GTP-binding Protein-like Domain of AGAP1 Is Protein Binding Site That Allosterically Regulates ArfGAP Protein Catalytic Activity*

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Background: The AGAPs are a subtype of ArfGAPs with a G-protein like domain (GLD) postulated to function as an allosteric binding site.

Results: The C terminus of RhoA binds to AGAP1 and stimulates GAP activity specifically for Arf1 and dependent on the GLD.

Conclusion: AGAP1 is allosterically regulated by specific proteins that bind to the GLD.

Significance: These results are the first example of protein-dependent allosteric regulation of an ArfGAP.

AGAPs are a subtype of Arf GTPase-activating proteins (GAPs) with 11 members in humans. In addition to the Arf GAP domain, the proteins contain a G-protein-like domain (GLD) with homology to Ras superfamily proteins and a PH domain. AGAPs bind to clathrin adaptors, function in post Golgi membrane traffic, and have been implicated in glioblastoma. The regulation of AGAPs is largely unexplored. Other enzymes containing GTP binding domains are regulated by nucleotide binding. However, nucleotide binding to AGAPs has not been detected. Here, we found that neither nucleotides nor deleting the GLD of AGAP1 affected catalysis, which led us to hypothesize that the GLD is a protein binding site that regulates GAP activity. Two-hybrid screens identified RhoA, Rac1, and Cdc42 as potential binding partners. Comunoprecipitation confirmed that AGAP1 and AGAP2 can bind to RhoA. Binding was mediated by the C terminus of RhoA and was independent of nucleotide. RhoA and the C-terminal peptide from RhoA increased GAP activity specifically for the substrate Arf1. In contrast, a C-terminal peptide from Cdc42 neither bound nor activated AGAP1. Based on these results, we propose that AGAPs are allosterically regulated through protein binding to the GLD domain.

The ArfGAPs are a family of proteins encoded by 31 genes in humans (1). The proteins have a common catalytic domain but are otherwise diverse and can be divided into 10 subgroups based on domain structure. The physiologic function of ArfGAPs is thought to be related to the biochemical activity of inducing hydrolysis of GTP bound to Arf family proteins (2), which are regulators of membrane traffic and actin. Consistent with this function, the ArfGAPs have been found to affect membrane traffic and actin (3–5). Mechanisms of regulation of the GAP activity and the relationship of GAP2 activity to biologic function have not been established.

The largest subgroup, the AGAPs, are encoded by 11 genes, and two, AGAP1 and AGAP2, were found to function in post-Golgi trafficking. AGAP1 binds through its PH domain to the clathrin adaptor protein AP-3 (6), which is important for the biogenesis of lysosomes and lysosomal-related organelles (7, 8). AGAP2, which has been implicated in glioblastoma (9), binds to the clathrin adaptor protein AP-1 and affects transferrin recycling (10) and retrograde transport from the early endosome to the trans-Golgi network (11). Both AGAP1 and AGAP2, as recombinant proteins, associate with endocytic structures containing AP-1 and AP-3. The potential role of clathrin adaptor binding on GAP activity was considered, but no effect of AP-1 on the activity of AGAP2 (10) or AP-3 on AGAP1 was detected. The GLD of AGAP1, which is related to Ras superfamily proteins with highest homology to Miro-family proteins (12–15), might have a regulatory role. The role of the GLD in regulating GAP activity has not been explored.

The molecular basis for the function of a G-protein domain within a multidomain protein has been well characterized for several proteins, including leucine-rich repeat kinase 1/2 (LRRK1/2) (16–21), which contains a G-protein domain called ROC, a dimerization domain called COR, and a kinase domain. GTP binding to the ROC domain induces dimerization that increases kinase activity (16, 20).

The GLD of AGAP2 was initially reported to bind nucleotides (22). However, in subsequent work no nucleotide was detected by chemical methods, and nucleotide binding was not detected either by methods that detect high affinity binding sites or by equilibrium binding methods that would detect low affinity binding sites (23). The GLD of AGAP2 was crystallized, and no nucleotide was found (24), although nucleotidase activ-
ity of the purified protein indicated the possibility of a low affinity (mm) binding site. With low affinity nucleotide binding, a plausible hypothesis is that AGAPs are regulated by a similar mechanism to LRRK with nucleotides driving dimerization and activation of the enzyme.

RhoA and RhoB are members of the Ras superfamily of GTP-binding proteins (25, 26). They have been studied mostly as regulators of actin cytoskeleton and cellular adhesions, but some evidence implicates these proteins in the endocytic pathway (27–30). RhoA activation has been reported to inhibit clathrin-mediated endocytosis by activating Rho kinase, resulting in the phosphorylation of endophilin (31, 32). Rho family proteins have been reported to function in similar endocytic traffic as AGAPs. RhoB, at least in part through PRK1, slows traffic from early and late endosomes to lysosomes (28, 29, 33–38). RhoA activation caused redistribution of lysosomes without affecting other endosomal compartments (39–42).

We tested the hypothesis that the GLD of AGAP1 has a role in regulating GAP activity of AGAP1. The GLD domain by itself did not affect activity. In a two-hybrid screen, Rho family GTP-binding proteins were identified as potential binding partners for the GLD domain. Characterization of the interaction revealed that the C-terminal 12 amino acids of RhoA were able to specifically interact with AGAP1 stimulating GAP activity. We conclude that AGAP family proteins can be allosterically regulated by proteins that bind to the GLD.

MATERIALS AND METHODS

Plasmids—Plasmids for bacterial expression of His10AGAP1, His10[347–804]AGAP1 (his10[ΔGLD]AGAP1), GST[69–317]AGAP1 (GST-GLD1), and FLAG-AGAP1 have been described (23). His10[S85N]AGAP1 was made using the QuickChange™ mutagenesis kit (Agilent Technologies, Santa Clara, CA). AGAP1-HA was subcloned into the NotI and EcoRI sites of pcDNA3.1(−) (Invitrogen). HA-AGAP2 and AGAP2-His6 have been described (11). Briefly, HA-AGAP2 was constructed from the full-length MGC image clone (IMAGE id 5218187; GenBank accession number NM014770). BamHI and XbaI sites were inserted in the N terminus and the C terminus of AGAP2, respectively. The cDNA with a sequence encoding an N-terminal hemagglutinin epitope tag was subcloned into pCDNA3.1(−), and 10% cholesterol was prepared as previously described (49). For recombinant His10[ΔGLD]AGAP1, recombinant protein was purified using a 5-ml Histrap HP™ column and further purified by gel filtration using a HiPrep 16/60 Sephacryl S-100 HR™ column (GE Healthcare) and developed in 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM MgCl2, and 5 mM β-mercaptoethanol. LARG DH/PH, His-RhoA, His-Rac1, and His-Cdc42 were expressed and purified as described (43, 47).

GAP Assay—The C50 (the concentration of ArfGAP that induces hydrolysis of 50% of the GTP bound to Arf in a fixed time) was determined as described in Luo et al. (44, 48). Lipids necessary for the reaction were provided as large unilamellar vesicles composed of 40% (mol %) phosphatidylcholine, 25% phosphatidylethanolamine, 15% phosphatidylserine, 9% phosphatidylinositol, 1% phosphatidylinositol 4,5-bisphosphate, and 10% cholesterol prepared as previously described (49). Total phospholipid concentration in the assays was 500 μM.

Synthesis of C-terminal Peptide of RhoA and Cdc42—Peptides were assembled on Wang resin utilizing Fmoc (9-fluorenylmethoxycarbonyl)/tert-butyloxycarbonyl chemistry. The coupling reactions were conducted by means of the 2(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-hydroxybenzotriazole method. Biotinylation at the N terminus was accomplished by the N,N[prime]-diisopropylcarbodiimide/hydroxy succinimide method. Cleavage of the peptide from the resin was achieved with a trifluoroacetic acid/water/triisopropylsilane mixture (92.5/2.5, v/v) at room temperature. After the resin had been removed by filtration, the filtrate was concentrated by flushing with nitrogen gas, and crude peptides were precipitated with diethyl ether. Crude peptides were reverse-phase HPLC on a preparative C4 column with a water/acetonitrile solvent system containing trifluoroacetic acid. Purified peptides were characterized by matrix-associated laser desorption ionization time-of-flight mass spectrometry and reversed-phase HPLC on an analytical C18 column. The purity of all peptides was found to be >95%. Peptide from RhoA was biotin-GGGLQARRGKKSG and from Cdc42 was biotin-GGGLEPPEKKSR.
**Allosteric Regulation of AGAP1**

Cell Culture and Immunoprecipitation (IP)—HeLa cell were maintained in Dulbecco's modified enriched media (DMEM) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C with 5% CO₂. Cells were transfected with plasmids directing expression of AGAP1-ΔGLD and or Myc-RhoA by Lipofectamine 2000 (Invitrogen). 24 h after transfection, HeLa cells were lysed in buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science). Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. 1 mg of cell lysate were incubated with 2 μg of rat anti-HA Ab or polyclonal anti-myc Ab for 1 h at 4°C. Immunocomplexes were precipitated by overnight incubation with γBind™ beads (GE Healthcare). After washing the beads with lysis buffer 3 times, the immunocomplexes were eluted in a 2-fold concentrate of SDS sample buffer and analyzed by SDS-PAGE and immunoblotting using specific Abs.

For the binding of the C-terminal peptide of RhoA or Cdc42 to AGAP1, HeLa cells were transfected with plasmids directing expression of AGAP1-ΔGLD and or FLAG-AGAP1. Cells were then lysed in the same buffer used in the IP experiments. Lysate were then incubated with 15 μM of each peptide overnight in the presence of streptavidin-coated beads (Sigma). Samples were collected by centrifugation at 750 × g for 5 min, washed in lysis buffer, and subjected to analysis by SDS-PAGE and immunoblot.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was carried out at Myriad Genetics (Salt Lake City, UT) using the GLD domain of AGAP2 as bait with a mating-based method. The corresponding cDNA for AGAP2 GLD domain (amino acids 30–250) was cloned into pGBT1 superB as fused to GAL4 DNA-binding domain. The bait plasmid was introduced into Myriad’s ProNet yeast strain PNY200 (MATα ura3-52 ade2-101 trp1-901 his3-Δ200 leu2-3,112 gal4Δ gal80Δ). The bait yeast cells were allowed to mate with Myriad’s ProNet MATa yeast cells, BK100 (MATa ura3-52 trp1-901 his3-Δ200 leu2-3,112 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) containing human brain, human spleen, or mouse embryo cDNA libraries. After mating, 5 million diploid yeast cells were obtained and selected on His- and Ade-lacking medium. The auxotrophy is suppressed if the bait and prey proteins interact. The prey plasmid was isolated from the positive colonies, and the interaction was confirmed by expression of third reporter gene (lacZ). cDNA in the positive prey plasmid was sequenced.

Miscellaneous—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). BODIPY-GTPγS was purchased from Invitrogen. A fluorescence assay for nucleotide binding to Rho family proteins using BODIPY-labeled guanine nucleotide has been described (43). Data were collected using a FluorMax 3 spectrophotometer (Jobin Yvon Horiba, Edison, NJ). The reactions were conducted at 30 degree, and fluorescence was measured at an excitation wavelength of 502 nm and an emission wavelength of 511 nm. Adobe Photoshop and Illustrator (Adobe Systems Inc., San Jose, CA) were used to prepare composite figures.

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**TABLE 1**

| Protein       | GTP     | GDP     | ATP     | ADP   |
|---------------|---------|---------|---------|-------|
| His10AGAP1    | 2.0 ± 1.0 | 2.0 ± 3.0 | 3.1 ± 1.4 | 2.0 ± 1.0 |
| [ΔGLD]AGAP1   | 2.1 ± 1.2 | 1.8 ± 1.3 |         |       |

**RESULTS**

GLD Domain Does Not Render AGAP1 Sensitive to Nucleotide—We determined GAP activity of full-length His10AGAP1 in the presence of GDP, GTP, ADP, or ATP (Fig. 1A). In these experiments, AGAP1 was titrated into the reaction to determine the amount of GAP that induced hydrolysis of 50% of the GDP bound to myristoylated Arf1 in a fixed time, 3 min. The value, referred to as the C50, is inversely proportional to catalytic power. The C50 was not affected by the presence of nucleotide. We then compared the activity of full-length His10AGAP1 and AGAP1 with the GLD deleted (His10- [ΔGLD]AGAP1) (Fig. 1B). The proteins had similar C50 values, and the C50 values were not affected by either guanine or adenine nucleotide (Table 1). We conclude that AGAP1 was not regulated by the same GTP-regulated homodimerization mechanism described for LRRK1/2 (20, 50).

GLD Is Protein Binding Site—We considered an alternate hypothesis that AGAP1 and AGAP2 were regulated by proteins binding to the GLD. Two-hybrid screens for binding partners to the GLD of AGAP2 identified Rho family GTP binding proteins as candidates, including RhoA, Rac1, Rac2, and Cdc42.
(Table 2). No other proteins were identified in the screen. The two-hybrid clones were missing critical elements for GTP binding (e.g., G1 motif GXXXXGK(T/S)) and the switch 1 region of the proteins but did contain at least part of switch 2 and the region immediately C-terminal to switch 2.

AGAP1 and AGAP2 have significant sequence homology (Fig. 2A), which led us to determine if both bound to Rho family proteins. Interaction between RhoA and both AGAP1 and -2 was examined by coimmunoprecipitation (Fig. 2, B–D). We used epitope-tagged recombinant proteins because of limitations of antibodies to the native proteins. RhoA was tested first. The two-hybrid results indicated the interaction may be nucleotide-independent. Nevertheless, switch 2 was still present in the two-hybrid clones so we could not a priori assert that either GTP- or GDP-bound forms of Rho were involved. The dominant negative ([T19N]RhoA) and wild type forms of RhoA were expressed. The constitutively active form was toxic. Both wild type RhoA (not shown) and [T19N]RhoA (Fig. 2B) coimmunoprecipitated with AGAP1-HA, although [T19N]RhoA was more efficient. We examined [T17N]Rac1 by the same approach but did not detect binding to AGAP1 (not shown). HA-AGAP2 and AGAP2-GFP were also examined (Fig. 2, C and D). As with AGAP1, binding to [T19N]RhoA was detected by coimmunoprecipitation using AGAP2 with either an N-terminal HA tag or a C-terminal GFP fusion. Thus, [T19N]RhoA coprecipitated with AGAP1 and AGAP2 independent of the type and position of the epitope tag used to detect the protein.

### Table 2

| Protein | Accession | Prey library | Amino acid coordinates |
|---------|-----------|--------------|------------------------|
| Cdc42   | NM_001791 | Human spleen | 39–192                 |
| Cdc42   | NM_001791 | Human spleen | 63–192                 |
| Rac1    | NM_006908 | Human brain  | 72–192                 |
| Rac1    | BC003828  | Mouse embryo | 66–193                 |
| Rac2    | NM_002872 | Human spleen | 27–193                 |
| RhoA    | NM_001664 | Human brain  | 41–193                 |
| RhoA    | NM_001664 | Human spleen | 48–194                 |

### FIGURE 2

**Interaction of AGAP1 and AGAP2 with RhoA.** A, a schematic of AGAP1 and AGAP2 is shown. The sequence identity for each domain is indicated. Ank, ankryin repeat. B–D, shown is detection of RhoA-AGAP interaction by coimmunoprecipitation. In panel B, HeLa cells expressing AGAP1-HA and myc[T19N]RhoA were lysed. In panel i, proteins were precipitated using an antibody to the myc epitope, and the relative quantities of AGAP1-HA in the precipitates (ppt) were determined by immunoblotting for the HA epitope. Blots for proteins in the total lysates are labeled IB HA and IB Myc. The blot for the HA-tagged AGAP1 that was precipitated with mycRhoA is labeled IP Myc and IB HA. In panel ii, proteins were precipitated with an antibody to the HA epitope, and the amount of RhoA in the precipitate was detected with an antibody to the myc epitope. In panel C, HeLa cells expressing AGAP2-GFP and myc[T19N]RhoA were examined. In panel i, proteins were precipitated with an antibody to GFP. In panel ii, proteins were precipitated with an antibody to myc. In panel D, cells expressing HA-AGAP2 (the epitope was fused to the N terminus of AGAP, as opposed to the epitope on the C terminus of AGAPs in other experiments presented in this figure) and myc[T19N]RhoA were examined. Proteins were precipitated with an antibody to the myc epitope. For all experiments, the input blots are 6% of the lysate used for the IP.
Characterization of the effect of Rho family protein on GAP activity of AGAP1 and AGAP2.

A, activation of AGAP1 is shown. GAP assays contained 0.2 μM [γ-32P]GTP-myArf1, 2.2 nM His10AGAP1, 500 μM large unilamellar vesicles, and either 1 mM GDP with 0.5 μM His-RhoA-GDP, His-Cdc42-GDP, and His-Rac1-GDP or 10 μM GTP-S with 0.5 μM His-RhoA-GTP-S, His-Cdc42-GTP-S, and His-Rac1-GTP-S. Activity is expressed as fold increase over His10AGAP1 only control. Statistical analysis of the data included one way analysis of variance followed by the Dunnett multiple comparison test using AGAP1 only as the control. * p < 0.05; **, p < 0.001; ns, not significant. B, activation of AGAP2 is shown. Experiment were performed essentially the same as in A, but 1.7 nM AGAP2-His6 was used instead of AGAP1. Statistical analysis was the same as for A. **, p < 0.01 compared with AGAP2 alone. C, heat sensitivity of RhoA effect is shown. The reactions were performed the same as in A, except 0.5 μM his-RhoA-GDP or his-Rac1-GDP was heated at 95 °C for 10 min where indicated. Data were analyzed by analysis of variance followed by Bonferroni’s multiple comparison test. **, p < 0.01 compared with AGAP1 alone; ns, not significant compared with AGAP1 alone; #, p < 0.05 compared with AGAP1 + RhoA. D, the effect of RhoA on ArfGAP1 is shown. GAP activity for ArfGAP1 (20 nM) incubated with 0.5 μM RhoA-GDP or RhoA-GTP was determined. The results were analyzed by analysis of variance followed by the Bonferroni’s multiple comparison test. ns, not significant compared with ArfGAP1 alone. E, shown is the effect of RhoA-GDP on [γ-32P]AGAP1. Either 2.2 nM His10AGAP1 or 2.2 nM His10ΔGLD]AGAP1 was used in the GAP assays. Statistical analysis was the same as for C. ***, p < 0.001 compared with AGAP1 alone; ns, not significant compared with [ΔGLD]AGAP1. F and G, shown is a comparison of substrates for RhoA-dependent AGAP1 catalytic activity. His10AGAP1 was titrated into a reaction containing myrArf1-GTP or myrArf6-GTP either in the absence (F) or presence (G) of 0.5 μM his-RhoA-GDP.
**TABLE 3**
Comparison of RhoA and Rac1 as activators of AGAP1

|        | RhoA (nM) | Rac1 (nM) |
|--------|-----------|-----------|
|        | 91 ± 24   | 197 ± 36* |

* p < 0.05.

**TABLE 4**
Effect of RhoA on the Arf specificity of AGAP1

| Protein | AGAP1 | AGAP1 + RhoA |
|---------|-------|-------------|
| Arf1    | 4.2 ± 0.9 | 0.32 ± 0.04* |
| Arf6    | 3.6 ± 0.7 | 23 ± 13* |

* p < 0.001 compared to no RhoA.
* p < 0.05 compared to no RhoA.

We focused on AGAP1 and RhoA because both have been implicated in lysosomal function.

**Rho Family Proteins Increase Activity of AGAP1**—We tested the hypothesis that Rho protein binding to AGAP1 regulates GAP activity by determining the effects of His-RhoA, His-Cdc42, and His-Rac1, bound to either GTP or GDP, on Arf GAP activity of purified full-length AGAP1 or AGAP2 using Arf1-GTP as a substrate (Fig. 3A and B). RhoA, bound to either GDP or GTP, was found to stimulate activity. Although Rac1 did not detectably interact with AGAP1 as measured by co-immunoprecipitation, we found it did activate GAP activity of both AGAP1 and AGAP2-His6 independent of the bound nucleotide. Titration revealed that Rac1 was less efficient than RhoA in activating AGAP1 (Table 3), consistent with a higher affinity for RhoA than for Rac. The difference in affinity could explain the ability to detect the interaction of RhoA and AGAPs, but not Rac with AGAPs, by IP. Cdc42 did not increase activity significantly.

Stimulation of AGAP1 GAP activity was dependent on properly folded Rho protein (Fig. 3C); RhoA heated to 95 °C for 5 min did not stimulate activity. The stimulation was also dependent on the GLD of AGAP1 (Fig. 3E). The activity of a recombinant protein comprised of the PH, ArfGAP, and Ank repeat domains, but lacking the GLD domain of AGAP1 (ΔGLD)AGAP1, was not stimulated by RhoA-GDP. In addition, the stimulation was specific for Arf isoforms (Fig. 3F and G, Table 4). RhoA increased activity of AGAP1 toward Arf1 but decreased activity toward Arf6. The effect was specific for AGAP1. ArfGAP1 activity was not affected by RhoA (Fig. 3D).

**C-terminal 12 Amino Acids of RhoA Are Sufficient for Effect on AGAP1**—The activation and binding of AGAP1 by RhoA is nucleotide-independent. This is similar to that described for other enzymes such as protein kinase C-related kinase/protein kinase N for RhoA and phosphatidylinositol phosphate kinase, Tor2, and CD2AP for Rac1. In these three instances, the Rho family proteins were found to bind through their C termini to targets (51–54). We determined whether RhoA may bind to AGAP1 by a similar mechanism. Biotin-conjugated peptides derived from the C termini of RhoA and Cdc42 were mixed with lysates of cells expressing AGAP1-HA or FLAG-AGAP1. The peptides were precipitated using streptavidin conjugated to agarose beads. AGAP1-HA and FLAG-AGAP1, detected by immunoblotting, were in precipitates with the RhoA peptide but not the Cdc42 peptide (Fig. 4A). The peptides were titrated into GAP assay reaction mixtures containing Arf1-GTP and His10AGAP1 (Fig. 4B). The peptide from RhoA increased activity by 44-fold with a half-maximal effect at 10 μM. The peptide from Cdc42 had no detectable effect. Unlike the full-length protein, the peptide was not heat-sensitive (Fig. 4C). The reason for the difference with full-length protein may be that the full-length may aggregate on heating, rendering the C terminus inaccessible, or the C terminus may be sequestered by another mechanism, e.g., oxidation of C-terminal cysteines. The short peptide would not be expected to aggregate on heating and does not contain cysteines.

**Effect of Peptide Does Not Depend on Myristoylation of Arf—** Myristoylated Arf is restricted to membrane surfaces. Activation by RhoA could be achieved by driving recruitment to the membrane surface containing Arf1-GTP. Alternatively, RhoA binding to AGAP1 could be inducing a conformational change independent of membrane recruitment. To test this idea, the effect of the peptide from RhoA on AGAP1 activity using a soluble mutant of Arf1, [L8K]Arf1, was determined. As for myrArf1, peptide from RhoA stimulated activity, whereas the peptide from Cdc42 had no effect (Fig. 4D).

**Nucleotides Do Not Have Detectable Effect on Allosteric Regulation**—Although we did not detect an effect of nucleotide on activity when determining GAP activity of AGAP1 in the absence of a GLD protein binding partner, it is plausible that nucleotides bind to the GLD to affect binding to Rho. We reexamined nucleotide binding to the AGAP1 as a first step in examining this possibility. The change of fluorescence of Bodipy-GTPγS, whose fluorescence increases upon protein binding, was determined. AGAP1 did not affect fluorescence (Fig. 5A). As a positive control, the change in fluorescence due to binding to the same concentration of RhoA, catalyzed by the RhoA exchange factor leukemia-associated Rho guanine nucleotide exchange factor is shown. The result that AGAP1 does not bind nucleotide is consistent with previous work in which binding of radioisotopically labeled GTP could not be detected, nor could nucleotide bound to AGAP1 be detected chemically (23). However, nucleotide binding could not be excluded on the basis of these negative results. To determine if the ability to bind nucleotide was necessary for RhoA-dependent activation, serine 85 on AGAP1 was mutated to asparagine ([S85N]AGAP1). Serine 85 is a residue in the P-loop that is critical for nucleotide binding in other G-proteins and is often mutated to asparagine to disrupt GTP binding. The effect of RhoA on [S85N]AGAP1 was determined. The mutant was activated by RhoA-GDP (not shown) and the C-terminal peptide from RhoA to a similar extent as the wild type protein (Fig. 5, B and C). Related to this finding is a recent report that, in contrast...
to previous reports, the ROC domain of LRRK2 affected kinase activity independently of nucleotide binding (21).

**DISCUSSION**

AGAP1 is the best documented case to date as far as we are aware of an ArfGAP being allosterically regulated by a protein interaction. The activity of other ArfGAPs, such as ArfGAP2 and ArfGAP3, are dependent on a second protein, such as coatomer (55–58). In these cases, the means of regulation are not clear. Coatomer may directly interact with the ArfGAP domain, and it is plausible that coatomer forms part of the substrate binding site. Cargo peptides have also been found to increase ArfGAP activity, but the effect required the presence of coat protein and, therefore, may have been mediated by an effect on the coat protein (56). Other mechanisms mediating the regulation of Arf GAPs that have been described involve lipids. ArfGAP1 is thought to preferentially bind to curved lipid surfaces, thereby being recruited to a surface containing the substrate Arf (59). Phosphoinositides are thought to also act as allosteric modifiers for the ArfGAP ASAP1 (60, 61). For AGAP1, given that there was specific activation for Arf1 over Arf6, the effect of the C-terminal peptide of RhoA is likely due to a conformational change affecting the active site rather than a change in recruitment to the membrane containing the substrate. The precise molecular mechanisms are now being examined.

We consider the increase in specificity for Arf1 on activation of AGAP1 to be significant for two reasons. First, the result supports the idea that the activation is due to a conformational change resulting in better substrate recognition with exclusion of irrelevant substrates. Our speculation is that at physiological concentrations of Arf and AGAP, the basal state AGAP does not regulate any of the Arfs because the proteins are present in insufficient concentrations. When stimulated, AGAP1 will specifically use Arf1 as a substrate. Second, these results highlight the fact that our current understanding of substrate specificity for the ArfGAPs may be flawed. If relatively inactive proteins are examined, real substrate specificity may be obscured. This phenomenon has been observed for another ArfGAP. Full-length ASAP1 is autoinhibited by an N-terminal extension of the BAR domain. The protein does not have strong preference for Arf1 over Arf6; however, with the autoinhibitory motif removed, ASAP1 uses Arf1 50–100-fold more efficiently than Arf6 (48, 62, 63). When trying to assess substrate specificity in cells, activity of an ArfGAP may be observed because of the large amount of protein expressed, but the results could be misleading because the allosteric activator is limiting.

Regulation of AGAP is unique among proteins with G-protein-like domains. Three modes of nucleotide-dependent dimerization have been described (16): (i) GTP-dependent dimerization of two proteins from the same family, e.g. signal recognition particle and signal recognition particle receptor; (ii) GTP-dependent formation of a dimer of identical G proteins (e.g. guanylate binding protein 1 or dynamin); (iii) dimerization through a domain adjacent to the G-protein.
Allosteric Regulation of AGAP1

Although LRRK dimerizes through a COR domain, the kinase is inactive until the G-protein domains associate on GTP binding. Our results support a different model for AGAPs. RhoA-GDP appears to make the active complex, and the effects of the GLD are independent of nucleotide binding. The AGAP dependence of activity is sigmoidal in the absence of Rho but hyperbolic in the presence of RhoA, consistent with the possibility that RhoA affects dimerization of AGAP1. We are currently testing the idea of RhoA regulating dimerization of AGAP1 and determining if other proteins may have a similar regulatory interaction with AGAPs.

The GLD of AGAP1 is different than other G protein domains in a second respect; we did not detect nucleotide binding. Although we cannot exclude nucleotide binding on the basis of our inability to detect it, the result may reflect another aspect of G protein domains. Although the focus is often on nucleotide binding, the G proteins are primarily protein binding motifs, not GTPases. Recent reexamination of LRRK1 has provided evidence that nucleotide binding is not necessary for the function (21). Given the conflicting reports, the role of GTP binding for dimerization in LRRK remains to be determined. These recent results together with ours raise the possibility that among G proteins and G protein domains, which evolved to bind to other proteins, some have retained protein binding while losing the ability to bind nucleotide.

The association of AGAP1 and RhoA is intriguing, with two possible connections to the biological function. The first connection is to lysosomal maturation. RhoA has been implicated in lysosomal distribution (39–42). AGAP1 binds to AP-3, which affects lysosome function (6, 65). Reduced expression of AP-3 has been examined in Natural Killer cells. The cytolytic granules, which are lysosome-related organelles, formed, but they were not able to distribute appropriately to immunologic synapse, the site of release of the contents of the cytolytic granules (66). AGAP2, which binds to AP-1, could also interact with a Rho family member to affect early endocytic trafficking. For instance, RhoB affects trafficking steps that might be AP-1-dependent. These links to membrane traffic are being examined.

The second connection between AGAPs and Rho family proteins is related to the actin cytoskeleton and focal adhesions. Epitope-tagged AGAP2 (67) associates with fatty acids, and overexpression of epitope-tagged AGAP1 causes disruption of stress fibers (23). AGAP2, which is reported to bind to focal adhesion kinase, also affects the morphology of focal adhesions and cell migration (67). In addition, Rock1 was identified as a potential binding partner of AGAP2 in two-hybrid screens using the PH domain as bait. A model that we will investigate is that the ternary complex of RhoA-AGAP2-Rock coordinates Arf and Rho signaling to control fatty acid and stress fiber dynamics as has been described for ARAP subtype ArfGAPs (47, 68, 69).

In summary, we have identified the GLD of AGAP1 as a protein binding motif that allosterically regulates catalytic activity. This mechanism for regulating an ArfGAP has not been previously described and could generalize to the other members of domain (e.g. LRRK1/2) with subsequent GTP-dependent association of the G-protein domain. Among these proteins, LRRKs appeared to be analogous to AGAPs in that they contain multiple domains including a catalytic domain (17, 21, 50, 64).

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3 Z. Nie and P. A. Randazzo, unpublished information.
the AGAP subfamily, the largest ArfGAP subfamily, as well as other multidomain proteins containing G-protein domains.

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