The Arf GAPs AGAP1 and AGAP2 distinguish between the adaptor protein complexes AP-1 and AP-3

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Summary

ADP ribosylation factors (Arf) regulate membrane trafficking at multiple intracellular sites by recruiting coat proteins to membranes. The site-specific regulation of Arf is thought to be mediated by regulatory proteins including the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Here, we test this hypothesis by comparing the site of action of the Arf GAP AGAP2 to the closely related AGAP1. AGAP1 has previously been found to associate with the adaptor protein complex AP-3 and regulate the function of AP-3 endosomes. We found that AGAP2 directly interacted with AP-1. AGAP2 colocalized with AP-1, transferrin receptor and Rab4 on endosomes. Overexpression of AGAP2 changed the intracellular distribution of AP-1 and promoted Rab4-dependent fast recycling of transferrin. Based on these results, we concluded that the closely related Arf GAPs, AGAP1 and AGAP2, distinguish between these related heterotetrameric adaptor protein complexes to specifically regulate AP-3 endosomes and AP-1 recycling endosomes.

Key words: ADP ribosylation factor, GTPase activating protein, Clathrin adaptor protein, Endocytosis, Transferrin

Introduction

ADP-ribosylation factors (Arfs) are members of the family of Ras-like GTP binding proteins. The Arfs are ubiquitously expressed in eukaryotic cells and highly conserved. Six mammalian Arfs have been identified. These are divided into three classes based on sequence homology: class I (Arf1, 2 and 3), class II (Arf4 and 5) and class III (Arf6) (Moss and Vaughan, 1998). The proteins were first identified and purified on the basis of a pathophysiologic activity as a cofactor for cholera toxin. Arf1 and Arf6, the best characterized members of this family of proteins, have physiologic functions in the regulation of membrane trafficking and actin cytoskeleton remodeling (Donaldson, 2003; Randazzo et al., 2000a; Randazzo et al., 2000b).

The effects of Arf1 on membrane traffic are mediated, at least in part, by coat proteins and coat protein adaptors, including coatomer, Golgi-associated, γ adaptin-homology, Arf binding proteins (GGA1/2/3), AP-4 and the clathrin adaptor proteins AP-1 and AP-3 (Bonifacino and Lippincott-Schwartz, 2003). AP-1 functions at the trans-Golgi network (TGN) and endosomes (Robinson, 1990; Stoorvogel et al., 1996). AP-1 is a heterotetrameric protein comprised of two approximately 100 kDa 'large' subunits (γ and β1), a 47 kDa 'medium' subunit (μ1) and a 19 kDa 'small' subunit (σ) (Boehm and Bonifacino, 2001; Robinson, 2004). The hinge region of the β subunit binds clathrin (Kirchhausen, 2002). Arf1•GTP is thought to bind to both the β and γ subunits (Austin et al., 2002). AP-1 also recognizes sorting motifs in cargo molecules. Tyrosine-based motifs bind μ1 (Ohno et al., 1996; Ohno et al., 1995). Acid dileucine motifs bind the γ-σ1 hemicomplex (Janvier et al., 2003).

In the current paradigm, the formation of a transport intermediate that mediates membrane traffic is initiated when Arf1 is activated to the GTP bound form (Spang, 2002; Springer et al., 1999). Arf1•GTP binds tightly to membranes and to vesicle coat protein or coat protein adaptor such as AP-1, recruiting the coat protein to the membrane. The coat protein then traps cargo and polymerizes, causing budding of the membrane. A fission event leads to release of a coated vesicle. Arf1 is then inactivated by the hydrolysis of GTP to GDP, releasing the coat from the vesicle, which is then competent to dock and fuse with an acceptor membrane (Bonifacino and Glick, 2004; McMahon and Mills, 2004; Rothman, 2002). GTP binding and hydrolysis by Arf1 are integral to these events. Furthermore, Arf1 functions with many coats at multiple sites. A description of the regulation of membrane traffic will require an understanding of how Arf1 is site-specifically regulated with particular coats.

The accessory proteins catalyzing nucleotide exchange (guanine nucleotide exchange factors) and GTP hydrolysis (GTPase-activating proteins, GAPs) are critical elements in the regulation of Arf1 (Donaldson and Jackson, 2000; Randazzo and Hirsch, 2004). Arf1 lacks detectable GTPase activity and, therefore, GTP hydrolysis is dependent on GAPs. The first GAP identified, Arf GAP1, was found to have a catalytic domain, the Arf GAP domain, comprised of a zinc-binding motif (Cukierman et al., 1995). At least 24 genes have subsequently been found that encode an Arf GAP domain. Products of two groups, comprised of 16 genes altogether, have documented Arf GAP activity. Many of these have multiple splice variants. One group, Arf GAP1/3 and Git1/2, have the Arf GAP domain at the extreme N-terminus of the protein

(Cukierman et al., 1995; Liu et al., 2001; Premont et al., 1998; Premont et al., 2000). The second group, called the AZAPs, have a catalytic core of PH, Arf GAP and Ank repeat domains. These include ASAP1/2/3, ACAP1/2/3, ARAP1/2/3 and AGAP1/2/3 (Andreev et al., 1999; Brown et al., 1998; Jackson et al., 2000; Krugmann et al., 2002; Miura et al., 2002). The AGAPs are Arf GAPs, M_r ~80,000, containing a GTP-binding protein-like, ankyrin repeat and PH domains. They use class I and II Arfs in preference to class III Arfs (Nie et al., 2002; Xia et al., 2003).

Recognition of the number of GAPs relative to the number of Arf isoforms led to the hypothesis that Arf GAPs are both isoform- and site-specific regulators of Arf, allowing for the independent regulation of a single Arf isoform at a number of intracellular sites (Bonifacino and Jackson, 2003; Nie et al., 2003b). Some support for this idea comes from comparing Arf GAP1 and AGAP1. The function of Arf GAP1 appears to be restricted to the Golgi, where it binds to coatomer and regulates Arf1 (Eugster et al., 2000; Majoul et al., 2001; Rein et al., 2002). AGAP1 has been found to bind, through its PH domain, to the heterotetrameric adaptor protein AP-3. Its function is restricted to regulating Arf1 in the AP-3 endosome in the cell periphery (Nie et al., 2003a).

Here, we extend the test of the hypothesis that Arf GAPs contribute to the coat-specific regulation of Arf1 by examining two Arf GAPs within a single group, AGAP1 and AGAP2. Our data support a model in which the AGAP isoforms distinguish between the heterotetrameric adaptor proteins AP-1 and AP-3. As a consequence, AGAP1 and AGAP2 independently regulate AP-3 endosomes and AP-1/Rab4 fast recycling endosomes.

Materials and Methods

Plasmids and antibodies

The open reading frame of AGAP2 was subcloned into the *Xho*I and *Not*I sites of pCI and pSI vectors (Promega) with the FLAG tag (DYKDDDDK) at the N-terminus by standard molecular biology procedures. The catalytic core, PZA2, was subcloned into the *Mlu*I and *Not*I sites of pSI and pCI vectors with the FLAG tag at the N-terminus. The [R618K]AGAP2 and [R618K]PZA2 were generated using the QuikChange kit from Stratagene. Polyclonal rabbit sera against AGAP2 were raised by Covance, using the peptide TPSITATPSPRRRSS linked to KLH. The specificity of the antisera was verified by western blot. It recognized the immunoprecipitated FLAG-AGAP2 as well as the endogenous AGAP2, and did not crossreact with AGAP1. The signal was blocked by the specific peptide used to raise the antisera, but not by a non-relevant peptide. Antisera to AGAP1 has been previously described (Nie et al., 2002). Polyclonal and monoclonal (M5) antibodies against FLAG epitope tag, and monoclonal antibody against the γ subunit of AP-1 were from Sigma. The antibodies against α, μ3, β1/2 and ε subunits were from BD Biosciences. GFP-Rab4, GFP-Rab5 and GFP-Rab11 were generous gifts from Juan Bonifacino at NICHD. GFP-[S22N]Rab4, GFP-[S25N]Rab11 and polyclonal anti-Rab4 antibody were generous gifts from Julie Donaldson and Roberto Weigert at NHLBI. Polyclonal anti-Rab11 was from Zymed.

Cells culture, transfection and immunofluorescence

NIH3T3, HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37°C with 5% CO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen) and harvested 24 hours after transfection for immunoblotting and immunoprecipitation. For immunofluorescence staining, cells were reseeded onto coverslips for 12 hours and fixed. For transferrin uptake, cells were starved for 30 minutes and then incubated with Rhodamine-conjugated transferrin (Molecular Probes) for 10 or 30 minutes. Cells were then washed three times with medium containing 10% FBS, once with ice-cold PBS, and fixed with 2% formaldehyde immediately. Transfected cells were visualized by staining for the FLAG-tag. Rhodamine-conjugated transferrin and Alexa-633 secondary antibodies were from Molecular Probes. FITC- and Texas Red-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Confocal microscopy was performed with a Zeiss LSM 510 NLO system mounted on an Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) using a 63× 1.4 NA Zeiss Plan-Apochromat oil immersion objective. Digital images were acquired using a multi-track configuration, where the emission signals for the far red, red and green fluorophores after sequential excitation with a HeNe laser tuned to 633 nm, another HeNe laser tuned to 543 nm, and an argon laser tuned to 488 nm were sequentially collected with a BP650-710 nm filter, a BP565-615 nm filter, and a BP500-530 nm filter, respectively.

Protein expression and purification

GST fusion proteins of AGAP2 were expressed in BL21 cells and purified using glutathione Sepharose 4B gel (Amersham Biosciences). The proteins bound to the glutathione beads were incubated with soluble extracts of bovine brain for the pulldown assays. Otherwise, the GST fusion proteins were eluted from the beads with glutathione and dialyzed against PBS with 1 mM dithiothreitol. PZA2 was also expressed as a His-tagged form and purified using the Talon kit (Clontech). AP-1 and AP-2 were purified from soluble extracts of bovine brain as described before (Prasad and Keen, 1991). Briefly, bovine brains were homogenized in buffer A (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide and protease inhibitors). The homogenate was centrifuged at 13,000 ***g*** for 40 minutes at 4°C in a GSA rotor. The supernatant was then centrifuged at 100,000 ***g*** for 1 hour at 4°C. The pellet was dispensed in buffer A using a Dounce homogenizer. The homogenate was clarified by centrifuging at 12,000 ***g*** for 10 minutes at 4°C in a SS34 rotor and the supernatant centrifuged at 100,000 ***g*** for 1 hour at 4°C, and this step was repeated for a total of three times. The final pellet was resuspended overnight at 4°C in buffer B containing 50% buffer A and 0.5 M Tris HCl (pH 7.0). The sample was centrifuged at 100,000 ***g*** for 1 hour at 4°C and the proteins in the supernatant were precipitated with 50% saturated ammonium sulfate and resuspended in buffer B. The proteins were then separated on a Superose 6 column equilibrated with buffer B. The fractions containing AP-1 and AP-2 were confirmed by western blot and the AP-1 and AP-2 were further separated on a hydroxyapatite column. The purified AP-1 and AP-2 were dialyzed against 20 mM Tris, pH 7.5 and 100 mM NaCl overnight at 4°C.

Immunoprecipitation and GST pulldown assay

Cells were harvested in lysis buffer containing 25 mM Tris, pH 8.0, 100 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitors. The cell lysates were cleared by centrifugation at 10,000 ***g*** for 10 minutes at 4°C. The lysates were incubated with anti-FLAG M2 gel overnight at 4°C. The beads were washed three times with lysis buffer and eluted with FLAG peptide for 30 minutes at 4°C. For the pulldown assay, GST fusion proteins of AGAP2 were incubated with soluble extracts of bovine brain in the presence of 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol and 0.1% Triton X-100 at 4°C overnight.

Miscellaneous

PCR was performed by standard molecular biology procedures. For the tissue distribution of AGAP1 and AGAP2 message by RT-PCR,

the cDNA was amplified from a human cDNA panel from Clontech. The forward primer is ACATCTACTCCATCTACgAgCTgC and the reverse primer is gCTgATTgTgCACggCAgACACC for AGAP1. The forward primer is AgATgggTgAAggCCTggAAgCCAC and the reverse primer is CgTTCCggATCgCCTggATggCCAC for AGAP2. Immunoblot signals were visualized using ECL Plus reagents (Amersham Biosciences). Lipids were obtained from Sigma and Avanti Polar Lipids and presented as mixed micelles with Triton X-100 or in vesicles. Vesicles contained 40% phosphatidylcholine, 25% phosphatidylethanolamine, 15% phosphatidylserine, 9.5% phosphatidylinositol, 10% cholesterol and 0.5% phosphatidylinositol 4,5-bisphosphate. Vesicles were prepared by extrusion through Whatman Nucleopore Etched Filters with pore size of 1 μm. GAP activity was measured as described before (Randazzo et al., 2001; Yoon et al., 2004). All experiments were performed at least three times with similar results. Values for all bar graphs are presented as mean±s.e.m. Figures are generated and statistical analysis performed using GraphPad Prism.

Results

Differential expression of AGAP2 in mammalian tissues and cell lines

The primary structure of AGAP2 is highly similar to that of AGAP1. They both contain, from the N-terminus, GTP-binding protein-like (GLD), pleckstrin homology (PH), Arf GAP (with a zinc-binding motif) and ankyrin repeat domains (Ank) (Fig. 1A). We began our study by comparing the tissue distribution of AGAP1 and AGAP2 message by RT-PCR. A human cDNA panel was used as template. AGAP1 cDNA was detected at a similar level in all the human tissues examined (Fig. 1B, top panel). AGAP2 cDNA was found at a much higher level in the brain and heart, compared with the level in the lung, liver, skeletal muscle and pancreas. The cDNA was barely detectable in the placenta and kidney (Fig. 1B, middle panel). As a control, the cDNA of the G3PDH was amplified to a comparable level from all tissues examined (Fig. 1B, lower panel).

We next determined whether AGAP2 protein was differentially expressed, comparing AGAP2 to AGAP1. We raised antibodies using peptides derived from the AGAP2 sequence. One antibody, 4572, recognized the endogenous as well as overexpressed AGAP2 by Western blot. The signal was abolished by the specific peptide from which the antisera were raised, but a non-relevant peptide from the AGAP2 sequence did not affect the signal recognized by 4572 (data not shown). We compared the expression of AGAP1 and AGAP2 in different cell lines. AGAP1, examined using the antibody 1158, was detected in all cell lines examined, with slightly higher levels in HeLa, AGS, HepG2 and U138 cells. AGAP2 was detected in relatively higher levels in Jurkat, A549, U87, H4, U118, U138 and U1620 cells (Fig. 1C).

AGAP1 and AGAP2 have similar biochemical properties

We next examined the Arf specificity and phosphoinositide specificity for AGAP2. Our previous study (Nie et al., 2002) showed that AGAP1 is specific for Arf1 and is activated by phosphatidic acid (PA) with phosphatidylinositol 4,5-bisphosphate (PIP_2). We measured the in vitro Arf specificity of AGAP2 in the presence of PA with either PIP_2 or PIP_3. Similar trends were seen with either lipid combination. Arf5 and Arf1 were used preferentially to Arf6 as substrate (Fig. 1D). PIP_2 stimulated AGAP2 ArfGAP activity better than did PIP_3 (Fig. 1D). We further compared the effect of a number of signaling phospholipids. When presented alone, either PA or PIP_2 activated AGAP2, although PIP_2 activated the enzyme to a slightly higher level. The other phosphoinositides, including phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$) and phosphatidylinositol 3,5-bisphosphate ($PI3,5P_2$), were less effective. When presented in combination, PA potentiated the activation of AGAP2 by all the phosphoinositides examined. However, even in the presence of PA, PIP_2 activated AGAP2 to a higher level than other phosphoinositides (Fig. 1E).

AGAP2 specifically binds to AP-1

AGAP1 has been found to bind the adaptor protein complex AP-3 and cause its dissociation from membranes in NIH3T3 cells (Nie et al., 2003a). Given the similarity in structure and basic enzymology between AGAP1 and AGAP2, we first considered the possibility that AGAP2 would also affect AP-3. However, we found no effect of AGAP2 overexpression on AP-3 in NIH3T3 fibroblasts (Fig. 2A, c,d). Under the same conditions, AGAP1 efficiently displaced AP-3 from endosomes (Fig. 2A, a,b). The effect of AGAP1 was observed in 53±3% cells overexpressing AGAP1. Next, we considered the possibility that AGAP2 interacted with another heterotetrameric adaptor protein. We first screened for interacting proteins by expressing FLAG-epitope-tagged AGAP1 and AGAP2 in HEK293 cells. The proteins were immunoprecipitated with anti-FLAG M2 agarose beads. FLAG-tagged AGAP1 and AGAP2 were then eluted from the beads with the FLAG peptide. The eluted proteins were resolved by SDS-PAGE and the presence of adaptor/coat proteins was determined by immunoblot. As shown in Fig. 2B, AGAP2 preferentially precipitated AP-1, as detected by antibodies against the γ subunit of AP-1 (Fig. 2B) and the β subunit of AP-1 (not shown). AGAP1 precipitated AP-3 more efficiently, as detected by antibodies against the δ subunit (Fig. 2B), and μ3 subunit (not shown). Both AGAP1 and AGAP2 showed weak association with AP-2 (Fig. 2B), and neither bound AP-4 (Fig. 2B). We performed GST pulldown assays to confirm the results of immunoprecipitation and to map out the domain(s) of AGAP2 responsible for binding to AP-1. The N-terminal GLD domain, the PH domain and the ArfGAP together with the ankyrin repeat domains (ZA, for zinc finger and ankyrin repeat domains) were fused with glutathione S-transferase (GST) and expressed in *E. coli* (BL21). The proteins were purified using glutathione-conjugated beads and incubated with a soluble extract of bovine brain. After incubation, the beads were centrifuged and proteins precipitating with the beads were detected by immunoblot. As shown in Fig. 2C, GST fusion protein of the PH domain of AGAP2 (GSTPH2) precipitated AP-1 from the bovine brain extract (Fig. 2C). GSTGLD2 also showed some weak association with AP-1. We did not detect an association of GSTZA2 with AP-1 (Fig. 2C). The adaptor protein complex AP-3 was precipitated to a much less extent than AP-1 (not shown), and neither AP-2 (Fig. 2C) nor AP-4 (not shown) precipitated with GSTPH2 (not shown). Therefore, analogous

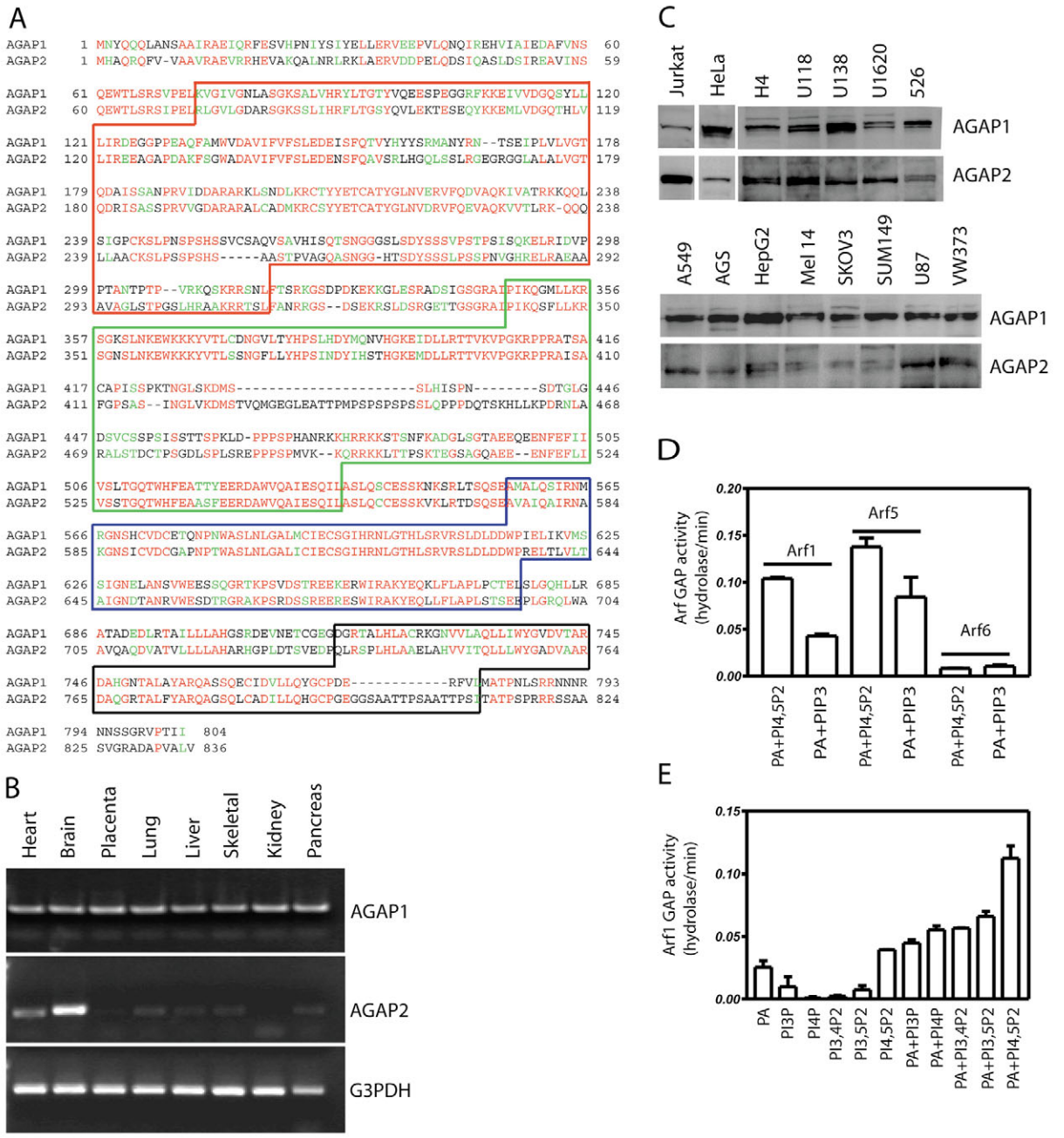

Fig. 1. Distribution and biochemical characterization of AGAP2. (A) Similarities between different domains of AGAP1 and AGAP2. Alignment was performed using MacVector. Identical residues were marked in red and similar residues were marked in green. The different domains were boxed with different colors, GLD (GTP-binding protein-like domain) in red; PH (pleckstrin homology domain) in green; ArfGAP (Arf directed-GTPase activating protein domain) in blue; Ank (ankyrin repeat domain) in black. (B) Distribution of AGAP1 and AGAP2 message by RT-PCR. The top panel shows the message of AGAP1, the middle panel shows the message of AGAP2, and the bottom panel shows the message of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in different human tissues. AGAP1, AGAP2 and G3PDH cDNAs were amplified for 34 cycles using a human cDNA panel from Clontech. AGAP1 cDNA was detected using primers to amplify the region between nucleotide 648 and 1369 of the open reading frame. AGAP2 cDNA was detected using primers to amplify the region between nucleotide 1303 to 1770 of the open reading frame. G3PDH cDNA was detected using primers provided by Clontech. (C) Differential distribution of AGAP1 and AGAP2 in different cell lines. Lysates from different cell lines were subjected to SDS-PAGE and western blot for AGAP1 (detected using the antibody 1158) and AGAP2 (detected using 4572). Jurkat, T lymphocyte; HeLa, cervical adenocarcinoma; H4, neuroglioma; U87, U118, U138 and U1620, glioblastoma; Mel 14 and 526, melanoma; A549, squamous carcinoma; AGS, gastric adenocarcinoma; HepG2, hepatocellular carcinoma; SKOV3, ovary cancer; SUM149, breast cancer; VW373, astrocytoma. (D) Arf specificity of AGAP2. Arf GAP activity of AGAP2 was measured as described in Materials and Methods, using 360 μM PA together with either 45 μM PIP_2 or 1 μM PIP_3 presented in micelles with 0.1% Triton X-100. (E) Phospholipid specificity of AGAP2. AGAP2 activity was measured in the presence of the lipids as indicated at concentrations as follows, PA, 360 μM; PI3P, 45 μM; $PI3,4P_2$, 45 μM; $PI3,5P_2$, 45 μM; $PI4,5P_2$, 45 μM presented in micelles with 0.1% Triton X-100.

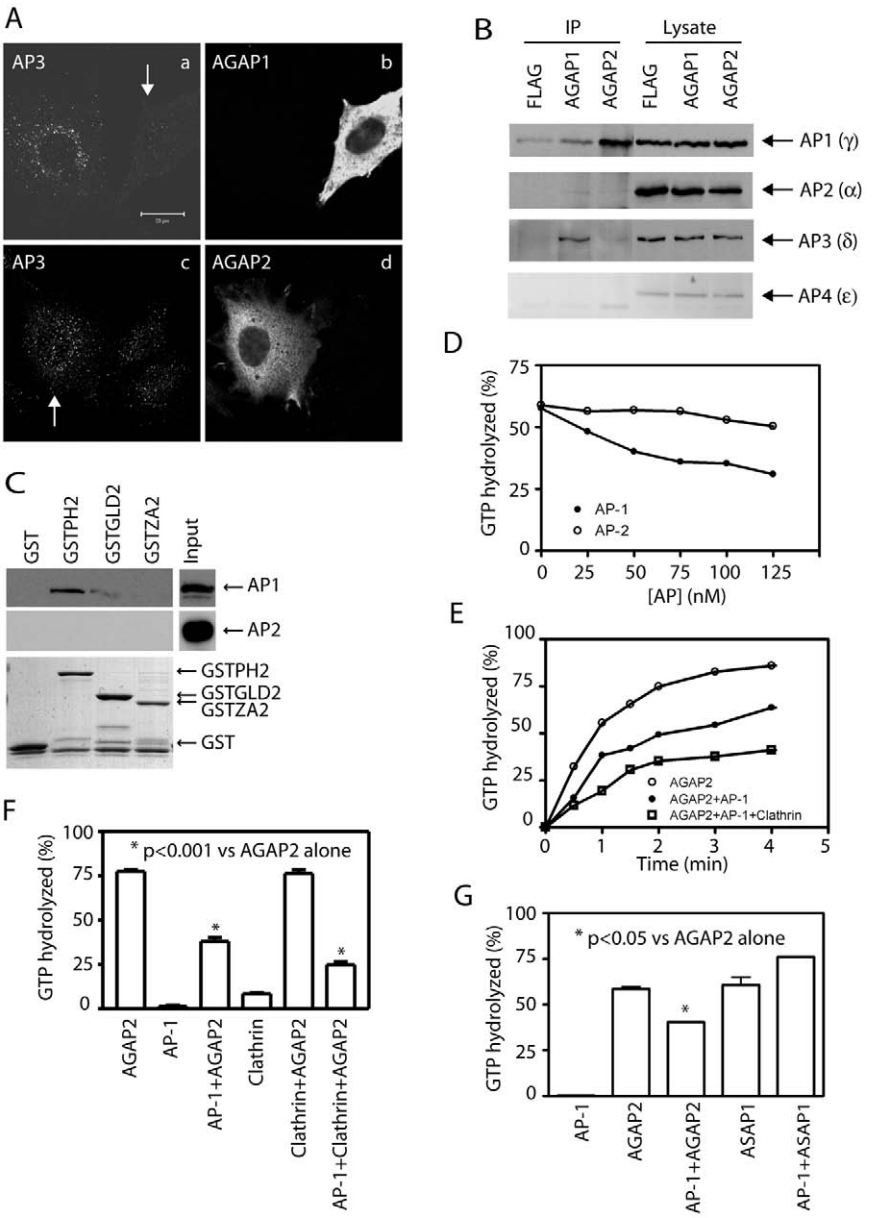

Fig. 2. Interaction of AGAP2 with adaptor protein complex AP-1. (A) Effect of AGAP1 and AGAP2 on the membrane association of AP-3. NIH3T3 cells were transfected with AGAP1 and AGAP2 for 24 hours. Cells were fixed and stained for AP-3 (using anti-δ antibody) and the FLAG tag (using a polyclonal anti-FLAG antibody) to visualize the transfected cells (arrows). (B) Co-immunoprecipitation of AP-1 with AGAP2. FLAG tagged AGAP1, AGAP2 or empty vector were transfected into HEK293 cells at 10 μg DNA/10cm dish, using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours after transfection and lysed into a buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 1% Triton X-100 and 10% glycerol. Protease inhibitors (Complete®, Roche) were included in the lysis buffer. AGAP1 and AGAP2 were immunoprecipitated through the FLAG tag using anti-FLAG M2 gel. Different coat protein complexes in the precipitates were detected by antibodies against the γ subunit for AP-1, α subunit for AP-2, δ subunit for AP-3 and ε subunit for AP-4. (C) Pulldown assay of AP-1 with different GST fusion proteins of AGAP2. Different domains of AGAP2, including the GLD2, PH2 and ZA2, were fused with the glutathione S-transferase (GST) and expressed in *E. coli*. GST was included as a control. The purified proteins were incubated with the soluble extracts of bovine brain at 4°C overnight. The beads were washed and the proteins precipitated were resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblots were performed using antibody to AP-1 (top panel) and AP-2 (middle panel). Only AP-1 was detected. The GST or the GST fusion proteins used for precipitation were shown in the bottom panel by Coomassie Blue staining. (D) Inhibition of AGAP2 activity by AP-1. His-tagged PZA2 domain of AGAP2 (25 nM) was preincubated with different concentrations of AP-1 or AP-2 for 30 minutes at room temperature before addition of [α^{32}P]GTP-labeled myristoylated Arf1. The phospholipids were presented in vesicles as described in Materials and Methods. The reaction was stopped after 2 minutes and the amount of GTP hydrolyzed quantified by a PhosphorImager (Molecular Dynamics). (E) Time course of GAP activity of AGAP2. The activity of AGAP2 was measured at the time intervals as indicated. For inhibition by AP-1 and clathrin, 100 nM of AP-1 and 100 nM of clathrin were incubated with AGAP2 at room temperature for 30 minutes before addition of [α^{32}P]GTP labeled myristoylated Arf1. (F) Effect of AP-1 and clathrin on AGAP2 activity. His-tagged PZA2 of AGAP2 was incubated with 88 nM AP-1, and/or 80 nM clathrin at room temperature for 30 minutes before addition of [α^{32}P]GTP labeled myristoylated Arf1. *P<0.001 compared with AGAP2 as analyzed by one-way ANOVA with Tukey post-test. (G) Differential effect of AP-1 on AGAP2 and ASAP1. AP-1 (75 nM) was incubated with AGAP2 or ASAP1 at room temperature for 30 minutes before the addition of [α^{32}P]GTP labeled myristoylated Arf1. GAP assay was performed as described in panel D. *P<0.05 compared with AGAP2 alone as analyzed by one-way ANOVA with Tukey post-test.

to AGAP1, the PH domain of AGAP2 appears to be the primary binding site on AGAP2 for AP-1.

Having established the association of AGAP2 with AP-1, we determined whether binding of AP-1 would also affect the function of AGAP2. When preincubated with His-tagged PZA domain of AGAP2, AP-1 inhibited the GAP activity of AGAP2 in a concentration-dependent manner (Fig. 2D). This effect was observed with AP-1 purified from coated vesicles or from a soluble extract from bovine brain. AP-2, purified from coated vesicles, did not inhibit the GAP activity of AGAP2 (Fig. 2D). Since AP-1 binds clathrin during coated vesicle formation, we examined whether clathrin binding to AP-1 would affect the inhibition of AGAP2 by AP-1. As shown in Fig. 2F, clathrin by itself did not affect the activity of AGAP2, and when in combination with AP-1, clathrin slightly increased the inhibition by AP-1. To further examine the inhibition of AGAP2 activity by AP-1, we determined the time-course for the GAP activity in the presence of AP-1. Consistent with the

single time point data (Fig. 2F), AP-1 or AP-1 plus clathrin inhibited the activity of AGAP2 (Fig. 2E).

The effect of AP-1 could be a result of the direct interaction of AP-1 with AGAP2. Another possibility might be that AP-1 was also binding to Arf1, sequestering it from the GAP. We performed two different assays to distinguish between these. First, AP-1 was incubated with Arf1 preloaded with [^{35}S]GTPγS. It has been shown that effector binding to Arf affects the dissociation of labeled GTP from Arf (Jacques et al., 2003). At concentrations up to 200 nM, AP-1 did not affect the dissociation of [^{35}S]GTPγS from Arf1 (not shown), suggesting it would not sequester Arf at the concentrations used to inhibit GAP activity. Second, we examined whether AP-1 affected the activity of ASAP1, the prototype of AZAP family of Arf GAPs. AP-1 inhibited the activity of AGAP2, but not that of ASAP1 (Fig. 2G). Therefore, the inhibition of AGAP2 activity most probably resulted from the direct interaction between AP-1 and AGAP2.

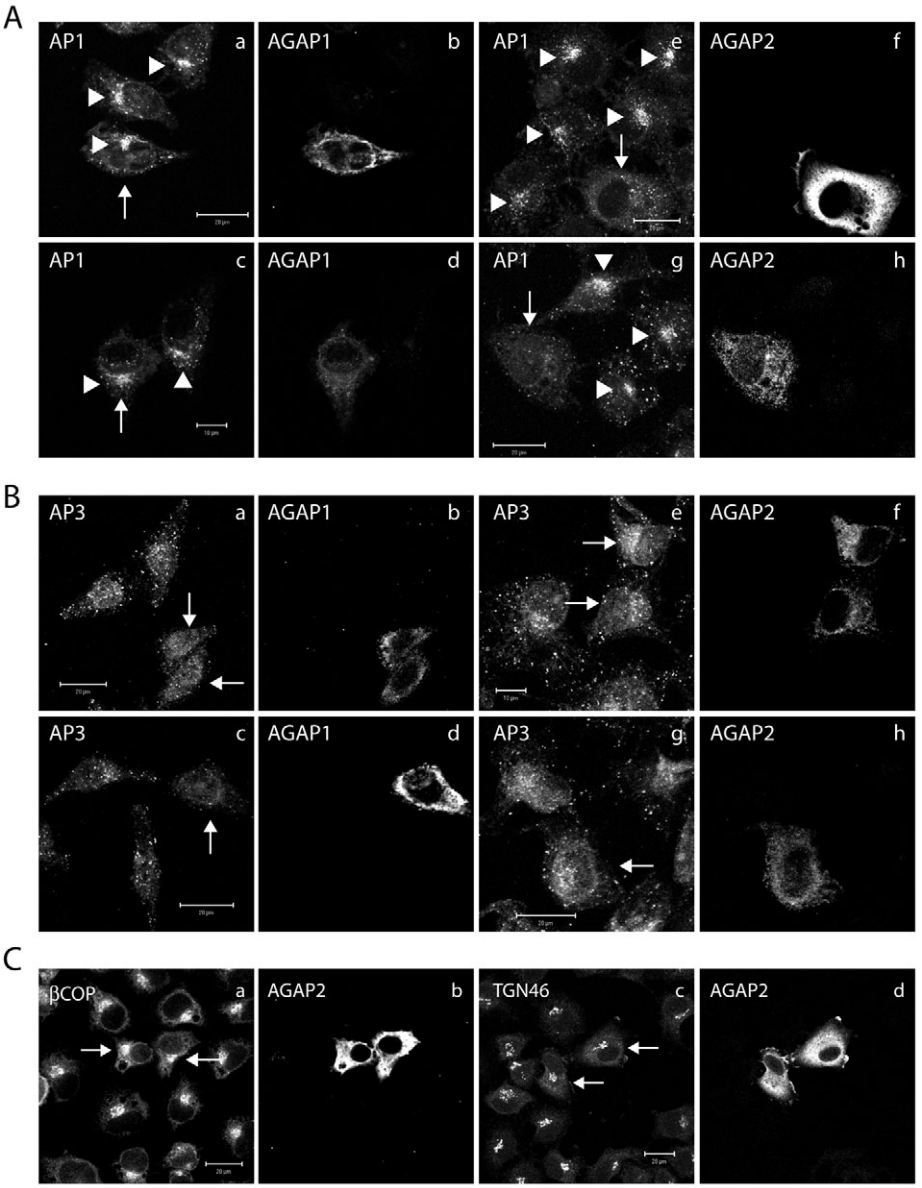

Fig. 3. Specific interaction between AGAP2 and AP-1. (A) Redistribution of AP-1 by AGAP2. HeLa cells were transfected with FLAG-tagged AGAP1 or AGAP2 for 24 hours. The cells were fixed and stained with antibodies against the γ subunit of AP-1. The transfected cells were detected using polyclonal anti-FLAG antibody. (B) Membrane dissociation of AP-3 by AGAP1. HeLa cells were transfected with FLAG-AGAP1 or AGAP2 for 24 hours. Cells were stained for the epitope tag and AP-3 using anti-δ antibody. (C) No effect of AGAP2 on the staining pattern of COPI or TGN46. HeLa cells were transfected with FLAG-AGAP2. The COPI coat was visualized by staining with antibody against βCOP (panels a, b). The TGN was visualized by staining with an antibody against TGN46 (panels c, d). Transfected cells were indicated by arrows.

AGAP2 affects AP-1 distribution in vivo

We next examined the in vivo consequences of the interaction between AGAP1 and AGAP2 with adaptor proteins. HeLa cells were transfected with FLAG-tagged AGAP1 or AGAP2, fixed 24 hours later and stained with antibodies against the γ subunit of AP-1 or the δ subunit of AP-3, and antibodies against the epitope tag. In non-transfected cells, AP-1 staining was mostly concentrated in the perinuclear region (Fig. 3A, arrowhead), consistent with its association with the TGN (Fig. 3A, a,c,e,g). AP-1 staining was also observed in small punctate structures, presumably endosomes (Fig. 3A). Overexpression of AGAP1 did not affect the subcellular distribution of AP-1 (Fig. 3A, a-d; transfected cells identified with arrows). In cells overexpressing AGAP2, AP-1 was redistributed in the punctate structures throughout the cells, but lost from the perinuclear region (Fig. 3A, e-h; transfected cells identified with arrows). This effect was observed in 62±3% of cells overexpressing AGAP2. By contrast, overexpression of AGAP1 (Fig. 3B, a-d), but not AGAP2 (Fig. 3B, e-h) caused the dissociation of AP-3 from endosomes, similar to that observed in NIH3T3 cells (Fig. 2A). Overexpression of AGAP2 did not affect the membrane association of βCOP (Fig. 3C, a,b), or the TGN localization of TGN46 (Fig. 3C, c,d), indicating that TGN remained intact.

The effect of AGAP2 on AP-1 localization was dependent on GAP activity. Two approaches were used to examine this possibility. First, we used [Q71L]Arf1, which is unable to hydrolyze GTP bound to it and thereby remains in the active form, to block the effect of AGAP2. When [Q71L]Arf1 was co-transfected with AGAP2, more

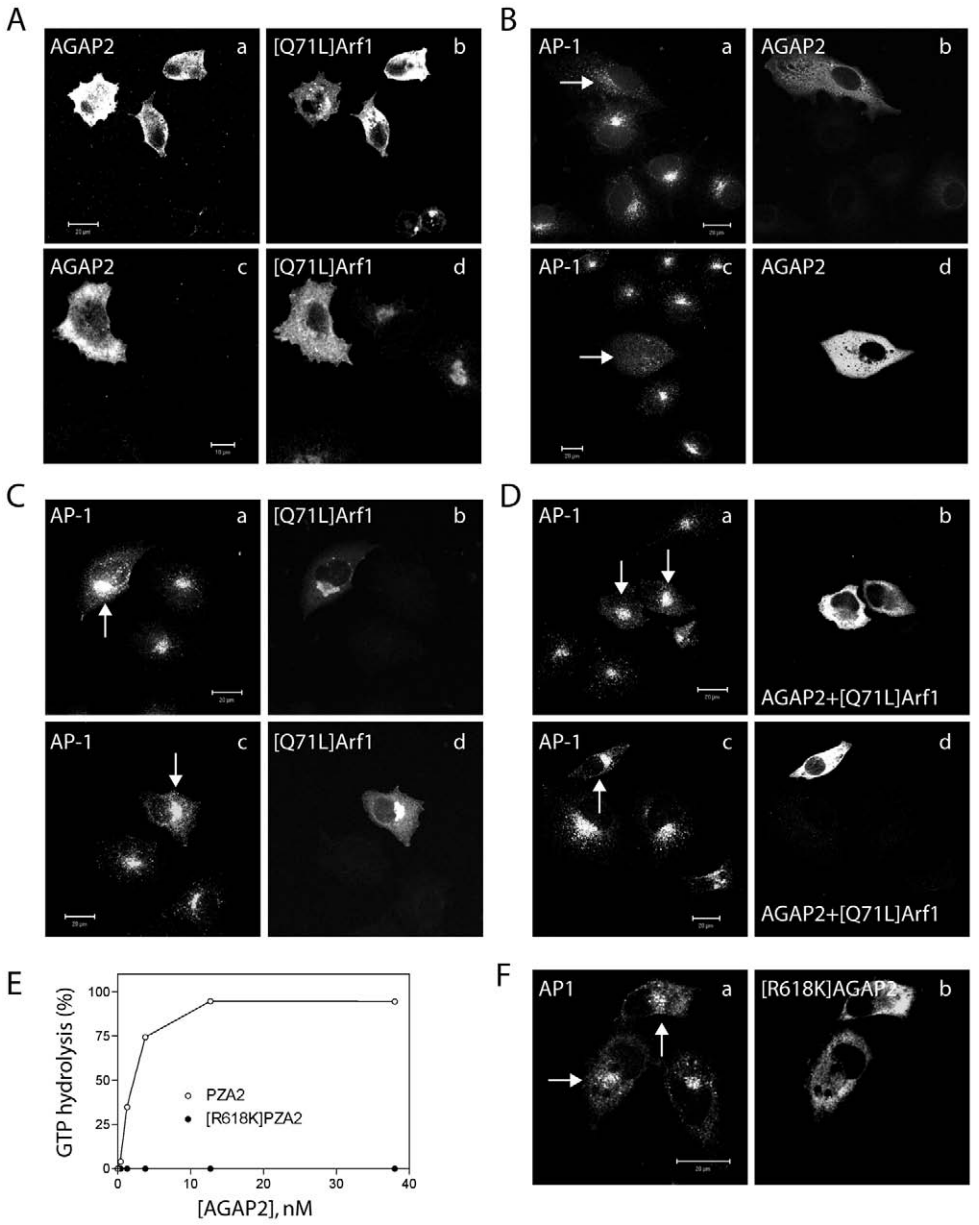

Fig. 4. Dependence of GAP activity of AGAP2 for interaction with AP-1. (A-D) Effect of [Q71L]Arf1 on AGAP2 induced AP-1 redistribution. HeLa cells were transfected with FLAG-AGAP2 (B), [Q71L]Arf1-HA (C) or both (A,D) for 24 hours. Cells were stained for the AP-1 and FLAG tag (B,D), or HA-tag (C), or stained for both FLAG and HA tag (A). (E) Requirement of the conserved arginine for GAP activity. The catalytic core of AGAP2, PZA2, or its point mutant with the conserved arginine mutated to lysine, [R618K]PZA2, were expressed as GST-fusion proteins in *E. coli* and purified. The proteins were eluted from the beads with glutathione and dialyzed overnight against PBS with 1 mM dithiothreitol. Increasing concentrations of PZA2 or [R618K]PZA2 were titrated into the GAP assay as described in Materials and Methods. (F) Effect of GAP dead AGAP2 on AP-1 association with TGN. FLAG-tagged [R618K]AGAP2 was transfected into HeLa cells. Cells were fixed 24 hours after transfection and stained for AP-1 and the FLAG tag. Transfected cells were indicated by arrows.

than 90% of the cells expressing AGAP2 also expressed [Q71L]Arf1 (Fig. 4A). AGAP2 alone disrupted the TGN localization of AP-1 (Fig. 4B) whereas [Q71L]Arf1 alone did not change or increased the TGN localization of AP-1 (Fig. 4C). When co-transfected, [Q71L]Arf1 blocked the effect of AGAP2 on AP-1 (Fig. 4D). Second, we introduced a mutation that changed a conserved arginine at position 618, found to be critical for GAP activity of other AZAP family proteins including AGAP1, to lysine. When tested in vitro, the mutant had less than 1/1000 the activity of wild-type protein (Fig. 4E). We expressed [R618K]AGAP2 in HeLa cells and examined the distribution of AP-1. In contrast to the effect on AP-1 of the wild type AGAP2, expression of [R618K]AGAP2 did not affect AP-1 distribution in HeLa cells (Fig. 4F).

Although an association between AP-1 and AGAP2 was detected both in vitro and, as judged by coimmunoprecipitation, in vivo, we did not observe substantial colocalization of the two proteins when AGAP2 was overexpressed as described above. In those experiments, transcription was driven by a CMV promoter using the pCI vector, leading to high protein levels. At high concentrations, the ectopically expressed AGAP2 could either mask its physiological localization or affect the compartment with which it normally associates. Therefore, we reassessed localization with AGAP2 expressed at lower levels (using a pSI plasmid with an SV40 promoter). Western blot detecting the epitope tag showed that transfection with these two vectors resulted in 5-10 times difference in the level of expression (Fig. 5C). Even at lower protein levels, ectopic expression of AGAP2 caused the redistribution of AP-1. However, in this case we observed that AP-1 and AGAP2 colocalized in the decentralized punctate structures (Fig. 5A, a,b). To determine the nature of these AGAP2 and AP-1 containing punctate structures, we examined the possible colocalization with other endosomal markers. AGAP2 colocalized with transferrin receptor (TfnR) in HeLa cells (Fig. 5A, c,d). To examine

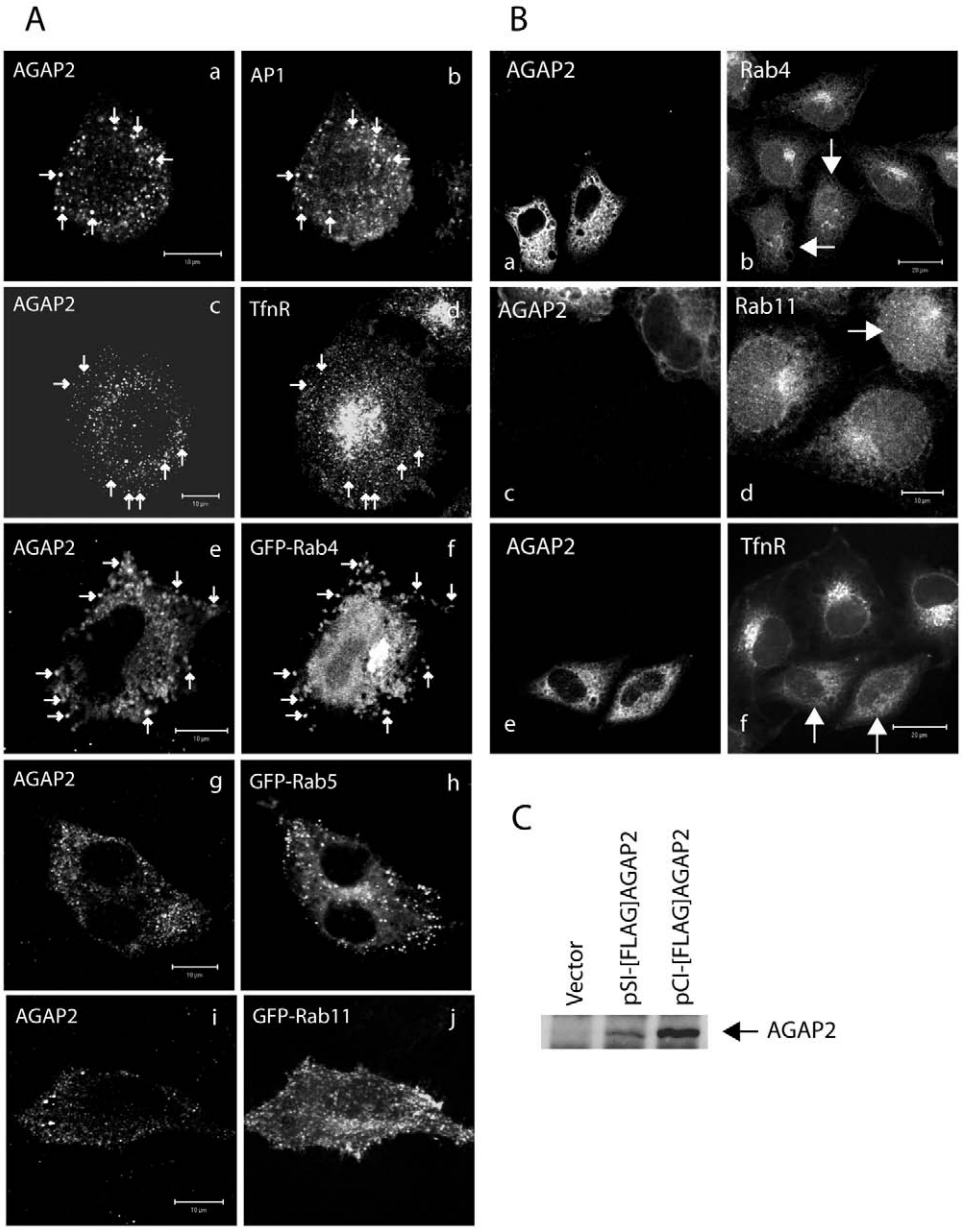

Fig. 5. Effect of AGAP2 on endosomal markers.
(A) Colocalization of AGAP2 with endosomal markers. (A, a-d) AGAP2 colocalized with AP-1 and transferrin receptor. FLAG-AGAP2 under the control of SV40 promoter (in pSI vector) was transfected into HeLa cells for 24 hours. The cells were fixed and stained for AP-1 and transferrin receptors (TfnR). The overexpressed AGAP2 was detected by staining for the FLAG tag. Colocalization of AGAP2 with AP-1 and TfnR was indicated by arrows. (A, e-j) Colocalization of AGAP2 with Rab4. FLAG-AGAP2 in pSI vector was transfected with GFP-Rab4, GFP-Rab5 and GFP-Rab11 into HeLa cells for 24 hours. The overexpressed AGAP2 was detected by staining for the FLAG tag. AGAP2 colocalized with Rab4 (arrows in e, f), but not with Rab5 (g,h) or Rab11 (i,j).
(B) Effect of AGAP2 on endogenous Rab4 and TfnR. HeLa cells were transfected with FLAG-AGAP2 driven by the CMV promoter (in pCI vector) for 24 hours. Endogenous Rab4 (B, a,b), Rab11 (B, c,d) and transferrin receptors (B, e,f) were visualized by staining with specific antibodies. Transfected cells were visualized by staining for the FLAG tag and indicated by arrows. (C) Relative expression level of [FLAG]AGAP2. HeLa cells were transfected with empty vector, pSI-[FLAG]AGAP2 or pCI-[FLAG]AGAP2 for 24 hours. Cells were harvested and lysed. The lysates were subjected to SDS-PAGE and western blot using polyclonal anti-FLAG antibody.

colocalization with Rab proteins, we expressed FLAG-tagged AGAP2 with GFP-tagged Rab4, Rab5 and Rab11. AGAP2 colocalized with GFP-Rab4 in punctate structures (Fig. 5A, e,f). AGAP2 did not colocalize with GFP-Rab5 (Fig. 5A, g,h) or GFP-Rab11 (Fig. 5A, i,j).

AGAP2 affects an AP-1/Rab4 endosomal compartment containing transferrin receptor

A receptor recycling compartment regulated by AP-1 and Rab4 has been previously described. Given AGAP2 colocalized with these two proteins, we determined whether AGAP2 could be involved as a regulator of this compartment. We started by expressing AGAP2 at high levels by using the CMV promoter in the pCI vector. We found that in addition to affecting AP-1 distribution, AGAP2 decreased the perinuclear concentration of endogenous Rab4 (Fig. 5B, a,b) and transferrin receptors (Fig. 5B, e,f). As a control, overexpression of AGAP2 did not affect the staining pattern of endogenous Rab11 (Fig. 5B, c,d).

Our results were consistent with AGAP2 affecting the AP-1/Rab4 compartment. This compartment has been reported to mediate rapid recycling of transferrin (Deneka et al., 2003). If AGAP2 were a regulator of this compartment, we predicted it would affect transferrin uptake. As a test for AGAP2 function in this compartment, we examined transferrin uptake in HeLa cells transfected with AGAP2. At 10 minutes after addition of transferrin, there was less transferrin in the cells overexpressing AGAP2 (Fig. 6A, a,b; Fig. 6E) than in controls. After 30 minutes, a similar amount of transferrin was taken up by non-transfected and AGAP2-transfected cells (Fig. 6A, c,d). The decreased accumulation could result from decreased uptake. Alternatively, the decreased accumulation could be due to accelerated exit from the Rab4/AP-1 recycling endosomes, thereby reducing apparent uptake at early times after

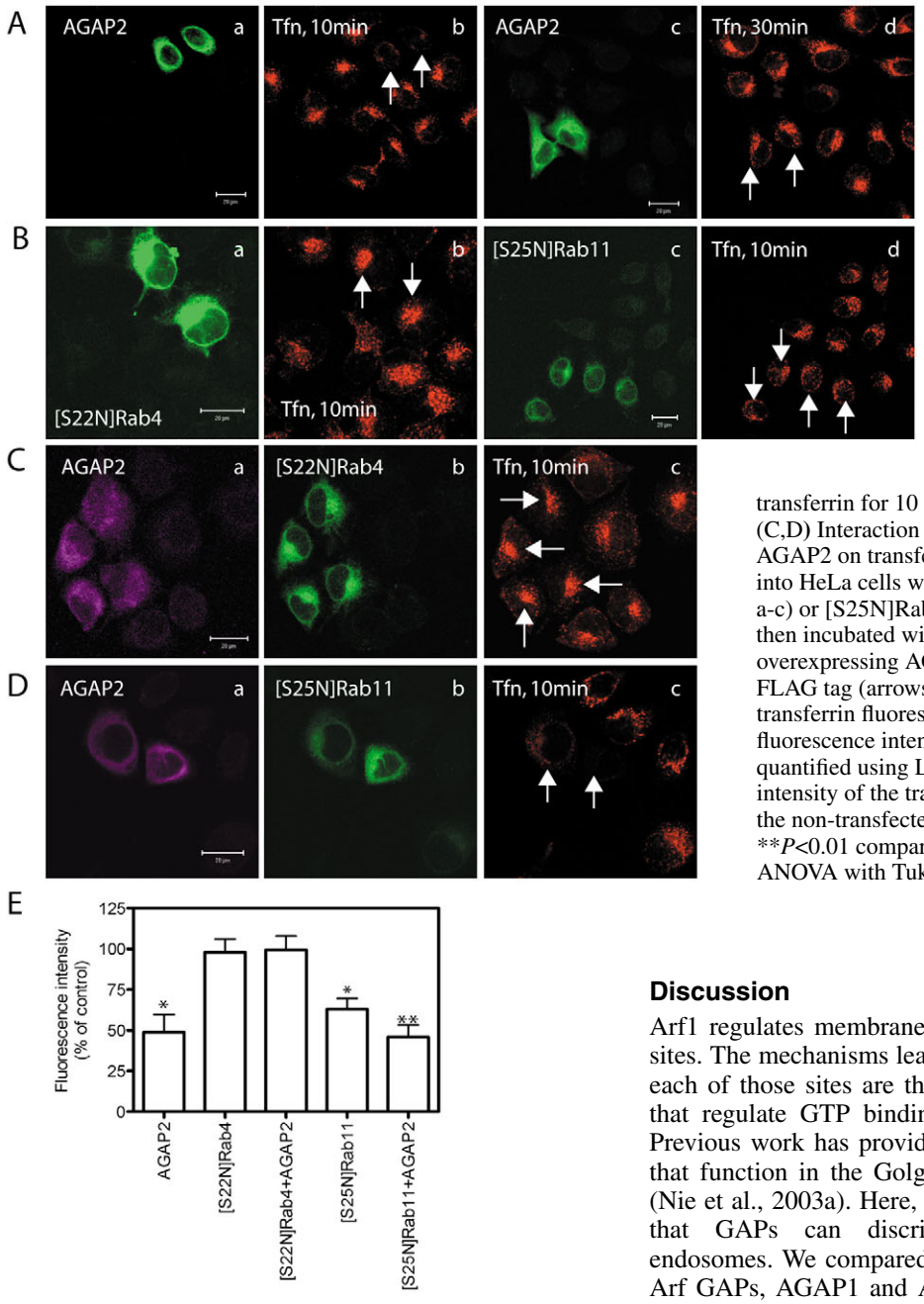

Fig. 6. Effect of AGAP2 on transferrin recycling. (A) AGAP2 promoted transferrin recycling. HeLa cells were transfected with FLAG-AGAP2 for 24 hours. The cells were starved for 30 minutes and then incubated with Rhodamine-conjugated transferrin for 10 minutes (a,b) or 30 minutes (c,d). (B) Effect of [S22N]Rab4 and [S25N]Rab11 on transferrin uptake. Cells were transfected with GFP-[S22N]Rab4 (a,b) and GFP-[S25N]Rab11 (c,d) for 24 hours. Cells were incubated with transferrin for 10 minutes before washing and fixing. (C,D) Interaction of [S22N]Rab4 and [S25N]Rab11 with AGAP2 on transferrin recycling. AGAP2 was transfected into HeLa cells with either GFP-tagged [S22N]Rab4 (C, a-c) or [S25N]Rab11 (D, a-c) for 24 hours. The cells were then incubated with transferrin for 10 minutes. Cells overexpressing AGAP2 were identified by staining for the FLAG tag (arrows). (E) Quantification of intracellular transferrin fluorescence intensity. Intracellular fluorescence intensity of transferrin from single cells was quantified using LSM510 software. The fluorescence intensity of the transfected cells was compared to that of the non-transfected cells on the same coverslip. *P<0.05; **P<0.01 compared with control as analyzed by one-way ANOVA with Tukey post-test.

transferrin addition. To test for the latter possibility, we examined the effect of the dominant negative form of Rab4, which blocks exit from the AP-1/Rab4 endosomes, on transferrin accumulation in the presence of AGAP2. When expressed alone, [S22N]Rab4 did not affect transferrin uptake at 10 minutes (Fig. 6B, a,b; Fig. 6E). When expressed together with AGAP2, [S22N]Rab4 completely reversed the effect of AGAP2 on the accumulation of transferrin in the recycling compartment (Fig. 6C, a-c; Fig. 6E), while [S25N]Rab11 (Fig. 6D, a-c; Fig. 6E) had no effect. Based on these results, we conclude that AGAP2 affected transferrin accumulation by accelerating the Rab4-dependent exit from the recycling compartment.

Discussion

Arf1 regulates membrane traffic at a number of intracellular sites. The mechanisms leading to its independent regulation at each of those sites are thought to involve accessory proteins that regulate GTP binding (GEFs) and hydrolysis (GAPs). Previous work has provided support for differences in GAPs that function in the Golgi (Cassel, 2003) and in endosomes (Nie et al., 2003a). Here, these results were extended to show that GAPs can discriminate between populations of endosomes. We compared the function of two closely related Arf GAPs, AGAP1 and AGAP2. Despite the high degree of structural similarity, AGAP1 and AGAP2 had different targets, specifically interacting with AP-3 and AP-1, respectively. The ability to discriminate between these two targets is a basis for specific regulation of distinct endosomal compartments. AGAP1 affected AP-3 endosomes; AGAP2 affected an AP-1/Rab4 rapid recycling compartment (Deneka et al., 2003) and, as a consequence, transferrin accumulation.

AGAP1 had previously been found to bind the heterotetrameric adaptor protein AP-3 (Nie et al., 2003a). The structural similarity of AGAP2 to AGAP1 led us to test for similar interactions with several heterotetrameric adaptor proteins and we consequently identified a specific interaction with AP-1. In testing for a cellular role of this interaction, we found that AGAP2 specifically associated with AP-1 positive endosomes. AGAP2 did not associate with AP-1 at the TGN nor did AGAP2 affect any TGN markers other than AP-1.

Instead, AGAP2 associated with a fast recycling compartment containing AP-1, Rab4 and transferrin receptor (Deneka et al., 2003). Consistent with a regulatory role, AGAP2 altered the distribution of Rab4 and transferrin receptor and decreased the rapid accumulation of transferrin. These results support the idea that AGAP2 works specifically with AP-1 directing activity to a particular AP-1 dependent endocytic compartment, such as the AP-1/Rab4/transferrin receptor compartment previously described (Deneka et al., 2003).

Based on this association of AGAP2 with a specific AP-1-dependent compartment, we have concluded that specific interaction with clathrin adaptor proteins may be one determinant of the site of AGAP action but is not the sole determinant. AGAP2 without GAP activity could bind to AP-1 (data not shown), but did not associate with AP-1 at the TGN, supporting the idea that AGAP2 binding to AP-1 is not the predominant determinant of site of action. Additional factors that could determine the site of action of AGAP2 include cargo proteins and phospholipids. The role of cargo proteins in directing Arf GAPs to specific cellular sites has been examined for both ACAP1, which binds directly to transferrin receptor (Dai et al., 2004), and Arf GAP1, which binds to KDEL receptor (Aoe et al., 1997). Phospholipids, particularly phosphoinositides, have also been implicated in targeting membrane trafficking proteins to specific sites (Lemmon, 2003). The PH domain of AGAP2 does interact with phosphoinositides and, therefore, could have a role in targeting. If multiple signals were involved, coincidence of these signals on a particular membrane could result in greater specificity for a site than might be achieved through any single targeting signal.

In addition to targeting, the PH domain of AGAP2 has two other functions that could be integrated for the purposes of temporal and spatial regulation of AGAP2 and, consequently, AP-1. First, the PH domain mediates binding to AP-1. Second, if the PH domain is like that of ASAP1, it folds with the GAP domain, regulating GAP activity (Kam et al., 2000; Che et al., 2005). With these functions contained within a single domain, phosphoinositide binding could influence AP-1 binding, AP-1 binding could influence phosphoinositide binding and, as described here, each can affect GAP activity. Describing these possible functional relationships will be an important step for understanding the molecular mechanisms by which Arf GAPs regulate membrane traffic and Arf, particularly considering that Arf directly activates enzymes catalyzing the production of acid phospholipids (Cockcroft et al., 2002; Honda et al., 1999).

The specific molecular mechanism by which AGAP2 affects the AP-1/Rab4 compartment is still being defined. The GAP activity is necessary for this effect. An AGAP2 mutant lacking GAP activity did not affect AP-1 distribution in cells whereas wild type AGAP2 did. By the current paradigm (Randazzo et al., 2000b; Hirsch et al., 2003), AGAP2 would induce the hydrolysis of GTP on Arf, preventing the association of coat protein with membranes and blocking the formation of transport intermediates. However, by this paradigm, expression of wild-type protein should cause the dissociation of AP-1 from membranes. Instead, AP-1 was redistributed to another vesicle population, Rab4 endosomes. Furthermore, AGAP2-induced reduction of transferrin accumulation was blocked by a dominant negative Rab4 mutant. Since the effect of this Rab mutant is to block exit from the fast recycling endosome (De Renzis et al., 2002), the effect of AGAP2 on transferrin accumulation could result from accelerated exit from the compartment. These results, together with direct association of AGAP2 with AP-1, have led us to consider whether AGAP2 could function as part of the coat, with a direct role in the formation of transport intermediates. Increased expression levels of AGAP2 could accelerate transport from a particular compartment. The possibility of a direct role in formation of transport intermediates has been discussed in the context of results obtained with other Arf GAPs. For instance, formation of COPI vesicles from the Golgi apparatus was found to be dependent on Arf GAP1 (Yang et al., 2002). Also, ASAP1 has been found to drive the tubulation of membranes, an early step in the formation of a transport intermediate (Z.N. et al., unpublished).

AP-1 at the TGN appeared to be more sensitive to AGAP2 than AP-1 in the Rab4 endosome. This result could be explained in a number of ways. One possibility is that AGAP2 is more active in the TGN, causing inactivation of Arf1 and, consequently, dissociation of AP-1, as described in the preceding paragraph. Because we were unable to visualize endogenous protein with the antibodies that we have available, we cannot exclude that AGAP2 is physiologically present in the TGN. However, because of the considerations discussed above, other explanations seem equally plausible. For instance, one possibility is that the AGAP2-AP-1 complex is site-specifically recruited to endosomes. An increase in AGAP2 concentration would drive, by mass action, an AGAP2-AP-1 complex to endosomes, sequestering AP-1 from the TGN. Another possibility is that the AGAP2-AP-1 complex is necessary to form a transport intermediate that exits from the TGN and docks with a Rab4 positive compartment that is normally transient but becomes evident with elevated AGAP2 concentrations.

AGAP1 has also been found to affect AP-1 as described in the first report implicating AGAPs as regulators of membrane traffic: however, there were important differences between the AGAP1 and AGAP2 effects on AP-1. In Nie et al. (Nie et al., 2002), overexpression of AGAP1 caused AP-1 to be redistributed to a compartment consisting of large punctate structures that contained Rab4 and Rab11. In the current study, AP-1 redistributed to a compartment of smaller punctate structures containing Rab4 but not Rab11. Thus, based on markers, AGAP1 and AGAP2 caused the redistribution of AP-1 to different structures. AGAP1 could have had an effect on AP-1 in Nie et al. (Nie et al., 2002) because it was expressed at higher levels than in any of the experiments described in this paper. The effect on AP-1 could have been indirect, due to a drastic disruption of another branch of the endocytic pathway. Indeed, we anticipate some indirect effects of AGAP2 on the endosomal pathway secondary to redistribution of AP-1 from the TGN to the Rab4 endosomes. Alternatively, an effect on AP-1 could have been the result of nonspecific targeting of AP-1 by AGAP1 at high protein concentrations. The fact that AP-1 is in different compartments is consistent with the interpretation that the effects of AGAP1 and AGAP2 on AP-1 are through different mechanisms. Nevertheless, the two papers (Nie et al., 2002; Nie et al., 2003), together with this paper, are consistent in providing evidence that AGAPs affect endocytic pathways, whereas Nie et al. (Nie et al., 2003) and this paper

support the hypothesis that AGAPs are able to discriminate between clathrin adaptor proteins.

AP-1 can be present in different membrane domains of endosomes. In epithelial cells, AP-1 was present on the transferrin-positive recycling endosomes (Folsch et al., 2003; Ang et al., 2004). AP-1B was also colocalized with exocyst subunits and Rab8 (Ang et al., 2003). It ensures polarized sorting by capturing cargo and promoting recruitment of targeting and fusion machinery (Traub and Apodaca, 2003). Our data indicate that AGAP2 affected the AP-1-containing endosomes at an early stage of endocytosis, i.e. the Rab4-positive membrane domains.

We found that AGAP2 has differential expression whereas AP-1 is ubiquitously expressed (Peyrard et al., 1998; Takatsu et al., 1998). This result has led us to speculate that AP-1 may have other GAP binding partners. Site-specific binding to different Arf GAPs would allow AP-1 to function at discreet sites within the cell while being independently regulated. If we consider the Arf GAP to be part of the coat, then different coats could be produced by changing the GAP-AP partners. Specificity could rely on the GAPs. In this speculative model, an Arf GAP other than AGAP2 could be associated with AP-1 at the TGN. Further studies are needed to identify additional Arf GAPs that may interact with AP-1 and to identify the determinants for Arf GAP site specificity. Elucidation of the molecular mechanisms by which AGAPs regulate membrane traffic will lead to a better understanding of how the Arf GAPs are involved in the highly selective transport of cargo between organelles in eukaryotic cells.

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