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A role for ADP ribosylation factor in the control of cargo uptake during COPI-coated vesicle biogenesis

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Abstract ARF-mediated hydrolysis of GTP has been demonstrated to regulate coat disassembly of Golgi-derived COPI transport vesicles (Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Helms, J.B. and Rothman, J.E. (1993) J. Cell Biol. 123, 1365–1371). In addition, a requirement for GTP hydrolysis at an early stage of COPI vesicle biogenesis has been established since cargo uptake is impaired in the presence of GTPγS (Nickel, W., Malsam, J., Gorgas, K., Ravazzola, M., Jenne, N., Helms, J.B. and Wieland, F.T. (1998) J. Cell Sci. 111, 3081–3090), a non-hydrolyzable analogue of GTP. We now demonstrate that the GTPase involved in the regulation of cargo uptake is ARF, revealing a multi-functional role of this GTPase in COPI-mediated vesicular transport. The molecular mechanism of cargo uptake as well as the functional implications of these findings on the overall process of COPI vesicle biogenesis are discussed.

Key words: COPI-coated vesicle; Cargo; Golgi; Coatomer; ADP ribosylation factor; Vesicular transport

1. Introduction

COPI-coated vesicles have been implicated in both anterograde and retrograde transport through the Golgi complex [3–8]. The basic machinery needed for the formation of these carriers has been characterized at the molecular level, thereby defining both the requirements for cytosolic and membrane-bound factors residing in the Golgi donor membrane (for a recent review see [9]). While the GTPase ARF [10–12] and the coat precursor coatomer [13,14] are the only cytosolic factors required for budding [15], the p24 family of transmembrane proteins has been implicated in coat recruitment to the donor membrane [16–18]. Coat assembly (i.e. subunit polymerization) is thought to be triggered by a bivalent interaction of coatomer with membrane-bound ARF and the cytoplasmic tails of p24 proteins [9,19,20]. This process may directly cause the formation of a coated bud and, eventually, the release of a fully coated transport vesicle. Based on this scheme, the core components of the COPI budding machinery were functionally reconstituted employing liposomes of a defined lipid composition containing the cytoplasmic domains of p24 proteins in form of lipopeptides. Addition to these liposomes of the purified coat components coatomer and ARF gave rise to liposome-derived coated vesicles, strictly dependent on the presence of GTP and an elevated temperature. Thus, these components constitute the basic machinery needed for the formation of COPI vesicles [21].

Less is known about the process of cargo uptake into COPI-coated vesicles. Golgi-derived COPI vesicles consist of at least two distinct populations carrying two types of cargo molecules: anterograde cargo destined for delivery to the cell surface and retrograde cargo destined for recycling back to the endoplasmic reticulum (ER) [8]. Transport of both soluble and membrane-bound retrograde cargo from the Golgi back to the ER is signal-mediated [22–25] and involves cargo receptors like the KDEL receptor [26,27]. Anterograde cargo is concentrated into COPII transport vesicles during export from the ER [28,29], or during transport from the ER to the Golgi complex [30]. In the latter case, this seems to be achieved by selective membrane removal from so-called vesicular-tubular clusters that are thought to mediate the final step of anterograde ER to Golgi transport [31–33]. However, the cisternal concentration of cargo does not further increase during transit through the Golgi and delivery to the *trans*-Golgi network (TGN) [28]. Because there is no reported evidence that anterograde cargo enters COPI vesicles in a signal-dependent manner, it has been proposed that anterograde cargo may enter departing COPI vesicles via a bulk process [34,35].

While the bulk flow model may still be valid for soluble cargo molecules [34–36], recent data suggest that the uptake of membrane-bound cargo by COPI transport vesicles is a regulated rather than a passive mechanism. This is because uptake by Golgi-derived COPI vesicles of this kind of cargo (both anterograde- and retrograde-directed transmembrane molecules) is impaired when coated vesicle formation is reconstituted in the presence of GTPγS [2]. These data suggest that membrane cargo must interact in one way or another with components of the budding machinery in order to be delivered to, and concentrated in, active budding zones. Apparently, this process is selectively inhibited by GTPγS.

We now report the identification of a GTPγS target protein involved in the regulation of cargo uptake. We found that ARF-catalyzed GTP hydrolysis is required at an early stage of COPI vesicle biogenesis in order to ensure the coupling of the budding process with the uptake of membrane cargo. This was demonstrated by the use of a mutant form of ARF, ARF-Q71L, that hydrolyzes GTP at a very slow rate [1]. When COPI vesicles were generated in the presence of cytosol (as a source for coatomer), GTP and ARF-Q71L, cargo uptake was markedly reduced. These data were confirmed employing a modified COPI budding assay based on purified coat proteins.

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2. Materials and methods

2.1. Antibodies

Monoclonal antibodies directed against the c-myc epitope of CD8-LT were purified from the hybridoma cell line 9E10. Polyclonal antibodies directed against hARF1 (#2048; [37]) were generously provided by Dr. Bernd Helms (BZH, Heidelberg). Polyclonal antibodies directed against δ-COP (#877) are described in [38,39]. Monoclonal antibodies directed against β-COP (M3A5) were purchased from Sigma (Deisenhofen, Germany). Secondary antibodies used for analysis of Western blots came from Dianova (Hamburg, Germany). All other reagents and chemicals were of analytical grade.

2.2. Purification of coatomer and N-myristoylated ARF

Rabbit liver coatomer was purified as described previously [39]. Recombinant N-myristoylated human ARF1 and the mutant form ARF-Q71L were purified to near homogeneity according to [40].

2.3. Preparation of cytosol and CHO Golgi membranes

Bovine brain cytosol was prepared according to [41] with the exception that a tangential filtration unit (Minitan, Millipore) was used to concentrate the cytosol preparation (as opposed to ammonium sulfate precipitation in the original protocol). The system was used according to the detailed instructions given in the manufacturer's manual. The flow rate along the membranes was adjusted to 700 ml/min resulting in a back pressure of 0.5 bar. Under these conditions, buffer was removed from the diluted protein solution at 10 ml/min. This procedure resulted in a cytosol preparation of about 40 mg/ml (i.e. about 10-fold concentrated compared to the starting material).

Golgi membranes were isolated from the CHO cell line CHO_{CD8-LT} [2] according to [41].

2.4. In vitro generation of COPI-coated vesicles from CHO Golgi membranes

COPI-coated vesicles were generated and purified to near homogeneity exactly as described by [41] with the only exception that Golgi membranes used as donor membranes were isolated from the cell line CHO_{CD8-LT} [2] rather than from CHO wild-type cells. When COPI-coated vesicles were generated employing purified coat proteins, a typical COPI budding assay was performed in 25 mM HEPES-KOH (pH 7.0), 2.5 mM magnesium acetate and 40 mM KCl in the presence of an ATP-regenerating system [41]. Golgi membranes isolated from CHO_{CD8-LT} were either pre-washed with 250 mM KCl or left untreated. Membranes were collected on a 35% sucrose cushion employing ultracentrifugation. A complete incubation consisted of 1 mg CHO_{CD8-LT} Golgi membranes, 90 μg of purified rabbit liver coatomer, 3 μg of N-myristoylated recombinant ARF-WT or ARFQ71L and either 20 μM GTPγS or 1 mM GTP in a total volume of 250 μl. After incubation for 60 min at 37°C samples were processed for purification of COPI-coated vesicles according to [41].

3. Results

In a previous study we reported that GTP hydrolysis is required for proper uptake of membrane-bound cargo by COPI-coated vesicles [2]. This finding directly established that a GTPase is involved in this process. Since the GTPase ARF functions in the recruitment of coatomer to Golgi membranes [42,43], and represents a stoichiometric component of the coat of COPI transport vesicles [12], we tested whether this protein has an additional role in cargo uptake during an early stage of budding.

3.1. An ARF GTP hydrolysis mutant, ARF-Q71L, inhibits cargo uptake into COPI-coated vesicles

Standard COPI budding assays [41] were performed using as donor membranes purified CHO Golgi membranes (containing the anterograde cargo marker CD8-LT; [2]), cytosol as a source for coat proteins (i.e. ARF and coatomer), and GTPγS (or GTP) to activate ARF. As a final purification step, samples were applied to a continuous sucrose gradient in which COPI-coated vesicles typically migrate at a position corresponding to 40–42% (w/w) sucrose (fractions 7 and 8 in Fig. 1 as detected by the coatomer subunit δ-COP; see also [41]). When incubations with GTP were directly compared with those containing GTPγS (Fig. 1A,B, [2]), GTP clearly promoted the uptake of CD8-LT, whereas COPI vesicles formed in the presence of GTPγS were found to be depleted of the marker. When the same assay was performed in the presence of cytosol, GTP and purified ARF-Q71L [1], we observed inhibition of cargo uptake (Fig. 1C, fractions 7 and 8) similar to incubations in the presence of cytosol and GTPγS (Fig. 1B, fractions 7 and 8). These data unambiguously demonstrate that ARF is involved in the regulation of cargo uptake and that this process requires ARF-mediated GTP hydrolysis.

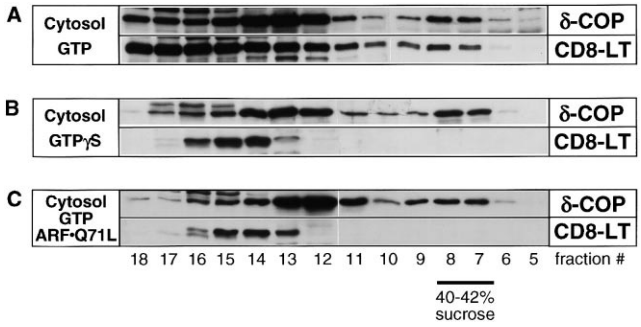

Fig. 1. In vitro generation of COPI-coated vesicles in the presence of cytosol. COPI-coated vesicles were generated as described under Section 2. Incubation of membranes with cytosol was performed either in the presence of 20 μM GTPγS, 1 mM GTP, or 1 mM GTP plus recombinant mARF-Q71L (final concentration: 12 μg/ml). Salt-released COPI vesicles were loaded onto an isopycnic sucrose gradient from 20 to 50% and membranes were sedimented for 18 h at $100\,000 \times g$. 250 μl fractions were collected from the bottom to the top. The membranes were collected by ultracentrifugation and analyzed by SDS-PAGE and Western blotting using the antibodies indicated. Typically, COPI vesicles band in fractions 7 and 8 corresponding to 40–42% (w/w) sucrose. A: Vesicles generated in the presence of GTP. B: Vesicles generated in the presence of GTPγS. C: Vesicles generated in the presence of GTP and ARF-Q71L.

3.2. Analysis of cargo uptake in a COPI budding assay employing purified coat proteins

In order to verify the experiments employing cytosol as a source for coatomer in a minimalized system, we made use of a modified budding assay that uses purified coat proteins. Furthermore, two kinds of Golgi donor membranes were used: (1) non-treated membranes and (2) salt-washed membranes (to remove Golgi-bound ARF-GAP [44]), which allowed us to compare conditions characterized by a normal (untreated membranes) or low (salt-washed membranes) membrane-bound ARF-GAP activity. Thus, cargo uptake can be correlated with GTP hydrolysis because in the absence of ARF-GAP, ARF does not hydrolyze GTP to a significant extent [45–47]. As a consequence, salt treatment promotes the accumulation (and, therefore, improves the overall yield) of COPI-coated vesicles generated in the presence of GTP [44].

The results with non-treated membranes are shown in Fig. 2. While the yield of COPI-coated vesicles is relatively low from incubations performed in the presence of the wild-type

form of ARF (ARF-WT) (as indicated by the presence of β-COP and ARF; Fig. 2A, fraction 7 and 8), higher amounts of COPI-coated vesicles are obtained from incubations containing either GTP/ARF-Q71L (Fig. 2B) or containing GTPγS/ARF-WT (Fig. 2C). Since COPI vesicles are purified based on their density, only coated vesicles are present in fractions 7 and 8 and, therefore, the amount of coat proteins detected in these fractions reflects their content of COPI vesicles. We have previously shown that these fractions do not contain significant membrane contaminants [2]. Thus, the ratio in fractions 7 and 8 of the amount of CD8-LT to the amount of coat protein directly indicates the enrichment of cargo relative to coat proteins in the purified vesicles. This ratio was highest in COPI vesicles generated with GTP, ARF-WT and coatomer (Fig. 2A), and significantly higher compared to COPI vesicles generated in the presence of either GTP/ARF-Q71L (Fig. 2B) or GTPγS/ARF-WT (Fig. 2C). The absolute yield of COPI vesicles isolated from incubations with GTP/ARF-WT is about 5- to 10-fold lower compared to the yield in the presence of either GTPγS/ARF-WT or GTP/ARF-Q71L. In summary: cargo is enriched in COPI vesicles generated under conditions that allow GTP hydrolysis, confirming the results obtained with COPI vesicles generated in the presence of cytosol and GTP (Fig. 1 and [2]).

Interestingly, cargo uptake was impaired under all three conditions when Golgi donor membranes were high-salt treated prior to incubation with purified coat proteins and guanine nucleotides (Fig. 3). Moreover, the yield of GTP/ARF-WT vesicles was now significantly increased indicating that high-salt treatment removes residual ARF-GAP activity from Golgi membranes, known to promote uncoating. However, this also reduces ARF-mediated GTP hydrolysis during early stages of the budding process and, hence, this condition is similar to incubations either with GTPγS or the slowly hydrolyzing ARF mutant, ARF-Q71L, as ARF alone has no intrinsic GTPase activity [45]. Therefore, GTP/ARF-WT COPI vesicles formed from high-salt treated Golgi membranes are expected to contain only small amounts of cargo which is indeed the case (Fig. 3A, fractions 7 and 8).

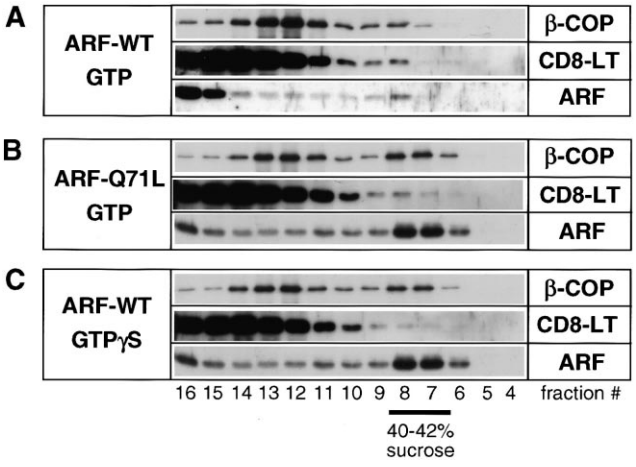

Fig. 2. In vitro generation of COPI-coated vesicles employing purified coat proteins. COPI-coated vesicles were generated and purified as described in Section 2. Western blotting analysis was performed using the antibodies indicated. A: Vesicles generated in the presence of 1 mM GTP and ARF-WT. B: Vesicles generated in the presence of 1 mM GTP and ARF-Q71L. C: Vesicles generated in the presence of 20 μM GTPγS and ARF-WT.

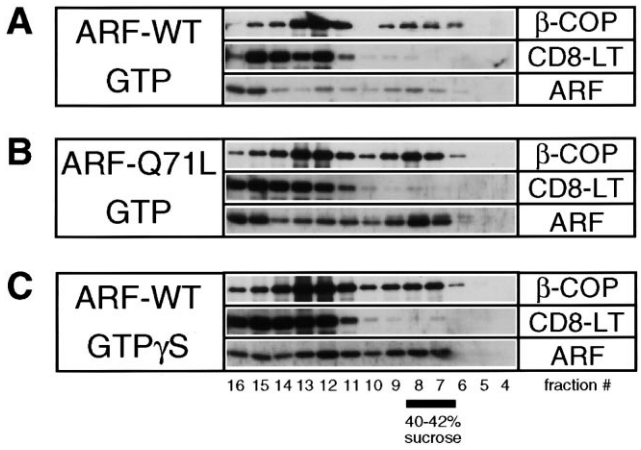

Fig. 3. In vitro generation of COPI-coated vesicles employing purified coat proteins and salt-washed Golgi membranes. Golgi membranes were pre-treated with 250 mM KCl and 2.5 mM magnesium acetate to remove membrane-bound cytosolic components. COPI-coated vesicles were generated and purified as described in Section 2. Western blotting analysis was performed using the antibodies indicated. A: Vesicles generated in the presence of 1 mM GTP and ARF-WT. B: Vesicles generated in the presence of 1 mM GTP and ARF-Q71L. C: Vesicles generated in the presence of 20 μM GTPγS and ARF-WT.

4. Discussion

In this paper we demonstrate that ARF1, a small GTPase known to be involved in the recruitment of coatomer to Golgi membranes [42,43] and in coat disassembly of COPI transport vesicles [1], has an additional function in the control of cargo uptake. This is shown employing a mutant form of ARF, ARF-Q71L, that hydrolyzes GTP at a very slow rate [1]. We made use of Golgi membranes isolated from a CHO cell line expressing the anterograde reporter molecule CD8-LT [2] and performed classical COPI vesicle budding assays [41] in order to analyze cargo uptake under various conditions.

Using both cytosol and purified ARF and coatomer as a source for coat proteins, we show that COPI-coated vesicles generated in the presence of GTPγS or GTP/ARF-Q71L are depleted of the anterograde cargo marker. In contrast, COPI vesicles generated in the presence of GTP/cytosol or GTP/ARF-WT/coatomer contain significant amounts of the anterograde cargo molecule indicating that they have been formed in a physiologically relevant manner. These results provide strong evidence for a multi-functional role of ARF in COPI-mediated vesicular transport because ARF serves: (1) to recruit coatomer [42,43], (2) to ensure proper uptake of cargo molecules (this study) and (3) to initiate coat disassembly prior to membrane fusion with the target organelle [1]. All of these functions are directly related to the ARF GTP-GDP cycle.

The fact that membrane-bound cargo uptake is impaired in the presence of GTPγS or GTP/ARF-Q71L indicates that this kind of cargo does not enter COPI-coated vesicles via a bulk flow mechanism. Bulk flow is defined as cargo inclusion into departing transport vesicles at a concentration prevailing in the corresponding donor membrane. Therefore, if cargo was taken up by a bulk mechanism, this uptake should not be selectively inhibited by GTPγS. However, when GTP hydrolysis is prevented by use of GTPγS, cargo uptake is markedly

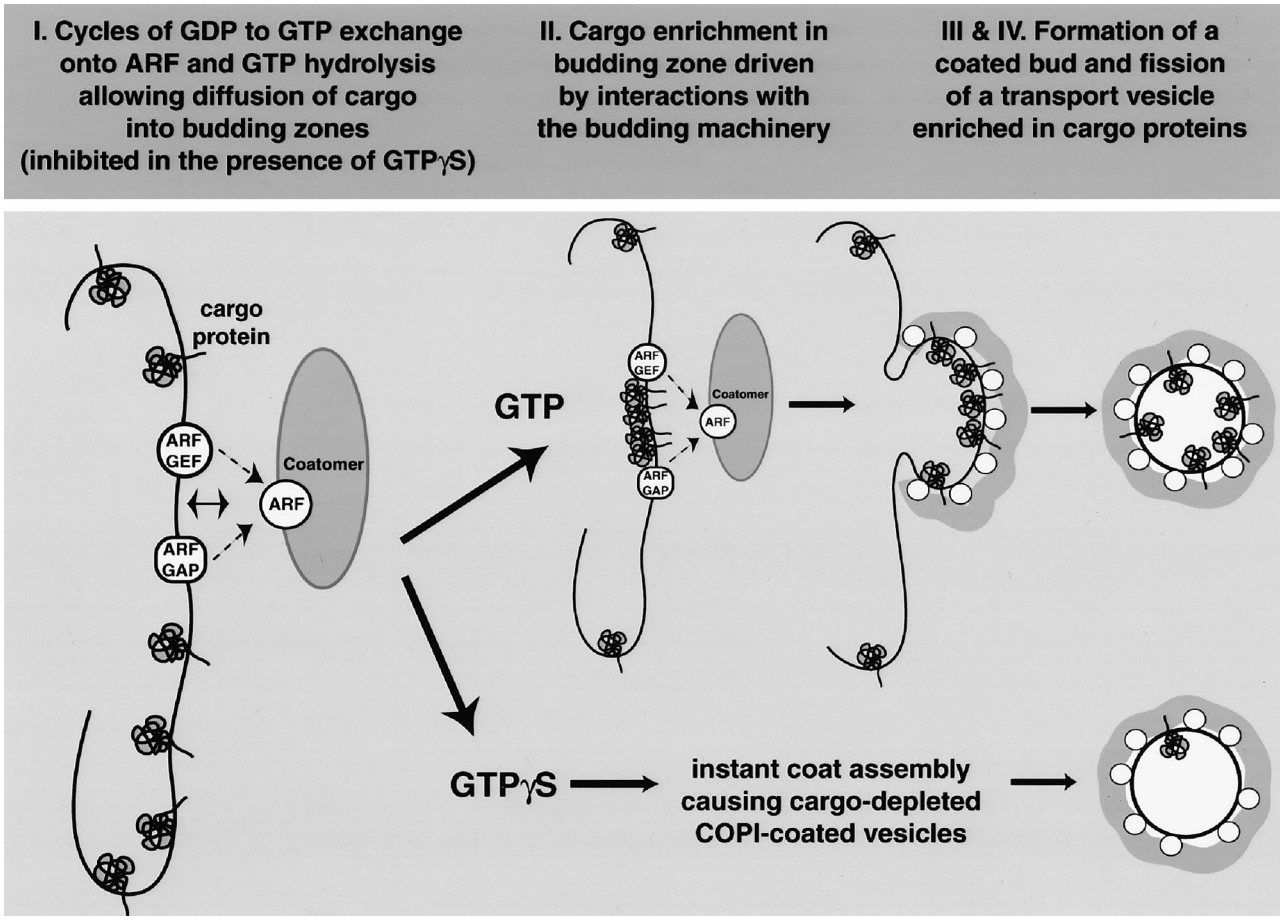

Fig. 4. Mechanism of cargo uptake by COPI-coated transport vesicles: a model. For details see text.

reduced and, therefore, we conclude that under physiological conditions (i.e. conditions permitting GTP hydrolysis) a mechanism exists that causes enrichment of cargo in active budding zones. Thus, the small amounts of cargo present in COPI vesicles generated in the presence of GTPγS (this study, [2]) may correspond to uptake via a bulk flow mechanism. The higher levels of cargo found in COPI vesicles generated under conditions that allow GTP hydrolysis indicate that cargo is enriched in these vesicles as compared to their donor membranes.

What might be the molecular mechanism underlying cargo uptake into COPI-coated vesicles, and how does inhibition of ARF-mediated GTP hydrolysis interfere with this process? A simple model based on the considerations mentioned above is illustrated in Fig. 4. If cargo molecules get enriched in the vesicular membrane during COPI vesicle biogenesis, then there is a need for them to diffuse into an active budding zone, where they must be trapped by interactions with components of the budding machinery (e.g. p24 proteins, coat proteins or other factors). What could be the role of ARF-mediated GTP hydrolysis in such a scenario? A straight-forward view would be that GDP/GTP exchange cycles and GTP hydrolysis open a time window that allows cargo to diffuse into budding zones. In this context it is of note that coatomer has been reported to potently stimulate ARF-GAP activity [48]. During this early phase of COPI vesicle biogenesis, a pre-budding assembly complex consisting of coat proteins and membrane factors will therefore trap cargo molecules. In this model, the window would close upon activation of p23/p24 proteins (possibly by an ARF-GTP-dependent mechanism, because the cytoplasmic tails of these proteins can only bind to coatomer following recruitment to the membrane of ARF-GTP) resulting in the induction of coat polymerization, the irreversible step in vesicle budding [20]. Thus, a coated bud forms and membrane fission results in the release of a COPI-coated transport vesicle enriched in cargo as compared to the parental membrane. In contrast, under conditions that do not permit GTP hydrolysis, coat assembly proceeds resulting in bud formation and coated vesicle release which, in turn, prevents cargo enrichment in the vesicle. This model is supported by the fact that only cargo molecules appear to be affected by GTPγS, whereas machinery molecules such as p23, p24 or the KDEL receptor are not [2]. In other words, under conditions that do not permit GTP hydrolysis, the assembly of the COPI machinery is uncoupled from the mechanism of cargo uptake, resulting in COPI-coated vesicles depleted of membrane cargo.

In contradiction to our results ([2], this study), Lanoix et al. recently reported that uptake by COPI vesicles of anterograde membrane cargo is not affected by reagents blocking GTP hydrolysis [49]. However, Lanoix et al. investigated uncoated vesicles (claimed to be derived from COPI-coated vesicles) which were characterized by a broad density distribution (corresponding to a density of 35 to 45% (w/w) sucrose) when analyzed on an isopycnic sucrose gradient. More specifically, due to the purification procedure employed, it appears likely

that the vesicle preparation of Lanoix et al. contains so-called Golgi remnants which can be released from Golgi membranes by salt treatment and typically migrate in a density gradient at a position corresponding to ~32–36% (w/w) sucrose [4,13]. This interpretation of their results would also explain the finding that this vesicle preparation seems to be enriched in resident Golgi proteins [49], a feature not expected for Golgi-derived transport vesicles. Since the preparation of uncoated vesicles of a defined origin is inherently associated with technical difficulties, we decided to analyze coated COPI vesicles (generated in the presence of GTP or GTPγS), which can be recovered as an essentially homogeneous vesicle preparation. Coated COPI vesicles sharply peak at a position corresponding to 40–42% (w/w) sucrose (i.e. well separated from Golgi remnants) and, therefore, can be assigned as bona fide COPI vesicles. Recently, uptake by COPI vesicles of various proteins has been studied using an entirely independent method that is based on microinjection of GTPγS or ARF-Q71L protein into living cells [50]. Regarding the findings presented in this study the authors reach essentially the same conclusions.

At first sight, the predicted enrichment of membrane-bound cargo in Golgi-derived transport vesicles does not seem to be consistent with the morphological observation that the classical membrane cargo marker VSV-G protein [51] has been reported not to be concentrated in Golgi-derived COPI buds and vesicles during transit through the Golgi [3]. However, cells infected with vesicular stomatitis virus express VSV-G protein at very high levels probably saturating the Golgi donor membranes with this kind of cargo. This experimental condition is likely to cause difficulties to distinguish between uptake by a bulk mechanism and uptake mediated by concentration into buds and vesicles. Likewise, uptake by COPI vesicles of soluble anterograde cargo has been analyzed by quantitative electron microscopy of whole cell sections [8]. The results implied that cargo is not concentrated during budding but, similar to experiments with VSV-G protein, a secretory protein was analyzed that is expressed at very high levels. In contrast, we used an anterograde membrane cargo marker expressed at moderate levels which appears to enter COPI vesicles through interactions with the budding machinery. Moreover, this phenomenon has proven to be a general one because an endogenous membrane marker for anterograde transport, the transferrin receptor, was demonstrated to behave similarly [2]. We, therefore, hypothesize that endogenous membrane-bound cargo molecules which are expressed at moderate levels become enriched in COPI transport vesicles in vivo and, upon delivery at the target membrane, diluted again resulting in approximately the same concentration as in the original donor compartment. This view is consistent with the finding that the concentration of cargo in the various cisternae of the Golgi stack is fairly constant [8,28]. Since cargo molecules pass through the Golgi en route to the cell surface, there is no obvious need to increase their concentration along the Golgi stack. On the other hand, a transient enrichment in transport vesicles may provide the basis for efficient transport.

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