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*

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The small GTPase Cdc42 interacts with multiple factors to transduce diverse intracellular signals. The factors that preferentially recognize the GTP-bound, active state of Cdc42 include a panel of GTPase-activating proteins (GAPs), the Cdc42/Rac interactive binding (CRIB) motif-containing molecules, and the RasGAP domain containing IQGAP1 and IQGAP2. In the present study, we have determined the kinetic parameters underlying the functional interactions between the Cdc42-binding domains of some of these factors and Cdc42 by monitoring the continuous release of γP and have compared the ability of the domains to bind to Cdc42. The catalytic efficiencies (Km/cat) of the GAP domains of Bcr, 3BP-1, and p190 on Cdc42 are found to be 60-, 160-, and over 500-fold less than that of Cdc42GAP, respectively, and the differences are due to, a large part, to differences in Kr. The Km values of the GAP domains compare well to the binding affinity to the guanylyl imidodiphosphate-bound Cdc42, suggesting a rapid equilibrium reaction mechanism. The affinity of the Cdc42-binding domains of the CRIB motif of Wiskott-Aldrich Syndrome protein and p21cdc42/rac-activated kinase 1, and the RasGAP-related domain of IQGAP1, which all inhibit the intrinsic GTPase activity of Cdc42, are found to be 4, 0.7, and 0.08 μM, respectively. These quantitative analysis provide insight that Cdc42GAP functions as an effective negative regulator of Cdc42 by fast, relatively tight binding to the GTP-bound Cdc42, whereas IQGAP1 interacts with Cdc42 as a putative effector with over 10-fold higher affinity than the CRIB domains and GAPs, and suggest that various GAPs and effectors employ distinct mechanism to play roles in Cdc42-mediated signaling pathways.

The Rho family small GTP-binding protein Cdc42 appears to be an essential component in the transduction of intracellular signals that induce actin-cytoskeleton reorganization and gene activation (1–3). Genetic analysis of bud-site mutants revealed that Cdc42 is a key component in an actin-dependent polarization process during budding (4), as well as in mating (5) in Saccharomyces cerevisiae. The mammalian homolog of yeast Cdc42 has been shown to regulate the formation of the actin structural folding in three dimensions (30, 31). The small GTPase Cdc42, like some of the other members of Rho family proteins (1–3), has been found to activate the Jun NH₂-terminal kinase (11–14), the p70 S6 kinase (15), serum response factors (16), and DNA synthesis pathway (17).

In cells receiving the appropriate stimuli, Cdc42 GTPase is converted to the active GTP-bound state by guanine nucleotide exchange factors (18), whereas the GTP-bound form is in turn rendered inactive due to its intrinsic GTPase activity that is further stimulated by the GTPase-activating proteins (GAPs)1 (19). The RhoGAP family of proteins represents one of the major groups of regulators for the small GTPase, of which over 17 members have been identified to date (19). Primary sequence analysis revealed an ~170-amino acid homology region, designated as RhoGAP domain, in these proteins (20). Biochemical analysis of the RhoGAP activity indicated that this domain contains the minimum structural unit that is necessary and sufficient for the GAP activity (21). The RhoGAP domains share about 20–40% amino acid identity, and the proteins in which they are contained are generally large and multifunctional. The RhoGAP family members include Cdc42GAP (also known as p50RhoGAP), which may form a complex with SH3 domain-containing signaling molecules such as PI-3 kinase and Sre through an additional proline-rich motif (22, 23); the breakpoint cluster region gene product (Bcr), which also possesses serine/threonine kinase (24) and guanine nucleotide exchange factor activities in its amino-terminal sequences (25); the RasGAP-binding phosphoprotein p190, which binds to GTP at its amino terminus (26, 27); and the SH3 domain-binding protein 3BP-1, which was identified by a screen for molecules interacting with the Abl oncoprotein with high affinity (28, 29). Recent x-ray crystallography studies of the RhoGAP domains of Cdc42GAP and p85, the regulatory subunit of PI-3 kinase, have provided further evidence that these critical domains involved in interaction with Rho GTPases adopt a highly conserved structural folding in three dimensions (30, 31).

The incoming signals of Cdc42 may bifurcate at the activated small G-protein itself through multiple effector targets to lead to the diverse biological effects. A panel of candidate effectors, identified by an overlay assay using the [γ-32P]GTP-bound Cdc42 (32), turned out to be a class of novel protein serine/threonine kinases termed p21-activated kinases (PAKs) (33–

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1 The abbreviations used are: GAP, GTPase-activating protein; Bcr, the breakpoint cluster region gene product; BSA, bovine serum albumin; GMP-PNP, guanylyl imidodiphosphate; GST, glutathione S-transferase; MESG, 2-amino-6-mercaptop-7-methylpurine ribonucleoside; PAK, p21cdc42/rac-activated kinase; WASP, the Wiskott-Aldrich Syndrome protein; CRIB, Cdc42/Rac interactive binding.
Interactions of Cdc42 and Its GAPs and Putative Effectors

35. Molecular cloning and data base searches led to the discovery of a family of proteins sharing a conserved Cdc42/Rac interactive binding (CRIB) motif of Pak (36), many of which have since been shown to be candidate effectors for Cdc42. The CRIB motif-containing putative effector molecules are known to lack essential residue in the activation binding to the active form of the GTPase (12, 34), and the Wiskott-Aldrich Syndrome protein (WASP), which binds to Cdc42-GTP and has been implicated in Cdc42-mediated actin polymerization process (37–39). Additional putative Cdc42 effector molecules have recently been identified by Cdc42-GTP affinity binding approach, and these include the IQGAP1 and IQGAP2, two RasGAP domain-containing proteins with selective affinity to the GTP-bound Cdc42 (40, 41). Moreover, in analogy to that suggested by the studies of p120RasGAP (42), the RhoGAPs may also serve as effectors of the small GTPases. Direct evidence of this comes from the observations that the RhoGAP domain of p85 can bind to the GDP-bound states of Cdc42 and Rac1 resulting in PI-3 kinase activation (43), and that the brain specific RhoGAP member n-chimaerin acts synergistically with Cdc42 and Rac1 to induce the effective formation of filopodia and lamellipodia (44).

The Cdc42-GAP and Cdc42-effector interactions have not been investigated in detail. In particular, little quantitative information of these seemingly important interactions has been made available and been compared. In the present work, we have developed a real-time spectroscopic method to study the kinetics of Cdc42-GTP hydrolysis in the context of its coupling to GAP domains of Cdc42GAP, Ber, 3BP-1, and p190, to the CRIB motifs of Pak1 and WASP, and to the Ras-GAP related domain of IQGAP1. By an enzyme-coupled phosphorylation reaction that allows direct measurement of γP, release from Cdc42, we show that the differences of catalytic efficiency of GAPs reside largely in their different Kd values, which compare well with their dissociation constants (Kd) for Cdc42-GTP. In addition, we have also obtained the affinity of Cdc42-GTP for the Cdc42-interactive domains of WASP, Pak1, and IQGAP1 based upon the property of the effectors to inhibit the intrinsic rate of GTP hydrolysis of Cdc42. The results indicate that Cdc42-GAP functions as an effective negative regulator of Cdc42 by fast, relatively high affinity binding to the GTP-bound Cdc42, whereas IQGAP1 interacts with Cdc42 as a putative effector with over 10-fold higher affinity than the CRIB domains and GAPs, and suggest that various GAPs and effectors employ distinct mechanism to play roles in Cdc42-mediated signaling pathways.

Experimental Procedures

Materials—GMP-PNP was from Boehringer Mannheim. GDP, GTP, bacterial purine nucleoside phosphorylase, and the ingredients to generate the phosphorylase substrate, 2-amino-6-mercaptop-7-methylpyrine ribonucleoside (MESG), were purchased from Sigma. MESG was synthesized following the published protocol (45) and was dried to a yellow solid in a lyophilizer for storage at -20 °C. An estimated ~60% product of the final synthesis procedure for MESG, and the linearity of its absorbance response at 360 nm for measuring a range of Pi concentrations from 1 μM to 70 μM was ensured by using the coupling reaction of purine nucleoside phosphorylase in a buffer containing standard phosphate solution (pH 7.6) (45).

Expression and Purification of Recombinant Proteins—Human Cdc42 protein was expressed in Escherichia coli as His-tagged fusion by the pET expression system 28 (Novagen). Briefly, the cDNA of Cdc42 was cloned into pET28 vector at the in-frame BamHI-EcoRI sites, and the resulting construct was transformed into competent BL21 cells. The induced expression and purification of His-tagged Cdc42 by isopropyl-1-thio-galactopyranoside and Ni²⁺-charged agarose beads were carried out following the instructions provided by Novagen. The glutathione S-transferase (GST) fusion of GAP domains and the Cdc42-interacting effector domains of Cdc42GAP, Ber, 3BP-1, p190, WASP, and IQGAP1 were expressed in E. coli using the pGEX system (21). The GAP domains of Cdc42GAP, Ber, 3BP-1, and p190 contain the amino acid residues 205–439, 1010–1271, 854–1153, and 1249–1513 of the native proteins, respectively, and the Cdc42-interacting domains of human Pak1, WASP, and IQGAP1 contain amino acid residues 51–135, 215–295, and 864–1657, respectively. The GST-Cdc42GAP and GST-Ber were generated as described previously (22, 29). The GST-Ber, GST-p190, GST-PK1, and GST-WASP constructs were produced by polymerase chain reaction amplification of cDNAs corresponding to the indicated amino acid regions with primers that contain the BamHI and EcoRI sites, followed by digestion and ligation into the pGEX-KG vector. The GST-IQGAP1 was generated by cloning the BamHI-EcoRI fragment of cDNA (nucleotides 2703–5670) derived from ph1-IQGAP1 (a kind gift of A. Bernardi) into pGEX-KG. Production and purification of the GST-fusions from E. coli were carried out as described by glutathione affinity chromatography (21). If necessary, the GST moiety of the fusions was cleaved by thrombin digestion, followed by incubation with α-aminobenzamidine immobilized on agarose beads (Sigma) to remove the thrombin (46).

All proteins prepared for measurements were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomasie Blue staining analysis, and the contents of each were judged at least 90% pure. Concentrations of the recombinant GAP and effector Cdc42-binding domains were determined using the BCA protein assay reagents from Pierce with bovine serum albumin (BSA) as standard, and the effective concentration of Cdc42 was measured using the MESG system under single-turnover conditions as described below.

Spectroscopic Measurements of GTPase Activity—The hydrolysis of GTP by Cdc42 was measured by the MESG system monitoring the absorbance increase of the reaction mixture at 360 nm, based on the method described by Webb and Hunter (47). Briefly, a 0.8-mmol solution containing 50 mM HEPES, pH 7.6, 0.1 mM EDTA, 0.2 mM MESG, 10 units of purine nucleoside phosphorylase, 200 μM GTP, and the indicated amount of recombinant Cdc42 (∼1 μM) was mixed in a 4 mM width, 10-mm pathlength cuvette, and the time courses of absorbance change at 360 nm were recorded using a Pharmacia Ultrasound spectrometer equipped with compatible computer software for data manipulations. Single-turnover GTPase reactions were initiated by addition of MgCl₂ to a final concentration of 5 mM. For measurements of GAP-catalyzed reactions, 5–50 μM of stock solution containing indicated amounts of GAP were added together with MgCl₂ to the reaction mixture. A control experiment in which Cdc42 was left out was carried out to provide a background of absorbance in each independent measurement to be subtracted from the sample signals. Moreover, experiments at a broad range of concentrations of GAPs were performed to minimize the possibility that the release of the reaction product, Pₐ, from Cdc42-GDP reaction, is the rate limiting step.

The concentrations of P, in the reaction solution are proportional to the net absorbance change by a factor of extinction coefficient ε₂₅₀,µm−¹cm−¹ at pH 7.6 (47), and were used to determine the concentration of Cdc42 after one round of single-turnover reaction. Because the phosphorylase coupling reaction is extremely fast with a rate constant of 40 s⁻¹ (45), the slope of the absorbance in the time course is treated as proportional to the rate of the GTPase activity of Cdc42: rate of Cdc42-GTP hydrolysis = kₑ × ΔA cm⁻¹min⁻¹.

Fluorescence Measurements of GTPase Activity—The fluorescence measurements were made using an SLM-Amino Series 2 Luminessence Spectrometer. To monitor the tryptophan fluorescence emission of Cdc42, samples containing the indicated amount of Cdc42-GTP in the absence or presence (0.94 mM) of Cdc42GAP in a buffer with 50 mM HEPES, pH 7.6, and 10 mM MgCl₂ were mixed and thermostated at 20 °C. The excitation was at 295 nm, and emission was at 330 nm. The increase of fluorescence intensity of a unique tryptophan residue (Trp-97) of Cdc42 due to the shift to a higher quantum yield of the GDP-bound state (48) was followed during the time course of GTP hydrolysis. The difference in fluorescence intensities (in defined arbitrary units) between the GTP- and GDP-bound forms of Cdc42 was calibrated by a factor of 48,000 M⁻¹ cm⁻¹. The background fluorescence as determined by omitting Cdc42 in the reaction mixture (which includes GAP and free GTP) was negligible comparing to the sample signal of Cdc42.

Filter-binding Assay of GTPase Activity—Recombinant Cdc42 was preloaded with [γ⁻³²P]GTP (10 μCi, 6000 Ci/mmol, NEN Life Science Products) in a 100-μl buffer containing 50 mM HEPES, pH 7.6, 0.2 mM MgCl₂, 0.5 mM EDTA, and 0.5 mg/ml BSA for 10 min at 25 °C, and then after the addition of MgCl₂ to a final concentration of 5 mM. An aliquot of the [γ⁻³²P]GTP-loaded Cdc42 was mixed with a reaction buffer containing 50 mM HEPES, pH 7.6, 0.2 mg/ml BSA, and 5 mM MgCl₂ in the presence or absence of GAP. At different time points the reaction was terminated as described (21) by filtering the reaction mixture through nitrocellulose filters, followed by washing with 10 ml of ice-cold buffer.
with 50 mM HEPES, pH 7.6, and 10 mM MgCl₂. The radioactivity retained on the filters was then subjected to quantitation by scintillation counting. In the competition assays to determine the binding affinity of GAP domains to Cdc42, GST-GAP domains at the indicated concentrations together with the indicated amount of Cdc42 preloaded with GMP-PNP or GDP were present in the reaction mixture in addition to the components described including 5 mM Cdc42-[γ-32P]GTP, and the reactions were terminated after a 5-min incubation at 20 °C.

Data Analysis—Kinetic data were analyzed by nonlinear regression using equations derived below with the program Enzfitter (Elsevier Biosoft). The rate constant (Kᵣ) of intrinsic GTP hydrolysis by Cdc42 was determined by fitting data obtained by the MESG system, by tryptophan fluorescence, or by filter-binding assay to a single exponential function. Since the amount of Cdc42 present in the GAP reaction is in great excess of GAPs, a modified Michaelis-Menten equation was used to derive kinetic parameters assuming GAP acting as the enzyme catalyst, Cdc42-GTP as the substrate, and the Cdc42-GDP and Pᵢ as the products.

\[ Vₐ = V_{max}([Cdc42]₀/[K_c + [Cdc42]₀]) + \epsilon K_c[Cdc42]₀ \]  
(1)

Vₐ is the initial rate of hydrolysis, \( \epsilon \) is the extinction coefficient at 360 nm for the phosphorylase reaction product, [Cdc42]₀ is the total Cdc42 concentration, K_c is the rate constant of intrinsic GTPase activity, and the term \( \epsilon K_c([Cdc42]₀) \) is a correction for the rate of intrinsic GTP hydrolysis by Cdc42. K_c is derived by fitting data obtained by the MESG system, by tryptophan fluorescence, or by filter-binding assay to a single exponential function. Since the amount of Cdc42 present in the GAP reaction is in great excess of GAPs, a modified Michaelis-Menten equation was used to derive kinetic parameters assuming GAP acting as the enzyme catalyst, Cdc42-GTP as the substrate, and the Cdc42-GDP and Pᵢ as the products.

\[ Vₐ = V_{max}([Cdc42]₀/[K_c + [Cdc42]₀]) + \epsilon K_c[Cdc42]₀ \]  
(1)

To determine the affinity of effector domains to Cdc42-GTP, the reaction mixture contained Cdc42-GTP and an effector domain added to the assay, respectively, and the derived equation, assuming that the rate of hydrolysis of Cdc42-GTP were extracted by fitting data of the initial rate of GTP hydrolysis, the time course of intrinsic Cdc42-GTPase reaction as reflected by the absorbance trace of Pᵢ release was monitored in the presence of phosphorylase and MESG substrate (Fig. 1A). In the presence of 0.1 mM EDTA when nucleotide exchange occurs fast, Cdc42 undergoes multiple turnover resulting in a linear increase of Pᵢ release. Upon the addition of 5 mM final concentration of MgCl₂, Cdc42 is stabilized at the GTP-bound state due to the presence of excess GTP (200 μM) and starts single-turnover GTP hydrolysis. The response of the absorbance increase is linear up to 70 μM Cdc42 (data not shown), and the conditions used here for the loading of GTP and the initiation of single-turnover GTPase reaction were adopted for all subsequent measurements.

To ensure that the observed rate of Cdc42-GTP hydrolysis measured by the MESG/phosphorylase method is valid, the time course for γPᵢ-elicited absorbance change was compared with that obtained by two other established GTPase assays for Cdc42, the [γ-32P]GTP filter binding and the Cdc42 tryptophan fluorescence methods (Fig. 1B). The time courses of Cdc42-GTP hydrolysis at 20 °C measured by the three different assays show excellent agreement, and fittings of the data to a single exponential function yield intrinsic rate constants (Kᵣ) of 0.073, 0.075, and 0.065 min⁻¹ by the MESG/phosphorylase, the fluorescence, and the filter binding methods, respectively (Table I).

These data validate the MESG/phosphorylase assay as a reliable means for measurement of the GTPase activity of Cdc42.

To see how Cdc42 would behave at more physiological temperatures, the rate of GTPase activity of Cdc42 was determined at 20, 30, and 37 °C (Fig. 1C). A ~1.6-fold increase in was observed when temperature was raised from 20 °C to 30 °C, or from 30 °C to 37 °C, and the GTP hydrolysis occurs ~2.6-fold faster at 37 °C than at 20 °C (Table I). To allow accurate assessment of the initial rates of GAP-stimulated GTP hydrolysis, all subsequent GAP-catalyzed GTPase assays were performed at 20 °C, which provides an optimal window of absorbance increase.

Kinetics of Interaction of Cdc42-GTP with Cdc42GAP—To determine the kinetics of Cdc42GAP-stimulated hydrolysis by Cdc42, we first made sure that the amount of Cdc42GAP employed is within the linear range with regard of the GAP-catalyzed rate increase. Fig. 2A shows the absorbance traces for three different reactions with the same amount of Cdc42 but by addition of different amounts of Cdc42GAP to initiate the reactions. The initial rates of the reactions were linear with Cdc42GAP concentrations up to 4 nM (Fig. 2A, insert). In the following experiments to investigate the interaction of Cdc42GAP with Cdc42-GTP, the initial rate of Pᵢ release was measured as a function of Cdc42 concentration at a fixed concentration of 3.6 nM Cdc42GAP (Fig. 2B). Since the amount of Cdc42-GTP in complex with E is much slower than the concentration of Cdc42-[γ-32P]GTP.

Inhibition of GAP-catalyzed GTP-hydrolysis % =

\[ 1 - K_c/K_c + [C]_0 \]  
(2)

To determine the affinity of effector domains to Cdc42-GTP, the GTPase-inhibitory reaction involving Cdc42-GTP and an effector domain (E) was treated as two simultaneous steps.

\[ E + Cdc42-GTP \rightarrow E-Cdc42-GTP \]  
(4)

The binding constants of the Cdc42-binding domain of effectors to Cdc42-GTP were extracted by fitting data of the initial rate of GTP hydrolysis by Cdc42 at various concentrations of the effector domain to the derived equation, assuming that the rate of hydrolysis of Cdc42-GTP in complex with E is much slower than Cdc42-GTP alone.

\[ Vₐ = 1/2εKᵣ([Cdc42]₀ - [E]₀) + (K_c + [E]₀) \]  
(3)

\[ Vₐ \text{ and } [E]₀ \text{ are the initial rate of GTP hydrolysis and the total concentration of effector domain added to the assay, respectively, and } K_c \text{ represents the binding affinity (dissociation constant) of the effector domain to Cdc42-GTP.} \]  

RESULTS

A Spectroscopic Assay for γPᵢ Release from Cdc42-GTP—The MESG/phosphorylase assay was developed to measure the kinetics of Pᵢ release from GTPases and ATPases (45), and has been applied to the studies of Ras and RasGAP interaction (47). To determine the suitability of this assay system for measuring the Cdc42-GTP hydrolysis, the time course of intrinsic Cdc42-GTPase reaction as reflected by the absorbance trace of γPᵢ release was monitored in the presence of phosphorylase and MESG substrate (Fig. 1A). In the presence of 0.1 mM EDTA when nucleotide exchange occurs fast, Cdc42 undergoes multiple turnover resulting in a linear increase of γPᵢ release. Upon the addition of 5 mM final concentration of MgCl₂, Cdc42 is stabilized at the GTP-bound state due to the presence of excess GTP (200 μM) and starts single-turnover GTP hydrolysis. The response of the absorbance increase is linear up to 70 μM Cdc42 (data not shown), and the conditions used here for the loading of GTP and the initiation of single-turnover GTPase reaction were adopted for all subsequent measurements.
Kinetics of Interaction of Cdc42-GTP with the GAP Domains of Bcr, 3BP-1, and p190—The interactions of the GAP domains of Bcr, 3BP-1, and p190 with Cdc42-GTP were examined by the MESG/phosphorylase system and compared with that of the Cdc42GAP. Similar to that shown in Fig. 2, Fig. 4 shows the initial rates of Pi release measured as a function of concentrations of Cdc42-GTP at fixed concentrations of GST-GAP domains. Data analysis revealed the $K_m$ values of Bcr, 3BP-1, and p190 to be 23.72, 50.47, and 59.19 m$M$, and the $V_{max}$ values to be 3.02, 7.55, and 2.52 m$M$/min, respectively (Table II). The catalytic efficiencies of the GAP domains of Bcr, 3BP-1, and p190 are at least 60, 160, and over 500-fold less than that of the GAP domain of Cdc42GAP, respectively, and the difference in $K_m$ seems to be a major factor for the varied ability of the GAPs to stimulate GTP hydrolysis of Cdc42.

Cdc42 Binding to GAPs—The interaction of GAPs with Cdc42 at steady state was investigated by measuring the ability of GMP-PNP-bound (a nonhydrolyzable GTP analog) or GDP-bound Cdc42 to inhibit competitively the GAP-stimulated hydrolysis of $[^{32}P]$GTP-bound Cdc42. The concentration range in which Cdc42-GMP-PNP or Cdc42-GDP inhibits GAP-mediated GTP hydrolysis is an indication of its affinity ($K_d$). MgCl$_2$. Arrow indicates the time point at which a final concentration of 5 mM MgCl$_2$ was added to the reaction mixture. The dashed line represents the period of addition and mixing. B, comparison of the time courses for single-turnover GTP hydrolysis by Cdc42 measured by the MESG system (squares), the tryptophan fluorescence change (diamonds), and the $[^{32}P]$GTP filter-binding assay (triangles). Experimental conditions for each assay were as described under “Experimental Procedures.” Data of GTP hydrolysis were best fitted into a single exponential to derive the intrinsic rate constant of Cdc42 ($K_c$).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Table I**

| Assay                  | 20 °C | 30 °C | 37 °C |
|------------------------|-------|-------|-------|
| $K_c$                  |       |       |       |
| P$_i$ release/MESG     | 0.073 ± 0.001 | 0.120 ± 0.004 | 0.193 ± 0.005 |
| Tryptophan fluorescence| 0.075 ± 0.002 | ND     | ND    |
| Filter binding         | 0.065 ± 0.001 | ND     | ND    |

The GTPase activity of Cdc42 was measured by the three different methods under otherwise identical conditions as described under “Experimental Procedures,” and the traces of GTP-hydrolysis depicted in Fig. 1B were fitted into a single exponential function to derive the intrinsic rate constant $K_c$. GTP-hydrolysis of Cdc42 measured at different temperatures by the P/MESG method as depicted in Fig. 1C were also analyzed similarly. ND, not determined. Results are representative of three independent experiments.
By fitting data to Equation 3 described under “Experimental Procedures” (Fig. 5), we determined that Cdc42GAP bound to Cdc42-GMP-PNP with an $K_d$ of 2.78 μM, Bcr bound with an $K_d$ of 24 μM, 3BP-1 bound with an $K_d$ of 46.6, whereas p190 bound with the lowest affinity of 55 μM (Table III). These $K_d$ values compare well with the $K_m$ values obtained for the GAPs (Table II), suggesting a rapid equilibrium binding of GAPs to Cdc42-GTP, which is not a rate-limiting step in the GAP-catalyzed reactions. The correlation between catalytic efficiency and binding affinity of the GAPs examined suggests that tight binding of a GAP domain may contribute to the stabilization of a transition state of Cdc42 in facilitating GTP hydrolysis.

To see if there is an effect of product inhibition in the time courses of GAP-stimulated GTP hydrolysis, we measured the affinity of Cdc42-GDP to various GAPs in a similar assay, and found that both Cdc42GAP and 3BP-1 bound to Cdc42-GDP with negligible affinity, while Bcr and p190 recognize Cdc42-GDP with ~100-fold and ~2-fold lower affinity, respectively, than the GMP-PNP-bound Cdc42 (Table III). We therefore conclude that there were minimal, if any, Cdc42-GDP-inhibitory effect on the GAP reactions of Cdc42. This is in contrast with the GAP-catalyzed reaction of RhoA in which the reaction product RhoA-GDP demonstrates a comparable affinity to GAP as the reactant Rho-GTP, suggesting a diversity of the mechanism of GAP-stimulated GTP hydrolysis among Rho proteins.

Interaction of Cdc42-GTP with Cdc42-binding Domains of Putative Effectors—We are interested in examining the interactions of putative effectors with Cdc42 and making comparisons with that of the GAPs, given that some RhoGAPs have been speculated to act as effectors for the G-protein function (43, 44). The CRIB domain of rat α-PAK and the RasGAP-related domain of IQGAP1 were previously observed to inhibit both the intrinsic and GAP-stimulated GTP hydrolysis of Cdc42 (33, 40), and recently, it was reported that the CRIB domain of mouse PAK3 has a similar effect on Cdc42 (49). To determine the direct binding affinity of the effector domains to Cdc42-GTP, we conducted GTPase assays of Cdc42 in the presence of various doses of purified Cdc42-binding domains of IQGAP1, PAK1, and WASP (Fig. 6). As shown in Fig. 6A, the intrinsic GTPase activity is inhibited by the addition of IQGAP1, and this inhibitory effect is dependent on the concentrations of the effector. The initial rates of GTP hydrolysis as a function of the concentrations of the inhibitory effector domains were fitted by a nonlinear regression (Equation 3) to extract the binding constants ($K_i$) to Cdc42-GTP (Fig. 6B), and $K_i$ values of 0.082, 0.78, and 4.15 μM for IQGAP1, PAK1, and WASP, respectively, were obtained (Table IV). Thus, IQGAP1 seems to bind to Cdc42-GTP with ~10-fold higher affinity than PAK1, and ~50-fold higher affinity than WASP. When the interaction of GAP domains with Cdc42 are compared with these effector domains, Cdc42GAP is the only GAP tested to fall in the range of effector interaction affinity (approximately micromolar range), while Bcr, 3BP-1, and p190 interact with...
Kinetic parameters of GAPs that regulate Cdc42 under single turnover conditions

The fluorescence measurements were carried out as described under "Experimental Procedures," the initial rates of tryptophan fluorescence change of the Cdc42-GTP resulting from the hydrolysis of GTP when mixed with 0.94 nM Cdc42GAP were determined at increasing concentrations of GAP domains used in the measurements were 0.96 nM, 11.4 nM, 34.7 nM, and 36.3 nM for Cdc42GAP, Bcr, 3BP-1, and p190, respectively, by the MESG/phosphorylase method, and 0.94 nM Cdc42GAP by the fluorescence method. These concentrations of GAPs fell within the linear range of the absorbance or fluorescence increase in response to increasing doses of GAPs. Results are representative of at least three independent measurements.

| GAP       | V_{max} (μM/min) | K_{m} (μM) | K_{cat} (min^{-1}) | K_{cat}/K_{m} (μM^{-1}) |
|-----------|------------------|------------|--------------------|--------------------------|
| Cdc42GAP  | 2.02 ± 0.09      | 3.08 ± 0.48| 2103.9 ± 93.7      | 683.1 ± 30.4             |
| Cdc42GAP* | 2.38 ± 0.10      | 2.61 ± 0.29| 2578.3 ± 108.3     | 987.8 ± 41.5             |
| Bcr       | 3.02 ± 0.16      | 23.72 ± 3.2| 265.5 ± 14.1       | 11.2 ± 0.6               |
| 3BP-1     | 7.55 ± 0.77      | 50.47 ± 9.42| 2173.7 ± 22.2      | 4.3 ± 0.4                |
| p190      | 2.52 ± 0.32      | 59.19 ± 12.4| 69.4 ± 8.8         | 1.2 ± 0.2                |

* Determined by the tryptophan fluorescence method.

**FIG. 3.** Single-turnover kinetic analysis of the interaction between Cdc42-GTP and Cdc42GAP by tryptophan fluorescence method. The fluorescence measurements were carried out as described under "Experimental Procedures," the initial rates of tryptophan fluorescence change of the Cdc42-GTP resulting from the hydrolysis of GTP when mixed with 0.94 nM Cdc42GAP were determined at increasing concentrations of Cdc42-GTP, and data were best-fitted into the modified Michaelis-Menten equation (Equation 1).

Cdc42 at least 5-fold less tightly than any of the effectors examined.

**FIG. 4.** Determination of the single-turnover kinetic parameters of the GAP domains of GST-Bcr, GST-3BP-1, and GST-p190. The initial rates of GTP hydrolysis as monitored by the γP, release with the MESG system were measured with a constant amount of the GAP domain of Bcr (11.4 nM, diamonds), 3BP-1 (34.7 nM, solid squares), or p190 (36.3 nM, triangles) and increasing concentrations of Cdc42-GTP, similar to that described in Fig. 2. K_{m} and V_{max} values were extracted by nonlinear fitting as in Fig. 2C. The intrinsic rates of GTP hydrolysis (open square) were fitted into a linear function to yield K_{c}.

**DISCUSSION**

Increasing evidence has emerged implicating Cdc42 as an essential component in a variety of cellular transduction pathways; this prominent member of Rho family small GTPases mediates the reorganization of actin polymerization process in mammalian and yeast cells and regulates critical cellular processes such as mitogen-activated kinase kinase (Fus3/Kss1 in yeast and JNK/p38 in mammals) activation, p70 S6 kinase activation, and cell cycle progression (1–3), and may also have a role in mammalian development (50). Studies aimed at understanding the biochemical mechanisms underlying the biological effects of Cdc42 have led to the discovery of numerous regulators and potential effector targets, which specifically interact with the activated GTP-bound form of Cdc42 (19, 36) and may serve to down-regulate or initiate one or more pathways bifurcating at the GTPase itself. In this paper we describe the quantitations of some of the interactions involving Cdc42 that have been implicated in the regulation or transduction of relevant signals, and provide for the first time a direct comparison of the kinetic and steady state parameters of the interactions with four mammalian RhoGAPs (Cdc42GAP, Bcr, 3BP-1, and p190) and three putative effectors (IQGAP1, PAK1, and WASP). Our data show that both the GAPs and the effectors interact with Cdc42-GTP by a fast equilibrium mechanism, and the affinities of the interaction correlate with the catalytic GAP activities of the GAPs or the GTPase-inhibitory activities of the effectors. The broad spectrum of binding affinities of these cellular factors to Cdc42 (K_{d} varying from 80 nM to 50 μM) suggest that they employ distinct mechanism to play roles, if any, in Cdc42-mediated signaling pathways.

Due to the relatively fast intrinsic GTP hydrolysis rate of Cdc42 (10-fold faster than Ras), the interactions of Cdc42 with GAPs and with putative effectors, which display GTPase-inhibitory protein function on Cdc42 similar to that by the GDP dissociation inhibitors in this respect (51), can be readily measured by the change in γP release. The MESG/phosphorylase assay allows us to monitor in real time the GTP hydrolysis process by converting the γP signal to an absorbance change at 360 nm, and offers a few advantages over two other available methods to measure GTPase activity of Cdc42; it provides faster, continuous, and more quantitative trace of GTP hydrol-
ysis compared with the \([\gamma-\text{32P}]\text{GTP}\) filter binding method, and it does not suffer from the possible interference by inner filter effect at high concentrations of fluorophore (i.e. tryptophan residue of Cdc42) or by nonphysiological artifact of fluorescent GTP-analogs such as the \(N\)-methylanthraniloyl-GMP-PNP of the fluorescence method (48, 49). As demonstrated in Figs. 1 and 3, this assay yielded similar kinetic parameters of the intrinsic GTPase activity as well as the GAP-stimulated GTPase activity of Cdc42 when the Cdc42 preloaded with GMP-PNP at the indicated concentrations was added. The reactions were terminated after 5 min at 20 °C.

TABLE III

| GAP         | \(K_d\) (\(\mu\text{M}\)) | [GMP-PNP] | [GDP] |
|-------------|-----------------|----------|-------|
| Cdc42GAP    | 2.78 ± 0.06     | >500     |
| Bcr         | 24.05 ± 0.04    | 266 ± 13 |
| 3BP-1       | 46.6 ± 1.5      | >1000    |
| p190        | 55.2 ± 1.1      | 127 ± 6  |

Fig. 5. Interaction of GAP domains with Cdc42 bound to GMP-PNP measured by inhibition of GAP-stimulated Cdc42-\(\gamma-\text{32P}\)GTP hydrolysis. \(-5 \text{ nM Cdc42-}\[\gamma-\text{32P}\]GTP\) together with 2 \text{nM Cdc42GAP (solid triangles), 14 nM Bcr (squares), 30 nM 3BP-1 (diamonds), or 34 nM p190 (open triangles) were present in the assay buffer containing 50 mM HEPES, pH 7.6, 0.2 mg/ml BSA, and 5 mM MgCl}_2 when Cdc42 preloaded with GMP-PNP at the indicated concentrations was added. The reactions were terminated after 5 min at 20 °C.

Fig. 6. Determination of the affinities of putative effector-domain binding to Cdc42 by inhibition of GTP hydrolysis of Cdc42. A, time courses of Cdc42-GTP hydrolysis in the presence of various amount of Cdc42-binding domain of IQGAP1 under single-turnover conditions measured by the MESG system. Reaction conditions were similar to that in Fig. 1. 6.5 \(\mu\text{M Cdc42}\) and the indicated amount of IQGAP1 domain were present when reaction was initiated by the addition of MgCl\(_2\). B, initial rates of GTP hydrolysis of Cdc42 as a function of effector domain concentrations. 6.5 \(\mu\text{M Cdc42}\) and the effector domain of IQGAP1 (squares), PAK1 (diamonds), or WASP (triangles) were present. Data were fitted to Equation 3 described under “Experimental Procedures” to derive the binding constants.

that Cdc42GAP represents the most active GAP specie toward Cdc42, with a catalytic efficiency (\(K_{\text{cat}}/K_m\)) at least over 60-fold higher than the other GAPs been examined. Given its wide spectrum of tissue distributions, it is likely that Cdc42GAP may serve as one of the major negative regulators of Cdc42 signaling pathway, whereas the significantly lower catalytic efficiencies and binding affinities of 3BP-1 and p190 (\(-160-\)and \(-600\)-fold lower, and \(-16\)- and \(-19\)-fold less tight, respectively) suggest that they are probably not involved in direct regulation of Cdc42. So far, \textit{in vivo} evidence of cellular functions of the GAPs has been lacking. Microinjections performed using the GAP domains suggest that while both Bcr and 3BP-1
TABLE IV
Affinity of the Cdc42-binding domain of effectors for Cdc42

| Effector domain  | \( K_i \) (m) |
|------------------|---------------|
| IQGAP1           | 0.082 ± 0.033 |
| PAK1             | 0.78 ± 0.10   |
| WASP             | 4.15 ± 0.06   |

measured in this study seems to indicate that, while it is possible for Cdc42GAP to act as a putative effector for Cdc42 with a \( K_i \) similar to that of WASP at \( m \) range, it is less likely that 3BP-1, or p190 functions to transduce signals from Cdc42 with a \( K_i \) value at over 50 \( m \). However, the relatively low binding affinity of some GAPs such as Bcr does not rule out their role in down-regulation or effector function of Cdc42 if the case for RasGAP, which has a \( K_i \) of \(-20 \) \( m \) for Ras, can be brought up as a comparison (56, 57).

It is important to note that current studies utilizing the limited Cdc42-interaction domains of GAPs and effectors provide only a simplified model which may differ from the situations in which full-length molecules are likely to behave. Recently, the amino-terminal sequences of p120RasGAP containing the SH2 and SH3 domains were found to be required for the full catalytic GAP activity toward Ras (58). Along these lines, many of the RhoGAPs and effectors are large and multifunctional, therefore a much more complicated intracellular or intermolecular interactions involving additional structural motifs of the molecules may contribute to the regulation of binding and/or catalytic interaction with Cdc42, in analogy to RasGAP. For example, we have previously observed that the carboxy-terminal fragment of Bcr containing the GAP domain is a much more active GAP than the full-length Bcr immunoprecipitated from cell lysates (21), and the GAP activity of the RhoGAP member n-chimaerin has been reported to be subject to regulation by lipid-binding (59). Thus, to measure and compare the binding and catalytic activities of intact RhoGAPs and effectors to Rho family GTPases will be of considerable interest in the future.

In summary, the data presented here provide direct comparison of the binding and catalytic activities of a panel of mammalian RhoGAP domains and putative effector domains to Cdc42. The superb catalytic efficiency of Cdc42GAP and high binding affinity of IQGAP1 constitute them as potentially important players in the regulation or transduction of Cdc42-mediated signals.

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