Site-Specific Cross-Linking Reveals a Differential Direct Interaction of Class 1, 2, and 3 ADP-Ribosylation Factors with Adaptor Protein Complexes 1 and 3

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ABSTRACT: We have used a site-specific photo-cross-linking approach to identify direct interactions between clathrin adaptor protein (AP)[1] complexes and small GTPases of the ADP-ribosylation factor (ARF) family and to explore the specificity of this interaction on immature secretory granule (ISG) membranes. ISG membranes are a well-characterized, highly enriched preparation of membranes that has previously been shown to have the membrane-associated factors for ARF1 recruitment that are not present on artificial liposomes. All three classes of ARF proteins could be recruited to ISG membranes, displaying differential requirements for GTPγS. We found that ARF1, ARF5, and ARF6 interacted directly with the β1-adaptin subunit of AP-1 in the presence of GTPγS. Furthermore, we observed a direct interaction between the switch 1 region of ARF1 and the N-terminal trunk domains of γ- and β1-adaptin. In addition, both ARF1 and ARF6 but not ARF5 interacted directly with the β3- and δ-adaptin subunits of AP-3. No interaction was observed between AP-2 and any of the ARF proteins. Our results delineate the specificity and provide evidence of a direct interaction between different ARF proteins and the AP complexes AP-1 and AP-3 on natural ISG membranes and show that residues in the switch 1 region of ARF proteins can selectively bind to the trunk domains of these complexes.

Vesicle formation in cells is driven by coat complexes recruited from the cytosol which deform the compartmental membrane into a vesicle. The assembly of coats on cellular membranes occurs through multiple protein−protein interactions between the coat subunits, membrane-associated proteins, and the cargo proteins (*1*). It is likely that coat assembly on distinct membranes arises from the contributions of all three components; however, the precise molecular details dictating the restricted and specific assembly of coats are not known. Several families of coats exist which assemble selectively on different intracellular compartments. Clathrin coats, composed of an inner layer of adaptors and an outer layer of clathrin, form on Golgi membranes, on immature secretory granules (ISGs),[1] on endosomes, and at the plasma membrane. While clathrin plays a structural role in the coat, the adaptors recognize and bind motifs in cargo molecules (*2*). Adaptor proteins belong either to the heterotetrameric adaptor protein (AP) complex family (AP-1 through AP-4) or to the more diverse group of monomeric proteins including β-arrestin and the Golgi-localizing, γ-adaptin ear homology domain, ADP-ribosylation factor (ARF) binding protein (GGA) family (GGA1 through GGA3) (for review see refs *3* and *4*).

Clathrin coat assembly on both cellular membranes and liposomes requires proteins from the ARF family of small GTPases. The six highly related mammalian ARF proteins are grouped into three classes on the basis of size and sequence homology (*5*). ARF1, ARF2, and ARF3 are members of class I, ARF4 and ARF5 belong to class II, and ARF6 is in class III. ARF1 recruitment is an early event in coat formation and is catalyzed by the membrane-bound guanine nucleotide exchange factor (GEF), resulting in conversion of the cytosolic GDP-bound form of ARF1 to the membrane-associated GTP form (*6*). The exchange of GDP for GTP results in a structural change in the orientation of the switch 1 (amino acids 45−54) and switch 2 (amino acids 70−80) regions of ARF1, resulting in exposure of the N-terminal myristic acid and increased association with membranes (*7*). These switch regions have been shown to bind effectors such as COPI (*8*), the GGAs (*9*), and AP-4 (*30*). Recent data suggest that class I and class II ARFs (ARF1 and ARF5) can both interact with AP-1 on Golgi membranes (*10*, *11*). In addition, a direct interaction between ARF1-GTP and the large subunits, γ- and β1-adaptin, of AP-1 has been demonstrated on immature secretory granule membranes (ISG) using site-specific cross-linking (*12*). Furthermore, ARF1-GTP is known to be required for the recruitment of AP-3 (*11*, *13*), and ARF5-GTP has been shown to be active in the recruitment of AP-1 and AP-3 (*14*).

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[1] Abbreviations: AP, adaptor protein; ARF, ADP-ribosylation factor; ISG, immature secretory granule; GEF, guanine nucleotide exchange factor; GGA, Golgi-localizing, γ-adaptin ear homology domain, ADP-ribosylation factor binding proteins; (Tmd)Phe, L-4′-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenylalanine; STI, soybean trypsin inhibitor.

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To gain information about the potential interactions of all three classes of ARF proteins and AP complexes and thereby increase our knowledge of how coats are initially formed on membranes, we used a photo-cross-linking approach (*8*). This approach identifies direct protein−protein interactions, in our case between ARF and the APs, only occurring in the environment of a bona fide intracellular membrane and not in solution or on liposomes (*12*). A photoactivatable analogue of phenylalanine was introduced into the switch 1 domain of ARF1, ARF5, and ARF6 and used to cross-link ARF-interacting molecules on ISG membranes. We then looked for interactions of ARF with AP-1 and AP-3 by specific immunoprecipitation with the relevant antibodies. We have used the ISG membrane as the acceptor membrane as the ISGs are a highly enriched, homogeneous membrane fraction to which ARF1-GTP can be recruited (*12*). In addition, the availability of the cross-linking technology has allowed us to re-explore the interaction of ARF1-GTP with AP-1 to map the region of the large subunits (γ- and β1-adaptin) that interacts with ARF1-GTP.

Using controlled proteolysis with trypsin, we show that the N-terminal domain of AP-1 β1-adaptin directly interacts with the switch 1 domain of ARF1-GTP. We demonstrate that, like class I ARF1, class II ARF5 and class III ARF6 can be recruited to ISG membranes. Using antibodies specific for AP-1, we show that while both γ-adaptin and β1-adaptin subunits of AP-1 interact efficiently with ARF1-GTP, only β1-adaptin interacts efficiently with ARF5-GTP and ARF6-GTP after recruitment to ISG membranes. Furthermore, AP-3 exhibits a similar degree of preference as AP-1 for ARF1. ARF1-GTP and, to a lesser extent, ARF6-GTP, bind directly to the δ and β3 subunits of AP-3. However, no interaction between ARF5-GTP and AP-3 was detected. These experiments provide insight into the direct interactions between the different classes of ARF proteins and the APs and contribute to our knowledge about how clathrin coats assemble on membranes.

EXPERIMENTAL PROCEDURES

Antibodies. Antibodies specific for the trunk of β1-adaptin were obtained from Transduction Laboratories and Sigma (100/1) and as a gift from Dr. T. Kirchhausen (Harvard University, Cambridge, MA). Antibodies specific for the γ-adaptin hinge were obtained from Sigma (100/3) or as previously described (STO25; *15*); antibodies specific for the γ-adaptin trunk were generated using a recombinant GST fusion protein encoding amino acids 112−564 of mouse γ1-adaptin (STO160) or were a gift from Dr. M. Robinson (Cambridge University, U.K.). An antibody specific for α-adaptin (100/2) was obtained from Sigma. The anti-δ-adaptin antibody was produced in rabbits immunized with a GST fusion protein containing amino acids 752−839 of δ-adaptin (*13*). The anti-σ3 antibody was produced in rabbits against a peptide (residues 166−180 of σ3B) (*16*). An additional antibody specific for δ-adaptin was also obtained from Dr. M. Robinson. Immunoprecipitations under native and denaturing conditions were performed as described (*12*).

Preparation of ISGs, Purified AP-1, and Bovine Adrenal Medulla Cytosol. ISGs were prepared from PC12 cells as previously described (*15*). Purified AP-1 and cytosol was prepared from bovine adrenal medulla as described (*12*). ARF-depleted cytosol was prepared by gel filtration (*17*) of bovine adrenal medulla cytosol using a Bio-Gel P60 equilibrated in binding buffer (25 mM HEPES−KOH, 25 mM KCl, 25 mM MgOAc, pH 7.2). Trypsin digestion was performed with TLCK-treated trypsin (Sigma) diluted in binding buffer for 10 min at 37 °C before the addition of soybean trypsin inhibitor (STI) using trypsin-to-protein ratios from 1:30 to 1:3.

Site-Directed Mutagenesis and in Vitro Transcription. The cDNAs for mouse ARF5 and human ARF6 tagged at their C-terminus with HA and polyhistidine, respectively, were obtained from Dr. S. Cockcroft (University College London, U.K.). Site-directed mutagenesis was performed using QuickChange according to the manufacturer's instructions (Stratagene, U.K.). Isoleucine 46 in the mouse ARF5 cDNA was mutated to phenylalanine using the primer 5′gagattgtcaccacctagcccactata3′ and its complement. Site-directed mutagenesis of the human ARF6 cDNA at isoleucine 42 to phenylalanine was done using the primer 5′cagtcggtgaccacctagcccactgtc3′ and its complement.

In vitro transcription of cDNAs encoding wild-type ARF1, ARF5-HA, and ARF6-His, mutated ARF1-Phe-46, ARF5-Phe-46, and ARF6-Phe-42 was performed with T7 RNA polymerase (Promega) (*8*, *18*).

Production of Supressor tRNA and in Vitro Translation. Supressor tRNA was produced by ligation of the supressor tRNA(−CA) with L-4′-(3-trifluoromethyl-3*H*-diazirin-3-yl)-phenylalanine, abbreviated (Tmd)Phe, to produce the amber supressor $tRNA^{(Tmd)Phe}$ (*8*, *18*). In vitro translation was performed using nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech) and analyzed as described (*12*). Before addition of the lysate to the binding assay, the lysate was spun for 15 min at 14000*g* at 4 °C.

Photo-Cross-Linking. Binding assays and photo-cross-linking assays were performed as described (*12*). Typically, 10 μL of the rabbit reticulocyte lysate containing the in vitro translated [^{35}S]methionine-labeled wild type or 25 μL of the lysate containing the mutated mRNAs was incubated with 125 μL of ISGs in the presence of 100 μM nucleotide [GTPγS or GDPβS (Roche)] at 37 °C for 30 min. The ISGs were isolated from the reaction by centrifugation at 100000*g* and exposed to UV light as described (*18*). After exposure to UV light the samples were either subjected to immunprecipitation or analyzed by either 7.5% or 15% SDS−PAGE and processed for autoradiography.

RESULTS

ARF1 Interacts with the Trunk Domains of both β1- and γ-Adaptin. Using the cross-linking assay previously described (*8*, *12*), which demonstrated that the β-COP subunit of COPI and both the β1 and γ subunits of AP-1 interacted directly with ARF1, we wanted to identify where ARF1 was binding to the AP-1 subunits. The four subunits of the heterotetrameric AP-1 are the large ~100 kDa subunits, γ-adaptin and β1-adaptin, μ1-adaptin (~50 kDa), and σ1-adaptin (~20 kDa). The other family members, AP-2, AP-3, and AP-4, have equivalent subunits: The γ-subunit equivalent in AP-2, AP-3, and AP-4 is α-, δ-, and ϵ-adaptin, respectively, the β, μ, and σ subunits are named according to the AP to which they belong (β2−β4, μ2−μ4, and σ2−σ4). The large

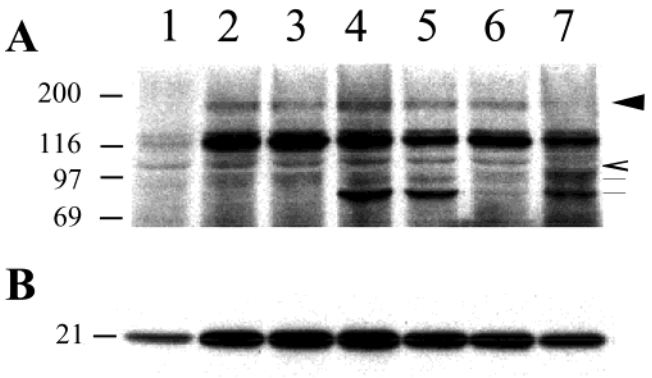

FIGURE 1: AP-1 core complexes are recruited to ISGs and directly interact with [^{35}S]ARF1-(Tmd)Phe-46. (A) A recruitment assay followed by cross-linking was performed with ISGs in the presence of GDPβS (lane 1) or GTPγS (lanes 2−7). Purified AP-1 or purified AP-1 incubated first with STI before the addition of 20 μg/mL trypsin was added to the assay before cross-linking (lanes 3 and 6, respectively). Purified AP-1 treated with 2 or 20 μg/mL trypsin, followed by STI, was added to the assay (lanes 4 and 5, respectively) before cross-linking. In lane 7, the recticulocyte lysate was treated with 20 μg/mL trypsin, followed by STI, after in vitro translation but before cross-linking. The proteolytic AP-1 products (80 and 90 kDa) cross-linked to ARF1-GTP are marked (−), a 100 kDa product detected in lane 7 is marked (<), and the arrowhead marks the 180 kDa cross-linked product. (B) Analysis of one-tenth of the total cross-linking reaction from panel A showing the [^{35}S]-ARF1-(Tmd)Phe-46 bound, but not cross-linked, to ISGs. SDS−PAGE was performed on (A) 7.5% or (B) 15% SDS−PAGE followed by autoradiography. The autoradiographs were exposed overnight on Biomax film (Kodak, U.K.).

subunits have three domains, an N-terminal trunk (comprising about two-thirds of the molecule), a short flexible hinge domain, and a C-terminal ear domain. Using chimeras of the large AP-1 and AP-2 subunits it has been shown that the N-terminal domain of AP-1 contains targeting information for recruitment to Golgi membranes (*19*). Experiments performed with AP-1 using trypsin to remove the appendages, i.e., the ear and the hinge domains of γ- and β1-AP, have demonstrated that the core complex (consisting of the small and medium subunits and the trunk of the large subunits) can be recruited to trans-Golgi membranes (*20*).

We exploited the ability of trypsin to cleave the large adaptor subunits in the flexible hinge domain located between the trunk and the head of both β1- and γ-adaptin. Controlled proteolysis of the heterotetrameric complex with trypsin liberates the appendages of β1- and γ-adaptin, reported to have molecular masses of approximately 40 and 32 kDa, respectively, leaving the core containing the trunk of β1- and γ-adaptin, as well as the intact μ1 and σ1 subunits. The trunk domain of the β1- and γ-adaptin subunits have molecular masses of approximately 60 and 70 kDa, respectively (*21*). We have confirmed that products of this size are produced in our assay conditions using either purified bovine AP-1, PC12 cytosol, or rabbit reticulocyte lysate as sources of AP-1 (data not shown).

To determine if the AP-1 core complex can be recruited to ISG membranes and bind ARF1, we supplemented the cross-linking assay with purified AP-1 either untreated (*12*) or treated with trypsin, followed by soybean trypsin inhibitor (STI). A set of cross-linked products with a molecular mass in the range of 120 kDa (Figure 1, lane 2) appears after untreated adaptors (recruited from both the reticulocyte lysate and the exogenous purified AP-1) on ISGs are cross-linked to in vitro translated [^{35}S]ARF1-(Tmd)Phe-46 in the presence of GTPγS. An additional band is detected at 180 kDa (Figure 1, lanes 2−6). Although addition of untrypsinized purified AP-1 before cross-linking does not cause a substantial increase in the intensity of the 120 kDa cross-linked products (see Figure 1, lane 3), immunoprecipitation with anti-γ-adaptin antibodies had previously revealed that an increased amount of AP-1 can be detected cross-linked to ARF1 (*12*). Addition of purified AP-1 which was preincubated with either 2 or 20 μg/mL trypsin for 10 min at 37 °C, followed by STI, resulted in the appearance of new cross-linked products at approximately 80 kDa and a minor band at 90 kDa (Figure 1A, lanes 4 and 5). As expected, AP-1 complexes incubated with STI before trypsin treatment gave results identical to those of the untreated AP-1 (Figure 1A, compare lanes 3 and 6). The recruitment of [^{35}S]ARF1-(Tmd)Phe-46 to ISGs was GTPγS dependent and unaffected by the addition of purified AP-1, trypsin-treated AP-1, or STI (Figure 1B, lanes 1−6). Furthermore, we tested if the endogenous AP-1 present in the rabbit reticulocyte lysate could also be recruited and cross-linked to ARF1 after trypsin treatment. To generate AP-1 core fragments, the rabbit reticulocyte lysate, after the in vitro translation reaction of ARF1-(Tmd)Phe-46, was treated with 20 μg/mL trypsin, followed by STI, prior to cross-linking. The trypsin treatment decreased slightly the amount of [^{35}S]ARF1-(Tmd)Phe-46 bound to the membranes (Figure 1B, lane 7), but the amount remaining is still active and sufficient to observe cross-linking. Pretreatment of the lysate with trypsin, followed by STI, resulted in the appearance of the cross-linked products at 80 and 90 kDa, as described above, and a new product at approximately 100 kDa (Figure 1A, lane 7). The appearance of the 100 kDa band correlates with the disappearance of the 180 kDa band and may represent the proteolytic product of other coat complexes present in the reticulocyte lysate, such as COPI (*12*), AP-3 (see below), or AP-4.

A complementary experiment was performed by trypsinization of the whole reaction, containing ISGs, [^{35}S]ARF1-(Tmd)Phe-46, rabbit reticulocyte lysate, and GTPγS, after cross-linking, with 10 μg/mL trypsin for 10 min at 37 °C followed by STI (Figure 2). Trypsin treatment of the reaction resulted in almost complete disappearance of the group of 120 kDa cross-linked products (Figure 2A, lane 1) and appearance of a set of cross-linked bands of molecular masses 80 and 90 kDa (Figure 2A, lane 3), again with the 90 kDa band being the minor band. Treatment with lower amounts of trypsin (1 μg/mL) under the same conditions results in the partial decrease in the intensity of the 120 kDa bands and the appearance of the 80 and 90 kDa bands (Figure 2A, lane 2). No smaller cross-linked products were detected even after prolonged exposure. These results are consistent with those shown in Figure 1 and strongly suggest that the trunk of the large subunits interacts with ARF1-GTP.

To confirm that the 80 and 90 kDa bands appearing after trypsinization were the large subunits derived from the AP-1 core complex, we performed immunoprecipitations with trunk-specific antibodies for the γ- and β1-adaptins (Figure 2B). Samples were treated identically as in Figure 2A, solubilized under denaturing conditions, and used for immunoprecipitation. Without trypsin treatment antibodies specific for γ-adaptin and β1-adaptin immunoprecipitated

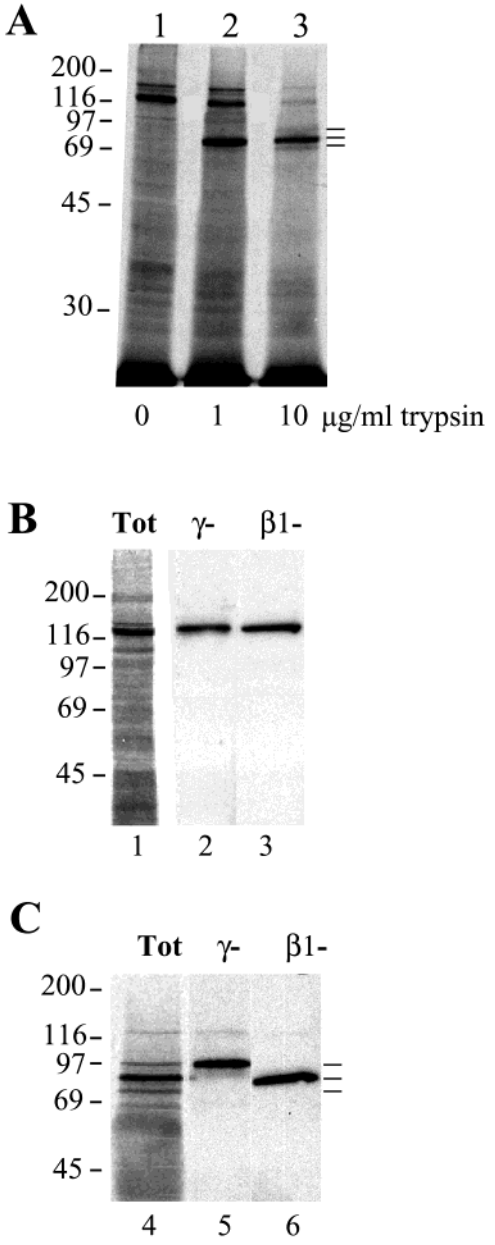

FIGURE 2: A trypsin-resistant core of AP-1 is cross-linked to ARF1-GTP via γ- and β1-adaptin. (A) A recruitment assay followed by cross-linking was treated with either no trypsin (lane 1), 1 μg/mL trypsin (lane 2), or 10 μg/mL trypsin (lane 3). After addition of STI the reactions were subjected to SDS−PAGE and autoradiography. The positions of the three bands that appear after trypsin digestion are marked. The autoradiograph was exposed to XAR film (Kodak, U.K.) for 1 week. (B) Untreated recruitment and cross-linking assays or (C) trypsin-treated recruitment and cross-linking assays were solubilized under denaturing conditions. One aliquot was solubilized and analyzed directly (Tot, lanes 1 and 4) while the remainder was subjected to immunoprecipitation with antibodies specific for the N-terminal domain of γ-adaptin (lanes 2 and 5) or β1-adaptin (lanes 3 and 6). Analysis was performed by 7.5% SDS−PAGE followed by autoradiography. Lanes 1 and 4 were exposed to XAR film overnight, while lanes 2, 3, 5, and 6 were exposed for 3 days. The films were scanned at the same settings for all exposures.

the 120 kDa cross-linked proteins γ-adaptin and β1-adaptin, respectively (Figure 2B, lanes 2 and 3). In the trypsinized reactions, conversely, immunoprecipitation of a cross-linked product at 90 kDa and at a slightly lower molecular mass of approximately 80 kDa could be observed with anti-γ- and anti-β1-adaptin antibodies, respectively (Figure 2C, lanes 5 and 6). As seen above (Figure 2A, lane 3) and in the total sample (Figure 2C, lane 4) it is apparent that the amount of the 90 kDa band, which is γ-adaptin cross-linked to ARF1, in the total cross-linked samples was much less than the 80 kDa band (β1-adaptin cross-linked to ARF1). The different intensities of the two bands are consistent with the ability of β1-adaptin to be cleaved more readily by trypsin than γ-adaptin (*21*). During the trypsin digestion it is also likely that the [^{35}S]ARF1-(Tmd)Phe-46 is cleaved. At high trypsin concentrations ARF cleavage may occur before cleavage of the γ-adaptin hinge, and this might explain why no full-length γ-adaptin cross-linked to ARF1 is detected in Figure 2, lane 3. The identity of the band at approximately 75 kDa has not been investigated.

It has previously been shown that the COPI subunits are also cross-linked to ARF1, and the β- and γ-COP subunits cross-linked to ARF1 have a molecular mass in the region of 120 kDa (*12*). We have found that trypsin treatment of purified COPI using the conditions described above results in the complete digestion of the β- and γ-COPI subunits (data not shown). Thus, the trunk of the AP-1 large subunits, which are resistant to further digestion with trypsin either before or after cross-linking, gives rise to the bands at 80 and 90 kDa. We have observed that the AP-1 core complex can be recruited to ISG membranes and undergoes a direct interaction with ARF1-GTP. Furthermore, recruitment and cross-linking can occur with a partially digested heterotetramer consisting of the trunk of β1-adaptin and intact γ, μ1, and σ1 subunits or a core complex consisting of the trunk of both β1- and γ-adaptin, intact μ1, and σ1 subunits. The appendages, comprising the hinge and C-terminal ears, are not required for recruitment to the ISG membrane or for interaction with ARF1-GTP. We could not detect any cross-linking product which would correspond to either the γ or β1 appendage cross-linked to ARF1 in experiments performed with preparations of trypsinized purified AP-1, which contain both the core complex and the free appendages followed by immunoprecipitation with hinge-specific antibodies (data not shown). Consistent with this finding, we have found that the appendage domain of γ-adaptin (the hinge/ear domains, residues 590−825 of mouse γ1-adaptin) expressed as a GST fusion protein does not cross-link to ARF1-GTP and does not compete for cross-linking of the intact AP-1 (data not shown).

ARF1 Interacts with AP-3 via Ile-46 in the Switch 1 Region. In cross-linking experiments containing [^{35}S]ARF1-(Tmd)Phe-46, ISGs, and GTPγS we reproducibly detected a faint cross-linked product at 180 kDa (see Figure 1, lane 2). Addition of increasing amounts of bovine adrenal medulla cytosol, previously depleted of endogenous ARFs using gel filtration, increased the intensity of the cross-linked 180 kDa band (Figure 3A, lanes 4−6). Three additional bands with a molecular mass between 100 and 70 kDa were also observed, but the appearance of these bands was not dependent on the addition of GTP (Figure 3A, compare lanes 3 and 6). As found for the cross-linked coat components at 120 kDa, the appearance of the 180 kDa band was dependent on the presence of GTPγS (Figure 3A, compare lanes 3 and 5) and UV light (data not shown). There was no detectable change in the amount of 120 kDa cross-linked bands with increasing cytosol in the presence of GTPγS. As expected, the amount

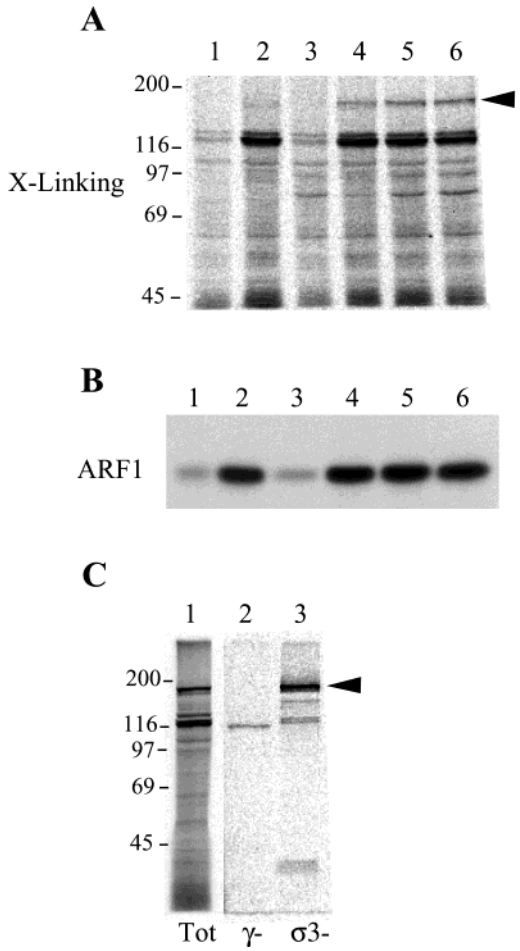

FIGURE 3: AP-3 interacts directly with ARF1-GTP. (A) A recruitment assay was performed with [^{35}S]ARF1-(Tmd)Phe-46 and ISGs in the presence of GDPβS (lanes 1 and 3) or GTPγS (lanes 2, and 4−6). 200 μg (lane 4), 500 μg (lanes 3 and 5), or 700 μg (lane 6) of ARF-depleted bovine adrenal medulla cytosol was added to the reaction during the recruitment, prior to cross-linking. The arrowhead indicates the 180 kDa band which is increased upon addition of cytosol. (B) Analysis of one-tenth of the cross-linking reaction showing the un-cross-linked [^{35}S]ARF1-(Tmd)Phe-46 bound to ISGs under conditions listed in panel A. (C) Immunoprecipitation of the total cross-linked reaction done with 200 μg of ARF-depleted cytosol (lane 1) with antibodies to γ-adaptin under denaturing conditions (lane 2) and σ3-adaptin under native conditions (lane 3). Analysis for (A) and (C) was performed by 7.5% SDS−PAGE and for (B) with 15% SDS−PAGE, followed by autoradiography. Panels A and B were exposed overnight; panel C, lane 1, was exposed for 1 week, and lanes 2 and 3 were exposed for 3 weeks and scanned as in Figure 2.

of [^{35}S]ARF1-(Tmd)Phe-46 bound to the ISGs membranes was also unaffected with increasing cytosol (Figure 3B, compare lane 2 with lanes 4, 5, and 6), confirming that the cytosol had been efficiently depleted of ARFs which, if present, would have competed for the binding of [^{35}S]ARF1-(Tmd)Phe-46 (*12*).

A likely candidate for the 180 kDa cross-linked product is the large δ subunit of AP-3 which has a molecular mass of 160 kDa and is the homologue of the γ subunit. It is also possible that β3-adaptin cross-linked to ARF1 is present within the 120 kDa region but cannot be identified in the total sample because of the complexity of the cross-linked products in that region. The potential direct interaction of ARF1 and AP-3 was investigated using cross-linking followed by immunoprecipitation with reagents specific for AP-3. We tested two polyclonal antibodies specific for δ-adaptin but were unable to observe a specific immunoprecipitation of the 180 kDa band. This could be attributed to a lower cross-reactivity of the antibodies with the bovine and rabbit protein. However, under native immunoprecipitation conditions using an antibody specific for the σ3 subunit, the homologue of the σ1 subunit of AP-1, we were able to immunoprecipitate the 180 kDa cross-linked band (Figure 3C, lane 3). In fact, the native immunoprecipitation with antibodies to the σ3 subunit resulted in the precipitation of the 180 kDa band and a cross-linked doublet with a molecular mass of approximately 120 kDa. The 120 kDa species migrates slighty slower than the 120 kDa cross-linked γ subunit (Figure 3C, lane 2) and may be the β3 subunit isoforms, β3A- and β3B-adaptin (*16*, *22*). The increase in the cross-linking signal obtained after the addition of cytosol may indicate that AP-3 is not as abundant in rabbit reticulocyte lysate compared to bovine adrenal medulla cytosol.

ARF5 and ARF6 Can Also Be Cross-Linked to AP-1. Among the six different members of the ARF family there is a high sequence conservation, suggesting that the different ARFs have the same overall structure (*5*). This is supported by the recent structural analysis of ARF1 and ARF6. Both ARF1 and ARF6, members of the class I and class III ARFs, respectively, have, in their GTP-bound form, a conserved structure in the switch region. We, therefore, examined the ability of ARF5 and ARF6 to be recruited to the ISG membrane and, if bound, their potential to directly interact with the clathrin coat adaptors AP-1, AP-2, and AP-3. Liang and colleagues have shown that recombinant purified preparations of all three classes of ARFs (ARF1, ARF5, and ARF6) can be recruited to isolated Golgi membranes. Once bound to isolated Golgi membranes ARF1, ARF5, and ARF6 could recruit both AP-1 and coatomer (*14*). Other lines of evidence support the notion that ARF1-GTP is required for AP-1 recruitment to Golgi membranes and ISGs both in vivo and in vitro (*12*, *23*), while AP-3 recruitment to Golgi membranes was shown to require purified recombinant myristoylated ARF1- and ARF5-GTP (*11*).

To perform cross-linking experiments on the surface of the ISGs, we had to first determine if the in vitro translated ARF5 and ARF6 could bind to ISG membranes and, if so, modify the ARF5 and ARF6 cDNAs to allow for the introduction of the cross-linker. We tested the binding of wild-type in vitro translated [^{35}S]Met-labeled ARF5 and ARF6 to ISG membranes and used in vitro translated [^{35}S]-Met-labeled ARF1 as a control (Figure 4). ARF5 and ARF6 were both able to bind to ISG membranes in a GTPγS-dependent manner; however, the binding of both ARF5 and ARF6 was lower than that of ARF1. The binding of ARF1, ARF5, and ARF6 was enhanced in the presence of GTPγS compared to GDPβS by 3.2-, 1.8-, and 2.6-fold, respectively. As ARF5 and ARF6 were able to bind to ISGs, we modified their cDNAs to enable use of the cross-linking technology. Using the wild-type ARF5 and ARF6 cDNAs, a point mutation was introduced into the codon for the isoleucine residue in ARF5 (Ile-46) and ARF6 (Ile-42) corresponding to isoleucine 46 found in the switch 1 domain of ARF1 to create an amber stop codon (*18*). Addition of the modified supressor tRNA, ligated to (Tmd)Phe, to the translation mixture allows supression of the amber stop codon and

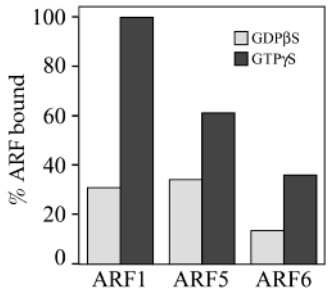

FIGURE 4: Wild-type ARF5 and ARF6 bind to ISGs. cDNA constructs for ARF1, ARF5, and ARF6 were in vitro transcribed and translated in a reticulocyte lysate. Recruitment of the wild-type ARFs from 10 μL of lysate to 125 μL of ISG was performed in the presence of GDPβS (light bars) or GTPγS (heavy bars). The amount of ARFs bound to the membranes was assayed after centrifugation of the ISGs, followed by 15% SDS−PAGE, and quantified using a Molecular Dynamics phosphorimager. The amount of ARF5 and ARF6 bound to ISGs was normalized to the amount of ARF1-GTP bound to allow averaging of two independent experiments performed in duplicate. The binding of ARF5 and ARF6 was then expressed as percent maximum of ARF1-GTP. The amount of ARF1-GTP, ARF5-GTP, and ARF6-GTP pelleting nonspecifically in the absence of ISG membranes was 23%, 7%, and 10%, respectively, of the ARF1-GTP in the presence of ISGs.

production of full-length ARF5 and ARF6 containing (Tmd)Phe at positions Ile-46 and Ile-42, respectively. We observed efficient translation of the mutated mRNA for ARF5 and ARF6 in the presence of [^{35}S]methionine and the modified supressor tRNA (Tmd)Phe (see Figure 5, lanes 1).

We performed recruitment and cross-linking assays on the ISGs after in vitro translation of the mutated mRNAs for ARF5 and ARF6 and compared the results to mutated ARF1. [^{35}S]ARF5-(Tmd)Phe-46 and [^{35}S]ARF6-(Tmd)Phe-42 bound to ISG membranes in a GTP-dependent fashion (Figure 5B,C, compare lanes 3 and 4) and remained bound after exposure to UV light (Figure 5B,C, lane 5). Like ARF1, low amounts of ARF5 and ARF6 sedimented in the absence of ISGs (Figure 5B,C, lane 2), and little ARF5 and ARF6 were detected on ISGs in the presence of GDPβS (Figure 5B,C, lane 3). The cross-linking products were analyzed and revealed differences in the 120−140 kDa region (Figure 6). In particular, the ratio of the 120 and 140 kDa bands initially seen cross-linked to ARF1 (Figure 6A, lane 4) was altered. [^{35}S]ARF5-(Tmd)Phe-46 was cross-linked with equal efficiency to the 140 and 120 kDa bands (Figure 6B, lane 4), whereas the 140 kDa band did not appear to be cross-linked efficiently by [^{35}S]ARF6-(Tmd)Phe-42 (Figure 6C, lane 4) compared to the 120 kDa band. The 140 kDa band most likely corresponds to γ-COP (*12*). In addition, the high molecular mass cross-linked band at 180 kDa seen with ARF1 (Figure 6A, lane 4) was detected after cross-linking with ARF6 (Figure 6C, lane 4) but was absent from the ARF5 reactions (Figure 6B, lane 4). All of the ARF mutants displayed the same behavior under control conditions; i.e., little cross-linking was observed in the absence of membranes (Figure 6, lane 1), reduced amounts in the presence of GDPβS (Figure 6, lane 2), and with GTPγS without exposure to UV light (Figure 6, lane 3). As seen for the wild-type ARF5 (Figure 4), [^{35}S]ARF5-(Tmd)Phe-46 recruitment, and subsequent cross-linking, was less dependent on GTP than that of ARF1 and ARF6 (compare Figure 6B, lanes 2 and 4, with Figure 6A,C, lanes 2 and 4).

After recruitment of ARF1, ARF5, and ARF6 to ISGs, followed by exposure to UV light, immunoprecipitation experiments were performed with antibodies specific to the AP-1, AP-2, and AP-3 complexes. Both AP-1 (*12*) and AP-3 (Figure 3) have been shown to interact with [^{35}S]ARF1-(Tmd)Phe46. As shown in Figure 6, under denaturing conditions antibodies to β1-adaptin could immunoprecipitate β1-adaptin labeled by the [^{35}S]methionine photoactivated ARF1, ARF5, and ARF6. However, γ-adaptin, which was cross-linked to ARF1 as expected, interacted only weakly with ARF5 and ARF6 (Figure 6, lanes 5). The 180 kDa band cross-linked to ARF1 and ARF6 in Figure 6A,C, likely to be δ-adaptin, was immunoprecipitated with anti-σ3 antibodies (Figure 7), confirming the interaction of AP-3 with ARF1 and showing that AP-3 can interact with both ARF1-GTP and ARF6-GTP. Similar to the results obtained with AP-3-ARF1-GTP cross-linking reactions, immunoprecipitation of the AP-3-ARF6-GTP cross-linked product revealed that the β3 isoforms were labeled. Finally, no AP-2 could be detected by immunoprecipitation with anti-α-adaptin after cross-linking in the presence of [^{35}S](Tmd)Phe ARF1, ARF5, or ARF6 (data not shown).

DISCUSSION

Past studies have documented the subcellular localization of ARF (*6*) and clathrin AP (*24*) family members. However, there is a lack of available information about the specificity of the interactions between the ARFs and the APs which

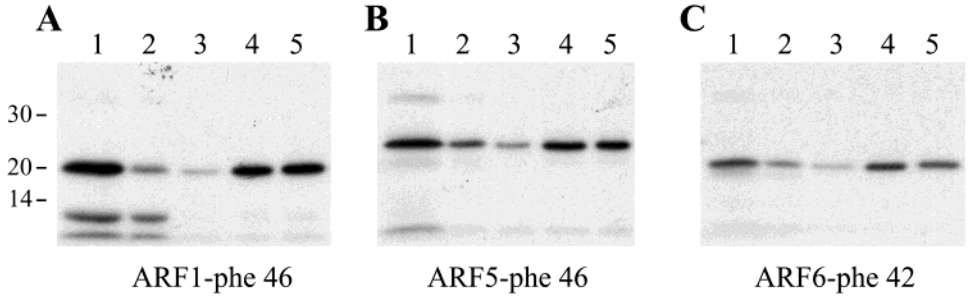

FIGURE 5: Expression and membrane recruitment of ARF1-(Tmd)Phe-46, ARF5-(Tmd)Phe-46, and ARF6-(Tmd)Phe-42 to ISGs. In vitro translation of amber stop codon mutants of ARF1-Phe-46, ARF5-Phe-46, and ARF5-Phe-42 in the presence of [^{35}S]methionine and supressor tRNA-(Tmd)Phe results in the production of [^{35}S]ARF1-(Tmd)Phe-46, [^{35}S]ARF5-(Tmd)Phe-46, and [^{35}S]ARF6-(Tmd)Phe-42 (panels A, B, and C, respectively, lane 1). 20% of the amount added to the recruitment reactions used in lanes 2−5 was loaded in lane 1. Recruitment to ISGs was performed in the presence of GDPβS (lane 3) or GTPγS (lane 4 and 5). Lane 2: recombinant ARFs-(Tmd)Phe and GTPγS but no ISGs were added. The samples in lanes 2, 3, and 5 were then exposed to UV light. After cross-linking the total reaction mixtures containing the ^{35}S-labeled ARFs were analyzed by 15% SDS−PAGE followed by autoradiography and exposed to Biomax film overnight.

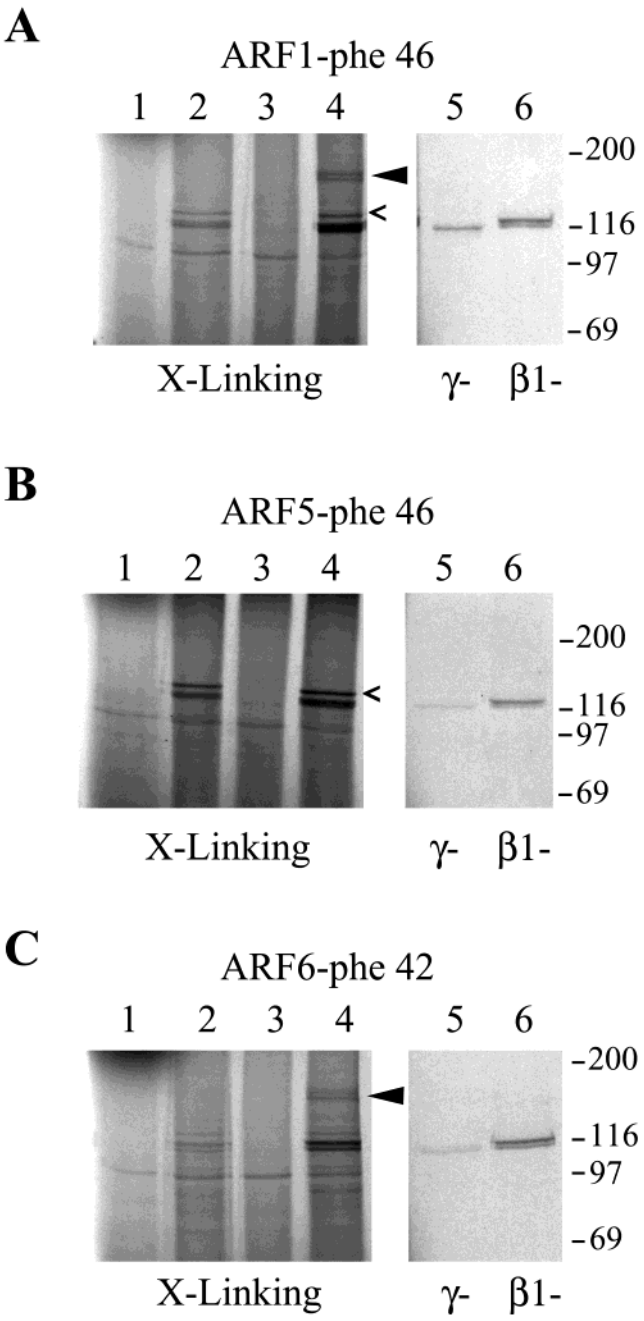

FIGURE 6: ARF1, ARF5, and ARF6 interact with β1-adaptin. Recruitment to ISGs was performed in the presence of GDPβS (lane 2) or GTPγS (lanes 3−6), followed by cross-linking (lanes 2 and 4−6) with [^{35}S]ARF1-(Tmd)Phe-46, [^{35}S]ARF5-(Tmd)Phe-46, and [^{35}S]ARF6-(Tmd)Phe-42 (panels A, B and C, respectively). No ISGs were added to the reaction in lane 1, and the samples in lane 3 were not exposed to UV light. After recruitment in the presence of GTPγS and exposure to UV light, samples identical to those in lane 4 were solubilized under denaturing conditions and subjected to immunoprecipitation with antibodies to γ-adaptin (lane 5) and β1-adaptin (lane 6). The positions of the 140 kDa product (<, panels A and B) and the 180 kDa product (panels A and C, arrowhead) are shown. Total samples or samples after immunoprecipitation were subjected to 7.5% SDS−PAGE followed by autoradiography. All panels were obtained from XAR film exposed to the autoradiograph for 4 weeks.

are required for coat formation on membranes. Here, we have exploited the site-specific photo-cross-linking approach (*8*) to probe for direct interactions of AP-1 and AP-3 with ARF family members on membranes. Previously, we showed a direct interaction between AP-1 and ARF1-GTP on ISG membranes (*12*). Both artificial liposomes and mature secretory granules, which form from the ISGs and do not have a clathrin coat, did not enable cross-linking of ARF1-GTP and AP-1 in the photo-cross-linking assay. These results suggested that ISGs have unique factors to facilitate the ARF-AP interaction. Here, using ISGs, we show a direct interaction of ARF1 with the core complex of AP-1 and intact AP-3 and identify interactions between β1-adaptin and ARF5-GTP as well as ARF6-GTP. Similarly, we show that AP-3 directly interacts with ARF1-GTP as well as ARF6-GTP but not with ARF5-GTP on ISG membranes. The interaction of ARF1-GTP and ARF6-GTP with AP-3 via the δ- and β3-adaptin subunits supports the recent data demonstrating a close structural homology between ARF1-GTP and ARF6-GTP (*27*).

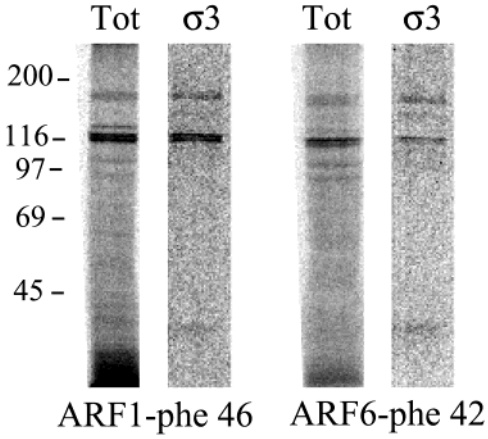

FIGURE 7: ARF1-GTP and ARF6-GTP directly interact with AP-3 subunits. Recruitment and cross-linking assays were performed as detailed in Figure 6 for samples in lane 4. After incubation and cross-linking, the samples containing ISGs, GTPγS, and either [^{35}S]-ARF1-(Tmd)Phe-46 or [^{35}S]ARF6-(Tmd)Phe-42 were analyzed (Tot) or solubilized under native conditions, immunoprecipitated with antibodies to the σ3-adaptin, and analyzed by 7.5% SDS−PAGE followed by autoradiography. The total lanes were exposed to XAR film for 1 week and the immunoprecipitates for 3 weeks.

We have further explored the AP-1-ARF1 interaction using controlled proteolysis of the AP-1 complex. Previously, using Golgi membranes, the core complex of AP-1 was shown to be recruited in the presence of GTPγS (*20*). However, as these experiments did not investigate the direct interaction of the AP-1 core complex with ARF1-GTP, it was possible that unidentified membrane-associated factors, perhaps in addition to ARF1-GTP, were promoting recruitment of the core complex. These factors were proposed to be binding to the N-terminal domain of the γ subunit (*25*). We show here that it is indeed the N-terminal domain of the large subunits, γ- and β1-adaptin, in the AP-1 core complex that interacts directly with the switch 1 domain of ARF1-GTP. Future experiments using the cross-linked photolabel as a tag may provide a means of identifying the precise location in the trunk of the AP-1 subunits that interacts with ARF1-GTP.

Recruitment of ARF to membranes requires interactions with GEFs and lipids in the membrane (*6*). ARF6 has been extensively characterized and has been shown to be present on the plasma membrane and not on Golgi membranes (*26*). However, as previous observations have shown that ARF1, ARF5, and ARF6 can bind to Golgi membranes in vitro (*14*), we asked if different classes of ARF could interact with APs on ISG membranes. We first demonstrated that ARF1-GTP,

ARF5-GTP, and ARF6-GTP all bind to ISGs, suggesting the presence of factors on ISG membranes that are sufficient for recruitment. In confirmation of previous results (*26*), the GTP dependence of ARF5 membrane binding was less pronounced than that of ARF1 or ARF6. Hence, the same group of cross-linked products was detected at a similar efficiency with ARF5-GTP and ARF5-GDP while, in contrast, for both ARF1-GDP and ARF6-GDP a very low signal of cross-linked products was obtained. Previous results demonstrated that ARF5 in the presence of GTPγS but not GTP could interact with AP-3 (*11*) on Golgi membranes and liposomes. Although ARF5 was recruited to ISGs, we did not detect a direct interaction of ARF5 in the presence of GTPγS with AP-3 on ISG membranes. The discrepancy between these observations may have to do with the assay conditions used or differences between the recombinant proteins produced in bacteria and in a mammalian in vitro translation system.

The GAT region of GGA1, GGA2, and GGA3 interacts with ARF1 in two-hybrid assays, pull-down assays, and in vivo experiments (*9*, *28*, *29*). In fact, two-hybrid experiments have shown that amino acid residues 49 and 51 of ARF1, contained within the switch 1 domain, are required for interaction of ARF1-GTP with the GGA3-GAT domain (*9*). Surprisingly, we did not observe any cross-linked products in our assays which might represent GGA1, GGA2, or GGA3 cross-linked to ARF1, ARF5, or ARF6, the predicted size of which would be 100 kDa. There could be several explanations for this result. The amount of GGAs present in the reticulocyte lysate could be significantly lower than that of the APs, leading to the cross-linked signal being below detection or to quenching of the interaction of ARF1-GTP with the GGAs by an excess of AP and COP proteins. Alternatively, the ARFs bound to the ISG membranes may not stably recruit the GGAs to the membrane, and thus cross-linking cannot occur efficiently. In support of the latter possibility, GGAs have been shown to rapidly dissociate from isolated membranes (*4*). Further experiments are required to determine if the GGAs can be recruited to ISGs and interact with ARF1 on ISGs.

Our results, which show interactions between GTP-bound ARFs and APs, do not address the mechanism of ARF1, ARF5, and ARF6 recruitment to ISG membranes. This was done by Liang and Kornfeld, who found, using Golgi membranes, that in the presence of GTPγS most of the recombinant myristoylated ARF1 was a substrate for a Golgi-localized, BFA-sensitive GEF but that recombinant myristoylated ARF5 and ARF6, as well as a pool of ARF1, were not substrates for GEF and were probably associating with Golgi membranes by interaction with the lipid bilayer (*14*). Importantly, both types of interactions required myristoylation. Our results confirmed the ability of all classes of ARF to interact with membranes, in a reaction most likely to be dependent on both GEFs and lipids for ARF1-GTP and lipids for ARF5-GTP and ARF6-GTP. In addition, only myristoylated ARF1, ARF5, and ARF6 were recruited to ISGs (C. Austin, unpublished data).

No cross-linking was detected in the absence of membranes between APs, present in the reticulocyte lysate, and ARF1, ARF5, or ARF6 in the presence of GTPγS, demonstrating that membranes are absolutely required for ARF and AP interactions. While ARF1 is present on ISG membranes and functions to recruit AP-1 (*15*), it is not known if ARF5 or ARF6 are present on ISG membranes, and the physiological significance of the recruitment of ARF5 and ARF6 to ISGs is unclear at present. Nonetheless, our results have demonstrated that once ARF proteins are bound to ISGs, there is a degree of specificity, for which the molecular basis is not known. Future experiments will explore the specificity of these interactions using hybrids of the various ARFs proteins in site-specific cross-linking experiments.

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REFERENCES

1. Kirchhausen, T. (2000) *Nat. Rev. Mol. Cell Biol. 1*, 187−198.
2. Brodsky, F. M., Chen, C.-Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001) *Annu. Rev. Cell Dev. Biol. 17*, 517−568.
3. Kirchhausen, T. (2000) *Annu. Rev. Biochem. 69*, 699−727.
4. Robinson, M. S., and Bonifacino, J. S. (2001) *Curr. Opin. Cell Biol. 13*, 444−453.
5. Tsuchiya, M., Price, S., Tsai, S., Moss, J., and Vaughan, M. (1991) *J. Biol. Chem. 266*, 2772−2777.
6. Jackson, C. L., and Casanova, J. E. (2000) *Trends Cell Biol. 10*, 60−67.
7. Roth, M. G. (1999) *Cell 97*, 149−152.
8. Zhao, L., Helms, J. B., Brugger, B., Harter, C., Martoglio, B., Graf, R., Brunner, J., and Wieland, F. T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4418−4423.
9. Puertollano, R., Randazzo, P. A., Presley, J. F., Hartnell, L. M., and Bonifacino, J. S. (2001) *Cell 105*, 93−102.
10. Zhu, Y., Drake, M. T., and Kornfeld, S. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 5013−5018.
11. Drake, M. T., Zhu, Y., and Kornfeld, S. (2000) *Mol. Biol. Cell 11*, 3723−3736.
12. Austin, C., Hinners, I., and Tooze, S. A. (2000) *J. Biol. Chem. 275*, 21862−21869.
13. Ooi, C. E., Dell'Angelica, E. C., and Bonifacino, J. S. (1998) *J. Cell Biol. 142*, 391−402.
14. Liang, J. O., and Kornfeld, S. (1997) *J. Biol. Chem. 272*, 4141−4148.
15. Dittié, A. S., Hajibagheri, N., and Tooze, S. A. (1996) *J. Cell Biol. 132*, 523−536.
16. Dell'Angelica, E. C., Ohno, H., Ooi, C. E., Rabinovich, E., Roche, K. W., and Bonifacino, J. S. (1997) *EMBO J. 16*, 917−928.
17. Beckers, J., and Rothman, J. E. (1992) *Methods Enzymol. 219*, 5−12.
18. Fischer, K. D., Helms, J. B., Zhao, L., and Wieland, F. T. (2000) *Methods 20*, 455−464.
19. Page, L. J., and Robinson, M. S. (1995) *J. Cell Biol. 131*, 619−630.
20. Traub, L. M., Kornfeld, S., and Ungewickell, E. (1995) *J. Biol. Chem. 270*, 4933−4942.
21. Schroder, S., and Ungewickell, E. (1991) *J. Biol. Chem. 266*, 7910−7918.
22. Simpson, F., Peden, A. A., Christoupoulou, L., and Robinson, M. S. (1997) *J. Cell Biol. 137*, 835−845.
23. Liang, J. O., Sung, T. C., Morris, A. J., Frohman, M. A., and Kornfeld, S. (1997) *J. Biol. Chem. 272*, 33001−33008.

24. Kirchhausen, T. (1999) *Annu. Rev. Cell Dev. Biol. 15*, 705−732.
25. Le Borgne, R., and Hoflack, B. (1998) *Biochim. Biophys. Acta 1404*, 195−209.
26. Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) *J. Biol. Chem. 271*, 21767−21774.
27. Pasqualato, S., Menetrey, J., Franco, M., and Cherfils, J. (2001) *EMBO Rep. 2*, 234−238.
28. Boman, A. L., Zhang, C., Zhu, X., and Kahn, R. A. (2000) *Mol. Biol. Cell 11*, 1241−1255.
29. Dell'Angelica, E. C., Puertollano, R., Mullins, C., Aguilar, R. C., Vargas, J. D., Hartnell, L. M., and Bonifacino, J. S. (2000) *J. Cell Biol. 149*, 81−94.
30. Boehm M., Aguilar, R. C., and Bonifacino, J. S. (2001) *EMBO J. 20*, 6265−6276.

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