Understanding the Catalytic Mechanism of GTPase-Activating Proteins: Demonstration of the Importance of Switch Domain Stabilization in the Stimulation of GTP Hydrolysis†

Nancy J. Fidyk‡ and Richard A. Cerione*,§

Department of Molecular Medicine, Department of Chemistry and Chemical Biology, and Graduate Field of Biochemistry and Molecular and Cellular Biology, Cornell University, Ithaca, New York 14853

Received July 8, 2002; Revised Manuscript Received October 28, 2002

ABSTRACT: Cdc42, a member of the Rho family of GTP-binding proteins, has been implicated in a variety of biological activities, including the organization of the actin cytoskeleton, changes in cell morphology and motility, intracellular trafficking, cell cycle progression, and cellular transformation. The cycling of Cdc42 between its on (GTP-bound) and off (GDP-bound) states is essential for its stimulation of cell growth and transformation, with an important aspect of this cycle being the regulation of the GTP hydrolytic activity of Cdc42 by its GTPase-activating protein (Cdc42GAP). On the basis of the structural determinations of the Cdc42−Cdc42GAP complex, as well as the Ras−RasGAP complex, it has been proposed that an arginine residue provided by the GAP (called the "arginine finger") stabilizes charges developing on the guanine nucleotide during the transition state for GTP hydrolysis and is an important contributor to GAP-stimulated catalysis. However, the 85 kDa regulatory subunit (p85) of the phosphoinositide 3-kinase (PI-3K) is homologous with the Cdc42GAP and contains the essential arginine residue, but is ineffective as a GAP. This argues that the introduction of the arginine finger is insufficient for GAP activity and that the GAP must fulfill an additional function, one possibility being the engagement and stabilization of the conformationally sensitive switch regions of Cdc42. In the study presented here, we have tested this idea by examining three residues within the Cdc42GAP, which are missing in the GAP homology domain of the 85 kDa regulatory subunit (p85) of the PI 3-kinase and are involved in specific interactions with switch domain residues of Cdc42. We show that the mutation of all three residues, as well as individual mutations of each of these residues, yields GAPs that are defective in stimulating GTP hydrolysis. We further demonstrate that the switch I residue tyrosine 32 plays an important role in GAP interactions and in the regulation of both intrinsic and GAP-stimulated GTP hydrolysis. Taken together, these findings indicate that stabilizing the switch domains of GTP-binding proteins is an important part of GAP-stimulated catalysis, and that the inability of p85 to participate in these interactions may at least in part explain its ineffectiveness as a GAP.

Cdc42, a member of the Rho subfamily of GTP[1]-binding proteins, has been shown to be involved in a wide variety of cellular processes. In mammalian cells, these include roles in cell cycle progression, apoptosis (*1*, *2*), the rearrangement of the actin cytoskeleton (*3*, *4*), activation of MAP kinase pathways (*5*), intracellular trafficking (*6*), and endocytosis (*7*). Like all other GTP-binding proteins, Cdc42 cycles between an active, GTP-bound state and an inactive, GDP-bound state. The cycling between the active and inactive states appears to be essential for many of the cellular activities of Cdc42, particularly, cell growth regulation. GTPase-defective forms of Cdc42 are growth inhibitory (*3*), whereas a point mutant [Cdc42(F28L)] that enables Cdc42 to undergo constitutive GTP−GDP exchange, but does not interfere with GTP hydrolysis, induces cellular transformation when expressed in NIH 3T3 cells (*8*).

Under normal circumstances, the GTP binding−GTP hydrolytic cycle of Cdc42 is tightly controlled by different classes of regulatory proteins. Members of the Dbl family of guanine nucleotide exchange factors or GEFs activate the GTP-binding protein by catalyzing the release of a tightly bound GDP molecule, thereby allowing cellular GTP to take its place in the active site (*9*, *10*). Guanine nucleotide dissociation inhibitors (GDIs) extract Cdc42 from the membrane where signaling takes place and also inhibit the GTP hydrolytic activity of Cdc42, as well as significantly

† This work was supported by grants from the National Institutes of Health (EY06429 and GM47458) to R.A.C.

* To whom correspondence should be addressed. Phone: (607) 253-3888. Fax: (607) 253-3659. E-mail: rac1@cornell.edu.

‡ Graduate Field of Biochemistry, Molecular and Cellular Biology.

§ Department of Molecular Medicine and Department of Chemistry and Chemical Biology.

[1] Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GTP, guanosine 5′−3′-*O*-triphosphate; GDP, guanosine 3′-*O*-diphosphate; Mant-GTP, 2′-*O*-(*N*-methylanthraniloyl)guanosine 5′-triphosphate; Mant-GDP, 2′-*O*-(*N*-methylanthraniloyl)guanosine 5′-triphosphate.

10.1021/bi026413p CCC: $22.00
Published on Web 12/05/2002

slow the dissociation of GDP (*11*, *12*). A third group of regulators are the GTPase-activating proteins (GAPs), which catalyze the hydrolysis of GTP to GDP and ensure that Cdc42 cycles back to its starting or basal state (*13*).

The ability of GAPs to enhance the rate of GTP hydrolysis by Cdc42 plays a key role in controlling Cdc42 signaling, and much effort has been directed toward understanding the underlying mechanism responsible for GAP catalytic activity. The X-ray crystallographic structure for Cdc42 in complex with AlF_3 and the catalytic domain of Cdc42GAP has provided important clues regarding the molecular basis for GAP-stimulated GTP hydrolysis (*14*). The structure clearly shows that the GAP introduces a conserved arginine residue into the active site of Cdc42, which helps to catalyze GTP hydrolysis by stabilizing the transition state of the hydrolytic reaction. This so-called "arginine finger", as originally identified from the structure of the Ras−RasGAP complex (*15*), is a common feature in the majority of the G protein−GAP complexes that have been determined thus far (*14*−*17*), and the importance of the electron-accepting arginine residue in the Cdc42GAP-stimulated GTP hydrolytic reaction has been confirmed through mutagenesis studies (*18*). However, it is clear that the introduction of an essential arginine residue is not the sole role of the Cdc42GAP, as the Cdc42GAP(R305A) mutant retains some catalytic capability, even though its activity is dramatically reduced relative to that of the wild-type Cdc42GAP (*18*). It was proposed, on the basis of the X-ray crystallographic structure of the Cdc42−Cdc42GAP complex, that the GAP may help to stabilize the otherwise flexible switch regions of Cdc42 and, in particular, promote a catalytically active conformation of the conserved Gln61 residue within switch II. Similar stabilization of switch II has been suggested to be imparted by the RasGAP (*15*) as well as by the RGS proteins, which act as GAPs for the heterotrimeric GTP-binding proteins (*19*).

Perhaps an even more interesting indication of the involvement of residues other than the arginine finger in GAP-stimulated GTP hydrolysis comes from a somewhat unique member of the Cdc42GAP (also sometimes termed RhoGAP) family, the 85 kDA regulatory subunit (p85) of the phosphoinositide 3-kinase (PI3-K). The sequence of the p85 protein is significantly similar to that of the limit functional (GAP) domain of Cdc42GAP; however, it shows no detectable GAP activity toward Cdc42 or other members of the Rho family of GTP-binding proteins (*20*). Sequence alignments of p85 and Cdc42GAP suggest that p85 in fact contains a putative, electron-accepting, arginine residue that would be expected to play the role of the arginine finger in GAP-catalyzed GTP hydrolysis. However, specific residues in Cdc42GAP that engage the switch domains of Cdc42 are not conserved in p85. The inability of p85 to make these contacts and thus potentially stabilize the switch domains of Cdc42 and other Rho-related proteins may account for its ineffectiveness as a GAP as well as highlight an important aspect of GAP-stimulated GTP hydrolysis. In this study, we have examined the importance of these residues in the ability of Cdc42GAP to stimulate the GTP hydrolytic reaction and provide evidence that supports a critical role for switch stabilization in the underlying mechanism of GAP-catalyzed GTP hydrolytic activity. In addition, through examination of the GAP contacts made in the switch regions of Cdc42, we have considered the role of a switch I residue in Cdc42, namely, tyrosine 32, in both intrinsic and Cdc42GAP-stimulated GTP hydrolysis.

MATERIALS AND METHODS

cDNA Constructs. Constructs encoding wild-type Cdc42, the GTPase-defective Cdc42(Q61L) mutant, and the limit Cdc42GAP were prepared using a pET15b expression vector (Novagen) as previously described (*18*). Four Cdc42GAP mutants were generated and designated GAP(R346L), GAP(N414V), GAP(N422M), and the triple GAP mutant (which contained each of the three point mutations). Five Cdc42 mutants were also generated: Cdc42(Y32A), Cdc42(Y32E), Cdc42(Y32F), Cdc42(Y32K), and Cdc42-(Y32S). All mutants were made in the pET15b expression vector using the Quickchange PCR mutagenesis system (Stratagene). Each construct was sequenced to verify that the appropriate mutation was generated.

Expression of the Limit Cdc42GAP. Constructs encoding the limit Cdc42GAP (residues 234−462, called simply Cdc42GAP from here on) were expressed in *Escherichia coli* as hexahistidine (six-His tag) fusion proteins and purified according to previously described protocols with some modification (*18*). GAP constructs were transformed into *E. coli* BL21(DE3) cells that were grown and induced using standard protocols. Bacterial pellets were resuspended in NiA buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole, and 1 mM NaN_3] and then lysed using an SLM-Aminco French Pressure Cell Press (Spectronic Instruments) set at 1500 psi. Lysates were cleared by centrifugation at 50000*g* for 30 min. The supernatant was loaded onto a 5 mL Hi Trap-chelating Sepharose Fast Flow column (Amersham/Pharmacia) charged with nickel sulfide (Sigma), and the recombinant proteins were eluted with a linear imidazole gradient (from 5 to 200 mM). The eluted GAPs were examined by SDS−polyacrylamide gel electrophoresis and then pooled and precipitated by a 30 min incubation at 4 °C in 55% ammonium sulfate. The suspensions were centrifuged at 12000*g* for 30 min at 4 °C, and the pellets were resuspended in TEDA buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 1 mM NaN_3]. The resuspended GAPs were treated with thrombin to remove the six-His tag, and the resultant mixtures were dialyzed overnight against TEDA buffer. The GAP samples were loaded onto a TEDA-equilibrated Q-Sepharose column and were eluted with a 0 to 300 mM NaCl gradient. The purified Cdc42GAPs were then concentrated and finally dialyzed against TEDA buffer containing 40% glycerol for storage at −20 °C. The final concentrations of the GAPs were typically ∼3.5 mg/mL.

Cdc42 Expression. All Cdc42 constructs were expressed in *E. coli* BL21(DE3) cells as six-His tag fusion proteins using standard protocols (see ref *18*). The recombinant, purified Cdc42 proteins were examined by SDS−polyacrylamide gel electrophoresis, pooled, concentrated, and then dialyzed against HMA buffer [20 mM HEPES (pH 8.0), 5 mM $MgCl_2$, and 1 mM NaN_3] containing 40% glycerol.

Single-Turnover Assays for GTP Hydrolysis. Single-turnover GTP hydrolysis assays were performed as previously described (*21*). Cdc42 (3 μM) was first incubated in 20 mM Tris-HCl (pH 8.0), 10 μM GTP with [γ-^{32}P]GTP, 1 mM NaN_3, 250 μM DTT, 120 mM NaCl, 0.2 mg/mL BSA, and 24 mM EDTA at room temperature for 30 min. The

[γ-^{32}P]GTP-bound Cdc42 was then incubated with Cdc42GAP (5 nM) in 20 mM Tris-HCl (pH 8.0), 2 mM GTP, 1 mM NaN_3, 0.2 mg/mL BSA, 50 mM $MgCl_2$, 80 mM NaCl, and 200 μM DTT to initiate GTP hydrolysis. Aliquots of the hydrolysis reaction mixture were removed at different time points, and the reaction was terminated by dilution into 3 mL of dilution buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM $MgCl_2$] at 4 °C. The diluted samples were then filtered through 25 mm BA-85 nitrocellulose filters (Schleicher and Schuell), and the filters were assessed for radioactivity using a Beckman scintillation counter.

Tryptophan Fluorescence Assays. Changes in the intrinsic fluorescence of tryptophan 97 of Cdc42 serves as a readout for GTP hydrolysis (*21*). Cdc42 (1 μM) in HMN buffer [20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 100 mM NaCl] was treated with 10 mM EDTA and 20 μM GTP to exchange bound GDP for GTP. Once equilibrated, 20 mM $MgCl_2$ was added along with Cdc42GAP (10 nM) to initiate the GTP hydrolytic reaction. The changes in tryptophan fluorescence (excitation at 280 nm and emission at 330 nm) were measured in an SLM 8000c spectrofluorimeter. Measurements of specific (GAP) activity were performed by maintaining the concentration of wild-type Cdc42 at 2 μM while varying the concentration of the Cdc42GAP.

Preparation of Cdc42·Mant-Labeled Nucleotide Complexes. Wild-type Cdc42 was preloaded with Mant-dGDP, and Cdc42(Q61L) was preloaded with Mant-dGTP as described previously (*22*). Mant-labeled deoxynucleotides were generated using previously described procedures (*23*). The Cdc42 protein was incubated at 4 °C for 4 h with a 10-fold molar excess of Mant-labeled nucleotide in the presence of 10 mM EDTA. The nucleotide exchange reaction was then quenched with 20 mM $MgCl_2$. Following exchange, the Cdc42 sample was applied to a G-25 superfine grade gel filtration column (Amersham Pharmacia) pre-equilibrated with HMA buffer to remove excess Mant-labeled nucleotide. The eluted Cdc42·Mant-labeled nucleotide complexes were then concentrated and stored in HMA buffer containing 40% glycerol.

Binding Assays. Formation of a complex between Cdc42 and the GAP mutants was monitored by measuring the fluorescence anisotropy of the bound Mant-labeled nucleotide as previously described (*22*). Cdc42·Mant-labeled nucleotide complexes (1 μM) were incubated in HMN buffer [20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 100 mM NaCl] and titrated with Cdc42GAP in the presence or absence of 60 μM $AlCl_3$ and 25 mM NaF. Anisotropy values were measured in a T-format using an SLM 8000C spectrofluorimeter. The dissociation constant (K_d) values for the Cdc42−Cdc42GAP complexes were obtained by fitting anisotropy data with a simple equilibrium model for a bimolecular interaction (*22*).

RESULTS

The three-dimensional structure for Cdc42 in complex with Cdc42GAP and AlF_3 suggested that GAP-stimulated GTP hydrolysis requires the introduction of an arginine residue into the active site of the GTP-binding protein (*14*). The catalytic arginine residue, termed the arginine finger, stimulates GTP hydrolysis by stabilizing the negative charge generated during the formation of the transition state, as well as by coordinating Gln61, a vital residue which properly positions the attacking water molecule. Nonetheless, we have previously shown that a GAP(R305A) mutant retains some catalytic activity (*18*). Moreover, the sequence of the 85 kDa regulatory subunit of PI 3-kinase (p85) is 21% identical and 47% similar with that of the Cdc42GAP within the limit functional (GAP) domain, and p85 contains the catalytically essential arginine finger (Figure 1A; the essential conserved arginine residue is in red); however, it does not exhibit measurable GAP activity toward Cdc42 (*20*), or toward any other Rho family member. Other functional members of this family such as BCR (29% identical and 46% similar), BEM3 (26% identical and 48% similar), and p190 (29% identical and 58% similar) share similar levels of identity and similarity to the GAP domain of Cdc42GAP. Sequence alignments between p85 and Cdc42GAP show that certain key residues contributed by the Cdc42GAP, which contact and potentially stabilize the switch domains of Cdc42, are not conserved in p85. These residues include arginine 346 and asparagine residues 414 and 422 of Cdc42GAP, where the corresponding residues in p85 are all nonpolar, specifically leucine 209, valine 277, and methionine 285, respectively (Figure 1A, in blue).

Figure 1B (left panel) shows that the Cdc42GAP (in green) engages the switch I and II domains of Cdc42 (in magenta; the remainder of the Cdc42 molecule is violet). The right panels of Figure 1B depict the hydrogen bonding contacts between arginine 346, asparagine 414, and asparagine 422 of the Cdc42GAP and the switch regions of Cdc42. We predicted that the corresponding residues in p85 would not be able to support these hydrogen bonds, thus providing a possible explanation for its lack of GAP activity. In this study, we set out to directly establish the importance of stabilizing the switch regions of Cdc42 in GAP-stimulated GTP hydrolysis by substituting arginine 346, asparagine 414, and asparagine 422 (individually and in tandem) for the corresponding residues found in p85. These substitutions were made within the limit functional domain of the Cdc42GAP, which lacks the 234 amino-terminal residues of the full-length GAP (*24*). The recombinant GAPs were each expressed with a six-His tag that was utilized during purification and was later removed by thrombin cleavage. Each of the purified recombinant GAPs was assessed by SDS−PAGE (Figure 1C).

Relative Rates of Cdc42GAP-Stimulated GTP Hydrolysis. The relative rates of GTP hydrolysis were determined in single-turnover assays measuring the extent of ^{32}P release and by monitoring the changes in the intrinsic tryptophan fluorescence of Cdc42, which accompanies the conversion of the GTP-bound state to the GDP-bound form of the protein. In the former case, Cdc42 was first loaded with [γ-^{32}P]GTP and then incubated in the presence of catalytic amounts of either wild-type Cdc42GAP or the different GAP mutants. The results presented in Figure 2A show that the GAP(R346L) mutant (blue line) was able to stimulate GTP hydrolysis as monitored by the release of $^{32}P_i$ with activity similar to that of the wild-type GAP (red line; in each plot, the green line depicts the intrinsic GTP hydrolytic activity of Cdc42). However, this was not the case for the other GAP mutants. While the GAP(N414V) mutant exhibited a moderate stimulation of GTP hydrolysis, both the GAP(N422M) mutant and the triple mutant were completely incapable of

A

```
Cdc42   283  PIVLRETVAYLQA-HALTTEGIFRRSANTQVVREVQQKYNMG----------------LPVDFDQYNE
p85     129  PPLLIKLVEAIEK-KGLECSTLYRTQ-SSSNLAELRQLLDCD----------------TPSVDLEMID
BCR    1068  PYIVRQCVEEIER-RGMEEVGIYRVSGVATDIQALKAAFDVN--------------NKDVSVMMSEMD
BEM2   1981  PTIVVKLLEEIEL-RGLDEVGLYRIPGSIGSINALKNAFDEE----------GATDNSFTLEDDRWFE
BEM3    927  PSVVYRCLEYLYKNRGIQEEGIFRLSGSSTVIKTLQERFDKEYDVDLCRYNESIEAKDDEASPSLYIG
p190   1262  PIFIERCIEYIEA-TGLSTEGIYRVSGNKSEMESLQRQFDQD---------------HNLDLAEKDFT

Cdc42   334  LHLPAVILKTFLRELPEPLLTFDL---YPHVVGFLNIDE---SQRVPATLQVLQT--LPEENYQVLRF
p85     179  VHVLADAFKRYLLDLPNPVIPAAVYSEMISLAPEVQSSE----EYIQLLKKLIRSPSIPHQYWLTLQY
BCR    1121  VNAIAGTLKLYFRELPEPLFTDEF---YPNFAEGIALSD--PVAKESCMLNLLLS--LPEANLLTFLF
BEM2   2038  VNAIAGCFKMYLRELPDSLFSHAMVNDFTDLAIKYKAHAMVNEEYKRMMNELLQK--LPTCYYQTLKR
BEM3    995  VNTVSGLLKLYLRKLPHLLFGDEQ---FLSFKRVVDENHNNPVQISLGFKELIESGLVPHANLSLMYA
p190   1314  VNTVAGAMKSFFSELPDPLVPYSM---QIDLVEAHKIND--REQKLHALKEVLKK--FPKENHEVFKY

Cdc42   394  LTAFLVQISAHSDQNKMTNTNCAVVFGPNLLWAKDAAITLKAIN--PINTFTKFLLDHQGELFPS
p85     243  LLKHFFKLSQTSSKNLLNARVLSEIFSPMLFRFSAASSDNTENLIKVIEILISTEWNER------
BCR    1182  LLDHLKRVAEKEAVNKMSLHNLATVFGPTLLRPSEKESKLPANPSQPITMTDSWSLEVMSQVQ--
BEM2   2104  IVFHLNKVHQHVVNNKMDASNLAIVFSMSFIN--------------QEDLAN-------------
BEM3   1060  LFELLVRINENSKFNKMNLRNLCIVFSPTLN--------------IPISMLQ-------------
p190   1375  VISHLNRVSHNNKVNLMTSENLSICFWPTLMRPDFSSMDALTATRSYQTIIELFIQQCPFF----
```

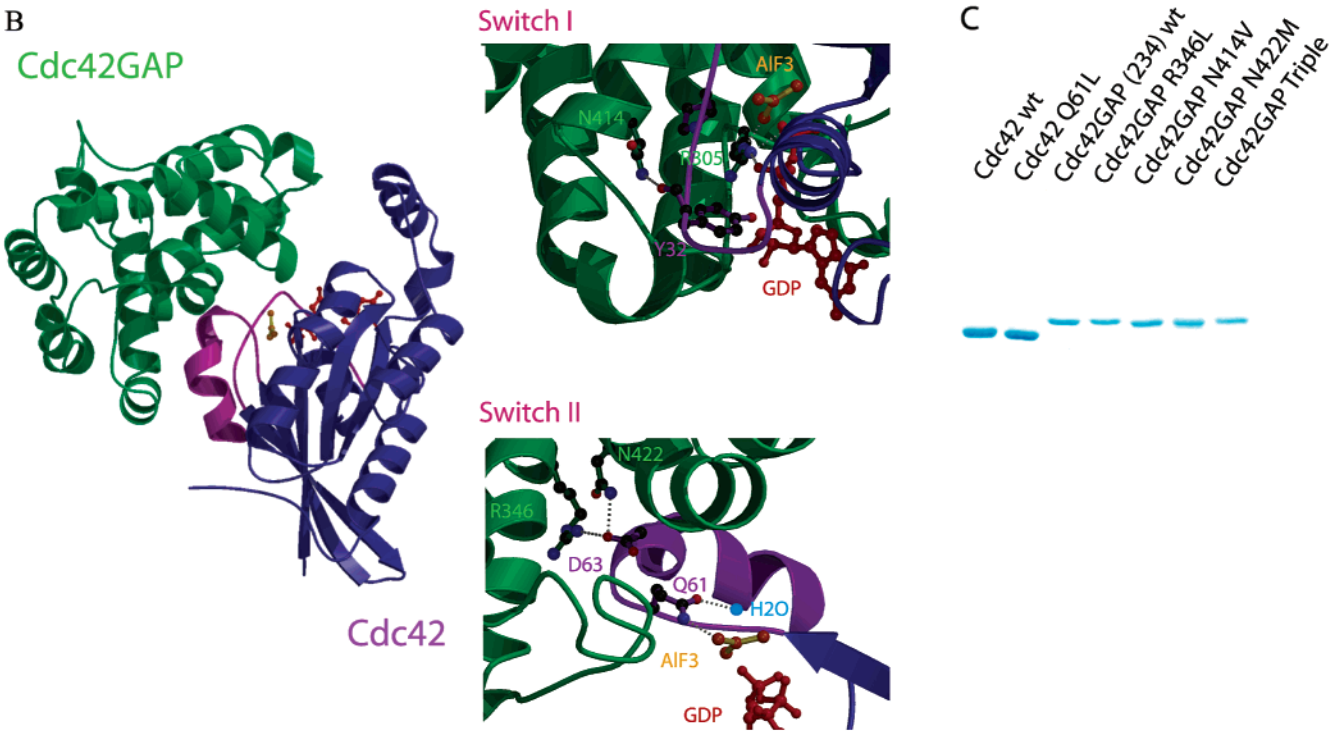

FIGURE 1: Cdc42GAP residues, which contact the switch domains of Cdc42 and are not conserved in p85. (A) Sequence alignments of the limit GAP domains were performed on the original GenBank entries for the indicated proteins using the Clustal algorithm of the DNAStar program. The highly conserved catalytic arginine residue is highlighted in red. Residues that are highlighted in blue are conserved among functional RhoGAP family members that at least retain their ability to hydrogen bond at this position. In p85, a RhoGAP family member for which GAP activity remains undetected, hydrophobic residues that are incapable of forming hydrogen bonds are found at these same positions. (B) The X-ray crystal structure of Cdc42 bound to Cdc42GAP illustrates how Cdc42GAP contacts the switch domains of Cdc42 using the aforementioned residues (*14*). Both arginine 346 and asparagine 422 of Cdc42GAP hydrogen bond to aspartic acid 63, a residue in the switch II domain of Cdc42. Asparagine 414 of Cdc42GAP hydrogen bonds to the backbone carbonyl group of tyrosine 32, a residue in the switch I domain of Cdc42. Panels A and B were generated using Molscript 1.4 (*34*), Bobscript 2.5 (*32*), Raster3D 2.5 (*34*), and Spock (*35*). (C) The recombinant proteins used in these studies were expressed with a six-His tag for aid in purification (see Materials and Methods), assessed by 12% SDS−PAGE, and visualized with Coomassie Blue staining.

stimulating GTP hydrolytic activity. These results suggested that asparagine 414 and, in particular, asparagine 422 were important contributors to Cdc42GAP activity.

Figure 2B shows the results obtained when assaying GTP hydrolytic activity by monitoring changes in the tryptophan fluorescence of Cdc42 (*21*). This assay represents a real-time measurement and can give accurate initial rate information (e.g., see Figure 3, below). The results obtained using this fluorescence read-out basically confirmed those from the single-turnover measurements of [γ-^{32}P]GTP hydrolysis.

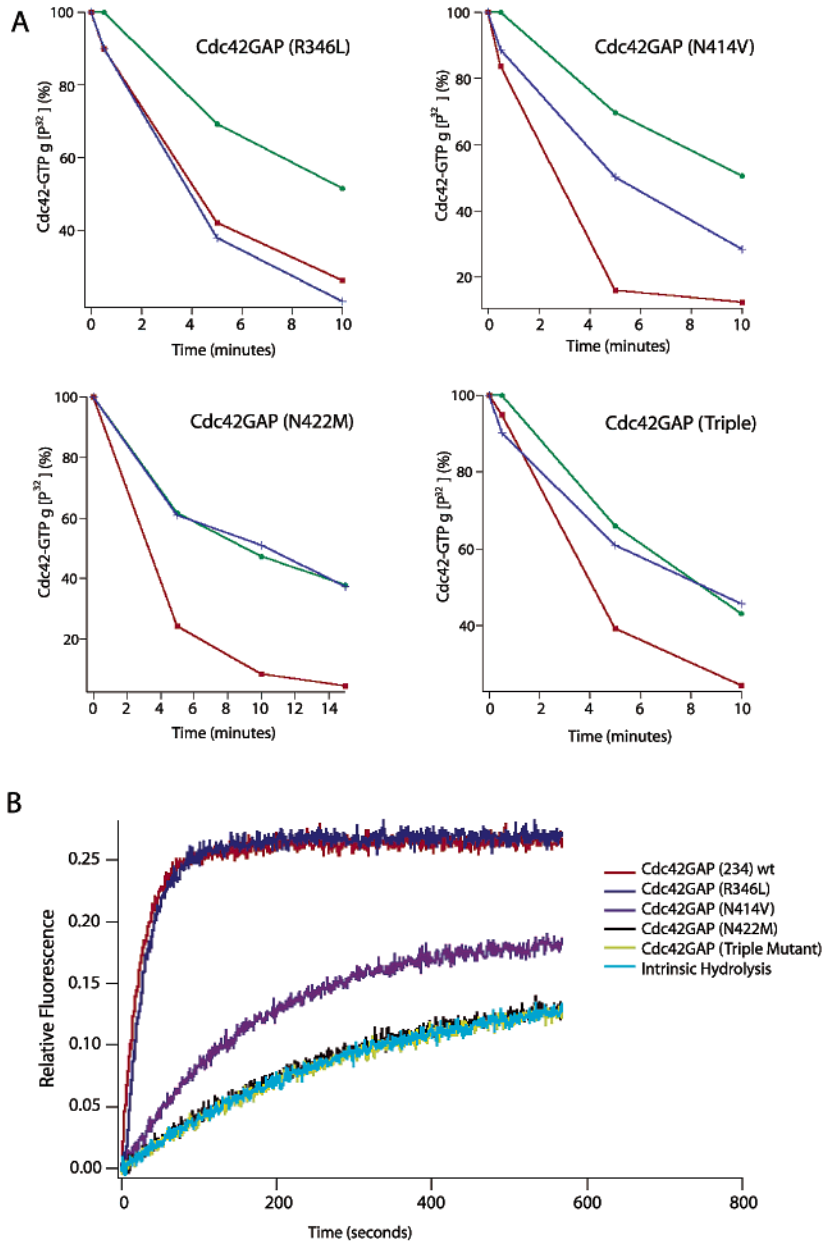

FIGURE 2: Assaying for GAP-stimulated GTP hydrolysis by mutant Cdc42GAPs. (A) Single-turnover assays. This filter binding assay was used to compare the activities of mutant Cdc42GAPs with that for wild-type Cdc42GAP. The green line depicts the intrinsic GTP hydrolysis of Cdc42; the red line depicts GTP hydrolysis stimulated by wild-type Cdc42GAP, and the blue line depicts the activity for the individual GAP mutants. Each plot is representative of three independent experiments. (B) Tryptophan fluorescence assay. The GTP hydrolysis rates catalyzed by the different Cdc42GAP mutants were measured following the changes in tryptophan 97 fluorescence as stated in Materials and Methods, and the data were normalized for direct comparison of the GAP activity for each protein. These data are representative of three independent experiments for each GAP mutant.

The GAP(R346L) mutant exhibited activity that was comparable to that exhibited by wild-type Cdc42GAP. However, the rate of GTP hydrolysis catalyzed by the GAP(N414V) mutant was clearly compromised relative to that of the wild-type GAP, and both the GAP(N422M) and triple mutants lacked any detectable catalytic capability under these conditions.

A plot of the initial rates of the GAP-catalyzed GTP hydrolytic activity determined from the fluorescence measurements, as a function of GAP concentration for wild-type Cdc42GAP and the different GAP mutants, is presented in Figure 3. These results show that when we examine catalytic activity over a range of GAP concentrations, we see that the GAP(R346L) mutant is actually ~5-fold less potent than the wild-type Cdc42GAP in its ability to stimulate the initial rate of GTP hydrolysis by Cdc42, while the GAP(N414V) and GAP(N422M) mutants show specific activities that are 30- and 300-fold reduced, respectively, compared to that of the wild-type Cdc42GAP. It is interesting that the asparagine to valine substitution at position 414 compromises the GAP-stimulated GTP hydrolytic activity to an extent similar to that of the arginine to alanine substitution at position 305. When all three mutations are combined in the same GAP molecule, the ability to stimulate GTP hydrolysis is severely impaired, with the specific activity of the triple mutant being reduced by a factor of >5000-fold.

Measurements of the Binding Affinities of Cdc42 for the Different Cdc42GAP Mutants. We next set out to determine

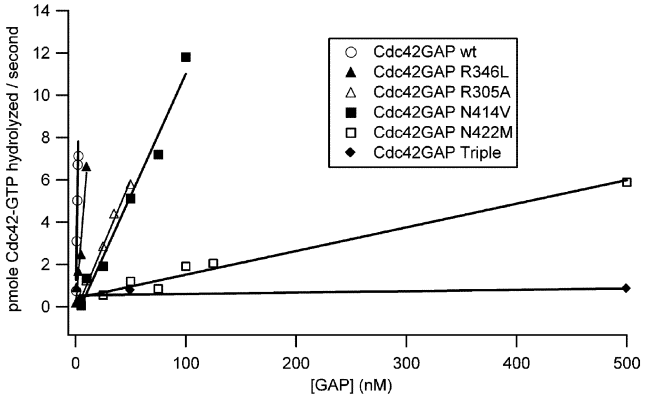

FIGURE 3: Specific activities for the different Cdc42GAP mutants. Rates of GTP hydrolysis were determined using the tryptophan fluorescence assay (*18*, *21*). The rates of GAP-stimulated GTP hydrolysis were corrected for intrinsic GTPase activity and plotted vs the amount of GAP protein added to the assay incubation. The concentration of Cdc42 was 2 μM. Data are representative of two to three independent experiments.

to what extent the reductions in the GAP-catalyzed GTP hydrolytic activity obtained with the different GAP mutants reflected an impaired ability to bind Cdc42. The binding of the different GAPs to Cdc42 was assessed by changes in the fluorescence anisotropy of Mant-guanine nucleotide analogues bound to Cdc42. Specifically, binding experiments were conducted with Cdc42 bound to Mant-dGDP in the presence of AlF_3, and with the GTPase-defective Cdc42(Q61L) mutant bound to Mant-dGTP. The AlF_3- and GDP-bound form of Cdc42 mimics the transition state for GTP hydrolysis, whereas the GTP-bound form of the Cdc42(Q61L) protein represents the ground state for the GTP hydrolytic reaction. Via comparison of the binding of the different GAP mutants to these two forms of Cdc42, it should be possible to determine whether the individual mutations specifically affect the ability of the GAP to stabilize the transition state for GTP hydrolysis, or if they impart a general reduction in the affinity of the GAP for Cdc42.

Figure 4A shows the titration profile for the Cdc42·Mant-dGDP complex with increasing amounts of the different GAPs in the presence of 60 μM $AlCl_3$ and 25 mM NaF [i.e., conditions that yield saturating amounts of AlF_3 (*22*)]. The K_d value measured for the wild-type Cdc42GAP was approximately 1 μM, which is within the range of other reported values (*18*, *22*, *25*). The GAP(R346L) and GAP(N414V) mutants both yielded K_d values of $\sim$1 μM, indicating that these point mutations did not significantly affect the ability of the GAP to stabilize the transition state complex for GTP hydrolysis. Experiments with the GAP(N422M) mutant revealed binding 4−5-fold weaker than that of the wild-type Cdc42GAP, while the triple GAP mutant showed a severe reduction in affinity (>100-fold reduction).

Figure 4B shows the corresponding sets of titration profiles obtained for the interactions of the different GAPs with the ground state form of Cdc42. The GAP(R346L) and GAP(N414V) mutants behaved like the wild-type GAP and again yielded K_d values of $\sim$1 μM. The GAP(N422M) mutant displayed an impaired binding ($K_d \sim 6$ μM), and the triple mutant exhibited a very weak interaction ($K_d \sim 120$ μM) with the GTP-bound (ground state) form of Cdc42. All measured K_d values are summarized in Table 1. Overall, these data indicate that the GAP(R346L) and GAP(N414V) mutants, while catalytically defective, do not significantly affect the stability of the transition state complex or binding by the GAP to the ground state, whereas the GAP(N422M) and the triple GAP mutant are compromised in their ability to bind Cdc42, which may at least in part account for the inability of these latter two GAP mutants to catalyze the GTP hydrolytic reaction of Cdc42.

Examination of a Critical Tyrosine Residue in the Switch I Region of Cdc42. Taken together, the work described in the preceding sections provides support for the necessity of GAP interactions with the switch domains of GTP-binding proteins in the mechanism of GAP-stimulated GTP hydrolysis. If in fact this is the case, it should be possible to demonstrate this with an appropriate point mutation(s) in one of the switch domains of the GTP-binding protein, as well as with different point-mutated GAPs. Of particular importance is the hydrogen bond interaction between asparagine 414 of the GAP and the backbone carbonyl group of tyrosine 32 of Cdc42. The X-ray crystallographic structure for the Cdc42·GDP·AlF_3−Cdc42GAP complex shows that asparagine 414 of the GAP positions tyrosine 32 within the switch I region of Cdc42 so that it is able to interact with arginine 305 (i.e., the arginine finger) (Figure 5A). The crystallographic structure suggests that the phenyl group of tyrosine 32, together with proline 34, places the arginine finger in a catalytic conformation, sandwiching it in the active site through π-cation interactions (*26*). The positioning of the arginine finger allows it to precisely coordinate glutamine 61 for nucleophilic attack.

These structural observations suggest that the side chain of tyrosine 32 plays a critical role in GAP-stimulated GTP hydrolysis and that substitutions at this position would be expected to produce Cdc42 mutants that have compromised abilities to respond to the GAP. To test this idea, Cdc42 was mutated at position 32 to various conserved and nonconserved residues. Phenylalanine and serine were chosen as conserved mutations where the phenyl and hydroxyl groups were preserved, respectively. Alanine, glutamic acid, and lysine substitutions for tyrosine 32 introduce a methyl group, a negatively charged residue, and a positively charged residue, respectively. All of the Cdc42(Y32) mutants were expressed in *E. coli* and purified to homogeneity (Figure 5B).

The abilities of the different Cdc42(Y32) mutants to hydrolyze GTP in the presence and absence of Cdc42GAP were analyzed by monitoring changes in the tryptophan fluorescence of Cdc42. The relative rates of hydrolysis were measured under conditions where the Cdc42GAP was functioning catalytically (2 nM Cdc42GAP per 2 μM Cdc42). The data presented in Figure 6 show that the intrinsic GTP hydrolytic activity for each of the Cdc42(Y32) mutants was reduced compared to that of wild-type Cdc42. Cdc42(Y32S) was capable of the highest level of intrinsic GTP hydrolytic activity, displaying $\sim$80% of the activity measured for wild-type Cdc42; on the other hand, the Cdc42(Y32F) mutant was 30% as active as wild-type Cdc42, and each of the other Cdc42(Y32) mutants exhibited $\sim$40% of the intrinsic GTP hydrolytic activity measured with wild-type Cdc42.

The different Cdc42(Y32) mutants were also impaired in their ability to be stimulated by Cdc42GAP (Figure 6). Only the more conserved substitutions, specifically, Y32S and Y32F, were able to respond to Cdc42GAP, although these

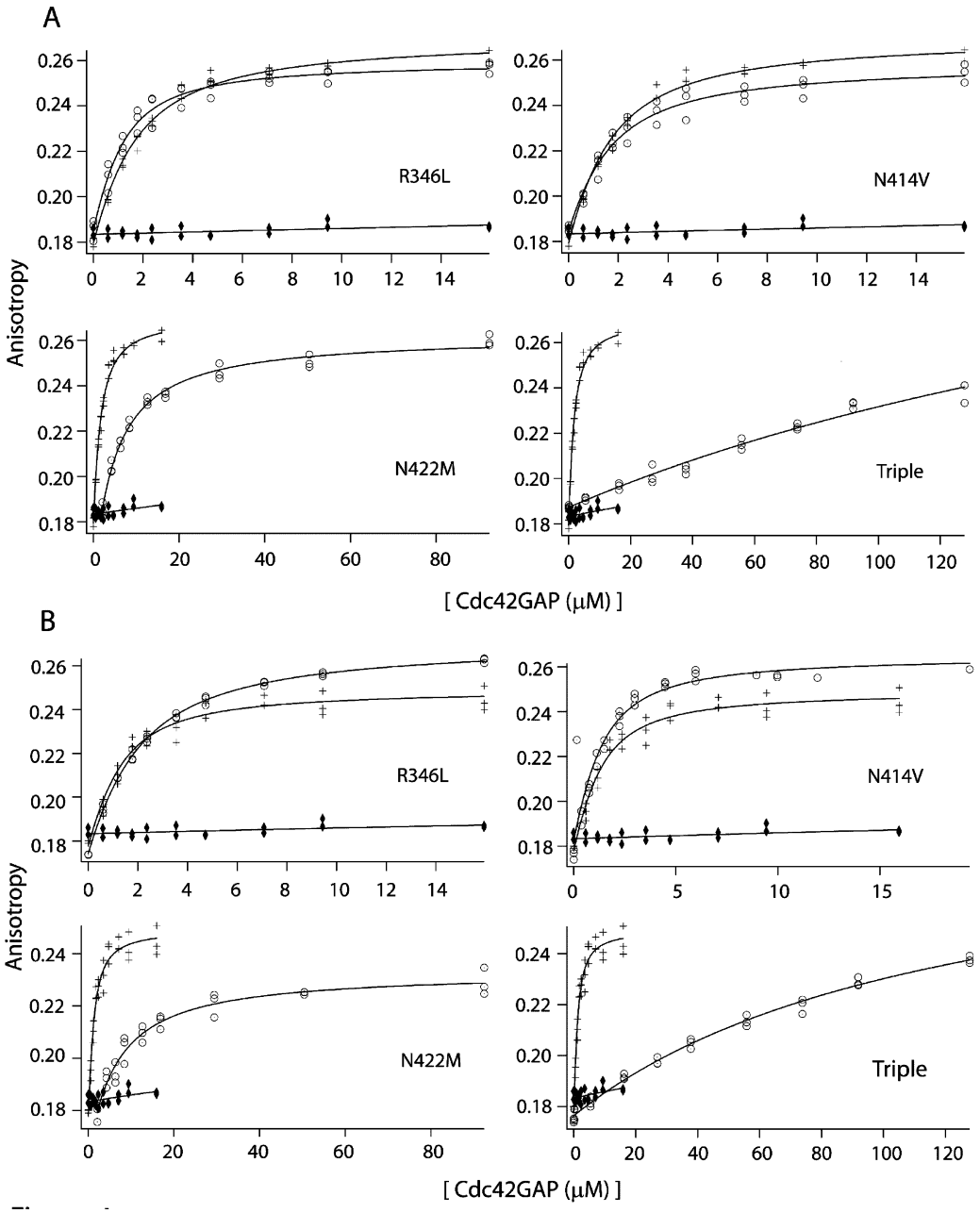

FIGURE 4: Fluorescence anisotropy measurements of Cdc42−Cdc42GAP interactions. (A) Cdc42·Mant-dGDP·AlF_3 (1 μM) was titrated with wild-type Cdc42GAP (+), the indicated Cdc42GAP mutants (○), or buffer alone (♦) as indicated in Materials and Methods. (B) The Cdc42(Q61L)·Mant-dGTP complex was titrated with wild-type Cdc42GAP (+), the indicated Cdc42GAP mutants (○), or buffer alone (♦). The anisotropy measurements were made and the data fit to a simple binding model (—) as described by Hoffman et al. (*22*).

Table 1: Summary of Binding Affinities for Cdc42−Cdc42GAP Complexes As Measured by Fluorescence Anisotropy and Catalytic (GAP) Activity Measured by the Change in the Tryptophan 97 Fluorescence

Cdc42GAP	ground state Cdc42Hs Q61L (μM)	transition state wild-type Cdc42Hs (μM)	relative hydrolysis activity
wild-type	0.766	1.25	100%[a]
R346L	1.52	0.706	5-fold decrease
N414V	0.817	1.25	28-fold decrease
N422M	6.22	4.69	~300-fold decrease
triple mutant	124.292	191.87	>5000-fold decrease

[a] One hundred percent refers to the specific activity measured for the wild-type Cdc42GAP [3.28 pmol s^{-1} (nM GAP)$^{-1}$] (see Figure 3).

mutants did not exhibit GAP-stimulated GTP hydrolytic activity that was comparable to that measured for wild-type Cdc42. The GTP hydrolytic activity of the Cdc42(Y32F) mutant was stimulated approximately 2-fold by Cdc42GAP, while the GTP hydrolytic activity of the Cdc42(Y32S) mutant was stimulated ~1.5-fold over the intrinsic hydrolytic activity.

The inability of the Cdc42(Y32) mutants to optimally hydrolyze GTP in the presence of Cdc42GAP is not due to a weakened affinity for Cdc42GAP. The bond between asparagine 414 of the GAP and tyrosine 32 of Cdc42 does not appear to influence the affinity of the Cdc42−Cdc42GAP complex. Previous results showed that when the asparagine was mutated to a valine, the GAP(N414V) mutant was able to bind to Cdc42 with wild-type affinity. To confirm that substitutions at position 32 do not affect binding affinity, we looked at formation of a complex between Cdc42(Y32K)

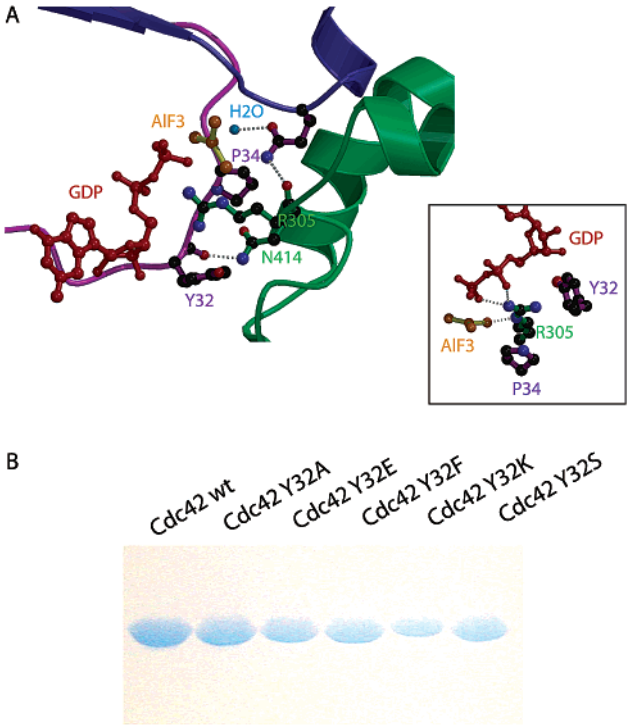

FIGURE 5: Importance of tyrosine 32 in the interactions between Cdc42 and Cdc42GAP. (A) Structural depiction of the role of tyrosine 32, from the switch I domain of Cdc42, in positioning arginine 305 of the Cdc42GAP. Panel A was generated using Molscript 1.4 (*34*), Bobscript 2.5 (*32*), Raster3D 2.5 (*34*), and Spock (*35*). (B) The Cdc42(Y32) mutants were each expressed with a six-His tag for aid in purification (see Materials and Methods). Recombinant proteins were examined by 12% SDS−PAGE and visualized with Coomassie Blue staining.

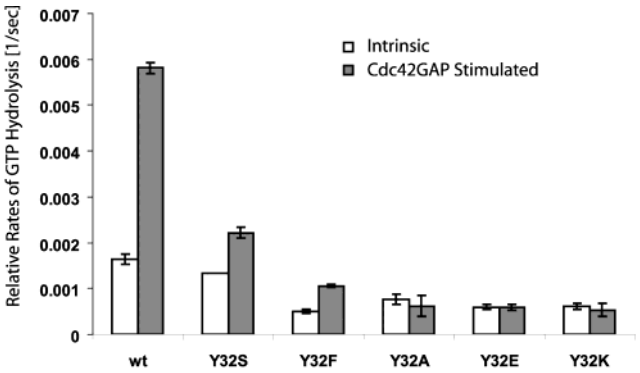

FIGURE 6: Mutational analysis of tyrosine 32 of Cdc42. Tyrosine 32 was systematically mutated to serine, phenylalanine, alanine, glutamic acid, and lysine residues. The GAP activity of these mutants was analyzed under one set of conditions [2 nM wild-type Cdc42GAP and 2 μM Cdc42 in HEPES buffer (pH 8.0) containing Mg^{2+} and NaCl]. Relative rates were determined by fitting changes in tryptophan 97 fluorescence, which accompanies GTP hydrolysis, with a single exponential and extracting the rate constants. Data analysis was performed using the Igor Software package from Wavemetrics, Inc.

and Cdc42GAP by monitoring changes in the fluorescence anisotropy of Mant-labeled guanine nucleotides and found no significant changes (data not shown). Taken together with the hydrolysis data, these results further highlight the importance of GAP-mediated stabilization of the switch domains in achieving optimal catalysis of the GTP hydrolytic reaction.

DISCUSSION

It has been accepted for some time that an essential arginine residue is critical for GAP-stimulated GTP hydrolysis (*15*). This residue, termed the arginine finger, is conserved among the different GAPs for Rho GTP-binding proteins (*14*, *16*) as well as being present in the switch I domain of the α-subunits of heterotrimeric G proteins (*27*, *28*). In the cases of RasGAP and Cdc42GAP, the arginine finger introduces a positive charge into the active site, stabilizing the negative charge, which develops during the transition state for GTP hydrolysis, and also positions glutamine 61 so that it can coordinate the nucleophilic attacking water molecule. The role of arginine 305 in Cdc42GAP activity has been further established through mutational analysis where the critical arginine was mutated to an alanine (*18*), resulting in a 45-fold reduction in specific activity when compared to that of the wild-type GAP. Still, although its catalytic capability was severely impaired, the GAP(R305A) mutant retained detectable activity. This indicates that while arginine 305 is indeed an important residue for proper Cdc42GAP function, it is not the sole contributor to catalytic activity.

Recent structural work has suggested the potential importance of stabilizing the switch domains in GAP-stimulated GTP hydrolysis. The X-ray crystal structure of the Cdc42−Cdc42GAP complex shows that the GAP makes a number of contacts with the switch I and II domains of Cdc42 (*14*). These conformationally sensitive regions, initially identified in Ras and subsequently shown to be present in all monomeric GTP-binding proteins, undergo conformational changes upon the exchange of GTP for GDP because they contain residues that interact with the bound nucleotide, the attacking water molecule, and the Mg^{2+} ion located in the active site. The GAP may help stabilize the switch domains to ensure that the GTP-binding protein is in a conformation that is optimal for hydrolysis. The idea that switch stabilization is an essential part of the GAP function is also suggested by work on the family of RGS (for regulator of G protein signaling) proteins, which serve as GAPs for the heterotrimeric or "large" G proteins. The X-ray crystallographic structure of the Gi1α·GDP·AlF_4^-·RGS4 complex shows that RGS4 interacts with the switch domains of Gi1α and does not contribute an arginine residue to the active site; i.e., the essential arginine is contributed by the large helical domain of the Gi1α (*19*). Thus, the ability of RGS4 to give rise to a marked stimulation of the GTP hydrolytic activity of Gi1α may be due to its ability to stabilize the switch domains.

The likely importance of switch stabilization in GAP-stimulated GTP hydrolysis is probably most strongly implicated by the fact that p85, the regulatory subunit of the PI 3-kinase, lacks detectable GAP activity. The p85 subunit is homologous with the limit functional domain of the Cdc42GAP (21% identical and 47% similar) and, importantly, contains the highly conserved arginine finger. However, comparison of the p85 GAP domain with the Cdc42GAP and other related members of the RhoGAP family shows that some residues used by the GAPs to contact the switch domains are not conserved in p85. These residues include arginine 346 and asparagines 414 and 422 in Cdc42GAP, which correspond to leucine 209, valine 277, and methionine 285, respectively, in p85. The hydrophilic residues found in

Cdc42GAP are involved in hydrogen bonds with both the switch I and II domains of Cdc42. Specifically, the side chain amino groups of arginine 346 hydrogen bond to glutamic acid 62 and aspartic acid 63 from switch II of Cdc42; asparagine 422 forms a hydrogen bond with aspartic acid 63, and asparagine 414 forms a hydrogen bond with the backbone carbonyl group of tyrosine 32, which is located in the switch I domain (*14*). These polar interactions cannot take place between Cdc42 and p85 because p85 has hydrophobic residues at these positions. Thus, it seemed attractive to consider that the inability of p85 to participate in these interactions with the switch domains of Cdc42 underlies its inability to stimulate GTP hydrolysis.

In this study, we have examined the importance of arginine 346, asparagine 414, and asparagine 422 for GAP-stimulated GTP hydrolysis. By mutating these residues to the hydrophobic residues found in p85, we generated impaired Cdc42GAP proteins. While the GAP(R346L) mutant was only modestly impaired (an ~5-fold reduction in GAP activity), the substitution of valine and methionine residues for asparagine at positions 414 and 422, respectively, had a marked effect on GAP activity. For example, the GAP(N414V) mutant exhibited a specific activity 30-fold lower than that of the wild-type GAP. While this mutant is still fully able to bind to Cdc42 with wild-type affinity, it is as catalytically impaired as the GAP(R305A) mutant, which lacks the arginine finger. However, unlike the GAP(R305A) mutant, which binds with a significantly weaker affinity (~10-fold) to the Cdc42·Mant-dGDP·AlF_3 species than to the Cdc42(Q61L)·Mant-dGTP species, the GAP(R346L) mutant bound with similar affinities to those two forms of Cdc42. Thus, the Cdc42−AlF_3 complex apparently does not distinguish every type of catalytically compromised GAP mutant and may work particularly well in the case of the GAP(R305A) mutant because it lacks the arginine finger that is needed to help coordinate the AlF_3 species (*14*). The specific activities of the GAP(N422M) mutant, as well as the triple mutant, were even more dramatically reduced (by 300- and >5000-fold, respectively) compared to the wild-type GAP. The GAP(N422M) mutant exhibited a 4−5-fold reduction in affinity for Cdc42, while the triple GAP mutant displayed an even greater reduction (>100-fold) in binding affinity. Since these two mutants are unable to bind to Cdc42 with wild-type affinity, their inability to stimulate GTP hydrolysis is probably at least in part a function of their reduced binding affinity. Nonetheless, these findings clearly argue for the importance of the GAP interaction with the switch domains of the GTP-binding protein for achieving maximal catalysis of the GTP hydrolytic reaction.

In light of these recent findings, it would be interesting to attempt to make p85 into a functional GAP protein. In fact, we have tried to make the corresponding triple mutant in p85 where the hydrophobic residues (Leu209, Val277, and Met285) were replaced with the hydrophilic residues found in wild-type Cdc42GAP (Arg346, Asn414, and Asn422, respectively). However, thus far, we have not been able to detect any GAP activity with this p85 triple mutant (data not shown). This suggests that there may be additional contacts needed for GAP activity for which we have not yet accounted, or that p85 requires the aid of other protein factors to stimulate GTP hydrolytic activity. It also is possible that p85 does not serve a GAP function but rather acts exclusively as a target and/or effector for Cdc42 or Rac (*20*). Nevertheless, the GAP homology domain within p85 has provided us with an important framework for delineating key aspects of GAP-mediated catalysis.

We have further examined the importance of the GAP interactions with the switch I domain of Cdc42 through analysis of tyrosine 32. This residue likely undergoes a π-cation interaction (*26*) between the negatively charged π-electrons of the phenyl group and the positively charged guanidinium group of the arginine finger. Mutation of tyrosine 32 produces Cdc42 molecules that either exhibit reduced levels of GAP-stimulated GTP hydrolysis or are absolutely unresponsive to the GAP. The nonconservative mutations (i.e., Y32K, Y32E, and Y32A), which most likely introduce unfavorable chemical interactions to the active site, are incapable of stimulating GTP hydrolysis. Only those Cdc42 mutants that retain properties of tyrosine 32 can be stimulated by the GAP (i.e., Y32S and Y32F). Thus, in the case of Cdc42 switch stabilization by Cdc42GAP, it is possible that asparagine 414 indirectly positions the arginine finger through its interactions with tyrosine 32 and thus could affect the active site geometry of the GTPase.

Through further analysis of the Cdc42(Y32) mutants, we have found that this tyrosine residue is also necessary for intrinsic GTP hydrolytic activity. Mutation of tyrosine 32 to any residue that does not contain a hydroxyl group results in Cdc42 proteins that exhibit an at least 60% reduction in GTP hydrolysis activity. These data raise the question of how tyrosine 32 contributes to intrinsic GTP hydrolysis. It is possible that tyrosine 32 acts as a pseudo-arginine finger, stabilizing the transition state during GTP hydrolysis by acting as the electron acceptor. This idea is supported by the structure for Cdc42 bound to Cdc42GAP(R305A), where the hydroxyl group of tyrosine 32 was found hydrogen bonded to the transition state analogue, AlF_3, in the absence of an arginine finger (*14*). Perhaps the flexibility of the switch domains in uncomplexed Cdc42 allows this solvent-exposed tyrosine residue to swing freely in and out of the active site and promote GTP hydrolysis. This is a mechanism for intrinsic GTP hydrolysis that might be used by other monomeric GTP-binding proteins, as this tyrosine residue is highly conserved.

As novel GAPs are being discovered and studied, new mechanisms, likewise, have been uncovered. A recently determined structure of Ran in complex with RanBP1 and RanGAP revealed that the GAP protein did not use the conventional arginine finger to stabilize the transition state, but rather utilized a tyrosine residue from the GTP-binding protein instead (*29*). In the case of the ArfGAP, it is thought that a third protein, an effector of Arf, may contribute the arginine finger (*30*). The systematic mutational analysis of the Rap1GAP showed that none of its conserved arginine residues were critical for normal function (*31*). The mechanism of GAP-stimulated GTP hydrolysis may differ on the surface, but there is an underlying theme, alignment of the hydrolytic machinery. GTP hydrolysis requires that a water molecule become activated so that it can attack the γ-phosphate. At the same time, the negative charge that is generated during the cleavage must be neutralized for this to be a catalytic process. Nature has found multiple ways to achieve GTP hydrolysis. In the future, it will be important to examine other members of the RhoGAP family to determine how

generally important the introduction of an arginine finger into the active site and the stabilization of the switch domains are for GAP function.

ACKNOWLEDGMENT

We are grateful for the expert secretarial assistance of Ms. Cindy Westmiller and Mrs. Debra Crane and to Guillermo Calero and Greg Hoffman for helpful discussions.

REFERENCES

1. Tu, S., and Cerione, R. A. (2001) Cdc42 is a substrate for caspases and influences Fas-induced apoptosis, *J. Biol. Chem. 276*, 19656−19663.
2. Chuang, T. H., Hahn, K. M., Lee, J. D., Danley, D. E., and Bokoch, G. M. (1997) The small GTPase Cdc42 initiates an apoptotic signaling pathway in Jurkat T lymphocytes, *Mol. Biol. Cell 8*, 1687−1698.
3. Johnson, D. I. (1999) Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity, *Microbiol. Mol. Biol. Rev. 63*, 54−105.
4. Erickson, J. W., and Cerione, R. A. (2001) Multiple roles for Cdc42 in cell regulation, *Curr. Opin. Cell Biol. 13*, 153−157.
5. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation, *J. Biol. Chem. 270*, 27995−27998.
6. Wu, W. J., Erickson, J. W., Lin, R., and Cerione, R. A. (2000) The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation, *Nature 405*, 800−804.
7. Yang, W., Lo, C. G., Dispenza, T., and Cerione, R. A. (2001) The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly, *J. Biol. Chem. 276*, 17468−17473.
8. Lin, R., Bagrodia, S., Cerione, R., and Manor, D. (1997) A novel Cdc42Hs mutant induces cellular transformation, *Curr. Biol. 7*, 794−797.
9. Cerione, R. A., and Zheng, Y. (1996) The Dbl family of oncogenes, *Curr. Opin. Cell Biol. 8*, 216−222.
10. Whitehead, I. P., Khosravi-Far, R., Kirk, H., Trigo-Gonzalez, G., Der, C. J., and Kay, R. (1996) Expression cloning of lsc, a novel oncogene with structural similarities to the Dbl family of guanine nucleotide exchange factors, *J. Biol. Chem. 271*, 18643−18650.
11. Fukumoto, Y., Kaibuchi, K., Hori, Y., Fujioka, H., Araki, S., Ueda, T., Kikuchi, A., and Takai, Y. (1990) Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins, *Oncogene 5*, 1321−1328.
12. Hoffman, G. R., Nassar, N., and Cerione, R. A. (2000) Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI, *Cell 100*, 345−356.
13. Boguski, M. S., and McCormick, F. (1993) Proteins regulating Ras and its relatives, *Nature 366*, 643−654.
14. Nassar, N., Hoffman, G. R., Manor, D., Clardy, J. C., and Cerione, R. A. (1998) Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP, *Nat. Struct. Biol. 5*, 1047−1052.
15. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants, *Science 277*, 333−338.
16. Rittinger, K., Walker, P. A., Eccleston, J. F., Smerdon, S., and Gomblin, S. (1997) Structure at 1.65 Å of RhoA and its GTPase-activating protein in complex with a transition-state analogue, *Nature 389*, 758−762.
17. Albert, S., Will, E., and Gallwitz, D. (1999) Identification of the catalytic domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases, *EMBO J. 18*, 5216−5225.
18. Leonard, D. A., Lin, R., Cerione, R. A., and Manor, D. (1998) Biochemical studies of the mechanism of action of the Cdc42-GTPase-activating protein, *J. Biol. Chem. 273*, 16210−16215.
19. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Structure of RGS4 bound to AlF4-activated G(i alpha1): stabilization of the transition state for GTP hydrolysis, *Cell 89*, 251−261.
20. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85, *J. Biol. Chem. 269*, 8727−8730.
21. Leonard, D. A., Evans, T., Hart, M., Cerione, R. A., and Manor, D. (1994) Investigation of the GTP-binding/GTPase cycle of Cdc42Hs using fluorescence spectroscopy, *Biochemistry 33*, 12323−12328.
22. Hoffman, G. R., Nassar, N., Oswald, R. E., and Cerione, R. A. (1998) Fluoride activation of the Rho family GTP-binding protein Cdc42Hs, *J. Biol. Chem. 273*, 4392−4399.
23. Hiratsuka, T. (1983) New ribose-modified fluorescent analogs of adenine and guanine nucleotides available as substrates for various enzymes, *Biochim. Biophys. Acta 742*, 496−508.
24. Barfod, 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.
25. 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.
26. Ma, J. C., and Doughertym, D. A. (1997) The Cation-π Interaction, *Chem. Rev. 97*, 1303−1324.
27. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) GTPase mechanism of G proteins from the 1.7-Å crystal structure of transducin alpha-GDP-AIF-4, *Nature 372*, 276−279.
28. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Structure of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis, *Science 265*, 1405−1412.
29. Seewald, M. J., Korner, C., Wittinghofer, A., and Vetter, I. R. (2002) RanGAP mediates GTP hydrolysis without an arginine finger, *Nature 415*, 662−666.
30. Goldberg, J. (1999) Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis, *Cell 96*, 893−902.
31. Brinkmann, T., Daumke, O., Herbrand, U., Kuhlmann, D., Stege, P., Ahmadian, M. R., and Wittinghofer, A. (2002) Rap-specific GTPase Activating Protein follows an Alternative Mechanism, *J. Biol. Chem. 277*, 12525−12531.
32. Esnouf, R. M. (1997) An extensively modified version of Molscript that includes greatly enhanced coloring capabilities, *J. Mol. Graphics 15*,132−134.
33. Merrit, E. A., and Bacon, D. J. (1997) Raster3D: Photorealistic molecular graphics, *Methods Enzymol. 277*, 505−524.
34. Kraulis, P. J. (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures, *J. Appl. Crystallogr. 24*, 946−950.
35. Christopher, J. A., and Baldwin, T. O. (1998) SPOCK: Real-time collaborative molecular modelling, *J. Mol. Graphics 16*, 285.

BI026413P