

# ARF1·GTP, Tyrosine-based Signals, and Phosphatidylinositol 4,5-Bisphosphate Constitute a Minimal Machinery to Recruit the AP-1 Clathrin Adaptor to Membranes

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At the *trans*-Golgi network, clathrin coats containing AP-1 adaptor complexes are formed in an ARF1-dependent manner, generating vesicles transporting cargo proteins to endosomes. The mechanism of site-specific targeting of AP-1 and the role of cargo are poorly understood. We have developed an *in vitro* assay to study the recruitment of purified AP-1 adaptors to chemically defined liposomes presenting peptides corresponding to tyrosine-based sorting motifs. AP-1 recruitment was found to be dependent on myristoylated ARF1, GTP or nonhydrolyzable GTP-analogs, tyrosine signals, and small amounts of phosphoinositides, most prominently phosphatidylinositol 4,5-bisphosphate, in the absence of any additional cytosolic or membrane bound proteins. AP-1 from cytosol could be recruited to a tyrosine signal independently of the lipid composition, but the rate of recruitment was increased by phosphatidylinositol 4,5-bisphosphate. The results thus indicate that cargo proteins are involved in coat recruitment and that the local lipid composition contributes to specifying the site of vesicle formation.

## INTRODUCTION

Sorting of membrane proteins is generally mediated by cytosolic coats which serve the dual role of creating a scaffold to form coated buds and vesicles and of selectively concentrating cargo proteins by interacting with cytosolic signals. The best studied systems are COPI in *intra*-Golgi and Golgi-to-endoplasmic reticulum (ER) transport, COPII in ER-to-Golgi transport, and clathrin with associated adaptor proteins in the formation of vesicles at the plasma membrane, the *trans*-Golgi network (TGN) and endosomes. There are different types of clathrin-associated adaptor proteins (APs), heterotetrameric complexes composed of two ~100-kDa adaptins, a ~50-kDa medium ( $\mu$ ), and a ~20-kDa small ( $\sigma$ )

chain (Robinson and Bonifacino, 2001). The adaptor complexes form the inner layer of the coat that specifies the site of coat formation and interacts with cargo molecules. AP-1 adaptors are primarily functional at the TGN generating vesicles destined for endosomes but have also been found on sorting endosomes and implicated in (basolateral) recycling to the plasma membrane (Futter *et al.*, 1998). AP-2 adaptors are found at the plasma membrane to form coated vesicles for endocytosis. AP-3 adaptors are involved in lysosomal transport from the TGN or endosomes. The different adaptor complexes recognize similar tyrosine and dileucine signals in cargo molecules, and in many cases the same signals are recognized by several adaptor types (Bonifacino and Dell'Angelica, 1999; Heilker *et al.*, 1999).

Recruitment of the different coats to their specific membranes appears to involve common basic mechanisms. With the exception of AP-2/clathrin coats, all the coats mentioned above require small GTPases that are activated from their soluble GDP-bound to their membrane-associated GTP-bound form by a guanine nucleotide exchange factor (GEF) at the correct membrane. For COPII coats in yeast, the GTPase Sar1p is activated by the GEF Sec12p in the ER membrane. In an assay with chemically defined liposomes containing acidic lipids like phosphatidic acid (PA), phosphatidylserine (PS), or phosphoinositides, these components were sufficient to recruit the subunits of COPII, first

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Abbreviations used: AP, adaptor protein; ARF1, ADP-ribosylation factor 1; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; GMP-PNP, guanylyl imidodiphosphate; GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); Lamp-1, lysosome-associated membrane protein-1; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphoinositide; PS, phosphatidylserine; TGN, *trans*-Golgi network.

Sec23p/24p and then Sec13p/31p, to form coated buds and vesicles (Matsuoka *et al.*, 1998b). In the presence of cargo membrane proteins (the v-SNAREs Sec22p or Bos1p), these were selectively incorporated (Matsuoka *et al.*, 1998a).

For COPI coats, the GTPase ARF1 (ADP-ribosylation factor 1) is activated by a Golgi-associated GEF. On liposomes made of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with unsaturated fatty acids or containing acidic phospholipids, ARF1-GTP $\gamma$ S and COPI complexes were sufficient to form coats and vesicles (Spang *et al.*, 1998; Bremser *et al.*, 1999). However, with saturated lipids of different compositions, COPI recruitment was only achieved in the presence of liposome-associated cargo sequences (Bremser *et al.*, 1999).

Recruitment of the clathrin adaptors AP-1 and AP-3 also involves ARF1, together with specific GEFs (e.g., BIG2; Shintotsuka *et al.*, 2002). ARF-GTP $\gamma$ S, AP-3, and clathrin were sufficient to generate coats on liposomes made from soybean lipids (containing 20% PC and various other lipids) and to bud coated vesicles (Drake *et al.*, 2000). Based on various studies (Dittié *et al.*, 1996; Mallet and Brodsky, 1996; Seaman *et al.*, 1996; Zhu *et al.*, 1998, 1999a), the following model for AP-1/clathrin coat formation has been proposed (Zhu *et al.*, 1998). After nucleotide exchange in ARF1 by a GEF at the site of coat initiation, ARF1-GTP will interact rapidly with putative docking protein(s) to generate high-affinity binding sites for AP-1. In turn, clathrin trimers will bind to immobilized AP-1 and laterally associate to form the characteristic lattice. Cargo molecules will associate with AP-1 despite the low affinity of interaction, because AP-1 is highly concentrated in the coat. GTP hydrolysis induced by an ARF GTPase activating protein will eventually inactivate the docking protein. As the growing coat soon interacts with multiple cargo proteins, it will stay membrane bound even as docking proteins and ARF1-GDP dissociate.

It has been proposed that the mannose-6-phosphate receptors form the major docking sites for AP-1 at the TGN (Le Borgne and Hoflack, 1997), a concept that has been challenged by studies with Golgi membranes devoid of mannose-6-phosphate receptors (Zhu *et al.*, 1999b). In addition, the finding that AP-1 could be recruited in an ARF1-dependent manner to protein-free soybean liposomes, which can be easily pelleted, in the presence of cytosol indicated that integral membrane proteins are not necessary (Zhu *et al.*, 1999a). Yet, the cytosol dependence of the process suggested the involvement of a soluble cytosolic factor(s) that peripherally attaches to the liposomes and functions as the AP-1 docking site. Peripheral membrane proteins have also been shown to bind to AP-1 on affinity chromatography (Mallet and Brodsky, 1996), and a Tris-strippable factor was shown to be required for AP-1 binding to immature secretory granules (Dittié *et al.*, 1996). AP-1 binding to liposomes was dependent on the lipid composition, which thus might play a role in the binding of a cytosolic factor to the membrane. A soybean lipid mixture containing 20% PC and acidic lipids was optimal, whereby PS, but to some extent also phosphatidylinositol (PI) or PA seemed to contribute (Zhu *et al.*, 1999a).

In the present study, we have analyzed the minimal requirements for the recruitment of AP-1 adaptor complexes to a membrane *in vitro* using chemically defined liposomes

in a floatation assay that does not require the liposomes to be pelletable. In particular, the contributions of cargo-sorting signals and lipids were tested. Stable AP-1 recruitment was found to require in addition to myristoylated ARF1-GTP also the presence of membrane-anchored tyrosine signals and specific phosphoinositides but no further cytosolic factors.

## MATERIALS AND METHODS

### Reagents

Guanlyl imidodiphosphate (GMP-PNP), guanosine 5'-O-(3-thiotriphosphate; GTP $\gamma$ S), and GTP were from Roche Diagnostics. Superose-6 (Prep grade) and ECL reagent were from Amersham Pharmacia Biotech (Piscataway, NJ). *N*-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (MMCC-DHPE) was from Molecular Probes (Eugene, OR). Egg PC, liver PI, liver PE, and brain PS were from Avanti Polar Lipids (Alabaster, AL), phosphatidylinositol 3-phosphate (PI3P), PI5P, and PI(3,4)P<sub>2</sub> from Echelon Research Laboratories Inc. (Salt Lake City, UT), PI(3,5)P<sub>2</sub> from Calbiochem (La Jolla, CA), and PI(3,4,5)P<sub>3</sub> from Matreya Inc. (Pleasant Gap, PA). mAb 100/3 (anti- $\alpha$ -adaptin), horseradish peroxidase-coupled anti-mouse IgG antibody, PI4P, PI(4,5)P<sub>2</sub>, soybean PC (azolectin, P-5638), mixed phosphoinositides (P-6023), GDP, and dipalmitoyl-PA were purchased from Sigma (Buchs, Switzerland). Peptides were synthesized on a Pioneer synthesizer (PerSeptive Biosystems, Framingham, MA) using Fmoc (fluorenylmethoxycarbonyl) protected amino acids with TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as coupling reagent. Cleaved and deprotected peptides were first purified via reverse phase HPLC (RP C18, Vydac, Hesperia, CA) and then verified by MALDI-TOF mass spectrometry (TOFSPEC-2E, Micromass, Manchester, UK). mAb 1D9 against ARF1 was a kind gift by Richard Kahn (Emory University, Atlanta, GA).

### Purification of AP-1 and ARF1

Clathrin-coated vesicles were purified from calf brains, freshly obtained at the local slaughterhouse as described (Campbell *et al.*, 1984). All the procedures were performed at 4°C. The coats were released by homogenizing vesicles with one volume of 1.5 M Tris-HCl (pH 7.0), 6 mM EDTA, 0.6 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 10  $\mu$ g/ml benzamidin and 2  $\mu$ g/ml pepstatin A, leupeptin, antipain, and chymostatin. After overnight incubation at 4°C membranes were spun for 30 min at 100,000  $\times$  g, and the supernatant was loaded in 2-ml portions on a 50  $\times$  1.6 cm Superose-6 column equilibrated with 0.5 M Tris-HCl (pH 7.0), 2 mM EDTA, 0.2 mM DTT and run at 0.5 ml/min. Mixed adaptors were collected between 55 and 64 ml of elution. To eliminate the remaining clathrin, mixed adaptors were dialyzed into 0.1 M MES, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, 0.2 mM DTT (pH 6.6) to form clathrin cages and centrifuged for 1 h at 400,000  $\times$  g. Although clathrin was only found in the pellet with most of AP-2 and AP180, AP-1 largely stayed in solution in accordance with its lower cage-promoting activity (Keen, 1989; Lindner and Ungewickell, 1992). The supernatant was dialyzed into 20 mM ethanolamine, pH 8.9, 2 mM EDTA, 1 mM DTT (MonoQ buffer; Ahle *et al.*, 1988) and loaded on a 2-ml CHT-II hydroxyapatite column (Bio-Rad, Cambridge, MA) that was equilibrated and washed with 0.5 M Tris-HCl, 2 mM K<sub>2</sub>PO<sub>4</sub>, pH 7.0, followed by 10 mM phosphate in the same buffer. AP-1 was eluted stepwise with 50 mM and 100 mM phosphate. Purified AP-1 was dialyzed against MonoQ buffer containing 0.5 mM PMSF and stored at 4°C with protease inhibitors. The 70-kDa protein was identified after Coomassie staining and in-gel digestion with trypsin (Perrot *et al.*, 1999) by analysis on a Reflex III MALDI-TOF instrument (Bruker, Bremen, Germany) using  $\alpha$ -cyano-hydroxy-cinnamic acid as matrix. Protein identification was done using the Mascot software (Matrix Science Ltd., London, UK).

Plasmids encoding bovine ARF1 with residues 3–7 from yeast Arf2p (Liang *et al.*, 1997) and yeast *N*-myristoyltransferase (pBB131; Duronio *et al.*, 1990) were generous gifts by Stuart Kornfeld and Jeffrey Gordon, respectively (both at Washington University, St. Louis, MO). After cotransformation of both plasmids into *Escherichia coli* BL21(DE3), myristoylated ARF1 was purified as described (Liang and Kornfeld, 1997). This ARF1 preparation bound to Golgi membranes (Martín *et al.*, 2000), indicating its efficient myristoylation. Nonmyristoylated ARF1 was also prepared and purified and showed the expected mobility shift on SDS gel electrophoresis (Franco *et al.*, 1995; Liang and Kornfeld, 1997). Proteins were quantified using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL) or the Bradford assay (Bio-Rad; for samples containing Tris), using bovine serum albumin as standard. Silver staining of polyacrylamide gels was performed as described (Morrissey, 1981).

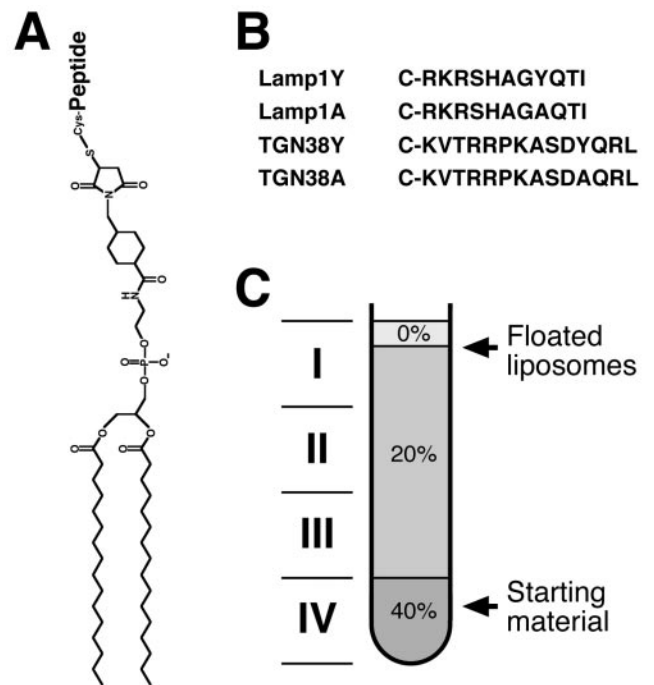
### Preparation of Peptidoliposomes

Five micromoles of egg PC (3.8 mg) were combined with 125 nmoles MMCC-DHPE (2.5 mol %). When indicated, other lipids were used to replace some of the PC. The organic solvent was evaporated under a stream of nitrogen. Dichloromethane was added and evaporated twice. Dried lipids were resuspended into 1 ml 10 mM HEPES (pH 6.5), 0.1 M NaCl, 0.5 mM EDTA and freeze-thawed five times in liquid nitrogen and then extruded 11 times through a 400-nm Nucleopore polycarbonate membrane (Corning, Corning, NY) using a homemade hand-driven extruder. The liposomes (0.3 ml) were immediately incubated with 120  $\mu$ g of peptide (i.e., about a fourfold excess over the coupling lipid, assuming half of it is exposed) for 1 h at room temperature, and then stored at 4°C with 0.02% (wt/vol) NaN<sub>3</sub> for up to 2 weeks. The coupling efficiency varied from ~30 to 50% as judged by measuring the amount of peptide associated with the liposomes the bicinchoninic acid assay after extensive dialysis of the liposomes against phosphate-buffered saline. We found it unnecessary to remove free peptides from the liposomes before the AP-1 recruitment assay (negligible inhibition of adaptor binding to immobilized peptides had also been observed in surface plasmon resonance assays; Heilker *et al.*, 1996).

### Liposome Recruitment Assay

Peptidoliposomes (200  $\mu$ l; 1  $\mu$ mol lipid) were first incubated for 30 min at 37°C with 5  $\mu$ g of ARF1 and either 0.2 mM GMP-PNP (or GTP $\gamma$ S), or 2 mM GTP or GDP. When GTP or GDP were used, 3 mM phosphate was also added to inhibit hydrolysis by a spurious phosphatase (Franco *et al.*, 1995). Samples were returned to ice and 10 mM MgCl<sub>2</sub> was added to stabilize the loaded ARF1 (Franco *et al.*, 1995) as well as 10  $\mu$ g of mixed adaptors or 0.5  $\mu$ g of AP-1. After 15 min on ice, samples of 250  $\mu$ l were mixed with 0.5 ml of 60% (wt/vol) sucrose in assay buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 10 mM KCl, 3 mM potassium phosphate, 2 mM MgCl<sub>2</sub>, 0.2 mM dithiothreitol; Höning *et al.*, 1997), overlaid with 3.07 ml of 20% sucrose in assay buffer and with 0.18 ml of buffer in a 4-ml centrifuge tube, and centrifuged in a TST60 rotor (Kontron, Zurich, Switzerland) at 55,000 rpm (300,000  $\times$  *g*<sub>av</sub>) for 1 h at 4°C. Four 1-ml fractions were collected from the top and precipitated with 8% (wt/vol) trichloroacetic acid. Acetone-washed pellets were analyzed by 7.5–15% PAGE and immunoblotting using antibodies to  $\gamma$ -adaptin (100/3) or ARF1 (1D9), a peroxidase-coupled secondary antibody, and ECL reaction. Quantitation was performed using a MultiImage Light Cabinet from Alpha Innotech Corporation (San Leandro, CA).

Cytosol was obtained from calf brain or bovine adrenals (gift of Kitaru Suda, Biozentrum, Basel, Switzerland) as the high-speed supernatant after homogenization (Campbell *et al.*, 1984), supplemented with protease inhibitors, and clarified by centrifugation before use. Peptidoliposomes (0.5  $\mu$ mol lipid) were incubated for 30 min at 37°C with 0.5 mg of cytosol, 5  $\mu$ g of ARF1, and 0.2 mM GMP-PNP in 200  $\mu$ l of assay buffer. Samples were returned to ice



**Figure 1.** Peptidoliposomes to assay AP-1 recruitment in vitro. The maleimide derivative of PE MMCC-DHPE was used to couple synthetic peptides via an N-terminal cysteine to a lipid (A). The peptides used correspond to the cytoplasmic domain of Lamp1 (B, Lamp1Y) or the segment of TGN38 that has previously been shown to contain the functional tyrosine motif (Boil *et al.*, 1996). Lamp1A and TGN38A are the control peptides with the critical tyrosine mutated to alanine. After incubation of peptidoliposomes with AP-1 and with or without ARF1, they were floated from the bottom of a sucrose step gradient (C). Four fractions were collected as indicated, with fraction I containing the floated liposomes with bound proteins and fraction IV including the loading zone with unbound proteins.

and mixed with 0.4 ml of 60% (wt/vol) sucrose in assay buffer, and liposomes were floated as described above.

### Nucleotide Exchange Assay

Nucleotide exchange was measured using [<sup>35</sup>S]GTP $\gamma$ S and the filtration assay according to Franco *et al.* (1995) under the experimental conditions used for the recruitment assay.

## RESULTS

### An Assay for AP-1 Recruitment to Model Membranes

To assess the interaction of AP-1 adaptors to sorting signals in the context of a chemically defined membrane, we coupled synthetic peptides via an N-terminal cysteine to a maleimide derivative of PE, thus creating lipid-anchored peptides. The reactive lipid was mixed with PC or various lipid mixtures at 2.5 mol %, and large unilamellar liposomes were produced by extrusion through a 400-nm pore-size filter. Peptides were then coupled via an N-terminal cysteine to the reactive lipid (Figure 1A). The peptides used (Lamp1Y and TGN38Y) corresponded to the C-terminal cytoplasmic



domain of Lamp-1 (lysosome-associated membrane protein-1) and a portion of the cytoplasmic domain of TGN38 (*trans*-Golgi network protein of 38 kDa), two proteins with well characterized tyrosine-containing sorting signals (Figure 1B). The same peptides with the tyrosines mutated to alanine (Lamp1A and TGN38A) were used as negative controls. Lamp-1 is sorted from the TGN via endosomes to lysosomes (Hunziker and Geuze, 1996) and has been demonstrated by immunogold electron microscopy in AP-1-positive clathrin-coated buds and vesicles at the TGN (Höning *et al.*, 1996). TGN38 cycles between the TGN and the plasma membrane. An interaction with AP-1 is less clearly established (Ohno *et al.*, 1995; Boll *et al.*, 1996; Stephens *et al.*, 1997).

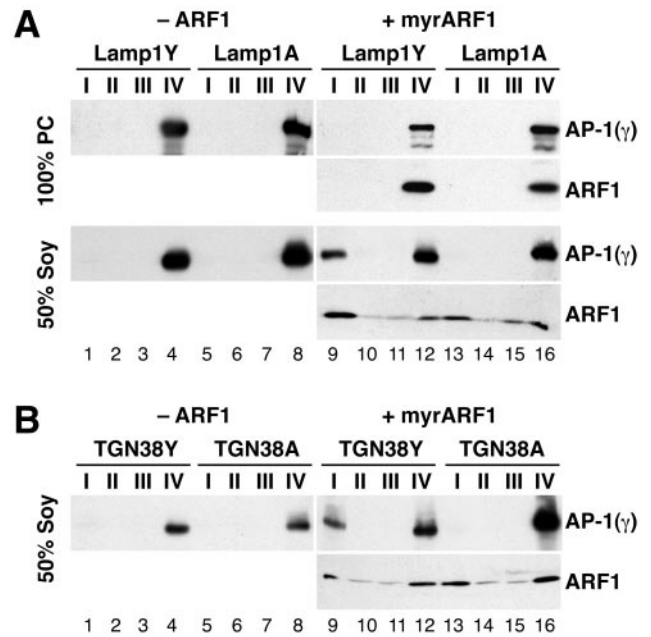
Adaptor complexes were isolated from calf brain coated vesicles by releasing the coat with 1 M Tris followed by gel filtration to remove the bulk of clathrin. This mixed adaptor preparation (containing both AP-1 and AP-2) was incubated with the peptidoliposomes. The mixture, supplemented with sucrose to a concentration of 40%, was then loaded below a 20% sucrose cushion and a small amount of sucrose-free buffer (Figure 1C) and centrifuged for 1 h at  $300,000 \times g$  to separate the liposomes and bound proteins from free adaptors. The gradient was collected from the top in four fractions (I–IV), with fraction I containing the floated liposomes with recruited proteins and fraction IV containing unbound material. Aliquots of the four fractions were analyzed by SDS-gel electrophoresis and probed by immunoblot analysis.

Because *in vivo* recruitment of AP-1 to the TGN requires the GTPase ARF1 in its active GTP-bound form (Stamnes and Rothman, 1993; Traub *et al.*, 1993), the potential requirement of ARF1 in our assay was tested by incubating purified ARF1 with the peptidoliposomes together with GTP or a nonhydrolyzable GTP analog (GMP-PNP or GTP $\gamma$ S) at 37°C for 30 min before addition of adaptors. It has previously been shown that liposomes induce guanine nucleotide exchange on ARF1 and thus activate it (Antonny *et al.*, 1997), a function performed *in vivo* by specific GEFs at the TGN.

### Recruitment of AP-1 Adaptors to Liposomes Requires a Tyrosine-based Signal, ARF1, and Specific Lipids

In previous *in vitro* assays, AP-1 was shown to bind to the cytoplasmic sequence of Lamp-1 immobilized on beads or on the sensor surface in surface plasmon resonance experiments (Höning *et al.*, 1996). In our assay, however, no recruitment of AP-1 could be observed to Lamp1Y presented on liposomes made of PC or of a 1:1 mixture of PC and soybean lipids (azolectin; Figure 2A, lanes 1–4).  $\gamma$ -Adaptin, a 100-kDa subunit of AP-1 complexes, was detected exclusively in fraction IV of the step gradients, which represents the loading zone. This result is consistent with the apparent dissociation rates of adaptors from immobilized tyrosine motifs in surface plasmon resonance experiments (Heilker *et al.*, 1996; Höning *et al.*, 1996), which would not allow interacting adaptors to stay bound to the peptidoliposomes during a 1-h floatation.

However, if purified myristoylated ARF1 with GMP-PNP was added to the Lamp1Y peptidoliposomes and incubated at 37°C before addition of adaptors, a significant fraction of



**Figure 2.** AP-1 recruitment to peptidoliposomes is signal-, ARF1- and lipid-dependent. (A) Peptidoliposomes made of 100% PC or 50% PC/50% soybean lipids and presenting Lamp1Y or Lamp1A peptides were incubated with a mixed adaptor preparation and with or without myristoylated ARF1 and GMP-PNP. After floatation on a sucrose step gradient, four fractions (I–IV, as shown in Figure 1C) were collected from the top and analyzed by immunoblotting for  $\gamma$ -adaplin or ARF1. (B) The same experiments were performed using peptidoliposomes made of 50% PC/50% soybean lipids and presenting TGN38Y or TGN38A peptides.

AP-1 was floated to the top of the gradient (fraction I) together with liposomes containing 50% soybean lipids (Figure 2A, lanes 9–12). AP-1 was not recruited to liposomes presenting Lamp1A peptides or to liposomes composed entirely of PC (lanes 9–16) even in the presence of ARF1·GMP-PNP.

AP-1 recruitment to the membrane was rather stable, because the middle fractions II and III of the gradient were entirely devoid of  $\gamma$  adaptin, indicating that bound adaptors did not significantly dissociate during the floatation. This is in contrast to the interaction of the bulk of ARF1 with liposomes. On nucleotide exchange, the active ARF1 exposes its myristoyl tail, which allows it to interact with lipid bilayers (Antonny *et al.*, 1997). The equilibrium between lipid-associated and soluble ARF1 is shifted by the addition of soy lipids in favor of the lipid-associated form: although ARF1 is not dragged out of the loading zone (fraction IV) by pure PC liposomes (in agreement with Helms *et al.*, 1993), approximately half of ARF1 was floated to fraction I in the presence of 50% soybean lipid, with considerable trailing into fractions II and III. The residual clathrin in the adaptor preparation was not corecruited with AP-1.

Like Lamp1Y, the tyrosine motif peptide TGN38Y was similarly able to recruit AP-1 only in the presence of ARF1·GMP-PNP and with liposomes containing 50% soybean lipids (Figure 2B). Again, recruitment depended on the tyrosine signal, because TGN38A was not functional. ARF1,

in contrast, was associated with liposomes irrespective of the peptides coupled to them. The results show that recruitment of AP-1 to liposomes requires activated ARF1, functional tyrosine motifs, and a particular lipid composition.

### Phosphoinositides Are Required to Recruit AP-1

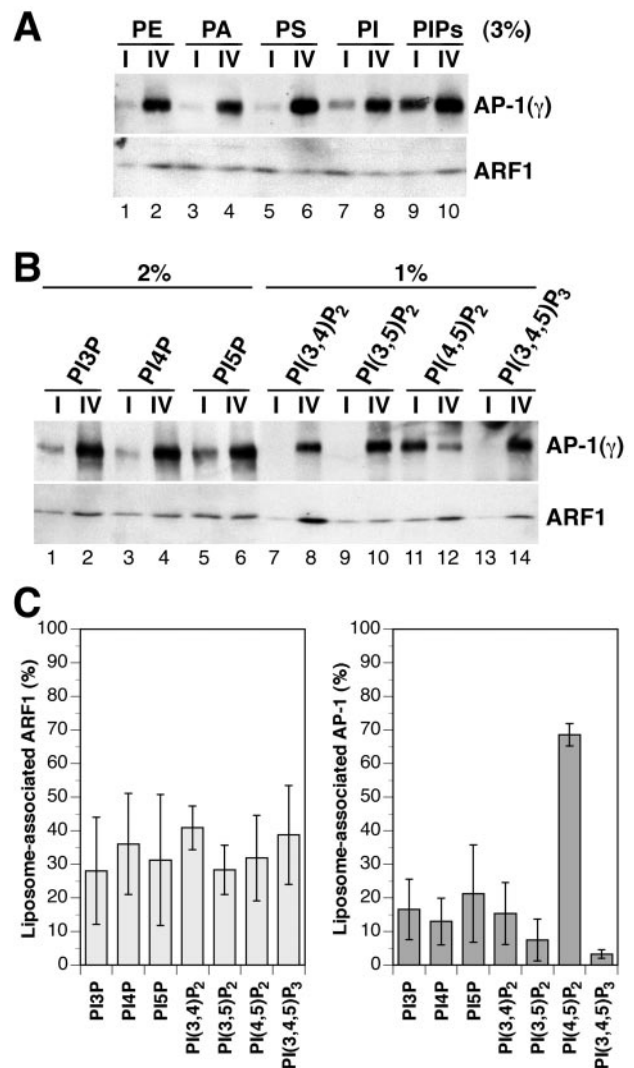
The soybean lipids used in Figure 2 contain 20% PC and an ill-defined mixture of other lipids. To identify which components are responsible for AP-1 recruitment, 3% of PE, PA, PS, PI, or a mixture of phosphoinositides (PIPs) were added to PC to produce peptidoliposomes presenting Lamp1Y in our assay (Figure 3A). AP-1 was not significantly recruited to the liposomes containing PE, PA, or PS and only slightly to those containing 3% PI. Most efficient recruitment was reproducibly observed to liposomes containing phosphoinositides.

To determine which phosphoinositides are capable of stimulating AP-1 recruitment, we compared Lamp1Y/PC peptidoliposomes containing 2% of the monophosphorylated phosphoinositides PI3P, PI4P, or PI5P, or 1% of the phosphatidylinositol bisphosphates PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, or PI(4,5)P<sub>2</sub>, or phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>). At these concentrations the phosphoinositides with one and two phosphates on the inositol ring introduce approximately the same negative charge to the membranes.

Among the monophosphorylated phosphoinositides, PI5P was the most effective in recruiting AP-1 (Figure 3, B and C), whereas PI3P and PI4P were only marginally functional. However, the most efficient AP-1 recruitment of all was obtained with PI(4,5)P<sub>2</sub>, even though it was used at only half the concentration of the monophosphorylated phosphoinositides. The other bis- or trisphosphorylated molecules were unable to sustain AP-1 recruitment. In contrast to the pronounced lipid dependence of AP-1 recruitment, the amount of ARF1 recovered in fraction I did not show significant differences for different lipids used.

### AP-1 Recruitment Depends on Myristoylated ARF1 in Its Active Conformation

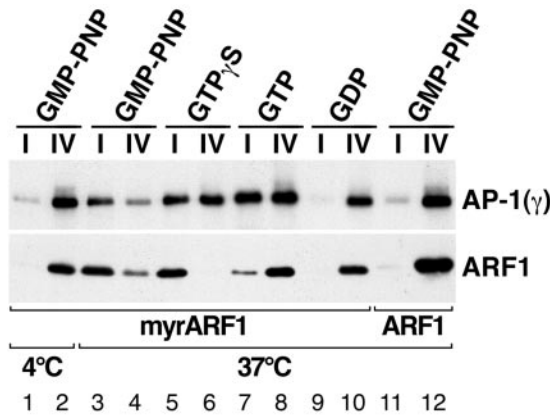
In the above experiments, GMP-PNP, a nonhydrolyzable analogue of GTP was used, indicating that GTP hydrolysis is not required for AP-1 recruitment to peptidoliposomes. In Figure 4, we further analyzed the nucleotide requirement using myristoylated ARF1 and liposomes with 10% mixed phosphoinositides and Lamp1Y peptides. No AP-1 recruitment and no ARF1 association with liposomes was detected when only GDP was added to the ARF1/peptidoliposome incubation (lanes 9 and 10), demonstrating that AP-1 binding required active ARF1. No significant differences in the efficiency of AP-1 recruitment were observed when GTP, GTP $\gamma$ S, or GMP-PNP were used as the nucleotide. In contrast, ARF1 association with liposomes reproducibly depended on the type of GTP analog used. ARF1-GTP $\gamma$ S floated more efficiently with liposomes than ARF1-GMP-PNP, whereas ARF1-GTP did so the least (lanes 3–8). This is possibly due to slight differences in conformation and/or to some hydrolysis of GTP. Both AP-1 recruitment and ARF1 association with peptidoliposomes depended on incubation of ARF1 with liposomes at 37°C because they were almost completely abolished at 4°C (Figure 4, lanes 1–4). This reflects the fact that nucleotide exchange is temperature dependent. As expected, unmyristoylated ARF1 was not functional in the assay (lanes 11 and 12).



**Figure 3.** Lipid requirement for AP-1 recruitment to peptidoliposomes. (A) Three percent of the indicated lipid was incorporated into PC peptidoliposomes exposing Lamp1Y. After incubation with a mixed adaptor preparation and with myristoylated ARF1-GMP-PNP, fractions I and IV of a flotation gradient were analyzed by immunoblotting. PIPs indicates a commercial mixture of phosphoinositides. (B) Two percent of PI-monophosphates and 1% of PI-bis- and trisphosphates were incorporated into PC peptidoliposomes exposing Lamp1Y and analyzed as in A. (C) The recruitment of AP-1 and ARF1 to liposomes containing different phosphoinositides (2% of PI-monophosphates and 1% of PI-bis- and trisphosphates) were densitometrically quantified. The amount recovered in fraction I is expressed in percent of the total in fractions I plus IV. The average and SDs of at least three experiments, including those shown in B, are presented.

### The Effect of Phosphoinositides Is Not via the Nucleotide Exchange Activity of Liposomes

The efficiency of AP-1 binding to peptidoliposomes with different lipid compositions did not correlate with the relative or absolute amounts of ARF1 that floated with the liposomes to the top fraction of the gradient (Figure 3). It

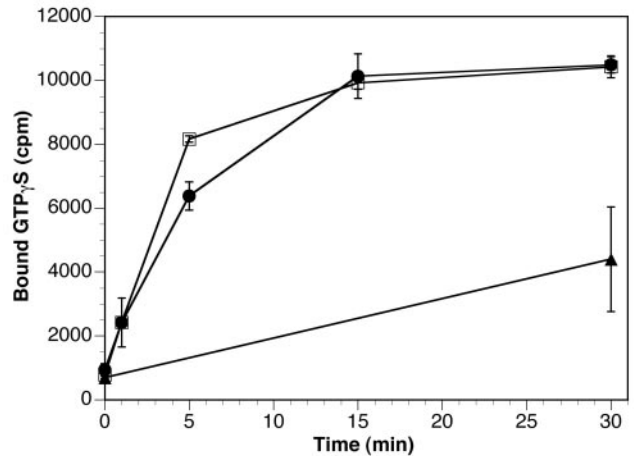


**Figure 4.** Nucleotide dependence of AP-1 recruitment to peptidoliposomes. The indicated nucleotide was incubated with myristoylated or nonmyristoylated ARF1 and peptidoliposomes containing 3% of mixed inositides and exposing Lamp1Y. The analysis was performed as in Figure 3.

appears that all acidic lipids increased ARF1 association to the liposomes compared with pure PC, whereas AP-1 recruitment was much more specific. Nevertheless, it was conceivable that the effect of the functional phosphoinositides on AP-1 recruitment was indirect by increasing the rate or extent of nucleotide exchange in ARF1, which in our assay is performed in an unphysiological manner by the liposome surface. To test this possibility, a nucleotide exchange assay was performed using liposomes made of PC only or of PC with 10% mixed phosphoinositides. ARF1 was incubated with these liposomes and [ $^{35}\text{S}$ ]GTP $\gamma$ S for different times, after which the samples were filtered and the amount of radioactivity bound to ARF1 was determined. As is shown in Figure 5, the rate of nucleotide exchange in the presence of liposomes is more than 10 times higher than in the absence of membranes. Yet, there is no significant difference in the kinetics or the final extent of GTP $\gamma$ S loading of ARF1 in the presence or absence of phosphoinositides that could explain the dramatic difference in AP-1 recruitment observed with these lipid compositions (compare Figure 4, lanes 3 and 6, with Figure 2A, lanes 9–12, top panel). Thus, the phosphoinositides must affect other aspects of ARF1 function or must act on the AP-1 adaptors.

#### A Minimal Machinery for AP-1 Recruitment

The mixed adaptor preparation used in the experiments described so far contains in addition to AP-1 also AP-2 adaptors, AP-180, and a number of unknown contaminating bands, which might directly or indirectly contribute to AP-1 recruitment. To identify the minimal set of proteins required, we purified AP-1 adaptors to near homogeneity. Figure 6A shows aliquots of the mixed adaptor preparation (lane 1) and of the purified AP-1 preparation (lane 2) containing the same amount of AP-1 (as judged by immunoblot analysis) on an SDS-gel stained with silver. All contaminating proteins except for one of ~70 kDa were removed below detection in the purified sample. By mass spectrometry, this copurifying contaminant was identified to be hsc70, the



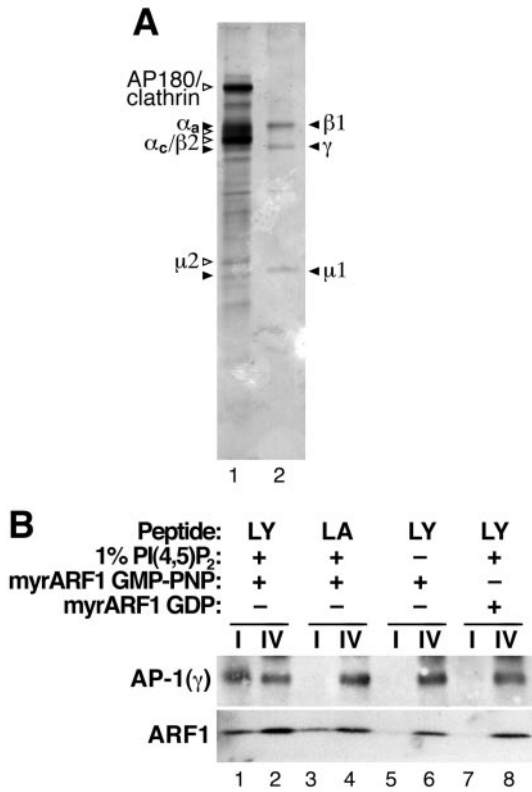
**Figure 5.** Nucleotide exchange on ARF1. Myristoylated ARF1 was incubated at 37°C with [ $^{35}\text{S}$ ]GTP $\gamma$ S and either buffer only ( $\blacktriangle$ ), PC liposomes ( $\bullet$ ), or PC with 10% mixed phosphoinositides ( $\square$ ). At the indicated times, samples were quickly filtered through a nitrocellulose filter. After washing, the radioactivity on the filter, corresponding to GTP $\gamma$ S bound to ARF1, was counted.

uncoating ATPase of clathrin-coated vesicles (Schlossman *et al.*, 1984; DeLuca-Flaherty and McKay, 1990), which is highly unlikely to contribute to coat recruitment and could not be detected in the floated fraction. Using this AP-1 preparation, again robust recruitment of AP-1 complexes was achieved to liposomes containing 1% PI(4,5)P $_2$  presenting the Lamp1Y peptides and in the presence of myristoylated ARF1 loaded with GMP-PNP (Figure 6B, lanes 1 and 2). Using Lamp1A lacking the tyrosine, liposomes lacking the phosphoinositides, or GDP-loaded ARF, each individually abolished AP-1 association with the liposomes. This result thus defines the minimal machinery to recruit AP-1 to a membrane to consist of a peptide with a functional tyrosine motif and anchored to a lipid membrane containing a small amount of PI(4,5)P $_2$ , and myristoylated ARF1 loaded with GTP or a nonhydrolyzable GTP analog.

#### Signal and Lipid Dependence of AP-1 Recruitment from Cytosol

Zhu *et al.* (1999a, 1999b) observed signal-independent AP-1 recruitment from cytosol to soybean liposomes in a pelleting assay. Therefore, using our floatation assay, we also investigated AP-1 recruitment from cytosol. Peptidoliposomes were mixed with cytosol supplemented with purified ARF1 and incubated for 30 min at 37°C before floatation of the liposomes as before. Consistent with the results by Zhu *et al.* (1999a), significant recruitment of AP-1 from brain cytosol to soybean liposomes presenting Lamp1A was observed (Figure 7A, lanes 3 and 4). This tyrosine-independent binding was even stronger using adrenal cytosol (which was used by Zhu *et al.* 1999a; Figure 7B, lanes 3 and 4). With both types of cytosol, however, AP-1 recruitment was clearly enhanced when functional Lamp1Y peptides were presented (Figure 7, A and B, lanes 1 and 2). If liposomes made of PC with 1% PI(4,5)P $_2$  or of pure PC were used, recruitment to Lamp1A was detectable, but very low (lanes 7 and 8, and 11 and 12,





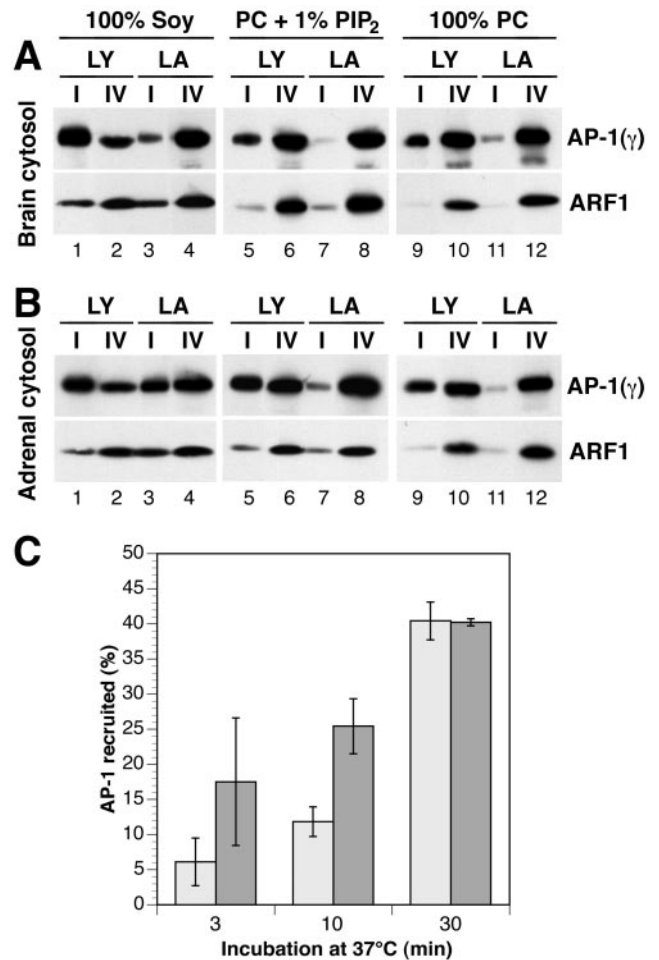
**Figure 6.** Recruitment of pure AP-1 to peptidoliposomes. (A) Aliquots of the mixed adaptor preparation (lane 1) and of hydroxyapatite-purified AP-1 (lane 2) containing the same amount of AP-1 (as judged by immunoblot analysis) were separated by SDS-gel electrophoresis and stained with silver. AP-1 subunits  $\beta 1$ ,  $\gamma$ , and  $\mu 1$  are indicated by filled arrowheads, whereas AP180 and AP-2 subunits  $\alpha_a$ ,  $\alpha_c$ ,  $\beta 2$ , and  $\mu 2$  are indicated by open arrowheads. (B) AP-1 recruitment assays were performed using liposomes made of PC with or without 1% PI(4,5)P<sub>2</sub> and exposing Lamp1Y (LY) or Lamp1A (LA) peptides in the presence of myristoylated ARF1 loaded with GMP-PNP or GDP. The analysis was performed as in Figure 3.

respectively), whereas recruitment to Lamp1Y-presenting liposomes was robust with ~40% (lanes 5 and 6, and 9 and 10).

The finding that AP-1 could be recruited from cytosol to pure PC liposomes with Lamp1Y peptides (lanes 5 and 6) is in contrast to our observation with purified AP-1 derived from clathrin coats, which was not recruited to pure PC membranes (Figure 2A). However, analysis of the time-course of AP-1 recruitment from cytosol to PC liposomes with or without 1% PI(4,5)P<sub>2</sub> revealed that the kinetics were significantly faster to peptidoliposomes containing 1% PI(4,5)P<sub>2</sub> than to those made of PC alone (Figure 7C).

## DISCUSSION

Vesicular transport requires the recruitment of coat components to the specific donor membrane in the cell and the



**Figure 7.** Recruitment of AP-1 from cytosol. AP-1 recruitment assays were performed using brain cytosol (A) or adrenal gland cytosol (B), and peptidoliposomes made of soybean lipids (lanes 1–4), PC with 1% PI(4,5)P<sub>2</sub> (lanes 5–8), or pure PC (lanes 9–12), exposing Lamp1Y (LY) or Lamp1A (LA) peptides. Cytosol supplemented with purified ARF1 and GMP-PNP was incubated with the peptidoliposomes for 30 min at 37°C before separation by gradient centrifugation. (C) To determine the kinetics, AP-1 recruitment assays were performed using brain cytosol and liposomes exposing Lamp1Y peptides prepared of either PC alone (white bars) or PC containing 1% PI(4,5)P<sub>2</sub> (dark bars) at different incubation times (average and SD of 3 determinations).

selection and incorporation of cargo proteins as well as of proteins necessary for vesicle targeting and fusion (e.g., the appropriate v-SNAREs). Two models for how this is accomplished have been proposed for different transport steps. Coat components may be targeted to the donor compartment by binding to a specific, high-affinity docking protein. Cargo molecules will diffuse into the coated area and be trapped by specific, but rather low-affinity interactions with coat molecules. Alternatively, it is the cargo itself that induces coat formation in combination with a site-specific feature like a particular lipid composition or a GEF for an accessory GTPase.

This second concept is attractive, because cargo selection and coat recruitment are coupled. This provides a mechanism to adjust vesicle formation to the amount of cargo to be transported, as has, for example, been observed experimentally for AP-2/clathrin coats in dependence of transferrin receptor overexpression (Iacopetta *et al.*, 1988; Miller *et al.*, 1991). However, the two models are not mutually exclusive. A docking protein is implicated in the nucleation of AP-2/clathrin coats, and there is evidence that synaptotagmin plays this role (Zhang *et al.*, 1994). Binding of AP-2 to synaptotagmin is stimulated by tyrosine-based endocytosis motifs, i.e., by cargo (Haucke and De Camilli, 1999). Because in addition both AP-2 and synaptotagmin bind to phosphoinositides, particularly PI(4,5)P<sub>2</sub> (Beck and Keen, 1991; Südhof and Rizo, 1996), it was proposed that the lipid composition might be an additional level of regulating AP-2 recruitment (Takei and Haucke, 2001).

Our results using liposomes show that a docking protein is not necessary for AP-1 recruitment. The minimal machinery in our assay consists of myristoylated ARF1-GTP (or GMP-PNP or GTPγS), membrane-anchored tyrosine-containing sorting motifs of cargo proteins and a small amount of specific phosphoinositides. In the absence of any other membrane-associated proteins, ARF1 thus must interact directly with AP-1 to stimulate its recruitment. Such an interaction has recently been shown between ARF1 and the β1 and γ-adaptors of AP-1 bound to immature secretory granules by cross-linking experiments (Austin *et al.*, 2000). Similarly, a direct interaction has been shown between ARF1 and COPI complexes (Zhao *et al.*, 1997). ARF1-GTP may dramatically increase AP-1 affinity for tyrosine signals or alternatively induce AP-1 to oligomerize, forming a surface patch with multiple cargo interactions already before addition of clathrin. AP-1 may thus behave similarly to COPI coatomer, which is induced to polymerize by a peptide corresponding to the cytoplasmic sequence of the COPI cargo protein p23 (Reinhard *et al.*, 1999).

The third component required for AP-1 recruitment besides ARF1 and cargo signals was a lipid composition containing phosphoinositides, particularly PI(4,5)P<sub>2</sub> and to a lesser extent PI(5)P, at physiologically low concentrations in the range of a few mole-percent. The phosphoinositide contribution is clearly specific and does not simply correlate with charge, because different isomers showed vastly different effectiveness and other acidic phospholipids at higher concentrations were inactive.

The lipid composition also affected the equilibrium distribution of activated ARF1 between the membrane-associated and the free form, as was apparent from the amount of ARF1 that was associated with the floated liposomes. However, all acidic lipids increased membrane association of ARF1, and there was no correlation between the recruitment of AP-1 and the fraction of floated ARF1. Phosphoinositides, which stimulated AP-1 recruitment, also did not affect the rate or extent of nucleotide exchange in ARF1 (in agreement with Antony *et al.*, 1997). Furthermore, recruitment of AP-3 or COPI, which are also ARF1 dependent, to liposomes was largely independent of the lipid composition (Bremser *et al.*, 1999; Drake *et al.*, 2000). The major effect of the lipid composition on AP-1 recruitment is thus unlikely to be exerted via ARF1, but rather via AP-1.

Phosphoinositides have indeed been shown to modulate tyrosine signal recognition of both AP-1 and AP-2 using a cross-linking assay with lipid/detergent micelles in the absence of ARF1. The interactions between the TGN38 motif and AP-2 (Rapoport *et al.*, 1997) as well as between the Lamp-1 motif and AP-1 (Rapoport *et al.*, 1998) were found to be enhanced by PI(3,4)P<sub>2</sub>. This phenomenon thus does not explain the lipid dependence of AP-1 recruitment in our system. However, the most efficient lipid for AP-1 recruitment, PI(4,5)P<sub>2</sub>, and the appropriate kinases for their synthesis have in fact been localized to the Golgi apparatus (Cockcroft and De Matteis, 2001). There, ARF1 was shown to regulate the synthesis of PI(4,5)P<sub>2</sub> by recruiting, and thus activating, PI 4-kinase and PI(4)P 5-kinase from the cytosol (Godi *et al.*, 1999; Jones *et al.*, 2000). Activation of ARF1 at the TGN may therefore contribute to preparing the ground with respect to the optimal lipid environment for AP-1 recruitment.

When a tyrosine signal was present, recruitment of AP-1 from cytosol was found not to be absolutely dependent on the lipid composition. This either reflects a difference between cytosolic and coat-derived AP-1 adaptors or contributions by unknown cytosolic factors. Yet, even in this system, the presence of PI(4,5)P<sub>2</sub> significantly enhanced the kinetics of the process. Generation of this phosphoinositide is thus a likely mechanism of regulating coat formation.

AP-1 recruitment in our assay is strongly dependent on tyrosine motifs presented on the membrane surface. The tyrosine motif of Lamp-1 has been shown to bind to both AP-1 and AP-2 in vitro (Höning *et al.*, 1996; Ohno *et al.*, 1996). The tyrosine motif of TGN38, also interacted with AP-2 adaptors in vitro (Ohno *et al.*, 1995) but only weakly with AP-1 (Boll *et al.*, 1996); yeast two-hybrid assays with μ1 yielded variable results (Ohno *et al.*, 1995, 1996; Rapoport *et al.*, 1997; Stephens *et al.*, 1997; Stephens and Banting, 1998). There is evidence that at least some membrane proteins are transported from the TGN to the basolateral surface via endosomes rather than in a direct vesicular transport route to the plasma membrane (Futter *et al.*, 1995; Leitinger *et al.*, 1995; Laird and Spiess, 2000; Orzech *et al.*, 2000). Together with the recent discovery of a μ1 isoform (μ1B) involved in basolateral sorting (Fölsch *et al.*, 1999; Ohno *et al.*, 1999), AP-1 adaptors are thus potentially involved in surface transport of basolateral proteins, including TGN38. AP-1 recruitment by the TGN38Y sequence in our assay might be related to this function.

In summary, our results define minimal requirements for AP-1 recruitment to a membrane and suggest the following modified model of the molecular events. Whereas in our assay ARF1 was activated by spontaneous nucleotide exchange on the lipid bilayer, ARF1 activation in the cell is a controlled and catalyzed process. Already ARF1-GDP may be concentrated at the membrane as indicated by its interaction with a putative PKA-activated receptor at the Golgi (Martín *et al.*, 2000). It is activated to ARF1-GTP by a specific brefeldin A-sensitive GEF like BIG2 (Shinotsuka *et al.*, 2002). The second factor specifying the site of AP-1 recruitment is likely to be the lipid composition in the TGN, i.e., the local production of PI(4,5)P<sub>2</sub>, which is further stimulated by ARF1-GTP activating appropriate lipid kinases. Productive AP-1 recruitment will only take place, when a sufficient concentration of cargo proteins with AP-1 recognition se-



quences is present. Interaction with ARF1, PI(4,5)P<sub>2</sub> and tyrosine signal may induce a conformational change in AP-1 inducing AP-1 oligomerization. The resulting structures will be stably attached to the membrane by multiple low-affinity interactions with cargo molecules and lipids. In our assay, this is reflected in the fact that, unlike ARF1, AP-1 attachment to the liposomes survived a 1-h floatation through a sucrose gradient without “bleeding” into the middle fractions. Subsequent binding of clathrin will then induce coat and membrane curvature. Because ARF1 is scarce in isolated clathrin-coated vesicles (Zhu *et al.*, 1998), it must dissociate at some point, most likely upon GTP hydrolysis. Interaction of ARF1-GTP with AP-1 might activate its GTPase activity. If AP-1 has not associated with other AP-1 complexes when GTP is hydrolyzed, it will be released from the membrane. Thus, ARF1 might function as a timer regulating coat assembly. It remains to be tested whether AP-1 acts as a GTPase-activating protein for ARF1, like the COPII components Sec23p/24p for Sar1 (Antonny *et al.*, 2001).

Our results do not exclude that docking proteins able to recruit AP-1 exist. In fact, we have reproduced the previous finding that AP-1 can be targeted to certain lipid compositions in a signal-independent, but cytosol-dependent manner. This might provide a mechanism for generating a basal level of cargo-independent vesicle budding as might be required to guarantee transport of lipids or recycling of v-SNAREs for endosome-to-Golgi transport when cargo proteins are few. Interestingly, the v-SNARE VAMP4 has been recently shown to bind AP-1 via a di-leucine motif (Peden *et al.*, 2001). Various membrane proteins thus may be able to nucleate AP-1/clathrin coats, as has also been proposed by Springer and Schekman (1998).

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