Structural Basis for the Specific Recognition of RhoA by the Dual GTPase-activating Protein ARAP3*

Received for publication, May 3, 2016, and in revised form, June 2, 2016. Published, JBC Papers in Press, June 15, 2016, DOI 10.1074/jbc.M116.736140

Hongyu Bao, Fudong Li, Chongyuan Wang, Na Wang, Yiyang Jiang, Yajun Tang, Jihui Wu, and Yunyu Shi

From the Hefei National Laboratory for Physical Science at Microscale, Collaborative Innovation Center of Chemistry for Life Sciences and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, 230027, China

ARAP3 (Arf-GAP with Rho-GAP domain, ANK repeat, and PH domain-containing protein 3) is unique for its dual specificity GAPs (GTPase-activating protein) activity for Arf6 (ADP-ribosylation factor 6) and RhoA (Ras homolog gene family member A) regulated by phosphatidylinositol 3,4,5-trisphosphate and a small GTPase Rap1-GTP and is involved in regulation of cell shape and adhesion. However, the molecular interface between the ARAP3-RhoGAP domain and RhoA is unknown, as is the substrates specificity of the RhoGAP domain. In this study, we solved the crystal structure of RhoA in complex with the RhoGAP domain of ARAP3. The structure of the complex presented a clear interface between the RhoGAP domain and RhoA. By analyzing the crystal structure and in combination with in vitro GTPase activity assays and isothermal titration calorimetry experiments, we identified the crucial residues affecting RhoGAP activity and substrates specificity among RhoA, Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division control protein 42 homolog).

The small GTPase Ras superfamily in human consists of at least 154 members divided into five principal families: the Ras, Rho, Rab, Arf, and Ran families (1). These proteins act as molecular switches, cycling between an inactive GDP-bound form and an active GTP-bound form, transducing signals from plasma membrane receptors to downstream signal molecules. These small GTPases generally have low intrinsic GTPase hydrolytic activity, and efficient hydrolysis requires interaction with family-specific groups of GAPs (GTPase-activating proteins) which can accelerate the cleavage step by several orders of magnitude. Arf6, an Arf (ADP-ribosylation factor) family member, is involved in endocytic pathways, endosomal recycling, cell migration, exocytosis, actin reorganization, plasma membrane reorganization, and cytokinesis (2). RhoA belongs to the Rho (Ras homologous) family, which primarily regulates cell shape, polarity, and motility by inducing stress fibers in cells that provides intracellular contractility (3–6). In addition, Arf6 plays a role in cancer cell invasion, and RhoA regulates cancer cell morphology and migration (7, 8).

ARAP3, a multidomain protein, belongs to the ARAP superfamily proteins and contains both functional ArfGAP and RhoGAP domains, a sterile α motif domain, five pleckstrin homology (PH) domains and an Ras-associating (RA) domain (see Fig. 1a). ARAP3 was originally identified as a PI3K effector in a screening for PtdIns(3,4,5)P3 binding proteins (9). ARAP proteins are regulated at different levels. It has been demonstrated that the ArfGAP activity of ARAP3 is induced by the phosphoinositols PtdIns(3,4,5)P3 and phosphatidylinositol 3,4-bisphosphate in vivo and in vitro, whereas its RhoGAP activity is unaffected in vitro (9, 10). Moreover, the RhoGAP activity of ARAP3 is up-regulated by direct binding of Rap1-GTP to the RA domain in vivo and in vitro (10). ARAP3 is unique because of its two different functional GAPs, ArfGAP and RhoGAP, which act specifically toward Arf6 and RhoA, respectively (9, 10). This property most likely provides an efficient way to coordinate the two signaling processes. However, the mechanisms underlying the specificity of ARAP3 for Rho and Arf GTPases have not been thoroughly characterized.

In this study, we focused on the structure and function of RhoGAP in ARAP3. It is known that ARAP3 regulates many cell functions through its RhoGAP activity, such as formation of lamellipodia upon growth factor stimulation (11), development of angiogenesis in PI3K signaling pathway (12), and inhibition of peritoneal dissemination of scirrhous gastric carcinoma cells (13). Because Rap1-GTP can up-regulate the RhoGAP activity of ARAP3, the role of ARAP3 in downstream Rap1-GTP cell processes is RhoGAP-dependent. In neutrophils, ARAP3 regulates chemotaxis and adhesion-dependent processes (14). In macrophages, following long term treatment by TGF-β, ARAP3 down-regulates the production of chemokines and slows cell migration (15). ARAP3 mediates the neutrophilic outgrowth from PC12 cells in response to treatment by basic fibroblast growth factors, nerve growth factor, and cAMP, all of which can up-regulate Rap1-GTP level in PC12 cells (16–18). However, the nature of the ARAP3-RhoGAP-RhoA interaction was still unknown.

To determine the structure and function of the ARAP3-RhoGAP domain, we solved the x-ray crystal structures of the

* This work was supported by National Basic Research Program of China 973 Program Grant 2011CB912701; Chinese National Natural Science Foundation Grants 31330018 and 31170693; Hefei Center for Physical Science and Technology Grant 2012FXZY002; and Strategic Priority Research Program Grants 31330018 and 31170693; Hefei Center for Physical Science and Technology Grant 2012FXZY002; and Strategic Priority Research Program Grant 2013HY02-01-02. Hongyu Bao, Fudong Li, Chongyuan Wang, Na Wang, Yiyang Jiang, Yajun Tang, Jihui Wu, and Yunyu Shi declare that they have no conflicts of interest with the contents of this article.

The atomic coordinates and structure factors (codes 5JD0 and 5JCP) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 To whom correspondence may be addressed. Tel.: 86-551-63603745; Fax: 86-551-63601443; E-mail: wujihui@ustc.edu.cn.

2 To whom correspondence may be addressed. Tel.: 86-551-63607464; Fax: 86-551-63601443; E-mail: yyshi@ustc.edu.cn.

3 The abbreviations used are: PH, pleckstrin homology; RA, Ras-associating; RMSD, root mean square deviation; PDB, Protein Data Bank; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; ITC, isothermal titration calorimetry; MESG, 2-amino-6-mercaptopurine riboside; PNP, purine nucleoside phosphorylase.
RhoGAP domain and of the complexed RhoA-RhoGAP domain with a substrate transition state analog GDP-AlF$_4$. Through these structures, we mapped the interface between RhoA and the ARAP3-RhoGAP domain. Using the complexed structure as a guide, we performed site-directed mutagenesis to analyze the role of interface residues on complex formation and on GTP hydrolysis. Meanwhile, we also found the residues responsible for the substrate specificity of ARAP3 among RhoA, Rac1, and Cdc42 in vitro.

**Results**

**Overall Structure of the ARAP3-RhoGAP Domain in Complex with RhoA in the Transition State**—To investigate the structure and function of the ARAP3-RhoGAP domain, we constructed different fragments according to the secondary structure prediction. The most promising construct is the fragment containing ARAP3 residues 906–1107, which binds RhoA with a dissociation constant ($K_d$) of 2.45 μM (Fig. 1d; see Table 2). After extensive crystallization trials, we determined the 2.30 Å crystal structure of the ARAP3-RhoGAP domain (Table 1). Although we failed to obtain crystals by mixing the ARAP3-RhoGAP domain with different RhoA constructs, we successfully obtained crystals by expressing a fusion protein of the ARAP3-RhoGAP domain and RhoA, using a 28-residue flexible linker (19, 20), which was purified in buffer containing GDP, Mg$^{2+}$, Al$_3^-$, and F$^-$ to mimic the transition state (21, 22).

Crystals of the ARAP3-RhoGAP domain belong to space group $P_4_3_2_1$ with two molecules in the asymmetric unit. The two ARAP3-RhoGAP domain molecules in the asymmetric unit are similar to one another with a C$\alpha$ atom root mean square deviation (RMSD) of 0.375 Å calculated by PyMOL. The overall structure of the ARAP3-RhoGAP domain exhibits a canonical RhoGAP domain structure, which consists of nine long $\alpha$-helices and two short $\alpha$-helices packed in an anti-paral-
lel way, labeled as αI–α10, with an extra α-helix at the C terminus (Fig. 1b). Most RhoGAP domains have a conserved motif in the loop between the α2 and the α3 helices. In this motif, a central arginine is the primary element required for GTPase rate enhancement and is called the arginine finger. The sequence of this motif in the ARAP3-RhoGAP domain is

\[
\text{motif from 17 to 30%}, \text{analyzed by the Dali server (23).}
\]

RhoGAP structures with an RMSD from 1.4 to 2.4 Å and identity from 17 to 30%, analyzed by the Dali server (23). The ARAP3-RhoGAP domain structure exhibits structural similarity to many other RhoGAP structures with an RMSD from 1.4 to 2.4 Å and identity from 17 to 30%, analyzed by the Dali server (23).

Crystals of RhoA-GDP-AlF₄⁻ complexed with the ARAP3-RhoGAP domain belong to space group P2₁₂₁₂₁, and the asymmetric unit contains two molecules. The structure was solved by molecule replacement, refined to 2.10 Å (Table 1), and the overall structure of one complex in the asymmetric unit is shown in Fig. 1c. The two complex molecules in the asymmetric unit are similar to one another with a Cα RMSD of 0.523 Å calculated by PyMOL. Residues 28–40 in the switch I region of RhoA, which cannot be observed because of missing electron density, are shown by the dotted line in Fig. 1c. In this final refined complex structure, the electron density map next to the GDP in the active center is almost square planar, modeled as AlF₄⁻ (Fig. 1f). The structures of the ARAP3-RhoGAP domain in the apo and the complexed state show no significant difference with a Cα atom RMSD of 0.523 Å calculated by PyMOL. The main differences between the apo and the complexed state structures of the ARAP3-RhoGAP domain are found in the α2-α3 loop and the α3 helix (Fig. 1e). The Arg-942 residue in the α2-α3 loop is moved closer to interact with and stabilize the orientation of GDP-AlF₄⁻. The α3 helix of ARAP3 RhoGAP domain has a 6.5° rotation between the apo and the complexed state, driving Arg-949ARAP3 closer to Asp-90RhoA to form tight salt bridges (Fig. 1e). RhoA-GDP-AlF₄⁻ superimposes well with the structure of RhoA-GTPγS (PDB code 1A2B; Fig. 1g) but has a conformational difference at the switch II region compared with RhoA-GDP (PDB code 1FTN; Fig. 1h). However, we could not compare the conformation of switch I from these structures because the electron density map of this region was missing from the structure we solved.

**Interface between ARAP3-RhoGAP Domain and RhoA**—ARAP3-RhoGAP domain complexed with RhoA in the transition state buries 2092 Å² of solvent-accessible surface. The interaction surface of the ARAP3-RhoGAP domain involves the α2-α3 loop and the α3, α4, and α8 helices. Residues contributing to the interaction interface from RhoA are located at the switch II region, the α3 helix, and the insert region (Fig. 2a). The complexed structure shows the conserved active center as reported previously. In the complexed structure, residue Arg-942 is pointed into the nucleotide binding pocket; the guanidinium group of Arg-942 interacts with the α- oxygen of GDP and two fluorides of AlF₄⁻, and the main chain oxygen of Arg-942 forms a hydrogen bond with the side chain amide group of the RhoA catalytic residue Gln-63. Gln-63RhoA is thus stabilized because the electron density map of this region was missing from the structure we solved.

![Image](image-url)

**Structure of the ARAP3-RhoGAP-RhoA Complex**

| Data collection and refinement statistics | RhoGAPARAP3 | RhoGAPARAP3/RhoA-GDP-AlF₄⁻ |
|------------------------------------------|--------------|-----------------------------|
| **Protein Data Bank code**               | 5JD0         | 5JC5                        |
| **Data collection**                      |              |                             |
| Wavelength (Å)                           | 0.9795       | 0.9795                      |
| Space group                              | P2₁₂₁₂₁      | P2₁₂₁₂₁                     |
| Cell dimensions (Å)                      | 108.86, 108.86, 94.46 | 56.79, 93.21, 164.28       |
| α, β, γ (°)                              | 90, 90, 90   | 90, 90, 90                  |
| Resolution range (Å)                     | 38.36–2.30   | 47.22.00–2.10 (2.18–2.10)   |
| Rmerge (%)                               | 13.8 (64.2)  | 10.8 (50.6)                 |
| Completeness (%)                         | 99.1 (100)   | 99.8 (99.9)                 |
| Redundancy                               | 2.1 (6.1)    | 2.1 (6.1)                   |
| **Refinement**                           |              |                             |
| Number of reflections (overall)          | 25,540       | 51,660                      |
| Number of reflections (test set)         | 1300         | 2645                        |
| Rwork/Rmerge (%)                         | 22.0/26.0    | 18.6/22.7                   |
| Number of atoms                          | 3088         | 5581                        |
| Ligands                                  | 0            | 68                          |
| Water                                    | 89           | 192                         |
| B-factors (Å²)                           | 44.8         | 38.3                        |
| Protein                                  | 0            | 27.8                        |
| Water                                    | 43.6         | 36.8                        |
| RMSDs                                    | 0.008        | 0.007                       |
| Bond length (Å)                          | 0.916        | 0.910                       |
| Ramachandran plot (%)                    | 97.0         | 98.0                        |
| Favored                                  | 3.0          | 2.0                         |
| Allowed                                  | 0            | 0                           |
| Outlier                                  | 0            | 0                           |

* The values in parentheses are for the highest resolution shell.
nucleophilic attack on the γ-phosphate of GTP; thus the GTP hydrolysis rate of RhoA is accelerated. The RhoGAP domain of ARAP3 interacts with RhoA mainly through electrostatic interactions. Negatively charged GDP·AlF$_4^-$ and negatively charged residues Glu-64 and Asp-65 of the switch II region and Asp-90 of the α3 helix of RhoA interact tightly with positively charged residues of the ARAP3-RhoGAP domain (Arg-942 of the α2-α3 loop, Arg-949 of the α3 helix, and Arg-982 and Arg-985 of the α4 helix) through salt bridges (Fig. 2b). We can observe the electrostatic interactions more clearly from the electrostatic representation of the complexed structure (Fig. 2c); residues Glu-64, Asp-65, and Asp-90 and GDP·AlF$_4^-$ forming the negatively charged surface of RhoA directly bind to the positively charged surface of the ARAP3-RhoGAP domain formed by residues Arg-942, Arg-949, Arg-982, and Arg-985. In addition to the interactions shown above, there are several hydrogen bonds formed between the ARAP3-RhoGAP domain and RhoA: the main chain oxygen of residue Gly-945$_{ARAP3}$ and the main chain nitrogen of residue Arg-947$_{ARAP3}$ form hydrogen bonds with residue Asn-94$_{RhoA}$ (see Fig. 4b); the main chain nitrogen of residue Ala-948$_{ARAP3}$ forms a hydrogen bond with residue Glu-97$_{RhoA}$ (see Fig. 4b); and the main chain oxygen of residue Leu-1057$_{ARAP3}$ forms a hydrogen bond with residue Tyr-66$_{RhoA}$. There are also water-mediated contacts between the main chain nitrogen of residue Gly-944$_{ARAP3}$ and Gly-14$_{RhoA}$ and between the main chain oxygen of residue Gly-944$_{ARAP3}$ and Asp-96$_{RhoA}$. In addition to electrostatic and hydrogen bonding interactions, hydrophobic interactions are also observed; residue Phe-972$_{ARAP3}$ of the α3′-α4 loop and residue Pro-1061$_{ARAP3}$ of the α8 helix make hydrophobic contacts with residue Met-134$_{RhoA}$ and residue Tyr-66$_{RhoA}$ in the switch II region.

Based on the complexed structure, we made site-directed mutations in ARAP3-RhoGAP to verify the importance of residues for the interaction between the ARAP3-RhoGAP domain and RhoA. We used ITC experiments to measure the binding...
affinity between RhoA and the wild type or mutants of the ARAP3-RhoGAP domain. The results indicated that substitution of residue Phe-972 in the RhoGAP domain by alanine reduced the binding affinity to a K_d of 4.37 μM, approximately half of the binding affinity of the wild type ARAP3-RhoGAP domain (Fig. 2d and Table 2). Moreover, a single mis-sense mutation of each of the residues Arg-949, Arg-982, or Arg-985 in the RhoGAP domain to glutamic acid was sufficient to abolish complex formation (Fig. 2d and Table 2). The GTPase assay results were consistent with the ITC findings. The GTP hydrolysis reaction rate, using 25 nM wild type ARAP3-RhoGAP and 35 μM RhoA-GTP, was 10-fold greater than that of the RhoA alone (Fig. 2e). A single point mutation of each of the residues R942A, R949A, R949E, R982E, and R985E in the RhoGAP domain dramatically reduced the GAP activity of the ARAP3-RhoGAP domain under the same conditions (Fig. 2e). Mutants R982A and R985A still retained slight GAP activity so they could weakly accelerate the GTP hydrolysis reaction rate of RhoA by ~3-fold (Fig. 2e). Intriguingly, mutant R949A also dramatically reduced the GAP activity of the ARAP3-RhoGAP domain (Fig. 2e), indicating that Arg-949ARAP3 is important for the ARAP3-RhoGAP domain to perform its GAP activity. Indeed, Arg-949ARAP3 interacts tightly with Phe-972ARAP3 and Asp-975ARAP3 through cation-π and electrostatic interactions (Fig. 2f), thus stabilizing the RhoGAP structure. All of these results demonstrate that both the arginine finger and the residues responsible for binding to RhoA are crucial to maintain the GAP activity of the ARAP3-RhoGAP domain.

Comparison with Other RhoGAP-RhoA Complexes in the Transition State—Although there are over 70 different RhoGPs, only two other RhoGAPs in complex with RhoA in the transition state have been solved (Fig. 3, b and c): ARHGAP1, which is also called p50RhoGAP or Cdc42GAP (PDB codes 1TX4 and 1OW3), and ARHGAP20 (PDB code 3MSX). The sequence of the RhoGAP domain of ARAP3 shows identity with ARHGAP1 (26%) and ARHGAP20 (25%). The most remarkable difference was found at the interface involving the switch I region of RhoA; in the p50RhoGAP-RhoA complex residues Tyr-34RhoA, Pro-36RhoA, Val-38RhoA, and Phe-39RhoA make extensive hydrophobic contacts with RhoGAP of ARHGAP1 (24), whereas in the ARHGAP20-RhoA complex residues Tyr-34RhoA, Val-35RhoA, Pro-36RhoA, and Phe-39RhoA make extensive hydrophobic interactions with RhoGAP of ARHGAP20. However, we could not observe these hydrophobic interactions between RhoA and RhoGAP of ARAP3, because the switch I region in our structure is poorly ordered, and the electron density map of residues 28–40 is missing. The missing electron density in the switch I region of RhoA indicates that the switch I region may not be concerned with the ARAP3-RhoGAP binding. Another notable difference was found at the N terminus of the α9 helix. The α9 helix of ARAP3-RhoGAP is shorter than ARHGAP1 and ARHGAP20 (Fig. 3d), and the α8-α9 loop (residues 1067–1071) is poorly ordered, losing the hydrophobic interactions with residues Leu-69 and Leu-72 in the α2 helix of RhoA (24).

Despite these differences, all three RhoGAP-RhoA complexes exhibit two interfaces in the same location. The first interface consists of the α3 helix of RhoGAP and the α3 helix of RhoA, and the second interface consists of the α4 helix of RhoGAP and the switch II region of RhoA. Residues in the first interface are less conserved than those in the second interface among ARAP3, ARHGAP1, and ARHGAP20 (Fig. 4a). There is a conserved alanine residue at the end of the α2-α3 loop of the RhoGAP domain (Fig. 4a), which interacts with Asn-94RhoA in ARHGAP1 and ARHGAP20 (Fig. 4b). However, in ARAP3, it is replaced by the Gly-945ARAP3 (Fig. 4b). There is also a conserved arginine residue on the α3 helix (Fig. 4a), which interacts with Glu-97RhoA only in ARHGAP20 (Fig. 4b). In the first interface, ARHGAP1 only has interactions with Asn-94RhoA and Glu-97RhoA, whereas the ARAP3-RhoGAP domain has extra interactions with Asp-90RhoA through salt bridges, and ARHGAP20 has interactions with Asp-90RhoA, Glu-93RhoA, Asn-94RhoA, and Glu-97RhoA (Fig. 4b). In the α4 helix, there are two conserved residues, lysine and arginine (Fig. 4a). The conserved arginine in the ARAP3-RhoGAP domain (Arg-985) interacts with Asp-65RhoA, whereas in both ARHGAP1 and ARHGAP20, it interacts with both Glu-64RhoA and Asp-65RhoA (Fig. 4c). In ARHGAP1 and ARHGAP20, the conserved lysine interacts with Asp-65RhoA (Fig. 4c). In ARAP3, however, the conserved lysine is not on the interface and is replaced by Arg-982 that interacts tightly with Glu-64RhoA (Fig. 4c).

**Structure of the ARAP3-RhoGAP-RhoA Complex**

| Rhogap | Gtpases with GDP-Mg2+ | ΔH | ΔS | K_D | N   |
|--------|----------------------|----|----|-----|-----|
| Wild type RhoA | 1.87 ± 0.08 | 31.9 | 2.45 ± 0.61 | 0.74 |
| F972A RhoA | 4.23 ± 0.19 | 37.7 | 4.37 ± 0.52 | 0.55 |
| R949E RhoA | ND | ND | ND | ND |
| R982E RhoA | ND | ND | ND | ND |
| R985E RhoA | ND | ND | ND | ND |
| Wild type Cdc42 | 10.33 ± 2.21 | 54.5 | 59.88 ± 11.31 | 0.67 |
| Wild type Cdc42 (S88D) | 5.50 ± 0.23 | 42.9 | 5.41 ± 0.95 | 0.73 |
| Wild type Rac1 | ND | ND | ND | ND |
| Wild type Rac1 (A88D,A95E) | 14.13 ± 0.50 | 69.8 | 18.87 ± 1.56 | 0.79 |

TABLE 2
Thermodynamic parameters of the wild type and mutants of the ARAP3-RhoGAP domain to different small GTPases by ITC experiments

The ITC experiments were carried out at 293 K, and all proteins were in buffer conditions: 20 mM Tris (pH 7.5), 200 mM NaCl, 5 mM MgCl2, and 10 mM NaF. ND, not detected. Errors in the table are standard errors.
ARAP3-RhoGAP domain binds the RhoA-like Rac1 mutant (A88D,A95E) with a $K_D$ of 18.87 nM (Fig. 5c and Table 2). Additionally, in GTPase activity experiments, the ARAP3-RhoGAP domain had greater efficiency in the presence of the RhoA-like Cdc42 or Rac1 mutants than with wild type Cdc42 and Rac1 (Fig. 5, d and e, Table 3). The best fit $K_m$ and $K_{cat}/K_m$ values of ARAP3-RhoGAP to RhoA-GTP examined by the 2-amino-6-mercapto-7-methylpurineriboside (MESG)/purine nucleoside phosphorylase (PNP) system are 11.08 μM and 16.93 min$^{-1}$ μM$^{-1}$, respectively. The $K_m$ values of the ARAP3-RhoGAP domain to Cdc42-GTP and Cdc42 (S88D)-GTP are 7.4- and 1.8-fold greater than that of RhoA-GTP, indicating that the binding affinity of the ARAP3-RhoGAP domain for the RhoA-like mutation of Cdc42 with 4.2-fold higher than for wild type Cdc42 and 1.8-fold weaker affinity for RhoA during GAP-stimulated GTP hydrolysis reactions. The catalytic efficiency of the ARAP3-RhoGAP domain with the RhoA-like Cdc42 mutant is 4.3-fold greater than that of wild type Cdc42 and is comparable with that of RhoA (only 1.5-fold weaker than that of RhoA). The kinetic parameters of the ARAP3-RhoGAP domain to wild type Rac1-GTP was not determined because of the very low binding affinity. However, the $K_m$ and $K_{cat}/K_m$ values of ARAP3-RhoGAP to Rac1 (A88D,A95E)-GTP are 69.40 μM and 5.68 min$^{-1}$ μM$^{-1}$, indicating that the ARAP3-RhoGAP domain binding affinity and catalytic efficiency toward the RhoA-like Rac1-GTP mutant is only 6.2- and 3-fold weaker than that of RhoA-GTP, respectively. Taken together, the specific GAP activity of the ARAP3-RhoGAP domain to RhoA over Cdc42 and Rac1 may be conferred by the interaction with the negatively charged residues Asp-90 and Glu-97 of RhoA.

Discussion

ARAP3 was first reported in 2002 (9), and since then the function of ARAP3 in different cells and tissues has been extensively investigated, and most of the ARAP3 functions are related to its RhoGAP activity. However, the molecular mechanism of the RhoA-specific GAP activity of the ARAP3-RhoGAP domain remained unclear. In this study, we solved the crystal structure of the ARAP3-RhoGAP domain in complex with RhoA$\cdot$GDP$\cdot$AlF$_4^-$. Based on the interaction surface, we found that single point mutations of Arg-942, Arg-949, Arg-982, or Arg-985 can dramatically reduce the binding affinity and the RhoGAP activity of the ARAP3-RhoGAP domain toward RhoA in vitro (Fig. 2, d and e, and Table 2). Over the years, researchers have made loss of function mutants of the ARAP3-RhoGAP domain by point mutation of Arg-942 or Arg-982 (9, 13, 16, 17); here we proved that residue Arg-942 is the catalytic arginine finger of RhoGAP and that residue Arg-982 is critical for the interaction between RhoGAP and RhoA (Fig. 2b). Only two other RhoGAP-RhoA complexed structures (ARHGAP1, PDB code 1TX4 and 1OW3; ARHGAP20, PDB code 3MSX) have been solved (Fig. 3, b and c). The ARAP3-RhoGAP domain shows 25% sequence identity with ARHGAP1 and ARHGAP20, and the complexed structures show differences at the binding interfaces, especially at the first interface (Fig. 4, a and b). Our complexed structure is an important contribution toward understanding the interactions between RhoGAPs and RhoA.

There are ~22 mammalian genes encoding Rho family small GTPases, but ~70 protein members of RhoGAPs have been found in mammals (25, 26). Most of the RhoGAPs show selec-
activity among the different Rho family small GTPases. ARAP1 and ARAP3 selectively use RhoA as their substrate (9, 27), but the ARAP2-RhoGAP domain lacks RhoGAP activity, because the catalytic arginine finger is replaced by glutamine. However, ARAP2 still has a direct interaction with RhoA-GTP (28). ARAP1 is an Arf1/Arf5 ArfGAP, whereas ARAP2 and ARAP3 are specific for Arf6 (9, 27, 28). The mechanism for the specificity of ARAP3 among Rho and Arf GTPases remains elusive.

In this study, we have found that residues Asp-90 and Glu-97 in the \(\alpha_3\) helix of RhoA are responsible for the substrate-specific GAP activity of the ARAP3-RhoGAP domain. In Cdc42, the related residues are Ser-88 and Glu-95, whereas in Rac1 they are Ala-88 and Ala-95 (Fig. 5a). The ARAP3-RhoGAP domain has interactions with both Asp-90 and Glu-97 (Fig. 4b) and exhibits higher binding affinity and GAP activity against a RhoA-like mutant Cdc42 (S88D) or Rac1 (A88D, A95E) than wild type Cdc42 and Rac1 (Fig. 5, b–e, and Tables 2 and 3). It is notable that the \(K_m\) values are comparable (the biggest difference is 2-fold) and the \(K_m\) values are the main factor for the differences of the catalytic efficiencies, further proving that ARAP3-RhoGAP chooses its specific target mainly through binding affinity. The same molecular mechanism for the specificity of RhoGAP activity was reported for the Myo9b-RhoGAP domain (29). As shown in Fig. 4b, ARHGAP1 interacts only via Glu-97 and ARHGAP20 interacts with both Asp-90 and Glu-97. Functionally, ARHGAP1 is a GAP with a preference for Cdc42 over Rho and Rac1 (30, 31), and ARHGAP20 is a GAP with a preference for RhoA (32), supporting our idea that RhoGAPs choose their specific target by recognizing differences in the \(\alpha_3\) helix among RhoA, Cdc42, and Rac1. In addition, \(\beta_2\)-chimaerin contains a Rac1-specific GAP domain, its specificity is conferred by interactions of Phe-315 and Glu-317.

**FIGURE 4.** All three RhoGAP-RhoA complexes exhibit two interfaces in the same location. a, sequence alignment of the RhoGAP domains of ARAP3, ARHGAP1, and ARHGAP20 located at the two interfaces. The secondary elements of ARAP3 RhoGAP are shown on the top of the sequences. The residues on the interaction interface forming polar interactions with RhoA within 4 Å are highlighted in red, green, and blue, respectively. b and c, comparison of the differences of interfaces 1 (b) and 2 (c) in detail. The hydrogen bonds are shown as black dashed lines, and the salt bridges are highlighted with red dashed lines. RhoA is colored in gray. The RhoGAP domains of ARAP3, ARHGAP, and ARHGAP20 are colored in cyan, yellow, and orange, respectively.
with Ala-88 and Ala-95 in the α3 helix of Rac1 (33). Glu-95 in Cdc42 and Glu-97 in RhoA would block that interaction and make them poor targets for 2-chimaerin. Thus, RhoGAPs may choose their specific target by the differences in the α3 helix of RhoA, Rac1, and Cdc42. Intriguingly, the GTPase substrate preference of p190-RhoGAP can be switched by phospholipids from RhoA and Rac1 to only Rac1 (34) and to only RhoA by phosphorylation (35). Phosphorylation can also switch the

---

**FIGURE 5.** The ARAP3-RhoGAP domain shows higher binding affinity and GAP activity toward RhoA-like Cdc42, and Rac1 mutations *in vitro*. 

| Domain       | Sequence                                         |
|--------------|--------------------------------------------------|
| RhoA         | AGQEDYDLRPLYPSDVMCSIDSPDLENPKEKWTPEVKHCPNVPIILVGNKD |
| Cdc42        | AGQEDYDLRPLYPSDVMCSIDSPDLENPKEKWTPEVKHCPNVPIILVGNKD |
| Rac1         | AGQEDYDLRPLYPSDVMCSIDSPDLENPKEKWTPEVKHCPNVPIILVGNKD |

---

**Structure of the ARAP3-RhoGAP-RhoA Complex**

---

**Table 3**

| Domain       | K_m (μM) | K_{cat}/K_m (min⁻¹ μM⁻¹) |
|--------------|----------|--------------------------|
| RhoA         |          |                          |
| Cdc42        | 50.88 ± 11.31 | 5.41 ± 0.95              |
| Cdc42(S88D)  | 50.88 ± 11.31 | 5.41 ± 0.95              |
| Rac1(A88D,A95E) | 16.87    |                           |
GTPase substrate preference of MgcRacGAP (male germ cell RacGAP, also named Rac GTPase-activating protein 1) from Rac1 and Cdc42 to RhoA (36). It is reasonable that there are other factors that regulate the substrate specificity of different RhoGAPs, and the detailed mechanisms need further investigation.

The ARAP3-ArfGAP activity can be activated by PtdIns(3,4,5)P$_3$ (9); two tandem PH domains in the N terminus, especially the first PH domain, are required for the interaction with PtdIns(3,4,5)P$_3$ (37). The mechanism of the regulation between PtdIns(3,4,5)P$_3$, PH domains, and the ArfGAP remains unclear. β2-Chimaerin, a Rac1 specific GAP, contains SH2-C1-RacGAP tandem domain. The N-terminal peptide binds the DAG (diacylglycerol)-binding site in the C1 domain and partially occupies the GAP active site. By binding to DAG through the C1 domain, the N-terminal peptide is released from the GAP active site, and GAP activity is enhanced (33). Whether the activation of ARAP3-ArfGAP by binding to PtdIns(3,4,5)P$_3$ adopts the same mechanism as β2-chimaerin requires further investigation. ARAP3 also contains an RA domain, and ARAP3 RhoGAP activity can be enhanced by direct binding of Rap1-GTP to the RA domain (10). ARHGAP20 has PH-RA-RhoGAP tandem domains; the RA domain can autoinhibit RhoGAP activity, and the autoinhibitory effect can be released by binding to Rap1-GTP (32). However, the mechanism of how Rap1-GTP regulates the ARAP3-RhoGAP activity remains obscure. ARAP3 has five PH domains, and the function of PH3 to PH5 needs further investigation. In summary, our work demonstrates the interface structure and is an important contribution to our understanding of the regulation and specific recognition of RhoA by ARAP3.

### Experimental Procedures

**Plasmid Constructions**—Full-length ARAP3 cloned in a pEG-FPC2 vector that was generously provided by Dr. Sonja Vermeiren (Babraham Institute, Cambridge, UK). The ARAP3 RhoGAP domain (residues 906–1107) was cloned into a His$_6$ tag (p28a) vector. Full-length RhoA, Cdc42, and Rac1 constructs were also cloned into the p28a vector. The linker sequence for the fusion protein was NLSSDSSLSSPSALN-STASNSPIEGLS (19, 20). The RhoGAP (residues 906–1107)-linker-RhoA (residues 2–181, F25N) fusion protein was constructed by overlap extension PCR and also cloned in p28a. The fusion protein harbored the stabilizing point mutation F25N in RhoA (21). All mutants were generated using the MutanBEST kit (Takara) and verified by DNA sequencing.

**Protein Expression and Purification**—All proteins were expressed in *Escherichia coli* Bl21 DE3 (Gold) cells (Novagen) that were cultured in LB medium at 310 K to an A$_{600}$ of 0.8–1.0, subsequently shifted to 289 K, and induced with 0.3–0.4 mM isopropyl β-D-thiogalactopyranoside for 24 h. RhoGAPs (wild type or mutant) were purified by nickel-nitritoltriacetic acid affinity chromatography after lysing cells in 20 mM Tris (pH 7.5) and 500 mM NaCl and eluted in buffer containing 30–500 mM imidazole (pH 7.5). The eluted His tag proteins were further purified using a Superdex 200 column (GE Healthcare) in buffer containing 20 mM Tris (pH 7.5) and 200 mM NaCl. The fusion protein was also purified by nickel-nitritoltriacetic acid affinity chromatography after lysing of cells in 20 mM Tris (pH 7.5), 500 mM NaCl, 5 mM MgCl$_2$, and 1 mM GDP, and eluting with buffer containing 30–500 mM imidazole (pH 7.5). Further purification used a Superdex 200 column (GE Healthcare) in buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, 5 mM MgCl$_2$, 1 mM AlCl$_3$, and 10 mM NaF. For ITC experiments, small GTPases and RhoGAPs were purified as described for the fusion protein (without AlCl$_3$). All purified proteins were concentrated and stored at 193 K. GDP and GTP were purchased from Sangon Biotech.

**Crystallization and Structure Solution**—RhoGAP was concentrated to ~10 mg/ml, and the buffer was exchanged to 20 mM KH$_2$PO$_4$ (pH 6.5), 200 mM NaCl, and 10 mM DTT. Crystals of RhoGAP formed in mother liquor containing 100 mM HEPES (pH 6.9) and 10% PEG8000. The fusion protein was concentrated to ~10 mg/ml, and the buffer was exchanged to 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM DTT, 5 mM MgCl$_2$, 1 mM AlCl$_3$, 10 mM NaF, and 2 mM GDP. Crystals of the fusion protein formed in the mother liquor containing 100 mM MES (pH 6.0), 15% (v/v) polyethylene glycol monomethy ether 550, and 4% (v/v) acetone. All crystals were grown at 293 K via the hanging drop method. X-ray diffraction data of the crystals were collected on Beamline 17U1 of the Shanghai Synchrotron Radiation Facility. The data were processed with HKL2000 and programs in the CCP4 suite. The structure of the RhoGAP domain and the fusion protein (complex) were solved by molecular replacement using the program Phaser (38). All the structural models were subsequently refined by programs REFMAC (39), PHENIX (40), and COOT (41), and ligands (GDP, AlF$_4^-$, and Mg$^{2+}$) were added manually. Crystallographic parameters are listed in Table 1. All structure figures were prepared with PyMOL (42). The transition state complex interface was calculated in PDBePISA (43).
Structure of the ARAP3-RhoGAP-RhoA Complex

Isothermal Titration Calorimetry—ITC assays were carried out on a MicroCal iTC200 calorimeter (GE Healthcare) at 293 K. The buffer for RhoGAPs (wild type or mutant) and full-length GTPases was 20 mM Tris (pH 7.5), 200 mM NaCl, 5 mM MgCl₂, and 10 mM NaF. The titration protocol consisted of 20 injections of 0.8–1.0 mM RhoGAPs (wild type or mutant) into 0.06–0.08 mM GTPases. Curve fitting to a one-binding-site (OriginLab) provided by the manufacturer. The parameters are summarized including the standard errors in Table 2.

GTPase Activity Assay—The initial rates and kinetic measurements of GAP-stimulated GTP hydrolysis were measured using the MESG and PNP system with a constant amount of RhoGAP and increasing concentrations of GTPases (44). MESG and PNP were purchased as part of the EnzChek® phosphate assay kit (Life Technologies, E6646). Briefly, the indicated amount of GTPase was incubated in buffer: 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 400 μM GTP, 0.2 mM MESG, and 1 unit of PNP. The reactions were started by adding 10 μl of a solution containing the indicated amounts of RhoGAP and MgCl₂ to the mixture. The final concentration of MgCl₂ was 5 mM. The time courses of absorbance change at 360 nm were recorded by DU 800 (Beckman Coulter) at 293 K. The kinetic parameters were nonlinear fitted by Origin 8.5.1 (OriginLab) using the modified Michaelis-Menten equation as described (44) and are summarized including the standard errors in Table 3.

Acknowledgments—We thank the staff of Beamline BL17U at Shanghai Synchrotron Radiation Facility for the assistance in data collection. We also thank Dr. Zhixin Wang for the advices on the MESG/PNP system GTPase activity assay experiments.

References
1. Wennerberg, K., Rossman, K. L., and Der, C. J. (2005) The Ras superfamily at a glance. J. Cell Sci. 118, 843–846
2. D’Souza-Schorey, C., and Chavrier, P. (2006) ARF proteins: roles in membrane traffic and beyond. Nat. Rev. Mol. Cell Biol. 7, 347–358
3. Bryan, B. A., Li, D., Wu, X., and Liu, M. (2005) The Rho family of small GTPases: crucial regulators of skeletal myogenesis. Cell. Mol. Life Sci. 62, 1547–1555
4. Ridley, A. J. (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. 16, 522–529
5. Čsépányi-Kómi, R., Lévay, M., and Ligeti, E. (2012) Small G proteins and their regulators in cellular signalling. Mol. Cell. Endocrinol. 353, 10–20
6. Riching, K. M., and Keely, P. J. (2015) Rho family GTPases: making it to the third dimension. Int. J. Biochem. Cell Biol. 59, 111–115
7. Sabe, H. (2003) Requirement for Arf6 in cell adhesion, migration, and cancer cell invasion. J. Biochem. 134, 485–489
8. Ridley, A. J. (2013) RhoA, RhoB and RhoC have different roles in cancer cell migration. J. Microsc. 251, 242–249
9. Krugmann, S., Anderson, K. E., Ridley, S. H., Risso, N., McGregor, A., Coadwell, J., Davidson, K., Eguinoa, A., Ellson, C. D., Lipp, M., Manifava, M., Tkistaxis, N., Painter, G., Thuring, J. W., Cooper, M. A., et al. (2002) Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. Mol. Cell 9, 95–108
10. Krugmann, S., Williams, R., Stephens, L., and Hawkins, P. T. (2004) ARAP3 is a PI3K- and Rap-regulated GAP for RhoA. Curr. Biol. 14, 1380–1384
11. Krugmann, S., Andrews, S., Stephens, L., and Hawkins, P. T. (2006) ARAP3 is essential for formation of lamellipodia after growth factor stimulation. J. Cell Sci. 119, 425–432
12. Gambardella, L., Hemberger, M., Hughes, B., Zudaire, E., Andrews, S., and Vermeren, S. (2010) PI3K signaling through the dual GTPase-activating protein ARAP3 is essential for developmental angiogenesis. Sci. Signal. 3, ra76
13. Yagi, R., Tanaka, M., Sasaki, K., Kamata, R., Nakanishi, Y., Kanai, Y., and Sakai, R. (2011) ARAP3 inhibits peritoneal dissemination of scirrhous gastric carcinoma cells by regulating cell adhesion and invasion. Oncogene 30, 1413–1421
14. Gambardella, L., Anderson, K. E., Nussbaum, C., Segonds-Pichon, A., Margarido, T., Norton, L., Ludwig, T., Sperandio, M., Hawkins, P. T., Stephens, L., and Vermeren, S. (2011) The GTPase-activating protein ARAP3 regulates chemotaxis and adhesion-dependent processes in neutrophils. Blood 118, 1087–1098
15. Moon, M. Y., Kim, H. J., Kim, J. G., Lee, Y. J., Kim, J., Kim, S. C., Choi, I. G., Kim, P. H., and Park, J. B. (2013) Small GTPase Rap1 regulates cell migration through regulation of small GTPase RhoA activity in response to transforming growth factor-beta. J. Cell. Physiol. 228, 2119–2126
16. Jeon, C. Y., Kim, H. J., Lee, Y. J., Kim, J. B., Kim, S. C., and Park, J. B. (2010) p190RhoGAP and Rap-dependent RhoGAP (ARAP3) inactivate RhoA in response to nerve growth factor leading to neurite outgrowth from PC12 cells. Exp. Mol. Med. 42, 335–344
17. Jeon, C. Y., Kim, H. J., Morii, H., Morii, N., Settlemaker, J., Lee, Y. J., Kim, J., Kim, S. C., and Park, J. B. (2010) Neurite outgrowth from PC12 cells by basic fibroblast growth factor (bFGF) is mediated by RhoA inactivation through p190RhoGAP and ARAP3. J. Cell. Physiol. 224, 786–794
18. Jeon, C. Y., Moon, M. Y., Kim, J. H., Kim, J. H., Kim, J. G., Li, Y., Jin, K., Kim, P. H., Kim, H. C., Meier, K. E., Kim, Y. S., and Park, J. B. (2012) Control of neurite outgrowth by RhoA inactivation. J. Neurochem. 120, 684–698
19. van Leeuwen, H. C., Strating, M. J., Rensen, M., de Laat, W., and van der Vliet, P. C. (1997) Linker length and composition influence the flexibility of Oct-1. EMBO J. 16, 2043–2053
20. Ismail, S. A., Vetter, I. R., Sot, B., and Wittinghofer, A. (2010) The structure of an Arf-ArfGAP complex reveals a Ca²⁺ regulatory mechanism. Cell 141, 812–821
21. Rittinger, K., Walker, P. A., Eccleston, J. F., Smerdon, S. J., and Gamblin, S. J. (1997) Structure at 1.65 Å of RhoA and its GTP-activating protein in complex with a transition-state analogue. Nature 389, 758–762
22. Graham, D. L., Lowe, P. N., Grime, G. W., Marsh, M., Rittinger, K., Smerdon, S. J, Gamblin, S. J., and Eccleston, J. F. (2002) MgF³⁻ as a transition state analog of phosphoryl transfer. Chem. Biol. 9, 375–381
23. Holm, L., and Rosenström, P. (2010) Dali server: conservation mapping in 3D. Nucleic Acids Res. 38, W545–W549
24. Dvorsky, R., and Ahmadian, M. R. (2004) Always look on the bright side of Rho: structural implications for a conserved intermolecular interface. EMBO Reports 5, 1130–1136
25. Tcherkezian, J., and Lamarche-Vane, N. (2007) Current knowledge of the large RhoGAP family of proteins. Biol. Cell 99, 67–86
26. Cherfils, J., and Zeghouf, M. (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol. Rev. 93, 269–309
27. Miura, K., Jacques, K. M., Stauffer, S., Kubosaki, A., Zhu, K., Hirsch, D. S., Resau, J., Zheng, Y., and Randazzo, P. A. (2002) ARAP1: a point of convergence for Arf and Rho signaling. Mol. Cell 9, 109–119
28. Yoon, H.-Y., Miura, K., Cuthbert, E. J., Davis, K. K., Ahvazi, B., Casanova, J. E., and Randazzo, P. A. (2006) ARAP2 effects on the actin cytoskeleton are dependent on Arf-specific GTPase-activating-protein activity and binding to RhoA-GTP. J. Cell Biol. 119, 4650–4666
29. Kong, R., Yi, F., Weng, P., Liu, J., Chen, X., Ren, J., Li, X., Shang, Y., Nie, Y., Wu, K., Fan, D., Zhu, L., Feng, W., and Wu, J. Y. (2015) Myo9b is a key player in SLIT/ROBO-mediated lung tumor suppression. J. Clin. Invest. 125, 4407–4420
30. Barford, E. T., Zheng, Y., Kuang, W. J., Hart, M. J., Evans, T., Cerione, R. A.,
and Ashkenazi, A. (1993) Cloning and expression of a human CDC42 GTPase-activating protein reveals a functional SH3-binding domain. *J. Biol. Chem.* **268,** 26059–26062

31. Wang, L., Yang, L., Burns, K., Kuan, C. Y., and Zheng, Y. (2005) Cdc42GAP regulates c-Jun N-terminal kinase (JNK)-mediated apoptosis and cell number during mammalian perinatal growth. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 13484–13489

32. Yamada, T., Sakisaka, T., Hisata, S., Baba, T., and Takai, Y. (2005) RA-RhoGAP, Rap-activated Rho GTPase-activating protein implicated in neurite outgrowth through Rho. *J. Biol. Chem.* **280,** 33026–33034

33. Canagarajah, B., Leskow, F. C., Ho, J. Y., Mischak, H., Saidi, L. F., Kazanietz, M. G., and Hurley, J. H. (2004) Structural mechanism for lipid activation of the Rac-specific GAP, beta2-chimaerin. *Cell* **119,** 407–418

34. Ligeti, E., Dagher, M. C., Hernandez, S. E., Koleske, A. J., and Settleman, J. (2004) Phospholipids can switch the GTPase substrate preference of a GTPase-activating protein. *J. Biol. Chem.* **279,** 5055–5058

35. Lévay, M., Settleman, J., and Ligeti, E. (2009) Regulation of the substrate preference of p190RhoGAP by protein kinase C-mediated phosphorylation of a phospholipid binding site. *Biochemistry* **48,** 8615–8623

36. Minoshima, Y., Kawashima, T., Hirose, K., Tomonaka, T., Kawai, T., Bao, Y. C., Deng, X., Tatsuka, M., Narumiya, S., May, W. S., Jr., Nosaka, T., Semb, K., Inoue, T., Satoh, T., Inagaki, M., et al. (2003) Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev. Cell* **4,** 549–560

37. Craig, H. E., Coadwell, J., Guillou, H., and Vermeren, S. (2010) ARAP3 binding to phosphatidylinositol-(3,4,5)-trisphosphate depends on N-terminal tandem PH domains and adjacent sequences. *Cell Signal.* **22,** 257–264

38. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* **40,** 658–674

39. Murshudov, G. N., Skubák, P., Lebedev, A. A., Panuš, N. S., Steiner, R. A., Nichols, R. A., Winn, M. D., Long, F., and Vagin, A. A. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* **67,** 355–367

40. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L.-W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66,** 213–221

41. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66,** 486–501

42. DeLano, W. L. (2002) PyMOL, DeLano Scientific, San Carlos, CA

43. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* **372,** 774–797

44. Zhang, B., Wang, Z.-X., and Zheng, Y. (1997) Characterization of the interactions between the small GTPase Cdc42 and its GTPase-activating proteins and putative effectors: comparison of kinetic properties of cDC42 binding to the Cdc42-interactive domains. *J. Biol. Chem.* **272,** 21999–22007