

# ARFGAP1 plays a central role in coupling COPI cargo sorting with vesicle formation

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Examining how key components of coat protein I (COPI) transport participate in cargo sorting, we find that, instead of ADP ribosylation factor 1 (ARF1), its GTPase-activating protein (GAP) plays a direct role in promoting the binding of cargo proteins by coatomer (the core COPI complex). Activated ARF1 binds selectively to SNARE cargo proteins, with this binding likely to represent at least a mechanism by which activated ARF1 is stabilized on Golgi membrane to propa-

gate its effector functions. We also find that the GAP catalytic activity plays a critical role in the formation of COPI vesicles from Golgi membrane, in contrast to the prevailing view that this activity antagonizes vesicle formation. Together, these findings indicate that GAP plays a central role in coupling cargo sorting and vesicle formation, with implications for simplifying models to describe how these two processes are coupled during COPI transport.

## Introduction

Coat proteins play a central role in the intracellular sorting of proteins by coupling vesicle formation with cargo sorting (Bonifacino and Glick, 2004). A favored current model for how this coupling is achieved, known as the priming complex model (Springer et al., 1999), proposes that the activated (GTP-bound) form of an ADP ribosylation factor (ARF)-like small GTPase promotes the binding of coat proteins onto the cytoplasmic domain of transmembrane cargo proteins to form a key intermediate that drives both cargo sorting and vesicle formation. Experimental evidence for this model has been derived mainly from studies on the COPII coat protein (Springer and Schekman, 1998), but whether coat protein I (COPI) cargo sorting uses the identical mechanism has been less clear. Cargo proteins that bind specifically to the activated form of ARF1 have not been identified. Moreover, several studies have shown that coatomer binds directly to the cytoplasmic domain of cargo proteins in the absence of activated ARF1 (Fiedler et al., 1996; Reinhard et al., 1999; Goldberg, 2000; Rein et al., 2002; Yang et al., 2002).

Even more puzzling has been the role of the GTPase-activating protein (GAP) that catalyzes the deactivation of ARF1 from its GTP-bound to its GDP-bound form. A prevailing view is that the translocation of coatomer between membrane

and cytosol is coupled to the GTPase cycle of ARF1 (Goldberg, 2000; Bigay et al., 2003; Reinhard et al., 2003), leading to the prediction that the GAP activity, which catalyzes the deactivation of ARF1, should antagonize the formation of COPI vesicles by releasing coatomer from membrane. However, subsequent studies have revealed that GTP hydrolysis on ARF1 is required for efficient cargo sorting (Nickel et al., 1998; Lanoix et al., 1999; Pepperkok et al., 2000), implying that GAP activity promotes cargo sorting that occurs during vesicle formation. Moreover, examining the requirements for purified soluble proteins to reconstitute COPI vesicles from Golgi membrane, we have found recently that ARFGAP1 promotes both vesicle formation and cargo sorting (Yang et al., 2002), implying that the GAP catalytic activity does not antagonize vesicle coating. However, two later studies that use liposomal membrane to reconstitute COPI vesicle formation have concluded otherwise, showing specifically that the GAP activity promotes vesicle uncoating and antagonizes vesicle formation (Bigay et al., 2003; Reinhard et al., 2003).

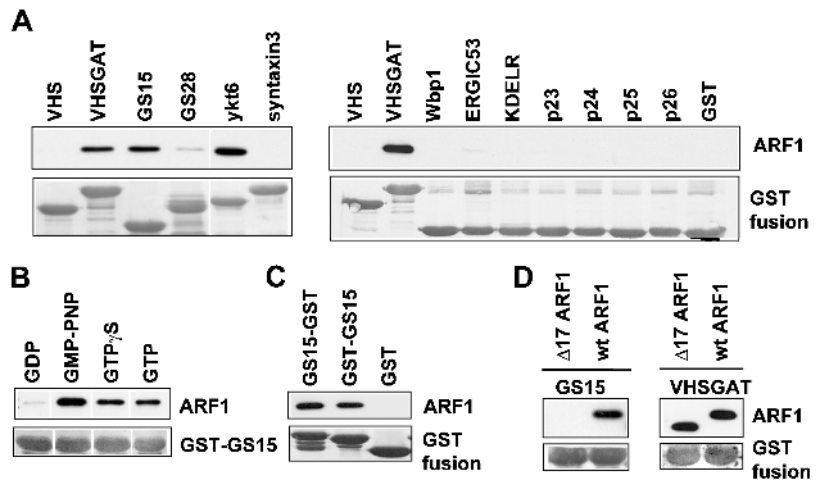
The importance in resolving this apparent disparity lies in the consequence for considering how cargo sorting and vesicle formation are coupled during COPI transport. As current models have been based on the view that the GAP catalytic activity possesses two opposing functions, promoting cargo sorting while inhibiting vesicle formation, these models have been necessarily complex, as they must postulate additional complex temporal and spatial mechanisms to segregate the supposed

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Abbreviations used in this paper: ARF, ADP ribosylation factor; COPI, coat protein I; GAP, GTPase-activating protein.

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Figure 1. **Activated ARF1 binds selectively to SNARE cargo proteins.** (A) Activated ARF1 binds selectively to SNARE cargo proteins. ARF1 loaded with GMP-PNP was incubated with the cytoplasmic domain of different cargo proteins expressed as GST fusion proteins on beads for pull-down assays. Beads were then assessed for bound proteins as indicated. GST fusion protein that contained VHS/GAT served as positive control, whereas one that contained VHS served as negative control. (B) Only activated ARF1 binds to the cytoplasmic domain of GS15. ARF1 loaded with different nucleotides as indicated was incubated with GST-GS15 on beads for pull-down assays. Beads were then assessed for bound proteins as indicated. (C) Activated ARF1 binds to the cytoplasmic domain of GS-15 regardless of its orientation of fusion to GST. ARF1 loaded with GMP-PNP was incubated with different fusion proteins [with GS15 appended to GST either at the carboxy terminus [GST-GS15] or amino terminus [GS15-GST]] on beads for pull-down assays. Beads were then assessed for bound proteins as indicated. (D) An activated form of ARF1 that lacks the first 17 amino-terminal residues no longer binds efficiently to the cytoplasmic domain of GS15. Different forms of ARF1 loaded with GMP-PNP were incubated with GST fusion proteins as indicated for pull-down assays. Beads were then assessed for bound proteins as indicated. White lines indicate that intervening lanes have been spliced out.



opposing functions of the GAP catalytic activity (Goldberg, 2000; Lanoix et al., 2001; Bigay et al., 2003). In the current paper, we seek a more precise understanding for the roles of ARF1, its GAP, and coatamer during cargo sorting, and also for the role of the GAP catalytic activity in vesicle formation from Golgi membrane.

## Results

When the cytoplasmic domain of the SNARE cargo proteins was fused onto GST and then gathered onto glutathione beads for incubation with purified COPII components and Sar1p, the COPII coat complex was found to bind these cargo proteins only in the presence of the activated form of Sar1p to form resulting priming complexes (Springer and Schekman, 1998). As this finding represented key direct evidence for the priming complex model of cargo sorting (Springer et al., 1999), we initially used the same approach to examine how ARF1 and its GAP may influence the binding of coatamer to the cytoplasmic domain of cargo proteins.

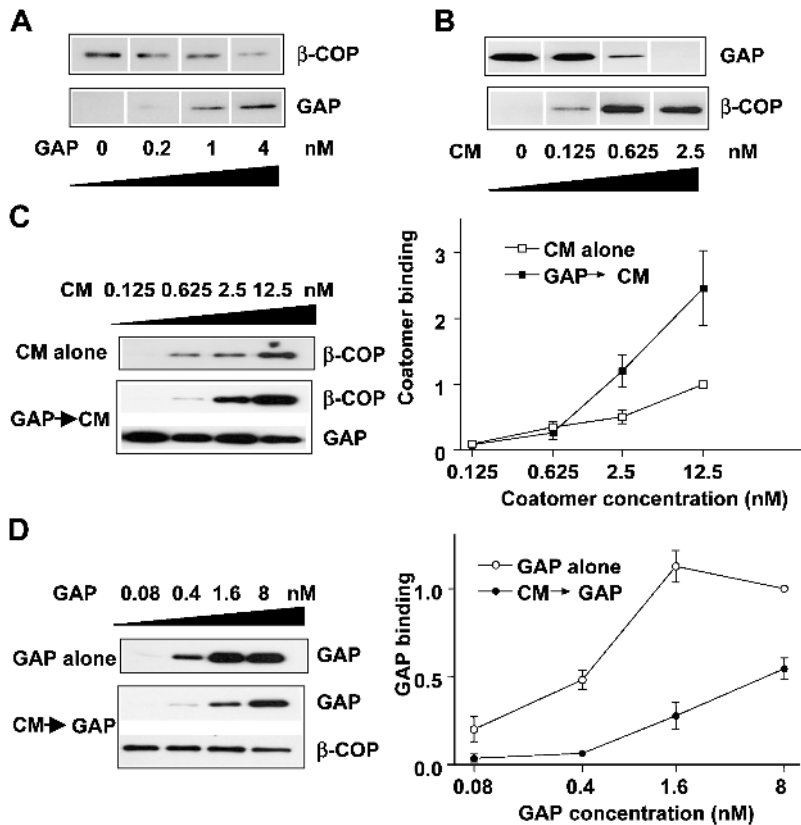
### Binding of activated ARF1 to a distinct set of cargo proteins

Model cargo proteins that have been well characterized to be transported by COPI were selected for detailed examination, including Wbp1 (a classic dilysine-containing cargo protein [Letourneur et al., 1994]), p23 and p25 (members of the p24 family of cargo receptors [Sohn et al., 1996; Dominguez et al., 1998]), and GS15 (a SNARE protein that has been shown to bind coatamer [Xu et al., 2002]). First, we confirmed that their cytoplasmic domains bound directly to coatamer in pull-down assays. However, we also found that activated ARF1 did not affect this binding (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404008/DC1>). In these incubations, and all subsequent ones unless stated otherwise, we used the full-length myristoylated form of ARF1 and also the full cytoplasmic domain of cargo proteins. Moreover, to show that ARF1

loaded with different GTP analogues represented its active form in a functional context, we found that ARF1 loaded with GTP, GTP $\gamma$ S, or GMP-PNP bound to an ARF1 effector domain of GGA (Dell'Angelica et al., 2000), whereas the GDP-bound form of ARF1 did not (Fig. S2).

As activated ARF1 did not play a direct role in recruiting coatamer to cargo proteins, we next determined whether activated ARF1 bound to any cargo protein. When ARF1 was activated with GMP-PNP, we found that it bound specifically to COPI-related SNAREs (Xu et al., 2002), such as GS15 and Ykt6, and GS28 to some extent (Fig. 1 A). Investigating this further, we found that ARF1 loaded with different analogues of GTP all bound GS15, but not ARF1 loaded with GDP (Fig. 1 B). As GS15 is a type II transmembrane protein (Xu et al., 1997), with the amino terminus residing in the cytoplasmic domain, we also tested whether the relative position of the SNARE sequence with respect to GST would affect its binding to ARF1. Similar binding was observed regardless of the relative position of the cytoplasmic domain of GS15 (Fig. 1 C).

Next, we compared the above results with that from two previous studies that had explored the interaction between ARF1 and cargo proteins. First, p23 had been detected to interact specifically with the deactivated form of ARF1 using a cross-linking approach (Gommel et al., 2001). However, using the pull-down approach, we could not detect such an interaction (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200404008/DC1>), suggesting that the interaction between p23 and deactivated ARF1 is much weaker than those detected between the SNARE cargo proteins and activated ARF1 (in Fig. 1 A). Second, a previous yeast study had shown that the interaction between SNARE cargo proteins and ARF1 was independent of the ARF1 activation status, but required its GAP (Rein et al., 2002). Although this result seemingly contradicted our results, we noted that the previous study had used a truncated form of ARF1 that lacked its first 17 amino acids. When comparing the full-length versus the truncated form of ARF1, we found that the full-length ARF1 bound GS15, whereas the



**Figure 2. Binding of coatomer to cargo proteins is directly enhanced by GAP.** (A) Simultaneous incubation of GAP and coatomer inhibits the binding of coatomer to cargo tail. GST-Wbp1 on beads was incubated with a fixed amount of coatomer (0.625 nM) and simultaneously with an increasing amount of GAP as indicated for pull-down assays. Beads were then assessed for bound proteins as indicated. (B) Simultaneous incubation of GAP and coatomer inhibits the binding of GAP to cargo tail. GST-Wbp1 on beads was incubated with a fixed amount of GAP (1 nM) and simultaneously with an increasing amount of coatomer as indicated for pull-down assays. Beads were then assessed for bound proteins as indicated. (C) Sequential incubation of GAP and then coatomer leads to optimal binding of coatomer to cargo tails. GST-Wbp1 on beads was incubated with either increasing levels of coatomer alone or sequentially with GAP followed by increasing levels of coatomer for pull-down assays. Beads were then assessed for bound proteins as indicated in gel images. For the graph, the level of coatomer on beads was then quantified, normalized to the level of GST fusion proteins, and then expressed as a fraction of the binding seen for the highest concentration of coatomer alone. The mean and standard error were derived from three independent experiments. (D) Sequential incubation of coatomer and then GAP inhibits the binding of GAP to cargo tails. GST-Wbp1 on beads was incubated with either increasing levels of GAP alone or sequentially with coatomer followed by increasing levels of GAP for pull-down assays. Beads were then assessed for bound proteins as indicated in gel images. For the graph, the level of GAP on beads was assessed similarly as done for coatomer above (C).

truncated ARF1 had markedly reduced capacity (Fig. 1 D). To rule out that this difference was due to differential activation of the two forms of ARF1, we found that both forms bound equally well to the GGA effector domain (Fig. 1 D). Thus, we concluded that the full-length form of ARF1 was needed for its activation-dependent interaction with SNARE cargo proteins.

### GAP regulates directly the binding of coatomer to cargo proteins

As ARFGAP1 has been suggested recently to function as a component of the COPI coat complex (Yang et al., 2002), we next tested whether it affected the binding of coatomer to cargo proteins. When GAP and coatomer were incubated simultaneously with GST-Wbp1 in pull-down assays, we initially observed antagonistic behavior, either when the level of coatomer was fixed and titrating in increasing level of GAP (Fig. 2 A; Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404008/DC1>) or when the level of GAP was fixed and titrating in increasing level of coatomer (Fig. 2 B and Table S1). To rule out that the observed antagonistic behavior was due to GAP and coatomer binding in solution so that they could no longer bind Wbp1 on beads, coprecipitation experiments indicated that GAP and coatomer did not interact with each other in solution (Fig. S4).

Next, we considered the possibility that simultaneous incubations masked temporal regulation that would allow GAP to exhibit its predicted role as a component of the COPI coat complex (Yang et al., 2002). When GST-Wbp1 on beads was first incubated with a fixed level of GAP and then increasing levels

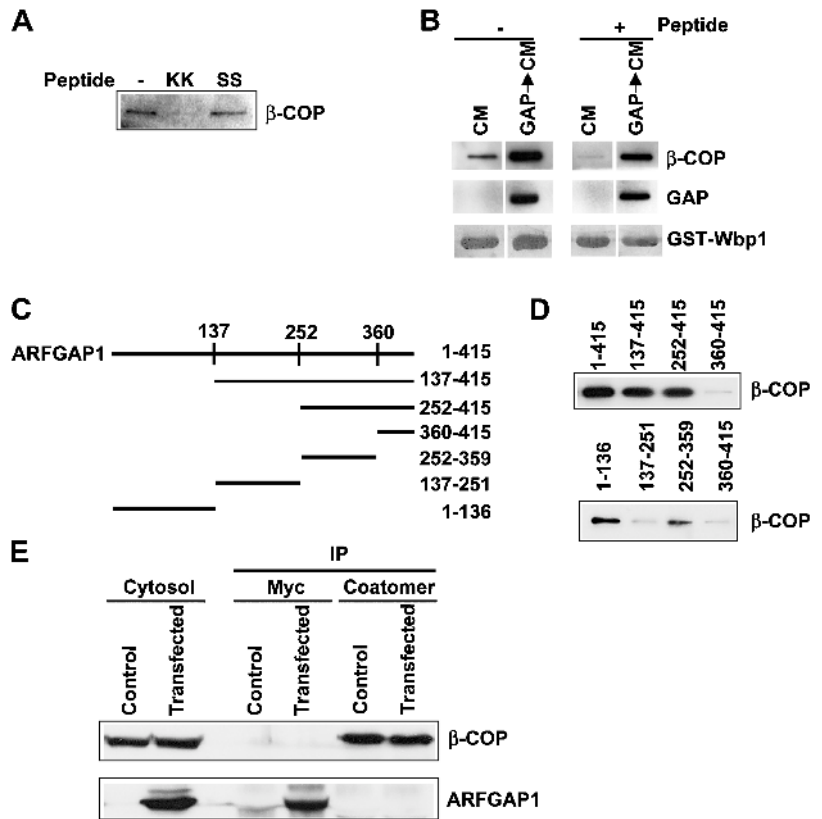
of coatomer, we observed that the binding of coatomer to GST-Wbp1 was enhanced (Fig. 2 C). In contrast, when the sequential incubation was performed with a fixed level of coatomer and then increasing level of GAP, we observed no such cooperative binding between GAP and coatomer (Fig. 2 D).

Next, we sought to elucidate how the sequential binding of GAP and then coatomer led to their cooperative binding to GST-Wbp1. First, to test whether GAP could mediate an indirect binding of coatomer to cargo proteins, we generated a form of coatomer that could no longer bind directly to the dilysine motif on Wbp1 by incubating purified coatomer with a free peptide that corresponded to the cytoplasmic domain of Wbp1. As expected, when saturated of all binding capacity for the dilysine sorting signal by incubating with an excess level of the Wbp1-free peptide, coatomer could no longer bind to GST-Wbp1 on beads in a pull-down assay (Fig. 3 A). As control, preincubation of coatomer with a control peptide, which contained the dilysine motif in Wbp1 replaced with serines, did not result in a similar inhibition. Subsequently, using the peptide-saturated form of coatomer, we found that it bound to GST-Wbp1 that had been prebound with GAP, whereas it did not bind GST-Wbp1 that had not been prebound with GAP (Fig. 3 B). Thus, these results suggested that GAP could mediate the indirect binding of coatomer to cargo proteins.

Second, we tested whether GAP had more than one binding site for coatomer by examining the binding of coatomer to different truncation mutants of GAP (Fig. 3 C). When these GAP mutants were fused to GST and then bound to glutathione beads followed by incubation with soluble coatomer, we found

**Figure 3. Elucidating how the sequential binding of GAP followed by coatomer results in their cooperative binding to cargo proteins.**

(A) Coatomer prebound with the Wbp1-free peptide does not bind to GST-Wbp1 on beads. Coatomer was incubated with a peptide that consists of either the cytoplasmic domain of Wbp1 (indicated as KK) or one with the di-lysines substituted with di-serines (indicated as SS) at 4°C for 1 h. GST-Wbp1 on beads was then added for a further incubation at 4°C for 1 h for pull-down assays, with the excess unbound free peptide still present to ensure that coatomer was fully saturated with the Wbp1-free peptide. (B) GAP supports the binding of saturated coatomer (with the Wbp1-free peptide) to GST-Wbp1 on beads. Coatomer, either unsaturated (indicated by no peptide added) or saturated (indicated by peptide added), were incubated with GST-Wbp1 on beads for pull-down assays, which involved either coatomer alone or sequentially with GAP followed by coatomer. In both cases, excess unbound free peptide was present to ensure that coatomer was fully saturated with the Wbp1-free peptide. Beads were then assessed for bound proteins as indicated. (C) Schematic of the different truncation mutants of GAP generated. (D) Two distinct domains in GAP mediating its direct binding to coatomer. Coatomer was incubated with different truncation mutants of GAP expressed as GST fusion proteins on beads for pull-down assays. Beads were then assessed for bound proteins as indicated. (E) GAP and coatomer do not interact with each other in cytosol. Co-precipitation experiments (lanes indicated by IP) were performed on cytosol that expressed myc-tagged ARFGAP1. The anti-ARFGAP1 antibody was not sensitive enough to detect endogenous ARFGAP1, as indicated by direct immunoblotting of cytosol (first two lanes).



that GAP contained at least two distinct binding sites for coatomer, one in its catalytic domain and another in the non-catalytic domain (Fig. 3 D). We also found that GAP and coatomer could not be coprecipitated together from the cytosol (Fig. 3 E), suggesting that the observed binding between GAP and coatomer likely represented interaction on membrane after GAP and coatomer have been recruited from the cytosol. Thus, when taken together with the above result that GAP could mediate the indirect binding of coatomer to Wbp1, we concluded that the sequential binding of GAP followed by coatomer resulted in their cooperative binding to Wbp1 by GAP mediating multiple complexes of coatomer binding indirectly to Wbp1.

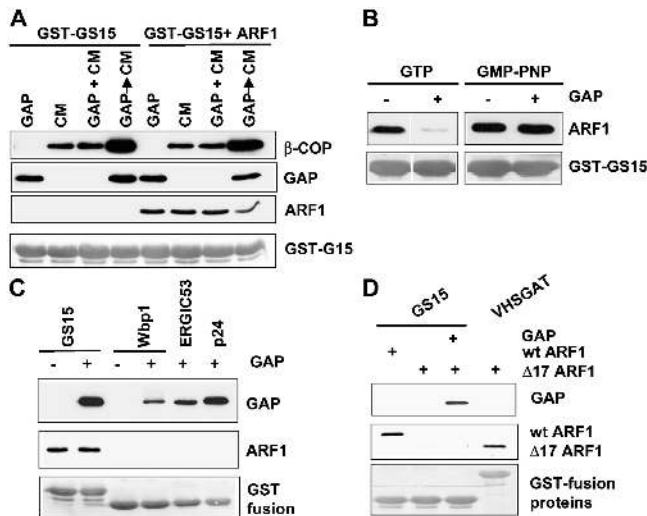
We then tested whether ARF1 played a role in this cooperative binding, using GS15 as the model system, as activated ARF1 only interacted with SNARE cargo proteins (see Fig. 1 A). First, extending the above findings that had used Wbp1 as the cargo protein, we found that the sequential incubation of GAP followed by coatomer also led to their cooperative binding to GS15 (Fig. 4 A). However, we found that prior binding by activated ARF1 did not enhance the binding of either GAP or coatomer to GS15, and this prior binding also did not further enhance the cooperative binding of GAP and coatomer to GS15 due to their sequential incubation (Fig. 4 A). In these experiments, we activated ARF1 with GMP-PNP rather than GTP to rule out the possibility that a lack of an observable effect by ARF1 was due to GAP deactivating ARF1 so that the effect of activated ARF1 became masked.

We also considered the possibility that the lack of an effect by ARF1 might be due to our experimental approach that did not allow ARF1 to interact with its GAP in a manner rele-

vant for cargo sorting. Because GTP hydrolysis on ARF1 has been shown previously to be critical for cargo sorting (Nickel et al., 1998; Lanoix et al., 1999; Pepperkok et al., 2000), we tested whether GAP could induce the release of ARF1 that had been bound to GS15. ARF1 was activated using GTP and then bound to GS15. Upon the addition of GAP, we found that ARF1 was released from GS15 (Fig. 4 B). This release was likely due to the GAP catalytic activity causing the deactivation of ARF1, as the release was blocked when the bound ARF1 was loaded with GMP-PNP.

A previous report had indicated that a yeast GAP could enhance the binding of ARF1 to SNARE cargo proteins (Rein et al., 2002). Examining whether the mammalian ARFGAP1 behaved similarly, we bound GAP to different cargo proteins as GST fusion proteins on beads. However, the prebound GAP did not enhance the binding of full-length activated ARF1 to any of these cargo proteins (Fig. 4 C). Moreover, we found that the truncated ARF1 could not be induced to bind GS15 in the presence of the mammalian ARFGAP1, even though we had used an activated form of the truncated ARF1 as indicated by its binding to the GGA effector domain (Fig. 4 D). Thus, we concluded that the mammalian ARFGAP1 only acted to antagonize the binding of ARF1 to a SNARE cargo protein, by its catalytic activity that resulted in the deactivation of ARF1.

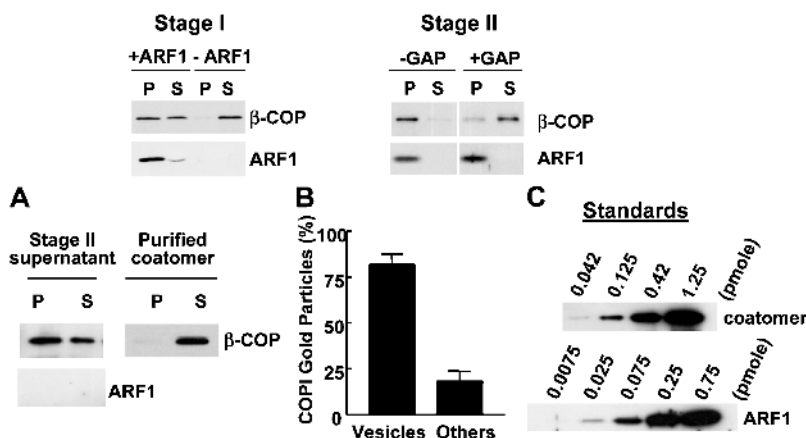
Thus far, using the same experimental approach that had revealed a necessary role for Sar1p in recruiting COPII components to cargo proteins to form priming complexes (Springer et al., 1999), we found that the GAP, rather than ARF1, played a role for COPI priming complexes. However, one potential caveat was that ARF1 is myristoylated and Sar1p is not, sug-



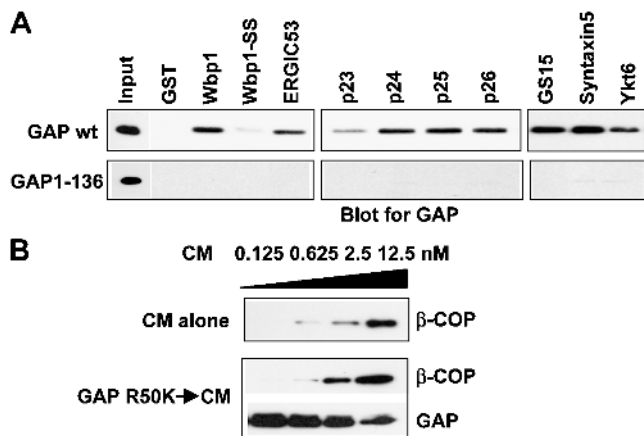
**Figure 4. Effect of ARF1 in complexes that contain cargo proteins, GAP, and coatomer.** (A) Prior incubation with ARF1 does not further promote the GAP-enhanced binding of coatomer to GS15. One set of experiments (indicated by GST-GS15) involved GST-GS15 on beads incubated with coatomer alone, GAP alone, both GAP and coatomer in simultaneous incubation, or GAP followed by coatomer in sequential incubation. Another set of experiments (indicated by GST-GS15 + ARF1) involved activated ARF1 bound to GST-GS15 on beads being incubated with GAP alone, coatomer alone, both GAP and coatomer in simultaneous incubation, or GAP followed by coatomer in sequential incubation (B) Addition of GAP releases activated ARF1 bound to GS15. ARF1 preloaded with either GTP or GMP-PNP was bound to GST-GS15 on beads for 1 h, and then incubated with or without GAP (200 nM) for another hour. Beads were then assessed for bound proteins as indicated. (C) GAP does not affect the binding of activated ARF1 to cargo proteins. The different cargo proteins as GST fusions on beads were incubated either with or without GAP and then incubated with activated ARF1 that had been previously loaded with GMP-PNP. Beads were then assessed for bound proteins as indicated. (D) GAP cannot induce truncated ARF1 ( $\Delta 17$ ) to bind GS15. GST-GS15 on beads was incubated either with or without GAP followed by incubation with the truncated ARF1 that had been loaded with GMP-PNP. Beads were then assessed for bound proteins as indicated. Incubation with GST-VHSGAT confirms that this truncated ARF1 represents an activated form. Incubation of activated wild-type ARF1 with GST-GS15 served as another positive control.

gesting the possibility that ARF1 could bind to lipids as an alternate way of participating in COPI priming complexes. To address this possibility, we examined the levels of ARF1 and coatomer released from Golgi membrane upon the reconstitution of COPI vesicles, as the level of ARF1 released from Golgi membrane in this manner would be predicted to participate in COPI priming complexes. However, a technical limitation was suggested by the previous observation that the COPI coating on vesicles reconstituted with GAP was not completely stable (Yang et al., 2002). Thus, the traditional technique of isolating these vesicles using prolonged equilibrium centrifugation resulted in many uncoated vesicles. As a way of overcoming this problem, we subjected the supernatant fraction of the second-stage incubation, which contained the reconstituted COPI-coated vesicles (Yang et al., 2002), to rapid high-speed sedimentation by ultracentrifugation at 200,000 g for 1 h, which would be predicted to pellet all membranes including small vesicles.

To validate this approach, we performed two experiments. First, the ultracentrifugation resulted in purified coatomer still residing in the supernatant fraction, while a significant fraction of coatomer derived from the supernatant fraction of the second-stage incubation resided in the pellet fraction (Fig. 5 A). Second, quantitative immunogold EM revealed that most membranes in the pellet were COPI vesicles (Fig. 5 B). Thus, as these results suggested that the level of coatomer in the pellet fraction after the ultracentrifugation was mostly indicative of those that participated in the formation of coated vesicles, we then compared this level (in Fig. 5 A) with known levels of purified coatomer as standards (Fig. 5 C) and found that it was at least 0.25 picomoles. In contrast, comparing the level of ARF1 in the supernatant fraction of the same experiment (in Fig. 5 A) with known levels of purified ARF1 as standards (Fig. 5 C), we found that its level was at least  $<0.025$  picomol. Thus, the level of soluble ARF1 released from Golgi membrane was at least 10-fold less than that of coatomer released as COPI vesicles, indicating that COPI cargo sorting was unlikely to require ARF1 to be present in all priming complexes.



**Figure 5. The level of ARF1 released from Golgi membrane is substoichiometric to the level of coatomer released as COPI vesicles.** (A) A rapid and quantitative method of assessing the level of coatomer on reconstituted coated vesicles. The two-stage incubation system (indicated by Stage I and Stage II) was performed. After the second-stage incubation that contained GAP, the supernatant fraction was subjected to ultracentrifugation (200,000 g for 1 h) followed by immunoblotting of pellet (P) and supernatant (S) fractions for proteins as indicated. Ultracentrifugation of purified coatomer served as a control. (B) The pellet fraction after the ultracentrifugation contains membranes that are mostly COPI vesicles. The pellet derived from the ultracentrifugation of the supernatant fraction after the second-stage incubation (as described above in A) was analyzed by immunogold EM using anti-COPI antibodies. Quantitation was performed by selecting random fields at 60,000 $\times$ , counting the gold particles on vesicles versus those on other membranes, and then calculating the fraction on these two respective membranes. The mean and standard error were derived from three independent experiments. (C) Quantitation of purified proteins. Increasing levels of purified proteins as indicated were immunoblotted.

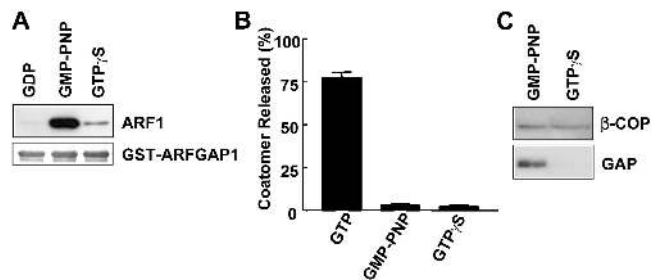


**Figure 6. The noncatalytic domain of GAP mediates its participation in COPI priming complexes.** (A) A truncated GAP, consisting of residues 1–136, does not interact with COPI cargo proteins. 6x-his tagged GAP, either wild-type or truncated, was incubated with different GST fusion proteins on beads for pull-down assays. Beads were then assessed for bound proteins using an anti-6x-his antibody. (B) A catalytic dead GAP still mediates enhanced binding of coatomer to cargo proteins. GST-Wbp1 on beads was incubated with either coatomer alone or sequentially with mutant GAP followed by coatomer for pull-down assays. Beads were then assessed for bound proteins as indicated.

As GAP contained both catalytic and noncatalytic functions (Huber et al., 1998), we also examined the role of these two functions in binding to cargo proteins. A truncated GAP that contained the first 136 (out of 415) residues had been previously shown to retain its catalytic activity (Goldberg, 1999). We found that this truncated GAP could not bind cargo proteins (Fig. 6 A). To complement this finding, we also examined the behavior of a GAP point mutant, generated by mutating an arginine in the catalytic domain to lysine (R50K), which has been shown previously to be catalytically dead (Szafer et al., 2000). This mutant behaved similar to the wild-type form in enhancing the binding of coatomer to Wbp1 in a sequential incubation (Fig. 6 B). Together, these results indicated that the noncatalytic function of GAP mediated its role in regulating the binding of coatomer to cargo proteins.

#### GAP catalytic activity required for vesicle formation from Golgi membrane

Finally, we sought to test more directly the prevailing view that the hydrolysis of GTP on ARF1 antagonizes vesicle coating (Goldberg, 2000; Bigay et al., 2003; Reinhard et al., 2003). In this respect, although we had shown previously that the addition of GAP was critical in reconstituting COPI vesicle formation from Golgi membrane (Yang et al., 2002), simply adding GAP to this reconstitution might not have dissected out a potential complex regulation of its catalytic activity, as a cryptic activity could still function in antagonizing vesicle coating through complex spatial and/or temporal possibilities as previously suggested (Goldberg, 2000; Lanoix et al., 2001; Bigay et al., 2003). However, under any complex scenario, a uniform prediction of the prevailing view would be that inhibiting GAP activity should ultimately promote vesicle formation rather than inhibiting this process. Thus, we sought ways of inhibiting the GAP activity and then assessing their effect on vesicle formation.



**Figure 7. Differential effects of GTP analogs on interaction between GAP and ARF1 and on COPI vesicle formation.** (A) Effect of different nucleotides on the interaction between GAP and ARF1. ARFGAP1 as a GST fusion protein on beads was incubated with ARF1 loaded with different nucleotides as indicated for pull-down assays. Beads were then assessed for bound proteins as indicated. (B) ARF1 loaded with GMP-PNP inhibits the release of coatomer from Golgi membrane in the vesicle reconstitution assay. The first-stage incubation was performed in the presence of different guanine nucleotides as indicated followed by the second-stage incubation using GAP. The level of coatomer released into the supernatant was quantified and then expressed as a fraction of total. The mean and standard error were derived from three independent experiments. (C) Reconstituted vesicles in the presence of GTPγS have decreased level of GAP as compared with reconstituted vesicles using GMP-PNP. The two-stage incubation system was performed in the presence of either GMP-PNP or GTPγS followed by pipette shearing to release vesicles from Golgi membrane, and then ultracentrifugation to collect coated vesicles in the pellet fraction.

First, we examined different nonhydrolyzable analogues of GTP to see how they affected the interaction between ARF1 and GAP (Fig. 7 A). When ARF1 that had been loaded with the different GTP analogues was incubated with GAP as a GST fusion protein bound to beads in pull-down assays, we found as expected that GMP-PNP supported this interaction, whereas GDP did not. Remarkably, ARF1 loaded with GTPγS showed reduced ability to interact with GAP, providing experimental confirmation for our previous suspicion that the use of GTPγS most likely blocked vesicle formation by preventing GAP from interacting with activated ARF1, rather than representing a true block in the GAP catalytic activity (Yang et al., 2002). Using GMP-PNP in the vesicle reconstitution assay, we also found that the release of COPI from Golgi membrane during the second-stage incubation was markedly inhibited, similar to that seen using GTPγS (Fig. 7 B). Moreover, consistent with the finding that GMP-PNP directly inhibited the GAP catalytic activity while GTPγS indirectly inhibited this activity by preventing GAP from interacting with ARF1, we found that the level of GAP was markedly reduced in the condition that used GTPγS as compared with the conditions that used GMP-PNP (Fig. 7 C). In this case, pipette-induced shearing was used to generate vesicles, as the use of a nonhydrolyzable analogue of GTP would have prevented vesicle formation in the absence of such additional maneuver (Yang et al., 2002).

To complement the above results, we also examined the behavior of the R50K GAP mutant. Incubating ARF1 activated by different GTP analogs with GST-R50K GAP on beads in pull-down assays, we found that the mutant GAP interacted similarly as seen above for the wild-type GAP (compare Fig. 8 A with Fig. 7 A). Subsequently, using the two-stage incubation reconstitution system, we found that COPI release from Golgi membrane during the second-stage incubation was also mark-

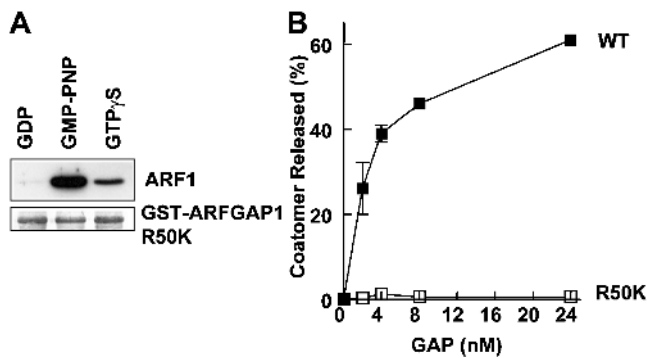


Figure 8. **A point mutation in GAP (R50K) that selectively abrogates its catalytic activity blocks vesicle formation.** (A) Interaction between the point mutant GAP (R50K) and ARF1 bound to different nucleotides. The point mutant GAP (R50K) as GST fusion protein on beads was incubated with different forms of ARF1 previously loaded with nucleotides as indicated for pull-down assays. Beads were then assessed for bound proteins as indicated. (B) The R50K point mutant GAP blocks the release of coatomer from Golgi membrane in the vesicle reconstitution assay. The two-stage incubation was performed using different GAPs at varying concentrations as indicated. The level of coatomer released into the supernatant was quantified and then expressed as a fraction of total. The mean and standard error were derived from three independent experiments.

edly inhibited using this mutant GAP, with dose–response analysis showing that this inhibition persisted even at high levels of the mutant (Fig. 8 B). Thus, taken together, the two complementary approaches that inhibited the GAP catalytic activity revealed that this activity promoted vesicle formation from Golgi membrane.

## Discussion

We have found that ARFGAP1, the GAP for ARF1 in COPI transport, plays a key role in the biogenesis of COPI vesicles in ways not previously suspected. Currently, the GAP catalytic activity is thought to promote COPI cargo sorting (Nickel et al., 1998; Lanoix et al., 1999) and inhibit vesicle formation (Bigay et al., 2003; Reinhard et al., 2003). However, we have revealed two additional activities of GAP that are relevant to understanding how COPI cargo sorting and vesicle formation are coupled. First, we have found that GAP plays a role in regulating the binding of coatomer to cargo proteins through its noncatalytic function, indicating that GAP rather than ARF1 is the more direct regulator in forming COPI priming complexes during cargo sorting. Second, we show that the GAP catalytic activity actually promotes vesicle formation rather than inhibiting this process when Golgi membrane instead of liposomal membrane is used to examine the formation of COPI vesicles. Together, these findings reveal that GAP plays a central role in coupling cargo sorting and vesicle formation.

Notably, the elucidation of how GAP functions in COPI priming complexes reveals that it does not require stoichiometric coupling to activated ARF1, as we have also found that most COPI cargo proteins form complexes that contain GAP and coatomer, but not ARF1. Thus, an obvious question in light of the general view that fundamental mechanisms of vesicular transport are highly conserved is why COPI transport does not

use an identical mechanism of cargo sorting as COPII transport. We suspect that the answer lies in the elucidated roles of the participating small GTPases. While Sar1p regulates COPII transport, ARF1 regulates multiple coat complexes (Randazzo et al., 2000). Moreover, ARF1 participates in other cellular events, such as actin rearrangement and intracellular signaling through the generation of lipid intermediates (Randazzo et al., 2000). Thus, a plausible explanation is that ARF1 is able to coordinate multiple cellular events by not having its activity being coupled solely to COPI transport. Consistent with this explanation, many more GEFs and GAPs have been identified for ARF1 as compared with those for Sar1p (Donaldson and Jackson, 2000; Randazzo et al., 2000), suggesting that more circumstances are needed in modulating the function of ARF1 as compared with that of Sar1p.

### SNARE cargo proteins as membrane receptors for activated ARF1

What might be the role of activated ARF1 binding to SNARE cargo proteins, if it does not participate directly in COPI priming complexes? ARF1 has been shown to interact with the p23 cargo protein (Gommel et al., 2001; Majoul et al., 2001). However, this interaction is specific for the deactivated form of ARF1 and requires a cross-linking approach to detect the interaction by biochemical means (Gommel et al., 2001). Moreover, we have not detected an interaction between p23 and inactive ARF1 using the pull-down approach. Thus, these observations suggest that the interaction between p23 and deactivated ARF1 is more likely to represent a mechanism by which cytosolic ARF1 is sufficiently stabilized on membrane to interact with membrane-bound GEFs for the activation of ARF1, as previously proposed (Gommel et al., 2001), rather than a mechanism of cargo sorting, for which protein interactions have been shown by many studies to be readily detectable by the pull-down approach (Springer and Schekman, 1998; Goldberg, 2000; Yang et al., 2002).

In considering an alternate explanation to describe the interaction between activated ARF1 and SNARE cargo proteins detected in the current paper, we are led by a previous study that has shown the stabilization of activated ARF1 on membrane to require interaction not only with lipids through its myristoyl sidechain, but also through protein interactions (Helms et al., 1993). Thus, we propose that the SNARE cargo proteins represent a class of membrane-bound “receptors” that are needed to stabilize activated ARF1 on membrane. Whether this interaction might also constitute another mechanism of cargo sorting for the SNARE cargo proteins will need to be addressed in more detail in the future.

We also note that our current findings on the binding of activated ARF1 to SNARE cargo proteins contradicts the results from a previous yeast study that has shown ARF1 to bind SNAREs independent of its activation state (Rein et al., 2002). However, this yeast study also shows that the binding of ARF1 to membrane is independent of its GTPase cycle (Rein et al., 2002), which contradicts the situation in the mammalian system where activation of ARF1 is required for binding to Golgi membrane (Donaldson et al., 1992). One explanation is that the

behavior of ARF1 in yeast is fundamentally different than that elucidated for the mammalian system. However, another possibility is based on technical considerations. The yeast membranes used in the previous study are microsomes developed originally to study the formation of COPII vesicles (Rein et al., 2002), suggesting the possibility that the ER membrane might contribute to the noted curious behavior of ARF1. Another possibility is that a truncated form of ARF1 that lacked the first 17 amino acids was used in the previous study (Rein et al., 2002), whereas in the current study we have found that ARF1 binds selectively to SNARE cargo proteins only when ARF1 is a full-length activated protein. Thus, these technical possibilities will need to be resolved before a firm conclusion can be made that the noted disparities for ARF1 and its GAP in yeast and mammalian systems represent true differences in function.

### **GAP activity promotes vesicle formation with implications for considering mechanisms of cargo sorting**

Using a COPI vesicle reconstitution system that involves incubating Golgi membrane with purified components, we have provided evidence recently that GAP functions to promote both cargo sorting and vesicle formation (Yang et al., 2002), implying that its catalytic activity does not antagonize vesicle coating. However, in two subsequent studies that use similar purified protein components, but in the context of liposomes rather than Golgi membrane, GAP is found to antagonize vesicle formation and promote vesicle uncoating (Bigay et al., 2003; Reinhard et al., 2003). In the current study, we have provided more precise ways of perturbing the GAP catalytic activity, such as the use of GMP-PNP and a GAP catalytic point mutant, as they block GAP activity without preventing GAP from interacting with ARF1. These perturbations profoundly affect the formation of COPI vesicles from Golgi membrane. Thus, the disparity begs the question as to why the GAP activity functions so differently on liposomes as compared with Golgi membrane. As the obvious difference is that biological membranes contain many membrane proteins, both integral and peripheral, a prediction is that one or more of these proteins play a role in aiding GAP to function in vesicle formation rather than in vesicle uncoating.

Our finding also indicates a need to revise the prevailing view that the translocation of coatamer between membrane and cytosol is strictly coupled to the GTPase cycle of ARF1 (Goldberg, 2000; Bigay et al., 2003; Reinhard et al., 2003). This "strict coupling" view has been challenged by a study using live cells (Presley et al., 2002). However, the conclusion of that study is limited by the caveat that ARF1 has multiple functions, including regulating multiple coat proteins and other cellular activities such as signaling and actin rearrangement (Donaldson and Jackson, 2000; Randazzo et al., 2000). Thus, one cannot rule out that a strict coupling mechanism indeed describes the pool of ARF1 that specifically participates in COPI transport. By addressing this caveat in the current study using a vesicle reconstitution system that specifically examines the role of ARF1 in COPI transport, we conclude that substantial experimental evidence now exists against the strict coupling view.

The significance of discarding the strict coupling view is that one no longer needs to consider the GAP catalytic activity, which is required for the deactivation of ARF1, as antagonistic to vesicle formation. This conclusion also suggests a different context to explain some key recent experimental observations regarding the regulation of GAP activity. In one report, coatamer is noted to enhance the catalytic activity of GAP, and a specific p24 family member inhibits this enhancement (Goldberg, 2000). These observations have led to a kinetic proofreading model whereby the GAP catalytic activity can be inhibited during vesicle formation, so that the two seemingly dichotomous functions of GAP, in promoting cargo sorting and inhibiting vesicle coating, can be satisfied (Goldberg, 2000). However, this model predicts a critical role for the p24 family member in vesicle formation, and yet, a yeast study has shown that the entire p24 family members can be deleted with minimal effects on transport (Springer et al., 2000). In another report, positive membrane curvature is noted to enhance the catalytic activity of GAP (Bigay et al., 2003), which has been interpreted in the context of vesicle uncoating. However, this interpretation predicts that the tip of the forming bud would be prone to releasing its coating, as it is subjected to increasingly high positive curvature that enhances GAP activity. To accommodate this problem, the study proposes that lateral interactions between coat components compensates for those ARF1 that would have been released at the bud tip, so that uncoating does not occur before vesicle fission (Bigay et al., 2003). Such an explanation essentially contradicts the general premise that membrane localization of coatamer is strictly coupled to the GTPase cycle of ARF1, as the explanation invokes a local exception to the rule. In contrast, for both cited examples, the noted difficulties in relating the experimental observations with mechanistic models would be alleviated if one views the GAP catalytic activity to promote vesicle formation.

## **Materials and methods**

### **Plasmids**

Constructs encoding for the cytoplasmic domain of cargo proteins were appended to the carboxy terminus of GST and were generated using either pGEX-KG or pGEX 4T-3 expression plasmids (Amersham Biosciences), or to the amino terminus of GST using pETGEX (from R. Scheckman, University of California, Berkeley, Berkeley, CA). Constructs encoding ERGIC53 (from H. Hauri, University of Basel, Basel, Switzerland), and p23, p24, p25, and p26 (from J. Gruenberg, University of Geneva, Geneva, Switzerland) were used in the PCR to append their cytoplasmic domains to GST. Other GST fusion constructs have been described previously: GST-Wbp1 (Cosson and Letourneur, 1994), GST-KDEL (Yang et al., 2002), GST-VHS and GST-VHSGAT (Dell'Angelica et al., 2000), GS15 (Xu et al., 1997), GS28 (Subramaniam et al., 1996), Ykt6 (Zhang and Hong, 2001), Syntaxin3 (Wong et al., 1999), and Syntaxin5 (Wong et al., 1999) fused to GST.

Construct encoding for ARFGAP1 fused to GST was generated in pGEX 4T-3, with silent mutations introduced in the ORF (AGA to CGC encoding for amino acid residues 5 and 7 in GAP, and AGG to CGC for residues 357 and 358) using the PCR-based mutagenesis approach, so that rare codon usage in bacteria is avoided (Makrides, 1996). His-tagged GAP1-136 was subcloned into pTrcHis (Invitrogen). Full-length ARFGAP1 point mutant (R50K) was generated through PCR mutagenesis of the wild-type GAP previously subcloned in the baculovirus transfer vector pVL1393 (BD Biosciences).

### **Proteins**

Previously described purification of proteins include: coatamer (Pavel et al., 1998), ARFGAP1 (Vitale et al., 2000), and ARF1 (Randazzo, 1997).



His-tagged, full-length, R50K point mutant GAP was expressed in a baculovirus system, as described previously for the wild-type form (Vitale et al., 2000). Truncated ARFGAP1 (1–136) and GST fusion proteins were expressed using a bacterial expression system and then purified, as described previously (Yang et al., 2002). Truncated ARF1 ( $\Delta$ 17) was obtained from P. Randazzo (National Institutes of Health, Bethesda, MD).

#### Other materials

Nucleotides (Sigma-Aldrich) and free peptides (New England Peptide) of the cytoplasmic domain of Wbp1 (KKLETFKKTN) and its corresponding diserine mutant (KKLETFSSTN) were obtained. Saturation of coatomer with the Wbp1-free peptide was accomplished by incubating 2.5 nM coatomer with 300  $\mu$ M free peptide. Antibodies used have been described previously: mouse CM1A10 against coatomer (Palmer et al., 1993), mouse M3A5 against  $\beta$ -COP (Allan and Kreis, 1986), rabbit antibody against ARF1 (Marshansky et al., 1997), and rabbit antibody against ARFGAP1 (Cukierman et al., 1995). The following antibodies were obtained from commercial sources: anti-six-histidine (6x-his) epitope (Santa Cruz Biotechnology, Inc.) and anti-myc epitope (9E10; American Type Culture Collection, Manassas, VA). Cytosol from transfected cells were prepared by permeabilizing cells with 0.2% saponin in PBS at RT for 10 min and then collecting the supernatant fraction after centrifugation at 15,000 g for 10 min. Transfections were performed using FuGENE 6 (Roche).

#### Loading ARF1 with guanine nucleotides

ARF1 (250 nM) was incubated at 32°C for 1.5 h with different nucleotides in a buffer containing 25 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.005 g BSA/L, and 0.1% Triton X-100. The different nucleotides used were: GTP (2 mM), GDP (2 mM), GMP-PNP (20  $\mu$ M), and GTP $\gamma$ S (20  $\mu$ M).

#### GST pull-down assays

Incubation of cargo proteins as GST fusion proteins on beads with soluble GAP (1 nM) and/or coatomer (2.5 nM) was performed at 4°C for 1 h using a previously described buffer (Lanoix et al., 2001) that consisted of 50 mM Hepes, pH 7.2, 300 mM NaCl, 90 mM KCl, 1 mM EDTA, and 0.5% NP-40. In titration experiments, various concentrations of the titrating component are indicated in specific figures. Incubation of ARFGAP1 as a GST fusion protein on beads with soluble coatomer (1.25 nM) was performed at 4°C for 1 h in a buffer containing 50 mM Hepes, pH 7.2, 90 mM KCl, 2.5 mM Mg(OAc)<sub>2</sub>, and 1% Triton X-100. All pull-down assays involving soluble ARF1, interacting with GST fusions of either cargo proteins or GAP on beads, were done using the condition described above for the loading of ARF1 with nucleotides, unless stated otherwise in figure legends. After the incubations, beads were pelleted by centrifugation (500 g for 2 min at RT) followed by three washes with the incubation buffer, and then analyzed by SDS-PAGE. Western blotting was performed using chemiluminescence (NEN Life Sciences) to detect the bound proteins. Coomassie blue staining was performed to detect the level of GST fusion protein on beads.

#### Reconstitution of COPI vesicles from Golgi membrane

The two-stage incubation system was performed as described previously (Yang et al., 2002). Ultracentrifugation of the supernatant from the second-stage incubation was performed at 200,000 g for 1 h using a rotor (model SW55; Beckman Coulter), after having scaled-up the two-stage incubation system by 10-fold. The resulting supernatant was recovered by TCA precipitation using BSA as carrier, with control experiments using a known quantity of purified protein revealing quantitative recovery of proteins.

#### Immunogold EM

Analysis of vesicles on grids was performed as described previously (Yang et al., 2002).

#### Image acquisition and display

Original gels were converted into digital images using a flatbed scanner (Perfection 1200U; Epson), and were then processed for figures using Adobe Photoshop 6.0 software. White lines in figures indicate that intervening lanes of gels were spliced out. Quantitation of gel bands was performed using Scion Image software.

#### Online supplemental material

Fig. S1 shows that activated ARF1 does not affect the binding of coatomer to different cargo proteins. Fig. S2 shows that the ARF1 effector domain of GGA interacts specifically with the activated forms of ARF1. Fig. S3 shows that different forms of ARF1 do not interact with p23 in pull-down assays. Fig. S4 shows that purified GAP and coatomer do not interact in

solution. Table S1 quantifies the relative level of binding seen in Fig. 2. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200404008/DC1>.

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