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ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes

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ABSTRACT **The coatomer is a cytosolic protein complex that reversibly associates with Golgi membranes and is implicated in modulating Golgi membrane transport. The association of β-COP, a component of coatomer, with Golgi membranes is enhanced by guanosine 5′-[γ-thio]triphosphate (GTP[γS]), a nonhydrolyzable analogue of GTP, and by a mixture of aluminum and fluoride ions (Al/F). Here we show that the ADP-ribosylation factor (ARF) is required for the binding of β-COP. Thus, β-COP contained in a coatomer fraction that has been resolved from ARF does not bind to Golgi membranes, whereas binding can be reconstituted by the addition of recombinant ARF. Furthermore, an N-terminal peptide of ARF, which blocks ARF binding to Golgi membranes, inhibits GTP[γS]- as well as the Al/F-enhanced binding of β-COP. We show that Golgi coat protein binding involves a sequential reaction where an initial interaction of ARF and GTP[γS] with the membrane allows subsequent binding of β-COP to take place in the absence of free ARF and GTP[γS]. The fungal metabolite brefeldin A, which is known to prevent the association of coat proteins with Golgi membrane, is shown to exert this effect by interfering with the initial ARF–membrane interaction step.**

Cytosolic coat proteins that reversibly bind to intracellular membranes play important roles in regulating vesicular traffic and organelle structure (1–3). Recent studies have led to the identification of a cytosolic protein complex, termed coatomer, which is found on non-clathrin-coated structures associated with Golgi membranes and Golgi-derived transport vesicles (4–7). The coatomer (≈800 kDa) consists of proteins designated α-, β-, γ-, and δ-COP, together with substoichiometric amounts of several other proteins (6, 7). Best characterized is the 110-kDa β-COP component (6, 8), which has homology in primary structure to the β-adaptin component of clathrin-coated vesicles (9). The association of β-COP (and presumably the entire coatomer) with the Golgi complex is sensitive to the fungal metabolite brefeldin A (10–12), which causes a rapid redistribution of the coat protein β-COP into the cytosol (10, 11). The brefeldin A-induced release of structural proteins such as the coatomer from Golgi membrane appears to underlie the dramatic effects of this drug on organelle structure and membrane traffic, including the formation of Golgi membrane tubules, mixing of Golgi membranes with the endoplasmic reticulum, and a block in secretion (13, 14).

GTP-binding proteins have been implicated in the regulation of multiple steps of membrane traffic through the secretory pathway (15). In *in vitro* Golgi transport assays, the addition of the nonhydrolyzable GTP analog guanosine 5′-[γ-thio]triphosphate (GTP[γS]) results in the accumulation of coated vesicles and in a concomitant inhibition of transport (16). A number of GTP-binding proteins (17, 18) have been localized to the Golgi complex, although their functions have not been defined. Distinct among these proteins is the ADP-ribosylation factor (ARF), originally identified as a cofactor required for *in vitro* cholera toxin-catalyzed ADP-ribosylation of the α subunit of the trimeric GTP-binding protein G_s ($G_{s\alpha}$) (19). ARF is an abundant cytosolic protein that reversibly associates with Golgi membranes (20, 21). ARF has been shown to be present on Golgi coated vesicles generated in the presence of GTP[γS], but it is not a component of the cytosolic coatomer (22).

The association of cytosolic ARF and coatomer (tracked with antibodies against the β-COP component) with Golgi-enriched membranes has been studied in a cell-free system (21). Binding of both ARF and β-COP is enhanced in the presence of GTP[γS] and inhibited by brefeldin A. GTP[γS] and brefeldin A act antagonistically: whereas pretreatment with GTP[γS] causes β-COP to be irreversibly bound and resistant to a subsequent addition of brefeldin A, treatment with brefeldin A inhibits coat binding and the inhibition is not readily reversed by the subsequent addition of GTP[γS] (21). In addition to GTP[γS], aluminum fluoride (a mixture of aluminum and fluoride ions, Al/F), known to activate trimeric G proteins (23), enhances the binding of β-COP to Golgi membranes (11, 21). This finding and the observation that βγ subunits of G proteins inhibit both ARF and β-COP binding (21) suggest that G proteins regulate coat protein binding.

Although ARF has been implicated as a Golgi "coat" protein, the precise cellular function of ARF is not known. Here we demonstrate that ARF plays an essential role in regulating coatomer binding. This activity of ARF provides a biochemical function for one of the multiple low molecular weight GTP-binding proteins that have been implicated in intracellular membrane traffic.

MATERIALS AND METHODS

Brefeldin A was obtained from Epicentre Technologies (Madison, WI) and stored in methanol as a 30 mM (8.4 mg/ml) stock solution at −20°C. GTP[γS] was purchased from Boehringer Mannheim. ATP (A3377 and A2383) and GTP were purchased from Sigma. Unless indicated A3377 ATP was used. Recombinant myristoylated ARF was purified from *Escherichia coli* coexpressing the human ARF1 gene and *N*-myristoyltransferase (24, 30). Peptides derived from sequences of human ARF1 and ARF4 were synthesized by Peptide Technologies (Washington) as described (25), and purified by reverse-phase HPLC to 95% purity.

Preparation of Golgi Membranes and Brain Cytosol. A Golgi-enriched membrane fraction was obtained from Chinese hamster ovary (CHO) cells by sucrose gradient centrif-

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Abbreviations: ARF, ADP-ribosylation factor; Al/F, mixture of aluminum and fluoride ions; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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ugation (26). Membranes were collected at the 0.84/1.02 M sucrose interface and stored at −80°C until use. Bovine brain cytosol was prepared as described by Malhotra *et al.* (4).

β-COP/ARF Binding Assay. Golgi membranes were incubated with bovine brain cytosol or comparable amounts of gel-filtered cytosol under conditions previously defined as favoring the GTP[γS]-induced accumulation of Golgi nonclathrin-coated vesicles (16, 21). Golgi membranes (10–12 μg of protein) and a saturating concentration of cytosol protein (3.0 mg/ml) were incubated at 37°C in a final volume of 0.2 ml in the presence of a reaction mixture consisting of 25 mM Hepes/KOH (pH 7.0), 25 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 M sucrose, 1 mM ATP, 5 mM creatine phosphate, and creatine kinase (10 units/ml). At the end of the incubation, the membranes were collected by centrifugation at 14,000 × *g* (Beckman TLA100.2) for 10 min at 4°C, and the sedimented material was suspended in SDS sample buffer and resolved by SDS/PAGE in Mini-protean 4–15% gradient ready gels (Bio-Rad). Proteins were transferred to nitrocellulose, and the nitrocellulose blot was cut in two sections. The upper section, containing β-COP, was incubated with a monoclonal antibody to β-COP (M3A5), and the lower part of the blot, containing ARF, was incubated with a monoclonal antibody to ARF (1D9) as described (21). Blots were sequentially incubated with rabbit antibody to mouse IgG and with ^{125}I-labeled protein A. Immunoblots were exposed to Kodak XAR film for 6–24 hr at −80°C with an intensifying screen. Quantitation of immunoblots was obtained by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Requirement of ARF for β-COP Binding. To investigate whether ARF was required for the binding of β-COP to Golgi membranes, we separated the coatomer from ARF by gel filtration of bovine brain cytosol on a Superose 6 FPLC column (Fig. 1). Fractions 28–32, containing the 800-kDa coatomer (7) and essentially free of ARF, were pooled. When this material, referred to as the coatomer fraction, was incubated with CHO Golgi membranes either in the absence or in the presence of GTP[γS] (25 μM), there was little binding of β-COP to the membrane (Fig. 2*A*). However, addition of purified, recombinant myristoylated ARF (10 μg/ml) restored GTP[γS]-dependent β-COP binding; ARF addition also caused a marginal increase in β-COP binding in the absence of GTP[γS]. The concentration of ARF required

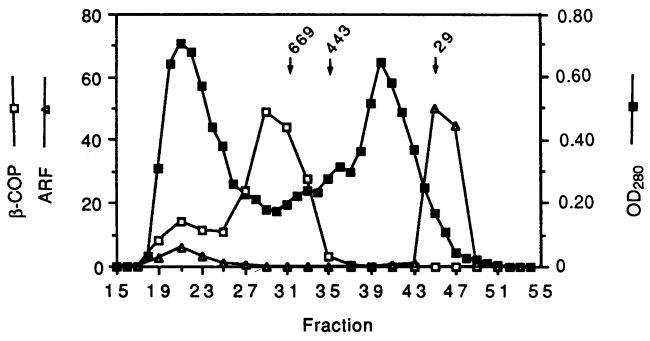

FIG. 1. Isolation of a coatomer fraction by gel filtration. Bovine brain cytosol (0.4 ml, 8 mg of protein) was applied to a 24-ml Superose 6 FPLC column (Pharmacia/LKB) and eluted with 0.1 M KCl/10 mM Tris, pH 7.8, at 0.4 ml/min. Fractions (0.4 ml) were collected and analyzed by SDS/PAGE and immunoblotting with antibodies to β-COP and ARF. The immunoblots were quantitated by PhosphorImager analysis and values are plotted in arbitrary units for β-COP and ARF. Arrows indicate the elution positions (OD_{280}) of molecular size markers of 669, 443, and 29 kDa.

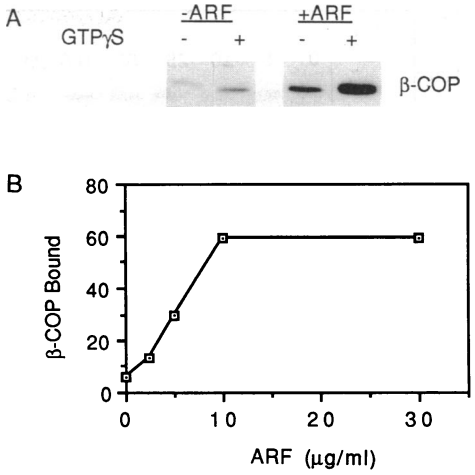

FIG. 2. ARF dependence of β-COP binding to Golgi membranes. (*A*) Golgi membranes were incubated for 15 min at 37°C with coatomer (0.1 ml of pooled fractions 28–32, from Fig. 1) in a reaction mixture in the presence or absence of GTP[γS] (25 μM) and recombinant ARF (10 μg/ml) before processing and immunoblotting with antibodies to β-COP. The amount of β-COP bound to the membranes was assessed by SDS/PAGE and immunoblotting with antibodies to β-COP. (*B*) Membranes were incubated as in *A* in the presence of GTP[γS] (25 μM) with increasing amounts of recombinant ARF. Shown is a quantitation of the immunoblot in arbitrary units.

for the reconstitution of GTP[γS]-dependent β-COP binding varied somewhat with different recombinant ARF preparations, showing a maximal effect between 10 and 30 μg/ml (see Fig. 2*B*). This concentration of ARF, although apparently high, is similar to that present in a typical Golgi transport (16) or coat protein binding (21) assay containing whole cytosol. β-COP binding could also be efficiently reconstituted with partially purified cytosolic ARF (data not shown). In the presence of ARF and GTP[γS], a significant portion of the β-COP present in the coatomer fraction (40–60%) bound to the membranes. The low level of GTP[γS]-enhanced binding of β-COP observed in the absence of added ARF (Fig. 2*B*) may be due to undetected levels of ARF in the coatomer fraction or in the membranes or may reflect some level of ARF-independent, GTP[γS]-induced coatomer binding.

Although recombinant ARF was able to reconstitute the binding of β-COP in the coatomer fraction, we sought to determine whether ARF function is also necessary for β-COP binding observed upon incubation of membranes with whole cytosol. To address this question, we tested the effect of an inhibitory N-terminal ARF peptide on GTP[γS]-induced binding of β-COP from unfractionated cytosol. This synthetic peptide, P-13 (corresponding to residues 2–17 of human ARF1), inhibits the ability of ARF to serve as a cofactor in the cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ and also inhibits an *in vitro* inter-Golgi transport assay (25). We observed that the binding of both ARF and β-COP to Golgi membranes in the presence of GTP[γS] (25 μM) was inhibited by P-13 with similar dose dependence for both proteins (Fig. 3*A*). As a control, we tested a series of ARF-derived peptides whose inhibitory activities in the cholera toxin ARF assay have been characterized (25) (see Fig. 3*B*). Peptide 26, which consists of residues 2–17 of another ARF gene product, ARF4, and P-27, a truncated form of P-13 (residues 4–17 of human ARF1), inhibited binding of both ARF and β-COP to Golgi membranes to an extent similar to that obtained with P-13. Peptides consisting of further N-terminal truncations of the inhibitory P-13 peptide, P-28 and P-29 (residues 6–17 and 8–17, respectively), and a C-terminal peptide, P-16 (residues 169–181 of human ARF1), were all unable to inhibit the binding of either ARF or β-COP. This specificity profile is

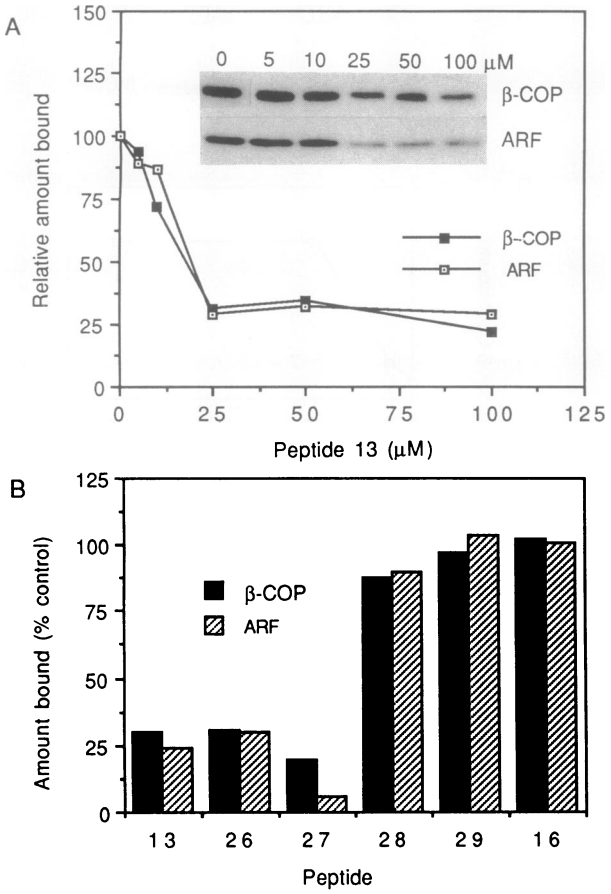

FIG. 3. Effect of inhibitory ARF peptides on GTP[γS]-dependent β-COP and ARF binding in whole cytosol. Binding of ARF and β-COP was assessed, as described in *Materials and Methods*, after incubation of Golgi membranes for 15 min at 37°C with cytosol in the presence of 25 μM GTP[γS] and increasing concentrations of peptide P-13 (*A*) or with different synthetic peptides derived from ARF proteins (50 μM each) (*B*). The synthetic peptides were as follows: P-13 (residues 2–17 of human ARF1), P-26 (2–17, human ARF4), P-27 (4–17, human ARF1), P-28 (6–17, human ARF1), P-29 (8–17, human ARF1), and P-16 (169–181, human ARF1).

similar to that observed in the cholera toxin and Golgi transport assays (25).

ARF and GTP Are Required for Fluoride-Dependent β-COP Binding. In addition to GTPγS, Al/F (a combination of 10 mM NaF and 30 μM $AlCl_3$) also increases β-COP binding to Golgi membranes, implicating a possible involvement of a trimeric G protein (11, 21). Although Al/F does not activate ARF (27), evidence that ARF is also required for Al/F-induced β-COP binding was provided by the finding that the addition of the inhibitory ARF peptide P-13 (50 μM) completely inhibited Al/F-dependent β-COP binding, whereas the inactive C-terminal ARF peptide had no effect (Fig. 4*A*).

The apparent requirement of ARF for β-COP binding in the presence of Al/F raised the question of whether ARF becomes activated under these conditions. Since activation of ARF requires GTP, it was expected that a nucleoside triphosphate would be required if active ARF is necessary for β-COP binding in the presence of Al/F. In initial experiments, we observed that the binding of β-COP to Golgi membranes in the presence of Al/F depended on the addition of ATP, whereas binding in the presence of GTP[γS] did not require ATP (data not shown). As many commercial preparations of ATP are contaminated with GTP, we further assessed the nucleotide requirement for the Al/F-promoted binding of β-COP to Golgi membranes. Addition of Sigma A-2383 ATP (0.5 mM), a preparation free of contaminating

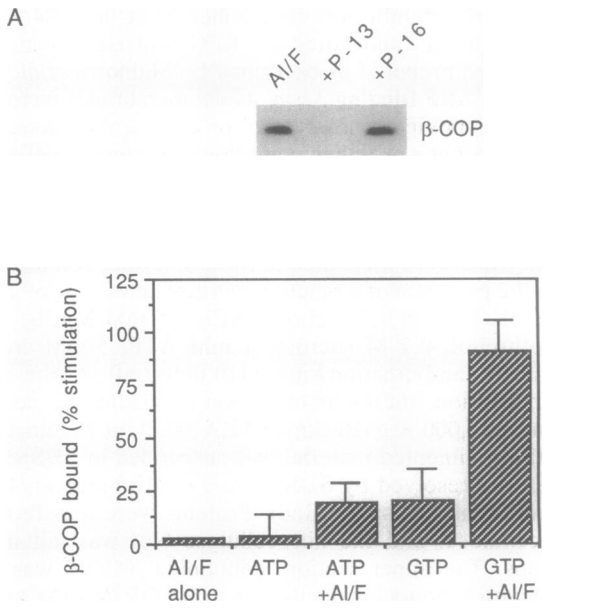

FIG. 4. Nucleotide requirements and peptide P-13 inhibition of Al/F-dependent β-COP binding. Membranes were incubated with cytosol and indicated additions for 15 min at 37°C before collection and processing for immunoblotting. (*A*) Incubation in the presence of 1 mM ATP (Sigma A3377) and Al/F, with or without peptides (50 μM). (*B*) Incubation with Al/F only, GTP-free ATP (Sigma A2383, 0.5 mM) alone or with Al/F, and GTP (10 μM) alone or with Al/F. Data are expressed as percent stimulation of binding above that observed in the absence of nucleotides and Al/F and are means ± SD of three to five experiments.

guanine nucleotide, did not support enhanced binding of β-COP upon addition of Al/F (Fig. 4*B*). However, the addition of only 10 μM GTP resulted in a full reconstitution of the Al/F-induced β-COP binding.

Sequential Interaction of ARF and β-COP with the Golgi Membrane. As GTP[γS] persistently activates many GTP-binding proteins, we investigated whether preincubation of membranes with ARF and GTP[γS] would allow a subsequent binding of β-COP to take place in the absence of free ARF or GTP[γS]. Golgi membranes were incubated with or without ARF and 1 μM GTP[γS] at 37°C for 10 min, and the free activators were removed by dilution and centrifugation. The resuspended membranes were subjected to a second incubation for 10 min in the presence of the coatomer fraction to allow β-COP binding. Preincubation with recombinant ARF and GTP[γS] led to a greatly enhanced β-COP binding in the second incubation (Fig. 5, lane 4). These results could not be due to a carryover from the first incubation, since after dilution and centrifugation, at most 0.1% of the initial concentration of GTP[γS] (1 μM) and ARF (30 μg/ml) was carried over into the second incubation. These concentrations of GTP[γS] (1 nM) and ARF (30 ng/ml) do not support enhanced coatomer binding (data not shown; see also Fig. 2*B*). The amount of β-COP bound was not further increased when 1 μM GTP[γS] and recombinant ARF were also included in the second incubation (lane 5), showing that the membranes became fully competent for β-COP binding following the first incubation. Both ARF and GTP[γS] were required, since each reagent alone had no effect above the control system that was preincubated in the absence of ARF and nucleotide (Fig. 5, lanes 2 and 3 vs. lane 1). These results suggest that ARF interacts with the membrane in a stage that precedes β-COP binding.

We asked at which stage does brefeldin A exert its effect that results in the inhibition of β-COP binding. When Golgi membranes were preincubated with GTP[γS] and ARF,

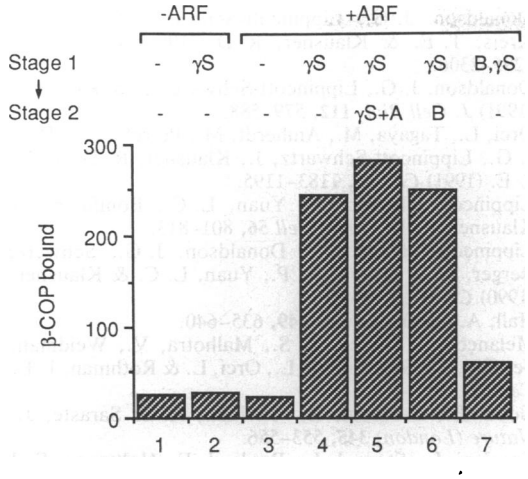

FIG. 5. Binding of β-COP to Golgi membranes following preincubation with GTP[γS] and ARF. Membranes were preincubated (Stage 1) in 0.1 ml of the standard reaction mixture with or without GTP[γS] (γS, 1 μM) in the presence or absence of recombinant ARF (30 μg/ml) for 10 min at 37°C. One sample (lane 7) was incubated with brefeldin A (200 μM) for 5 min prior to GTP[γS] addition (B,γS). The membranes were then diluted 5-fold with buffer containing 0.2 M sucrose and were pelleted at 14,000 × *g*. To assay for β-COP binding, the pellet was suspended in a total volume of 0.2 ml containing reaction mixture and 0.1 ml of a pooled coatomer fraction (see Fig. 2) and incubated at 37°C for 15 min (Stage 2). During the stage 2 incubation, GTP[γS] and ARF were added to one sample (lane 5) and brefeldin A (200 μM) was added to another (lane 6). The amount of β-COP bound after the second incubation is expressed in arbitrary units.

addition of brefeldin A to the second incubation had no effect on β-COP binding (Fig. 5, lane 6). In contrast, preincubation of Golgi membranes and ARF with brefeldin A for 5 min prior to the addition of GTP[γS] inhibited subsequent β-COP binding (lane 7). These results indicate that brefeldin A inhibits the GTP[γS]-dependent ARF–membrane interaction step rather than the ensuing binding of β-COP.

DISCUSSION

This study establishes an essential role of ARF in the regulation of binding of β-COP to the Golgi membrane. Thus, a β-COP fraction that had been resolved from ARF by gel filtration showed little β-COP binding activity, whereas addition of recombinant myristoylated ARF effectively restored both basal and GTP[γS]-dependent β-COP binding (Fig. 2). Supporting evidence for the role of ARF was provided by the finding that an inhibitory N-terminal ARF peptide (25) blocked ARF as well as β-COP association with the membrane (Fig. 3). Due to the unavailability of antibodies to other coatomer proteins, only the β-COP component could be followed in these experiments. However, Rothman and colleagues (3, 6, 7, 12) have shown that all the components of the cytosolic coatomer are present on Golgi-derived coated vesicles generated in the presence of GTP[γS]. We therefore assume in the following discussion that the observed binding of β-COP reflects the association of the entire coatomer complex to the Golgi membrane.

Further details about the role of ARF were provided by experiments where binding could be divided into two stages. These experiments showed that preincubation of membranes with ARF and GTP[γS] allows subsequent binding of β-COP to take place in the absence of free ARF and GTP[γS] (see Fig. 5). We conclude from this observation that the guanine nucleotide-dependent reaction involves only the membrane and ARF and that the interaction of the Golgi membrane with ARF and GTP[γS] makes the membrane fully competent for coatomer binding. These results clearly eliminate the coatomer or cytosol components (other than ARF) as possible sites of GTP[γS] action. However, we cannot exclude the possibility that the "activation" of the membranes which occurs in the presence of GTP[γS] and ARF involves other GTP-binding proteins, such as Rab-6 (17) and/or trimeric G proteins (18). In principle, an assessment of whether ARF is the sole GTP-binding protein required for coatomer binding could be investigated by testing the effect of GTP[γS]-prebound ARF on coatomer binding to Golgi membranes. However, this has proven technically difficult because, although ARF can be loaded with GTP[γS] in the presence of dimyristoyl phosphatidylcholine and cholate, binding under these conditions is rapidly reversible (19).

Previous observations suggested a possible role of G proteins in regulating coat protein binding to Golgi membranes (21). One argument in support of this was the ability of Al/F to substitute for GTP[γS] as an activator of β-COP binding. As Al/F is a potent activator of G proteins, but not of ARF (27), we investigated whether ARF has a role in Al/F-induced β-COP binding. The ability of inhibitory ARF peptides to block Al/F-induced binding of β-COP (Fig. 4) strongly implicates ARF in this process. A possible clue to the mechanism by which ARF supports coatomer binding in the presence of Al/F is provided by the finding that binding also depends on the presence of micromolar concentrations of GTP. As Al/F only activates the trimeric G proteins in the GDP-liganded state (28), it is likely that requisite GTP acts by activating ARF and/or other small GTP-binding proteins in the membrane, rather than a G protein. The requirement for both Al/F and GTP for enhanced coatomer binding might reflect the need to activate a G protein, in addition to ARF. One role of the G protein could be to enhance the formation of the active ARF–GTP complex.

It follows from the above that a GTP and Al/F-dependent activation may be expected to result in an increase in the amount of ARF bound to the membrane. While we did not detect enhanced ARF binding to our membranes in the presence of Al/F (21), Serafini *et al.* (22) did observe an increased amount of ARF associated with Golgi-derived coated vesicles that were generated in the presence of Al/F. The reason for these differences is not known but they may reflect detection limitations that are encountered in studies using Golgi-enriched membrane preparations as compared with studies using isolated coated vesicles.

A model of the ARF/coatomer binding cycle is shown in Fig. 6. This model suggests that, like other GTP-binding proteins, ARF cycles between GDP- and GTP-bound states, and that during activation cytosolic ARF–GDP interacts with a membrane receptor, whereupon GDP/GTP exchange converts it to the active, GTP-bound form that initiates the process of coatomer binding. A subsequent hydrolysis of GTP reverses the activation, resulting in the release of coatomer from the membrane.

The ARF–membrane interaction may be a site of action of drugs that alter membrane traffic through their ability to modulate coat protein assembly. As suggested by the two-stage incubation experiments, brefeldin A acts by inhibiting the initial ARF–membrane interaction step preceding coatomer binding. Previous studies have indicated that the target of brefeldin A is on the Golgi membrane (29). While the exact mechanism of action of brefeldin A remains to be defined, an intriguing possibility is that ARF and brefeldin A compete for a common membrane receptor. The action of Al/F, which results in enhanced coatomer binding both in intact cells and *in vitro*, could be explained if Al/F action results in either a stimulation of GDP/GTP exchange on ARF or an inhibition of the GTPase reaction (see Fig. 6). Further understanding of these mechanisms will require the identification of additional components (such as putative ARF

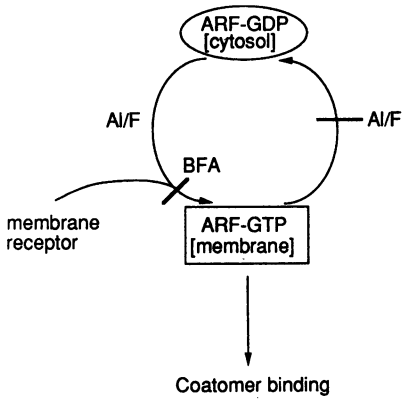

FIG. 6. A model of the ARF–coatomer binding cycle. The binding of coatomer to Golgi membrane is initiated by the binding of ARF–GTP to a membrane receptor. Brefeldin A (BFA) and Al/F may act to modulate the amount of membrane-bound ARF–GTP, resulting in an inhibition and enhancement of coatomer binding, respectively. (See text for details.)

exchange factors and GTPase-activating proteins) that are involved in the regulation of the ARF/coatomer binding cycle.

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