Cytosolic and Membrane-associated Proteins Involved in the Recruitment of AP-1 Adaptors onto the Trans-Golgi Network*

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The AP-1 adaptor complex is recruited from the cytosol onto the trans-Golgi network membrane, where it co-assembles with clathrin into a coat that drives vesicle budding. The GTPase ARF1 has been shown to be required for AP-1 recruitment, and here we demonstrate that we can reconstitute full GTP·γS-dependent recruitment of adaptors onto an enriched trans-Golgi network membrane fraction by adding purified AP-1 and recombinant myristylated ARF1, indicating that these are the only soluble proteins required for binding. To identify some of the membrane proteins involved in recruitment, we have incubated permeabilized metabolically labeled cells with cytosol under conditions that promote adaptor binding, then cross-linked the samples with 3,3'-dithiobis(sulfosuccinimidylpropionate), denatured by boiling in SDS, and immunoprecipitated with antibodies against the various subunits. Under these conditions, the adaptor subunits co-precipitate not only with each other and with clathrin, but also with three novel proteins: p75, which is specifically cross-linked to γ-adaptin; p80, which is specifically cross-linked to β'-adaptin; and p60, which is specifically cross-linked to AP47. These proteins are all candidates for components of the adaptor docking site on the trans-Golgi network membrane.

The first step in all vesicular trafficking pathways is the recruitment of coat proteins from the cytosol to a donor membrane compartment, where they drive the formation of a transport vesicle which can then fuse with an acceptor membrane compartment. A number of different types of coats have now been identified, each of which must be recruited onto a specific membrane. For instance, the AP-1 adaptor complex is recruited onto the TGN, while the AP-2 adaptor complex is recruited onto the plasma membrane. In both cases soluble clathrin then binds to the membrane-associated adaptors, and the two components co-assemble into a coat (1). The coatomer complex, which facilitates the formation of COPI-coated vesicles, is recruited onto the Golgi stack, the intermediate compartment, and, at least in vitro, the ER and endosomes (2–5). The Sec23p-Sec24p complex and the Sec13p-Sec31p complex are recruited onto the ER, where they facilitate the formation of COPII-coated vesicles (6). Recently an adaptor-related protein complex has been identified which, like AP-1, is associated with the TGN, but which appears to become incorporated into a novel type of non-clathrin-coated vesicle (7).

The recruitment of each of these coats onto the appropriate membrane is essential for the formation of transport vesicles bearing the right sort of cargo. For instance, clathrin-coated vesicles budding from the TGN selectively include cation-independent and cation-dependent mannose 6-phosphate receptors (MPRs), which bind newly synthesized lysosomal enzymes on the lumenal side of the membrane. In this way the enzymes are packaged into a unique type of vesicle, which can then deliver its contents to a prelysosomal compartment. There is evidence that the AP-1 adaptor complex can interact with the cytoplasmic domain of the cation-independent MPR, and that this interaction causes the receptor and its bound ligand to be sequestered into the budding coated vesicle (8, 9). Similarly, membrane proteins like the low density lipoprotein receptor have been shown to bind to the AP-2 adaptor complex, which would provide a molecular basis for the selective inclusion of such proteins into the coated vesicles that bud from the plasma membrane (10). It seems unlikely, however, that these interactions are sufficient to account for the precision with which each type of coat is recruited onto its target membrane. The two MPRs, for instance, are mainly found in late endosomes in many cells (11), yet the AP-1 complex is recruited onto the TGN and, to a lesser extent, onto an early endosomal compartment which may be functionally related to the TGN (12). Thus, the ability of adaptors to associate with a particular membrane cannot depend simply on binding to membrane proteins like the MPR and the low density lipoprotein receptor, and there must be additional components of the recruitment machinery.

One such component, required for AP-1 binding, is the small GTPase ARF1. When cells are treated with the drug brefeldin A, which prevents the nucleotide exchange of ARF proteins and causes them to become cytosolic rather than membrane-associated, AP-1 adaptors also rapidly redistribute to the cytoplasm (13, 14). Recruitment of cytosolic AP-1 adaptors onto the TGN in vitro is enhanced by the addition of GTP·γS, which activates ARFs and drives them onto the membrane. When ARF is depleted from the cytosol by gel filtration, the adaptors are no longer capable of binding to the TGN membrane, but addition of recombinant myristylated ARF1 restores this ability (15, 16). However, ARF1 alone also cannot account for the specificity of recruitment of AP-1 adaptors, since it is associated with other membranes in addition to the TGN, including the Golgi stack and intermediate compartment where it is required for coatomer recruitment. Two models have been proposed to explain the role of ARF1 in AP-1 recruitment. The first model predicts that ARF1 and the MPR together constitute an adaptor binding site on the TGN (12). The second model postulates that there is a specific AP-1 docking protein which resides in...
the TGN, which is distinct from the MPR and which is activated by ARF1 (16). Once on the membrane, the adaptors would then be able to bind to the MPR, but the docking protein would be responsible for their initial recruitment. However, this docking protein has yet to be identified.

Thus, it is clear that adaptor recruitment is a relatively complicated event involving several protein-protein interactions. This idea is supported by our recent studies on chimeric adaptor subunits, which reveal that adaptor complexes contain two or more targeting signals, each of which may interact with a different component of the recruitment machinery (17). In the present study, we have begun to dissect the various components of the machinery required for AP-1 recruitment. First, we have investigated the soluble protein requirements to find out whether AP-1 adaptors and ARF1 alone are sufficient for recruitment or whether additional cytosolic factors are needed. Second, we have used a cross-linking approach to identify membrane-associated proteins that interact with each of the AP-1 subunits as a means of finding candidate docking proteins.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Antibodies used in this study include the species-specific γ-adaptin antibody mAb100/3 (18) (Sigma), rabbit antisera specific for the β′, medium, and small chain subunits of the AP-1 adaptor complex (17), a rabbit antisera raised against clathrin heavy chain (7), and rabbit antisera raised against the three domains of γ-adaptin expressed as fusion proteins. The hinge domain-specific antisera has already been described (7); the antisera against the N-terminal and ear domains were raised in a similar manner using polymerase chain reaction to amplify the appropriate sequences from γ-adaptin cDNA and cloning them into pGEXIX. The N-terminal domain sequence consists of amino acids 1–595, while the ear domain sequence consists of amino acids 703–822. The ear domain construct was soluble and could be purified by glutathione affinity chromatography, while the N-terminal domain construct was insoluble and was purified from inclusion bodies by preparative gel electrophoresis, as described previously (17). All rabbit antisera were affinity-purified before use. Immunofluorescence was carried out as described previously (19) using methanol/acetone-fixed MDBK cells.

**Preparation of Enriched TGN Membranes—**A postmitochondrial supernatant was prepared from rat liver as described by Mullock et al. (20) using 40 ml of STM buffer (0.25 mM sucrose, 10 mM TES-NaOH, pH 7.4, 1 mM MgCl$_2$) for each ~15 g of liver. Preliminary experiments using a continuous Ficoll gradient prepared in STM buffer indicated that the AP-1 adaptor binding compartment fractions are a broad peak between 6 and 18% Ficoll; therefore, 10 ml of the postmitochondrial supernatant were loaded onto a step gradient consisting of 15 ml of 6% Ficoll layered over 10 ml of 18% Ficoll and underlayered with 4 ml of 45% Nycodenz, all dissolved in STM buffer. The gradients were centrifuged at 50,000 rpm in a Beckman VTi 50 vertical rotor, and the material from the 6–18% interface was collected and stored in liquid nitrogen in 1-ml aliquots containing 2 mg/ml protein.

**Preparation of Soluble Proteins—**Cytosol was prepared by homogenizing either pig brain or rat brain in cytosol buffer (25 mM HEPES-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM diithiothreitol, 1 mM glucose) and centrifuging at 50,000 rpm for 15 min in a Beckman TLA 100.3 rotor as described previously (21). AP-1 adaptor complexes were purified from pig brain clathrin-coated vesicles by hydroxylapatite chromatography (22), dialyzed into cytosol buffer, and stored in liquid nitrogen. Recombinant myristylated ARF1 was prepared from BL21 bacteria that had been co-transformed with plasmids encoding ARF1 and N-myristyltransferase. Expression was induced in the presence of 50 μM sodium myristate (23), and the protein was purified by anion exchange followed by gel filtration, as described by Weiss et al. (24). Fractions from the gel filtration column containing ARF1 were concentrated by ultrafiltration to ~1.4 mg/ml and stored at −70 °C.

**In Vitro Recruitment—**Recruitment studies were carried out using both enriched TGN membranes and permeabilized NRK cells. The TGN membranes, prepared as described above, were thawed immediately before use, diluted by the addition of 3 volumes of STM buffer, and pelleted by centrifugation at 50,000 rpm for 15 min in a Beckman TLA 100.2 rotor. Aliquots containing 500 μg of protein were incubated in 50-μl volumes for 10 min at 37 °C. The incubation mixture contained 100 mM EGTA, 1 mM ATP, 1 mM creatine phosphate, and 50 μg/ml creatine phosphokinase in cytosol buffer. GTPγS, when added, was used at a concentration of 100 μM. Other components of the incubation mixture that were used in some experiments included pig or rat brain cytosol prepared as described above and clarified by centrifugation at 100,000 rpm for 15 min in a Beckman TLA 100.2 rotor immediately before use; purified AP-1 adaptors, used at a concentration of 20 μg/ml and clarified as described above; and purified recombinant ARF1, also used at a concentration of 20 μg/ml. Recruitment was stopped by transferring the tubes containing the incubations onto ice and adding 950 μl of ice-cold STM buffer. The membranes were collected by centrifugation in a Beckman TLA 100.2 rotor at 50,000 rpm for 15 min, and the pellets were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer. Electrophoresis, Western blotting, and antibody labeling were all carried out as described previously (19).

Immunogold labeling was carried out on frozen thin sections of enriched TGN membranes that had been incubated with pig brain cytosol in the presence of GTPγS as described above but scaled up 3-fold. After the final centrifugation step, the membranes were resuspended into a small volume of cytosol buffer without the diithiothreitol and then fixed in 250 mM HEPES-NaOH (pH 7.2) containing 4% paraformaldehyde and 0.1% glutaraldehyde for 2 h at room temperature. The fixed membranes were pelleted, embedded in 10% gelatin, and processed for electron microscopy and labeled as described previously (7).

NRK cells were used for cross-linking studies. The cells were metabolically labeled by growing them overnight at ~50% confluence in methionine-free medium with 10% dialyzed fetal calf serum, 5 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin to which 0.25 mCi of Tran35S-label (ICN Flow) had been added for each 10-cm dish. The cells were then washed in cytosol buffer, permeabilized by plunging them into liquid nitrogen, scraped from the dish, and each dish was aliquoted into four Eppendorf tubes. Each tube was incubated with 100 μl of pig brain cytosol and then pelleted and washed as described previously (7, 13). The final pellets were resuspended in 100 μl of cross-linking buffer (25 mM HEPES-NaOH, pH 7.4, 1 mM MgCl$_2$, 0.25 mM sucrose), and the cross-linker 3,3′-dithiobis(sulfosuccinimidyl-propionane) (DTSSP) was added from a 100 mM stock solution to give a final concentration of 2 mM. Cross-linking was allowed to proceed at room temperature for 30 min, after which the cells were pelleted, resuspended in 100 μl of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and boiled for 3 min. The samples were then diluted into radioimmune precipitation buffer and immunoprecipitated as described by Simpson et al. (7).

**RESULTS**

**Soluble Proteins Involved in Recruitment—**To facilitate our study of adaptor recruitment onto the TGN, we have generated new antibodies against the γ-adaptin subunit of the AP-1 complex and a new fractionation method for enriching for AP-1-binding membranes. Until recently, the only antibody available against any of the AP-1 components has been mAb100/3, a mouse monoclonal directed against the hinge domain of γ-adaptin that reacts with most mammalian species but not with rodents (18). Although the species specificity of this antibody has proved useful for some experiments (e.g. one can monitor the recruitment of exogenous adaptors onto TGN membranes without any background from endogenous γ-adaptn by using rodent membranes and non-rodent cytosol), for other experiments this property has proved to be a limitation. Therefore, we have raised more widely cross-reacting antibodies against all three domains of γ-adaptin expressed as glutathione S-transferase fusion proteins: the N-terminal domain, the hinge, and the ear. Fig. 1, a–c, shows Western blots of brain homogenate probed with the three antibodies and reveals that they all detect a band of the appropriate size. However, although all of the antibodies work on blots, only anti-H, the hinge-specific antibody, was found to be useful for immunofluorescence, as shown in Fig. 1, d and e. Western MDBC cells double labeled with anti-H and mAb100/3 and reveals that the two labeling patterns are virtually identical. We have also developed a rapid fractionation method for
isolating a membrane fraction enriched in AP-1 binding. A rat liver postmitochondrial supernatant is centrifuged on a Ficoll step gradient (Fig. 2a) and the fractions assayed by Western blotting with anti-γH. Fig. 2, b and c, shows that endogenous γ-adaptin is enriched in the 6%/18% interface (Fraction 1), with about 3-fold the signal per μg of protein than in the postmitochondrial starting material. This preparation also binds exogenous AP-1 in a GTPγS-dependent manner, as shown in Fig. 2d. In this experiment the membranes were incubated with pig brain cytosol, with or without GTPγS, then pelleted and blotted to detect newly recruited γ-adaptin using mAb100/3. Addition of GTPγS resulted in a 30-fold increase in the signal associated with the pellet. Fig. 2e shows a gel of the purified proteins used in these experiments, while Fig. 3 shows Western blots of two separate experiments. When the membranes were incubated with AP-1 adaptors alone (lanes 1 and 2), the signal is enhanced ~2-fold by the addition of GTPγS. This basal level of GTPγS-dependent recruitment seen in the absence of cytosol may be due to residual ARF still associated with the membrane (25). Omitting the membranes (lane 3) strongly reduces the amount of signal associated with the pellet. In contrast, when the membranes are incubated with AP-1 adaptors plus rat brain cytosol (lanes 4–6), binding is much more dependent on GTPγS. Omitting the GTPγS results in a signal that is not significantly different from that obtained in the absence of membranes (compare lane 4 with lane 6), while the signal goes up 10-fold or more when GTPγS is added. This same pattern is seen when recombinant myristylated ARF1 is added to the adaptors in place of cytosol (lanes 7–9). Thus, the effect of cytosol on AP-1 recruitment can be completely reproduced by the addition of ARF1, indicating that no other soluble factors are needed.

Membrane-associated Proteins Involved in Recruitment—To identify membrane-associated proteins that interact with AP-1 adaptor subunits, a cross-linking approach was used. For these experiments, permeabilized NRR cells were used as the source of membranes instead of the TGN-enriched fraction, because the cells could be metabolically labeled. The cells were grown overnight with [35S]methionine, permeabilized by freezing and
thawing, and incubated with pig brain cytosol in the presence of GTPγS, then treated with the reversible cross-linker DTSSP. The samples were boiled in SDS to disrupt any noncovalent protein-protein interactions, diluted into radioimmune precipitation buffer, immunoprecipitated using anti-γH, and boiled in sample buffer containing β-mercaptoethanol to reverse the cross-linking.

Fig. 4a shows that in the absence of cross-linker, the only prominent labeled band in the immunoprecipitate is γ-adaptin, running just below the 97-kDa standard. When the samples were cross-linked before boiling in SDS, three other proteins were found to co-precipitate. The largest and the smallest of these proteins are of the right mobility to be the β′-adaptin and AP19 subunits of the AP-1 adaptor complex, and their identity was confirmed by Western blotting (data not shown). In addition, an unknown protein of ~75 kDa (p75) also co-precipitated with γ-adaptin in a cross-linker-dependent manner. When the cross-linked sample was run on a two-dimensional gel in which reducing agents were omitted in the first dimension (Fig. 4b), three prominent bands were found to run off the diagonal, β′-adaptin, γ-adaptin, and p75. This confirms that all three bands are part of a cross-linked complex or complexes. Somewhat surprisingly, neither ARF nor the cationic-independent protein assumed to be a cytosolic protein, because even though the cross-linker-dependent binding, suggesting either that some of the binding observed in the absence of additional soluble factors may be nonspecific or that negative as well as positive regulation may be occurring in the presence of ARF.

β′-adaptin was still found to co-precipitate with γ-adaptin in a cross-linker-dependent manner, but not p75 (first two lanes). This result rules out the possibility that p75 is another cytosolic protein which is co-recruited with the AP-2 adaptors onto the membrane. Similarly, treating cytosol with cross-linker in the absence of membranes caused β′-adaptin but not p75 to co-precipitate with γ-adaptin (last two lanes). These results indicate that p75 is only associated with γ-adaptin when it is on the membrane and not when it is in its soluble form.

Can p75 also be co-immunoprecipitated using antibodies against the other AP-1 adaptor subunits? Fig. 6 shows that when the immunoprecipitations were carried out using antibodies against AP19, AP47, or β′-adaptin, the amounts of p75 that co-precipitated were considerably reduced. However, for each subunit there was a set of additional bands which co-precipitated in a cross-linker-dependent manner. Thus, proteins assumed to be β′-adaptin and γ-adaptin were found to co-precipitate with AP19, β′-adaptin and (to a lesser extent) γ-adaptin as well as an unknown protein of ~60 kDa (p60) with AP47, and γ-adaptin, AP47, and an unknown protein of ~80 kDa (p80) with β′-adaptin.

DISCUSSION

A number of studies have shown that coat protein recruitment is regulated by small GTP-binding proteins. Thus, Sar1p

FIG. 3. Cytosolic factors involved in AP-1 adaptor recruitment. a, SDS-polyacrylamide gel of the purified proteins used in these experiments, recombinant myristylated ARF1 (0.5 μg) and AP-1 adaptors from pig brain (1.0 μg). β′-Adaptin, γ-adaptin, and AP47 are indicated. b, Western blots of two separate experiments in which purified AP-1 adaptors were added to TGN-enriched membranes and newly recruited γ-adaptin was detected with mAb100/3 as in Fig. 2d. Addition of AP-1 alone (lanes 1–3) results in a ~2-fold increase in binding in the presence of GTPγS. Addition of AP-1 plus either rat brain cytosol (lanes 4–6) or ARF1 (lanes 7–9) results in binding that is much more strongly GTPγS-dependent, increasing 10-fold or more. There is also a decrease in GTPγS-independent binding, suggesting either that some of the binding observed in the absence of additional soluble factors may be nonspecific or that negative as well as positive regulation may be occurring in the presence of ARF.

FIG. 4. Cross-linking of proteins associated with γ-adaptin. NRK cells were labeled overnight with [35S]methionine, permeabilized by freezing and thawing, incubated with pig brain cytosol plus GTPγS, and then treated with the reversible cross-linker DTSSP and boiled in 1% SDS before immunoprecipitation with anti-γH. As controls, either the cross-linker or the antibody were omitted. On a reducing gel (a) with γ-adaptin in the presence of cross-linker, indicated with dots: β′-adaptin, running at ~105 kDa; p75, running at ~75 kDa; and AP19, running at ~19 kDa. When a two-dimensional gel was run (b), omitting the reducing agent in the first dimension, both β′-adaptin and p75 were found to run off the diagonal with γ-adaptin.

FIG. 5. Controls for the co-precipitation of p75 with γ-adaptin. a, anti-γE as well as anti-γH were both found to bring down p75 (arrowheads) with γ-adaptin in a cross-linker-dependent manner. b, cytosol was prepared from metabolically labeled NRK cells and proteins were either allowed to be recruited onto unlabeled TGN-enriched membranes before cross-linking (first two lanes) or cross-linked directly (last two lanes). In both cases, p75 no longer co-precipitated with γ-adaptin, indicating that it is a membrane-associated protein.

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Proteins Involved in AP-1 Recruitment

**Fig. 6. Cross-linking and immunoprecipitation using antibodies against other AP-1 adaptor subunits.** Metabolically labeled NRK cells were treated as in Fig. 4a and immunoprecipitated with antibodies against AP19, AP47, and β′-adaptin as well as γ-adaptin. The antigen recognized by each antibody is indicated with a black dot. Arrowheads, other adaptor subunits that co-precipitate in the presence of cross-linker; white stars, position of p75; black stars, other bands that co-precipitate with each antibody in the presence of cross-linker. Thus, a protein of ~60 kDa (p60) comes down with AP47, and a protein of ~80 kDa (p80) comes down with β′-adaptin.

is required for COPII binding (6) and ARF1 for COPI and AP-1 adaptor binding (15, 16), while the relevant GTP-binding proteins that might facilitate the binding of AP-2 adaptors and the adaptor-related complex to membranes have not yet been identified, but are likely to be ARFs because (at least in vitro) both events are sensitive not only to GTPγS but also to brefeldin A (7, 21). In the case of COPI and COPII coats, the coat proteins themselves and the small GTP-binding proteins are the only soluble components that are required for recruitment, and here we show that the same is true for AP-1 adaptors. However, this does not rule out the possibility that there might be peripheral membrane proteins derived from the cytosol that play a role in recruitment, as suggested in a recent study on the binding of AP-1 adaptors to immature secretory granules (25). The role of such proteins in coat protein recruitment is highlighted by the finding that Sec16p, a peripheral membrane protein associated with the ER, is essential for COPII recruitment, even though it does not need to be added in soluble form (26).

As a first step toward identifying membrane proteins involved in adaptor recruitment, both peripheral and integral, we have made use of the cross-linker DTSSP, followed by denaturation and immunoprecipitation, to bring down proteins associated with membrane-bound AP-1 adaptors. Under the conditions we have used, the adaptor subunits also become cross-linked to each other and to clathrin. It is clear, however, that the cross-linking is incomplete, since although the four subunits of the adaptor complex are known to be stoichiometric with each other, the major band brought down by each antibody was its own antigen. The relative amounts of the other subunits that co-precipitated in the presence of cross-linker provide some clues as to which subunits are likely to be in direct contact with each other. For instance, the AP47 antibody brought down considerably more β′-adaptin than γ-adaptin, suggesting that AP47 may be directly associated with β′-adaptin and only indirectly associated with γ-adaptin. This possibility is supported by our studies on adaptor subunit interactions using the yeast two-hybrid system, in which β′-adaptin was found to interact with γ-adaptin and AP47 but not AP19, and γ-adaptin with β′-adaptin and AP19 but not AP47 (17).

In addition to adaptor subunits and clathrin, unknown bands also co-precipitated with the various antibodies. Most striking of these was p75, which appeared as a strong band in immunoprecipitates using antibodies against two different γ-adaptin epitopes and as a weaker band in the immunoprecipitates using antibodies against the other subunits. These observations suggest that p75 interacts specifically with γ-adaptin. Experiments making use of radiolabeled cytosol revealed that p75 only co-precipitates with membrane-bound γ-adaptin and that it is not co-recruited onto the membrane with the AP-1 adaptor complex. Thus, p75 is a strong candidate for a component of the adaptor docking site on the TGN membrane.

Two additional proteins were found to co-precipitate with other adaptor subunits in the presence of cross-linker, p60 with AP47 and p80 with β′-adaptin; these may be additional components of the adaptor docking site. Studies making use of chimeric α- and γ-adaptins have revealed that the adaptors must contain at least two targeting signals, which presumably act by binding to docking sites on the membrane. One of these targeting signals is in the C-terminal “ear” domains of α and γ, and the other is in in the adaptor “head,” which contains the N-terminal domains of the α/γ and β subunits as well as the medium and small chains (17, 27). There is a strong correlation between the presence of a particular medium and small chain and targeting of the complex to a particular membrane, indicating that one or both of these subunits may contain targeting information. Thus, p60 may also play a role in the specificity of adaptor recruitment. In contrast, no correlation was found between the β subunits and targeting; however, p80 could still be involved in adaptor docking as a general component of the recruitment machinery, acting both at the TGN and at the plasma membrane. Interestingly, Mallet and Brodsky have also identified an ~80-kDa protein that associates with AP-1 adaptors using a different approach.²

Attempts to further characterize p75, p60, and p80 have so far proved unsuccessful. There are no strong bands at these positions on gels of purified clathrin-coated vesicles, indicating that the proteins bind adaptor subunits only transiently. Although the bands appear heavily labeled in our immunoprecipitates, the intensity of the signal relative to that of the adaptor subunits is somewhat misleading, since most of the adaptors in the immunoprecipitate are likely to be derived from the unlabeled cytosol. Thus, even in highly scaled-up preparations, we have been unable to obtain sufficient quantities of these proteins for sequence analysis. We are at present making use of alternative methods for identifying proteins that interact with adaptor subunits, in particular the yeast two-hybrid system. Antibodies will then be raised against such proteins to determine whether they can be cross-linked to newly recruited adaptor subunits, and whether they correspond to any of the proteins identified in the present study.

Adaptors and other coat proteins are recruited onto their target membranes with extremely high precision, and it is possible that the only way to achieve such accuracy is by multiple interactions involving different membrane proteins and different coat subunits. Some of these interactions may also be important in the regulation of membrane traffic pathways, controlling the extent of coat protein recruitment and thus of vesicle formation. Thus, the AP-1 adaptor docking site may consist of not one protein but several, ARF1 (possibly acting indirectly by stimulating phospholipase D) (28, 29),

2 W. Mallet and F. Brodsky, personal communication.
MPRs, and other as yet uncharacterized proteins including p75, p60, and p80. Each of these individual proteins might be present on more than one membrane, but only the TGN membrane would have all of them in sufficiently high concentrations to ensure that the AP-1 adaptor complex is always targeted to the correct location.

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