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ARF-GAP–mediated interaction between the ER-Golgi v-SNAREs and the COPI coat

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In eukaryotic cells, secretion is achieved by vesicular transport. Fusion of such vesicles with the correct target compartment relies on SNARE proteins on both vesicle (v-SNARE) and the target membranes (t-SNARE). At present it is not clear how v-SNAREs are incorporated into transport vesicles. Here, we show that binding of ADP-ribosylation factor (ARF)–GTPase-activating protein (GAP) to ER-Golgi v-SNAREs is an essential step for recruitment of Arf1p and coatomer, proteins that together form the COPI coat. ARF-GAP acts catalytically to recruit COPI components. Inclusion of v-SNAREs into COPI vesicles could be mediated by direct interaction with the coat. The mechanisms by which v-SNAREs interact with COPI and COPII coat proteins seem to be different and may play a key role in determining specificity in vesicle budding.

Introduction

Proteins travelling from the Golgi to the ER are included in coatomer protein (COP)*I-coated vesicles. The small GTPase ADP-ribosylation factor (Arf)1p and coatomer are necessary and sufficient to drive COPI vesicle formation from enriched Golgi membranes in vitro and from chemically defined liposomes reflecting the lipid composition of the Golgi apparatus (Spang et al., 1998; Spang and Schekman, 1998; Lanoix et al., 1999). In vitro experiments demonstrated that COPI-, COPII-, and clathrin-coated vesicles can form from liposomes in the absence of cargo proteins and transport factors (Matsuoka et al., 1998b; Spang et al., 1998; Takei et al., 1998). However, in vivo cargo and transport factors need to be included in COPI vesicles for efficient transport, which is important for the survival of the cell. The most prominent class of transport factors are SNARE proteins, which are essential in the process of consumption of vesicles (for review see Rothman, 1994; Hay and Scheller, 1997; Nichols and Pelham, 1998; Pelham, 1999). The fusion of membranes requires the formation of SNARE complexes that span the two membranes (Nichols et al., 1997; Ungermann et al., 1998). Thus, subfamily members need to be present on the vesicle (v-SNARE) and on the target membrane (t-SNARE). Although motifs on transmembrane domain containing cargo proteins have been defined, it remains unclear how SNAREs and membrane proteins lacking an obvious motif are recruited into sites where vesicles emerge. Nevertheless, SNAREs cycle very efficiently between different compartments (Ballensiefen et al., 1998; Ossipov et al., 1999). One example for cargo proteins without an apparent retrieval motif are yeast mannosyltransferases that cycle between ER and Golgi (Todorow et al., 2000).

The exit from the ER is well studied, and at least for membrane proteins it became clear that there is an active mechanism for sorting; secretory membrane proteins and SNAREs are sorted into COPII vesicles and enriched there to concentrations greater than in the ER (Mizuno and Singer, 1993; Balch et al., 1994; Rexach et al., 1994; Bednarek et al., 1995; Martinez-Menarguez et al., 1999). Springer and Schekman (1998) have shown that components of the COPII coat bind specifically to the v-SNAREs Bet1p and Bos1p, whereas no interaction was observed with the other SNAREs involved in ER-Golgi transport. The binding seems to be mediated through a structural motif rather than primary sequence requirements. This was the first indication that coat proteins may be required for the uptake of v-SNAREs into vesicles. Further evidence was provided by Matsuoka et al. (1998a) who showed that SNAREs could be taken up into COPII vesicles that formed from chemically defined liposomes. Although these studies

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*Abbreviations used in this paper: ARF, ADP-ribosylation factor; COP, coatomer protein; GAP, GTPase-activating protein; GST, glutathione *S*-transferase.

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showed that the coat might play an active role in the recruitment of SNAREs, the results are restricted to the COPII coat. Since every vesicle coat identified to date has its own characteristics, this role may or may not be a common theme. The COPII coat consists of the small GTPase Sar1p, the Sec23/24p complex, and the Sec13/31p complex. Sec23p is the GTPase-activating protein for Sar1p, whereas Sec24p might play a role in cargo uptake (Pagano et al., 1999; Roberg et al., 1999). Sec13/31p is thought to have a more structural role in "shaping" the COPII coat. The small GTPase Arf1p and the heptameric protein complex coatomer are referred to as the COPI coat. Coatomer subunits interact with Arf1p, ARF–GTPase-activating protein (GAP), and cargo proteins (Letourneur et al., 1994; Harter et al., 1996; Cosson et al., 1998; Eugster et al., 2000). Clathrin-coated vesicles use a set of adaptor proteins that interact specifically with clathrin (for review see Scales et al., 2000).

We investigated interactions between v-SNAREs and COPI components that may lead to the sorting into COPI-coated vesicles. An initial idea about how this process might be regulated came from studies by Aoe et al. (1997) on the cycling of the KDEL receptor, which carries no obvious retrieval motif. They showed that the KDEL receptor interacts with ARF-GAP. However, this interaction was restricted to a receptor that was loaded with a KDEL motif–containing protein, thus marking the receptor for uptake into COPI-coated vesicles. Recently, Lanoix et al. (2001) showed that ARF-GAP mediates sorting of p24s in different subpopulations of vesicles. Although SNAREs would not need to be marked by another protein, the interaction with an ARF-GAP might facilitate the sorting. Here we report the interaction of COPI coat components and ER-Golgi v-SNAREs and provide evidence concerning how this interaction may be regulated in a catalytic manner by the ARF-GAPs, Glo3p and Gcs1p (Poon et al., 1996, 1999).

Results

ARF-GAP recruits Arf1ΔN17p to microsomal membranes

We aimed to elucidate the effect of the ARF-GAP Glo3p on the budding of COPI-coated vesicles from the Golgi apparatus. Therefore, we used an in vitro system using microsomal membranes, Arf1ΔN17p, and ARF-GAP. Arf1ΔN17p and mutants of Arf1ΔN17p were expressed in *Escherichia coli* as His_6-tagged proteins where the first NH_2-terminal 17 amino acids were replaced by the His_6 affinity tag. This facilitated the purification greatly and reduced the unspecific background caused by the high hydrophobicity and lipid modification of the NH_2 terminus (Paris et al., 1997). These ARF proteins have been used in all experiments described below. We incubated microsomal membranes with Arf1ΔN17p, nucleotides, and ARF-GAP. After the incubation at 4 or 25°C, the soluble proteins were separated from the membranous fraction. The membranes were solubilized and analyzed by immunoblot (Fig. 1 A). Unexpectedly, the recruitment of Arf1ΔN17p was solely dependent on the presence of ARF-GAP. Neither temperature nor the nucleotides seemed to alter the binding behavior. To investigate whether Glo3p could act on Arf1ΔN17p before recruitment of

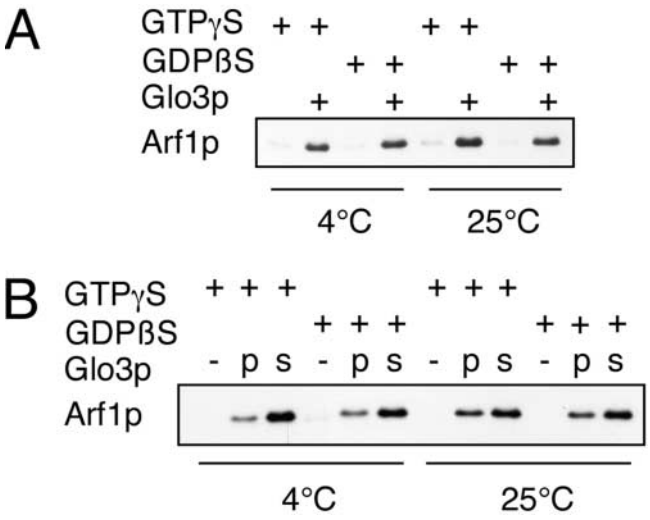

Figure 1. **Arf1ΔN17p recruitment to membranes is dependent on ARF-GAP.** (A) Microsomes from wild-type cells were incubated with Arf1ΔN17p, nucleotides, and ARF-GAP (Glo3p) as indicated for 30 min. The Arf1ΔN17p that was recruited to the microsomes was separated from the unbound fraction by centrifugation and visualized by immunoblot. (B) The experiment was performed as described in A. Glo3p was only present only during a preincubation step (p) or throughout the recruitment reaction (simultaneous; s).

Arf1ΔN17p to the membranes, we repeated the experiments and compared the levels of bound Arf1ΔN17p to membranes that were only preincubated with ARF-GAP (Fig. 1 B, p compared with s). Although clearly less Arf1ΔN17p was bound to the microsomes when ARF-GAP was present only during the preincubation step, significant amounts were immobilized. In this scenario, a slight temperature dependence was detectable. Some Glo3p was recruited to the microsomes. However, the amount did not change significantly when ARF-GAP was solely present during the preincubation or simultaneously with Arf1ΔN17p (unpublished data). We repeated these experiments with Gcs1p, a second ARF-GAP, which has overlapping functions with Glo3p in retrograde transport from the Golgi to the ER (Poon et al., 1999). Gcs1p was also able to facilitate the binding of Arf1ΔN17p to microsomes (unpublished data). These results suggested that ARF-GAP interacts with proteins or lipids in the microsomal fraction and not with Arf1p before the recruitment of Arf1ΔN17p to the membrane.

ARF-GAPs recruit COPI components to v-SNAREs

If the interpretation of the results presented above is correct, we hypothesized that the interacting proteins on the microsomes might be SNAREs. This could then provide a platform on which the vesicle coat could assemble. This mechanism would ensure the enclosure of SNAREs in the forming bud. To test this possibility, we expressed the cytosolic domains of ER-Golgi v-SNAREs fused to glutathione *S*-transferase (GST) in *E. coli* (Springer and Schekman, 1998). At least two of these fusion proteins were efficiently included in COPII-coated vesicles that budded from liposomes, indicating that they reflect the behavior of their in vivo counterparts (Matsuoka et al., 1998a). We chose to concentrate on the v-SNAREs that cycle between the ER and the Golgi be-

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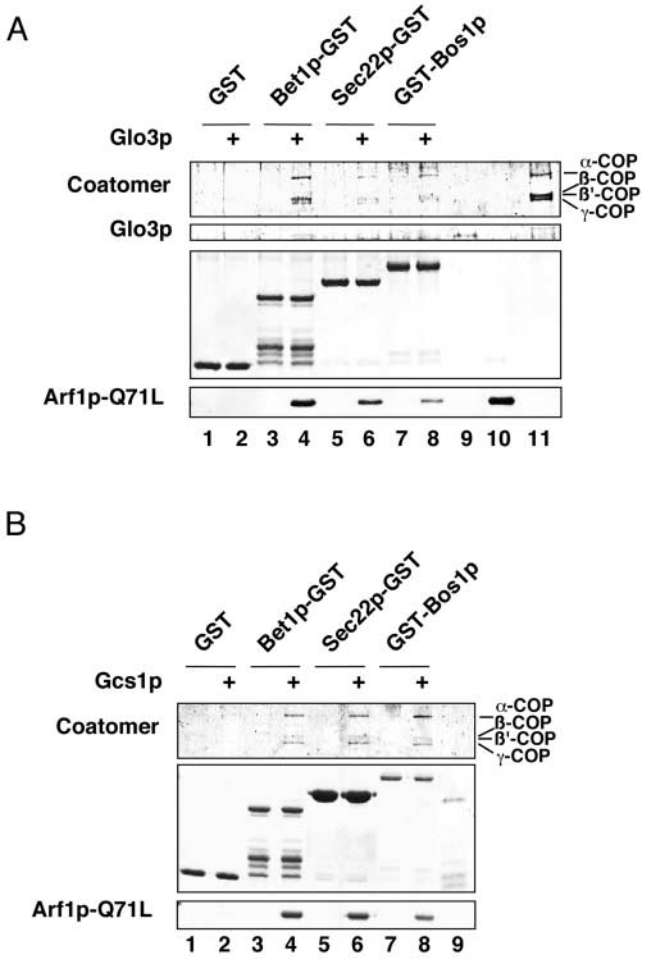

Figure 2. **ARF-GAPs mediate Arf1ΔN17p-Q71L and coatomer binding to the cytosolic domains of v-SNAREs.** SDS-PAGE analysis of in vitro binding of coatomer (top) and Arf1ΔN17p-Q71L (bottom) to GST (lanes 1 and 2) or GST fusions to v-SNAREs (lanes 3 and 4, Bet1p-GST; lanes 5 and 6, Sec22p-GST; lanes 7 and 8, GST-Bos1p). GST or GST fusion proteins were immobilized onto glutathione-agarose. Where indicated, 20 nM Glo3p (A) or Gcs1p (B) were added to the reaction, and 50% of the amount added is shown in lane 9. The guanine nucleotide on Arf1ΔN17p-Q71L was exchanged to GTP before the binding reaction, and 1.2 μM preexchanged Arf1ΔN17p-Q71L was added to the binding reaction. All reactions contained GTP. The coatomer concentration was 40 nM in the assay. After the binding reaction, the unbound proteins were removed by centrifugation. The proteins immobilized on the glutathione-agarose were separated by SDS-PAGE followed by SyproRed staining and analysis using the red fluorescent mode of a Storm PhosphorImager. In A, lanes 10 and 11 represent 20% of Arf1ΔN17p-Q71L and 50% of coatomer present in the reaction, respectively. In Bet1p-GST and Sec22p-GST, GST is the COOH-terminal fusion partner, whereas in GST-Bos1p it is in the NH_2-terminal position.

cause they are enriched in COPI and COPII vesicles. Furthermore, this would allow us to compare our results to what is known about the uptake into COPII vesicles. SNARE–GST proteins, or GST as a control, were incubated with Glo3p, dominant-active Arf1ΔN17p (Arf1ΔN17p-Q71L), and coatomer (Fig. 2 A). We used the hydrolysis-deficient Arf1p mutant in order to circumvent a possible effect due to guanine nucleotide hydrolysis (Kahn et al., 1995). The proteins did neither bind to a GST-Tlg1p and GST-Sso1p fusion proteins nor to GST alone (unpublished data; Fig. 2 A). Tlg1p and Sso1p are t-SNAREs in the late Golgi/endosomal system (Holthuis et al., 1998). Arf1ΔN17p-Q71L and coatomer bound to Bet1p-GST, Sec22p-GST, and Bos1p-GST only in the presence of Glo3p. The localization of the GST within the SNARE molecule (NH_2- or COOH-terminal) did not alter the behavior of the fusion proteins in the assay. Bet1p recruited always more COPI components than Sec22p and Bos1p. Denatured Glo3p abolished the interaction between Arf1ΔN17p, coatomer, and SNAREs (unpublished data). The replacement of Glo3p by Gcs1p did not alter the binding qualitatively, but differences in the amount of recruited coatomer and Arf1ΔN17p-Q71L were detectable (Fig. 2 B). However, both ARF-GAPs seemed functionally interchangeable in this assay. The recruitment of Arf1ΔN17p-Q71L and coatomer was strongly dependent on the presence of ARF-GAP. Arf1ΔN17p-Q71L bound almost stoichiometrically to the SNAREs. The amount of recruited coatomer seemed low compared with Arf1ΔN17p-Q71L, although the whole heptameric coatomer complex was recruited. For simplicity, we only show the larger four subunits. In contrast, Glo3p and Gcs1p were nearly undetectable on a SyproRed-stained gel (Fig. 2, A, lanes 4, 6, and 8 compared with lane 9, and B, lanes 4, 6 and 8 compared with lane 9). However, their presence could be confirmed by immunoblot (unpublished data).

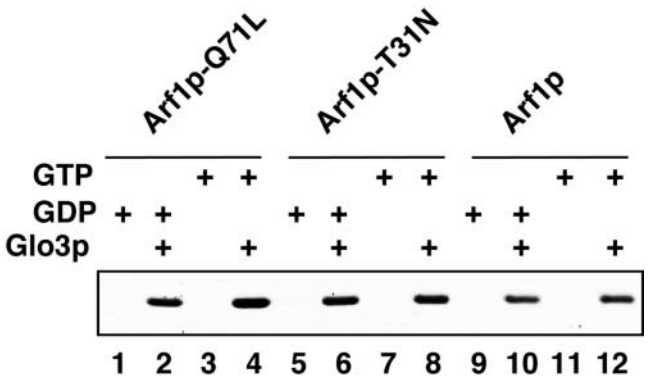

Figure 3. **Recruitment of Arf1ΔN17p to Bet1p-GST does not require GTP hydrolysis.** Arf1ΔN17p-Q71L, Arf1ΔN17p-T31N, and Arf1ΔN17p were preincubated with either GTP or GDP before the binding to Bet1p-GST. The same guanine nucleotides and Glo3p were present in the binding reaction as indicated.

Recruitment of Arf1ΔN17p does not depend on its activation

For the first set of the pull down assays, we used a dominant-active form of Arf1NΔ17p, which we preexchanged with GTP in order to ensure that the recombinant protein was in its GTP-bound form. We considered the possibility that the v-SNAREs may act as an ARF receptor, recruiting Arf1ΔN17p to the membrane where the exchange of GDP to GTP and thus the activation of the ARFs take place. If so, one would expect that the dominant negative mutant of Arf1ΔN17p (Arf1ΔN17p-T31N [Kahn et al., 1995]), which is in its GDP-bound form, should be recruited to Bet1p-GST. Indeed, equal amounts of Arf1ΔN17p could be found

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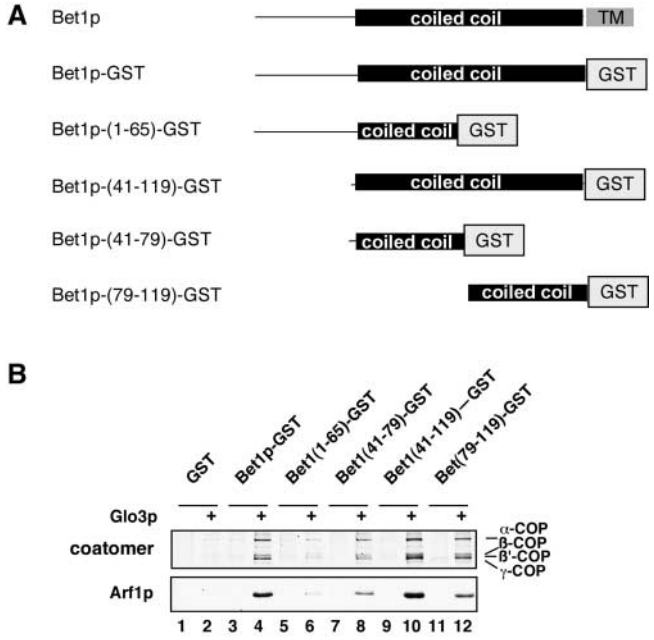

Figure 4. **Binding site of COPI on Bet1p-GST.** (A) Schematic depiction of Bet1p and GST fusions of Bet1p. (B) The interaction site for Arf1ΔN17p and coatomer to Bet1p is comprised within the COOH-terminal 79 amino acids of the cytosolic domain. Although significant amounts were bound to the COOH-terminal 41 amino acids, sequences toward the NH_2-terminal part may be required and vice versa. Shown are incubations either in the presence of coatomer or Arf1ΔN17p with GST fusion proteins of Bet1p. Glo3p was added where indicated. Arf1ΔN17p was preincubated with GTP, and GTP was also present during the binding reaction.

in association with Bet1p, independent of the nucleotide bound to Arf1ΔN17p (Fig. 3, lanes 2 and 4 compared with 6 and 8). Since this interaction was still dependent on the presence of an ARF-GAP, we concluded that the GAP activity was not required for the binding of Arf1ΔN17p. In addition, no difference in the recruitment levels was detected when wild-type Arf1ΔN17p was used instead of the dominant mutants (Fig. 3, lanes 10 and 12). The lack of nucleotide dependence was not due to the high protein concentration in the in vitro assay. 20-fold diluted assays did not result in a preference for Arf1ΔN17p-GTP versus Arf1ΔN17p-GDP or vice versa (unpublished data). Furthermore, this binding reaction was performed at 4°C, a temperature at which the enzymatic activity should be reduced. Finally, we tried to elute Arf1ΔN17p-GTP with an excess of GDP and Arf1ΔN17p-GDP with an excess of GTP-γ-S from the SNAREs immobilized to beads. However, the GTPase would not come off, indicating that a nucleotide exchange did not remove it from the SNAREs. Arf1ΔN17p-GDP might interact with v-SNAREs on the membrane and then recruit an ARF-GEF to the same place. Similar results were obtained when ARF-GAP–dependent binding of the different Arf1ΔN17p proteins to microsomal membranes was assayed (unpublished data). However, these results were obtained with Arf1ΔN17p, which lacks the domain that greatly changes its conformation during nucleotide exchange. Thus, it is also possible that in vivo Arf1p needs to be activated and membrane bound before interaction with SNAREs.

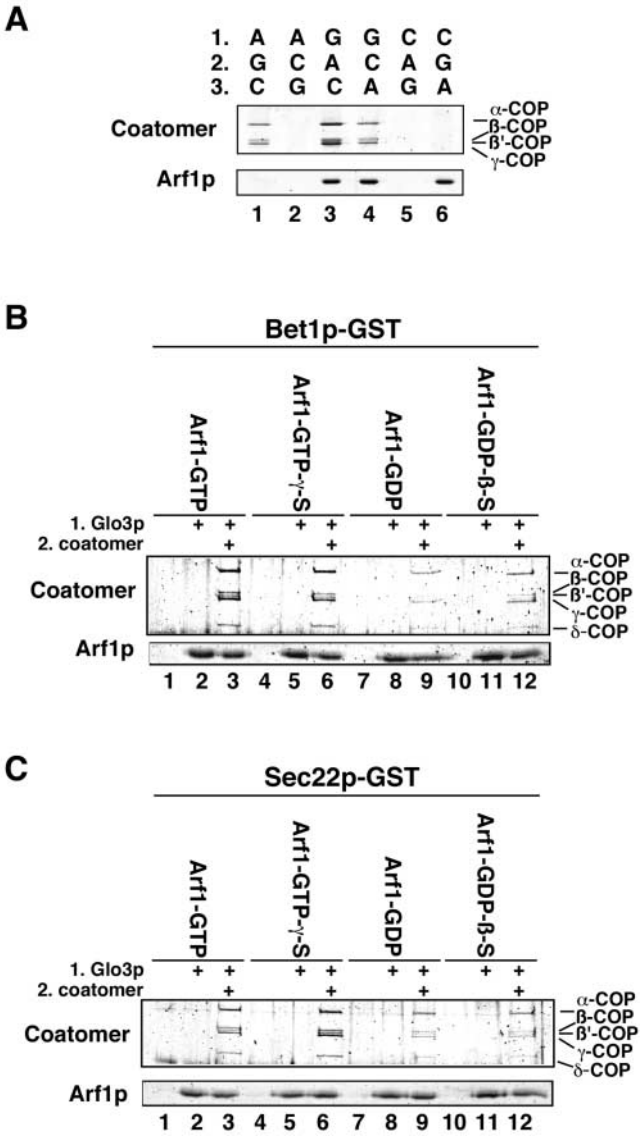

Figure 5. **Sequential binding of ARF-GAP and COPI components to v-SNARE–GST fusion proteins.** (A) Bet1p-GST immobilized on glutathione-agarose beads was incubated with Arf1ΔN17p (A), Glo3p (G), or coatomer (C) for 45 min at 4°C. The beads were washed with BBP and then incubated with a second protein for another 45 min at 4°C. This procedure was repeated a third time. The numbers 1, 2, and 3 indicate the order of addition. Arf1ΔN17p was preexchanged with GTP, and GTP was present during all incubations. (B and C) Arf1p-GTP stimulates coatomer recruitment to v-SNAREs. Bet1p-GST (B) or Sec22p-GST (C) were immobilized on glutathione-agarose beads. The SNAREs were incubated with Arf1ΔN17p and Glo3p for 45 min at 4°C. The guanine nucleotide on Arf1ΔN17p was preexchanged to GTP, GTP-γ–S, GDP, or GDP-β–S before the binding reaction. The same nucleotide was present during the binding reactions. The unbound proteins were removed by three washes in BBP, and coatomer was added to the indicated samples. The recruitment of coatomer was allowed to take place for 45 min at 4°C.

COPI components bind to the membrane proximal region of Bet1p

A retrieval signal, KKXX, allows the direct interaction of certain recycled membrane proteins with coatomer. However, this sequence is not present in the cytosolic domain of

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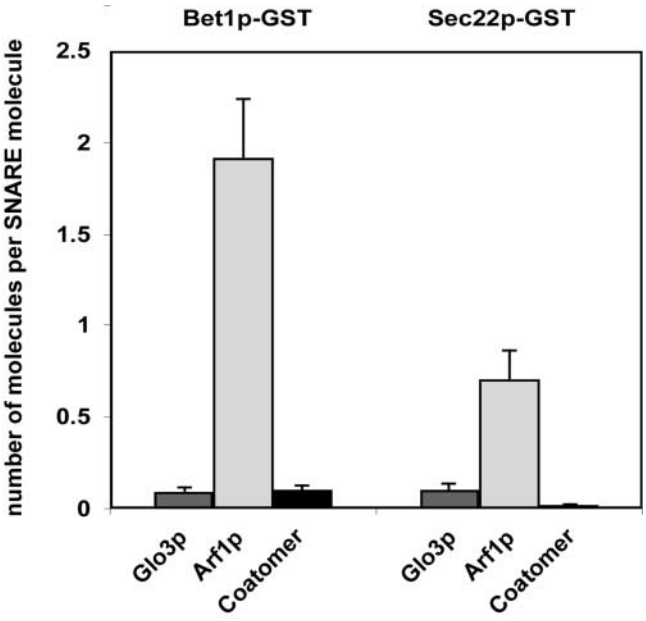

Figure 6. **Quantification of proteins recruited to v-SNAREs.** SyproRed–stained bands from nine independent binding experiments were quantified using ImageQuant (Amersham Pharmacia Biotech). The average and standard derivation is given. On the y axis, the number of molecules per SNARE molecule is represented. The dark gray bars indicate the number of Glo3p per v-SNARE molecule, the light gray bars represent the number of Arf1ΔN17p, and the black bars represent coatomer molecules.

Bet1p. We investigated whether truncations of Bet1p-GST were still able to bind Arf1ΔN17p and coatomer (Fig. 4 B). The different constructs used are summarized in Fig. 4 A. All three, ARF-GAP, Arf1ΔN17p, and coatomer, bound to the 79 COOH-terminal amino acids fused to GST (Bet1p-[41–119]). The amount of recruited protein was comparable to that bound to full-length Bet1p-GST. Binding to Bet1p-(79–119) was quite significant, indicating that Arf1ΔN17p and coatomer might bind to the most membrane proximal part of Bet1p. However, additional sequences in Bet1p might be required to enhance the efficiency. Recruitment to Bet1p-(49–71) was less efficient and almost undetectable to Bet1p-(1–65). No difference in the quantity of recruitment of Arf1ΔN17p and coatomer was observed, suggesting that the interaction is solely dependent of the ability of ARF-GAP to provide a platform on which Arf1ΔN17p and coatomer could bind.

ARF-GAP interaction with v-SNAREs is required before COPI recruitment. If ARF-GAPs were required for the recruitment of Arf1ΔN17p and coatomer, one could expect that they would need to bind first to the SNAREs. Thus, we incubated Bet1p-GST first with Glo3p, Arf1ΔN17p-Q71L, or coatomer and sequentially added the other proteins. The unbound proteins were removed with three washes between subsequent incubations. In experiments where the ARF-GAP was present in the first incubation only both Arf1ΔN17p and coatomer, irrespective at which point they were added, were recruited (Fig. 5 A, lanes 3 and 4). In contrast, in reactions where the ARF-GAP was added as second or third component only proteins that were added after the ARF-GAPs could be immobilized in a complex with Bet1p-GST (Fig. 5 A, lanes 1, 2, 5, and 6). These results indicate that ARF-GAPs are necessary and sufficient to recruit COPI components to SNAREs and that they may play an important role in inserting v-SNAREs in COPI vesicles. However, since Glo3p or Gcs1p are hardly detected in the assay they may prime or activate the v-SNARE for subsequent recruitment of coatomer and Arf1ΔN17p.

To determine the stoichiometry of binding, we quantified the amount of Glo3p and COPI components that bound to Bet1p-GST and Sec22p-GST (Fig. 6). Standard protein concentrations of each protein were present on the gels that were used for quantification. Although about two Arf1ΔN17p molecules were bound per molecule Bet1p-GST, less than one molecule Arf1ΔN17p was detected on Sec22p-GST. In contrast, coatomer was only present on 1/10 or a 1/20 of the SNARE molecules, depending on the v-SNARE. As expected from the results described above, one molecule Glo3p was bound to every 10 v-SNAREs. Thus, Glo3p may only act catalytically on the v-SNAREs to mediate Arf1ΔN17p binding.

The next step in COPI vesicle formation after the binding of Arf1p to SNAREs (or cargo) should be the recruitment of coatomer. Unlike our findings that the immobilization of Arf1ΔN17p on GST–SNAREs was independent on the guanine nucleotide bound to Arf1ΔN17p (Fig. 2), the recruitment of coatomer to Arf1ΔN17p should be stimulated by Arf1ΔN17p-GTP. To test this hypothesis, we preexchanged Arf1ΔN17p with GTP, GTP-γ–S, GDP, or GDP-β–S and performed a binding assay in the presence or absence of ARF-GAP to Bet1p-GST (Fig. 5 B) or Sec22p-GST (Fig. 5 C). At the end of the incubation period, unbound proteins were removed by three washes, and coatomer was added to one half of samples where Arf1ΔN17p had been immobilized onto glutathione-agarose beads. The levels of bound coatomer were assessed as described above. Significant amounts of coatomer could be detected in the samples where Arf1ΔN17p bound to GTP, or its nonhydrolyzable analogue, were present (Fig. 5, B and C, lanes 3 and 6). In contrast, only background levels of coatomer were observed in samples containing Arf1ΔN17p-GDP or Arf1ΔN17p–GDP-β–S (Fig. 5, B and C, lanes 9 and 12). These results indicate that the guanine-nucleotide dependence of the Arf1p–coatomer interaction is reflected in our in vitro system. In addition, it provides further evidence that ARF-GAP might play a crucial role in the inclusion of v-SNARE molecules in COPI vesicles.

ARF-GAPs render SNAREs more protease resistant

So far our results indicated that the continuous presence of ARF-GAPs is not required for subsequent Arf1ΔN17p recruitment. Since SNAREs are known to undergo conformational changes, we wondered if ARF-GAP would be able to provoke such a conformational alteration in a catalytic manner. To test this assumption, we subjected Bet1p-GST to a protease digestion regime (Fig. 7, A and B). Most of the Bet1p-GST was digested after 30 min, and after 60 min no Bet1p-GST was detectable (Fig. 7 A, −Glo3p, and B, ■). However, after preincubation of Bet1p-GST with Glo3p the digestion of Bet1p-GST was delayed dramatically. Even after 60 min, >40% of the SNARE was unaffected (Fig. 7 A, +Glo3p, and B, □). We repeated these experiments with Sec22p-GST and

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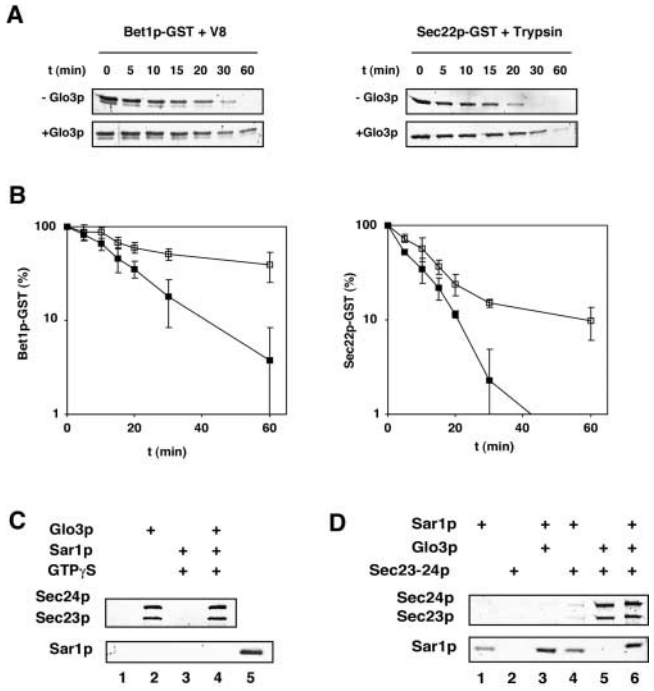

Figure 7. **Glo3p induce conformational changes on v-SNARE–GST fusion proteins.** (A) Bet1p-GST and Sec22p-GST were mock treated (−Glo3p) or pretreated with ARF-GAP (+Glo3p). The ARF-GAP was removed before the addition of the proteases to the SNAREs. Samples were withdrawn at the indicated time points and analyzed by SDS-PAGE and SyproRed staining. B shows a quantification of A. The standard derivation was calculated from six and nine experiments for Bet1p-GST and Sec22p-GST, respectively. (C) Sec23/24p complex can bind to Bet1p-(1–65)–GST even in the absence of Sar1p upon activation by Glo3p. Binding reactions were performed under the same conditions as above. Lane 5 shows 20% of the Sar1p that was present in the binding reactions. Sec23/24p complex was added to all reactions. (D) Binding of Sar1p and Sec23/24p complex is enhanced in the presence of Glo3p. Binding assays to Bet1p-GST were performed under optimized conditions for COPII binding as described by Springer and Schekman (1998). GTP-γ–S was present in all reactions in C and D.

trypsin and came to similar results (Fig. 7, A and B). The stability of Sec22p-GST was enhanced greatly after preincubation with ARF-GAP. In all experiments, the samples without Glo3p were mock preincubated to exclude any buffer influence. The efficient removal of Glo3p was monitored by immunoblot. Since Glo3p was removed from the reaction mixture before the protease treatment, these results are consistent with a catalytic action of ARF-GAP on SNAREs that might induce a conformational change on SNAREs.

ARF-GAP facilitates COPII binding to Bet1p-(1–65)

If ARF-GAP induces structural shift on ER-Golgi v-SNAREs that would allow COPI recruitment, we predicted that this change should also allow COPII components to bind. The rationale was that the conformational changes that would allow for uptake in COPI vesicles might be the same as for COPII vesicle inclusion. We compared COPI and COPII recruitment to Bet1p-GST. The COPII coat consists of Sar1p, the Sec23/24p complex, and the Sec13/31p complex. Indeed, ARF-GAP was sufficient to recruit Sec23/24p even in the absence of Sar1p (unpublished data). Springer and Schekman (1998) reported that immobilization of the Sec23/24p complex on a v-SNARE is dependent on the binding of the small GTPase Sar1p. To extend the results, we used Bet1p-(1–65), which does not bind either COPI (Fig. 4 B) or COPII (Springer and Schekman, 1998). Surprisingly, under our experimental conditions the Sec23/24p complex was recruited to Bet1p-(1–65) in a Glo3p-dependent manner in the absence of Sar1p (Fig. 7 C). In this setting, very little Arf1ΔN17p or coatomer was recruited (Fig. 4 B, lane 6; unpublished data). These results indicate that Glo3p may modify Bet1p-(1–65) in a way that it became a good interactor for Sec23/24p. Furthermore, they suggest that the mechanism of recruitment of Sec23/24p mediated by Sar1p differs from that mediated by Glo3p. However, in vivo this part is most likely played by Sar1p. Under the experimental conditions above, no Sar1p binding, independent of Glo3p, was observed, which may have been due to the high concentration of Triton X-100 in the assay. These data support our previous results that ARF-GAP may act catalytically on v-SNAREs. In addition, they suggest that the conformational change affects most likely the entire cytoplasmic tail of Bet1p. Furthermore, they confirmed that even though the action of ARF-GAP modifies Bet1p-(1–65) the binding site of Arf1ΔN17p is not comprised in this fragment.

To be able to compare our results with those described by Springer and Schekman (1998), we repeated the binding assay under their experimental conditions, which employed lower detergent concentrations. Arf1ΔN17p and coatomer bound to Bet1p-GST and did not bind to Bet1p-(1–65) as observed before (unpublished data). As reported by Springer and Schekman (1998), Bet1p recruited Sec23/24p complex in a Sar1p-dependent manner. However the binding efficiency could be enhanced greatly by the presence of Glo3p in the assay (Fig. 7 D, lanes 4 and 5 compared with lane 6). These results indicate that ARF-GAP might induce conformational changes on Bet1p that would allow subsequent binding of multiple coat components at different sites.

ARF-GAPs catalyze the recruitment of Arf1ΔN17p

So far we have presented evidence that ARF-GAP might induce conformational changes on SNAREs in a catalytic manner. To strengthen the argument, we added to Bet1p-GST either Glo3p that had been blocked with affinity purified anti-Glo3p antibodies or added sequentially Glo3p and the affinity purified antibodies (Fig. 8 A, lanes 4 and 8). In the first case, as expected the antibodies eliminated Glo3p–Bet1p-GST interaction and thus prevented subsequent Arf1ΔN17p binding (Fig. 8 A, lane 3 compared with 4). In the second scenario, two outcomes were possible: either Arf1ΔN17p would require bound Glo3p, then the antibody should block Arf1ΔN17p binding, or Glo3p could trigger a possible conformational change on Bet1p, which then should enable Arf1ΔN17p recruitment. In the latter case, the addition of the antibodies should have no or little effect on Arf1ΔN17p binding. Indeed, when we sequentially added Glo3p, anti-Glo3p antibodies, and Arf1ΔN17p we found almost the same amount of Arf1ΔN17p bound to Bet1p independent of the addition of anti-Glo3p antibodies (Fig. 8 A, lane 7 compared with 8). Together, these results indicate that there are two separable func-

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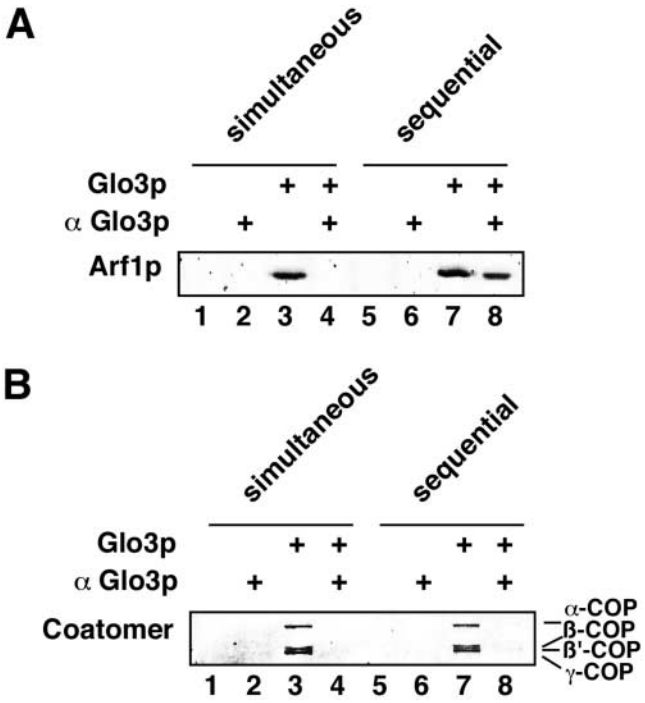

Figure 8. **Glo3p presence on Bet1p-GST is not required for Arf1ΔN17p binding.** (A) Anti-Glo3p antibodies do not block Arf1ΔN17p recruitment. Glo3p was preincubated with affinity purified anti-Glo3p antibodies and then added to binding reaction involving Arf1ΔN17p and Bet1p-GST (lane 4, simultaneous). In the controls, either Glo3p or anti-Glo3p antibodies were added (lanes 2, 3, 6, and 7). In lane 8, Bet1p-GST was first incubated with Glo3p and then with anti-Glo3p antibodies followed by Arf1ΔN17p (sequential). All reactions contained Arf1ΔN17p, GTP, and Bet1p-GST. (B) Coatomer binds to Bet1p-GST via Glo3p. We employed the same protocol as described in A, replacing Arf1ΔN17p with coatomer.

tions of Glo3p. The first activity involves the recruitment of Arf1p to SNAREs and most likely demands a conformational change. The second activity is the GAP activity, which is probably necessary later in the vesicle budding process.

The results presented would be consistent with a direct coatomer–Glo3p interaction. To test this suggestion, we repeated the antibody inhibition experiment with coatomer. After blocking bound Glo3p with antibodies, only very little coatomer was still recruited (Fig. 8 B, lane 8), whereas under the same conditions Arf1ΔN17p was efficiently immobilized on Bet1p-GST (Fig. 8 A, lane 8). Thus, Glo3p acts catalytically on v-SNAREs to recruit Arf1p but not coatomer.

Discussion

We have investigated whether ARF-GAPs may play a role in recruiting v-SNAREs into vesicles. Our results indicate that the ARF-GAPs, Glo3p, and Gcs1p may play a pivotal role in this process. They are necessary and sufficient to allow the interaction of the small GTPase Arf1p where the NH_2-terminal 17 amino acids were deleted (Arf1ΔN17p) with v-SNARE–GST fusion proteins. Arf1ΔN17p binding to the v-SNAREs did not require the continued presence of ARF-GAP. Instead, a prior contact between the v-SNAREs and the ARF-GAP was essential and sufficient for subsequent binding of Arf1ΔN17p to SNAREs. ARF-GAP (Glo3p or Gcs1p) would bind to v-SNAREs present on the Golgi membrane and would induce a conformational change (Fig. 9). This could lead to two different possibilities. The altered conformation of the SNAREs might lower the affinity of the ARF-GAPs for the v-SNAREs, allowing the complex to dissociate (Fig. 9 A). In the next step, Arf1p would be able to interact with v-SNAREs. ARF-GAP might start recruiting cargo proteins like the loaded HDEL receptor. If there is no cargo to be included into vesicles, ARF-GAP might bind to Arf1p, which could result in an abortive complex. Alternatively, in vivo SNAREs, ARF-GAP, and Arf1p may form a complex (Fig. 9 B). Only after coatomer binds to this activated complex could GTP hydrolysis on Arf1p occur. We favor the first hypothesis because GTP hydrolysis could not take place prematurely, thus leading to an efficient budding process. Although we did not detect a difference between dominant-activated and dominant-inactivated Arf1ΔN17p in vitro, this step might still be dependent on a prior immobilization of Arf1p to the Golgi membrane, since the binding domain in Bet1p is close to the membrane. This process may allow the selection of the site where the next vesicle should emerge. Thus, SNAREs, Arf1p, ARF-GAP, and coatomer could form a primer that would subsequently lead to diffusion of cargo into the bud formation area and finally to vesicle emergence as suggested by Springer et al. (1999). In our view, the conformational change in the SNAREs that is brought about by the interaction with ARF-GAP is necessary for efficient uptake in COPI vesicles. This mechanism would ensure the enclosure of v-SNAREs with high fidelity. Recently, Lanoix et al. (2001) have shown that ARF-GAP plays a role in sorting Golgi resident proteins into different subpopulations of COPI vesicles. This is in very good agreement with our data. However, they postulate that ARF-GAP exists in a high affinity and a low affinity state for Arf1p. The high affinity state stimulates GTP hydrolysis by Arf1p in the presence of coatomer but in the absence of cargo. Thus, Arf1p should undergo futile GDP-GTP cycles. ARF-GAP reaches the low affinity state by interaction with cargo, which slows down the Arf1p GTPase activity, allowing a COPI vesicle to form. Since we do not have any data for different affinities of ARF-GAP for Arf1p, we do not include this view in our model. Nor do we think that coatomer must be present for the abortive ARF-GAP–Arf1p complex to form in the absence of cargo. Nevertheless, the work of Lanoix et al. (2001) and our own data suggest a role of ARF-GAP in early events of COPI vesicle formation.

The COPI-dependent priming event is different from COPII budding where it seems that the small GTPase Sar1p may play a crucial role in bud site selection (Springer and Schekman, 1998). The GAP for Sar1p is Sec23p, a subunit of the COPII coat. However, interestingly the conformational change on the SNAREs that might be induced by Glo3p is sufficient to recruit the Sec23/24p complex. Thus, this first interaction does not determine high specificity rather than preparing a platform. COPII vesicle budding requires Sec12p, a resident ER membrane protein, to promote Sar1p nucleotide exchange. Thus, this event is precluded at the cis-Golgi membrane. Furthermore, it seems unlikely that ARF-GAP would prime the v-SNAREs for uptake in COPII

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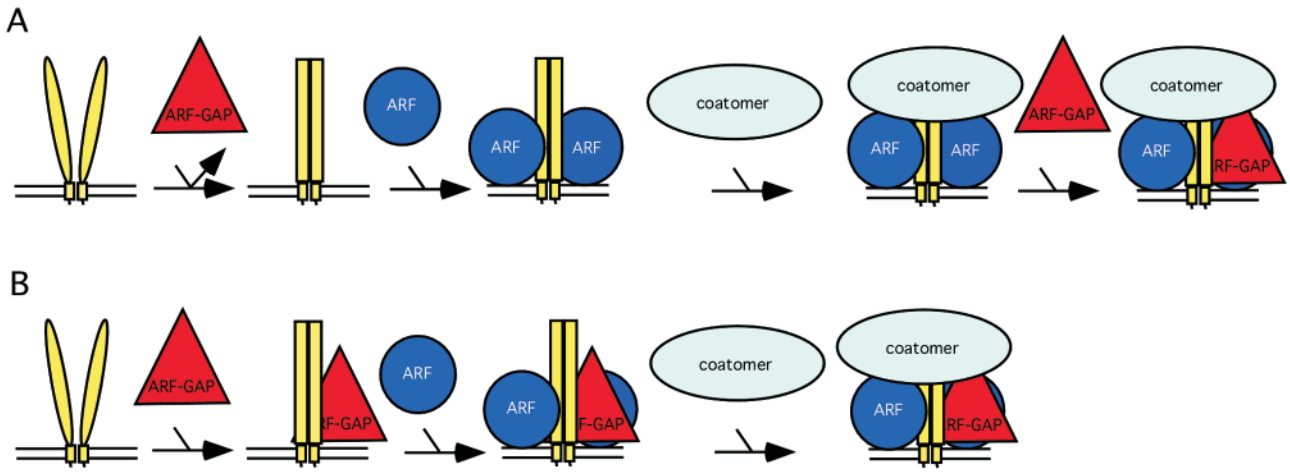

Figure 9. **Schematic drawing of possible mechanisms for the "priming step" of ER-Golgi v-SNAREs.** In A, ARF-GAP only interacts temporally with the SNAREs and reenters the complex after Arf1p and coatomer have been recruited to the SNAREs. The mechanism in B would postulate the continuous presence of ARF-GAP in the growing SNARE–Arf1p–coatomer complex. For detailed explanations see text.

vesicles. Therefore, in vivo this conformational change at the ER exit sites might be brought about by another factor, most likely Sar1p.

Springer and Schekman (1998) have reported a preference of Sar1p for Bet1p and Bos1p, although interactions with Sec22p were not detectable. Here again the situation is different for the retrograde transport from the Golgi to the ER. We observed interactions between Arf1ΔN17p and all three v-SNAREs, although there was a clear preference for Bet1p. A likely explanation would be that for the anterograde vesicle fusion Sec22p is dispensable and thus does not have to be present. Although Bos1p is not required for the consumption of retrograde transport vesicles, it has to be retrieved with high efficiency in order to undergo another round of transport.

Do v-SNAREs act as the elusive ARF receptors on membranes? Our results do not allow any conclusion in this respect. The microsome experiments with Arf1ΔN17p do point in this direction. However, we have not yet been able to obtain the same result with full-length myristoylated Arf1p. Furthermore, there might be more than one way to recruit Arf1p to membranes.

Our data suggests that for the inclusion of v-SNAREs in a COPI vesicle an ARF-GAP needs to interact first with the v-SNAREs. This initial interaction may serve to recruit Arf1ΔN17p to an exit site. This model would predict that ARF-GAP might have two functions. The first function would be a chaperone-like activity that induces a conformational change on the SNAREs (Fig. 9). One possibility would be that ARF-GAP mediates or facilitates the formation of helix bundles as has been described for the synaptic exocytotic SNARE complex (Sutton et al., 1998). This helix bundle formation may be artificially facilitated in our system due to the dimerization abilities of GST. Thus, two SNARE molecules would already be in close proximity to interact. SNAREs exist as oligomeric protein complexes in vitro and most likely also in vivo (Swanton et al., 1998; Ungermann et al., 1999; Xu et al., 2000). Although the SNARE–GST fusion proteins might represent homodimers, in vivo SNAREs probably form heteromeric complexes. The other possibility is a conformational change within one SNARE molecule that would render it more compact and thus less susceptible to protease digestion.

The second function of the ARF-GAP would then be the GAP activity itself. These two activities should be separated in order to allow the formation of a productive budding complex and marking the budding area into which additional cargo could diffuse. Recently, Goldberg (1998, 1999) has shown that coatomer stimulates the activity of ARF-GAP and that certain cargo molecules retard the coatomer-stimulated rate of GTP hydrolysis. Based on these data, Goldberg concluded that ARF-GAPs possess a proof reading activity. However this model is limited to proteins containing a coatomer-binding site for which cargo recognition may be coupled to GTP hydrolysis on Arf1ΔN17p. In contrast, the interaction between v-SNAREs, Glo3p, and Arf1ΔN17p probably does not require GTP hydrolysis. Thus, this interaction should be of a different nature. In addition, ER-Golgi v-SNAREs bind very weakly to coatomer if at all. However, this interaction could be mediated by ARF-GAP. The coatomer binding to the v-SNAREs was increased in the presence of GTP-bound Arf1ΔN17p, indicating that GTP-dependent interaction of Arf1p and coatomer occurs also in a SNARE–Arf1p–coatomer complex. It should be pointed out that Szafer et al. (2000, 2001) have reported a stimulatory role of ARF-GAP in the absence of coatomer. Thus, without the crystal structure of full-length Arf1p with full-length ARF-GAP the nucleotide requirements and staging of hydrolysis will remain open for a wide range of speculations. In summary, in this study we emphasize a novel additional role for ARF-GAPs that is required before the proofreading activity. Both activities could be linked in vivo.

A role of ARF-GAP in cargo uptake into COPI vesicles has been suggested by Aoe et al. (1998) and more recently by Lanoix et al. (2001). Aoe et al. (1999) showed that the noncatalytical domain of ARF-GAP is responsible for the interaction with the KDEL receptor. The authors concluded that the KDEL receptor and ARF-GAP regulate retrograde transport. These data are not in conflict with our results, since we invoke an additional function for ARF-GAP. ARF-GAP may

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regulate budding from the cis-Golgi in a spatially and temporally regulated manner. An initial interaction with the v-SNAREs could determine the site of vesicle emergence. Subsequent interaction with the ligand-occupied KDEL receptor would ensure that cargo is included into the vesicle or alternatively may sense if there is a need for vesicle formation. This would allow the establishment of equilibrium of vesicle and membrane flow between the Golgi and ER.

Materials and methods

Microsome binding assay

Microsomes were prepared as described by Barlowe et al. (1994). The membranes (containing ~20 μg protein) were incubated with 0.2 mM nucleotides, 1 μg Arf1ΔN17p, and with or without 50 ng Glo3p in a final volume of 25 μl. The reactions were performed for 30 min at either 4 or 25°C. Membrane-bound Arf1ΔN17p was separated from unbound GTPase by three washes in B88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM $Mg[OAc]_2$), and each wash was followed by a centrifugation at 14,000 rpm and 3 min in a cooled microfuge. The membranes were solubilized and analyzed by SDS-PAGE and immunoblot. The blots were developed with an ECL kit (Amersham Pharmacia Biotech).

Purification of proteins

The ARF-GAPs, Glo3p and Gcs1p, were purified according to Poon et al. (1996) with minor modifications. In brief, ARF-GAP synthesis was induced with IPTG in *E. coli* containing the appropriate plasmids. Cells were harvested and lysed in either 6 M guanidinium-Cl or 8 M urea. The ARF-GAPs were purified over Ni-NTA agarose (QIAGEN) in the presence of 8 M urea. Proteins were renatured, after adjusting the protein concentration to 10 μg/ml, by dialysis against 2× 25 mM Hepes, pH 7.2, 150 mM KOAc, 1 mM DTT, 100 μM $ZnCl_2$, 20% glycerol followed by 2× 25 mM Hepes, pH 7.2, 150 mM KOAc, 100 μM DTT, 10 μM $ZnCl_2$, and 20% glycerol. The activity of the ARF-GAPs was determined as described in Albert et al. (1999).

Coatomer was purified as described by Hosobuchi et al. (1992). Arf1ΔN17p-Q71L (Arf-DA), Arf1ΔN17p-T31N (Arf-DN), and Arf1ΔN17p were purified as His_6-tagged fusion proteins over Ni-NTA (QIAGEN). For these proteins, the first 17 amino acids were deleted in order to remove the hydrophobic helix and the myristoylation site in *ARF1* (Paris et al., 1997).

The different SNARE–GST fusion proteins were expressed in *E. coli* and purified over glutathione-agarose as described by Springer and Schekman (1998).

Affinity purification of anti-Glo3p antibodies

The antibodies were purified according to Olmsted (1981) with some modifications. In brief, purified His_6-tagged Glo3p was isolated by preparative SDS-PAGE. The proteins were blotted onto nitrocellulose and stained with Ponceau S. The Glo3p band was cut out, blocked with 5% nonfat milk in TBS, and incubated with anti-Glo3p serum in a wet chamber for 2 h. After removal of the serum, the strip was washed with TBS. The antibodies were eluted by incubation of the strip with 0.2 M glycine, pH 2.5, for 10 min. The pH of the antibody solution was adjusted to neutral pH with 100 mM Tris-HCl, pH 8.0.

Nucleotide exchange reaction

The binding assays were preceded by a nucleotide exchange reaction. His_6-tagged variants of Arf1ΔN17p were incubated with 0.7 mM of guanine nucleotide or nonhydrolyzable analogs in 25 mM Hepes, pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM DTT, and 0.1% Na-cholate for 30 min at 37°C.

SNARE binding assay

SNARE–GST fusion proteins (5 μg) were immobilized on 25 μl 50% glutathione-agarose slurry (Amersham Pharmacia Biotech) for 30 min at 4°C. The unbound proteins were removed with three washes with BBP (25 mM Hepes, pH 6.8, 1 mM DTT, 0.5 mM $MgCl_2$, 300 mM KOAc, 0.2% Triton X-100). Beads were incubated with 20 nM Glo3p or Gcs1p, 1.2 μM preexchanged Arf1ΔN17p, and 40 nM coatomer in BBP for 1 h at 4°C. The total reaction volume was 100 μl. In sequential experiments, washes with BBP were included between the different incubations. Affinity purified anti-Glo3p antibodies and nucleotide were added as indicated. After the binding, beads were washed three times with BBP, once with 20 mM Hepes, pH 6.8, and then heated to 65°C for 10 min in SDS sample buffer. Eluted proteins were analyzed by SDS gel electrophoresis, SyproRed staining, and scanning with a Storm PhosphorImager (Amersham Pharmacia Biotech). Bands were quantified using ImageQuant software (Amersham Pharmacia Biotech).

Protease protection assay

Bet1p-GST and Sec22p-GST were immobilized on glutathione-agarose as described above. The immobilized SNARE–GST fusion proteins were incubated with or without 20 nM Glo3p for 1 h at 4°C. The ARF-GAP was removed by three washes with BBP. Bet1p-GST beads and Sec22p-GST beads were incubated for indicated times at 37°C under agitation with 0.5 μg/ml V8 protease or 2.5 μg/ml trypsin, respectively. After the incubation period, 5 μl 5× SDS sample buffer was mixed with 10 μl sample and heated immediately for 5 min at 95°C. Eluted proteins were analyzed as described before.

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