-length recombinant ARF1-GAP, ~3.5 nmol peptides were coupled to beads and incubated with 200 ng purified ARF1-GAP for 1 h at 4°C with rotation in 300 mM Na/90 mM KCl buffer (50 mM Hepes, pH 7.3, 300 mM NaCl, 90 mM KCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitors). In all cases, beads were washed three times with buffer, and bound ARF1-GAP was then analyzed by SDS-PAGE and Western blotting using specific antibodies.

Western blotting and quantitation

After SDS-PAGE, proteins were transferred 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 20 min at room temperature in blocking buffer. HRP-labeled secondary antibodies were revealed by the ECL system.

Indirect immunofluorescence and electron microscopy

Immunofluorescence was performed as described previously (Dominguez et al., 1998). After immunoisolation, magnetic beads with bound membranes or vesicles were washed briefly with 0.1 M cacodylate buffer (pH 7.4) and then fixed for 1 h in the same buffer containing 1% glutaraldehyde. The beads were washed in cacodylate buffer and triple distilled water (tridest) and incubated in 1% OsO₄ in tridest for 30 min. After washing with tridest (three times for 5 min), beads were incubated in 3% uranyl acetate for 30 min, washed with tridest, and dehydrated in ethanol (70–100%). Excess ethanol was removed, and epon was added. This was replaced by fresh epon after 30 min. The epon was polymerized overnight at 60°C. The solidified epon was sectioned into 60-nm slices and contrasted by incubating for 1 min with lead citrate (1%) followed by 10 min with 3% uranyl acetate. Samples were observed on a ZEISS electron microscope.

Thin frozen sections of NRK cells were prepared and labeled with specific antibodies followed by protein A gold as described previously (Röttger et al., 1998; Lanoix et al., 1999). For vesicle labeling, vesicles were processed for negative staining as described (Lanoix et al., 1999) and labeled with specific antibodies followed by protein A gold. Between incubations of both the vesicles and the sections, blocking was performed by fixing the samples with 4% paraformaldehyde, blocking with 0.15% glycine, 0.1% BSA, and 0.1% fish skin gelatin in PBS followed by incubation with nongold-conjugated protein A in a concentration of 10 μ g/ml in the blocking buffer. Control experiments showed that this blocking was sufficient. Quantitation of the vesicle labeling was performed by counting the number of vesicles containing at least two gold particles.

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References

- Antonny, B., D. Madden, S. Hamamoto, L. Orci, and R. Schekman. 2001. Dynamics of the COPII coat with GTP and stable analogues. *Nat. Cell Biol.* 3:531–537.
- Aridor, M., S.I. Bannykh, T. Rowe, and W.E. Balch. 1999. Cargo can modulate COPII vesicle formation from the endoplasmic reticulum. *J. Biol. Chem.*

- 274:4389–4399.
- Barnard, R.J., A. Morgan, and R.D. Burgoyne. 1997. Stimulation of NSF ATPase activity by α -SNAP is required for SNARE complex disassembly and exocytosis. *J. Cell Biol.* 139:875–883.
- Chabre, M. 1990. Aluminofluoride and beryllifluoride complexes: new phosphate analogs in enzymology. *Trends Biochem. Sci.* 15:6–10.
- Cosson, P., and F. Letourneur. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science.* 263:1629–1631.
- Cukierman, E., I. Huber, M. Rotman, and D. Cassel. 1995. The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. *Science.* 270:1999–2002.
- Dominguez, M., K. Dejgaard, J. Füllekrug, S. Dahan, A. Fazel, J.P. Paccaud, D.Y. Thomas, J.J. Bergeron, and T. Nilsson. 1998. gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer. *J. Cell Biol.* 140:751–765.
- Donaldson, J.G., R.A. Kahn, J. Lippincott-Schwartz, and R.D. Klausner. 1991. Binding of ARF and β -COP to Golgi membranes: possible regulation by a trimeric G protein. *Science.* 254:1197–1199.
- Fiedler, K., M. Veit, M.A. Stamnes, and J.E. Rothman. 1996. Bimodal interaction of coatomer with the p24 family of putative cargo receptors. *Science.* 273:1396–1399.
- Finazzi, D., D. Cassel, J.G. Donaldson, and R.D. Klausner. 1994. Aluminium fluoride acts on the reversibility of ARF1-dependent coat protein binding to Golgi membranes. *J. Biol. Chem.* 269:13325–13330.
- Füllekrug, J., T. Sukanuma, B.L. Tang, W. Hong, B. Storrie, and T. Nilsson. 1999. Localization and recycling of gp27 (hp24 γ_3): complex formation with other p24 family members. *Mol. Biol. Cell.* 10:1939–1955.
- Glick, B.S., and V. Malhotra. 1998. The curious status of the Golgi apparatus. *Cell.* 95:883–889.
- Goldberg, J. 1999. Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis. *Cell.* 96:893–902.
- Goldberg, J. 2000. Decoding of sorting signals by coatomer through a GTPase switch in the COPI coat complex. *Cell.* 100:671–679.
- Gommel, D., L. Orci, E.M. Emig, M.J. Hannah, M. Ravazzola, W. Nickel, J.B. Helms, F.T. Wieland, and K. Sohn. 1999. p24 and p23, the major transmembrane proteins of COPI-coated transport vesicles, form hetero-oligomeric complexes and cycle between the organelles of the early secretory pathway. *FEBS Lett.* 447:179–185.
- Griffiths, G., S.D. Fuller, R. Back, M. Hollinshead, S. Pfeiffer, and K. Simons. 1989. The dynamic nature of the Golgi complex. *J. Cell Biol.* 108:277–297.
- Hay, J.C., J. Klumperman, V. Oorschot, M. Steegmaier, C.S. Kuo, and R.H. Scheller. 1998. Localization, dynamics, and protein interactions reveal distinct roles for ER and Golgi SNAREs. *J. Cell Biol.* 141:1489–1502.
- Huber, I., M. Rotman, E. Pick, V. Makler, L. Rothen, E. Cukierman, and D. Cassel. 2001. Expression, purification, and properties of ADP-ribosylation factor (ARF) GTPase activating protein-1. *Methods Enzymol.* 329:307–316.
- Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* 9:3153–3162.
- Lanoix, J., J. Ouwendijk, C.C. Lin, A. Stark, H.D. Love, J. Ostermann, and T. Nilsson. 1999. GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles. *EMBO J.* 18:4935–4948.
- Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S.D. Emr, H. Riezman, and P. Cosson. 1994. Coatomer is essential for retrieval of di-lysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207.
- Love, H.D., C.C. Lin, C.S. Short, and J. Ostermann. 1998. Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport. *J. Cell Biol.* 140:541–551.
- Makler, V., E. Cukierman, M. Rotman, A. Admon, and D. Cassel. 1995. ADP-ribosylation factor-directed GTPase-activating protein. Purification and partial characterization. *J. Biol. Chem.* 270:5232–5237.
- Malsam, J., D. Gommel, F.T. Wieland, and W. Nickel. 1999. A role for ADP ribosylation factor in the control of cargo uptake during COPI-coated vesicle biogenesis. *FEBS Lett.* 462:267–272.
- Marzioch, M., D.C. Henthorn, J.M. Herrmann, R. Wilson, D.Y. Thomas, J.J. Bergeron, R.C. Solari, and A. Rowley. 1999. Erp1p and Erp2p, partners for Emp24p and Erv25p in a yeast p24 complex. *Mol. Biol. Cell.* 10:1923–1938.
- McBride, H.M., V. Rybin, C. Murphy, A. Giner, R. Teasdale, and M. Zerial. 1999. Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell.* 98:377–386.
- Nagahama, M., L. Orci, M. Ravazzola, M. Amherdt, L. Lacomis, P. Tempst, J.E. Rothman, and T.H. Sollner. 1996. A v-SNARE implicated in intra-Golgi transport. *J. Cell Biol.* 133:507–516.
- Nilsson, T., M. Jackson, and P.A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707–718.
- Otter-Nilsson, M., R. Hendriks, E.I. Pecheur-Huet, D. Hoekstra, and T. Nilsson. 1999. Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion. *EMBO J.* 18:2074–2083.
- Pelham, H.R., and J.E. Rothman. 2000. The debate about transport in the Golgi—two sides of the same coin? *Cell.* 102:713–719.
- Quinn, P., G. Griffens, and G. Warren. 1984. Density of newly synthesized plasma membrane proteins in intracellular membranes II. Biochemical studies. *J. Cell Biol.* 98:2142–2147.
- Rabouille, C., N. Hui, F. Hunte, R. Kieckbusch, E.G. Berger, G. Warren, and T. Nilsson. 1995. Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. *J. Cell Sci.* 108:1617–1627.
- Reinhard, C., C. Harter, M. Bremser, B. Brugger, K. Sohn, J.B. Helms, and F. Wieland. 1999. Receptor-induced polymerization of coatomer. *Proc. Natl. Acad. Sci. USA.* 96:1224–1228.
- Rojo, M., R. Pepperkok, G. Emery, R. Kellner, E. Stang, R.G. Parton, and J. Gruenberg. 1997. Involvement of the transmembrane protein p23 in biosynthetic protein transport. *J. Cell Biol.* 139:1119–1135.
- Röttger, S., J. White, H.H. Wandall, J.C. Olivo, A. Stark, E.P. Bennett, C. Whitehouse, E.G. Berger, H. Clausen, and T. Nilsson. 1998. Localization of three human polypeptide GalNAc-transferases in HeLa cells suggests initiation of O-linked glycosylation throughout the Golgi apparatus. *J. Cell Sci.* 111:45–60.
- Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.
- Slusarewicz, P., N. Hui, and G. Warren. 1994. Purification of rat liver Golgi stacks. In *Cell Biology: A Laboratory Handbook*. J.E. Celis, editor. Academic Press, Orlando, FL. 509–516.
- Sohn, K., L. Orci, M. Ravazzola, M. Amherdt, M. Bremser, F. Lottspeich, K. Fiedler, J.B. Helms, and F.T. Wieland. 1996. A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatomer binding. *J. Cell Biol.* 135:1239–1248.
- Sönnichsen, B., R. Watson, H. Clausen, T. Misteli, and G. Warren. 1996. Sorting by COP I-coated vesicles under interphase and mitotic conditions. *J. Cell Biol.* 134:1411–1425.
- Springer, S., A. Spang, and R. Schekman. 1999. A primer on vesicle budding. *Cell.* 97:145–148.
- Springer, S., E. Chen, R. Duden, M. Marzioch, A. Rowley, S. Hamamoto, S. Merchant, and R. Schekman. 2000. The p24 proteins are not essential for vesicular transport in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 97:4034–4039.
- Storrie, B., R. Pepperkok, and T. Nilsson. 2000. Breaking the COPI monopoly on golgi recycling. *Trends Cell Biol.* 10:385–390.
- Subramanian, V.N., F. Peter, R. Philp, S.H. Wong, and W. Hong. 1996. GS28, a 28-kilodalton Golgi SNARE that participates in ER-Golgi transport. *Science.* 272:1161–1163.
- Szafer, E., E. Pick, M. Rotman, S. Zuck, I. Huber, and D. Cassel. 2000. Role of coatomer and phospholipids in GTPase-activating protein-dependent hydrolysis of GTP by ADP-ribosylation factor-1. *J. Biol. Chem.* 275:23615–23619.
- Szafer, E., M. Rotman, and D. Cassel. 2001. Regulation of GTP hydrolysis on ADP-ribosylation factor-1 at the Golgi membrane. *J. Biol. Chem.* In press.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
- Velasco, A., L. Hendricks, K.W. Moremen, D.R. Tulsiani, O. Touster, and M.G. Farquhar. 1993. Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. *J. Cell Biol.* 122:39–51.
- Weiss, M., and T. Nilsson. 2000. Protein sorting in the Golgi apparatus: a consequence of maturation and triggered sorting. *FEBS Lett.* 486:2–9.
- Weiss, O., J. Holden, C. Rulka, and R.A. Kahn. 1989. Nucleotide binding and cofactor activities of purified bovine brain and bacterially expressed ADP-ribosylation factor. *J. Biol. Chem.* 264:21066–21072.
- Yoshihisa, T., C. Barlowe, and R. Schekman. 1993. Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science.* 259:1466–1468.