

COPI vesicles accumulating in the presence of a GTP restricted Arf1 mutant are depleted of anterograde and retrograde cargo

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We dedicate this work to the memory of Thomas Kreis

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SUMMARY

Microinjection of the slowly hydrolyzable GTP analogue GTP γ S or the ectopic expression of a GTP restricted mutant of the small GTPase arf1 (arf1[Q71L]) leads to the rapid accumulation of COPI coated vesicles and buds in living cells. This effect is blocked at 15°C and by microinjection of antibodies against β -COP. Anterograde and retrograde membrane protein transport markers, which have been previously shown to be incorporated into COPI vesicles between the endoplasmic reticulum and Golgi complex, are depleted from the GTP γ S or arf1[Q71L]

induced COPI coated vesicles and buds. In contrast, in control cells 30 to 60% of the COPI carriers co-localize with these markers. These *in vivo* data corroborate recent *in vitro* work, suggesting that GTP γ S and arf1[Q71L] interfere with the sorting of membrane proteins into Golgi derived COPI vesicles, and provide the first *in vivo* evidence for a role of GTP hydrolysis by arf1 in the sorting of cargo into COPI coated vesicles and buds.

Key words: COPI, Membrane traffic, Sorting

INTRODUCTION

Coated vesicles are thought to mediate sorting and transport of material between adjacent membranes in the secretory pathway. At least two vesicular coat complexes, COPII and COPI, are involved in trafficking between the ER and the Golgi (Schekman and Orci, 1996; Rothman and Wieland, 1996; Lowe and Kreis, 1998; Kaiser and Ferro-Novick, 1998; Bannykh et al., 1998). COPII is required for the concentration and export of secretory cargo from the ER (Kuehn et al., 1998; Matsuoka et al., 1998). COPI mediates retrograde transport of recycling proteins returning from the Golgi to the ER (Pelham, 1994; Letourneur et al., 1994; Cosson and Letourneur, 1997). Both coat complexes can interact with the cytoplasmic tails of membrane proteins proposed to function as cargo receptors (Kuehn et al., 1998; Bremser et al., 1999; Cosson and Letourneur, 1997; Sohn et al., 1996; Fiedler and Rothman, 1997; Tisdale et al., 1997; Dominguez et al., 1998) and are thus thought to be important for the sorting of cargo into transport vesicles at the respective donor membranes.

COPI and COPII have also been directly implicated in vesicle formation. The recruitment of COPI and COPII precursors to donor membranes and the assembly of a functional coat complex is regulated by small ras like GTPases; arf1 for COPI and sar1 for COPII. After GTP loading of the respective GTPase by a GTP exchange factor, precursors of the coat complexes are recruited to the donor membrane, followed

by the assembly of a complete COPI and COPII coat which then drives the formation of a transport vesicle (Springer et al., 1999; Rothman, 1996). COPI and COPII, with their respective small GTPases, have been shown to be the minimal cytosolic proteins required for the formation, respectively, of COPI and COPII buds *in vitro* (Orci et al., 1993; Ostermann et al., 1993; Bednarek et al., 1995).

Hydrolysis of GTP by arf1 or sar1 has been demonstrated to be required for the uncoating of the respective vesicles, an essential step for the fusion and delivery of vesicular cargo at the target membrane (Barlowe et al., 1994; Tanigawa et al., 1993). Thus, incubation of donor membranes with cytosol in the presence of the poorly hydrolyzable GTP analogue GTP γ S leads to the accumulation of coated transport vesicles, which cannot fuse with their target membranes. This phenomenon has been extensively exploited in the past to purify and subsequently characterize COPII and COPI coated vesicles at the molecular level. These studies have suggested that COPII functions at the ER level, most likely in the packaging of secretory cargo into transport vesicles destined for the Golgi complex (Barlowe, 1998). In agreement with this, expression of dominant negative mutants of sar1a block ER export but not subsequent transport steps (Kuge et al., 1994; Pepperkok et al., 1998).

In contrast to the data on COPII, the data on the role of COPI in membrane traffic is controversial (Schekman and Mellman, 1997). COPI has been shown to be required for ER to Golgi and intra-Golgi transport of secretory proteins (see

Lowe and Kreis, 1998, for a recent review). However, yeast genetics has suggested that COPI may be primarily or exclusively involved in the retrieval (to the ER) of proteins cycling between the ER and the Golgi complex (Letourneur et al., 1994; Gaynor et al., 1994; Pelham, 1994). Consistent with this, COPI vesicles generated *in vitro*, in the presence of GTP γ S, have been shown to carry predominantly retrograde rather than anterograde cargo (Sonnichsen et al., 1996). In contrast to these data, ultrastructural analyses of unperturbed cells have suggested that COPI buds and vesicles carry anterograde or retrograde cargo (Orci et al., 1997). COPI coated vesicles have also been shown to bud from a variety of cellular membranes including the ER, vesicular tubular transport complexes involved in ER to Golgi transport, the Golgi complex and endosomes (Bednarek et al., 1995; Scales et al., 1997; Malhotra et al., 1989; Whitney et al., 1995; Aniento et al., 1996), suggesting COPI may act at multiple sites within the cell.

We have now studied the regulation of COPI vesicle formation and uptake of putative cargo molecules in living cells. COPI vesicles and buds accumulating in the presence of GTP γ S or arf1[Q71L] do not carry detectable amounts of the anterograde or retrograde membrane transport markers tested. Based on these results we propose a model which suggests that hydrolysis of GTP by arf1 plays an essential role in the sorting of cargo into COPI vesicles and buds.

MATERIALS AND METHODS

Cell culture, microinjection and immunofluorescence labeling

Vero cells (African green monkey kidney cells, ATCC CCL81) were maintained and infected with ts-O45 VSV (Indiana serotype) as described earlier (Kreis, 1986). Microinjection was performed on a computer automated microinjection system (AIS from Zeiss) as described (Pepperkok et al., 1993). Immunofluorescence was performed as described (Pepperkok et al., 1993) with antibodies against calnexin (Hammond and Helenius, 1994), ERGIC53 (Schweizer et al., 1988), γ -adaptin (Robinson and Kreis, 1992), mammalian KDEL-receptor (Tang et al., 1993), β -COP (Pepperkok et al., 1993), β' -COP (Lowe and Kreis, 1995), rab1a (Saraste et al., 1995), and VSV-G (Kreis, 1986).

Anti-p23 antibodies were obtained by immunisation of rabbits with a peptide representing the last 16 C-terminal amino acids of p23 (Rojo et al., 1997). Purification and characterization of antibodies was carried out as described by Pepperkok et al. (1993).

Fluorescence microscopy and image analyses

Images of immunofluorescence-labeled cells were recorded on a Zeiss inverted fluorescence microscope (Axiovert TV135) equipped with a cooled, slow scan CCD camera (Photometrics CH250, 1317 \times 1035 pixels, Tucson, AZ, USA), controlled by a Power Macintosh 8100/100. Images were further processed with the software package IPLab spectrum V3.0 (Signals Analytics Corp., Vienna, VA, USA) before printing on either T_{max}100 film using a slidewriter IS200 (Focus Graphics, Forster City, CA, USA), or on paper using a Xante laser press 1800 (Conware Informatic AG, Baar, Switzerland).

Co-localization of COPI vesicles/buds with different marker proteins was determined as already described (Pepperkok et al., 1998; Griffiths et al., 1995). For each cell analysed, a square area (200 \times 200 image pixels, equivalent to about 15-30% of the total projected cell area) was randomly chosen, digitally zoomed using the IPLab software, and all vesicular structures positive for COPI marked by

circular overlays (with a diameter equivalent to 250 nm in microscope coordinates). The different membrane compartment marker proteins investigated were indicated in the same way. Two structures were scored as co-localizing when their respective overlays overlapped by more than 50%.

The number of COPI vesicles/buds within a cell was determined by counting the number of distinct COPI structures in a randomly chosen square area (200 \times 200 image pixels, equivalent to about 15-30% of the total projected cell area using the overlay features of the IPLab software. The data displayed were normalised to the cell size.

Electron microscopy

Vero cells grown on glass coverslips (as for the immunofluorescence studies) were injected with 500 μ M GTP γ S and BSA coupled to 9 nm colloidal gold. Injected cells were incubated for 30 minutes at 37°C, fixed for 60 minutes in 1% glutaraldehyde in 200 mM cacodylate, pH 7.4, postfixed with 1% OsO₄ for 60 minutes, stained for 60 minutes with 2% uranyl acetate in 50 mM maleate buffer, pH 5.2, dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon. The coverslips were removed from the Epon and the blocks were sectioned parallel to the substrate. Sections were contrasted with uranyl acetate and lead citrate and examined on a Zeiss EM10 electron microscope.

For quantitation of Golgi cisternae, 20 EM images of either control injected (BSA alone) or GTP γ S injected cells were examined for the appearance of Golgi stacks. The number of cisternae apparent in each Golgi stack were then counted and an average number of cisternae per stack was determined for both conditions.

RESULTS

Accumulation of COPI-coated vesicles and buds in cells injected with GTP γ S

To characterize the mechanisms regulating the formation of COPI coated vesicles and the sorting of cargo into these transport carriers directly *in vivo*, we microinjected two slowly hydrolyzable analogues of GTP – GTP γ S and GMPPNP – into cells. Compared to control injected cells both nucleotides induced a redistribution of COPI (Fig. 1B; GMPPNP not shown), without affecting the distributions of COPII, γ -adaptin and the small GTPase rab1a (data not shown; see also Pepperkok et al., 1998). Quantification by immunofluorescence and digital image analysis revealed that the number of distinct COPI positive vesicular profiles increased by a factor of 3.6 or 4 in the presence of GTP γ S or GMPPNP, respectively (Table 1), and appeared evenly distributed throughout the cytoplasm (Fig. 1B). These structures are reminiscent of normal COPI coated buds or vesicles present, albeit less abundantly, in untreated control cells (Fig. 1A). GTP and ATP γ S, on the other hand, had no or little effect on the distribution of coat proteins or the number of COPI vesicles and buds in the injected cells (Table 1). The GTP γ S stimulated accumulation of COPI labeled buds and vesicles throughout the cell could be completely blocked by incubation of cells at 15°C, a condition known to inhibit ER to Golgi transport (Table 1; Fig. 1D). In these experiments COPI labeling was restricted to the Golgi region and ER to Golgi transport complexes (TCs; Scales et al., 1997) where it appeared increased compared to non-treated control cells (Fig. 1). In cells incubated with brefeldin A prior to microinjection of GTP γ S, relatively few distinct COPI labeled structures were detected

Fig. 1. Distribution of β -COP after microinjection of GTP γ S. Cells were injected at either 37°C (A-C,) or 15°C (D,E) with 500 μ M GTP γ S and coumarin-conjugated BSA (B-D), with coumarin-conjugated BSA alone (A) or with 500 μ M GTP γ S and anti-EAGE antibodies (E). Cells were incubated for 30 minutes at 37°C (A,B,C,E) or 15°C (D), fixed and stained for β -COP, or they were incubated for another 60 minutes at 37°C in the presence of BFA prior to fixation and staining (C). Microinjected cells (marked by asterisks) were detected by microinjected coumarin BSA (not shown). Arrowheads point to distinct COPI coated structures and arrows in A and B show COPI staining in the juxtannuclear Golgi region. Bar, 10 μ m.

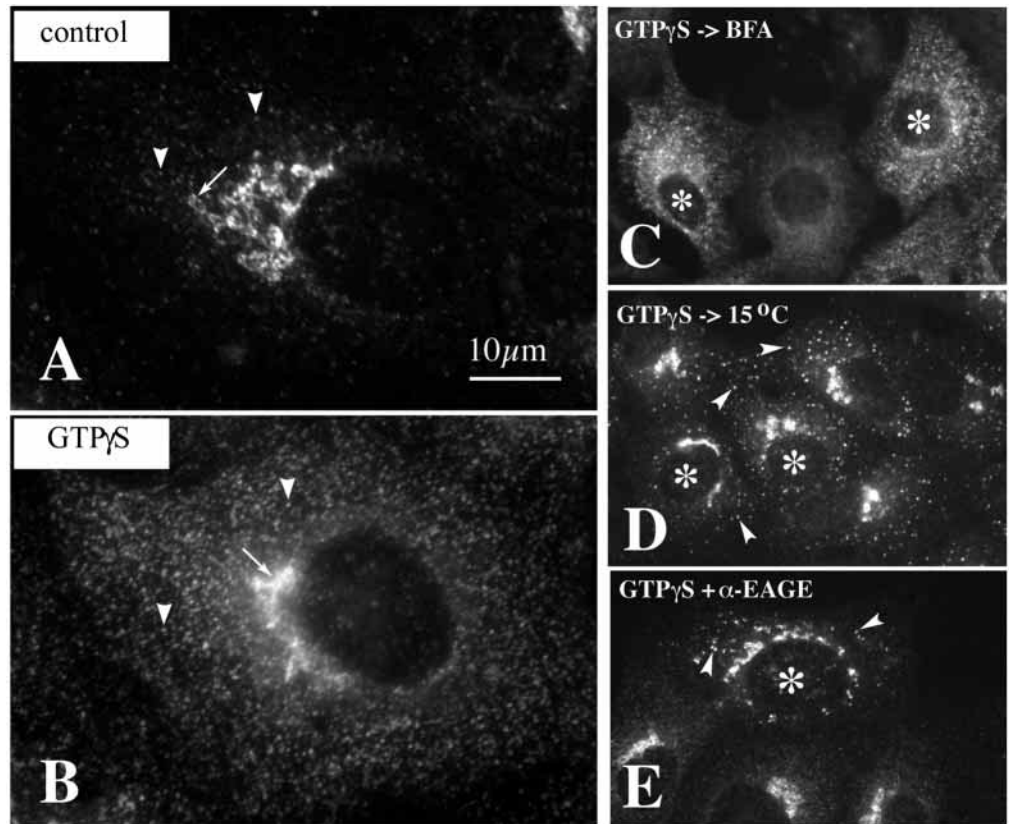


Table 1. Quantification of distinct COPI positive vesicular profiles

Material injected	Incubation temperature	No. of COPI vesicles and buds*
A		
Coumarin BSA	37°C	100±13
	15°C	56±14
500 μ M GTP γ S	37°C	357±68
	15°C	66±14
500 μ M GMPPNP	37°C	401±101
500 μ M ATP γ S	37°C	139±36
500 μ M GTP	37°C	96±21
B		
500 μ M GTP	15→37°C	177±31
500 μ M GTP γ S	15→37°C	368±31
500 μ M GTP γ S+anti-EAGE	15→37°C	46±8
Anti EAGE	15→37°C	51±13
C		
arf1 [Q71L]	37°C	289±12
arf1 [wt]	37°C	93±4
sar1a [H79G]	37°C	31±13
sar1a wt	37°C	107±9

*The number of distinct COPI vesicles and buds was determined as described in Materials and Methods. The number in coumarin BSA injected cells at 37°C was set to 100.

A: Cells were injected and subsequently incubated for 30 minutes at temperatures indicated, fixed and stained for β -COP.

B: Cells were injected at 15°C and then shifted to 37°C for 30 minutes before fixation and staining.

C: Cells were injected with plasmids encoding the respective proteins, incubated for 90 minutes then fixed and stained for COPI.

(not shown), consistent with the hypothesis that brefeldin A prevents binding of COPI to membranes (Helms and Rothman, 1992; Donaldson et al., 1992). In cells microinjected with GTP γ S prior to treatment with brefeldin A, the effect of brefeldin A was fully inhibited and an increased number of COPI labeled buds and vesicles was observed similar to cells treated with GTP γ S alone (Fig. 1C). Microinjection of anti- β -COPI antibodies, which inhibits ER to Golgi transport, reduced the number of distinct COPI vesicular structures in control cells by 50% and completely blocked the accumulation of COPI vesicles and buds in the presence of GTP γ S (Fig. 1E; Table 1).

To corroborate the data obtained by immunofluorescence we also investigated the microinjected cells at the ultrastructural level (Fig. 2). The morphology of the Golgi complex was clearly affected in cells injected with GTP γ S. Control injected cells (Fig. 2A,B) showed typical Golgi stack morphology with an average of 4.2±1.0 apparent cisternae per Golgi profile. In contrast, Golgi membranes in cells injected with GTP γ S (see especially Fig. 2C,E) had fewer cisternae, with an average of 2.7±1.0 cisternae per Golgi profile. Furthermore, in those GTP γ S injected cells where residual Golgi stacks could be identified, the stack structures tended to be shorter and fragmented (Fig. 2E). Also, Golgi membranes often appeared as a 'tangle' of tubules and vesicles (Fig. 2F-H). Most importantly GTP γ S injected cells also showed an increased number of 60-80 nm vesicular profiles distributed throughout the cytoplasm (see Fig. 2D-F). Taken together these data suggest that upon microinjection of GTP γ S, COPI coated vesicles and buds accumulate in vivo.

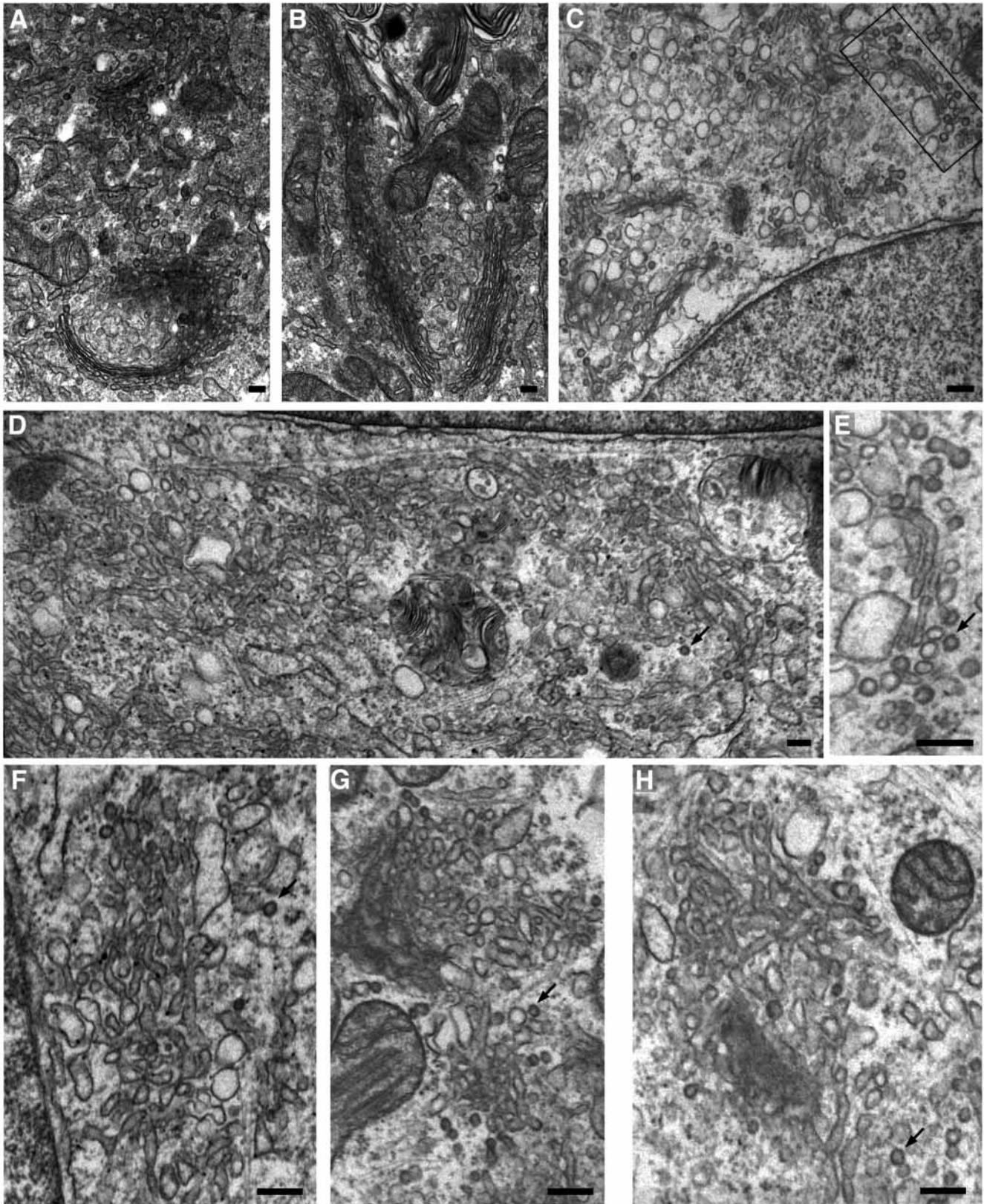


Fig. 2. Electron microscopy analysis of microinjected cells. Vero cells were microinjected with either BSA coupled to 9 nm gold (A,B) or 500 μ M GTP γ S and BSA-9nm gold (C-H), incubated for 30 minutes at 37°C, fixed and processed for electron microscopy. While all injected cells show at least some elements of Golgi structure, those injected with GTP γ S showed smaller, disrupted stacks (especially seen in C and at higher magnification of the boxed region of C shown in E), many small vesicular profiles throughout the cytoplasm (D) and many Golgi remnants appearing as a tangle of tubular profiles (F,G,H). Arrows show potential COPI coated vesicles. Bar, 200 nm.

COPI coated buds and vesicles accumulating in the presence of GTP γ S or GTP restricted arf1[Q71L] are depleted of anterograde and retrograde marker proteins

We next investigated the cargo present in the COPI coated buds and vesicles accumulating in the presence of GTP γ S. Transport of cargo was arrested at the ER to Golgi interface by incubating cells at 15°C prior to injection of either coumarin-conjugated BSA (control) or GTP γ S. At this temperature all the markers used in this study (ts-O45-G, ERGIC53, KDEL-R, p23, and data not shown: CD8) accumulated in the juxta-nuclear Golgi region and in distinct cytoplasmic structures labeled with COPI, regardless of the material injected (Fig. 3). These COPI structures appeared to be larger than the COPI vesicles or buds accumulating in the presence of GTP γ S at 37°C (compare for example Fig. 1B to D) and are coincident with the transport complexes (TCs) moving secretory cargo from the ER to the Golgi complex (previously described by Presley et al., 1997; Scales et al., 1997; Shima et al., 1999). Following a brief (6 minutes) incubation at 37°C (31°C when ts-O45-G was used) 50% to 60% of the distinct COPI positive structures were labeled with antibodies against ts-O45-G, ERGIC53, KDEL-R (Figs 3A and 4; see also Griffiths et al., 1995; Scales et al., 1997). Again, most of these COPI structures appeared to be larger than COPI vesicles and buds accumulating in the presence of GTP γ S and most likely represent ER to Golgi transport complexes. At later time points after the temperature shift less than 10% of the COPI structures contained ts-O45-G, which was transported to the plasma membrane (not shown). The other marker proteins tested had rapidly reached the steady state distribution obtained for cells always kept at 37°C, and 50% to 30% of the COPI structures were positive for ERGIC53 and KDEL-R or p23 (Fig. 3A). No co-localization (<5%) of distinct COPI structures was observed with γ -adaptin, galactosyltransferase or internalized rhodamine-conjugated transferrin, or internalized fluorescein-conjugated dextran (not shown).

The results were significantly different when cells were injected with GTP γ S prior to shifting to 31°C or 37°C. Injection of GTP γ S at 15°C and subsequent incubation at this temperature had no effect on the distribution of COPI and the membrane transport markers tested compared to control cells (Fig. 1D, and data not shown). However, only less than 20% of the COPI vesicles and buds accumulating in the presence of GTP γ S following a 6 minute shift to 31°C or 37°C were labeled with antibodies recognizing the membrane transport markers (Figs 3B and 5). Co-localization of markers with COPI was only observed in COPI structures larger than the COPI vesicles and buds forming in response to GTP γ S. These larger structures most likely represent remaining TCs, in which all the markers tested accumulated during the 15°C transport block. Transport of ts-O45-G to the plasma membrane was arrested in tubular structures, which completely overlapped with the distribution of KDEL-R and ERGIC 53 (Fig. 5, and data not shown; see also Pepperkok et al., 1998). The overall distribution of p23 appeared to be less affected by GTP γ S compared to ts-O45-G, ERGIC53 and KDEL-R (Figs 4 and 5). However, the number of distinct p23 positive structures appeared to be slightly increased in GTP γ S injected cells consistent with a block in transport between the ER and Golgi in the presence of GTP γ S, observed for all markers tested

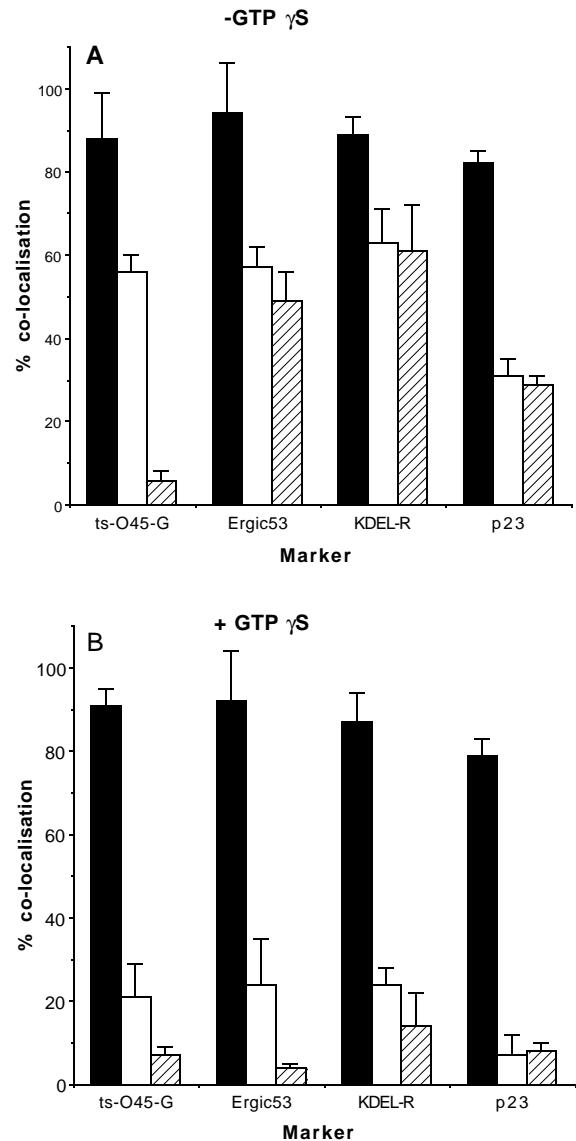


Fig. 3. Quantitation of the co-localization of COPI vesicles and buds with ts-O45-G, KDEL-R, ERGIC 53 or p23 after shift from 15°C to 31°C or 37°C. Infected (ts-O45-G) or uninfected cells were incubated at 15°C for 2 hours. Then they were microinjected with 500 μ M GTP γ S and coumarin-BSA at 15°C, either left at 15°C for an additional 30 minutes (filled bar) or incubated for 6 (open bar) or 30 minutes (hatched bar) at 31°C (for ts-O45-G) or 37°C (other markers). Subsequently they were fixed and immuno-stained. The number of COPI vesicles and buds co-localising with the various markers were determined as described in Materials and Methods. Results are shown for control injections with coumarin-conjugated BSA alone (A) and for injections with GTP γ S and coumarin-conjugated BSA (B).

here (Pepperkok et al., 1998). At later time-points after the temperature shift co-localization of COPI vesicles and buds with the used membrane transport markers was even less (less than 10%) compared to the 6 minute shift (Fig. 3B). Similar results were obtained when cells were injected at 37°C without prior arrest of ER to Golgi transport at 15°C (not shown).

The data described so far show that GTP γ S induces in living cells accumulation of COPI coated vesicles and buds

which are depleted of membrane transport markers, suggesting GTP γ S interferes with the incorporation of cargo into COPI vesicles. Similar results have been obtained with an *in vitro* system reconstituting COPI vesicle budding from Golgi membranes (Nickel et al., 1998). However, the cellular targets of GTP γ S have remained elusive in this study. GTP γ S may affect several different GTP binding proteins which could be involved in sorting of cargo into COPI vesicles. Therefore, we investigated the putative target protein(s) of GTP γ S affecting the regulation of COPI vesicle and bud formation in further detail. A prime candidate was arf1, of which mutants in a GTP restricted form are available (arf1[Q71L]; Zhang et al., 1994; Dascher and Balch, 1994). To test this hypothesis, arf1[Q71L] was transiently expressed in Vero cells by microinjection of a plasmid expression vector. Quantitative immunofluorescence analysis revealed a 3- to 5-fold excess of the mutant over endogenous arf1 two hours after microinjection (not shown). Expression of arf1[Q71L] had virtually identical effects on the distribution of COPI as microinjection of GTP γ S. The number of COPI coated vesicles and buds increased about threefold (Table 1; Fig. 6C) when compared to cells injected with the wild type arf1 (Fig. 6E) and COPI bound to membranes became resistant to brefeldin A (not shown). In contrast, microinjection of purified GTP restricted sar1a[H79G] reduced the number of distinct COPI coated structures about threefold (Table 1; Fig. 6A).

Similar to the microinjection of GTP γ S, expression of arf1[Q71L] had also dramatic effects on the distribution of membrane transport marker proteins. Transport of CD8 to the plasma membrane was completely blocked (not shown). ERGIC53 (Fig. 6D), CD8 (co-expressed with mutant arf1, not shown) and KDEL-R (not shown) accumulated in tubular structures similar to those, which occurred in the presence of GTP γ S (see Fig. 5). Less than 10% of the COPI vesicles and buds, accumulating in the presence of arf1[Q71L], co-localized with CD8, ts-O45-G, KDEL-R or ERGIC 53 (Fig. 6, and data not shown). In contrast, expression of wild-type arf1 (arf1-wt) had no effect on the distribution of COPI or the membrane transport markers (Fig. 6F, and data not shown) or the kinetics of the appearance of CD8 at the cell surface when compared to neighboring non-injected cells (data not shown). Microinjection of purified sar1a[H79G] but not sar1a wild type arrested all membrane transport markers tested in the ER (Fig. 6B, and data not shown).

DISCUSSION

COPI vesicles, synthesized *in vitro* in the presence of GTP γ S, have been extensively used in the past to characterize the vesicular coat complex COPI at the molecular level (see for example Malhotra et al., 1989; Ostermann et al., 1993; Sonnichsen et al., 1996). Using purified donor membranes, cytosol and GTP γ S, COPI vesicle budding at the donor membrane could be reconstituted and has helped to identify the minimal number of cytosolic components required for the formation of COPI vesicles.

In this work we have shown that *in vivo* COPI coated buds and vesicles accumulating in the presence of either GTP γ S or a GTP restricted form of arf1 (arf1[Q71L]) are depleted of

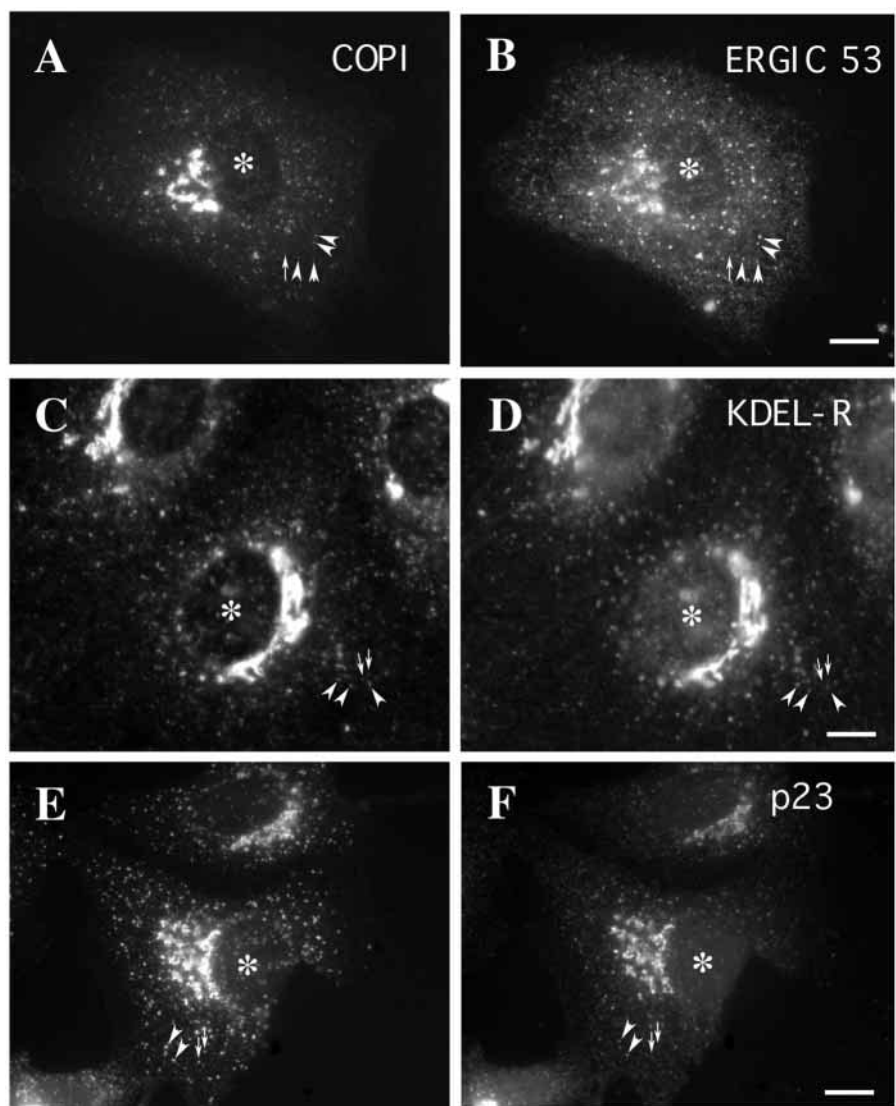


Fig. 4. Co-localisation of COPI with membrane transport markers after shift from 15°C to 37°C. Vero cells were incubated at 15°C for 2 hours. They were then microinjected with coumarin-conjugated BSA at 15°C and shifted to 37°C for 6 minutes before cells were fixed and stained for COPI (A,C,E), Ergic 53 (B), KDEL-R (D) or p23 (F). Injected cells (marked by asterisks) were identified by injected coumarin BSA (not shown). Arrowheads point to COPI coated structures co-localising with the markers. Arrows show COPI structures not co-localising with the markers. Bars: 10 μ m (A and B); 15 μ m (C-F).

anterograde and retrograde marker proteins. An increased number of 60 to 80 nm vesicles or buds throughout the cell cytoplasm were observed in response to GTP γ S when cells were analyzed by electron microscopy. In parallel experiments, using quantitative immunofluorescence, an accumulation of distinct COPI vesicular-like structures, which failed to uncoat in the presence of brefeldin A, was revealed. Almost identical results were obtained by the expression of a GTP restricted arf1 mutant (arf1[Q71L]) but not by the expression of wild-type arf1 or a GTP restricted sar1 mutant. GTP γ S, at the concentrations used here, had little or no effect on the distribution of the small GTPases rab1 or sar1 (data not shown and Pepperkok et al., 1998). Together these data suggest that, similarly to in vitro experiments, GTP γ S induces in vivo the formation of COPI vesicles and buds which can not uncoat. They also suggest that a major GTP γ S target(s) in our experiments has been arf1.

The GTP γ S induced accumulation of COPI vesicles and buds throughout the cell was blocked by microinjection of anti- β -COP antibodies or by incubation of cells at 15°C, two conditions known to inhibit ER to Golgi transport. In these experiments the COPI labeling was restricted to the Golgi region and ER to Golgi transport complexes (TCs; Scales et al., 1997) where it appeared to be increased compared to non-treated control cells (see Fig. 1). This suggests that both conditions do not interfere with the recruitment of COPI to membranes and possibly not with the subsequent formation of COPI buds. However, they appear to inhibit the release of COPI vesicles. Thus these data raise the question whether the 15°C transport block could be explained by the inability to release COPI transport vesicles from donor membranes. COPI has been proposed to function in the retrieval of membrane proteins from post ER membranes of the secretory pathway (Cosson and Letourneur, 1997). Failure to retrieve these factors, because functional COPI vesicles can not form at 15°C, could indirectly prevent transfer of secretory cargo to the Golgi complex. More experiments will be required to address this question more precisely.

Surprisingly, most of the GTP γ S induced COPI vesicles and buds did not co-localize to detectable levels with any of the anterograde or retrograde membrane transport markers tested. These were arrested in tubular structures and the juxtannuclear Golgi region. In contrast, in control cells between 30 to 60% of COPI coated transport intermediates co-localized with the membrane transport markers. These COPI carriers appeared to be larger than the GTP γ S induced COPI vesicles and buds. They most likely represent COPI coated ER to Golgi transport complexes (TCs), which have been described to transport newly synthesized material from the ER to the Golgi complex

(Shima et al., 1999). Our observations are unlikely to be explained by an inaccessibility of the markers to the antibodies used for immuno-staining, because identical results were obtained with antibodies recognizing cytoplasmic or luminal

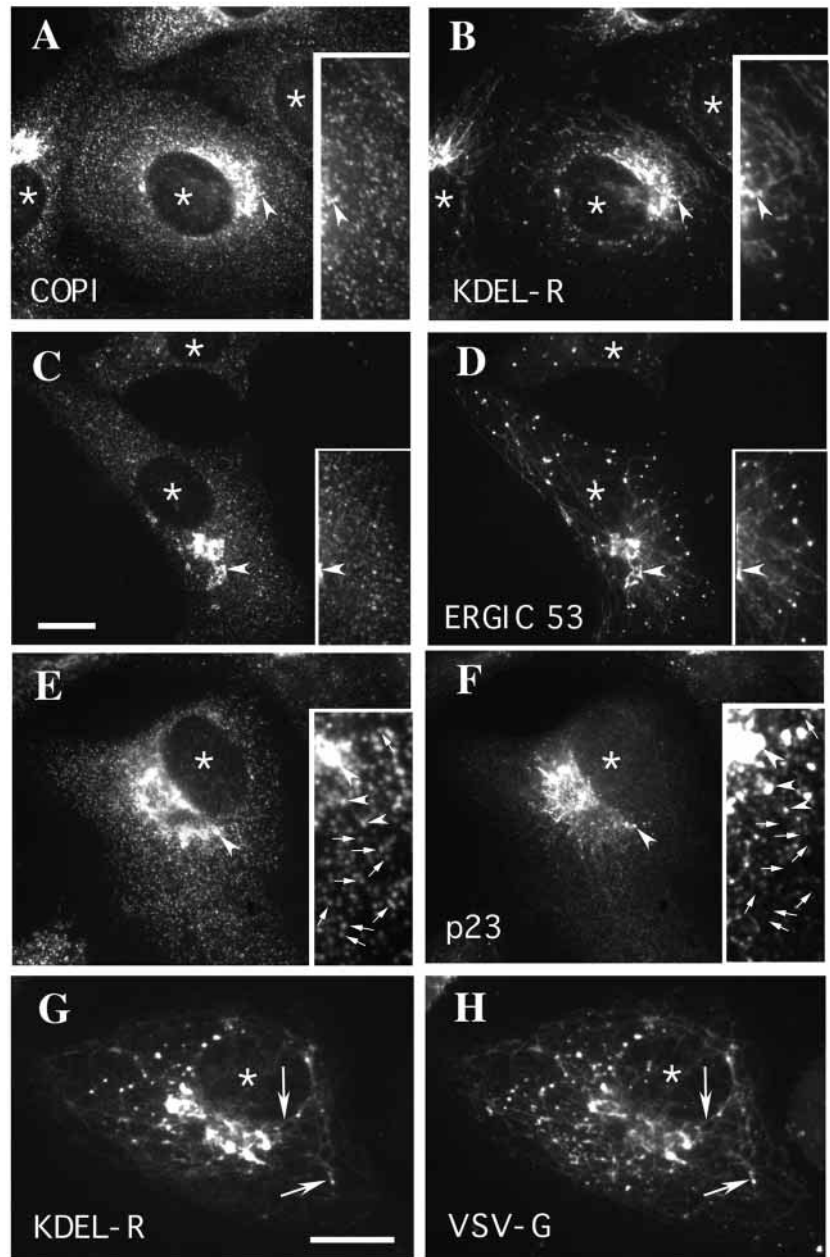


Fig. 5. Co-localisation of COPI vesicles and buds with membrane transport markers after shift from 15°C to 37°C in the presence of GTP γ S. Ts-O45 infected (G,H) or uninfected cells (A-F) were incubated at 15°C for 2 hours. They were then microinjected with 500 μ M GTP γ S and coumarin BSA at 15°C and incubated for 6 minutes at 37°C, fixed and double stained for COPI (A,C,E) and KDEL-R (B), ERGIC53 (D) or p23 (F) or double stained for ts-O45-G and KDEL-R (G,H). Injected cells (marked by asterisks) were identified by co-injected coumarin BSA (not shown). Insets in A-F show 2-fold magnifications of the respective areas. Arrowheads in A to D point to reference points. Arrowheads in E and F point to COPI positive structures co-localising with p23. Arrows in E and F indicate the position of COPI positive structures which do not significantly co-localise with p23. Arrows in G and H point to overlapping structures. Bar, 10 μ m.

epitopes (not shown). Also, the overall fluorescence intensity of the staining for the markers in GTP γ S injected cells was not significantly reduced compared to control cells. A reduction in fluorescence intensity would have been expected if significant amounts of the membrane markers had been incorporated into COPI vesicles and buds, but were inaccessible to the antibodies used. Therefore, our results demonstrate that COPI vesicles and buds can form *in vivo* in the presence of GTP γ S or arf1[Q71L], without having detectable amounts of putative cargo molecules incorporated. Previous electron microscopy analyses of unperturbed cells have shown however, that the markers ts-O45-G, KDEL-R and ERGIC 53 can be incorporated into COPI vesicles (Griffiths et al., 1994, 1995; Orci et al., 1997; Klumperman et al., 1998); and in our control experiments between 30 to 60% of the COPI coated ER to Golgi transport complexes co-stained with either of the markers used. Thus a simple explanation of our results here is that the hydrolysis of arf1 is essential for the sorting of these marker molecules into COPI buds and vesicles. This conclusion is consistent with *in vitro* work, analyzing the content of Golgi derived COPI vesicles synthesized in the presence of GTP in comparison to those synthesized in the presence of GTP γ S, suggesting that GTP γ S inhibits the incorporation of secretory cargo into COPI vesicles (Nickel et al., 1998). The target(s) of GTP γ S have remained elusive in these earlier studies. Our data here show now *in vivo* that expression of arf1[Q71L] has similar, if not identical, effects as GTP γ S. This is also in agreement with most recent *in vitro* data showing that GTP hydrolysis by arf1 mediates sorting of membrane proteins into COPI vesicles (Lanoix et al., 1999; Malsam et al., 1999). The markers used in this study have been shown to be also present in COPI vesicles generated *in vitro* in the presence of GTP γ S (Ostermann et al., 1993; Sohn et al., 1996; Sonnichsen et al., 1996; Nickel et al., 1998). This appears to be in contrast to our observations here. However, in these studies the uptake of markers into COPI vesicles has been quantified by biochemical methods averaging over the entire population of COPI vesicles obtained. It is therefore possible that a sub-population of the COPI vesicles generated *in vitro* in the presence of GTP γ S contain the markers in significant amounts, thus increasing the average signal detected. This would be consistent with our findings here,

where we still detect, in the presence of GTP γ S, a minor sub-population of COPI carriers (less than 10%) efficiently co-localizing with the markers. In the majority of COPI vesicles and buds the markers remained however below the detection threshold in agreement with our model suggesting that hydrolysis of arf1 is essential for cargo uptake into COPI vesicles.

What could the precise role of arf1 in the sorting of cargo into COPI vesicles and buds be? COPI interacts with the cytoplasmic tails of membrane proteins ending in a KKXX or related motifs (Cosson and Letourneur, 1997; Sohn et al., 1996;

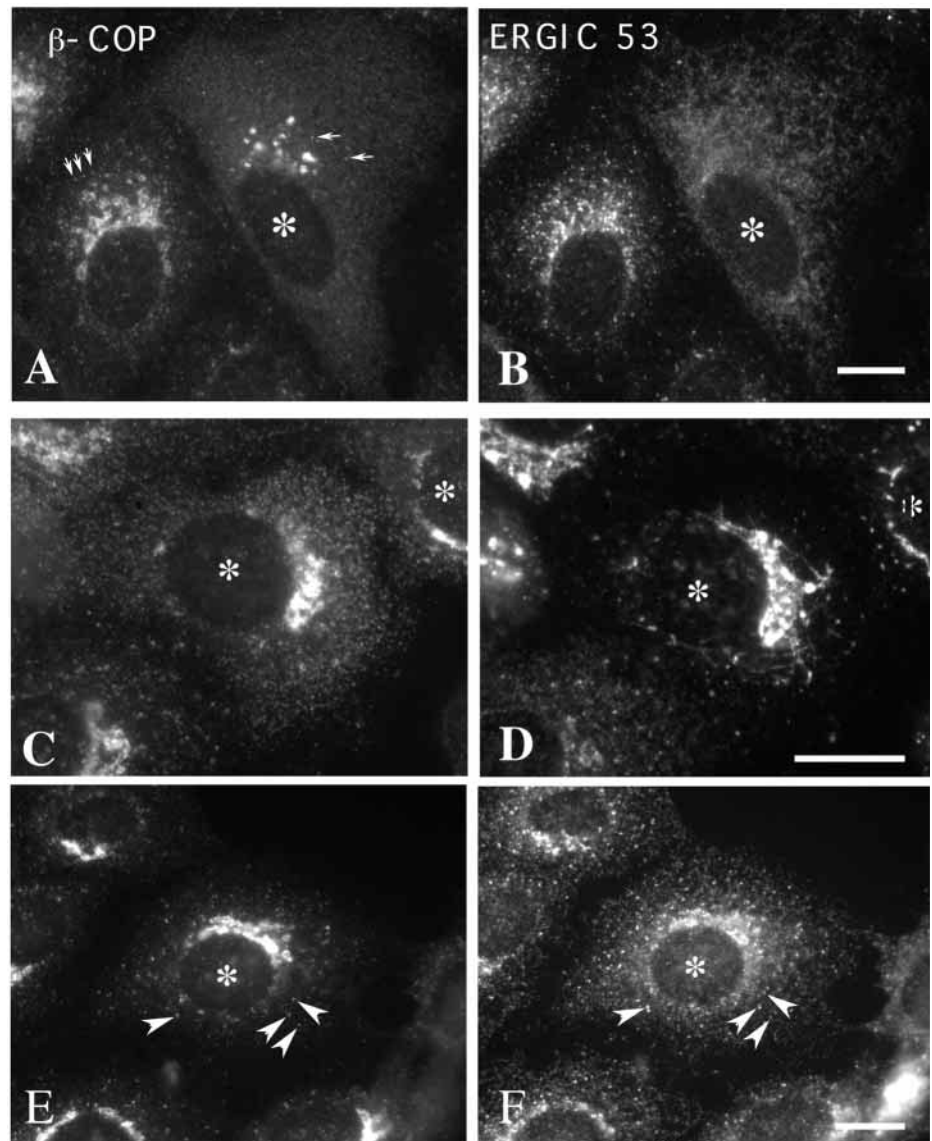


Fig. 6. Redistribution of COPI and ERGIC53 in the presence of arf1[Q71] or sar1a[H79G]. Vero cells were microinjected with coumarin-conjugated BSA and plasmids encoding sar1a[H79G] (A,B), arf1[Q71L] (C,D) or arf1 wildtype (E,F). After 90 minutes of incubation at 37°C cells were fixed and double stained for COPI (A,C,E) and ERGIC 53 (B,D,F). Microinjected cells (marked by asterisks) were identified by the co-injected coumarin BSA (not shown). The number of COPI vesicles/buds was increased or decreased relative to wildtype injected cells in the presence of arf1[Q71L] or sar1a[H79G], respectively. Arrows in A point to COPI positive structures and arrowheads in E and F indicate the position of COPI positive structures co-localising with ERGIC 53. Bars, 20 μ m.

Fiedler and Rothman, 1997; Dominguez et al., 1998; Tisdale et al., 1997).

The cytoplasmic tails of these proteins have been shown to be required for the budding of COPI vesicles from lipid bilayers (Bremser et al., 1999). A model has been proposed suggesting that a bivalent interaction of COPI with membrane bound arf1 and the cytoplasmic tails of cargo or putative cargo receptors couples the uptake of cargo to COPI vesicle formation (Bremser et al., 1999). When integrated with our *in vivo* data, it seems likely that arf1 directly regulates this interaction of COPI with cargo or cargo receptors. In a first step arf1 would be activated by an arf1 specific GTP exchange factor. This then would result in the recruitment of arf1-GTP and coatomer, the precursor of COPI, to membranes. GTP hydrolysis by arf1 would then facilitate the interaction of COPI with the cytoplasmic tails of cargo receptors, possibly by inducing a conformational change in COPI or the receptors themselves. Finally, this should allow the incorporation of cargo into a COPI coated bud and the release of a functional transport vesicle. In this model the inhibition of GTP hydrolysis by arf1 should prevent the incorporation of cargo molecules into COPI vesicles as it has been described here.

Alternative mechanisms for COPI vesicle formation have also been proposed. They suggest that only arf1 and coatomer but not membrane proteins are necessary for COPI vesicle formation (Spang et al., 1998; Springer et al., 1999; Roth, 1999). Also, arf1 has been shown to be an effective activator of phospholipase D (PLD; Brown et al., 1993; Cockcroft et al., 1994) and COPI binding to membranes has been demonstrated to be facilitated by acidic phospholipids (Ktistakis et al., 1996; Spang et al., 1998), which are a downstream product of activated PLD. Therefore, it is also possible that in our experiments the requirement for membrane proteins (cargo or cargo receptors) in the budding of COPI vesicles may have been bypassed by injection of GTP γ S or expression of arf1[Q71L]. In both cases the production of acidic phospholipids by PLD, which is activated by arf1[Q71L], might have facilitated COPI vesicle formation without the incorporation of cargo.

Although more work *in vivo* and *in vitro* will be necessary to test and distinguish these models, the identification of arf1 as a key molecule involved in the sorting of cargo into COPI vesicles should help to unravel this essential step in vesicular membrane transport.

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