

# The Role of ADP-ribosylation Factor and Phospholipase D in Adaptor Recruitment

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**Abstract.** AP-1 and AP-2 adaptors are recruited onto the TGN and plasma membrane, respectively. GTP $\gamma$ S stimulates the recruitment of AP-1 onto the TGN but causes AP-2 to bind to an endosomal compartment (Seaman, M.N.J., C.L. Ball, and M.S. Robinson. 1993. *J. Cell Biol.* 123:1093–1105). We have used subcellular fractionation followed by Western blotting, as well as immunofluorescence and immunogold electron microscopy, to investigate both the recruitment of AP-2 adaptors onto the plasma membrane and their targeting to endosomes, and we have also examined the recruitment of AP-1 under the same conditions. Two lines of evidence indicate that the GTP $\gamma$ S-induced targeting of AP-2 to endosomes is mediated by ADP-ribosylation factor-1 (ARF1). First, GTP $\gamma$ S loses its effect when added to ARF-depleted cytosol, but this effect is restored by the addition of recombinant myristoylated ARF1. Second, adding constitutively active Q71L

ARF1 to the cytosol has the same effect as adding GTP $\gamma$ S. The endosomal membranes that recruit AP-2 adaptors have little ARF1 or any of the other ARFs associated with them, suggesting that ARF may be acting catalytically. The ARFs have been shown to activate phospholipase D (PLD), and we find that addition of exogenous PLD has the same effect as GTP $\gamma$ S or Q71L ARF1. Neomycin, which inhibits endogenous PLD by binding to its cofactor phosphatidylinositol 4,5-bisphosphate, prevents the recruitment of AP-2 not only onto endosomes but also onto the plasma membrane, suggesting that both events are mediated by PLD. Surprisingly, however, neither PLD nor neomycin has any effect on the recruitment of AP-1 adaptors onto the TGN, even though AP-1 recruitment is ARF mediated. These results indicate that different mechanisms are used for the recruitment of AP-1 and AP-2.

**P**ROTEINS are transported from one membrane compartment of the cell to another by means of carrier vesicles. The first step in the formation of these vesicles is the recruitment of cytosolic proteins onto a “donor” membrane compartment, where they assemble into a coat. This coat may serve two purposes: to deform the membrane into a budding vesicle and to select the vesicle cargo by interacting with the cytoplasmic domains of some of the proteins in the donor membrane (Schekman and Orci, 1996).

The process of coat recruitment is still not well understood, although it is thought that there are specific docking sites on the membrane for coat proteins. In addition, in most cases a small GTP-binding protein has been shown to be involved in coat recruitment, somehow priming the membrane for the subsequent binding of coat proteins. Thus, the coatomer or COPI coat, which is recruited onto

the membranes of the Golgi stack and intermediate compartment, requires ADP-ribosylation factor (ARF)<sup>1</sup> for its membrane association, and studies making use of purified components have implicated the most abundant of the ARF isoforms, ARF1, in this event (Donaldson et al., 1992; Palmer et al., 1993). Similar studies have shown that ARF1 also allows AP-1 adaptors to be recruited onto the TGN membrane, after which clathrin binds to the adaptors and the two components coassemble to form a clathrin-coated bud (Stamnes and Rothman, 1993; Traub et al., 1993). Recently, an adaptor-related complex, called AP-3, has been identified; AP-3 is also recruited onto the TGN, but it is not associated with clathrin. Although studies have not yet been carried out using purified components, AP-3 recruitment is affected by reagents that act on ARF, indicating that it too requires ARF (Simpson et al., 1996, 1997). In contrast, the COPII coat, which is associated with the ER, requires another small GTP-binding protein, Sar1p, to bind to the membrane (Barlowe et al., 1994).

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1. *Abbreviations used in this paper:* AFR, ADP-ribosylation factor; BFA, brefeldin A; NRK, normal rat kidney; PA, phosphatidic acid; PIP<sub>2</sub>, phosphatidylcholine 4,5-bisphosphate; PLD, phospholipase D.

There is one other well-characterized type of coat in the cell, which mediates the formation of endocytic-coated vesicles at the plasma membrane and which consists of clathrin and AP-2 adaptors. The subunits of the AP-2 adaptor complex are closely related to those of the AP-1 complex and more distantly related to those of the AP-3 complex. However, unlike AP-1 and AP-3, AP-2 recruitment does not appear to be dependent on a conventional ARF. Thus, the drug brefeldin A (BFA), which prevents the nucleotide exchange of most ARFs, causes AP-1, AP-3, and coatamer to redistribute to the cytoplasm when added to living cells, while the distribution of AP-2 remains unchanged (Donaldson et al., 1990; Robinson and Kreis, 1992; Simpson et al., 1997). Similarly, in an *in vitro* system, BFA prevents the binding of AP-1, AP-3, and coatamer to their target membranes, without affecting the binding of AP-2 to the plasma membrane (Orci et al., 1991; Robinson and Kreis, 1992; Seaman et al., 1993; Simpson et al., 1996). Intriguingly, however, GTP $\gamma$ S, a poorly hydrolyzable analogue of GTP, which stimulates the recruitment of other coats onto their target membranes, does not stimulate the binding of AP-2 adaptors to the plasma membrane but instead causes them to become associated with a late endosomal compartment. This endosomal association can be prevented by the addition of brefeldin A, indicating that it is ARF dependent (Seaman et al., 1993). These observations suggest that docking sites for AP-2 adaptors exist both on the plasma membrane and on the endosomal compartment, but that normally they are only active at the plasma membrane, and that somehow GTP $\gamma$ S, presumably acting via ARF, switches on endosomal docking sites that normally are inactive. Whether recruitment of AP-2 adaptors onto the plasma membrane might also involve an (unconventional) ARF or ARF-related protein is not known.

So far, five ARFs have been identified in humans (Price et al., 1988; Bobak et al., 1989; Kahn et al., 1991; Tsuchiya et al., 1991), in addition to the less closely related ARL (ARF-like) family (Clark et al., 1993). ARF1 has been localized to the Golgi region in mammalian cells and has been shown to associate with isolated Golgi membranes (Stearns et al., 1990; Serafini et al., 1991). In other reports, ARFs 1, 3, and 5 have been detected on crude Golgi/microsomal fractions (Tsai et al., 1992) and, together with ARF4, have also been found on an endosome-enriched fraction (Whitney et al., 1995), while ARF6 has been localized to the plasma membrane (Cavenagh et al., 1996) and endosomes (Peters et al., 1995). All of the ARFs have been shown to be BFA sensitive, with the exception of ARF6, the least related of the ARF family (Tsuchiya et al., 1991). ARF6 also differs from the other ARFs in that it appears to be constitutively membrane associated, instead of cycling back and forth between membranes and cytosol (Peters et al., 1995; Cavenagh et al., 1996).

The mechanism by which ARF promotes the recruitment of cytosolic coat components onto the membrane is still not understood. One possibility is that ARF may interact directly with coat proteins, an idea supported by the finding that ARF1 can bind to one of the coatamer subunits (Zhao et al., 1997). A second possibility was proposed in a study on AP-1 recruitment, in which it was suggested that ARF might interact with the putative AP-1 docking site, inducing a conformational change that would allow the

docking site to bind the adaptor complex (Traub et al., 1993). A third possibility is that ARF may interact with neither the coat proteins nor their docking sites, but exert its effect through some other mechanism. ARF has been reported to activate phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (Brown et al., 1993; Cockcroft et al., 1994), and a recent study suggests that PLD may play a key role in coatamer recruitment (Ktistakis et al., 1996).

Here we use an *in vitro* system to investigate both the recruitment of AP-2 adaptors onto the plasma membrane and their alternative targeting to the endosomal compartment. As a comparison, we have also used our *in vitro* system to study the recruitment of AP-1 adaptors. We have raised a panel of monospecific anti-ARF antibodies and have used these and recombinant ARF to investigate the potential role of ARF(s) in adaptor recruitment. We find that ARF1 can mediate not only the recruitment of AP-1 adaptors onto the TGN, but also the GTP $\gamma$ S-induced recruitment of AP-2 adaptors onto endosomes. However, ARF1 does not appear to be required for the recruitment of AP-2 adaptors onto the plasma membrane. To investigate the possibility that ARF may facilitate adaptor recruitment through the activation of PLD, we have tested the effects of exogenous bacterial PLD and of neomycin, which inhibits endogenous ARF-dependent PLD by binding to its cofactor phosphatidylcholine 4,5-bisphosphate (PIP<sub>2</sub>). We have found that neither reagent has any effect on AP-1 recruitment. However, exogenous PLD stimulates AP-2 recruitment onto the endosomal compartment, while neomycin inhibits this event. Interestingly, neomycin also has a strong effect on plasma membrane AP-2 binding, indicating that the recruitment of AP-2 adaptors onto both membranes may require PLD.

## Materials and Methods

### Production of Anti-ARF Antibodies

The full-length coding sequences for ARFs 1, 4, 5, and 6 were amplified by PCR from human brain cDNA (CLONTECH Laboratories, Palo Alto, CA) and ligated into pBluescript. DNA manipulations were carried out essentially as described by Sambrook et al. (1989). Either full-length or variable regions of the ARFs were then ligated into pGEX-3X using BamHI and EcoRI or KpnI restriction sites, which were introduced by PCR using the following primers: ARF 1 forward: 5' CGC GGA TCC CCA GCA ATG ACA GAG AGC GTG TG 3', reverse: 5' CCG GAA TTC AAT CCC GGA GCT CGT CCT CGG C 3'; ARF 4 forward: 5' GCG GGA TCC CCA TGG GCC TCA CTA TCT CCT CC 3', reverse: 5' CGG GGT ACC AAC TAA TTT TGT TGT AAC AAG CCT 3'; ARF 5 forward: 5' CGC GGA TCC CCA GTA ATG ACC GGG AGC GGG TC 3', reverse: 5' CCG GAA TTC AAT CCC GCA GCT CGT CCT CCT G 3'; ARF 6 forward: 5' CGC GGA TCC CCT GCG CCG ACC GCG ACC GCA TC 3', reverse: 5' CCG GAA TTC AGT CCC TCA TCT CCC GGT CAT T 3'.

MC1061 cells were transformed with the plasmids and expression of the GST fusion proteins was induced with 0.1 mM IPTG. The fusion proteins were affinity purified on glutathione-Sepharose (Pharmacia LKB Biotech., Piscataway, NJ), using the method of Smith and Johnson (1988), and injected into rabbits using 0.5 mg in complete Freund's adjuvant for the first injection and 0.5 mg in incomplete Freund's adjuvant for two subsequent injections (Page and Robinson, 1995). Affinity purification of the resulting antisera was carried out essentially as previously described (Page and Robinson, 1995). Briefly, 10 ml of each serum was first adsorbed against 1 mg of GST coupled to CNBr-activated Sepharose (Pharmacia LKB Biotech.) and then affinity purified on 1 mg of its own fusion protein coupled to CNBr-activated Sepharose. Bound antibodies were eluted with

0.2 M glycine, pH 2.3, containing 0.1% gelatine. To select for monospecific antibodies, each of the affinity-purified sera was adsorbed at least twice against a mixture of the other full-length ARF-GST fusion proteins (1 mg each) coupled to CNBr-activated Sepharose.

To test the specificity of the antibodies, 50  $\mu$ g of each full-length ARF-GST fusion protein was digested with 500 ng activated Factor X according to Smith and Johnson (1988) to generate GST-free ARFs. The digests were subjected to SDS-PAGE on a 15% SDS gel, and Western blots were probed with the various antisera.

### Expression and Purification of Recombinant ARF1

Recombinant myristoylated wild-type and mutant ARF1 were prepared from BL21 *Escherichia coli* that had been transformed with plasmids encoding *N*-myristoyl transferase and either wild-type ARF1, Q71L ARF1, or T31N ARF1 (Dascher and Balch, 1994). The transformed cells were induced with 0.3 mM IPTG in the presence of 50  $\mu$ M myristate and grown at 27°C for 3 h (Franco et al., 1995). The cells were lysed, and the expressed ARF1 was purified using DEAE-Sephacel and AcA54 Ultrogel columns as described by Weiss et al. (1989). This particular strain of transformed bacteria has been shown to myristoylate 10–60% of the protein (Ranazzo et al., 1993).

### Rat Liver Fractionation

Fractionation of rat liver membranes was carried out essentially as described by Branch et al. (1987). 15 g of rat liver was homogenized in 40 ml of cold 0.25 M sucrose, 10 mM TES, pH 7.4, 1 mM MgCl<sub>2</sub> (STM), and a postmitochondrial supernatant was prepared by centrifuging the homogenate at 1500 g for 10 min. 5-ml samples of this were loaded onto 34-ml linear gradients prepared from 0.25 M sucrose, 10 mM TES pH 7.4, 1 mM EDTA, and 45% Nycodenz in 10 mM TES pH 7.4, 1 mM EDTA. The gradients were centrifuged at 206,000 g for 60 min in a vertical rotor (model VTi50; Beckman Instruments, Fullerton, CA), and 1-ml fractions were collected by upwards displacement.

### Preparation of Cytosol

Pig brain cytosol was prepared in cytosol buffer (25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mg/ml glucose, 1 mM DTT) as described by Seaman et al. (1993). The cytosol was clarified before use by centrifuging at 350,000 g for 15 min in a rotor (model TL100.2; Beckman Instruments) and was used at a final protein concentration of  $\sim$ 8 mg/ml.

For ARF depletion, 4 ml of cytosol was fractionated at 30 ml/h on a Superose 6 column (45  $\times$  1.6 cm) equilibrated in cytosol buffer containing 0.2 mM PMSF. Fractions containing ARFs were identified by Western blotting, and the remaining ARF-depleted fractions pooled and concentrated to the original volume using a Centricon-10 concentrator (Amicon, Beverly, MA). ARF immunoreactivity was undetectable in the ARF-depleted fractions.

### In Vitro Recruitment

Recruitment experiments were carried out both on enriched rat liver membrane fractions and on permeabilized normal rat kidney (NRK) cells. 400- $\mu$ l aliquots of the membrane fractions, prepared as above, were diluted threefold with STM and collected by pelleting at 90,000 g for 15 min in a rotor (model TL100.2; Beckman Instruments). Membrane pellets were resuspended in 50  $\mu$ l of cytosol containing 1 mM ATP, an ATP-regenerating system (5 mM creatine phosphate, 80  $\mu$ g/ml creatine phosphokinase), and 100  $\mu$ M EGTA and incubated at 37°C for 10 min. GTP $\gamma$ S, when included, was used at a concentration of 100  $\mu$ M. After the incubation, the membranes were diluted with 1 ml of cold STM and collected by centrifugation as before. The pellets were resuspended in 50  $\mu$ l of SDS-PAGE sample buffer, and 10- $\mu$ l aliquots were subjected to SDS-PAGE and Western blotting. Blots were probed with the brain-specific rabbit anti- $\alpha$ -adaplin antibody, A706-727 (Ball et al., 1995), or the species-specific mouse anti- $\gamma$ -adaplin monoclonal antibody, mAb100/3 (Sigma Chemical Co., Poole, UK) (Ahle et al., 1988) and rabbit anti-mouse IgG (Sigma Chemical Co.), followed by <sup>125</sup>I-protein A (Amersham Corp., Indianapolis, IN). Quantification was carried out using a phosphorimager (FujiX Bas2000 Bio-Imaging Analyzer; Fuji Photo Film Co., Tokyo, Japan). Each experiment was carried out at least three times to ensure that the results were reproducible.

In some experiments, recombinant ARF1, bacterial PLD, and/or neomycin were added. Recombinant ARF1 was added to a final concentration of between 20  $\mu$ g/ml and 200  $\mu$ g/ml, depending on the experiment. Endogenous ARF1 in our cytosol preparations was estimated to be  $\sim$ 10  $\mu$ g/ml by Western blotting. Bacterial PLD (type VII from *Streptomyces* species) was obtained from Sigma Chemical Co. It had a specific activity of 1,200 U/mg, 1 U being defined as the amount of enzyme required to liberate 1  $\mu$ mol of choline per hour at pH 5.6 at 30°C. It was added to a final concentration of between 0.5 and 37.5  $\mu$ g/ml, depending on the experiment. As a comparison, in a recent study by Ktistakis et al. (1996), bacterial PLD was added to a final concentration of 4  $\mu$ g/ml, although the specific activity was measured differently (50 U/mg, 1 U being defined as the amount of enzyme that will hydrolyze 1.0  $\mu$ mol of phosphatidylcholine per min at 37°C). Neomycin was also obtained from Sigma Chemical Co. and was added at concentrations ranging from 0.03 to 3.0 mM.

Methods for recruitment onto permeabilized NRK cells have been previously described (Robinson and Kreis, 1992; Seaman et al., 1993).  $\alpha$ - and  $\gamma$ -adaplin were labeled for immunofluorescence with A706-727 and mAb100/3 followed by FITC anti-rabbit IgG or Texas red anti-mouse IgG (Amersham Corp.), respectively. Each experiment was repeated at least three times.

### Assay for PLD Activity

PLD activity was determined essentially as described by Bi et al. (1997). Briefly, NRK cells grown to  $\sim$ 90% confluency were labeled overnight with 25  $\mu$ Ci/ml [<sup>3</sup>H]palmitic acid (Amersham Corp.), and then cells from two 9-cm-diam dishes were washed with cytosol buffer, frozen in liquid nitrogen and rapidly thawed, scraped from the dishes, and incubated as eight aliquots, each with 50  $\mu$ l cytosol containing 1% butanol for 20 min. GTP $\gamma$ S and neomycin were added as indicated. Cellular lipids were then extracted, and the organic phase was subjected to thin-layer chromatography (TLC) as described by Bi et al. (1997). Radioactivity was quantified using a phosphorimager, and the phosphatidyl butanol produced in each tube was normalized using other radiolabeled lipids to control for variability in recovery and/or loading. Another two dishes of cells were grown under identical conditions but without the [<sup>3</sup>H]palmitic acid, permeabilized as above, and used to assay AP-2 recruitment under the same conditions.

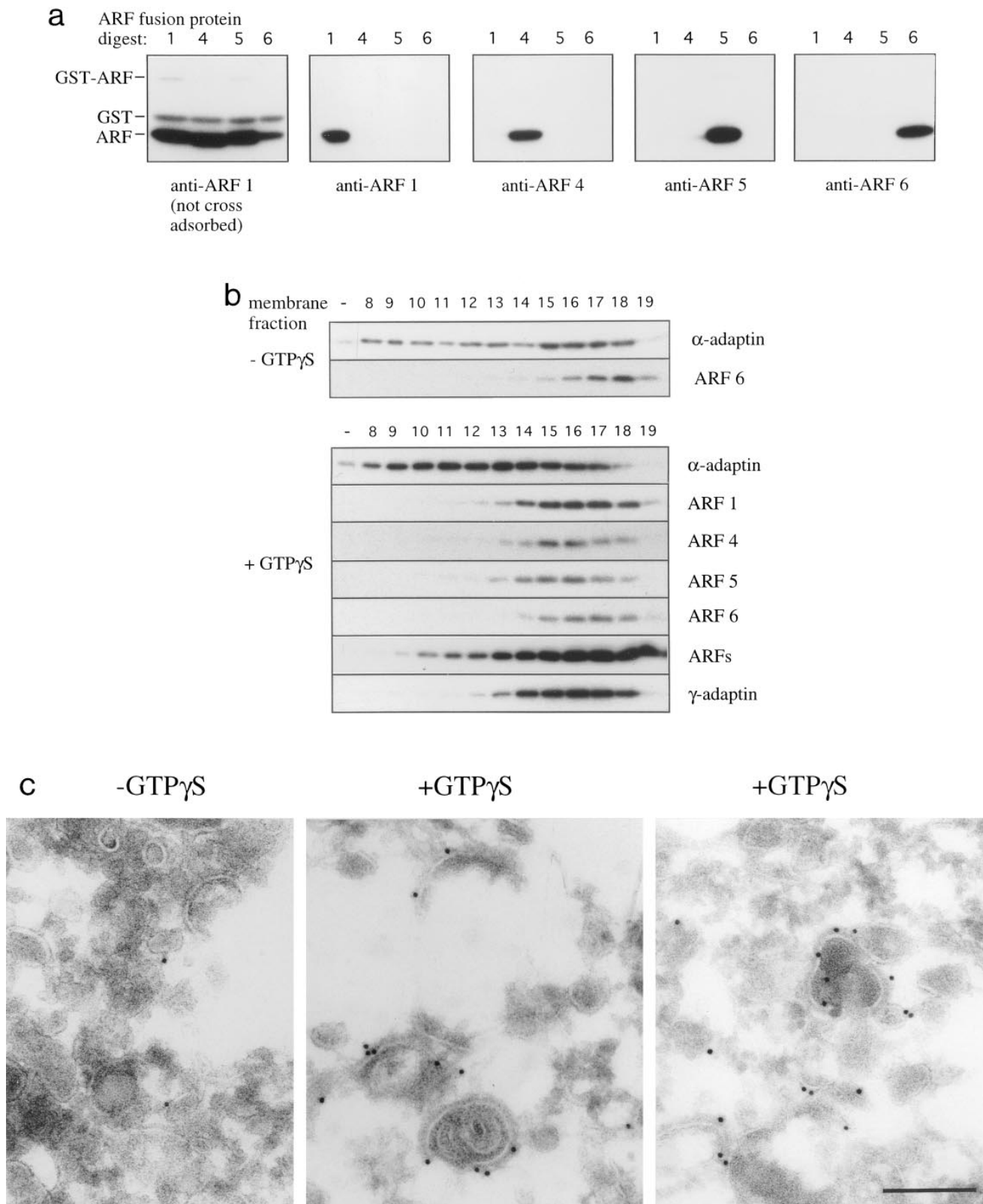
### Electron Microscopy

For immunogold localization of newly recruited AP-2 adaptors, NRK cells grown in 60-mm dishes were permeabilized by immersion into liquid nitrogen before incubation with cytosol, as previously described (Seaman et al., 1993). At the end of the incubation, the cells were washed gently with cytosol buffer and then fixed for 1 h with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.25 M Hepes, pH 7.2, before scraping, pelleting, and embedding in 10% gelatine. For examination of rat liver membranes, recruitment was carried out on gradient fractions as above, and the pellets were fixed and embedded in 10% gelatine. Cryosections were prepared, immunolabeled, and contrasted as described by Simpson et al. (1996). Newly recruited AP-2 adaptor complexes were detected using the A706-727 anti- $\alpha$ -adaplin antibody. The anti-Igp110 antibody was a kind gift from Paul Luzio (University of Cambridge, Cambridge, UK) (Reaves et al., 1996).

## Results

### Recruitment of AP-2 Adaptors onto Rat Liver Membranes

Previous studies have shown that the recruitment of adaptor complexes onto membranes can be reconstituted in vitro using permeabilized NRK cells as a source of target membranes and pig brain cytosol as an exogenous source of adaptors. After incubation at 37°C in the presence of an ATP-regenerating system, newly recruited adaptors can be detected by immunofluorescence using species- or tissue-specific antibodies that do not recognize the endogenous adaptors (Robinson and Kreis, 1992; Seaman et al., 1993). We have adapted this system to allow a more quantitative assessment of adaptor recruitment, using enriched



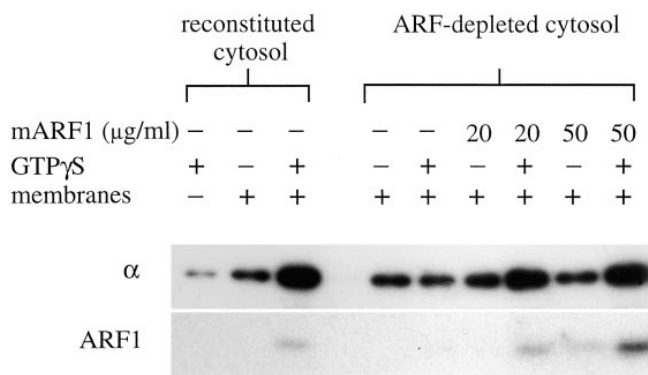
**Figure 1.** Recruitment of cytosolic proteins onto rat liver membranes. (a) To determine the specificity of the anti-ARF antibodies raised in this study, samples of ARF-GST fusion proteins were digested with Factor X and the digests subjected to SDS-PAGE on 15% gels and Western blotted. Identical panels were probed with affinity-purified and cross-adsorbed anti-ARF antisera as indicated. Non-cross-adsorbed anti-ARF1 labels all four ARF isoforms, while after cross-adsorption each antibody is specific for its own isoform. ARF3, which is 97% identical to ARF1, was not included in this study. (b) Membranes from a Nycodenz gradient were incubated with pig brain cytosol in the presence or absence of GTP $\gamma$ S. The incubation mixture in this experiment and in all subsequent experiments also contained ATP and an ATP-regenerating system. After the incubation, membranes were pelleted and assayed by Western blotting for the

rat liver fractions as the source of target membranes. To investigate whether any of the ARFs cofractionate with membranes that bind adaptors, we have raised antisera against ARFs 1, 4, 5, and 6 and then cross-adsorbed them with the other ARF isoforms. Fig. 1 *a* shows Western blots probed with the various ARF antisera and demonstrates that each of the adsorbed antisera recognizes only its appropriate ARF, while an antiserum raised against ARF1 and not adsorbed recognizes ARFs 1, 4, 5, and also 6 to a lesser extent.

To generate target membranes for adaptor recruitment, rat liver postmitochondrial supernatants (5 ml) were fractionated on a 34-ml Nycodenz gradient, and 1-ml fractions were incubated with pig brain cytosol in the presence of an ATP-regenerating system, either with or without GTP $\gamma$ S. The various ARF isoforms were detected by probing Western blots with the antisera shown in Fig. 1 *a*, while newly recruited adaptor complexes were detected with a brain-specific anti- $\alpha$ -adaptin antibody (A706-727) (Ball et al., 1995) for AP-2 adaptors and a species-specific anti- $\gamma$ -adaptin antibody (mAb100/3) (Ahle et al., 1988) for AP-1 adaptors (Fig. 1 *b*).

In the absence of GTP $\gamma$ S, newly recruited AP-2 adaptors were found to bind to membranes that were broadly distributed throughout the upper half of the gradient, with a slight peak in fractions 15–18. ARF6 could also be detected in the absence of GTP $\gamma$ S and was found to peak in fractions 15–19. The other ARF isoforms, and newly recruited AP-1 adaptors, were unable to be detected under these conditions (data not shown). In the presence of GTP $\gamma$ S, the binding of AP-2 adaptors was found to be enhanced and was shifted somewhat to less dense membranes. This shift is consistent with GTP $\gamma$ S inducing the recruitment of AP-2 onto a different (i.e., endosomal) compartment (Seaman et al., 1993). GTP $\gamma$ S also facilitated the binding of the other ARF isoforms and AP-1 adaptors to membranes. The membranes that bound AP-1 and the membranes that bound the various ARFs had similar fractionation profiles, peaking between fractions 14 and 18 or 19. The nonadsorbed ARF1 antibody, which cross-reacts with all of the known ARFs and possibly also with other as yet unidentified ARFs, produced a strong signal that also peaked between fractions 14 and 19 but which extended into the lighter portion of the gradient. In contrast, the membranes that bound AP-2 in the presence of GTP $\gamma$ S had a different fractionation profile, with a broad peak between fractions 9 and 17. These results suggest either that low levels of a conventional ARF may facilitate the GTP $\gamma$ S-dependent recruitment of AP-2 onto these membranes, or alternatively that a novel ARF could be involved.

To confirm that the AP-2 detected in this assay was in



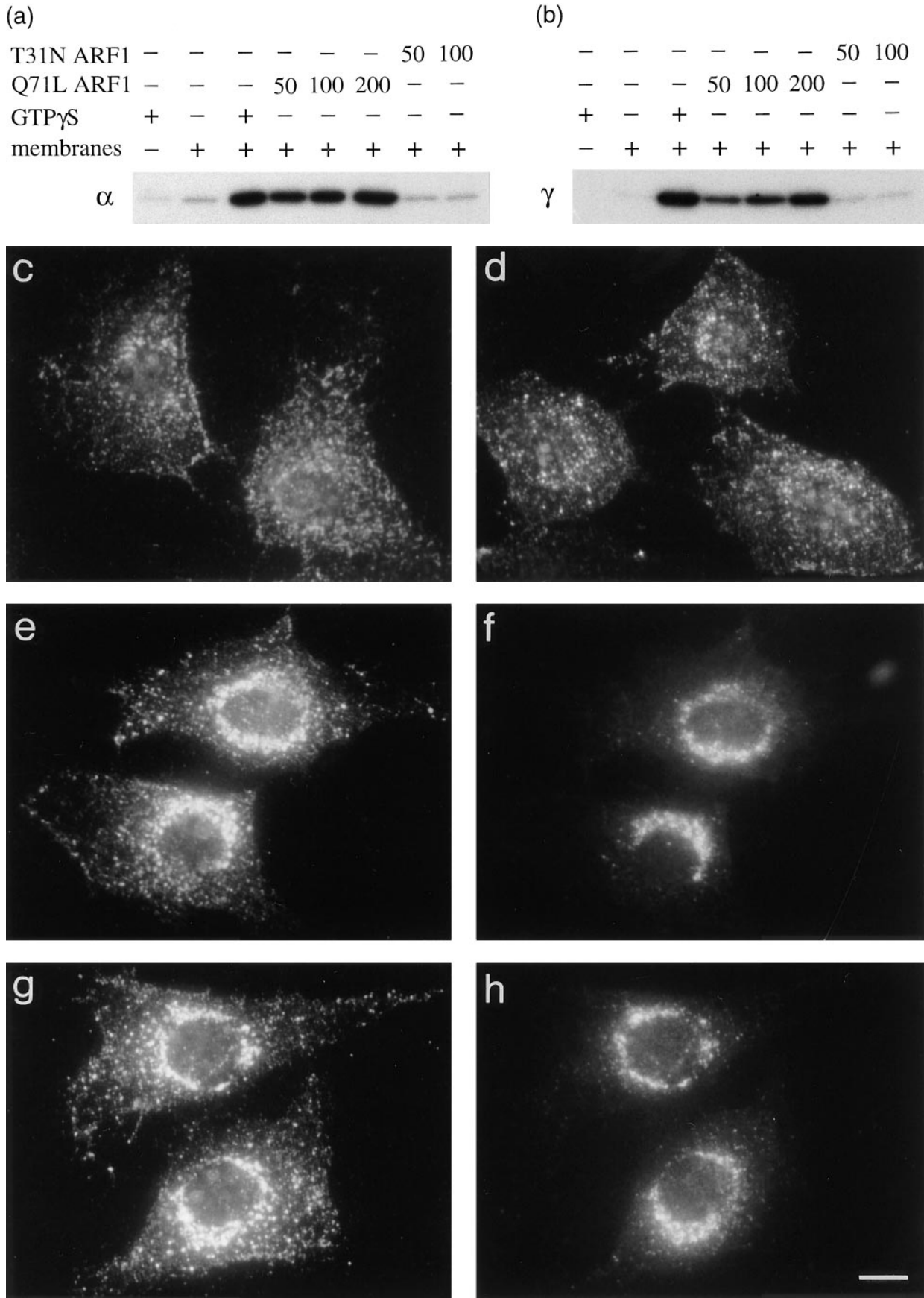
**Figure 2.** Role of ARF1 in AP-2 recruitment. Aliquots of peak fractions from a Nycodenz gradient were incubated with either reconstituted cytosol or with high-molecular weight, ARF-depleted fractions of cytosol that had been gel filtered. GTP $\gamma$ S and recombinant myristoylated ARF1 were included as indicated. Newly recruited AP-2 adaptors ( $\alpha$ ) and membrane-associated ARF1 were detected by Western blotting. When reconstituted cytosol was added, AP-2 recruitment onto the membranes was enhanced by the addition of GTP $\gamma$ S. No such enhancement was seen with the ARF-depleted cytosol, but it was restored by the addition of recombinant myristoylated ARF1.

fact membrane associated, fractions were fixed after incubation with pig brain cytosol and processed for immunoelectron microscopy (Fig. 1 *c*), using the brain-specific anti- $\alpha$ -adaptin antibody followed by 10-nm protein A gold. Without GTP $\gamma$ S, low levels of newly recruited AP-2 were observed associated with small vesicles. In the presence of GTP $\gamma$ S, enhanced labeling was seen that tended to be associated with larger membranous structures. These structures often contained internal membranes and are likely to correspond to the endosomal compartment to which AP-2 is targeted in permeabilized cells (Seaman et al., 1993), while the smaller vesicles labeled in the absence of GTP $\gamma$ S are likely to be derived from the plasma membrane.

### Role of ARF1 in the Binding of AP-2 Adaptors

To determine whether low levels of a conventional ARF, such as ARF1, might be involved in the binding of AP-2 to endosomes, we generated ARF-depleted cytosol by gel filtration and then added back defined components. Fig. 2 (*first three lanes*) shows an experiment in which the ARF-depleted cytosol was mixed with low molecular weight, ARF-containing fractions to generate reconstituted cytosol and then added to membranes in the presence or absence of GTP $\gamma$ S. The addition of GTP $\gamma$ S increased the

various ARF isoforms, or for newly recruited AP-2 or AP-1 adaptor complexes using brain-specific anti- $\alpha$ - or species-specific anti- $\gamma$ -adaptin antibodies, respectively. Significant amounts of both  $\alpha$ -adaptin and ARF6 are associated with membranes even in the absence of GTP $\gamma$ S, although they show only partial overlap. In the presence of GTP $\gamma$ S, the binding of AP-2 is increased and the fractionation profile shifted to less dense membranes. The other ARF isoforms and AP-1 are also recruited onto membranes and show similar fractionation profiles to each other but not to AP-2. (*c*) Frozen thin sections were prepared from samples equivalent to 8–19 in *b*. The sections were labeled with brain-specific anti- $\alpha$ -adaptin, followed by 10-nm protein A gold. In the absence of GTP $\gamma$ S, AP-2 is recruited onto small vesicles, presumably derived from the plasma membrane, while in the presence of GTP $\gamma$ S, AP-2 is recruited onto larger structures that often contain internal membranes, characteristic of endosomes. Bar, 200 nm.



**Figure 3.** Effects of ARF1 mutants on adaptor recruitment. Aliquots of pooled peak fractions from a Nycodenz gradient were used as acceptor membranes for the recruitment of (a) AP-2 ( $\alpha$ ) or (b) AP-1 ( $\gamma$ ). These membranes were incubated with cytosol containing either GTP $\gamma$ S, Q71L ARF1, or T31N ARF1 and then subjected to SDS-PAGE and Western blotting. Q71L ARF1, but not T31N ARF1, stimulates the recruitment of both types of adaptors even in the absence of GTP $\gamma$ S. (c-h) Permeabilized NRK cells were incubated with either cytosol alone (c) or cytosol containing 100  $\mu$ g/ml T31N ARF1 (d), GTP $\gamma$ S (e and f), or 100  $\mu$ g/ml Q71L ARF1 (g and h), and newly recruited AP-2 or AP-1 adaptors were detected using anti- $\alpha$  (c, d, e, and g) or anti- $\gamma$  (f and h) antibodies. Q71L ARF1 has a GTP $\gamma$ S-like effect on both types of adaptors, indicating that in both cases the GTP $\gamma$ S is acting via ARF. Bar, 10  $\mu$ m.

binding of AP-2 adaptors to the membranes and also caused trace amounts of ARF1 to bind. In contrast, membranes incubated with ARF-depleted cytosol alone showed only a basal level of AP-2 recruitment, which was not increased with GTP $\gamma$ S (Fig. 2, *fourth and fifth lanes*). To determine whether ARF1 could substitute for the ARF-containing fractions, recombinant myristoylated ARF1 was included with ARF-depleted cytosol. The last four lanes of Fig. 2 show that the recombinant ARF1 protein fully restored the GTP $\gamma$ S-enhanced binding of AP-2 adaptors and was itself recruited onto the membrane as well, although at lower levels.

Because GTP $\gamma$ S is likely to have multiple effects, we also tested whether the Q71L mutant of ARF1, which hydrolyzes GTP poorly (Tanigawa et al., 1993), could mimic the effect of GTP $\gamma$ S on AP-2 recruitment. For these experiments, unfractionated cytosol was used since the Q71L mutant is expected to have a dominant effect. Fig. 3 *a* shows that 50  $\mu$ g/ml of the mutant ARF1 strongly stimulated the binding of AP-2 to membranes, giving levels of recruitment approaching that seen with GTP $\gamma$ S. Higher concentrations (100 and 200  $\mu$ g/ml) of Q71L ARF1 increased AP-2 binding slightly further. Equivalent amounts of the dominant negative ARF1 mutant, T31N (Dascher and Balch, 1994), did not enhance AP-2 binding over levels seen without GTP $\gamma$ S. Q71L ARF1 also promoted the membrane recruitment of  $\gamma$ -adaptin (Fig. 3 *b*), as expected since ARF1 has been demonstrated to have a role in AP-1 adaptor recruitment to the TGN (Stamnes and Rothman, 1993; Traub et al., 1993). However, the effect of the mutant ARF1 on AP-1 was more moderate than on AP-2 adaptors in that even 200  $\mu$ g/ml Q71L ARF1 did not stimulate AP-1 recruitment to quite the levels seen with GTP $\gamma$ S.

To confirm that Q71L ARF1 causes AP-2 adaptors to bind to the perinuclear endosomal compartment, a recruitment experiment was carried out using permeabilized NRK cells, and the cells were labeled for immunofluorescence using the tissue-specific  $\alpha$ -adaptin antibody. After incubating the cells with cytosol alone, a punctate plasma membrane pattern was seen (Fig. 3 *c*). This same type of labeling was seen when T31N ARF1 was included (Fig. 3 *d*). Only faint AP-1 recruitment was detected under either of these conditions (not shown). When GTP $\gamma$ S was included in the incubation, intense perinuclear labeling of  $\alpha$ -adaptin, characteristic of endosomal targeting, was observed (Fig. 3 *e*). A very similar pattern was seen when Q71L ARF1 was included instead of GTP $\gamma$ S (Fig. 3 *g*). With either GTP $\gamma$ S or Q71L ARF1, perinuclear labeling of newly recruited  $\gamma$ -adaptin was also detected (Fig. 3, *f* and *h*), consistent with its TGN localization (Robinson and Kreis, 1992). However, this pattern was distinct from the perinuclear labeling seen for AP-2 adaptors (see Seaman et al., 1993).

Thus, these data indicate that ARF1 can mediate the binding of AP-2 adaptors to the endosomal compartment, even though there is relatively little ARF1 associated with this compartment.

### ***Phospholipase D Can Stimulate AP-2 Recruitment***

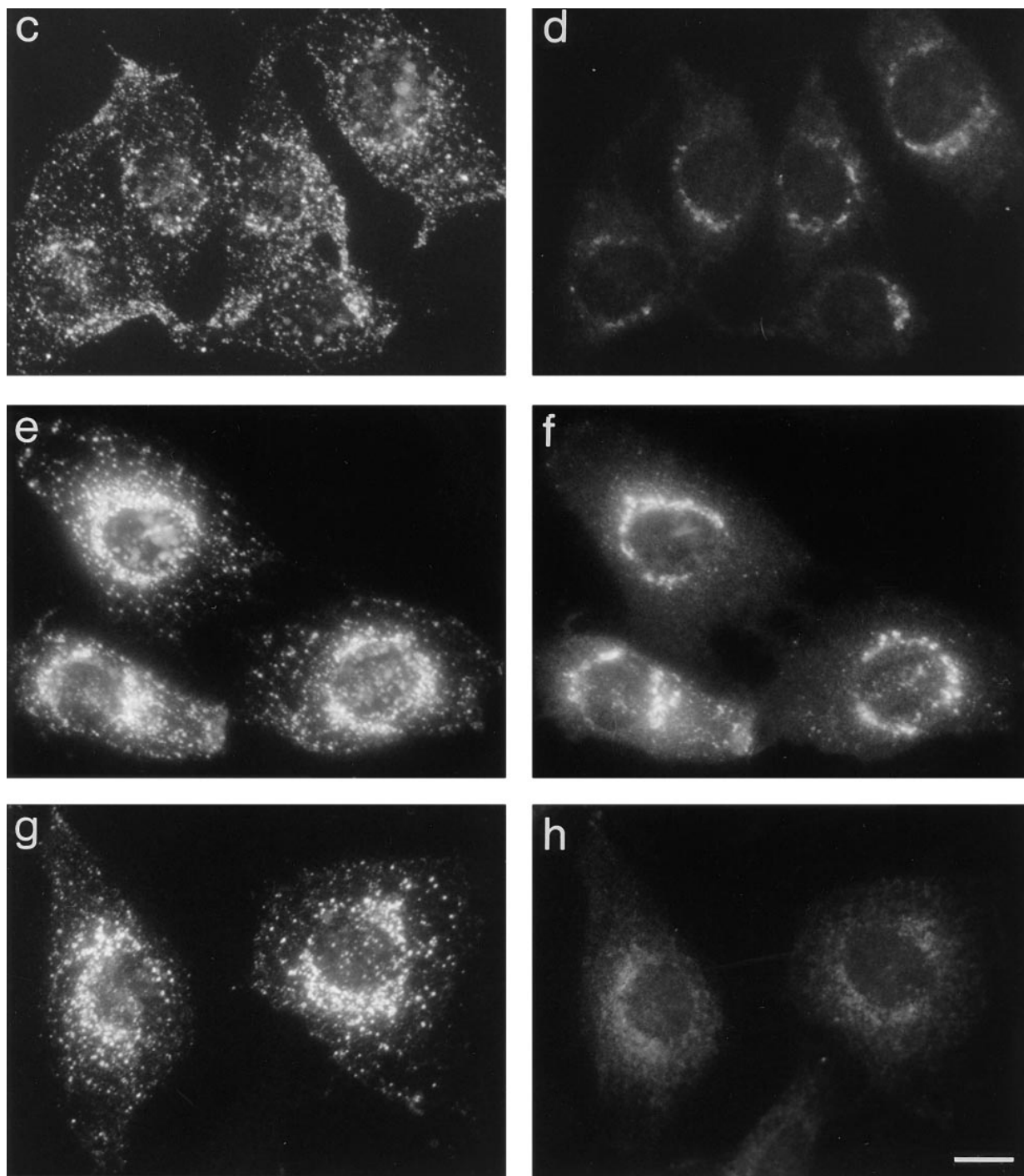
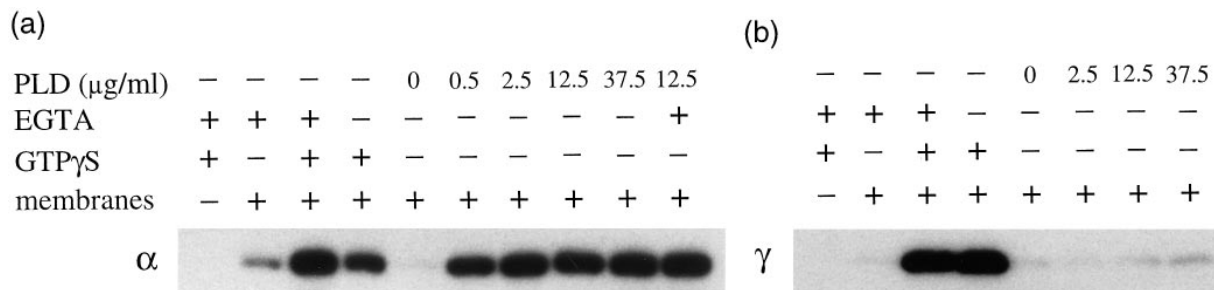
How is ARF1 acting to promote the recruitment of AP-2

adaptors onto endosomes? The relatively low levels of ARF1 associated with the endosomal compartment suggest that it may be acting catalytically. One possibility is that it may be acting via PLD since previous reports have shown that ARF is an effective activator of this enzyme (Brown et al., 1993; Cockcroft et al., 1994). PLD hydrolyzes phosphatidylcholine to phosphatidic acid (PA), which along with PIP<sub>2</sub> has been proposed to contribute to coatamer binding to membranes (Ktistakis et al., 1996). If ARF1 acts in AP-2 recruitment via activation of PLD, then the addition of exogenous active PLD should bypass the requirement for activated ARF to give enhanced recruitment.

Fig. 4 *a* shows an experiment assessing the recruitment of cytosolic AP-2 adaptors onto rat liver membranes in the presence of varying amounts of bacterial PLD. Although EGTA is normally added to the incubation mixture, most of these incubations were carried out in the absence of EGTA since bacterial PLD is reported to require Ca<sup>2+</sup> for maximum activity (Imamura and Horiuti, 1979). Under these conditions, the membrane association of AP-2 adaptors in both the absence and the presence of GTP $\gamma$ S is reduced, although a GTP $\gamma$ S enhancement of recruitment is still observed (Fig. 4 *a*, *first five lanes*). When the effect of adding exogenous PLD was tested, we found that bacterial PLD can effectively substitute for GTP $\gamma$ S in promoting AP-2 recruitment. At low concentrations of PLD (e.g., 0.1  $\mu$ g/ml), the addition of 1 mM EGTA was found to inhibit PLD-induced recruitment by  $\sim$ 70%, but at concentrations of 2.5  $\mu$ g/ml or above, 1 mM EGTA only inhibited by  $\sim$ 40% (data not shown). In addition, because EGTA itself promotes AP-2 recruitment, at high concentrations of PLD similar amounts of AP-2 were found to be recruited onto the membrane in the absence and in the presence of EGTA (Fig. 4 *a*, *last lane*).

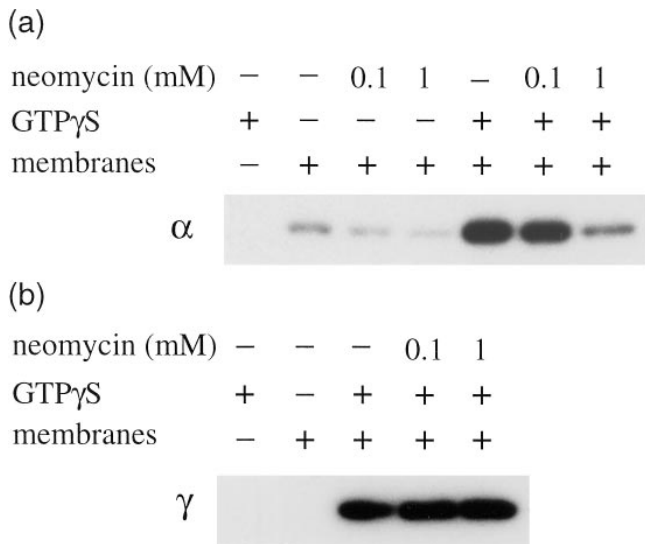
Perhaps surprisingly, since its membrane association is believed to be ARF1 mediated, the recruitment of AP-1 adaptors was not appreciably enhanced by PLD, even at the highest concentrations used (Fig. 4 *b*). Thus, although GTP $\gamma$ S and Q71L ARF1 stimulate both AP-1 and AP-2 recruitment, the finding that only AP-2 recruitment is stimulated by PLD suggests that the mechanisms of ARF1-dependent AP-1 and AP-2 recruitment may differ.

The effect of PLD was also examined using permeabilized NRK cells. For these experiments, 12.5  $\mu$ g/ml PLD plus 100  $\mu$ M EGTA were used to avoid any Ca<sup>2+</sup>-induced mistargeting (see Seaman et al., 1993). As previously shown, in the absence of GTP $\gamma$ S or PLD, AP-2 adaptors were recruited onto the plasma membrane (Fig. 4 *c*) and AP-1 adaptors showed a faint perinuclear localization (Fig. 4 *d*), while in the presence of GTP $\gamma$ S, AP-1 recruitment was increased (Fig. 4 *f*) and AP-2 adaptors bound to a perinuclear compartment (Fig. 4 *e*). When bacterial PLD was included in the incubation mixture, the enhanced AP-2 recruitment detected biochemically on rat liver membranes (Fig. 4 *a*) was seen to correlate with perinuclear staining (Fig. 4 *g*), morphologically similar to that induced by GTP $\gamma$ S (Fig. 4 *e*). Some punctate plasma membrane staining was also observed, but this did not seem to be increased in the presence of PLD. Double labeling of the same cells for  $\gamma$ -adaptin confirmed the lack of effect of PLD on AP-1 adaptor recruitment (Fig. 4 *h*).



**Figure 4.** Effect of exogenous PLD on adaptor recruitment. (a and b) Membrane pools (see Fig. 3) were incubated with cytosol containing bacterial PLD, EGTA (100  $\mu\text{M}$ ), and GTP $\gamma$ S in combinations as indicated, and either  $\alpha$  (a) or  $\gamma$  (b) recruitment was detected by Western blotting. PLD mimics the GTP $\gamma$ S effect on AP-2 recruitment, but not on AP-1 recruitment. (c-h) Recruitment onto permeabilized NRK cells was carried out with cytosol alone (c and d), with GTP $\gamma$ S (e and f) or with 12.5  $\mu\text{g/ml}$  PLD (g and h). The same cells were double labeled for immunofluorescence using either the anti- $\alpha$  (c, e, and g) or anti- $\gamma$  (d, f, and h) antibodies. PLD only has a GTP $\gamma$ S-like effect on the AP-2 adaptors. Bar, 10  $\mu\text{m}$ .





**Figure 5.** Effect of neomycin on the recruitment of adaptors onto rat liver membranes. Rat liver membrane pools (see Fig. 3) were incubated with cytosol in the absence or presence of GTP $\gamma$ S and varying concentrations of neomycin. The effect of neomycin on AP-1 and AP-2 recruitment was assessed by Western blotting of the membrane samples using anti- $\alpha$  (a) or anti- $\gamma$  (b) antibodies. Neomycin, which indirectly inhibits PLD activity, decreases the amount of AP-2 recruitment both in the absence and in the presence of GTP $\gamma$ S but has no appreciable effect on AP-1 recruitment.

### Neomycin Inhibits Both Endosomal and Plasma Membrane AP-2 Recruitment

The effect of exogenous bacterial PLD on AP-2 recruitment suggests that GTP $\gamma$ S exerts its effect through the cell's endogenous PLD. If this is the case, then inhibitors of the endogenous PLD activity would be expected to prevent or reduce the GTP $\gamma$ S-stimulated AP-2 recruitment. Although multiple PLD activities have been described, it has been reported that the mammalian ARF-dependent PLD requires PIP<sub>2</sub> as a cofactor for optimum activity (Hammond et al., 1995). Thus, we investigated the effects of including the drug neomycin, a high-affinity ligand of PIP<sub>2</sub> that inhibits membrane-bound PLD activity (Liscovitch et al., 1994), in the incubation.

When 1 mM neomycin was added, the amount of newly recruited AP-2 was significantly reduced both in the absence and in the presence of GTP $\gamma$ S (Fig. 5 a). 0.1 mM neomycin gave a lesser but still detectable inhibition. We also assessed the effect of neomycin on AP-2 recruitment induced by the dominant active Q71L ARF1 since it is formally possible that ARF and PLD may independently lead to the binding of AP-2 adaptors to endosomes. However, neomycin had the same effect on Q71L-induced recruitment as on GTP $\gamma$ S-induced recruitment, indicating that the activities of ARF1 and PLD are in fact coupled (data not shown). In contrast, the recruitment of AP-1 adaptors was unaffected by neomycin, even at 1 mM (Fig. 5 b).

These observations were confirmed by immunofluorescence labeling of permeabilized cells after adaptor recruitment (Fig. 6). The normal punctate plasma membrane recruitment of AP-2 adaptors seen in the absence of GTP $\gamma$ S

was much reduced by the presence of 1 mM neomycin in the incubation (Fig. 6, compare a and c). In the same cells, neomycin did not appear to affect the basal levels of AP-1 (Fig. 6, b and d). Similarly, the GTP $\gamma$ S-induced perinuclear targeting of AP-2 adaptors was also strongly inhibited (Fig. 6, compare e and g), while AP-1 recruitment in the same cells was apparently unaffected by treatment with neomycin (Fig. 6, f and h). Thus, these experiments confirm a role for PLD activity in the binding of AP-2 to endosomal membranes *in vitro*, and in addition they implicate PLD in the normal recruitment of AP-2 adaptors onto the plasma membrane.

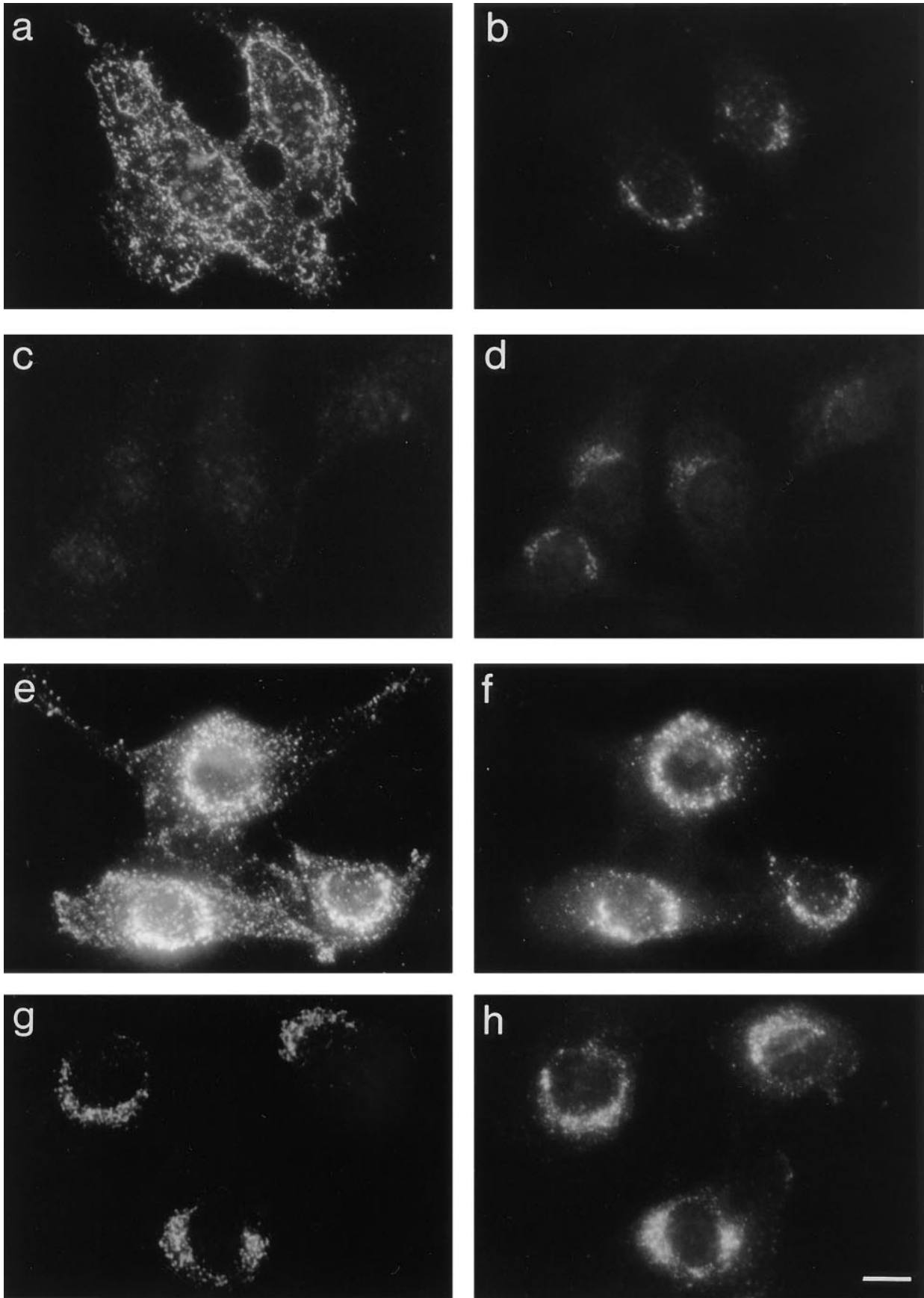
To compare the effect of neomycin on AP-2 recruitment with its effect on PLD activity, NRK cells were grown overnight with [<sup>3</sup>H]palmitic acid to label membrane lipids. They were then permeabilized and incubated for 20 min with increasing concentrations of neomycin plus 1% butanol so that PLD activity could be measured by assaying for the production of phosphatidyl butanol. At the end of the incubation, lipids were extracted and analyzed by TLC. A similar experiment was carried out on unlabeled NRK cells to assess the effect of the same concentrations of neomycin on AP-2 recruitment. Fig. 7 shows the results of the two experiments.

Because whole cells were used, the effect of GTP $\gamma$ S on AP-2 recruitment was less pronounced than on membrane fractions, although recruitment could be inhibited by incubating the cells at 4°C (Fig. 7 a). When neomycin was added to the cells, there was a steady decline in recruitment with increasing concentrations of the drug, and at 3 mM neomycin, AP-2 binding was essentially undetectable. Increasing concentrations of neomycin also inhibited PLD activity, but the effect was less pronounced, and even at 3 mM the production of phosphatidyl butanol in GTP $\gamma$ S-treated cells was only diminished by ~40% (Fig. 7 b). However, it is worth noting that the addition of GTP $\gamma$ S only stimulated PLD activity by ~30% over the level found in the absence of GTP $\gamma$ S. This result suggests that much of the PLD activity is ARF independent and might not be susceptible to neomycin. In addition, it is possible that PIP<sub>2</sub> may play a direct role in AP-2 recruitment, as well as an indirect role via PLD (see Discussion).

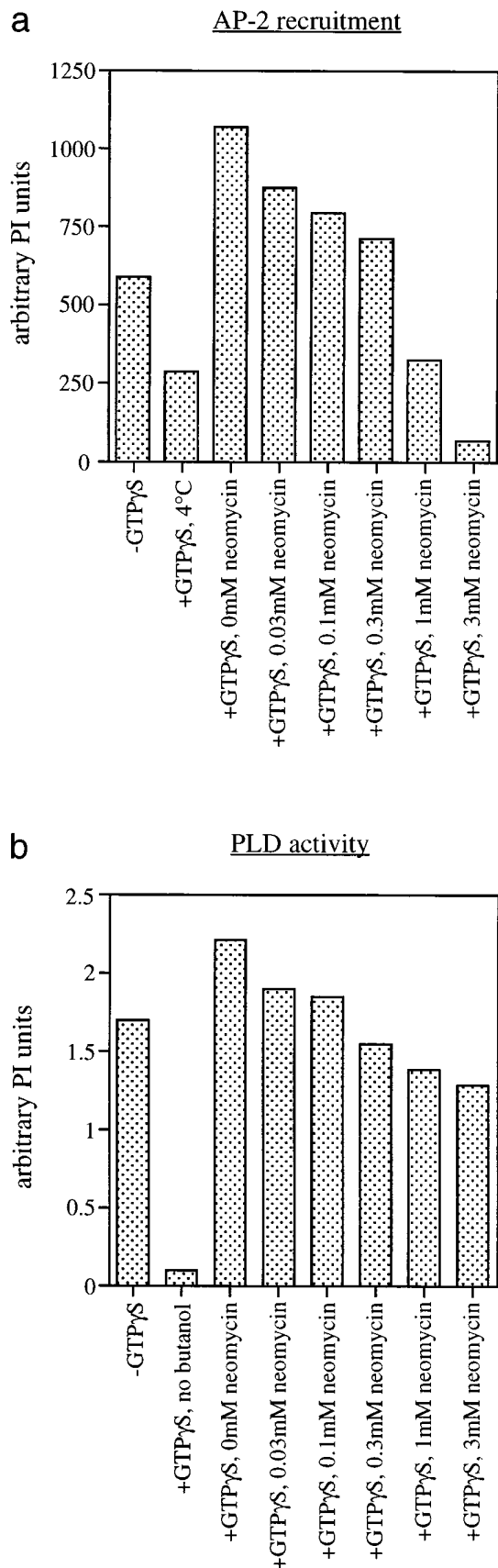
### Characterization of AP-2-binding Membranes

The immunofluorescence results presented so far demonstrate that GTP $\gamma$ S, Q71L ARF1, and PLD all cause AP-2 adaptors to bind to a perinuclear compartment. However, there are many organelles concentrated in the perinuclear region of the cell. To confirm that all three treatments cause the adaptors to bind to the same compartment, we investigated the localization of the newly recruited AP-2 adaptors by subcellular fractionation and by immunogold electron microscopy.

Rat liver membrane pools from Nycodenz gradients were incubated with cytosol in the presence of GTP $\gamma$ S, Q71L ARF1, or PLD, and the recruitment of adaptor complexes onto each pool was assessed by Western blotting and quantified using a phosphorimager. Membranes binding AP-1 and AP-2 adaptors in the presence of GTP $\gamma$ S had distinct fractionation profiles, as shown previously (Fig. 8 a; see also Fig. 1 b). AP-2 adaptors recruited



*Figure 6.* Effect of neomycin on adaptor recruitment in permeabilized cells. Permeabilized NRK cells were allowed to recruit adaptors from cytosol in the absence (*a–d*) or presence (*e–h*) of GTP $\gamma$ S. Neomycin (1 mM) was included in *c*, *d*, *g*, and *h*. For each of the conditions, the cells were double labeled for immunofluorescence using anti- $\alpha$  (*a*, *c*, *e*, and *g*) or anti- $\gamma$  (*b*, *d*, *f*, and *h*) antibodies. Photographs in the presence and in the absence of neomycin were taken and printed under identical conditions. Neomycin inhibits the recruitment of AP-2 adaptors both onto the plasma membrane in the absence of GTP $\gamma$ S and onto the endosomal compartment in the presence of GTP $\gamma$ S, without showing any effect on AP-1 recruitment. Bar, 10  $\mu$ m.



**Figure 7.** Comparison of the effects of neomycin on AP-2 recruitment and on PLD activity. (a) Permeabilized NRK cells were allowed to recruit adaptors from cytosol either without GTP $\gamma$ S,

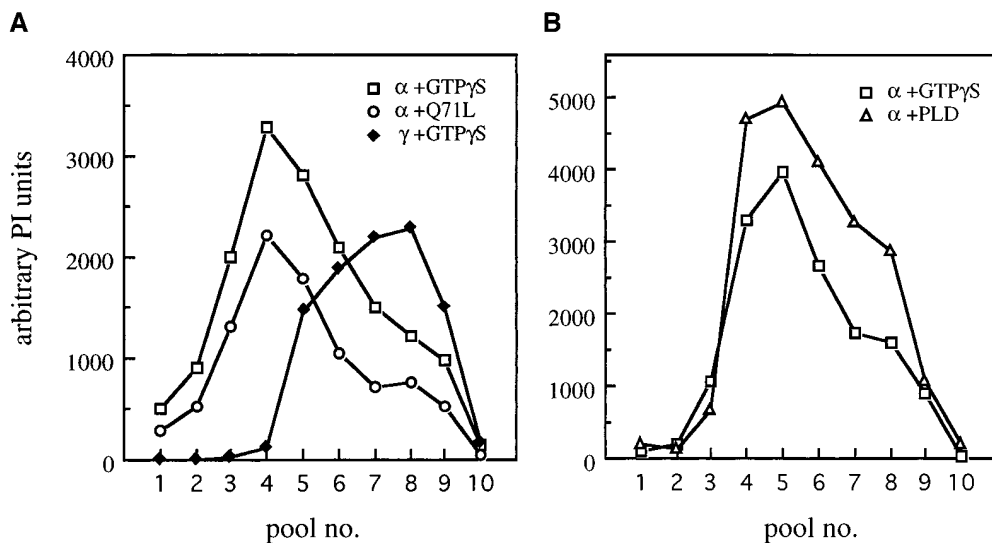
in the presence of Q71L ARF1 gave a very similar profile to that seen with GTP $\gamma$ S (Fig. 8 a). In a separate experiment, fractions were incubated with cytosol containing either GTP $\gamma$ S or exogenous PLD and then assayed for newly recruited AP-2, and again the profiles were found to be similar (Fig. 8 b).

To investigate the localization of the AP-2 adaptors at an ultrastructural level, we carried out a recruitment experiment using permeabilized NRK cells incubated with cytosol in the presence of GTP $\gamma$ S, Q71L ARF1, or PLD and then prepared frozen thin sections of the cells for immunogold electron microscopy. When the recruitment was carried out in the presence of GTP $\gamma$ S, the AP-2 adaptors (labeled with large gold particles) were detected on endosome-like structures, often with internal membranes, and on smaller tubulovesicular elements, often in close proximity to the Golgi stack (Fig. 9, a and b). A proportion of these structures were also positive for lgp110, a marker for late endosomes and lysosomes (small gold particles) (Reaves et al., 1996). These data are consistent with the previous identification of this compartment as a late endosome (Seaman et al., 1993). When either Q71L ARF1 or PLD were included in the cytosol, AP-2 adaptors were recruited to structures of a similar appearance to those observed with GTP $\gamma$ S, including multivesicular bodies that sometimes colabeled for lgp110 and smaller tubulovesicular profiles (Fig. 9, c-f). We could detect no significant morphological difference in the membrane structures onto which AP-2 adaptors were recruited under these three conditions, indicating that GTP $\gamma$ S, Q71L ARF1, and PLD all activate the same pathway.

## Discussion

Previous studies have shown that the recruitment of both AP-1 and AP-2 adaptors in vitro is affected by GTP $\gamma$ S. In the case of AP-1 adaptors, GTP $\gamma$ S stimulates their recruitment onto the TGN, the membrane with which they are normally associated, and this event has been shown to be mediated by ARF1 (Robinson and Kreis, 1992; Stamnes and Rothman, 1993; Traub et al., 1993; Seaman et al., 1996). In contrast, GTP $\gamma$ S causes AP-2 adaptors to be recruited onto an endosomal compartment, even though

with GTP $\gamma$ S at 4°C, or with GTP $\gamma$ S plus increasing concentrations of neomycin and then assayed by Western blotting and phosphorimager quantification for AP-2 recruitment. The relatively weak effect of GTP $\gamma$ S is due to the use of whole cells rather than membrane fractions. Neomycin inhibits AP-2 recruitment in a dose-dependent manner, with essentially complete inhibition at 3 mM. (b) NRK cells were labeled overnight with [ $^3$ H]palmitic acid, permeabilized, and then incubated with cytosol containing 1% butanol either without GTP $\gamma$ S or with GTP $\gamma$ S plus increasing concentrations of neomycin. Lipids were extracted and subjected to TLC, and PLD activity was assayed by quantifying the production of phosphatidyl butanol. In one sample, the butanol was omitted as a control. PLD activity is stimulated ~30% by the addition of GTP $\gamma$ S and inhibited by neomycin, although even at 3 mM it is only inhibited ~40%. This probably reflects the existence of multiple types of PLD in the cell, not all of which can be inhibited by neomycin, and may also indicate that neomycin has a direct effect on AP-2 recruitment, as well as an indirect effect by inhibiting PLD.



**Figure 8.** Fractionation of membranes binding adaptors under various conditions. Pools of pairs of membrane fractions from Nycodenz gradients were incubated with cytosol in the presence of GTP $\gamma$ S or 100  $\mu$ g/ml Q71L ARF (a) or in a separate experiment in the presence of GTP $\gamma$ S or 12.5  $\mu$ g/ml PLD (b). Newly recruited AP-2 or AP-1 adaptors were detected on Western blots using anti- $\alpha$ - or anti- $\gamma$ -adaplin antibodies, respectively. The signal in each sample was quantified using a phosphorimager. As also shown in Fig. 1 b,  $\alpha$ - and  $\gamma$ -adaplin-binding membranes have distinct fractionation profiles. However, the profile of the  $\alpha$ -adaplin-binding membranes is similar whether GTP $\gamma$ S, Q71L ARF1, or PLD is added.

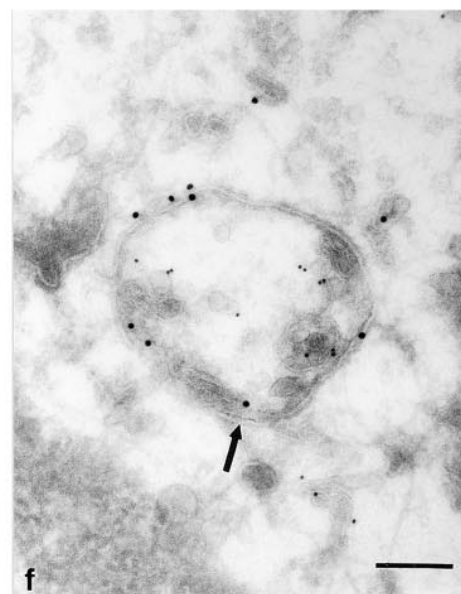
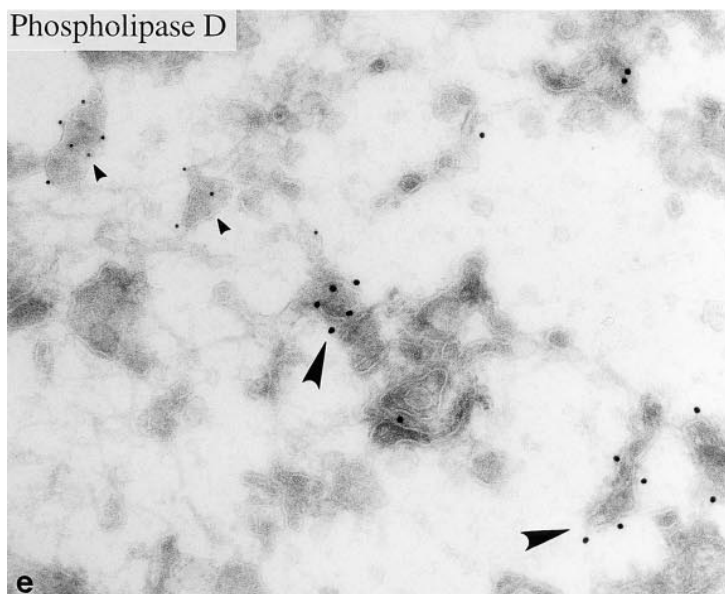
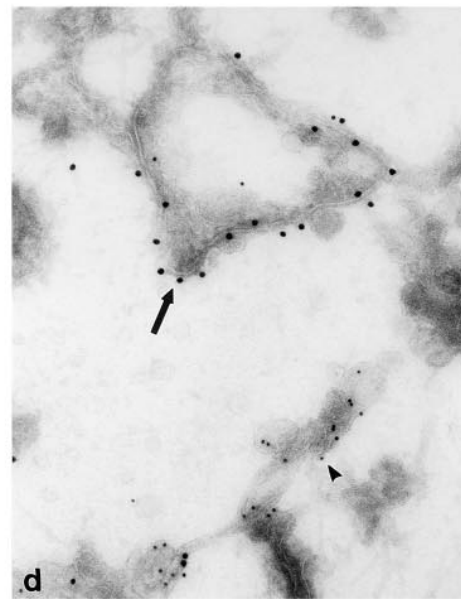
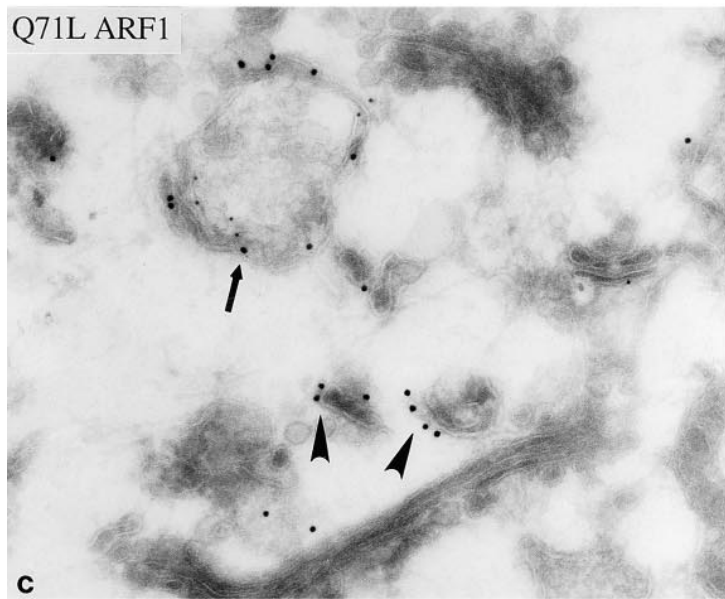
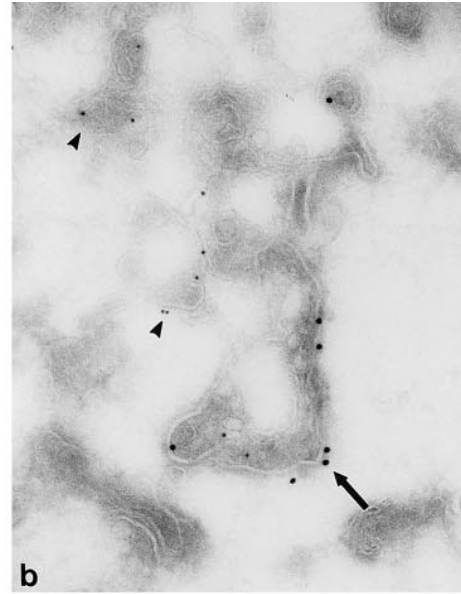
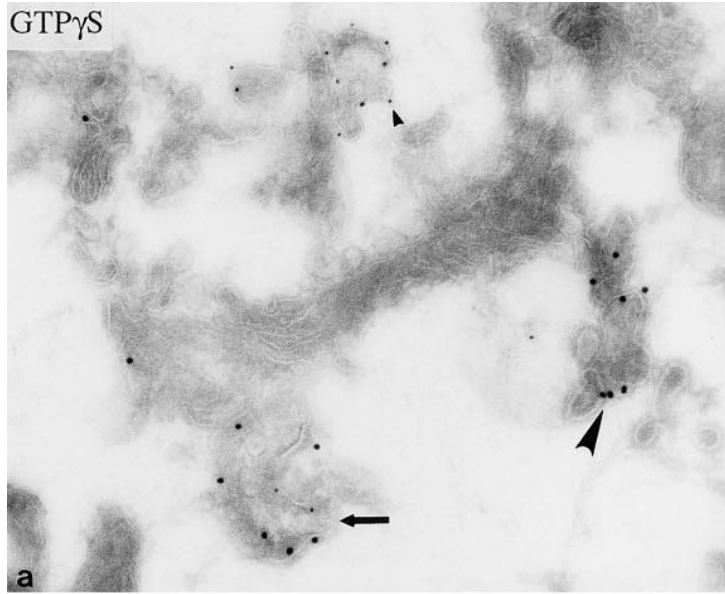
they are normally associated with the plasma membrane (Seaman et al., 1993). Excess  $\text{Ca}^{2+}$  was found to have a similar effect (Seaman et al., 1993), and a recent study reported that AP-2 adaptors can also bind to lysosomes *in vitro*, although in this case the recruitment was carried out under essentially physiological conditions, so it is not clear why the adaptors bound to this compartment (Traub et al., 1996). In addition, in living cells certain cationic amphiphilic drugs such as chlorpromazine cause AP-2 adaptors to bind to late endosomes and/or lysosomes (Wang et al., 1993). Taken together, these studies indicate that AP-2 docking sites are present on membranes of the endolysosomal system as well as the plasma membrane and that a number of stimuli can switch them on. Whether the sites on these membranes are normally completely inactive, perhaps acting as a storage pool that can be mobilized to the plasma membrane, or whether there is a very low level of recruitment onto endosomes and lysosomes to facilitate a particular trafficking pathway, is still not known.

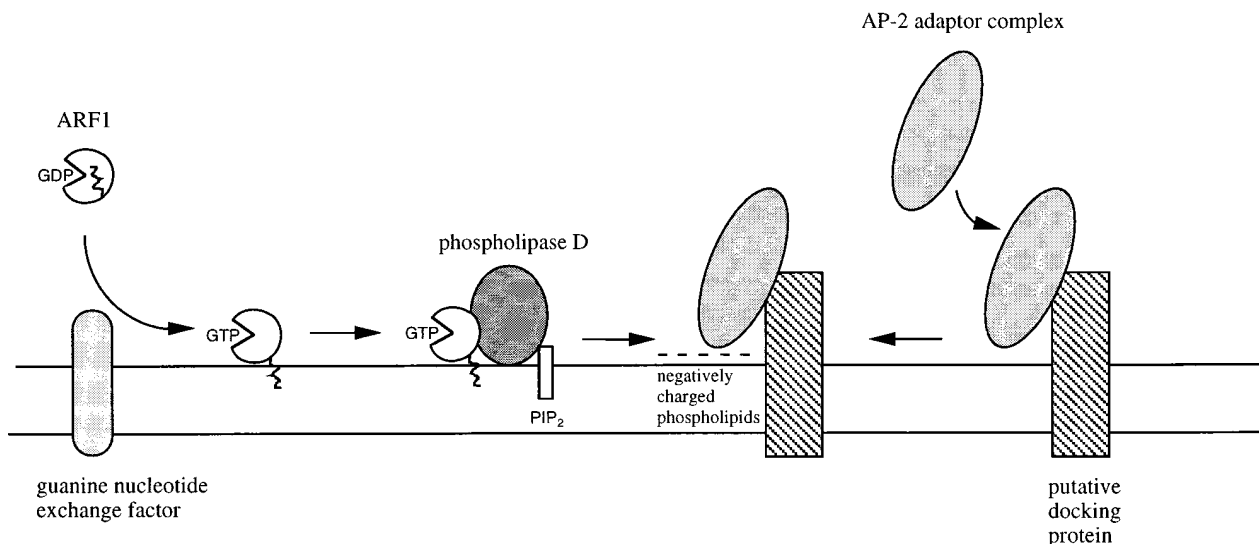
In this study, we began by focusing on the effect of GTP $\gamma$ S on AP-2 adaptors. We found that when ARFs and other low molecular weight GTP-binding proteins were removed from cytosol by gel filtration, the GTP $\gamma$ S effect was lost but could be restored by the addition of recombinant ARF1, indicating that ARF1 is the target of the GTP $\gamma$ S. Further evidence for a role for ARF1 came from our finding that the constitutively active ARF1 mutant, Q71L ARF1, can substitute for GTP $\gamma$ S. Whether other ARFs are equally effective at driving AP-2 onto endo-

somes is still not known. The endosomal membranes that recruit AP-2 adaptors have relatively little ARF1 or any of the other ARFs associated with them, suggesting that ARF is acting catalytically rather than stoichiometrically. Studies from other labs have shown that ARF activates PLD (Brown et al., 1993; Cockcroft et al., 1994), and PLD has been implicated in the recruitment of coatmer onto Golgi membranes (Ktistakis et al., 1996). Similarly, we found that exogenous PLD causes AP-2 adaptors to bind to endosomes, indicating that ARF1 promotes recruitment onto endosomes by activating endogenous PLD. Interestingly, two other treatments that cause AP-2 to bind to endosomes, elevated  $\text{Ca}^{2+}$  levels and cationic amphiphilic drugs, have also been reported to activate PLD (Brown et al., 1995; Liscovitch et al., 1994; Siddiqi et al., 1995). In contrast, exogenous PLD has no apparent effect on AP-1 adaptors, even though both GTP $\gamma$ S and Q71L ARF1 greatly stimulate the recruitment of AP-1 adaptors onto the TGN.

Further evidence for the involvement of PLD in AP-2 recruitment comes from our studies on the effect of neomycin. Neomycin inhibits PLD indirectly by binding to  $\text{PIP}_2$ , a cofactor for optimum enzyme activity (Hammond et al., 1995). In our *in vitro* system, it inhibits not only the recruitment of AP-2 onto endosomes but also the recruitment of AP-2 onto the plasma membrane, without affecting the recruitment of AP-1 onto the TGN. These observations suggest that under physiological conditions, endogenous PLD associated with the plasma membrane may play a

**Figure 9.** Immunogold EM labeling of newly recruited AP-2 adaptors. Frozen thin sections were prepared of permeabilized NRK cells that had been allowed to recruit proteins from cytosol in the presence of either GTP $\gamma$ S (a and b), Q71L ARF1 (100  $\mu$ g/ml) (c and d), or PLD (12.5  $\mu$ g/ml) (e and f). The sections were labeled with antibodies against newly recruited  $\alpha$ -adaplin (15-nm gold) and lgp110 (8-nm gold). The arrows show membranes that are positive for both antigens, the large arrowheads show membranes that are positive for  $\alpha$ -adaplin only, and the small arrowheads show membranes that are positive for lgp110 only. The same types of membranes are labeled under all three conditions. Bar, 200 nm.





**Figure 10.** Schematic model for the recruitment of AP-2 adaptors onto endosomal membranes. This model is based on the model of Traub and Kornfeld for AP-1 adaptor recruitment (Traub et al., 1993), but with several important modifications. We propose that ARF1 does not bind directly to the putative docking protein, but instead activates PLD, either directly or indirectly. Activated PLD, in the presence of PIP<sub>2</sub>, may lead to rapid local changes in the concentrations of negatively charged phospholipids such as PA and PIP<sub>2</sub>. These negatively charged phospholipids, together with the putative docking protein, would constitute an AP-2 binding site.

role in the recruitment of AP-2 adaptors. However, it is difficult to dissociate the effects of PIP<sub>2</sub> and PLD because once the PLD is activated, it generates PA from phosphatidylcholine, and the PA can in turn activate phosphatidylinositol 4-P 5 kinase to generate more PIP<sub>2</sub> (Jenkins et al., 1994). Thus, either one or both of these lipids may be involved in AP-2 recruitment.

There are several observations that suggest that PIP<sub>2</sub> may be at least as important as PA. First, AP-2 adaptors have been shown to bind with high affinity to inositol phosphates and inositol phospholipids (Beck and Keen, 1991; Gaidarov et al., 1996). Second, AP-2 adaptors require ATP in order to be recruited onto membranes (Seaman et al., 1993; Traub et al., 1996), and one possible role for the ATP may be in the production of PIP<sub>2</sub>. Third, neomycin has a stronger inhibitory effect on AP-2 recruitment than on PLD activity, and although this result may be due at least in part to interference from other types of PLD that do not require PIP<sub>2</sub>, it also suggests that PIP<sub>2</sub> may play a more direct role in AP-2 recruitment. Fourth, we have found that neomycin inhibits the recruitment of AP-2 adaptors onto endosomes not only in cells that have been treated with GTP $\gamma$ S or Q71L ARF1, both of which presumably work by stimulating the cell's endogenous PLD, but also in cells that have been treated with exogenous PLD from bacteria (data not shown). Since it is unlikely that bacterial PLD requires PIP<sub>2</sub> as a cofactor, this result also indicates that PIP<sub>2</sub> plays a direct role in AP-2 recruitment. It may also be significant that AP-2 adaptors bind unusually tightly to hydroxylapatite (i.e., calcium phosphate) (Pearse and Robinson, 1984), suggesting that AP-2 adaptors may have an affinity for phosphate groups in general.

Fig. 10 depicts a model for the recruitment of AP-2 adaptors onto the endosomal compartment. This model is based on one proposed by Traub and Kornfeld for AP-1 recruit-

ment (Traub et al., 1993), and some of its aspects can be adapted to describe other coating events as well. ARFs are recruited from the cytosol through interaction with a BFA-sensitive nucleotide exchange factor (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993; Peyroche et al., 1996), which catalyzes the exchange of bound GDP for GTP. This leads to the exposure of the myristoyl group on ARF, facilitating its association with the membrane. The active ARF1, in conjunction with PIP<sub>2</sub>, stimulates an endogenous PLD resulting in a local increase in PA. PA then stimulates phosphatidylinositol 4-P 5 kinase to produce PIP<sub>2</sub>, which in turn further stimulates PLD to generate more PA and in turn more PIP<sub>2</sub>. Such positive feedback could rapidly lead to a local increase in these acidic phospholipids. It seems unlikely that AP-2 binding is mediated by acidic phospholipids alone since AP-2 is only recruited onto a specific subset of membranes. Thus, we favor the hypothesis that there is an AP-2-specific docking protein or protein complex, localized both on the plasma membrane and on endosomes and lysosomes, which is responsible for the initial binding of AP-2 to the membrane. This docking site may act only transiently, after which AP-2 may become more stably associated with the membrane through interactions with acidic phospholipids, and possibly also through interactions with the cytoplasmic domains of membrane proteins that carry internalization signals.

According to this model, all of the proteins leading to coat binding, i.e., ARF, PLD, and the putative docking protein, could act catalytically and would not necessarily be found to any great extent in the resulting coated pits or vesicles. Indeed, there is no evidence for stoichiometric quantities of any of the known ARFs, or of any other proteins, in purified clathrin-coated vesicles. This model also suggests that there may not necessarily be a direct interac-

tion between ARF and the docking protein, in contrast to the model proposed by Traub and Kornfeld for AP-1 recruitment (Traub et al., 1993).

In some respects, this model is similar to models proposed for proteins such as rab and ARFs, which use lipid modifications to associate with membranes. The lipids do not actually direct such proteins to a particular membrane since proteins with the same lipid modification may associate with different membranes. Instead, it has been proposed that such proteins initially interact with a specific docking site (which in the case of ARF may also be its nucleotide exchange factor), which causes a conformational change so that the lipid becomes inserted into the membrane bilayer (Donaldson and Klausner, 1994; Pfeffer, 1994). Similarly, AP-2 adaptors may interact with certain lipid head groups after their initial binding to a specific docking site. If the lipid head groups are rapidly turning over, as is the case for both PA and PIP<sub>2</sub>, this would suggest a means for releasing the adaptors once the coated vesicle had formed (De Camilli et al., 1996). Possibly such lipids are normally generated on the plasma membrane rather than on endosomes, but addition of reagents such as GTP $\gamma$ S, constitutively activated ARF1, Ca<sup>2+</sup>, cationic amphiphilic drugs, or exogenous PLD may cause unusually high levels of these lipids to be generated on endosomes and/or lysosomes. Whether the production of such lipids at the plasma membrane depends upon an ARF or another small GTP-binding protein is still not known, although ARF6 is a potential candidate. ARF6, like the other ARFs, has been shown to stimulate PLD (Brown et al., 1995), but it differs from the other ARFs in that its membrane association is unaffected by GTP $\gamma$ S or BFA. Overexpressed ARF6 has been localized to the plasma membrane and endosomes, and constitutively active or constitutively inactive forms of ARF6 cause cells to accumulate plasma membrane or endosomal membranes, respectively, consistent with a role in endocytosis (Dsouza-Schorey et al., 1995; Peters et al., 1995; Cavenagh et al., 1996).

How well can this model be extrapolated to account for other membrane coating events? It seems likely that the recruitment of AP-2 adaptors onto the plasma membrane also requires the activation of PLD and the generation of acidic phospholipids, even though a conventional ARF is probably not involved. Other coats that require ARF for membrane binding, such as AP-1 adaptors and coatomer, might be expected to use the same mechanism as that shown in Fig. 10. Indeed, there is evidence that PLD mediates coatomer binding to Golgi membranes (Ktistakis et al., 1996), and coatomer has also been shown to interact with inositol phosphates (Fleischer et al., 1994). In addition, membrane recruitment of the AP-3 adaptor-related complex, which is BFA sensitive and therefore likely to require an ARF (Simpson et al., 1996), was significantly enhanced in the presence of exogenous PLD (data not shown). Like AP-2, AP-3 requires ATP for its recruitment in vitro (Simpson et al., 1996) and binds tightly to hydroxylapatite (Simpson, F., and M.S. Robinson, unpublished observations). However, from the data presented here for AP-1 adaptors, it is clear that the model may not always be entirely applicable and that there may be variations on the general mechanism. For example, although there is much

evidence to indicate that ARF1 mediates AP-1 binding to membranes (Seaman et al., 1996; Stamnes and Rothman, 1993; Traub et al., 1993), we found that neither exogenous PLD nor neomycin had any effect on AP-1. In addition, AP-1 recruitment does not require ATP (Simpson et al., 1996), indicating that PIP<sub>2</sub> is not involved, it does not bind particularly well to hydroxylapatite (Pearse and Robinson, 1984), and there is no evidence that it binds inositol phosphates or inositol phospholipids.

Thus, it seems clear that different binding mechanisms have evolved for different coats. One common theme may be that all coats make use of a small GTP-binding protein, although the role of such a protein in the recruitment of AP-2 onto the plasma membrane is still open to question. However, the same GTP-binding protein may regulate different coats in different ways. Thus, although ARF1 may promote the binding of AP-2 adaptors and coatomer by activating PLD, it must use some other mechanism to promote the binding of AP-1 adaptors. A number of activities have been attributed to ARF, and a number of interactions with both proteins and phospholipids have been reported (Bowman and Kahn, 1995; Kanoh et al., 1997). As coat proteins and small GTP-binding proteins coevolved, it seems likely that different coats became specialized to make use of different regulatory mechanisms, while still retaining a general requirement for ARF or an ARF-related protein.

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