ADP-ribosylation factor 1 (ARF1) mediates clathrin coat formation on PC12 immature secretory granules (ISGs). We have used two approaches to investigate whether ARF1 interacts directly with the clathrin adaptor protein, AP-1. Using an in vitro recruitment assay and co-immunoprecipitation, we could isolate an AP-1-ARF1 complex. Then we used a site-directed photocross-linking approach to determine the components that act downstream of ARF1 in clathrin coat formation on ISGs. Myristoylated ARF1, with a photolabile phenylalanine analogue incorporated into its putative effector domain (switch 1), showed a specific, GDP-dependent interaction with both the γ- and β-adaptin subunits of AP-1 on ISGs. These experiments provide evidence for a direct interaction of ARF1 with AP-1. On mature secretory granules myristoylated ARF1 does not bind, and hence clathrin coat formation cannot be initiated, supporting the hypothesis that molecules involved in coat recruitment are removed during ISG maturation.

The assembly of clathrin coats on the cytoplasmic face of intracellular membranes occurs at specific sites. Ultimately, the site of coat assembly becomes a transport vesicle as the membrane is deformed by the rearrangement of the clathrin triskelion, driven by interaction with other coat constituents (for recent review see Refs. 1 and 2). The mechanism underlying assembly of the clathrin coat at specific sites involves a complex set of protein-protein and protein-lipid interactions that is not fully understood. At all intracellular sites where clathrin assembles, the minimum machinery is the adaptor proteins (APs)1 and clathrin. Four types of adaptors, AP-1 through AP-4 (1, 3, 4) have been identified to date. All adaptors are heterotetrameric complexes of two large subunits with a molecular mass of ~100 kDa, a medium subunit (~50 kDa) and small subunit (~20 kDa). The first identified and most studied adaptors are AP-1 and AP-2. AP-1 consists of the large subunits γ and β1, medium chain μ1, and small chain σ1 and is found primarily on the Golgi complex (5) and immature secretory granules (6), whereas AP-2, which consists of the α, β2, μ2, and ω2 subunits, is localized mainly to the plasma membrane and endosomes (7). The localization of AP-1 and AP-2 to different compartments can, in part, be attributed to their ability to bind to different sequences in the cytoplasmic domains of trans-membrane receptors (for recent reviews see Refs. 8 and 9).

ARF1 recruits AP-1 to both ISG membranes (6) and the trans-Golgi network (10, 11). ARFs are a family of small molecular mass (~20 kDa) GTP-binding proteins that associate with lipid bilayers and membranes via a myristoylated, hydrophobic amino terminus (12, 13). ARF in the GDP-bound form is cytosolic. Interaction of ARF with its exchange factor allows GDP to be exchanged for GTP, and this causes a major structural change in the switch 1 domain of ARF1 (14), thus exposing the myristic acid and promoting interaction of ARF with the membrane.

ARF1 has been identified as a component of Golgi-derived nonclathrin coated (COP-coated) vesicles (15) and is required for the formation of COP1 vesicles (16). However, unlike COP-coated vesicles, ARF is not present in clathrin-coated vesicles in sufficient quantities to be a stoichiometric component of the coat, although it has been shown to recruit AP-1 in a stoichiometric manner (17). Furthermore, ARF1 in the presence of GTP-γS generates high affinity binding sites for AP-1 that are stable even in the presence of high concentrations of Tris (17). To investigate whether ARF1 interacts with AP-1, we used an in vitro recruitment assay with [35S]myrARF1, followed by co-immunoprecipitation with antibodies directed against AP-1 and discovered an AP-1-ARF1 complex on ISG membranes.

A second approach using site-specific cross-linking was employed to start to understand the nature of the ARF1 interaction with AP-1 on ISG membranes. Recent work using this approach has demonstrated that the switch 1 domain of ARF1 interacts directly with COP1 (18). ARF1, with amino acid Ile-46 replaced with the photocrossatable analogue of phenylalanine ((Tmd)Phe) (19), cross-linked to both β- and γ-COP (20). Using the ARF1-(Tmd)Phe-46 mutant, we performed cross-linking experiments with ISGs as the acceptor component for ARF and AP-1. We have found that on ISG membranes ARF1 can be cross-linked to AP-1 in a GDP-dependent manner via the γ- and β1-adaptin subunits of AP-1, and this interaction is strictly dependent on the presence of ISG membranes. Mature secretory granules (MSGs) or liposomes do not facilitate a direct interaction between ARF and AP-1. Our data show that the switch 1 domain of ARF1, encompassing Ile-46, interacts with AP-1 as well as COP1.

EXPERIMENTAL PROCEDURES

ISG Preparation—ISGs and MSGs were prepared from PC12 cells by velocity and equilibrium sucrose gradient centrifugation, as described
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Previously (6). Each gradient generated 3 ml of ISGs and 2 ml of MSGs, 1/24 of which was used per reaction.

AP-1 Purification—Bovine adrenal AP-1 was purified essentially as described (21, 22) with some modifications: the precipitated coat proteins that had been stripped from the isolated clathrin-coated vesicles using 0.5 M NaCl, 5% Triton X-100 (TNTE buffer) for denaturing conditions, the samples were boiled for 3 min in the presence of 1% SDS and then diluted as for the native conditions except that the Triton X-100 concentration was increased to 0.9%. Rabbit anti-mouse antisera was added to all incubations containing mAbs. The immune complexes were bound by protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). After washing in TNTE buffer and once with 20 m NaHCO3, pH 7.5, samples were solubilized in sample buffer and analyzed by Western blotting and autoradiography.

Removal of AP-1 and COPI from Lysate—We used a method previously shown to deplete 80% of COPI from rat liver cytosol (30). 110 μl of the translation mix containing in vitro translated [35S]ARF-(Tmd)(Tmd)Phe-46 was placed over a 40-μl 0.5 M sucrose cushion (in binding buffer) and centrifuged at 300,000 × g using a TLA-100 rotor (Beckman) for 2 h at 4 °C.

Preparation of Liposomes—Liposomes were prepared using soyabean mixed lipids (Azolectin), containing 20% l-α-phosphatidylcholine (Sigma P5638), according to the reverse-phase evaporation method (31). The resulting liposomes were filtered through a 0.8-μm Millex®-PF filter (Millipore). 72.5 mg of liposomes were used per sample.

RESULTS

Purified Myristoylated ARF1 Regulates AP-1 Binding to the ISGs—It has previously been shown, using a cell-free assay system and partially purified components, that ARF1 is required for γ-adaptin binding to PC12 ISGs (6). We have reproduced the ARF1-dependent AP-1 recruitment to ISGs using purified components (Fig. 1). Quantification relies on the use of the mAb 100/3 that recognizes bovine but not rat γ-adaptin; hence only the bound exogenous bovine γ-adaptin is detected. Both purified bovine AP-1 and purified recombinant myrARF1 were titrated into the binding assay (Fig. 1A). We used bovine AP-1 because we could obtain large amounts of tissue for the purification. Our previous experiments have shown that bovine AP-1 binds to ISGs as efficiently as rat AP-1 (6). Human, bovine, and mouse AP-1 are 99% identical; thus it was assumed that the human ARF1 would bind as efficiently to rat ISGs as the endogenous rat ARF1. Increasing the myrARF1 concentration up to 20 μg/ml resulted in an increase in the γ-adaptin bound to the ISGs at all concentrations of AP-1 used except 1 μg/ml. At 1 μg/ml AP-1, the γ-adaptin binding was saturated at 4 μg/ml myrARF1, this may be due to limiting amounts of AP-1. In the absence of added ARF1 there is a basal level of γ-adaptin binding (Fig. 1B); this is likely to be due to endogenous ARF1 already bound to the ISGs and is similar to the level observed in the presence of ARF1 but in the absence of any nucleotide. The addition of GTPyS increases γ-adaptin binding to ISGs 3-4-fold but only in the presence of ARF1 (Fig. 1B). We did not observe GTP-dependent binding of nonmyristoylated ARF1 to the membranes (results not shown). Hence, the only cytosolic component required for purified AP-1 to bind to the ISGs is GTP-bound myrARF1.

In Vitro Translation of [35S]MyrARF1 Interacts with AP-1 on the ISGs in a GTP-independent Manner—To monitor ARF1 binding to the ISGs, we wanted to generate [35S]myrARF1 by in vitro translation using rabbit reticulocyte lysate. To demonstrate that the ARF1 was myristoylated during the translation reaction, ARF1 mRNA was in vitro translated in the presence of [3H]myristic acid. This resulted in a 1H-labeled ~20-kDa band whose migration was identical to purified recombinant myrARF1 (Fig. 2A, lane 1). Others have also observed N-myristoyltransferase activity (28, 32, 33) in the rabbit reticulocyte lysate. In vitro translation of ARF1 mRNA in the presence of [35S]methionine resulted in a broader ~20-kDa band (Fig. 2A, lane 2) that, after a lower exposure time, is visible as a doublet (not shown) corresponding to myristoylated and unmyristoylated ARF1.
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nonmyristoylated [35S]ARF1. These results confirm that ARF1 is myristoylated during the in vitro translation reaction.

To determine whether AP-1 and ARF1 form a complex, ISG binding assays were performed in the presence of [35S]ARF1. After incubation of [35S]ARF1 with the ISGs in the presence of GTPyS or GDPβS, immunoprecipitation of γ-AP was performed with polyclonal, STO-25 to determine whether the [35S]myrARF1 was immunoprecipitated under native conditions with either the anti-γ-AP antiserum, STO-25 (γ-AP, lanes 1 and 2) or preimmune serum (PI, lanes 3 and 4). Samples were analyzed by 12% SDS-PAGE, quantified using a PhosphorImager, and subjected to autoradiography. The results are expressed as percentages of the signal observed with 2 μg/ml AP-1 and 20 μg/ml myrARF1 in the presence of GTPyS. All assays are done in duplicate. Similar results were obtained in at least four independent experiments.

FIG. 1. Recombinant human myrARF1 recruits purified AP-1 to ISGs in a GTPyS-dependent manner. A, PC12 ISGs were incubated with 1 μg/ml (closed circles), 2 μg/ml (open circles), or 4 μg/ml (closed squares) of purified bovine adrenal AP-1 and increasing amounts of recombinant myrARF1 (2–20 μg/ml GTPyS) at 37 °C for 30 min in the presence of GTPyS. The amount of γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting with the bovine specific mAb antibody, 100/3. The membrane-bound γ-adaptin was quantified using a PhosphorImager, and results are expressed as a percentage of the signal observed with 2 μg/ml AP-1 and 20 μg/ml myrARF1. B, binding assay, similar to that in panel A, using 2 μg/ml AP-1 in the presence (+) or absence (−) of 20 μg/ml myrARF-1, in the presence (shaded bars) or absence (open bars) of GTPyS. The amount of [35S]myrARF immunoprecipitated compared with the percentages of [35S]myrARF immunoprecipitated with the corresponding purified recombinant proteins (not shown). No labeling was observed in this region in the absence of mRNA. After incubation at 37 °C for 30 min, the membranes were pelleted, solubilized, and then immunoprecipitated under native conditions with either the anti-γ-AP antiserum, STO-25 (γ-AP, lanes 1 and 2) or preimmune serum (PI, lanes 3 and 4). Samples were analyzed by 12% SDS-PAGE, quantified using a PhosphorImager, and subjected to autoradiography. The results are expressed as percentages of the signal observed with 2 μg/ml AP-1 and 20 μg/ml myrARF1 in the presence of GTPyS. All assays are done in duplicate. Similar results were obtained in at least four independent experiments.

Fig. 2. AP-1 forms a complex with myristoylated [35S]ARF on ISGs. A, in vitro translated ARF1, labeled with either [3H]myristic acid (lane 1) or [35S]methionine (lane 2) was subjected to 15% SDS-PAGE and fluorography. Arrows indicate the positions of the myristoylated and nonmyristoylated ARF1 as defined by Coomassie Blue staining of the corresponding purified recombinant proteins (not shown). No labeling was observed in this region in the absence of mRNA. B, in vitro translated [35S]ARF1 was incubated with ISG membranes, in the presence of either GTPyS (lanes 1 and 3) or GDPβS (lane 2 and 4). After incubation at 37 °C for 30 min, the membranes were pelleted, solubilized, and then immunoprecipitated under native conditions with either the anti-γ-AP antiserum, STO-25 (γ-AP, lanes 1 and 2) or preimmune serum (PI, lanes 3 and 4). Samples were analyzed by 12% SDS-PAGE, quantified using a PhosphorImager, and subjected to autoradiography. B shows a representative fluorograph showing ~20 kDa [35S]myrARF1 as indicated by an arrow. C, three experiments similar to that in B were performed and quantified using a PhosphorImager. Values represent the percentages of [35S]myrARF immunoprecipitated compared with the amount immunoprecipitated with STO-25 in the presence of GTPyS (hatched bars) or GDPβS (open bars). Error bars represent the standard error of the mean.

Approximately 1% of the total membrane bound [35S]myrARF1 added to the assay could be co-immunoprecipitated with anti-γ-AP antibodies. These results demonstrate that AP-1 and myrARF1 do form a complex on the ISG membrane.

Membrane-bound [35S]ARF-(Tmd)Phe-46 Cross-links to 100–120-kDa Coat Proteins—To investigate whether ARF1 interacts directly with AP-1, we decided to use site-directed photocross-linking, as used previously by others to demonstrate a direct interaction of the nonclathrin coat, COPI and ARF1 (18). Full-length photoactivatable [3S]ARF-(Tmd)Phe-46 was generated by mutating the codon for Ile-46, in the human ARF1 cDNA, to amber, followed by in vitro translation (of the corresponding mRNA) in the presence of a suppressor tRNA ligated to the photolabile group, (Tmd)Phe, which recognizes this stop codon (18). [3S]ARF-(Tmd)Phe-46 was then incubated with ISGs with or without AP-1 in the presence of GTPyS. The GTPyS-dependent binding of [3S]ARF-(Tmd)Phe-46 to the ISG
FIG. 3. Photocross-linking of membrane-bound [35S]ARF-(Tmd)Phe-46. A, in vitro translated [35S]ARF-(Tmd)Phe-46 was incubated in the absence (lanes 9 and 10) or presence (lanes 1–8) of ISG membranes, with GDP (lanes 1 and 5) or GTPγS (lanes 2–4 and 6–10), in the absence (lanes 1–4 and 9) or presence (lanes 5–8 and 10) of 4 μg/ml purified AP-1 or with 40 μg/ml recombinant myrARF1 (lanes 4 and 8). After incubation at 37 °C for 30 min, the membranes were sedimented and irradiated (except samples lanes 2 and 6) and trichloroacetic acid precipitated. Then ¼ were analyzed by 7.5% SDS-PAGE and autoradiography (upper panel) to visualize the cross-linked products, and ¼ was analyzed by 15% SDS-PAGE and autoradiography (lower panel) to visualize the uncross-linked [35S]ARF-(Tmd)Phe-46 bound to ISG membranes. In the upper panel, the region of the ISG specific photocross-linked products is labeled X. Upper and lower panels are representative of at least three independent experiments and were exposed for 1 week and 1 day, respectively. B, photocross-linked products of [35S]ARF-(Tmd)Phe-46 (samples identical to that in panel A, lane 3) were immunoprecipitated with preimmune serum (lane 2) or polyclonal antisera, to γ-adaptin (γ-AP, lane 3 and 4), β-COP (lane 5), or γ-COP (lane 6) under native (n) or denaturing (d) conditions. Lane 1 represents the total photocross-linked products before immunoprecipitation. Samples were analyzed by 7.5% SDS-PAGE and subsequent autoradiography for 2 weeks. Results are representative of at least three independent experiments.

membranes (Fig. 3A, lower panel, compare lane 1 versus lane 3 and lane 5 versus lane 7) excludes the possibility that replacement of Ile-46 with the (Tmd)Phe severely alters the GTP-dependent ARF binding to ISGs. The migration of the [35S]ARF-(Tmd)Phe-46 bound to the ISG membranes is identical to the migration of myrARF1. Irradiation resulted in at least three cross-linked products with estimated molecular masses of 120–140 kDa (region marked X, Fig. 3A, upper panel, lanes 3 and 7). Addition of AP-1 appeared to increase the intensity of the 120-kDa bands (Fig. 3A, lane 7). This was consistent with cross-linking of ARF (~20 kDa) to one or both of the large subunits of AP-1 (~100 kDa). The photocross-linked products are specific because they were not observed in the presence of GDP (Fig. 3A, upper panel, lanes 1 and 5), or in the absence of irradiation (Fig. 3A, upper panel, lanes 2 and 6). Displacement of the membrane-bound [35S]ARF-(Tmd)Phe-46 using excess unlabeled recombinant wild type myrARF1 (Fig. 3A, lower panel, lanes 4 and 8) abolished the photocross-linking (Fig. 3A, upper panel, lanes 4 and 8). Importantly, the cross-linked products were not observed in the absence of ISG membranes either in the presence or absence of added AP-1 (Fig. 3A, upper panel, lanes 9 and 10), demonstrating that only membrane-bound myrARF1 interacts with the 100–120-kDa proteins and that ARF1:AP-1 does not interact in solution.

Immunoprecipitation of the specific cross-linked products confirmed an interaction of myrARF1 with AP-1. Immunoprecipitation of the whole AP-1 complex with the γ-AP polyclonal, STO-25 (6) under native conditions resulted in a [35S]-labeled doublet (Fig. 3B, lane 3), whereas immunoprecipitation of the dissociated subunits under denaturing conditions showed a single band of lower intensity corresponding to γ-AP (Fig. 3B, lanes 4). Because the pattern of cross-linked products derived from [35S]ARF-(Tmd)Phe-46 bound to ISGs were similar to that observed with Golgi membranes in the presence of coatamer (20), antisera to β- and γ-COP were used to characterize the other cross-linked products observed with the ISG membranes. Similar to the result obtained for Golgi membranes (20), anti-β-COP immunoprecipitated a band of 120 kDa (Fig. 3B, lane 5), and the anti-γ-COP immunoprecipitated two bands of approximately 120 and 140 kDa (Fig. 3B, lane 6).

Membrane-bound [35S]ARF-(Tmd)Phe-46 Can Directly Interact with γ- and β-AP—To increase the efficiency of cross-linking of [35S]ARF1-(Tmd)Phe-46 to AP-1 on the ISGs, purified bovine AP-1 was titrated into the photocross-linking binding assay (Fig. 4A, lanes 1–3). The cross-linking pattern showed an increase in the intensity of the labeled 120-kDa bands with increasing AP-1 (Fig. 4A, top panel, lanes 1–3). Immunoprecipitation of the whole AP-1 complex with 100/3 confirmed an increase in the efficiency of cross-linking of [35S]ARF1-(Tmd)Phe-46 to AP-1 (Fig. 4A, middle panel, lanes 1–3). Increasing the [35S]ARF1-(Tmd)Phe-46 caused a further increase in the intensity of the cross-linked products (Fig. 4A, top panel, lanes 6 and 7), which is due to increased cross-linking to AP-1 (Fig. 4A, middle panel, lanes 6 and 7). Because the mAb 100/3 only recognizes bovine and not rat AP-1, it was surprising that, in the absence of exogenous bovine AP-1, 100/3 could immunoprecipitate a cross-linked product (Fig. 4A, middle panel, lane 1). This suggested that there might be AP-1 in the rabbit reticulocyte lysate used to generate the [35S]ARF1-(Tmd)Phe-46. Western blot analysis demonstrated that indeed this was the case (data not shown). Using purified bovine AP-1 as a standard and assuming equal cross-reactivity of 100/3 with rabbit AP-1 it was estimated that the rabbit reticulocyte lysate contains ~16 μg/ml AP-1, giving a final concentration of ~1 μg/ml rabbit AP-1 in the binding assay. AP-2 and COP1 were also detected in the lysate using polyclonal anti-β-COP and
PHOTOCROSS-LINKING SAMPLES, IDENTICAL TO...

**Fig. 4. Photocross-linking of membrane-bound [35S]ARF-(Tmd)Phe-46 to the large subunits of AP-1.** A, in vitro translated [35S]ARF-(Tmd)Phe-46 was either kept constant (see *Experimental Procedures*) and incubated in the presence of ISG membranes with increasing amounts of purified bovine AP-1 (lanes 1–3), or the AP-1 concentration was kept constant (4 μg/ml) and the in vitro translated [35S]ARF-(Tmd)Phe-46 (lanes 4–7) was varied. All samples contained GTPγS. After sedimentation and irradiation, the products (upper panel) were subjected to 7.5% SDS-PAGE and autoradiography, and the region of the specific photocross-linked products is shown (X). Alternatively, cross-linked samples were immunoprecipitated with the mAb anti-γ-AP (100/3) under native conditions and then analyzed by 7.5% SDS-PAGE and autoradiography (middle panel) for 1 month. Lower panel, the uncross-linked membrane-bound [35S]ARF-(Tmd)Phe-46 was visualized by analysis of 1/10 of the products of the upper panel by 15% SDS-PAGE and autoradiography. The upper and lower panels were exposed for 6 days. Photocross-linking samples, identical to those in lane 1, underwent two rounds of immunoprecipitation (the second round was performed on the remaining supernatant from the first round) with B, polyclonal anti-γ-AP antisera, STO-25, either under native (n) or denaturing (d) conditions, C, mAb anti-γ-AP, 100/3 and mAb anti-β-AP, D, mAb anti-β-AP and mAb anti-γ-AP, 100/3 and as indicated. Second round immunoprecipitations with the same antibody as the first round were performed to indicate efficacy of the first round. In B–D, all autoradiographs were obtained after a 3-day exposure.

anti-γ-COP and may explain the source of β- and γ-COP cross-linked to ARF1 on the ISG membranes. Thus, the rabbit reticulocyte lysate acts like a cytosol, providing the adaptor proteins and coatomer for recruitment to ISGs. Increasing the amount of [35S]ARF1-(Tmd)Phe-46 results in an increased binding (Fig. 4A, bottom panel, lanes 4–7), whereas increasing AP-1 had no effect on the amount of [35S]ARF1-(Tmd)Phe-46 bound to the membrane (Fig. 4A, bottom panel, lanes 1–3). This suggests that AP-1 does not influence [35S]ARF1-(Tmd)Phe-46 binding to the membrane. These results confirm a direct interaction of [35S]ARF1-(Tmd)Phe-46 with both exogenous rabbit and bovine AP-1 on ISG membranes.

Immunoprecipitation with STO-25 under native and denaturing conditions (Fig. 3B, lane 3 and 4) implied that both γ- and β1-AP may interact with ARF. The photocross-linking assay in the absence of added bovine AP-1 contains endogenous rat AP-1 from the PC12 ISGs and exogenous rabbit AP-1 from the rabbit reticulocyte lysate. Anti-γ-AP antiserum, STO-25, either under native (n) or denaturing (d) conditions, C, mAb anti-γ-AP, 100/3 and mAb anti-β-AP, D, mAb anti-β-AP and mAb anti-γ-AP, 100/3 and as indicated. Second round immunoprecipitations with the same antibody as the first round were performed to indicate efficacy of the first round. In B–D, all autoradiographs were obtained after a 3-day exposure.
binding assay compared with ISGs (Fig. 6, lower panel) using the crude coat proteins. As a result, no cross-linking of \[ ^{35}S \]ARF1-(Tmd) to \[ ^{35}S \]ARF1-(Tmd)Phe-46 with mAb, 100/3 (Fig. 5, middle panel, lanes 5–7). However, cross-linking of \[ ^{35}S \]ARF1-(Tmd)Phe-46 to AP-1 was still detected in the absence of exogenous bovine AP-1 (Fig. 5, top panel, lanes 5, 6). The resulting supernatant (Supe.) was subjected to 7.5% SDS-PAGE and autoradiography for 10 days. In the top panel, the region of the ISG specific photocross-linked products is labeled (X). This panel was obtained after a 1-week exposure. Middle panel, samples identical to those in the top panel, lanes 5–7 were immunoprecipitated with mAb anti-γ-AP. 100/3 and mAb anti-β-COP, M3A5, respectively, and chemiluminescence, and for \[ ^{35}S \]ARF-(Tmd)Phe-46 by 15% SDS-PAGE and autoradiography for 8 h. B, in vitro translation mix, either untreated (crude) or centrifuged (depleted), containing equal amounts of \[ ^{35}S \]ARF-(Tmd)Phe-46 were used for the photocross-linking experiments as in Fig. 3A. Experiments were performed in the presence of either GDPβS (lanes 1 and 4) or GTPγS (lanes 2, 3, and 5–8), with or without AP-1 as indicated.

**DISCUSSION**

myrARF1 is required for the recruitment of AP-1 to ISGs (6) and ultimately for clathrin-coated vesicle formation during ISG maturation. Previous work showed that ARF1 is a limiting factor in the GTP-stimulated recruitment of AP-1 onto isolated Golgi membranes, and although ARF1 is not a stoichiometric component of the clathrin coat, it appears to function in a stoichiometric manner to generate high affinity binding sites for AP-1 (17). We have extended these findings by demonstrat-
ing a direct interaction between AP-1 and myrARF1 in the GTP-bound form. We show that AP-1 and ARF1 form a complex using co-immunoprecipitation of \(^{35}\)S]myrARF1 with γ-adaptin from solubilized ISG membranes. We could estimate that 1% of the membrane bound myrARF1 is in a complex with AP-1. We exploited a site-directed photocross-linking approach to demonstrate that myrARF1 in the GTP form interacts directly with both the γ- and β1 subunits of AP-1. The interaction of ARF1, via Ile-46, which is in the switch 1 domain, the putative effector region of ARF1 (14), with AP-1 is consistent with data of Liang and Kornfeld (36); using chimeras of mammalian and yeast ARF they showed residues 35–94 were essential for AP-1 recruitment to the Golgi. Because the reticulocyte lysate contains significant amounts of AP-1, COPI, and AP-2 (data not shown), we removed the endogenous coat proteins from the lysate using high speed centrifugation and demonstrated that interaction of myrARF1 and AP-1 was dependent on the addition of exogenous AP-1. In addition, we could not detect an interaction of \(^{35}\)S]ARF-(Tmd)Phe-46 and AP-1 in solution, either in the cross-linking assay in the absence of ISGs or by co-sedimentation of ARF1 with AP-1 during the centrifugation.

The photocross-linking method is qualitative and does not allow a quantification of the stoichiometry between ARF and AP-1. Cross-link yields are typically <1%, because the reactive carbene predominantly interacts with the water molecules (19). The photoprobe is highly specific, because a protein of very close proximity would be required to compete with the abundant water molecules. Because the rabbit reticulocyte lysate used for the in vitro translation also contains ~1 μg/ml of unlabeled ARF proteins, as determined with the pan-ARF antibody, the specific activity of the \(^{35}\)S]ARF-(Tmd)Phe-46 is further reduced. We are unable to detect by immunoblotting, with antibodies to AP-1 or COPI, the population of coat proteins that were photo-cross-linked to myrARF1. Thus, we are unable to quantify the extent of the interaction between myrARF1 and AP-1 or COPI using the photocross-link approach.

Using the reticulocyte lysate, in the presence of GTPγS, we observed cross-linking of COPI to \(^{35}\)S]ARF-(Tmd)Phe-46, which was dependent upon membranes. The amount of β-COP cross-linked is at least as great as AP-1. β-COP is present, but not enriched, in the ISG fraction. Although Golgi contamination in the ISG fractions has been excluded with assays for trans-Golg network 38 (37), sialyltransferase assays and labeling with \(^{35}\)S]sulfate 38, endosomal membrane contaminants maybe present in the ISG fraction (39), which may explain the cross-linking of γ- and β-COP. Alternatively, the observations of Martínez-Menárguez et al. (40), showing low but significant labeling of ISGs with COPI in the exocrine pancreas by immunoelectron microscopy, suggest that COPI binding sites may be present on ISG membranes. However, clathrin coats were far more abundant than the COPI coats, suggesting that COPI-mediated transport is a minor pathway and may be a remnant of the retrograde machinery from the Golgi complex.

Interaction of both AP-1 and COPI with the switch 1 domain of ARF1 suggests that common structural features exist between these multisubunit coat protein complexes. A phylogenetic analysis by Schledzewski et al. (41) provides evidence that the clathrin adaptors and F-COP (a subcomplex of COPI containing the subunits β, γ, δ, and ζ) have arisen from coordinated gene duplications of common ancestral genes and predicts that β-COP is homologous to β-adaptin and γ-COP to γ-adaptin. The structural similarities between the adaptors and COPI could explain the cross-linking data of Zhao et al. (20) showing cross-linking of \(^{35}\)S]ARF-(Tmd)Phe-46 to β- and γ-COP on Golgi membranes and the results presented here.

How can the switch 1 domain of ARF1 interact with two subunits of AP-1 or COPI? There is increasing evidence to suggest that ARF oligomerizes; crystals of ARF-GDP were found in a dimeric form (42, 43), and cross-linking of ARF with itself has been detected using \(^{35}\)S]ARF-(Tmd)Phe-49 (20). Furthermore, others have determined that there are multiple copies of membrane associated ARF-GTP per cotumer (15, 45, 46). Thus, it is possible that dimers of myrARF1 exist, where the switch I region of one molecule of ARF interacts with the γ- or β-subunit of one AP-1 or COPI and the other ARF molecule with the other large subunit.

ISG maturation involves homotypic fusion and changes in the size and dense core (47) of the secretory granule. Unlike MSgs, which lack a clathrin coat, ISGs are partially coated vesicles (48, 49), with AP-1 containing clathrin coats (50). Clathrin coats have been seen enveloping small vesicular structures on ISGs (49), leading to the hypothesis that clathrin-coated vesicles serve to remove components of the secretory granule. We show that myrARF1 does not bind to MSGs and, using photocross-link, that AP-1 and myrARF1 do not interact on the MSG, suggesting that there are proteins on the ISG, required for myrARF1 binding and subsequent AP-1/ARF1 interaction, which cannot be supplied by the reticulocyte lysate or bovine brain cytosol. These proteins may be transmembrane proteins such as mannose-6-phosphate receptor or furin, both of which are present on ISGs but absent from MSGs (37, 39). In support of this others have shown that mannose-6-phosphate receptors can stabilize ARF-1 and ARF1 interaction on trans-Golg network membranes (44), and a bivalent interaction of coatomer with membrane-bound ARF and cytoplasmic tails of cargo or putative cargo receptors has been demonstrated (45).

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