Biochemical Dissection of AP-1 Recruitment onto Golgi Membranes

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Abstract. Recruitment of the Golgi-specific AP-1 adaptor complex onto Golgi membranes is thought to be a prerequisite for clathrin coat assembly on the TGN. We have used an in vitro assay to examine the translocation of cytosolic AP-1 onto purified Golgi membranes. Association of AP-1 with the membranes required GTP or GTP analogues and was inhibited by the fungal metabolite, brefeldin A. In the presence of GTPγS, binding of AP-1 to Golgi membranes was strictly dependent on the concentration of cytosol added to the assay. AP-1 recruitment was also found to be temperature dependent, and relatively rapid at 37°C, following a lag period of 3 to 4 min. Using only an adaptor-enriched fraction from cytosol, purified myristoylated ARF1, and Golgi membranes, the GTPγS-dependent recruitment of AP-1 could be reconstituted. Our results show that the association of the AP-1 complex with Golgi membranes, like the coatomer complex, requires ARF, which accounts for the sensitivity of both to brefeldin A. In addition, they provide the basis for a model for the early biochemical events that lead to clathrin-coated vesicle formation on the TGN.

CLATHRIN-coated vesicles are believed to mediate the selective exit of both lysosomal and secretory granule constituents from the TGN (24, 38, 54). As these vesicular intermediates bud from the surface of the TGN, new clathrin-coated pits must be generated by the recruitment of cytosolic adaptors and clathrin onto the Golgi membrane. Clathrin-coated vesicles also form at the cell surface during receptor-mediated endocytosis (38). While both plasma membrane- and Golgi-derived clathrin-coated vesicles contain clathrin as the major protein component, the vesicles can be differentiated on the basis of the copolymerizing adaptors. Immunofluorescence studies have demonstrated that the distribution of the AP-1 adaptor heterotetramer, composed of 100-kD γ- and β′-adaptin subunits, and two smaller polypeptides of 47 and 20 kD, is restricted to the Golgi complex (2). In contrast, the AP-2 adaptor, composed of 100-kD α- and β-adaptin subunits, and two different polypeptides of 50 and 17 kD, shows a punctate fluorescence pattern consistent with a plasma membrane localization (2, 43). Encoding cDNAs for α-adaptin (44), β-adaptin (23, 40), and γ-adaptin (45) subunits have been isolated. The α- and γ-adaptins exhibit only weak amino acid homology (45), whereas the β-and β′-adaptins appear to be closely related (2). In addition, the purified 100-kD β-adaptin subunit derived from brain AP-2 associates with preassembled clathrin cages (1). These observations have led to the suggestion that the β/β′-adaptins function in clathrin recognition and binding while the α- and γ-adaptin subunits recognize defined sequences on cytoplasmically oriented receptor tails, and thereby link the overlaying clathrin lattice to the membrane (38).

Using different in vitro assays, it has been shown that adaptors can bind to the cytoplasmic tail of the 275-kD mannose-6-phosphate/insulin-like growth factor II receptor (13), as well as to the asialoglycoprotein receptor (3). However, it is not clear why cytoplasmic AP-1 and AP-2 adaptors do not bind to suitably oriented cytoplasmic domains of receptors present in inappropriate intracellular membrane compartments, such as endosomes. Clearly, adaptor recruitment within the cell must be specifically regulated.

A clue to this regulation has come from studies utilizing the fungal metabolite, brefeldin A (BFA).[1] BFA induces a rapid and remarkable disassembly of the Golgi complex (12, 27), and one of the earliest events following BFA addition is the redistribution of a coatomer subunit, β-COP, into the cytoplasm (7). The TGN–endosomal compartment is also perturbed by BFA treatment, albeit more subtly (28, 59). Interestingly, an approximate fivefold increase in the cell surface expression of mannose 6-phosphate receptors has been noted on BFA treatment (6, 59). This is indicative of missorting of the mannose 6-phosphate receptors in the TGN, resulting in passage to the plasma membrane. More recently, it has been shown that BFA directly inhibits the association of γ-adaptin, and hence AP-1, with the Golgi complex (46, 58). This effect of BFA can be prevented in permeabilized cells by pretreatment with the poorly hydrolyzable GTP analogue, GTPγS. These studies suggested that guanine nucleotide–binding proteins regulate clathrin coat assembly, and imply that adaptor recruitment to the TGN is a more complex process than previously anticipated.

1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BFA, brefeldin A; GppNHp, 5′ guanylyl-imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

In this paper we describe a biochemical assay that has allowed us to follow AP-1 translocation from the cytosol onto purified rat liver Golgi membranes. Our results confirm that the association of AP-1 with purified Golgi membranes requires GTP or GTP analogues. We have also found that binding of AP-1 is temperature dependent, relatively rapid, and dependent on the concentrations of both Golgi and cytosol. Furthermore, incubation of an adaptor-enriched fraction of rat liver cytosol with recombinant myristoylated ADP-ribosylation factor (ARF) was able to reconstitute AP-1 translocation onto purified Golgi membranes. Our results provide the basis for a model for adaptor recruitment to the Golgi surface, and the assay provides a means for further dissection of the components involved in the specific recruitment of AP-1 to the TGN.

Materials and Methods

Materials

Aprotinin, ATP, creatine kinase, DTT, 5'-guanylyl-imidodiphosphate (GppNHp), leupeptin, pepstatin A, PMSF, and soybean trypsin inhibitor were from Sigma (St. Louis, MO). ATPγS, GDP, GTP, GTPγS, and creatine phosphate were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). BFA was obtained from either Epicenter Technologies (Madison, WI) or Sigma and a 10 mg/ml stock solution in ethanol was stored at −20°C. We used trypsin from Cooper Biomedical (Malvern, PA) and the relative molecular weight standards for SDS-PAGE, CNBr-activated Sepharose 4B, PD-10 columns, and Superose 6 were from Pharmacia Fine Chemicals (Piscataway, NJ). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH), the ECL reagents for chemiluminescent detection from Amersham Corp. (Arlington Heights, IL), and X-Omat XAR and XRP5 film from Eastman Kodak Co. (Rochester, NY). All other reagents were the highest grade commercially available. Male Sprague Dawley rats from SASCO (Omaha, NE) were starved overnight before use. The synthetic peptides corresponding to residues 2–17 and 6–17 of bovine ARF1 were synthesized and purified by reverse phase HPLC by Jay Ponder (Washington University, St. Louis, MO). Recombinant myristoylated ARF1, expressed in *Escherichia coli* BL21 together with *N*-myristoyltransferase and purified by sequential chromatography on DEAE-Sephacel and Ultragel AcA 54 (10), was generously provided by Julie Donaldson and Richard Klausner (National Institutes of Health, Gaithersburg, MD).

Antibodies

The anti-clathrin heavy chain mAb, mAb TD.1 (36), and the anti-α-adaptin mAb AP.6 (5) were kindly provided by Frances Brodsky (University of California, San Francisco, CA). TD.1 was used at a final concentration of 2.5 μg/ml for immunoblotting. The mAbs 100/2 (directed against α-adaptin), and 100/1 (recognizing both β- and β'-adaptin subunits) (2), were generously provided by Ernst Ungewickell (Washington University). mAb 100/2 was diluted to 1 μg/ml and mAb 100/1 to 0.5 μg/ml for use on immunoblots. A rabbit polyclonal serum raised against denatured α-mannosidase II was a gift of Kelley Moremen (University of Georgia, Athens, GA) and used at a dilution of 1:6,000 for blotting. The anti-β-COP antibody, M3A5 was obtained from Thomas Kreis (University of Geneva, Geneva, Switzerland) and used at a dilution of 1:500. HRP-conjugated anti-mouse and anti-rabbit immunoglobulins were purchased from Amersham Corp. (Arlington Heights, IL).

Subcellular Fractionation

Rat liver Golgi membranes were prepared by established methods (52), after homogenization in 10 mM Hepes-KOH, pH 7.4, 500 mM sucrose, 5 mM EDTA supplemented with 1 mM PMSF, 0.1 TIU/ml aprotinin, and 5 μg/ml leupeptin. The final Golgi membrane pellet was resuspended to approximately 2–3 mg protein/ml in 250 mM sucrose in 10 mM Hepes-KOH, pH 7.4, 1 mM EDTA, quick-frozen in dry ice in small aliquots, and stored at −80°C. Protein concentrations of the various fractions were determined by the Coomassie blue method (4) using BSA as a standard. The specific activity of galactosyltransferase in these Golgi membrane preparations was increased approximately 50-fold when compared to the liver homogenate. For the experiments presented in Fig. 5 (*C* and *D*) the purified Golgi membranes were first stripped of endogenous AP-1. The membranes were diluted to 50 μg/ml in 25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, and incubated at 37°C for 15 min. The tube was then chilled on ice, and the membranes collected by centrifugation at 10,000 *g* for 15 min at 4°C. The resulting stripped Golgi membranes were resuspended to 1 mg protein/ml in 10 mM Hepes-KOH, pH 7.0, 250 mM sucrose for subsequent use in the binding assay.

Liver cytosol was prepared from fresh rat livers homogenized in 3 vol of ice cold 25 mM Hepes-KOH, pH 7.4, 250 mM sucrose, 2 mM EDTA supplemented with 1 mM PMSF, 0.1 TIU/ml aprotinin, and 5 μg/ml leupeptin. A postnuclear supernatant, obtained after centrifugation at 3,000 *g* for 10 min at 4°C, was centrifuged at 12,000 *g* for 20 min at 4°C to yield a crude postmitochondrial supernatant. Cytosol was prepared from this fraction by centrifugation at 100,000 *g* for 60 min at 4°C. The resulting supernatant fraction was aspirated and stored in small aliquots at −80°C following quick freezing in dry ice. Rat brain cytosol was prepared similarly. Before use in the Golgi binding assay, an aliquot was thawed and immediately desalted on ice over a PD-10 column equilibrated in 25 ml of 25 mM Hepes-KOH, pH 7.0, 250 mM sucrose, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT. The gel-filtered cytosol was then centrifuged at 150,000 *g* for 60 min at 4°C, and the resulting supernatant used in the binding assay.

A coat protein-enriched fraction from rat liver cytosol was prepared by gel filtration. Frozen rat liver was used to prepare the cytosol fraction as outlined above, and prior to chromatography, the cytosol was centrifuged at 150,000 *g* for 60 min at 4°C to remove aggregated material. Routinely, 4 ml of the clarified rat liver cytosol, containing approximately 120 mg protein, was loaded at 60 ml/h onto a preparative Superose 6 column packed into a HR 16/50 column previously equilibrated in 25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 5 mM magnesium acetate. Fractions of 1 ml were collected beginning 30 min after loading. Fractions 27–33 contained the AP-1 peak, and were pooled, adjusted to 1 mM DTT, 0.5 mM PMSF, 0.05 TIU/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A and soybean trypsin inhibitor, and stored on ice until used. Fractions 7–13, containing the clathrin pool, were pooled separately, supplemented with the protease inhibitors, and also stored on ice.

Immunodepletion of AP-2 from Rat Liver Cytosol

mAb AP.6 was coupled to CNBr-activated Sepharose-4B as recommended by the manufacturer. A control matrix of CNBr-activated Sepharose-4B quenched with 100 mM Tris-HCl, pH 8.0, was prepared in parallel. The AP.6-Sepharose matrix contained 2.0 mg antibody/ml packed gel. Before immunodepletion, 250 μl of control or AP.6-Sepharose were packed into polypropylene columns, and equilibrated with 10 column volumes of 10 mM Hepes-KOH, pH 7.4, 250 mM sucrose on ice. 3 ml of freshly prepared rat liver cytosol was then passed over each column on ice and the effluent reapplied for a second passage over the columns. 2 1.5-ml aliquots of the resulting cytosol were quick frozen in dry ice and stored frozen at −80°C. For use in the binding experiments, an aliquot of both control and AP.6-treated cytosol was thawed and passed over a second 250 μl column of control or AP.6-coupled Sepharose equilibrated in 10 mM Hepes-KOH, pH 7.4, 250 mM sucrose on ice. The cytosol fractions were then immediately desalted over PD-10 columns on ice and centrifuged at 150,000 *g* for 60 min at 4°C. The resulting control and AP.6-treated cytosol fractions were diluted in the in vitro binding assay to a final concentration of 5 mg/ml.

Golgi Binding Assay

Assays were performed in final volume of 400 μl in 1.5 ml microfuge tubes. The typical assay mixture contained 25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT. Cytosol, purified Golgi membranes, nucleotides, or nucleotide analogues were added to the concentrations noted in the individual figure legends. Routinely, the assay contained 25 μg/ml purified Golgi membranes and 5 mg/ml gel-filtered cytosol. When used, the ATP regeneration system consisted of 1 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase. BFA was added from a stock solution in ethanol, and an equal volume of ethanol was added to tubes which did not receive BFA. All additions to the assay tubes were made on ice. The binding assay was initiated by transferring the tubes to a 37°C waterbath and usually terminated after 15 min by placing the tubes on ice. The Golgi membranes were then recovered by centrifugation at 8,800 *g* for 15 min at 4°C. In most instances, the supernatant was aspirated and discarded and the pellets centrifuged again at 15,000 *g* for 2.5

min at 4°C. The residual supernatant was aspirated and each pellet solubilized in 20 μl of SDS-PAGE sample buffer for subsequent SDS-PAGE analysis.

For experiments using the purified recombinant ARF1, the reaction volume was reduced to 200 μl. Golgi membranes were added to a final concentration of 80 μg/ml, the coat protein–enriched pool from the Superose 6 column added to approximately 1 mg/ml and, when present, the clathrin-containing pool was at 0.05 mg/ml. The concentration of the purified ARF added to each tube is detailed in the figure legends. GTPγS was added to a final concentration of 200 μM and the reactions were for 15 min at 37°C. All the experiments reported in this study have been repeated at least twice with essentially similar results.

Electrophoresis and Immunoblotting

Discontinuous SDS-PAGE was performed as described (26) using the mini-Protean II system. Gels were prepared from an acrylamide/*bis*-acrylamide stock solution of 30:0.4 rather than the usual 30:0.8, as the reduced cross-linking provided better resolution of the β- and β′-adaptin subunits. In most instances, 8% gels were used and samples were loaded after boiling for 3 min in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% sucrose, 5% 2-mercaptoethanol, and 0.003% bromophenol blue. After electrophoresis, gels were either stained in Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid and destained by diffusion in the same solution without the Coomassie blue, or transferred onto nitrocellulose.

For transfer onto nitrocellulose, the gels were first incubated for 10–15 min in cold (4°C) transfer buffer consisting of 15.6 mM Tris, 120 mM glycine. After the incubation, the blot was assembled and transfer was at 250 mA for 60 min. The blotted proteins were visualized by brief staining in Ponceau S, and the position of the relative molecular weight standards marked before complete destaining in transfer buffer. The membranes were blocked overnight in 5% skim milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST). This and all subsequent steps were performed at room temperature. Blocked blots were incubated with the specified antibodies, as detailed in the figure legends, for 2 h with constant shaking. All antibodies were diluted in 0.5% milk in TBST. After washing the blots four times with TBST, the membranes were incubated with the HRP-conjugated secondary antibody for 60 min, followed by four washes in TBST. The ECL reaction was for 1 min as recommended by the manufacturer and the chemiluminescent signals were visualized on autoradiographic film. When blots were to be reprobed with a second antibody, the nitrocellulose was first stripped of previously bound antibodies by incubation in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 50°C for 30 min. The nitrocellulose membrane was then washed three times with TBS and blocked overnight in 5% milk in TBST. The blocked blots were then incubated with antibodies as outlined above. For quantitative analysis, autoradiographs were analyzed using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) equipped with Image-Quant software.

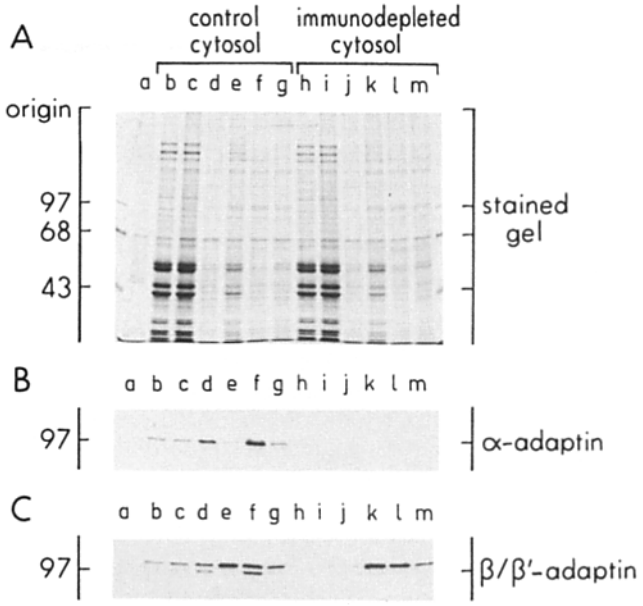

Figure 1. Cytosolic adaptor binding to Golgi membranes. Tubes contained 25 μg/ml Golgi membranes (lane *a*), 5 mg/ml control gel-filtered cytosol (lane *b*), Golgi membranes and control cytosol (lanes *c–g*), 5 mg/ml AP-2–depleted cytosol (lane *h*), or Golgi and AP-2–depleted cytosol (lanes *i–m*). Either no further additions (lanes *a–c*, *h*, and *i*), or an ATP regenerating system (lanes *d* and *j*), or 100 μM GTPγS (lanes *e* and *k*), or an ATP-regenerating system and 100 μM GTPγS (lanes *f* and *l*), or an ATP regenerating system, 100 μM GTPγS and 100 μg/ml BFA (lanes *g* and *m*) were added before incubation at 37°C for 15 min. Reactions were terminated by chilling on ice and pellets collected by centrifugation. Equal aliquots of the pellets were resolved on duplicate 8% gels and either stained with Coomassie blue (*A*) or transferred onto nitrocellulose. *B* is from an autoradiograph of the blot probed with mAb 100/2, and *C* from an autoradiograph of the blot reprobed with mAb 100/1. Only the relevant portions of the autoradiographs are shown, and the positions of the relative molecular weight standards, in kD, are indicated on the left.

Results

Binding of Adaptors to Purified Golgi Membranes

We have used the pool of adaptors within the cytosol to follow AP-1 translocation onto Golgi membranes. The assay involves the incubation of gel-filtered rat liver cytosol with purified rat liver Golgi membranes at 37°C followed by recovery of the membranes by centrifugation for subsequent analysis by SDS-PAGE and immunoblotting. The results of a representative experiment are shown in Fig. 1. As several different points have been addressed in this experiment, the relevant findings are discussed separately below.

Identification of AP-1 and AP-2. Since we made use of rat cytosol, an anti-β/β′-adaptin antibody, mAb 100/1, was used to follow AP-1 recruitment in this study. This antibody detects both β- and β′-adaptin subunits (2), and thus should recognize both AP-1 and AP-2. In assays using gel-filtered cytosol, two immunoreactive bands could be seen (Fig. 1 *C*, lanes *d* and *f*). This was unexpected however, as all previous studies on non-neuronal adaptors have failed to detect major mobility differences between β- and β′-adaptin on standard SDS-PAGE (2, 43). Therefore, to determine the identity of the two mAb 100/1-reactive bands, we removed the cytosolic pool of AP-2 by immunodepletion using the anti-α-adaptin antibody, mAb AP.6. mAb AP.6 binds to native AP-2 adaptors (5), and our immunodepletion procedure resulted in the removal of approximately 90% of α-adaptin from liver cytosol, as determined by immunoblotting with mAb 100/2 (data not shown).

Comparison of the data from assays using control (Fig. 1, lanes *b–g*) and immunodepleted (lanes *h–m*) cytosol revealed that the appearance of the lower β/β′-adaptin band was correlated with the presence of α-adaptin immunoreactivity (lanes *d* and *f*). Furthermore, when AP-2 was depleted from the cytosol, only the upper β/β′-adaptin band was evident (Fig. 1, lanes *h–m*). These results clearly show that of the two mAb 100/1-reactive bands seen in rat liver, the upper band corresponds to the β′-adaptin subunit of the AP-1 complex and the lower to authentic β-adaptin.

Specificity of AP-1 recruitment. Rat liver Golgi membranes, after a 15-min incubation at 37°C, demonstrated little detectable AP-1 or AP-2 (Fig. 1, lanes *a*). When gel-

filtered rat liver cytosol was incubated at 37°C for 15 min in the absence of nucleotides, a considerable pellet was recovered as seen on Coomassie blue staining (Fig. 1, lanes *b*). We have ascertained by immunoblotting that one of the prominent 50-kD bands in this precipitate is β-tubulin (data not shown). Similar precipitates have also been detected recently by others on warming of ATP-depleted or gel-filtered cytosol (25, 32). Analysis of the duplicate gel transferred to nitrocellulose and probed sequentially with mAbs 100/1 and 100/2 showed that the precipitate contained both AP-1 and AP-2 complexes (Fig. 1, lanes *b*). However, on addition of the Golgi membranes (Fig. 1, lanes *c*) no additional immunoreactivity was detected in the pellet fractions. Therefore, in the absence of nucleotides, a cytosolic precipitate formed that contained both AP-1 and AP-2 complexes.

By supplementing the gel-filtered cytosol and Golgi mixture with an ATP regeneration system, the precipitation phenomenon could be prevented (Fig. 1, lanes *d*), but a significant increase in both AP-1 and AP-2 recovered in the pellet was seen (lanes *d*). By contrast, on adding the poorly hydrolyzable analogue of GTP, GTPγS, only trace levels of AP-2 were noted in the Golgi pellet (Fig. 1, lanes *e*). However, a dramatic increase in the amount of AP-1 was evident. Addition of a combination of an ATP regeneration system and GTPγS resulted in the translocation of both AP-1 and AP-2 onto the membranes (Fig. 1, lanes *f*). This recruitment could be inhibited by the simultaneous addition of 100 μg/ml BFA (Fig. 1, lanes *g*).

In contrast to these findings, binding assays containing the AP-2-depleted cytosol showed only the upper 100-kD β′-adaptin subunit of AP-1 (Fig. 1, lanes *k–m*). AP-1 was recruited in the presence of GTPγS (Fig. 1, lanes *k* and *l*) and this was inhibited by addition of 100 μg/ml BFA (lane *m*). We have concluded that incubation of Golgi membranes with cytosol and GTPγS resulted in the recruitment of only AP-1, while on the addition of ATP or an ATP regenerating system, AP-2 was also recruited onto the membrane preparation.

The identity of the membrane compartment with which AP-2 interacts is unknown, but endosomal vesicles are possible acceptor membranes and are a major contaminant of the rat liver Golgi membrane preparation (22). AP-2 binding clearly required ATP hydrolysis, because ATPγS was unable to recruit AP-2 onto the Golgi membranes (data not shown). It is unlikely that the ATP-dependent membrane association of AP-2 was simple aggregation of the adaptor complex. We found that the membrane association of AP-2 was inhibited by BFA, but BFA has no direct effect on clathrin or AP-2 (58). This was also in contrast to the action of BFA on intact cells, where BFA does not have a major effect on endocytosis, and hence on AP-2 function (12, 28). Understanding the nature and significance of this membrane association of AP-2 will require further studies.

Effects of Guanine Nucleotides and BFA on AP-1 Translocation

The effects of adding different guanine nucleotides to the binding assay are shown in Fig. 2. Gel-filtered cytosol incubated at 37°C with 1 mM GDP demonstrated an AP-1/AP-2–containing precipitate (column *2*) similar to that observed in the absence of any nucleotides (Fig. 1). Upon addition of Golgi membranes to the cytosol–GDP mixture, no appreciable binding of AP-1 above this background level was observed (column *3*). However, in the presence of 1 mM GTP, the precipitation was prevented and neither AP-1 nor AP-2 were seen in the pellet fraction of cytosol incubated at 37°C (column *4*). On adding Golgi membranes to the GTP-containing cytosol, a modest translocation of AP-1 was observed (column *5*). By substituting GTP with the poorly hydrolyzable analogue, GTPγS (100 μM), an eightfold increase in the amount of AP-1 associated with the Golgi pellet was seen (column *7*). Unexpectedly, the results obtained with the non-hydrolyzable analogue GppNHp (100 μM) (column *9*) were similar to those observed with 1 mM GTP. Note that some AP-1 was also found in the pellets of cytosol incubated together with GTPγS but without added Golgi membranes (column *6*). We have found this background level to vary somewhat between individual experiments, and that it is exacerbated by the presence of 1% ethanol in the assay. It should also be mentioned that no significant recruitment of AP-2 was observed in the presence of GTP or the GTP analogues alone.

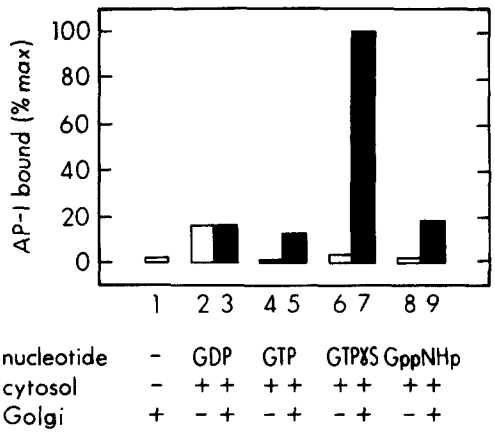

Figure 2. Adaptor translocation in the presence of guanine nucleotides. Tubes containing 25 μg/ml Golgi membranes and 5 mg/ml gel-filtered cytosol were prepared on ice as indicated in the figure. The final nucleotide concentrations were 1 mM GDP (column *2* and *3*), 1 mM GTP (columns *4* and *5*), 100 μM GTPγS (columns *6* and *7*), or 100 μM GppNHp (columns *8* and *9*). After incubation at 37°C for 15 min, the reactions were terminated and analyzed for β′-adaptin by immunoblotting with mAb 100/1. The amount of β′-adaptin (*AP-1*) was determined by densitometry and the data are presented as a percent of maximal AP-1 found in each pellet.

The GTPγS-induced translocation of cytosolic AP-1 onto the Golgi membranes was concentration dependent (Fig. 3 *A*). Half-maximal stimulation occurred at approximately 2.5 μM and concentrations of GTPγS above 100 μM did not result in additional binding of AP-1 (data not shown). An analysis of the opposing effects of GTPγS and BFA on AP-1 translocation is shown in Fig. 3 *B*. The simultaneous presence of both 100 μM GTPγS and 50 μg/ml BFA in the assay resulted in reduced levels of AP-1 recovered on the Golgi membranes (Fig. 3 *B*, column *4*) compared to the AP-1 recruited in the absence of BFA (column *3*). When applied simultaneously, the extent of BFA-mediated inhibition could be increased by reducing the concentration of GTPγS in the assay (data not shown). If GTPγS was added 10 min before BFA, no inhibition of AP-1 binding was observed (Fig. 3 *B*, column *5*). Conversely, on incubation of liver cytosol and

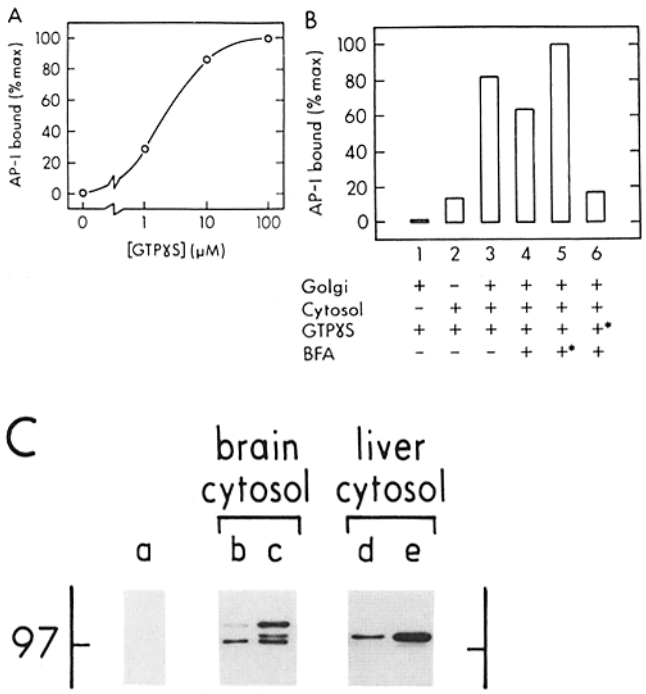

Figure 3. Effects of GTPγS and BFA on AP-1 translocation. (*A*) Tubes containing 25 μg/ml Golgi membranes, 5 mg/ml gel-filtered cytosol, and increasing concentrations of GTPγS were incubated at 37°C for 15 min. The reactions were terminated and analyzed by immunoblotting with mAb 100/1 followed by densitometry. Data are presented as percent of maximal AP-1 found in each pellet. For each GTPγS concentration, the amount of AP-1 found in the pellet of cytosol and GTPγS incubated alone at 37°C for 15 min has been subtracted. (*B*) Tubes containing 25 μg/ml Golgi membranes, 5 mg/ml gel filtered cytosol, 100 μM GTPγS, or 50 μg/ml BFA were prepared on ice as indicated in the figure. The tubes were transferred to 37°C, and after 10 min BFA (column *5*) or GTPγS (column *6*) were added as indicated by the asterisks. After an additional 5 min at 37°C, the reactions were terminated and analyzed by immunoblotting with mAb 100/1 followed by densitometry. The data are presented as the percent of maximal AP-1 found in each pellet. (*C*) Tubes containing 25 μg/ml purified Golgi membranes (lane *a*), 4 mg/ml gel-filtered rat brain cytosol (lane *b*), Golgi membranes and brain cytosol (lane *c*), 4 mg/ml gel-filtered rat liver cytosol (lane *d*), or Golgi membranes and liver cytosol (lane *e*) were prepared on ice. GTPγS (100 μM) was added and the tubes incubated at 37°C for 15 min. An autoradiograph of the blot probed with mAb 100/1 is shown. The position of the relevant molecular weight standard is indicated. Note the reduced electrophoretic mobility of the rat brain β′-adaptin subunit.

Golgi membranes with BFA for 10 min before the addition of GTPγS (Fig. 3 *B*, column *6*), no adaptor binding above background levels (Fig. 3 *B*, columns *1* and *2*) was observed. These effects, dependent on the order of addition of GTPγS and BFA, are entirely consistent with the action of these agents in permeabilized cells (8, 46, 58).

We have also performed binding assays with gel-filtered rat brain cytosol substituted for the rat liver cytosol. In brain, the ratio of AP-1 to AP-2 is reversed compared with non-neuronal tissues, as AP-2 appears to play an important role in synaptic vesicle recycling (33). Furthermore, brain β′-adaptin is easily distinguished from β-adaptin on SDS-PAGE (2), and from the endogenous liver β′-adaptin. Incubation of rat brain cytosol with 100 μM GTPγS at 37°C for 15 min resulted in the appearance of low levels of both the 110-kD β′-adaptin and 100-kD β-adaptin bands in a pellet (Fig. 3 *C*, lane *b*). When Golgi membranes were added, there was a large increase in the β′-adaptin immunoreactivity noted in the Golgi pellet with no change in the amount of β-adaptin (Fig. 3 *C*, lane *c*). A third mAb 100/1-reactive band was also apparent, which aligned with the non-neuronal β′-adaptin recruited from rat liver cytosol (Fig. 3 *C*, lane *e*). Taken together, these data demonstrate that GTPγS induced the translocation of cytosolic AP-1 to the membrane rather than stabilizing endogenous Golgi-associated AP-1 (see below). They also reveal the specificity of the AP-1 recruitment in the presence of GTPγS, even when the cytosol fraction contained a large excess of AP-2.

Effects of Golgi and Cytosol Concentration on AP-1 Recruitment

An analysis of AP-1 recruitment with increasing Golgi membrane concentration is shown in Fig. 4 *A*. At a fixed concentration of 5 mg/ml gel-filtered cytosol and 100 μM GTPγS, the AP-1 associated with the Golgi pellet fraction was saturable, with half-maximal binding obtained with approximately 10 μg/ml purified Golgi membranes. Since the extent of AP-1 recruitment should depend on the concentration of both the Golgi membranes and the cytosol, these results suggested that cytosol became limiting at Golgi membrane concentrations above 25 μg/ml. Indeed, when AP-1 membrane translocation was assayed as a function of cytosol concentration, AP-1 binding increased linearly over a concentration range of 1–5 mg/ml (Fig. 4 *B*) and began to saturate at 10 mg/ml. To examine the possibility that the cytosolic pool of AP-1 might be limiting in these assays, we analyzed the supernatant fractions from this particular binding assay for AP-1 content. No significant differences in the amount of AP-1 were detected in the supernatants from tubes that contained the added Golgi membranes compared to those incubated without membranes (data not shown). We have estimated that less than 5% of the cytoplasmic AP-1 was recruited onto the membranes. This clearly demonstrates that a vast excess of AP-1 remained in the cytosol, and the simplest interpretation of the data is that a limiting factor other than AP-1 is required for the translocation of AP-1 onto Golgi membranes. However, we cannot exclude other possibilities, for example, a minor subpopulation of the total immunoreactive cytosolic AP-1 pool may be functional.

Kinetics of AP-1 Translocation onto Golgi Membranes Stripped of Endogenous AP-1

Golgi membranes, as isolated from rat liver, contain a significant level of bound β′-adaptin. This β′-adaptin remained associated with the Golgi membranes when maintained on ice (Fig. 5 *A*, lane *b*), but 90% was released during a 15-min incubation at 37°C, even when GTPγS was present (Fig. 5 *A*, lane *k*). However, when the Golgi membranes were incubated at 37°C in the presence of cytosol and GTPγS, the amount of Golgi-associated β′-adaptin remained constant (Fig. 5 *A*, lanes *d–i*), and was comparable to the level of β′-adaptin observed when the incubation was performed on ice (lane *c*). We interpret this to mean that during the incubation at 37°C, the endogenous Golgi-bound AP-1

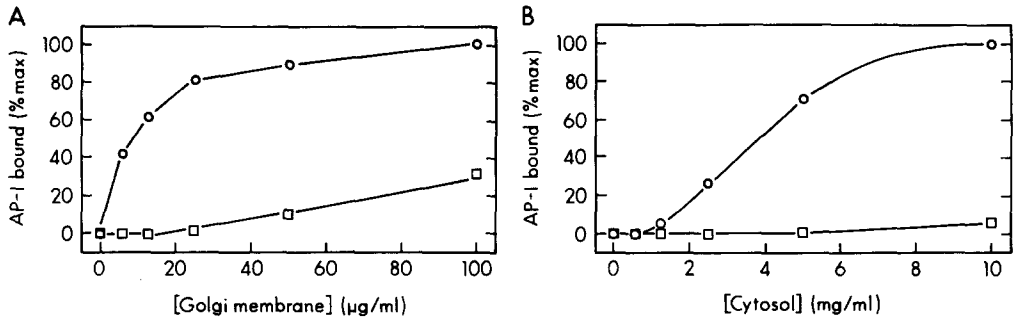

Figure 4. Titration of Golgi membranes and cytosol in the binding assay. (*A*) Increasing concentrations of Golgi membranes were incubated in the absence (□) or presence (○) of 5 mg/ml gel-filtered rat liver cytosol for 15 min at 37°C. GTPγS was present at 100 μM and the resulting Golgi pellet fractions were analyzed by immunoblotting with mAb 100/1, followed by densitometry. Data are presented as the percent of maximal AP-1 found in each pellet. A value corresponding to the amount of AP-1 found in the pellet of cytosol incubated at 37°C for 15 min without added Golgi membranes has been subtracted. (*B*) Increasing concentrations of gel-filtered cytosol were incubated in the absence (□) or presence (○) of 25 μg/ml Golgi membranes 37°C for 15 min. GTPγS was present at 100 μM and the resulting pellet fractions were analyzed by immunoblotting with mAb 100/1, followed by densitometry. Data are presented as the percent of maximal AP-1 found in each pellet.

dissociated from the membranes at the same time that cytoplasmic AP-1 was being recruited onto the membranes, with the result that little change in the overall amount of Golgi-associated AP-1 was evident.

The rate at which the endogenous AP-1 was released from the Golgi membranes upon incubation at 37°C is shown in Fig. 5 *B*. These experiments were performed in the absence of cytosol and within 1–2 min of warming the Golgi membranes to 37°C, a decrease in the level of endogenous AP-1 was seen (Fig. 5 *B*, lanes *c* and *d*). After 15 min, only 3–4% remained associated with the pelleted membranes (Fig. 5 *B*, lane *g*). The inclusion of 100 μM GTPγS in the incubation mixture did not alter the rate or extent of adaptor dissociation from the membranes (Fig. 5 *B*, lanes *h–m*). Using mAb TD.1, directed against the terminal domain of the clathrin heavy chain, we noted that clathrin dissociated from the Golgi membranes with very similar kinetics to the AP-1 adaptors. However, β-COP, a component of the coatomer complex (49) detected using mAb M3A5, remained stably associated with the Golgi membranes. In addition, the level of immunoreactivity of the integral Golgi membrane protein, α-mannosidase II, was unchanged during the incubation. We then used this 37°C incubation procedure to strip endogenous AP-1 from the Golgi membranes to allow an examination of the kinetics of AP-1–Golgi interaction.

Binding of AP-1 to the stripped Golgi membranes was followed in both the absence and presence of 100 μM GTPγS. The efficacy of the 37°C incubation in removing the endogenous Golgi-associated AP-1 can be seen by comparing the membranes before (Fig. 5 *C*, lane *a*) and after (lane *c*) the 15-min incubation. Incubation of the gel-filtered cytosol on ice without added nucleotides, resulted in a pellet containing predominantly AP-2 (Fig. 5 *C*, lane *b*). When the stripped Golgi membranes were incubated together with gel-filtered cytosol on ice, we found a reproducible increase in the AP-1 immunoreactivity in the pellet (Fig. 5 *C*, lane *d* compared to lanes *b* and *c*). This may represent temperature-independent binding of cytosolic AP-1 onto pre-activated acceptor structures remaining on the Golgi membranes. Upon incubation of the cytosol and Golgi membrane mixture at 37°C, the level of β′-adaptin associated with the Golgi membranes decreased over time to the non-specific background level observed with cytosol alone (Fig. 5 *C*, lanes *e–j* compared with lane *k*).

When the incubations were performed in the presence of 100 μM GTPγS, the temperature-independent association of AP-1 with the purified Golgi membranes was again seen (Fig. 5 *D*, lane *d*). A low level of AP-2 was also noted in the membrane pellet. On incubating the Golgi–cytosol mixture at 37°C, the amount of AP-1 associated with the Golgi pellet remained constant for the first 2 min, whereas the membrane-associated AP-2 decreased significantly (Fig. 5 *D*, lanes *e–g*). After 5 min at 37°C, the AP-1 associated with the Golgi pellet had increased 2.5-fold (Fig. 5 *D*, lane *h*) and after 15 min, when equilibrium was reached, the increase was fivefold (lane *j*). From these data, we infer that the lag period of 1–4 min is necessary for recruitment and/or assembly of GTP-requiring components on the Golgi membrane that must precede the translocation of AP-1 from the cytosol onto the membrane. It should also be noted that maximal recruitment of AP-1 always approximated the level of AP-1 found originally on the untreated Golgi membranes (Fig. 5 *D*, compare lane *j* to *a*).

Stability of GTPγS-recruited Golgi-associated AP-1

Since the endogenous AP-1 found on the Golgi membranes dissociated from the membranes at 37°C, we were curious to determine whether cytosolic AP-1, recruited in the presence of GTPγS, was more stably associated with the Golgi membrane. Adaptors were first bound to Golgi membranes by incubation at 37°C for 15 min in the presence of either an ATP regenerating system, or GTPγS or a combination of both. The membranes were then recovered by centrifugation and washed in 1 M Tris-HCl, pH 7.0. Similar washing of purified clathrin-coated vesicles extracts both clathrin and adaptors from the vesicle surface (21). Fig. 6 shows that AP-1 and AP-2 bound to the membranes in the presence of ATP, were completely extracted by Tris, and found in the supernatant fraction (lane *a*). On the other hand, approximately 70% of the AP-1 recruited to the Golgi membrane in the presence of GTPγS was found in the pellet fraction (Fig. 6, lane *d*), indicating a more stable membrane association. When the Golgi membranes, previously incubated together with cytosol, an ATP regenerating system and GTPγS were washed with Tris, two distinct populations of adaptors were recovered. These corresponded to the AP-1/AP-2 pool, recovered in the supernatant (Fig. 6, lane *e*) and the stably associated AP-1 pool, found in the pellet (Fig. 6, lane *f*).

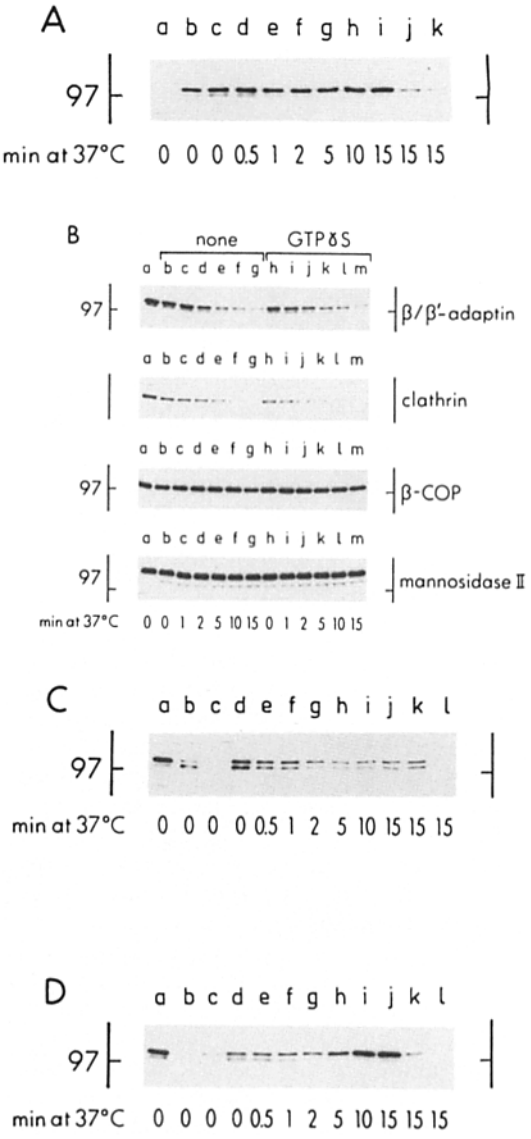

Figure 5. Kinetics of AP-1 adaptor translocation onto Golgi membranes. (*A*) Tubes containing 5 mg/ml gel-filtered cytosol (lanes *a* and *j*), 25 μg/ml Golgi membranes (lanes *b* and *k*) or Golgi membranes and 5 mg/ml gel-filtered cytosol (lanes *c–i*) were prepared on ice. GTPγS was then added to 100 μM, and the tubes either kept on ice (lanes *a–c*) or incubated at 37°C for 0.5 min (lane *d*), 1 min (lane *e*), 2 min (lane *f*), 5 min (lane *g*), 10 min (lane *h*), or 15 min (lanes *i–k*). Pellets were analyzed by immunoblotting with mAb 100/1 and an autoradiograph of the blot is shown with the relevant molecular weight standard indicated on the left. (*B*) Tubes containing 50 μg/ml purified Golgi membranes (lanes *b–g*) or purified Golgi membranes and 100 μM GTPγS (lanes *h–m*) were either maintained on ice (lanes *b* and *h*) or incubated at 37°C for 1 (lanes *c* and *i*), 2 (lanes *d* and *j*), 5 (lanes *e* and *k*), 10 (lanes *f* and *l*), or 15 min (lanes *g* and *m*). Reactions were terminated as in *A* and analyzed by immunoblotting. Lane *a* contained an equivalent amount (10 μg) of untreated Golgi membranes. The blots were sequentially probed with mAb 100/1 anti-β/β'-adaptin, mAb TD.1 anti-clathrin heavy chain, mAb M3A5 anti-β-COP, and anti-α-mannosidase II antiserum as indicated. Autoradiographs from each blot are shown with the relevant molecular weight standards, in kD, indicated on the left. (*C*) Tubes containing 25 μg/ml untreated (lane *a*) or stripped (lanes *c* and *l*) Golgi membranes, 5 mg/ml gel filtered cytosol (lanes *b* and *k*), or a combination of Golgi membranes and cytosol (lanes *d–j*) were prepared on ice. The tubes were either maintained on ice (lanes *a–d*) or incubated at 37°C for 0.5 (lane *e*), 1 (lane *f*), 2 (lane *g*), 5 (lane *h*), 10 (lane *i*), or 15 min (lanes *j–l*). Reactions were terminated and analyzed as in *A*. (*D*) Tubes were prepared and analyzed exactly as in *C* but with GTPγS added to a final concentration of 100 μM.

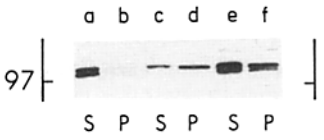

Figure 6. Reversibility of AP-1–Golgi interaction. AP-1 was bound to Golgi membranes in tubes containing 5 mg/ml gel-filtered cytosol, 25 μg/ml Golgi membranes, and either an ATP regenerating system (lanes *a* and *b*), 100 μM GTPγS (lanes *c* and *d*), or a combination of the ATP regenerating system and GTPγS (lanes *e* and *f*). After incubation at 37°C for 15 min, the membranes were collected by centrifugation and resuspended in 1 M Tris-HCl, pH 7.0 on ice. The membranes were again collected by centrifugation, the supernatants aspirated, and the pellets resuspended to the same volume. Equal aliquots of the supernatant (*S*, lanes *a*, *c*, and *e*) and pellet (*P*, lanes *b*, *d*, and *f*) fractions were resolved on an 8% gel and analyzed by immunoblotting with mAb 100/1. Position of the relevant molecular weight standard, in kD, is indicated on the left.

AP-1 Translocation onto Trypsinized Golgi Membranes

We also examined whether the recruitment of AP-1 was dependent on cytoplasmically oriented proteins on the Golgi membrane. In our initial experiments, Golgi membranes were digested with increasing concentrations of trypsin, and the extent of proteolysis was monitored using the antibodies against the cytoplasmically oriented β/β'-adaptin subunits and α-mannosidase II as a lumenal Golgi marker. Limited tryptic digestion resulted in the rapid disappearance of the intact 100-kD β'-adaptin subunit, with the concomitant appearance of the 60-kD trunk fragment (48). At 50 μg/ml, further tryptic degradation of the trunk destroyed the mAb 100/1 epitope completely, but addition of soybean trypsin inhibitor before the addition of 50 μg/ml trypsin was completely effective in preventing proteolysis (data not shown).

In contrast to the proteolysis of the β'-adaptin, α-mannosidase II remained insensitive to increasing concentrations of trypsin (data not shown). Only traces of the characteristic 115- and 75-kD tryptic fragments (35) were evident in the presence of 50 μg/ml trypsin. Thus, in addition to revealing the extent of proteolysis, these studies also confirmed that the Golgi membranes were correctly oriented as isolated, with very little leakage evident. When Golgi membranes were digested with trypsin and then added to the standard binding assay, the level of β'-adaptin bound to the membranes decreased as a function of trypsin concentration (Fig. 7), and was abolished at 50 μg/ml. The decreased recovery of AP-1 in the pellets was not due to continued proteolysis during the subsequent binding assay, because the addition of soybean trypsin inhibitor, used to inhibit the proteolysis reaction before addition of 50 μg/ml trypsin, resulted in slightly higher levels of AP-1 bound than untreated control samples. These results suggest that proteins located on the cytoplasmic face of the purified Golgi membranes are required for AP-1 binding.

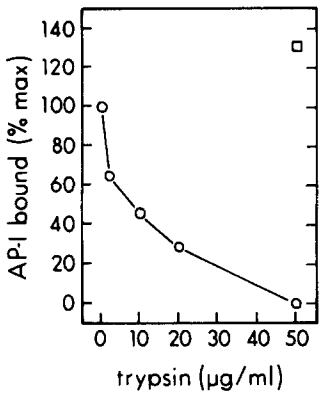

Figure 7. AP-1 translocation onto trypsin-digested Golgi membranes. Purified Golgi membranes were digested with increasing concentrations of trypsin (○) at 37°C for 15 min. A control sample contained 100 μg/ml soybean trypsin inhibitor before addition of 50 μg/ml trypsin (□). Proteolysis was terminated by the addition of soybean trypsin inhibitor, and equal aliquots of the digested membranes were added to the binding assay to yield a final concentration equivalent to 25 μg/ml undigested membranes. The assay also contained 5 mg/ml gel-filtered cytosol and 100 μM GTPγS and was incubated at 37°C for 15 min. Samples were analyzed by immunoblotting with mAb 100/1 and densitometry. The data are presented as the percent of AP-1 bound to Golgi membranes relative to the untreated control. A value corresponding to the amount of AP-1 found in the pellet fraction of cytosol incubated alone at 37°C for 15 min has been subtracted from each point.

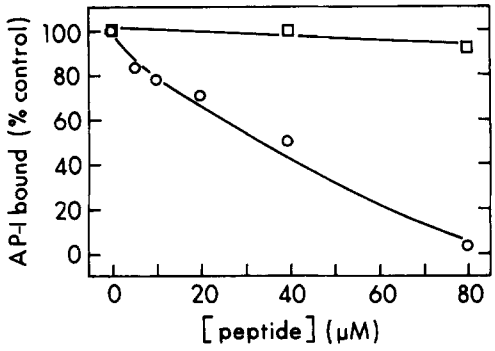

Figure 8. Effects of synthetic ARF peptides on AP-1 recruitment. AP-1 translocation was determined in the presence of 50 μg/ml Golgi membranes, 5 mg/ml gel-filtered liver cytosol, 200 μM GTPγS, and increasing concentrations of peptides corresponding to residues 2–17 (○) or residues 6–17 (□) of bovine ARF1. Assays were for 15 min at 37°C, and the pellets analyzed by immunoblotting with mAb 100/1 followed by densitometry. The data is presented as the percent of AP-1 found in the Golgi pellet fractions relative to the untreated control.

Role of ARF in AP-1 Recruitment onto the Golgi Membrane

Taken together, our results, and those of others (46, 58), indicate that the translocation of AP-1 from the cytosol to the Golgi membrane is dependent on a BFA-sensitive GTP-binding protein. Given this remarkable similarity to ARF-regulated coatomer recruitment (9), ARF is an obvious candidate for mediating the GTPγS-dependent recruitment of AP-1 onto the TGN. To explore this possibility, we initially made use of synthetic peptides corresponding to the amino-terminal sequence of mammalian ARF1. These peptides have been used by several groups (9, 20) to indicate a specific role for ARF in vesicular trafficking. Addition of a peptide corresponding to residues 2–17 of ARF1 inhibited GTPγS-stimulated AP-1 translocation onto the purified Golgi membranes (Fig. 8). The concentration causing 50% inhibition was approximately 40 μM. A synthetic peptide corresponding to residues 6-17 of ARF1 was without effect, even at a concentration of 80 μM. These findings support the notion that ARF is involved in clathrin-coated vesicle formation.

To obtain more conclusive evidence that ARF can support the translocation of AP-1 onto Golgi membranes, we modified our basic binding assay so that the effects of adding purified recombinant myristoylated ARF could be assessed. Rat liver cytosol was separated on a preparative Superose 6 column, and an example of a typical fractionation is shown in Fig. 9. The column resolved the clathrin triskelia and the coat proteins from the bulk of the cytosolic components. Fractions 28–31 (Fig. 9, lanes *k* and *l*) usually contained the majority of the cytoplasmic pools of both AP-1 and AP-2, as well as some coatomer as determined by the presence of β-COP, and were pooled separately from fractions 7–13 (lanes *d–f*), which contained the clathrin triskelia.

Incubation of purified Golgi membranes with a combination of the clathrin and coat protein–enriched fractions in the presence of GTPγS for 15 min at 37°C did not result in any increase in the level of β′-adaptin found in the Golgi pellet fraction (Fig. 10 *A*, compared lane *c* to *a*). Evidently, GTPγS was not promoting AP-1 binding by directly acting on the adaptor heterotetramer or the purified Golgi membranes. Thus it was clear that the additional cytosolic component required for AP-1 translocation must be the GTP-requiring component. By supplementing the Golgi membrane and coat protein mixture with increasing concentrations of recombinant ARF1, dose-dependent AP-1 recruitment was observed (Fig. 10 A, lanes *d–h*). The maximal amount of AP-1 recruited with 20 μg/ml ARF1 (Fig. 10 *A*, lane *h*) was less than that seen in the presence of 5 mg/ml cytosol (lane *b*). As the coat protein–enriched fraction used in these experiments contained coatomer as well (Fig. 9), we also followed β-COP in this experiment. Reprobing the blot with mAb M3A5, indicated that coatomer was also being recruited onto the Golgi membranes under the same conditions that promoted AP-1 translocation (Fig. 10 *A*, lanes *d–h*). This demonstrated that the additional cytosolic requirements for AP-1 and coatomer binding were met by addition of myristoylated ARF1.

Note that the level of AP-1 associated with the Golgi pellet incubated together with 20 μg/ml recombinant ARF and GTPγS, but without added adaptors (Fig. 10 *A*, lane *i*), was significantly higher than the level found on the Golgi membranes alone (lane *a*). As we have shown that endogenous AP-1 dissociated from the Golgi membranes at 37°C, we believe that in the presence of the purified ARF, some of the released adaptors were recruited back onto the Golgi membranes. It appears that a similar phenomenon was observed in Fig. 3 *B*, when analyzing AP-1 translocation from rat brain cytosol. In addition to the 110-kD brain-specific β′-adaptin, we also noted the endogenous 100-kD Golgi-derived β′-adaptin on the membranes. This may be the result of both the higher ARF concentration (55) and the lower AP-1 concentration found in brain.

To explore a possible role of clathrin in the translocation of AP-1 onto the Golgi, we compared ARF-mediated AP-1 recruitment in the absence and presence of cytosolic clathrin (Fig. 10 *B*). Again, the purified Golgi membranes, incubated at 37°C for 15 min with only GTPγS, contained low levels of AP-1 (Fig. 10 *B*, lane *a*). No increase in AP-1 as-

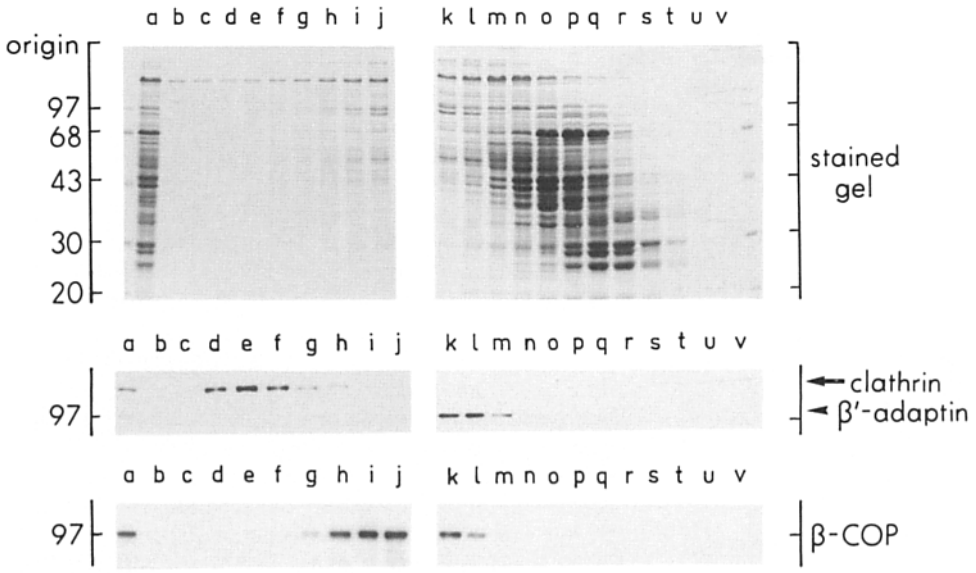

Figure 9. Fractionation of rat liver cytosol. Cytosol was resolved on a preparative Superose 6 column at 1 ml/min and 1-ml fractions were collected beginning 30 min after loading. A sample of 30 μg of the unfractionated cytosol (lane *a*) and 6-μl aliquots of every third column fraction from fraction 1 to fraction 61 (lanes *b–v*) were resolved on 11% gels and either stained with Coomassie blue (*top panel*) or transferred to nitrocellulose. The middle panel is from autoradiographs of the blots probed with a mixture of mAb TD.1 and mAb 100/1. The arrow indicates the position of the clathrin heavy chain and the arrowhead the position of the β'-adaptin subunit. The lower panel is from autoradiographs of the blots reprobed with mAb M3A5. Positions of the relative molecular weight standards, in kD, are indicated. Note that β-COP eluted in fractions 19–31 (lanes *h–l*) between the clathrin in fractions 7–16 (lanes *d'-g*) and the β'-adaptin in fractions 28–34 (lanes *k–m*).

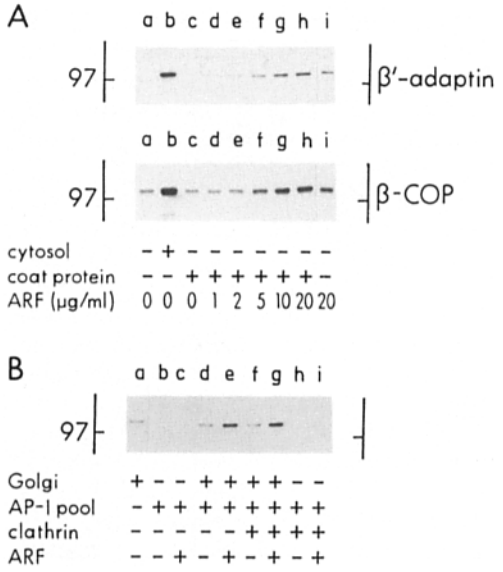

Figure 10. Role of ARF in AP-1 adaptor recruitment. (*A*) Tubes containing 80 μg/ml purified Golgi membranes, 200 μM GTPγS (lane *a*) and, in addition, 5 mg/ml gel-filtered cytosol (lane *b*) or a mixture of the coat protein–enriched pool (1 mg/ml) and the clathrin pool (0.05 mg/ml) (lanes *c–h*) were prepared on ice. Increasing concentrations of purified recombinant ARF1 were added to yield final concentrations of 1 (lane *d*), 2 (lane *e*), 5 (lane *f*), 10 (lane *g*), and 20 μg/ml (lanes *h*). A control tube contained the Golgi membranes, GTPγS, and 20 μg/ml ARF1 without the coat protein and clathrin pools (lane *i*). Tubes were incubated at 37°C for 15 min and the Golgi pellets analyzed by immunoblotting. The upper panel is from an autoradiograph of the blot probed with mAb 100/1 and the lower panel is an autoradiograph from the blot reprobed with mAb M3A5. The position of the relevant molecular weight standard is indicated on the left. (*B*) Tubes containing 200 μM GTPγS and 80 μg/ml Golgi membranes (lanes *a* and *d–g*), 1 mg/ml coat protein–enriched pool (lanes *b–e*), the coat protein–enriched pool and 0.05 mg/ml clathrin-containing pool (lanes *f–i*), and 20 μg/ml recombinant ARF1 (lanes *c*, *e*, *g*, and *i*) were incubated at 37°C for 15 min. The pellets were collected by centrifugation, and analyzed by immunoblotting with mAb 100/1. An autoradiograph of the blot is shown with the position of the relevant molecular weight standard indicated on the left.

sociated with the Golgi pellet was observed when the coat protein-containing pool was added to 1 mg/ml (Fig. 10 *B*, lane *d*). However, a fivefold increase in the AP-1 bound to the membranes resulted from adding 20 μg/ml purified ARF to the coat protein–Golgi membrane-GTPγS mixture (lane *e*). The level of AP-1 associated with the Golgi pellet after incubation of a mixture of the coat protein and clathrin pools, Golgi membranes and GTPγS at 37°C for 15 min (Fig. 10 *B*, lane *f*) was the same as that found on the Golgi membranes alone (lane *a*). On adding 20 μg/ml ARF, there was a sixfold increase in the AP-1 in the Golgi pellet (Fig. 10 *B*, lane *g*). As controls, no β'-adaptin was found in pellets from reactions containing the coat protein-enriched fraction plus 200 μM GTPγS (Fig. 10 *B*, lane *b*), coat proteins, ARF and GTPγS (lane *c*), coat proteins, clathrin and GTPγS (lane *h*) or coat proteins, clathrin, ARF, and GTPγS (lane *i*) incubated at 37°C for 15 min. We conclude that the translocation of AP-1 onto Golgi membranes is not dramatically influenced by cytosolic clathrin. These results, then, confirm that cytoplasmic adaptors and ARF are the minimal requirements for binding of AP-1 to Golgi membranes.

Discussion

Adaptors, like clathrin (14), occur in two biochemically distinct intracellular pools, a cytosolic and a membrane-associated pool. Clathrin coat formation is thought to occur via a cyclical process, with adaptors and clathrin being recruited from the cytosolic pool to the site of a nascent-

coated pit. This is followed by polymerization, budding, and finally release of the components back into the cytoplasmic pool by uncoating of the clathrin-coated vesicle. In this study, we have examined the translocation of the Golgi-specific AP-1 adaptor complex onto Golgi membranes. Our results confirm that GTP is required for this membrane association, and indicate that ARF is the GTP-requiring component. The BFA sensitivity of AP-1 recruitment onto Golgi membranes (46, 58) provided the initial indication of ARF involvement. The most recent studies examining the mode of action of BFA have concluded that the drug prevents ARF from exchanging nucleotides on the Golgi membrane by inactivating an ARF-specific nucleotide exchange protein (10, 16, 41). Therefore, it appears likely that any BFA-inhibitable process is likely to involve ARF. The finding that GppNHp was less effective than GTPγS in promoting AP-1 translocation is also consistent with a role for ARF in this process. Several years ago, GTPγS was shown to be at least 10-fold more effective than GppNHp in inhibiting intercisternal protein transport (34). It is now known that in that assay, the GTP analogues were acting directly on ARF (53). More recently, it has been shown directly that GTPγS is approximately 10-fold more potent at inducing ARF membrane association than GppNHp (42). These observations all pointed to a specific role for ARF in clathrin-coated vesicle formation on the TGN. We have directly confirmed this requirement for ARF by first showing that a synthetic peptide, corresponding to the amino-terminal region of ARF1, could inhibit AP-1 binding in our assay. More importantly, we have been able to reconstitute AP-1 translocation using Golgi-enriched membranes, a coat protein–enriched fraction from rat liver cytosol and purified ARF. It therefore appears that these are the minimal requirements for AP-1 recruitment to the Golgi. The same conclusion was reached in a complementary study recently published by Stamnes and Rothman (51).

Our analysis of the cytosol dependence of AP-1–Golgi translocation indicated that a limiting factor was present in cytosol. We also showed that only an AP-1–containing fraction, ARF and Golgi membranes were necessary to recruit the adaptor onto the membranes. Theoretically, it would seem reasonable to exclude ARF as the limiting cytosolic factor based on the abundance of ARF in brain, where it may constitute up to 1% of total protein (18). The mammalian ARF family is comprised of at least six members, and several different ARF proteins can be expressed in a single cell type (57). Our experiments and those of Stamnes and Rothman (51) have shown that high concentrations of recombinant mammalian ARF1 are capable of supporting AP-1–Golgi translocation in vitro, in addition to the formation of coatomer-coated vesicles (37). Within the cell, however, these processes may be regulated by distinct ARF members, and these may be present in different concentrations. In fact, a recent study has shown that mammalian ARF1, ARF3, and ARF5 all exhibit different Golgi membrane–binding characteristics (56). The alternative possibility is that a single ARF member is limiting in our assays. The absolute concentration of ARF in rat liver cytosol has not been determined, but is considerably less than that found in brain (55). We have shown that AP-1 translocates to the Golgi under conditions known to facilitate coatomer-coated vesicle accumulation (31, 34, 37, 49). EM shows that large quantities of these coatomer-coated vesicles accumulate (31, 34, 37). As the stoichiometry of ARF to coatomer in these vesicles is 3:1 (49), at the end of a 15-min incubation at 37°C a significant proportion of the cytosolic ARF pool may be irreversibly associated with these coatomer-coated vesicles. Also, coatomer-coated vesicles probably form throughout the Golgi complex, while the formation of clathrin-coated vesicles is restricted to the TGN, which might restrict ARF accessibility. Whether ARF is indeed the limiting factor we noted in cytosol can now be analyzed using coatomer-depleted cytosol. Since ARF still binds to Golgi membranes after removing coatomer by immunodepletion, but no coatomer-coated vesicles are formed (37), ARF would not be expected to be limiting under these conditions.

We observed a lag of several minutes before the AP-1 adaptors began to associate with Golgi membranes at 37°C. Thereafter, recruitment of AP-1 onto the Golgi membranes proceeded rapidly, and was complete within 15 min. Recently, the time course of the association of purified bovine brain ARF (41) and mammalian ARF5 (15) with purified Golgi membranes was examined. No association of ARF with the membranes was seen at 0°C. This probably reflects the catalytic nature of the nucleotide exchange process. At 37°C, ARF5 translocates on the membranes with a $t_{1/2}$ of approximately 10 min (15). Therefore, the lag period that preceded the increase in Golgi-associated AP-1 in our study most likely represents the time required to generate GTPγS-bound ARF on the Golgi membrane. This is also consistent with the finding that incubation of purified Golgi membranes with recombinant ARF1 and GTPγS at 37°C for 10 min allowed the subsequent binding of coatomer in the absence of ARF1 and GTPγS (9).

A model depicting our view of the early events in clathrin-coated vesicle assembly on the TGN is represented diagrammatically in Fig. 11. Both the coat components (clathrin and AP-1) and GDP-bound ARF are known to occur in a cytoplasmic reservoir pool. There is also evidence that ARF is recruited to the cytoplasmic face of the Golgi membrane through interaction with a nucleotide exchange protein, reported to be the target of BFA (10, 16, 41). The catalyzed exchange of GTP for GDP is thought to result in the exposure of the myristic acid on the amino terminus of ARF, and facilitate intercalation of ARF into the lipid bilayer. Indeed, purified GTP-bound ARF associates stably with phosphatidylcholine liposomes in vitro (17, 19, 41). The simplest mechanism for adaptor recruitment would be via a direct ARF interaction. However, since our experiments have shown that both AP-1 and coatomer were recruited onto the Golgi membranes by recombinant ARF1, the question of specificity and how clathrin coat formation remains confined to the TGN is raised. We envisage that a specific AP-1–docking protein occurs in the TGN that assists in the formation of a ternary ARF-docking protein–AP-1 complex. Alone, the docking protein would exhibit minimal affinity for the cytosolic pools of either ARF or AP-1. However, the interaction of GTP-bound ARF with the putative docking protein within the plane of the membrane might lead to a conformational change that would facilitate recruitment of AP-1 into ternary complex. A specific ARF-docking protein interaction is consistent with data showing two biochemically distinct pools of ARF on Golgi membranes (17). It is also incumbent on this model that a mechanism would exist to localize the puta-

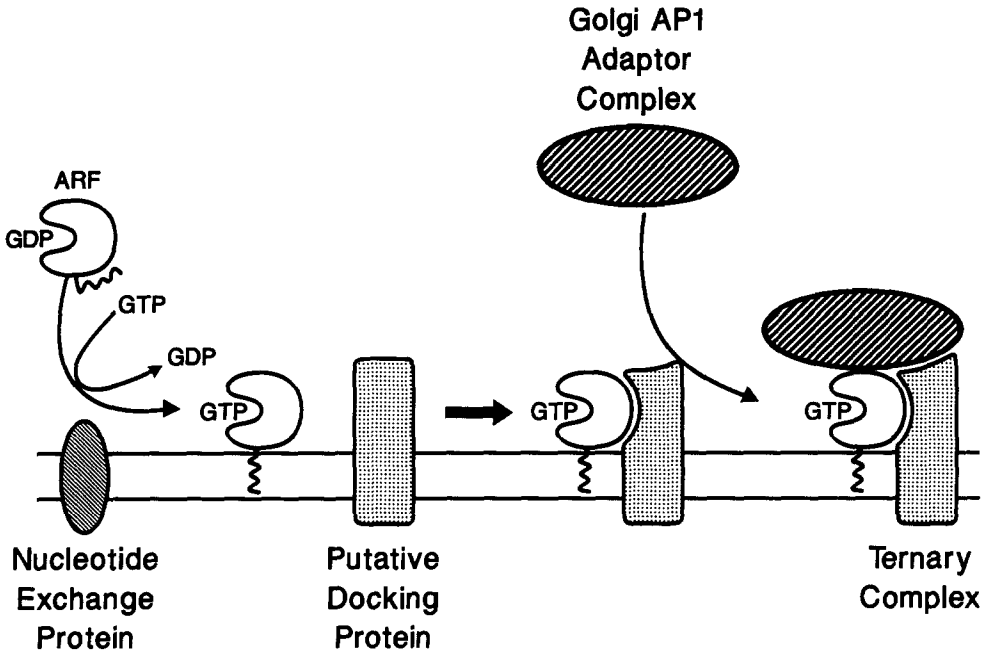

Figure 11. Schematic model for the early events in Golgi-derived clathrin-coated vesicle formation. Both the ARF nucleotide exchange protein and the putative docking protein are drawn as transmembrane proteins, although at present, no direct evidence for this is available.

tive docking protein to the TGN to prevent mislocalized ARF-mediated clathrin coated vesicle formation. Our proteolysis experiments have demonstrated the necessity for cytoplasmically oriented Golgi membrane proteins for AP-1 binding. Unfortunately, we cannot distinguish whether the trypsin treatment abolished the function of a nucleotide exchange protein, a putative docking protein, or both. Note that this model does not preclude additional levels of specificity imparted by distinct nucleotide exchange proteins for each member of the ARF family.

Rothman and colleagues have proposed a similar model for the events leading to coatomer-coated vesicle assembly (17, 47). They have documented that on addition of GTPγS to a mixture of cytosol and purified Golgi membranes, large quantities of coatomer-coated vesicles can be generated (31, 34, 37). These coated vesicles contain ARF as a major component (49), and GTP hydrolysis only appears to be required after association of the coatomer-coated vesicles with an acceptor membrane. The hydrolysis of GTP is thought to facilitate coat disassembly before membrane fusion (47). Given the requirement for ARF in AP-1 recruitment, it could be assumed that clathrin coat assembly might proceed similarly. However, several observations lead us to propose that clathrin-coated vesicle formation may occur by a distinct, but related mechanism. Our data showed that exogenous AP-1, recruited onto the Golgi by 100 μM GTPγS, was resistant to extraction with 1 M Tris-HCl, pH 7.0. In contrast, coatomer-coated vesicles can be efficiently separated from residual Golgi membranes by washing in 250 mM KCl (31). We also found that endogenous Golgi-associated AP-1 dissociated from the membranes on warming to 37°C, but β-COP remained membrane bound. This suggests that the interactions that maintain these different coat proteins on the Golgi membrane are different.

Furthermore, clathrin-coated vesicles have not been noted in significant quantities in preparations of cytosol and Golgi membranes incubated with GTPγS to accumulate coatomer-coated vesicles (11, 31, 34, 37). Because our study has demonstrated that these conditions also facilitate AP-1 translocation onto the Golgi membranes, it appears that under the conditions of those studies, clathrin-coated vesicle formation was arrested in the presence of GTPγS. The clathrin coat differs fundamentally from coatomer in that clathrin and AP-1 are distinct complexes in the cytosol, but must polymerize in a coordinated manner during clathrin-coated vesicle formation. Interestingly, clathrin and adaptors demonstrate no affinity for each other within cytosol, and are readily resolved by gel filtration. Thus, once cytosolic AP-1 has translocated onto the TGN, some modification is required to facilitate subsequent clathrin binding. This reaction may require additional components not provided in the standard assay, and hence, result in arrest. Alternatively, the formation of an ARF-docking protein–AP-1 ternary complex may be sufficient to recruit a clathrin triskelion from the cytosol. Once assembled, GTP hydrolysis may be required to allow further coat assembly. At present our data cannot exclude either of these possibilities.

It is also interesting to compare our results with the data available on the mode of association of AP-2 and clathrin with the plasma membrane. Because of the high degree of both sequence and structural homology between the AP-1 and AP-2 complexes, it might be assumed that they function similarly. We observed a large pool of AP-1 in liver cytosol and found that AP-1 translocation onto Golgi membranes was limited by a factor other than cytosolic AP-1. By contrast, the cytosolic concentration of AP-2 limits the rate and extent of clathrin coat formation at the cell surface directly (50). Furthermore, AP-2 has been found to bind to plasma membranes in a temperature-, cytosol- and energy-independent manner in two unrelated in vitro assays (29, 50). A specific receptor for AP-2 has been suggested (30), and recently, a plasma membrane–derived peptide fragment has been identified as a putative AP-2 receptor (39). The interaction between this putative AP-2 receptor and AP-2 is rapid and spontaneous at 4°C. These differences between AP-1 and AP-2 recruitment may underlie some mechanistic diver-

gence. As AP-2 is insensitive to BFA (12, 46, 58), and associates with plasma membranes spontaneously, perhaps the translocation of AP-1 and AP-2 are differently regulated.

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