AGAP1, an Endosome-associated, Phosphoinositide-dependent ADP-ribosylation Factor GTPase-activating Protein That Affects Actin Cytoskeleton

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We have identified three members of the AGAP subfamily of ASAP family ADP-ribosylation factor GTPase-activating proteins (Arf GAPs). In addition to the Arf GAP domain, these proteins contain GTP-binding protein-like, ankyrin repeat and pleckstrin homology domains. Here, we have characterized the ubiquitously expressed AGAP1/RhA1099. AGAP1 had Arf GAP activity toward Arf5, Arf6, Phosphatidylinositol 4,5-bisphosphate and phosphatidic acid synergistically stimulated GAP activity. As found for other ASAP family Arf GAPs, the pleckstrin homology domain was necessary for activity. Deletion of the GTP-binding protein-like domain affected lipid dependence of Arf GAP activity. In vivo effects of AGAP1 were distinct from other ASAP family Arf GAPs. Overexpressed AGAP1 induced the formation of and was associated with punctate structures containing the endocytic markers transferrin and Rab4. AP1 was redistributed from the trans-Golgi to the punctate structures. Like other ASAP family members, AGAP1 overexpression inhibited the formation of PDGF-induced ruffles. However, distinct from other ASAP family members, AGAP1 also induced the loss of actin stress fibers. Thus, AGAP1 is a phosphoinositide-dependent Arf GAP that impacts both the endocytic compartment and actin.

The Arfs are a subfamily of GTP-binding proteins, within the Ras superfamily, comprised of the Arf and Arf-like (Arl) proteins. The Arf proteins can be divided into classes I, II, and III on the basis of primary structure (1, 2). Arf proteins were first identified as a cofactor for cholera toxin-catalyzed ADP-ribosylation of the heterotrimeric G-protein Ga (3). In normal physiology, Arfs regulate membrane traffic (2, 4, 5) and also affect actin cytoskeleton dynamics (6–8). A single Arf gene product can function at multiple sites within a cell and affect both membrane trafficking and actin remodeling. Furthermore, multiple Arfs can exist within a single cell. Therefore, regulatory mechanisms are likely specific to Arf gene products and cellular site. Because efficient function of Arf requires the controlled binding and hydrolysis of GTP by Arf, the accessory proteins that catalyze this cycle, guanine nucleotide exchange factors (GEFs) (9) and GTPase-activating proteins (GAPs) (4, 10, 11), are candidates for mediating these two levels of regulation. The roles of Arf guanine nucleotide exchange factors and GAPs in Arf and site-specific regulation are being examined.

The first Arf GAP identified was Arf GAP1 (12). The protein consists of a catalytic domain and a targeting domain, both of which are necessary for the regulation of Arf1 in the Golgi apparatus (13). Other Arf GAPs, such as the GITs (14–18), and the ASAP-type Arf GAPs, ASAPs (19, 20), ACAPs (21), and ARAPs (22, 23, 24), have more complex structures. ASAP-type Arf GAPs are defined by a core of a pleckstrin homology (PH), Arf GAP and ankyrin (ANK) repeat domains. The ASAP proteins are further divided on the basis of additional domains. The ACAPs have a coiled coil domain (21), the ASAPs have Src homology 3 and proline-rich domains (19), and the ARAPs have Rho GAP, Ras-associating domains, and five PH domains (22, 23). Each of the ASAPs has been found to affect the actin cytoskeleton. ACAPs have effects secondary to the inactivation of Arf6 (21). ASAP1 affects the dynamics of proteins that comprise focal adhesions (24). ARAPs have two effects; one is to inactivate Rho, and the other is to increase Cdc42 activity as a consequence of Arf GAP activity (22, 23).

Here, we examine a member of a fourth subgroup within the ASAPs, the AGAPs. This subgroup contains a GLD (GTP-binding protein-like domain) N-terminal to the core of PH, Arf GAP, and ANK repeat domains. The proteins are also distinguished from other ASAP family members in having a split PH domain. We found that AGAP1, like other ASAP family members, is a phosphoinositide-dependent Arf GAP and affects the actin cytoskeleton. However, distinct from other Arf GAPs, AGAP1 induces and localizes to Rab4/AP1 containing structures presumed to be endosomes and specifically alters stress fibers. Both effects require Arf GAP activity; the effect on stress fibers is partly dependent on the GLD of AGAP1. Based on these results, AGAP1 is a possible link between endocytic traffic and the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

Plasmids—KIAA1099 cDNA clone was kindly provided by Dr. Taka-hiro Nagase at the Kazusa DNA Research Institute (Chiba, Japan). The coding region for AGAP1 or different domains were amplified by polyn-
AGAP1 Regulates Actin

**A. AGAP1**

1. **MNVVQDLANS AARAIRFQP RSVMHTYS YLLRVEPEF VQNOQREBV TAEIEAPNS**
2. **QWTLRSEVP ELCXQVHN AGKALYVR YLTOOTGEV SPVQCKREKV UPQDPSYVL**
3. **LDIRBGKRES AGAPMDFAV IFVPELETR SPVTYHTS EFVMAENTAR 1P1VUWTO**
4. **BSSAPREE DEEPEEETE CATLYQVEF VFQVQATLV LTERQG**
5. **EVTYKPAEV EEHPEEHPEE EAHECTPEE EPEHEE**
6. **IAVAKPKEV TANLLEKEK KPECVKEV KPELREKKE**
7. **LNQBMKEVY TLQGLOVITY HPSLDHVIDQ VHQORIDLLR TTQVWDQK**
8. **SSPNETGLK DSMQIHIPN DATLQGDSV SQPISIHT PSKQPFSFPH ANWKHRK**
9. **STAFFQADL SOTAQSAEAN PFPIVPLLQ QNPIFYATTY BERAMYAQE BQFPLQ**
10. **CESQHAGL AQHOAMLGQ CVQCTQHNSK HASLQALAGM CIRCQGH**
11. **LOTLQHEVQY LOLDQWNLQK LKQMNSSE ANWILQVMK QRTIKQGLV MKEEKN**
12. **AKWQKGLPA PLTLPSLQ QHLRATAD EELTAIIA HSQDENVET COQBEPTAL**
13. **HACRQYHQV LQLLIMQV YUDTADAM CPALYANQAS SQQICDVLQ YQOQSDPV**
14. **MATHPQHLD NWHRQSGK FY**

**G-protein like**

AGAP1: 73-217 (72%, K-Ras)
AGAP2: 70-230 (28%, Rab9)
M-RIP: 91-249 (27%, K-Ras)

**C. AGAP1**

1. **KVQYVHLAQGRKALVGRELWLOVYKSLFTQVEPPRQKEIUVDDQ**
2. **K-Ras**
3. **M-DIQGRAPHK**
4. **LQIVPFN**

**D. ETK:**

1. **VILESMFL**
2. **PIQGKLQ**
3. **PIQGKLQ**
4. **M-RIP:**

**Fig. 1. Primary structure of AGAP1.** A, amino acid sequence of AGAP1. The structural domains were identified using Pfam. The GLD is underlined, PH domain is in bold, and Arf GAP domain is underlined. B, schematic structural domains of AGAPs. The percentages of identity of the GLD to different G-proteins are indicated in parentheses. The starting and ending residues of each domain are numbered as identified by Pfam. C, comparison of AGAP1 GLD to K-Ras. Alignment was performed with ClustalW program. Consensus GTP binding motifs are in bold. Switch I and Switch II regions are boxed. D, comparison of PH domains of AGAPs with that of human Bruton tyrosine kinase (BTK). Alignment was performed with ClustalW program and subsequent manual adjustment. Consensus motif for D3-phosphorylated phosphoinositol binding (30, 31) is shown at the bottom, and the conservative mutation from arginine to lysine is indicated with an arrow.
The reverse primer for human cDNA was GCTGATGTGCACGGCAGACACC (nucleotides 1369 to 1347 of KIAA1099). Thermal cycling was performed under the conditions specified by the manual from Clontech Laboratories, Inc. PCR products were fractionated on a 1% agarose gel and stained with ethidium bromide.

**Cell Culture and Immunofluorescence**—NIH 3T3, COS7, U87, and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS at 37 °C with 5% CO₂. PC6 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS at 37 °C with 5% CO₂. Cells were transfected using LipofectAMINE 2000 (Invitrogen) and harvested 24 h.
The antisera were used subsequently for Western blot. Inhibition of actin polymerization with phalloidin and anti-FLAG antibody (25). At least 100 cells were seeded in phosphate-buffered saline for 10 min and stained with rhodamine-phalloidin, and transfected cells were detected using an antibody to the epitope tag. Cells were later for reseeding, immunoblotting, or immunoprecipitation. Cells were visualized by staining with rhodamine-phalloidin, and transfected cells were detected using an antibody to the epitope tag. NLS-AGAP1 signal by the peptide. The peptide (1 mg/ml) to which the antisera were raised was incubated with the antisera for 1 h at room temperature. The antisera were used subsequently for Western blot.

later for reseeding, immunoblotting, or immunoprecipitation. Cells were deprived of serum for 12 h before growth factor stimulation. Cells were visualized by staining with rhodamine-phalloidin, and transfected cells were detected using an antibody to the epitope tag. Cells were examined by confocal microscopy using a Zeiss Pascal confocal mounted on an Axioscop 2 microscope equipped with an 100× Plan-Neofluor oil immersion lens (Carl Zeiss). For PDGF-induced dorsal ruffle assays, cells were incubated for 6 h and then treated with 10 ng/ml PDGF for 4 min. For transferrin uptake, NIH 3T3 cells were incubated with regular phosphate-buffered saline for 10 min before incubation with transferrin for 30 min. For transferrin uptake, NIH 3T3 cells were incubated with regular phosphate-buffered saline for 10 min before incubation with transferrin for 30 min. For transferrin uptake, NIH 3T3 cells were incubated with regular phosphate-buffered saline for 10 min before incubation with transferrin for 30 min.

Protein Expression and Purification—N-terminal FLAG epitope-tagged AGAP1 (amino acids 1–804), [ΔGLD]AGAP1 (amino acids 347–804), [ΔGLD,ΔPH]AGAP1 (amino acids 556–804), or GLD (amino acids 69–317) were overexpressed in HEK293T cells by transient transfection. The cells were lysed in 20 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, and 1% Triton X-100. The lysates were incubated with anti-FLAG affinity gel at 4 °C overnight. The tagged proteins were eluted with Flag peptide for 30 min at 4 °C. For GST fusion proteins AGAP1 (1–804), [ΔGLD]AGAP1 (347–804), or GLD (69–317) were expressed in BL21 cells and purified with glutathione-Sepharose 4B gel (Amersham Biosciences). Both GST fusion proteins and FLAG epitope-tagged proteins were used for Arf GAP assays with nearly identical results. Arf proteins used in the assays were purified as described before (26).

Point mutants and truncation mutants of AGAP1 were expressed in HEK293T and BL21 cells. Based on homology modeling, the change in these proteins AGAPs for Arf GAP with GTP-binding protein-like, ANK repeat and PH domains. In this nomenclature, KIAA1099 and KIAA0167 are AGAP1 and AGAP2.

The GLDs of the AGAPs, though highly similar to Ras family proteins, have some critical differences in primary structure. The GLD of AGAP1 is 51% similar and 27% identical to K-Ras, AGAP2 is 42% similar and 28% identical to Rab9, and M-RIP is 47% similar and 27% identical to H-Ras (Fig. 1B). Three GTP binding motif consensus sequences are well conserved, GXXXGK(S/T) (residues 78–85 in AGAP1; see Fig. 1C, bold), DXXG (residues 124–127 in AGAP1; see Fig. 1C, bold), and TCAT (residues 210–213 in AGAP1; see Fig. 1C, bold). However, glutamine 61 of Ras (threonine 61 in Rap) is replaced with a proline in AGAPs, and there is no identifiable NXKD motif. In AGAP1, the stretches over switch I and II (Fig. 1C, box) have 25 and 17% identity to K-Ras (29). Residues in switch I critical for

**RESULTS**

The AGAP Subfamily of ASAP-type Arf GAPs—We have defined ASAP-type Arf GAPs to be proteins containing a core of a PH, Arf GAP, and ANK repeat domains. Database searches revealed three cDNA-encoding proteins containing these structures, KIAA1099 (accession number AB029022), KIAA0167 (accession number D79989), and M-RIP (accession number AF359283). These three proteins are distinguished from other ASAP family proteins in two ways. First, they contain an N-terminal GLD (GTP-binding protein-like domain). Second, the PH domain is split (Fig. 1A, bold in box). We propose calling these proteins AGAs for Arf GAP with GTP-binding protein-like, ANK repeat and PH domains. In this nomenclature, KIAA1099 and KIAA0167 are AGAP1 and AGAP2.

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**Detection of Nucleotide by HPLC—Nucleotides copurified with proteins were detected by the method described (27) with slight modifications. Proteins were denatured by the addition of 1 M formic acid and separated from nucleotides by centrifugation. The supernatant was lyophilized and resuspended in a buffer containing 50 mM sodium phosphate, pH 6.5, 0.2 mM tetrabutylammonium hydrogen, 3% (v/v) acetonitril, and 0.2 mM NaN3. The nucleotides were then fractionated on a C18 column developed by isocratic elution with the suspension buffer. Absorbance was monitored at 252 nm. Under this condition, the retention times for GDP, GTP, ADP, and ATP are 6.2, 9.5, and 17 min, respectively. Arf was used as a positive control.

**Equilibrium Binding—Nucleotide binding was measured using GST-GLD and GST-AGAP1 fusion proteins bound to 10 μl of glutathione beads.** The immobilized proteins were incubated with 0.25 μM radiolabeled GTP, GTP-γS, or ATP in 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, either 0.5 mM MgCl2 or 2 mM MgCl2 (high Mg2+), 0.1% Triton X-100, 360 μM PA, and 45 μM PIP2, in a total volume of 50 μl. After 5 min at room temperature, the reactions were centrifuged briefly at 500 × g, and 40 μl of the supernatant was removed. The radioactivity in the supernatant and the remaining pellet was quantified by scintillation spectroscopy.

Similarly, ArfGTP-γS binding to AGAP1 was measured by immobilizing GST-AGAP1 on glutathione beads, incubating the protein under the same conditions as the GAP assay with Arf[32P]GTP-γS, and determining the amount of Arf[32P]GTP-γS associated with the beads by scintillation spectroscopy.
interacting with the Ras effector Raf, Tyr-32, Thr-35, and Asp-38 in Ras are Glu, Glu, and Arg in AGAP1.

The PH domains of the AGAPs are distinct from those in other ASAP family Arf GAPs. They are split and, therefore, extend over 189 residues (Fig. 1A, bold in box). AGAP1 contains nine of the ten residues identified in the phosphatidylinositol 3,4,5-trisphosphate (PIP3) binding consensus sequence (30, 31) including a tyrosine that is present in all identified PIP3 binding PH domains (Fig. 1D). The difference is conservative, with a lysine substituting for an arginine (Fig. 1D, arrow).

We have characterized the product of the AGAP1 gene, a protein with 804 amino acids (Fig. 1A) and a calculated molecular mass of 89 kDa. Expressed sequence tag database search revealed a mouse homologue of AGAP1 (accession BF322239), which is 96% identical (179/187) to AGAP1 at the amino acid level and 87% (489/559) identical to AGAP1 at the nucleic acid level for the 187 amino acids encoded by the expressed sequence tag. Based on reverse transcriptase-PCR using cDNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, AGAP1 is expressed in all the tissues tested (data not shown).

AGAP1 Is a PA/PIP2-dependent Arf1 GAP—AGAP1 expressed in bacteria as a GST fusion protein and in mammalian cells as an epitope-tagged protein was purified and examined as an Arf GAP. Both bacterially and mammalian-expressed AGAP1 contained Arf1 GAP activity. Activity was dependent on phospholipids. In the absence of other phospholipids, 10 and 45 \( \mu \text{M} \) PIP3 stimulated GAP activity to a greater extent than did PIP2. However, phosphatidic acid potentiated PIP2 stimulation, which was not observed for PIP3 (Fig. 2A). An interaction between PA and PIP2 was indicated by two-way ANOVA analysis. The synergy was specific for PA. Phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC) had less of an effect on PIP2-stimulated activity (Fig. 2B). Repre-
sentative proteins from each class of Arf, Arf1, Arf5, and Arf6, were used as substrates for AGAP1 under optimal conditions. Both bacterially expressed GST-AGAP1 (Fig. 2C) and mamma-
lian-expressed FLAG-AGAP1 (not shown) used Arf1 as the preferred substrate.

We next examined the structural requirements for Arf GAP activity as defined for other ASAP family members. Different AGAP1 constructs were expressed in HEK293T cells, immuno-
precipitated with M2 gel, and eluted with FLAG peptide. Equal amounts of protein were used in these assays. We found that AGAP1 was similar to other ASAP family members in three structural requirements as follows. (i) A zinc finger was necessary. The zinc finger motif could be disrupted by substituting a serine for a cysteine. This mutation resulted in a protein with no activity (Fig. 2D). (ii) The arginine five residues C-terminal to the last cysteine defining the zinc finger was also required for activity. A conservative substitution of a lysine resulted in a protein with no activity (Fig. 2D). This mutation was unlikely to cause the protein to misfold. Homology modeling (Fig. 2E) was carried out against the crystal structure of the Arf GAP domain of PAP3 (32), using Homology/Discover module within Insight II (Accelrys, San Diego, CA). Based on this result, a conservative mutation of arginine 599 to lysine is expected to have minimal effect on the stability and structure of the protein. Furthermore, mutation of arginine 599 to lysine did not affect the ability of GST-AGAP1 to bind to Arf\(\gamma\)S. GST-[\(\Delta\)GLD]AGAP1 (1.5 \(\mu\)M) and GST-[R599K\(\Delta\)GLD]AGAP1 (1.5 \(\mu\)M) bound 21.4 ± 1.8% and 19.9 ± 2.5% of the Arf\(\gamma\)S bound in an \textit{in vitro} binding assay (33). (iii) The PH domain was necessary. Whereas the protein comprised of the PH, Arf GAP (zinc finger motif), and ANK repeats ([\(\Delta\)GLD]AGAP1) had activity comparable with full-length AGAP1, a protein comprised of the Arf GAP and ANK repeats ([\(\Delta\)GLD, \(\Delta\)PH]AGAP1) did not have any detectable activity (Fig. 2D). Based on analogy with PAP\(\alpha\), we had no reason to suspect that [\(\Delta\)GLD,\(\Delta\)PH]AGAP1 was misfolded. Similar results were ob-

Fig. 5. Effect of AGAP1 on trans-
ferrin (Tfn) distribution in NIH 3T3
cells. NIH 3T3 cells were transfected with
FLAG-AGAP1 (A–C), FLAG-[\(\Delta\)GLD]A-

GAP1 (D–F), FLAG-[R599K]AGAP1 (G–I),
and FLAG-GLD (J–L) for 24 h. Cells were replated on fibronectin-coated coverslips in
regular Dulbecco’s modified Eagle’s me-
dium with 10% FBS for 6 h. Cells were
incubated with serum-free medium for 30
min before incubation with Tfn for 30 min.
The cells were then washed and fixed for
staining with M5 antibody to visualize trans-
fected cells. Distribution of Tfn in re-
presentative untransfected cell is shown in
panel M. Both AGAP1 (A–C) and [\(\Delta\)GLD]A-
GAP1 (D–F) induced redistribution of Tfn
into the punctate structures, as indicated
by arrows in panels A, B, D, and E, respec-
tively. [R599K]AGAP1 (G–I) and GLD
(J–L) did not affect the distribution of Tfn
under the experimental conditions.
tained with the GST-tagged proteins purified from bacteria, except for [C594S]AGAP1, which was insoluble (not shown), consistent with the suspected unfolding of the zinc-finger-containing domain.

GLD Affects the Phospholipid Dependence of Arf GAP Activity—Because of the similarity of the GLD to K-Ras, we examined AGAP1 for analogous function. First we tested for nucleotide binding under a number of conditions, including the presence or absence of detergent, and/or phosphoinositides, in the presence of 1 mM MgCl2 or Mg2+ buffered to 1 μM with EDTA. No high affinity binding of GDP, GTP, or GTPγS was detected by a nitrocellulose filter binding assay under any of these conditions. The presence of nucleotide on purified AGAP1 was examined by denaturing the protein in formic acid and detecting released nucleotide by HPLC. No GDP, GTP, ADP, or ATP was detected on purified GLD. We also could not detect any low affinity ATP, GTP, or GTPγS binding in equilibrium dialysis experiments (not shown). Finally, no GTPase activity was detected in full-length AGAP1 or the isolated GLD (not shown). Similarly, we could not detect any effect on the Ras phosphorylation cascade. Overexpression of AGAP1 in PC6 or COS7 cells had no effect on MAP kinase phosphorylation observed in cells maintained in serum-free medium, either in the presence or absence of epidermal growth factor (34) or nerve growth factor (35). AGAP1 also had no effect on the Ras phosphorylation induced by coexpressing v-Ras (36) (data not shown). However, stimulation by PIP2 did not require, nor was it synergistic with, PA. At concentration range from 11.25 to 90 μM, PIP2 activated [ΔGLD]AGAP1 GAP activity to a similar extent regardless of whether PA was present (Fig. 2F).

In Vivo Analysis of AGAP1 Function—To examine the in vivo function of AGAP1, we first examined the expression of endogenous AGAP1 by Western blot using an antibody raised to a peptide corresponding to the C terminus of AGAP1. In lysates from U87, HeLa, and HEK293T cells, two forms of the protein were found. One band was detected at the predicted molecular mass and one additional band was at a slightly greater molecular mass than predicted. This difference could represent either a covalent modification or splicing variation. In NIH 3T3 cells, the molecular mass was slightly greater than predicted for AGAP1 (Fig. 3A). A species of the same molecular mass was noted in skeletal muscle (data not shown). The difference in molecular mass is most likely the result of different splicing of the message. In addition, immunoprecipitated FLAG-AGAP1 and purified GST-ΔGLD/AGAP1 were also recognized by the antisera. These specific bands were blocked with addition of the peptide to which the antisera were raised (Fig. 3B). These results indicated that the bands around 98 kDa and above as recognized by the antisera represent the endogenous AGAP1.

AGAP1 associated with an endocytic compartment. U87 and NIH 3T3 cells expressing FLAG-epitope tagged AGAP1 were fixed and stained with an antibody to the FLAG epitope. Signal was detected in punctate structures in the periphery of the cell (see Fig. 7A and Fig. 8B, arrows). At higher expression levels the signal was more diffuse. To determine whether the punctate structures represented a membrane-bound organelle, we co-stained cells for a number of known organellar markers. Overexpression of AGAP1 caused redistribution of GFP-Rab4 (Fig. 4, A–C and M) and AP1 (Fig. 4, J–L and O) to the punctate structures that contained AGAP1. GFP-Rab11 showed predominant perinuclear localization when coexpressed with AGAP1, with limited colocalization with AGAP1 (Fig. 4, G–I and N). The punctate structures did not contain, nor did AGAP1 affect, the distribution of Rab5 (Fig. 4, D–F), AP2, EEA1, or β-COP (not shown). The colocalization of AGAP1 with Rab4 and Rab11, but not Rab5, was also observed when the cells were stained with antibodies against the different Rab proteins (not shown). The effect on Rab4 and AP1 depended on GAP activity. [R599K]AGAP1, which lacks GAP activity, neither associated with the punctate structures nor colocalized with AP1 and Rab4. The effect on AP1 and Rab4 did not depend on the GLD. [ΔGLD]AGAP1 induced the punctate structures containing Rab4 and AP1 (not shown). The isolated GLD did not induce the structures. Given that both Rab4 and AP1 are associated with endocytic traffic, the punctate structures are likely an endocytic intermediate affected by AGAP1.

Fig. 6. AGAP1 inhibits PDGF-induced dorsal ruffles in NIH 3T3 cells. A, effect of AGAP1 on PDGF-induced dorsal ruffles. NIH 3T3 cells were transfected with different constructs of AGAP1 at 3 μg DNA/35-mm well using LipofectAMINE 2000 for 24 h. Cells were then trypsinized and re-seeded on fibronectin-coated coverslips for 8 h before they were treated with PDGF at 10 ng/ml for 4 min. Dorsal ruffles were visualized using rhodamine-phalloidin. One hundred cells were counted for control and different transfections in each experiment. Data are the mean ± S.E. of one experiment representative of three with similar results. *, p < 0.05; **, p < 0.01 compared with FLAG empty vector transfection control as analyzed by one-way ANOVA with Tukey post-test. B, expression level of different AGAP1 constructs in NIH 3T3 cells. Cells were transfected as in A and were harvested 24 h after transfection. Cell lysates were used for SDS-PAGE and Western blot and detected by M5 antibody against the FLAG tag.

Z. Nie and P. A. Randazzo, unpublished observation.
To further test for a role of AGAP1 in endocytic traffic, we looked at its effect on transferrin uptake. NIH 3T3 cells were loaded with rhodamine-conjugated transferrin for 30 min. In untransfected NIH 3T3 cells, transferrin was in punctate structures distributed evenly throughout the cytosol (Fig. 5M). Overexpression of AGAP1 resulted in the accumulation of transferrin into larger punctate structures in the cell periphery, similar to those containing Rab4 and AP1 (Fig. 5, A–C). This effect of AGAP1 was dependent on its Arf GAP activity. [AGLD]AGAP1 showed similar effect in accumulating transferrin in these punctate structures (Fig. 5, D–F). [R599K]AGAP1, which is deficient in GAP activity, did not
induce redistribution of transferrin (Fig. 5, G–I). The GLD domain does not have a direct role in this effect of AGAP1. Overexpression of GLD had no observable effect on the distribution of transferrin (Fig. 5, J–L).

All ASAP family members examined to date affect the actin cytoskeleton when overexpressed. Here, we examined whether AGAP1 also had effects on actin. We used NIH 3T3 cells as a model, because they are well characterized in this regard. When overexpressed in NIH 3T3 cells, AGAP1 inhibited PDGF-induced ruffling effectively (Fig. 6A). This effect was dependent on Arf GAP activity. [ΔGLD]AGAP1 was as effective as the full-length AGAP1. [ΔGLD,ΔPH]AGAP1, which also lost GAP activity, had only a small effect on ruffling. [R599K]AGAP1, which also lacked GAP activity, was about 50% as effective as wild type protein indicating that interactions, in addition to the GAP activity, might also be involved in inhibiting ruffling. These differences in the effects of the protein were not a result of differences in

Fig. 8. Effects of AGAP1 in U87 cells. A, expression level of different AGAP1 constructs in U87 cells. U87 cells were transfected with different constructs at 3 μg DNA/35-mm well for 24 h, cleared cell lysates were used for Western blot, and the overexpressed proteins were detected by M5 antibody against the FLAG tag. B, morphological alterations of U87 cells. The left panel shows the staining of actin with rhodamine-phalloidin, and the right panel shows the transfected cells stained with M5 antibody. Twenty-four h after transfection, cells were re-seeded on glass coverslips in Dulbecco's modified Eagle's medium with 10% FBS for an additional 6 h before they were fixed and stained. C, structural requirements for AGAP1-induced loss of stress fibers. U87 cells were transfected with FLAG-tagged AGAP1 constructs at 3 μg DNA/35-mm well for 24 h, re-seeded on glass coverslips in regular Dulbecco's modified Eagle's medium with 10% FBS for 6 h, fixed, and stained as described in B. Data are the mean ± S.E. of three experiments. *, p < 0.05; **, p < 0.01 compared with FLAG empty vector transfection control; &, p < 0.05 compared with AGAP1 as analyzed by one-way ANOVA with Tukey post-test.
protein expression. \([R599K]AGAP1, \{\Delta GLD, \Delta PH\}AGAP1,\) and GLD, which did not block ruffling, were expressed at a similar or higher level to AGAP1 and \{\Delta GLD\}AGAP1, which did block ruffling (Fig. 6B).

Examination of NIH 3T3 cells also revealed a specific effect of AGAP1 on the actin cytoskeleton. Cells had fewer stress fibers and a thickened cortical actin around the cell periphery (Fig. 7A, medium arrow). This effect on stress fibers was specific for AGAP1. Two other members of this family, ASAP1 and ACAP1, had only a small effect on stress fibers in NIH 3T3 cells (Fig. 7B). In the case of AGAP1, although there was a loss of stress fibers, the cells remained flat. Further analysis of the structural requirements of AGAP1 to induce this effect on actin revealed several characteristics of the protein. First, multiple domains of AGAP1 contributed to this effect. The maximum effect was seen with full-length protein. \{\Delta GLD\}AGAP1, a functional Arf GAP, was less effective than wild type protein (Fig. 7C). The isolated GLD, though to a lesser extent, also affected stress fibers. Therefore, both the GLD and the Arf GAP domain contributed to this effect on actin stress fibers. Second, Arf GAP activity contributed to the effect of the protein on stress fibers. \([R599K]AGAP1,\) which lacked GAP activity, had less of an effect. Expression levels are unlikely to cause these differences (Fig. 7D). The GLD also induced morphological changes that were distinct from the effects on stress fibers. Cells expressing the isolated GLD were more likely to be elongated with filopodia (Fig. 7A, arrowhead). This effect of the GLD was attenuated in the context of the full-length protein.

The in vivo effects of AGAP1 were similar in U87 cells. FLAG-tagged AGAP1 constructs were expressed in U87 cells (Fig. 8A). First, as in NIH 3T3 fibroblasts, both AGAP1 and \{\Delta GLD\}AGAP1 induced the accumulation of the punctate structures presumed to be endocytic intermediates (Fig. 8B, arrow). Second, overexpression of AGAP1 in U87 cells also resulted in loss of actin stress fibers (Fig. 8C). Although all examined proteins had an effect, the full-length wild type AGAP1 was most effective. Third, when GLD was overexpressed in U87, the cells were more likely to appear in an elongated form, and about 50% cells had pseudopodia or long processes (Fig. 8B, arrowhead). This effect of GLD was attenuated or blocked in the context of the full-length protein.

DISCUSSION

Here we describe a fourth subgroup of the ASAP family of Arf GAPs, the AGAPs. These proteins have a G-protein like domain, in addition to the core of PH, Arf GAP, and ANK repeat domains seen in other ASAP family members. We characterized one gene product, AGAP1. AGAP1 had PA- and PIP,-dependent Arf GAP activity. The GLD imparted the PA dependence. AGAP1 induced the formation of punctate structures that contained Rab4 and AP1 and were an intermediate in transport from other ASAP-type Arf GAPs are the GLD and the split PH domain. Although the GLD of AGAP1 is highly similar to K-Ras at 27% identity, it lacks NKXD, the motif involved in binding the nucleotide ring (37). Consistent with this structural deficiency, our data indicate that AGAP1 is not a GTP-binding protein. We cannot exclude the possibility that under a specific set of conditions, the equivalent of NKXD is contributed by another part of AGAP1 or from an interacting protein. With the high level of homology with K-Ras, we also tested for a role of AGAP1 in the Ras-dependent MAP kinase pathway. However, we did not find any effects.

Although we could not detect Ras-like function (38, 39), we did find that GLD affected lipid dependence. Like ASAP1 (40), AGAP1 was activated synergistically by PA and PIP,, the presence of the GLD contributed to the effect of PA. With the deletion of the GLD domain, PA was no longer required, nor did it potentiate the effect of PIP for GAP activity. One explanation is that GLD is conferring an inhibitory effect on the Arf GAP activity. Interaction of PA with GLD could remove this inhibition. Another possibility is that the GLD interacts with the PH domain, affecting the lipid binding properties of the latter. PH domains have been found to fold together with adjacent domains in other proteins (41). We have found some indication that this is true for ASAP1 (42). The insert in the PH domain of AGAP1 could mediate this or other interactions and confer another level of regulation of GAP activity.

The association of AGAP1 with Rab4-containing endosomes distinguishes AGAP1 from other Arf GAPs. The localization was dependent on a functional Arf GAP domain. However, ARAP1 binding to AGAP1 is not likely involved in targeting, because [R599K]AGAP1 also binds Arf but does not associate with endosomes. We speculate that the split PH domain of AGAP1 has a role in targeting to the endosomal structures. The colocalization with Rab4 suggests that AGAP1 functions in transport between early and recycling endosomes (43). The colocalization of AP1 also suggests a role in endosome function; however, in this case, the movement would be from the trans-Golgi network (44). The coincidence of AP1 with Rab4 may indicate that the overexpression of AGAP1 slows transport out of the recycling endosome or some intermediate, causing the accumulation of material in this particular compartment. This “trapping” of an intermediate would explain the colocalization of AP1 and Rab4, which is not normally observed. Furthermore, the distribution of endogenous AGAP1 is still unknown and may not be identical to that of the overexpressed AGAP1 in the endocytic compartment containing Rab4 and AP1. A difference in the distribution of endogenous and ectopically expressed protein is not unprecedented. Different distribution patterns have been observed with endogenous and overexpressed ASAP1. Although ASAP1 is known to associate with, impact the turnover of focal adhesions, overexpressed ASAP1 is not detected in these structures (19, 24). Thus, although our data do implicate AGAP1 as a regulator of the endocytic compartment, we need to investigate the cellular distribution of endogenous AGAP1 and its relationship to the observed effects of overexpressed protein.

The other distinguishing effect of AGAP1 is reduction of stress fibers. ARAP1 will induce a loss in stress fiber but only when serum is excluded from the cell culture medium. ASAP1 and ACAP1 had much less of an effect. Like the effects on membrane traffic, the effect on actin required Arf GAP activity. Distinct from the membrane traffic effects, this effect of AGAP1 was dependent on both the GLD and Arf GAP activity. The common requirement for Arf GAP activity could result from a number of mechanisms. For instance, the effect on actin may be secondary to an effect on membrane traffic. However, the difference in GLD dependence indicates a more complex mechanism. Possibly, the common requirement ensures that the membranes and actin are affected coordinately, but a distinct machinery actually remodels actin. The effects of the isolated GLD on cell morphology indicate that this may be the case. We are identifying associated proteins in an effort to further define
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the molecular mechanisms underlying the cellular effects of AGAP1.

Among the effects of expression of the isolated GLD was the induction of filopodia and cell elongation. These effects raise the possibility that AGAP1 may influence activity of Rho family GTP-binding proteins. Filopodia are also induced by activation of Cdc42, and overexpression of CEP, a CDC42 effector, induces a phenotype that is similar to that seen on overexpression of AGAP1 (45). It is conceivable that the effects of the GLD are mediated by CEP, Cdc42, or another protein in that pathway.

In summary, we have characterized a phosphoinositide-dependent Arf GAP that is associated with Rab4-containing endosomes and affects a specific aspect of actin remodeling. We speculate that this protein coordinates changes in membranes and actin.

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