Coatomer, a complex of seven proteins, appears to be the precursor of the coat structure of non-clathrin-coated Golgi-derived vesicles. Another component of this vesicle coat is the cytosolic protein ADP-ribosylation factor (ARF). Like coatomer, ARF appears to reversibly associate with Golgi membranes.

We now report that ARF is required for coatomer binding to Golgi membranes and that myristoylated, but not non-myristoylated, ARF is the required species. We utilize an antibody directed against the β-subunit of coatomer (β-COP) to follow coatomer binding. ARF and β-COP bind stoichiometrically to Golgi membranes. ARF-dependent β-COP binding requires a membrane-associated protein, is saturable, and is enhanced in the presence of stable GTP analogues like guanosine 5'-O-(3-thiotriphosphate) (GTPγS). ARF and β-COP bind sequentially to Golgi membranes, since β-COP can be bound to reisolated membranes that had been previously incubated with ARF and GTPγS. We conclude that membrane-bound ARF confers to Golgi membranes all of the requirements for specific β-COP binding.

The coatomer was identified in cytosol as a soluble complex of about 700 kDa containing polypeptides of 160, 110, 98, and 61 kDa, designated α, β, γ, and δ-COP, respectively, and three smaller polypeptides (p36, p35, and p20; Waters et al., 1991). These same coat proteins (COPs) are found as components of Golgi-derived non-clathrin-coated vesicles (Serafini et al., 1991b). Accordingly, the coatomer was proposed to be an unassembled precursor of the vesicle coat. Evidence in support of this has recently been obtained by Orci et al. (1993) where it was found that the addition of coatomer to incubations of Golgi membranes and cytosol that was immunodepleted of coatomer resulted in coated vesicle production. Further characterization of the Golgi-derived vesicle coat revealed the presence of stoichiometric amounts of low molecular mass GTP-binding proteins which were identified as members of the ADP-ribosylation factor (ARF) family (Serafini et al., 1991a). Although these two subunits of the vesicle coat, coatomer, and ARF, have been identified as components of cytosol, the mechanism by which they bind to Golgi membranes and assemble into coated buds and vesicles is poorly understood. In the present paper, we have characterized an early step in this process, namely the binding of coatomer and ARF to Golgi membranes.

ARF was initially identified as a cofactor required for the cholera toxin-catalyzed ADP-ribosylation of the α subunit of the trimeric G protein G₁₃, (Schleifer et al., 1982; Kahn and Gilman, 1984). The localization of ARF to the Golgi complex in mammalian cells and the secretion phenotype in yeast of an arf1 mutation (Stearns et al., 1990), as well as its subsequent identification as a structural component of Golgi-derived coated vesicles (Serafini et al., 1991a) has implicated ARF in the process of Golgi transport. Synthetic peptides corresponding to the N-terminal region of ARF that inhibit ARF function, as determined by ARF-dependent ADP-ribosylation of G₁₃, by cholera toxin, also inhibit an assay that reconstitutes transport between cis and medial Golgi (Kahn et al., 1992). This inhibition correlated with a block in the assembly of coated vesicles, suggesting that ARF is required for coated vesicle budding. Similar studies with other in vitro assays have implicated ARF in ER to Golgi transport (Birch et al., 1992) and in endosome-endosome fusion (Lenhardt et al., 1992). Mammalian ARFs have a conserved glycine at amino acid residue 2 (Tsucui et al., 1991), which is the site of attachment of myristate (Kahn et al., 1988). The role of myristoylation in ARF function is not clear; however, it is thought to be required for membrane attachment (Serafini et al., 1991a; Kahn et al., 1992).

β-COP and ARF bind reversibly to Golgi membranes in a manner that is affected in vitro by guanine nucleotides and brefeldin A (Orsi et al., 1991; Duden et al., 1991; Donaldson et al., 1991a, 1991b). Stable GTP analogues, such as GTPγS, promote the association of β-COP and ARF with membranes whereas brefeldin A appears to inhibit their binding. Although ARF is a GTP-binding protein (Kahn and Gilman, 1986), increased β-COP and ARF binding in the presence of GTPγS may also be due to the involvement of a trimeric G protein or another low molecular mass GTP-binding protein. Examples of both of these types of GTP-binding protein have been localized to the Golgi apparatus; G₁₃ (Efroleague et al., 1990).

1 The abbreviations used are: ARF, ADP ribosylation factor; BFA, brefeldin A; GMP-PNP, β,γ-imidoguanosine 5'-triphosphate; GTPγS, guanosine 5'-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
and rabbp (Goud et al., 1990). The involvement of a trimeric G protein is indicated by the observation that purified βγ subunits of G proteins inhibit the binding of β-COP and ARF (Donaldson et al., 1991a). Furthermore, the AIF1 ion which activates trimeric G proteins (Gilman, 1987) but not GTP-binding proteins of low molecular mass, including ARF (Kahn, 1991) and mastoparan both promote β-COP association with Golgi membranes (Donaldson et al., 1991a; Kitagakis et al., 1992). A trimeric G protein has previously been implicated in Golgi transport since AIF1 inhibits intra-Golgi transport in vitro (Melançon et al., 1987).

We have found that the binding of β-COP to Golgi membranes requires a cytosolic factor in addition to the coatmer, and we demonstrate that this factor is ARF.

MATERIALS AND METHODS

Brefeldin A (Sigma) was stored at -20 °C as a 10 mM stock in methanol. Peroxidase and alkaline phosphatase-conjugated secondary antibodies were obtained from Bio-Rad, L-1-lysylamido-2-phenylalanine-conjugated cholera toxin and ketone-trypsinogen was obtained from Sigma.

Preparation of Cytosol, Golgi Membranes, Coatmer, and ARF—Bovine brain cytosol (18 mg of protein/ml) was prepared as described by Malhotra et al. (1989). Golgi-enriched membranes were prepared from rabbit livers (Pel-Freez Biologicals, Rogers, AR) as described by Malhotra et al. (1989). ARF was purified from 1 mg of washed ARF-Sepharose (Pharmacia) as described (Clary et al., 1989). ARF was coupled to CNBr-activated Sepharose according to the manufacturer’s instructions (Pharmacia, LKB Biotechnology Inc.).

Preparation of Affinity Purified Antibodies against ARF—Non-myristoylated ARF1 was used to elicit polyclonal antibodies in rabbits by East Acres Biologicals (Southbridge, MA). An IgG fraction was isolated from immune serum by chromatography on protein A-agarose columns as described by Ey et al. (1987). Immunoprecipitated IgG, ARF was coupled to CNBr-activated Sepharose according to the manufacturer’s instructions (Pharmacia, LKB Biotechnology Inc.). The IgG fraction (190 mg of protein, at 10 mg/ml in phosphate-buffered saline (PBS)) was passed three times over an ARF-Sepharose column (1 mg ARF/ml, 1 × 15 cm, 12-ml bed volume) at 18 ml/h. The column was then washed with 300 ml of PBS, and the ARF-specific IgG was eluted sequentially with 0.1 M glycine, pH 2.5, and 0.1 M triethylamine, pH 11.5, at 18 ml/h. The chromatography was essentially as described by Ey et al.

Immunodepletion of Coatmer from Bovine Brain Cytosol—CM1A10, a mouse monoclonal IgG, was bound to protein G-agarose by incubation of CM1A10 ascites (7.4 mg IgG/ml) with 1/6 volume of protein G-agarose (Calbiochem, San Diego, CA) for 16 h at 4 °C with mixing. The agarose beads were washed four times with 10 volumes of 0.2 M sodium borate, pH 9.0, at 4 °C, and the bound antibody was coupled to the protein G-agarose by incubation with 20 mM dimethylsulfoxide in 0.2 M sodium borate, pH 9.0, at room temperature for 30 min with mixing. The cross-linking reaction was terminated by extensive washing with 0.2 M ethanolamine, pH 8.0. The CM1A10-protein G-agarose (8.8 mg IgG/ml of 50% suspension) was stored in PBS at 4 °C. Bovine brain cytosol (18 mg of protein/ml) was immunodepleted of coatmer by sequential incubations with CM1A10-protein G-agarose (13 μg IgG/ml of protein/incubation) for 6 h at 4 °C.

Immunoprecipitation of Coatmer from Bovine Brain Cytosol—Bovine brain cytosol (1.8 mg of protein) was incubated with 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl, pH 7.4, (immunoprecipitation buffer) and 15 mg of CM1A10 (0.4 μg/ml of myristoylated ARF) that had been covalently coupled to 1 ml M-agarose (13 μg of IgG, 0.75 μl of protein G-agarose) in a volume of 1 ml, overnight at 4 °C with mixing. The agarose beads were then recovered by centrifugation and washed five times with 1 ml of immunoprecipitation buffer. The immunoprecipitated material was subjected to SDS-PAGE using a 9% gel under reducing conditions followed by Coomassie Blue staining.

Western Blot Analysis—Proteins were fractionated by SDS-PAGE under reducing conditions (Laemmli, 1970) and electroblotted on to nitrocellulose in 25 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS at 22 V/cm for 1 h. β-COP was detected using the mouse monoclonal antibody M2A5 (0.4 μg/ml) and goat anti-mouse IgG (1 μl of coatmer) by imaging with a Scan Jet Plus scanner (Hewlett Packard, CA) and integration of images using the ScanAnalysis software from BioSoft (Cambridge, United Kingdom). Analysis of samples for both β-COP and ARF was achieved by fractionation using 8–15% polyacrylamide gels and immunodepletion of β-COP and ARF on the top and bottom halves of nitrocellulose blots, respectively. β-COP and ARF Binding Assays—Incubations were carried out in 0.5-ml silanized tubes (Marsh, Rochester, NY) for 20 min at 37 °C in the presence of 2 mg/ml soybean trypsin inhibitor, 25 mM HEPES/ KOH, pH 7.2, 25 mM KCl, 0.2 mM sucrose, 2.5 mM magnesium acetate and unless indicated otherwise 50 μM ATP, 2 mM creatine phosphate, and 8 IU/ml creatine kinase. GTP-γ-S and GTP were used at 20 μM. Incubation volumes were 50 μl, unless indicated otherwise. The amounts of salt-extracted rabbit liver Golgi membranes, coatmer, ARF, bovine brain cytosol, coatmer-depleted bovine brain cytosol, or the 10–70-kDa fraction of bovine brain cytosol present in incubations are indicated in the figure legends. Assays containing 3H-myristoylated ARF were 100 μl in volume. After incubation these were divided into two 50-μl aliquots which were used separately to determine the amount of membrane-bound β-COP or N-myristoylated ARF. Incubations were washed twice in micrococcal nuclease on 150 μl of 15% sucrose, 25 mM HEPES-KOH, pH 7.2, 20 mM KCl, centrifugation, and using 0.5-ml tubes that had been previously incubated with 10 mg/ml bovine serum albumin and washed with water, for 30 min in a microcentrifuge at 4 °C. To measure the amount of ARF. For the determination of membrane-bound N-myristoylated ARF, membrane pellets were counted after solubilization with 40 μl of 0.5% Triton X-100 in PBS, in a scintillation counter.
For trypsin-treatment of membranes, salt-extracted rabbit liver Golgi membranes (80 µg of protein/ml final concentration) were incubated with 1-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin (0–80 µg/ml final concentration) for 5 min at 20 °C in a volume of 40 µl. Soybean trypsin inhibitor, coatomer, myristoylated ARF, and GTP-γS were then added in a volume of 10 µl, to give 2 mg/ml, 10 pg/ml, 20 pg/ml, and 20 µM final concentrations, respectively. The mixtures were then incubated and processed as described above. In mock trypsin-treated samples, soybean trypsin inhibitor was added before trypsin.

For two-stage incubations where the Golgi membranes were reisolated after the first incubation (Stage I), Stage I reactions (200 µl) contained salt-extracted rabbit liver Golgi membranes (160 µg of protein/ml) and, where indicated, myristoylated ARF (40 µg/ml). After incubation the reaction mixtures were overlaid on sucrose step gradients of 35% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4 (80 µl), and 25% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, (350 µl) in 5 × 41-mm tubes. Gradients were centrifuged in the SW55Ti rotor with adaptors for these tubes for 1 h at 30,000 revolutions/min at 4 °C. The membranes were recovered from the 25–35% interface in a volume of 80 µl, and 10 µl of this (assumed to contain 4 µg of the original membrane protein) was used in each Stage II incubation. The amounts of membranes and ARF in Stage I incubations are indicated in the figure legends as if they were present in Stage II.

RESULTS

Characterization of Antibodies against Coatomer and ARF

Immunoprecipitation of Coatomer from Bovine Brain Cytosol with CM1A10—CM1A10 is a monoclonal IgG2a produced after immunization of mice with native coatomer. This antibody does not react with coatomer subunits in a Western blot analysis, so the antigenic subunit is not known. CM1A10 was characterized by immunoprecipitation of coatomer from cytosol. Bovine brain cytosol was subjected to immunoprecipitation using CM1A10-protein G-agarose. Analysis of the immunoprecipitated material by SDS-PAGE revealed the presence of major Coomassie Blue-staining bands that corresponded to the α, β, γ, and δ subunits of coatomer (Fig. 1a). The presence of β-COP in the immunoprecipitate was confirmed by Western blot analysis using the monoclonal antibody M3A5 (data not shown). The immunoprecipitate from cytosol also contained major Coomassie Blue-staining bands of relative molecular mass 55,000 and 25,000. These components appeared to be bovine IgG chains precipitated from cytosol since they reacted with antibodies against bovine IgG, but not with antibodies against mouse IgG, in a Western blot analysis (data not shown). Furthermore, the amount of CM1A10 antibody that was released from CM1A10-protein G-agarose was relatively low (Fig. 1a). Incubation of bovine brain cytosol with protein G-agarose that did not contain antibody appeared to result in the precipitation of IgG related components only (data not shown). Thus, CM1A10 appears to be specific for the coatomer.

Western Blot Analysis of Bovine Brain Cytosol with Antibodies against ARF—An IgG fraction reacting with ARF was affinity purified from a rabbit antiserum by sequential chromatography on protein A-agarose and ARF-Sepharose. These antibodies appeared to be specific for bovine brain ARF in a Western blot analysis (Fig. 1b).

β-COP Binding to Golgi Membranes Requires Myristoylated ARF

Incubation of purified coatomer with Golgi membranes resulted in the binding of a small amount of β-COP to membranes (Fig. 2a). A similar level of binding was observed in the presence of GTP or GTP-γS. The level of β-COP binding was significantly increased when cytosol that had been immunodepleted of coatomer was included in incubations.
tions with coatamer, membranes, and GTPγS (Fig. 2a). With this condition approximately 25% of the β-COP present in the incubation bound to membranes. The coatamer-depleted cytosol contained approximately 15% of the original level of coatamer, as determined by Western blot analysis for β-COP (Orci et al., 1993); however, the remaining coatamer did not appear to contribute a significant amount of β-COP in these experiments (Fig. 2a). High levels of β-COP binding were also obtained when a low molecular mass fraction of cytosol (10–70 kDa) that contains ARF, prepared by gel filtration chromatography, or when recombinant myristoylated ARF were incubated with coatamer, membranes, and GTPγS (Fig. 2a). Myristoylated ARF restored the ability of GTPγS to enhance β-COP binding relative to the binding in the presence of GTP.

This enhancement of approximately 5-fold was similar to that observed with the 10–70-kDa fraction of cytosol and coatamer-depleted cytosol (Fig. 2a) and is in agreement with results obtained with unfractionated cytosol (data not shown; Donaldson et al., 1991a). The level of β-COP binding obtained with GTPγS in the presence of coatamer-depleted cytosol, the 10–70-kDa fraction of cytosol, or myristoylated ARF was similar. This correlated with a similar level of ARF binding in these incubations (Fig. 2b).

ARF which was not myristoylated did not stimulate β-COP binding to membranes (Fig. 2a) nor did it appear to bind to membranes (Fig. 2b), indicating that the myristyl moiety is required for ARF binding and suggesting that ARF-dependent β-COP binding requires ARF in a membrane-bound but not soluble form.

Less than 1% of the β-COP present was recovered as a “pellet” from incubations that did not contain membranes (data not shown). This level was not increased by the presence of GTPγS, coatamer-depleted cytosol, the low molecular mass fraction of cytosol, or ARF. Thus, these experiments reflect interaction of β-COP with membranes and not simple aggregation of β-COP-containing complexes.

To determine whether β-COP and ARF associate with Golgi membranes in a stoichiometric fashion, ARF was titrated into incubations containing an amount of coatamer that was saturating with respect to the range of ARF concentration used. Where the amount of ARF bound increased linearly with increasing ARF concentration (Fig. 3a) there was a corresponding linear increase in the amount of β-COP bound (Fig. 3b), indicating that these components bind stoichiometrically to membranes. The data presented in Fig. 3 indicate a stoichiometry of 14.7 mol of membrane-bound ARF/1 mol of membrane-bound β-COP. This ratio varied between experiments when different preparations of salt-washed Golgi membranes and myristoylated ARF were used. Overall, the range of membrane-bound ARF was 11 to 17 mol/mol of membrane-bound β-COP.

ARF binding to Golgi membranes requires a membrane-associated protein (Serafini et al., 1991a) so it was expected that β-COP binding would also be diminished by protease pretreatment of Golgi membranes. Fig. 4a shows that ARF-dependent β-COP binding is significantly reduced by pretreatment of Golgi membranes with trypsin (Fig. 4a). This corresponded to a decrease in ARF binding (Fig. 4b). The decrease in β-COP and ARF binding appeared to be due to protease activity during the trypsin-pretreatment of membranes and not to the presence of trypsin during the subsequent binding reaction, since Western blot analysis of the supernatants recovered after the binding reactions showed that the unbound β-COP and ARF were not degraded (data not shown). Also, mock trypsin treatment of membranes did not appear to affect the levels of β-COP (Fig. 4a) and ARF (not shown) binding.

ARF-dependent β-COP binding appears to be a saturable process (Fig. 5). The near-saturating amount of β-COP bound at the highest coatamer concentration tested, 150 µg/ml in Fig. 5, corresponded to 0.39 ± 0.13 pmol (± standard deviation, n = 8) of β-COP bound/microgram of salt-extracted Golgi membrane protein. The apparent saturability was not due to kinetics of β-COP or ARF binding, since increasing the time of incubation did not lead to an increased level of membrane-bound β-COP (not shown).

Relatively high levels of ARF-dependent β-COP binding were observed in the presence of the stable GTP analogues, GTPγS and GMP-PNP (Fig. 6). In the presence of GTPγS the binding did not appear to be significantly affected by the addition of ATP or by the depletion of any endogenous ATP with hexokinase. Low levels of ARF-dependent β-COP binding in the absence of stable GTP analogues was observed with either GTP or ATP. There was no additive effect of combining GTP with ATP.

**ARF Binding to Golgi Membranes Precedes β-COP Binding**

Recombinant myristoylated ARF binds to Golgi membranes without a requirement for other soluble proteins (Helms and Rothman, 1992; Helms et al., 1993). This suggested the possibility that β-COP might bind without a requirement for other factors to membranes that had been “preloaded” with ARF. When membranes were preincubated with myristoylated ARF and GTPγS (Stage I) a high level of β-COP binding...
After incubation the membranes were recovered by centrifugation, and membrane-bound β-COP was determined by Western blot analysis and densitometry. The means of duplicate measurements are shown. Error bars indicate half of the difference between duplicates. Panel b, the Western blot in panel a was also reacted with affinity purified antibodies against ARF. The regions of the blot containing ARF and β-COP corresponding to binding assays with trypsin-treated membranes are shown.

**FIG. 4.** ARF-dependent β-COP binding to Golgi membranes requires a membrane-associated protein. Panel a, salt-extracted Golgi membranes (80 µg of protein/ml) were treated with trypsin in the absence (●) or presence (○) of soybean trypsin inhibitor for 5 min at 20 °C. The trypsin-treated membranes were incubated with coatomer (10 µg/ml), myristoylated ARF (20 µg/ml), and GTPγS. After incubation the membranes were recovered by centrifugation, and membrane-bound β-COP was determined by Western blot analysis and densitometry. The means of duplicate measurements are shown. Error bars indicate half of the difference between duplicates. Panel b, the Western blot in panel a was also reacted with affinity purified antibodies against ARF. The regions of the blot containing ARF and β-COP corresponding to binding assays with trypsin-treated membranes are shown.

**DISCUSSION**

We have shown that β-COP binding to Golgi membranes requires myristoylated ARF and that ARF appears to account for the binding of β-COP to Golgi membranes. This phenomenon was observed in a subsequent incubation (Stage II) without the readdition of ARF or GTPγS (Fig. 7a). The addition of GTP or GTPγS in Stage II did not affect the level of β-COP binding relative to the addition of no guanine nucleotide (data not shown). Preincubation with ARF and GTP did not give rise to an increased level of β-COP binding relative to prein-cubation of membranes with GTP or GTPγS alone. This is in agreement with the observation that the level of ARF binding with GTP is relatively low (Helms et al., 1993). These data indicate that ARF and β-COP bind sequentially to Golgi membranes and that GTPγS is required only for ARF binding. A surprising observation was that β-COP binding to membranes that had been preincubated with ARF and was observed in a subsequent incubation (Stage II) without the readdition of ARF or GTPγS (Fig. 7a). The addition of GTP or GTPγS in Stage II did not affect the level of β-COP binding relative to the addition of no guanine nucleotide (data not shown). Preincubation with ARF and GTP did not give rise to an increased level of β-COP binding relative to preincubation of membranes with GTP or GTPγS alone. This is in agreement with the observation that the level of ARF binding with GTP is relatively low (Helms et al., 1993). These data indicate that ARF and β-COP bind sequentially to Golgi membranes and that GTPγS is required only for ARF binding. A surprising observation was that β-COP binding to membranes that had been preincubated with ARF and GTPγS did not require incubation at 37 °C and occurred with incubation on ice (Fig. 7a). This is in striking contrast to incubations containing ARF and coatomer, where there is no apparent ARF-dependent β-COP binding with incubation on ice (preincubation with GTPγS in Fig. 7a). In agreement with this, the binding of myristoylated ARF, in the absence of coatomer, to Golgi membranes is temperature-sensitive (Helms et al., 1993).

The fungal metabolite brefeldin A inhibits the binding of ARF and β-COP in incubations with cytosol (Donaldson et al., 1991a) and the binding of recombinant ARF to Golgi membranes (Helms and Rothman, 1992). We were interested to determine whether brefeldin A also prevents binding of β-COP following ARF binding in a two-stage experiment. The presence of brefeldin A during the preincubation of membranes with ARF and GTPγS in Stage I led to a significant reduction in the subsequent level of β-COP binding during Stage II (Fig. 7b). This correlated with a reduced level of ARF binding, as determined by Western blot analysis (data not shown). The effect of brefeldin A was reversible since β-COP binding was restored by the addition of ARF and GTPγS to the Stage II incubation (Fig. 7b). Brefeldin A had no effect on the level of β-COP binding during Stage II to membranes that had been preincubated with ARF and GTPγS in the absence of brefeldin A (Fig. 7b), indicating that this drug affects only ARF binding.

**FIG. 5.** ARF-dependent β-COP binding to Golgi membranes is a saturable process. Salt-extracted Golgi membranes (15 µg of protein/ml) were incubated with GTPγS and increasing amounts of coatomer and myristoylated ARF. In each incubation, the ratio of coatomer/ARF was 1:2 (w/w). After incubation the membranes were recovered by centrifugation, and membrane-bound β-COP was determined by Western blot analysis and densitometry.

**Fig. 6.** Nucleotide requirement of β-COP binding to Golgi membranes. Salt-extracted Golgi membranes (80 µg of protein/ml) were incubated with coatomer (10 µg/ml) in the presence or absence of myristoylated ARF (20 µg/ml) and with various combinations of nucleotides, nucleotide analogues, or hexokinase as indicated. After incubation, membranes were recovered by centrifugation, and membrane-bound β-COP was determined by Western blot analysis and densitometry. An ATP-regenerating system was present with ATP (50 µM). GTPγS and GTP were present at 20 µM. GMP-PNP was present at 50 µM. Glucose (25 mM) was included in incubations with hexokinase (hexo; 4 units/ml). Data are representative of three experiments.

The fungal metabolite brefeldin A inhibits the binding of ARF and β-COP in incubations with cytosol (Donaldson et al., 1991a) and the binding of recombinant ARF to Golgi membranes (Helms and Rothman, 1992). We were interested to determine whether brefeldin A also prevents binding of β-COP following ARF binding in a two-stage experiment. The presence of brefeldin A during the preincubation of membranes with ARF and GTPγS in Stage I led to a significant reduction in the subsequent level of β-COP binding during Stage II (Fig. 7b). This correlated with a reduced level of ARF binding, as determined by Western blot analysis (data not shown). The effect of brefeldin A was reversible since β-COP binding was restored by the addition of ARF and GTPγS to the Stage II incubation (Fig. 7b). Brefeldin A had no effect on the level of β-COP binding during Stage II to membranes that had been preincubated with ARF and GTPγS in the absence of brefeldin A (Fig. 7b), indicating that this drug affects only ARF binding.

**DISCUSSION**

We have shown that β-COP binding to Golgi membranes requires myristoylated ARF and that ARF appears to account for the binding of β-COP to Golgi membranes. This phenomenon was observed in a subsequent incubation (Stage II) without the readdition of ARF or GTPγS (Fig. 7a). The addition of GTP or GTPγS in Stage II did not affect the level of β-COP binding relative to the addition of no guanine nucleotide (data not shown). Preincubation with ARF and GTP did not give rise to an increased level of β-COP binding relative to prein-cubation of membranes with GTP or GTPγS alone. This is in agreement with the observation that the level of ARF binding with GTP is relatively low (Helms et al., 1993). These data indicate that ARF and β-COP bind sequentially to Golgi membranes and that GTPγS is required only for ARF binding. A surprising observation was that β-COP binding to membranes that had been preincubated with ARF and was observed in a subsequent incubation (Stage II) without the readdition of ARF or GTPγS (Fig. 7a). The addition of GTP or GTPγS in Stage II did not affect the level of β-COP binding relative to the addition of no guanine nucleotide (data not shown). Preincubation with ARF and GTP did not give rise to an increased level of β-COP binding relative to prein-cubation of membranes with GTP or GTPγS alone. This is in agreement with the observation that the level of ARF binding with GTP is relatively low (Helms et al., 1993). These data indicate that ARF and β-COP bind sequentially to Golgi membranes and that GTPγS is required only for ARF binding. A surprising observation was that β-COP binding to membranes that had been preincubated with ARF and
An important observation was that $\beta$-COP binding to Golgi membranes containing prebound ARF with incubation on ice. This is a characteristic typical of receptor-ligand interactions (Silverstein et al., 1977) and suggests that membrane-bound ARF may act as a $\beta$-COP/coatomer receptor. The data available at present do not allow a distinction to be made between the possibilities that $\beta$-COP/coatomer binds directly or indirectly to ARF. However, it is attractive to propose the involvement of a third stoichiometric component, provided by membranes in binding assays, as this could account for the observed...
stoichiometric binding of ARF and β-COP as well as the conclusion that only a fraction of membrane-bound ARF participates in β-COP binding. Coatomer would be envisioned as binding to a site created by the interaction of ARF and this component. A complex formed between ARF, coatomer, and a membrane-associated component could be the unit from which coated buds and vesicles are formed. We note from other work (Orci et al., 1993) that membrane-bound ARF was associated with coated buds and vesicles only when incubations were performed in the presence of coatomer. In the absence of coatomer, the majority of membrane-bound ARF was distributed over the Golgi cisternae. This suggests the possibility that a complex of ARF, coatomer, and a membrane-associated component is assembled independent of bud formation and that this complex then becomes incorporated into a growing bud.

Acknowledgments—We thank Sidney Whiteheart and Gary Tani-gawa for comments on the manuscript.

REFERENCES