Interaction of Rac1 with GTPase-activating Proteins and Putative Effectors

A COMPARISON WITH Cdc42 AND RhoA*

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The intrinsic GTPase activity of the Rho family GTP-binding protein Rac1 is drastically stimulated upon interaction with its GTPase-activating proteins (GAPs) and is significantly inhibited when coupled to certain effector targets such as the p21-activated kinases (PAKs) and IQGAPs. Here we have characterized the interaction of Rac1 with a panel of mammalian GAPs and putative effectors by measuring the kinetic and binding parameters involved and made comparisons with similar interactions for Cdc42 and RhoA. In contrast, with Cdc42 (for which the GAP domain of p50RhoGAP is 50-fold more efficient than those of p190, Bcr, and 3BP-1) and with RhoA (toward which only p50RhoGAP and p190 displayed high efficiencies), the catalytic efficiencies ($K_{cat}/K_m$) of the GAP domains of p50RhoGAP, p190, Bcr, and 3BP-1 on Rac1 are found to be comparable in a range between 0.9 and 2.6 min$^{-1}$ µM$^{-1}$. However, similar to the cases of Cdc42 and RhoA, the $K_m$ values of the GAP domains on Rac1 compare well to the binding affinity to the guanylyl imidodiphosphate-bound Rac1, which ranges from 10.5 to 40.5 µM, suggesting a rapid equilibrium reaction mechanism. The dissociation constants of the p21-binding domains of PAK1, PAK2, and the RasGAP-related domain of IQGAP1, which all cause significant reduction of the intrinsic rate of GTP hydrolysis upon binding to Rac1-GTP, are found to be 0.71, 0.26, and 2.13 µM for Rac1-GTP, compared with that determined for Cdc42-GTP at 2.9, 20.5, and 0.39 µM, respectively, under similar conditions. These results suggest that p50RhoGAP, p190, Bcr, and 3BP-1 are all capable of acting as a negative regulator for Rac1-mediated signaling, and that, although PAK1 and IQGAP1 can couple tightly with both Rac1 and Cdc42, PAK2 is likely to be a specific effector for Rac1 instead of Cdc42.

Rac1 belongs to the mammalian Rho family of Ras superfamily small GTP-binding proteins, which includes the additional members of RhoA, RhoB, RhoC, RhoE, RhoG, Cdc42, TC10, and Rac2 (1, 2). The Rho family proteins appear to be key components in the transduction of intracellular signals that induce actin-cytoskeleton reorganization and gene activation (1–3). The initial studies of the role of Rac, Rho, and Cdc42 in fibroblast cells have provided evidence that Rac1 regulates lamellipodium formation and membrane ruffling (4), RhoA regulates the formation of actin stress fibers and focal adhesion complexes (5), and Cdc42 regulates filopodium formation (6, 7). All three GAPs were subsequently shown to be required for G1 to S phase cell cycle progression (8) and to be capable of mediating gene transcription through the Jun N-terminal kinase module of the mitogen-activated protein kinase pathways (9–11). Rac1, in particular, may also be involved in the regulation of exocytic and endocytic pathways, and has a role in activation of the NAPDH oxidase enzyme complex (12, 13).

The biochemical mechanisms underlying the biological functions of Rho GAPs have been under intensive scrutiny. Like other types of G-proteins, the GDP-bound forms of Rho proteins are in an inactive state while the GTP-bound forms serve to transduce signals to the immediate downstream effector targets (14). A family of GTPase-activating proteins (GAPs)$^1$ that stimulates the intrinsic GTPase activity and thereby facilitates the deactivation of the small G-proteins has emerged over the past few years by molecular cloning and biochemical studies (15). Primary sequence analysis revealed an ~170-amino acid homology region, designated the RacGAP domain, in GAP family molecules that was found to be necessary and sufficient for GAP activity (16). Recently available x-ray crystal structures of the RacGAP domains of the p85 regulatory subunit of phosphatidylinositol 3-kinase and the p50RhoGAP (also known as Cdc42GAP) suggest that these critical domains involved in interaction with Rho GAPases adopt a highly conserved structural folding in three dimensions (17, 18). Moreover, the structure of the complex of Cdc42 with p50RhoGAP further revealed that the mechanism of Rho protein interaction with RacGAPs are different from the Ras-RasGAP or the heterotrimeric G-protein-RGS interaction in a manner that the small G-protein contacts mostly through its switch I and II regions with a unique shallow pocket of RacGAP domain formed between the B and F helices, the A-A1 loop, and the F-G loop (19). However, detailed mechanistic proposal regarding how RacGAPs enhance the GTPase activity of Rho proteins remains lacking, especially when it is realized that certain unique features of each Rho protein-GAP pair apparently contribute to the specificity of individual reactions (20, 43).

The recent identification of putative effectors of Rho family GAPases has helped to provide molecular links to the diverse biological activities of Rho proteins. Among a growing panel of the small G-protein targets, p21-activated kinase (PAK) was initially found to be activated upon binding to the GTP-bound

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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; MESG, 2-aminomethylphosphoric ribonucleoside; PAK, p21-activated kinase; PBD, p21-binding domain.
forms of Rac1 and Cdc42 (22, 23), and later was implicated in mediating signaling from Rac1 and Cdc42 to the Jun N-terminal kinase (24, 25) and in certain cases, to actin cytoskeleton (26). IQGAP1, a RasGAP domain containing protein with selective affinity to the GTP-bound Rac1 and Cdc42 (27), binds to F-actin directly and cross-links the actin filaments into interconnected bundles (28), therefore may serve as a link between these GTPases and the actin cytoskeleton. Furthermore, in analogy to that suggested by the studies of p120RasGAP, which was implicated as an effector for Ras signaling in defined cases (29), there is also evidence that certain RhGAPs may act as potential effectors of Rho GTPases (30, 31).

To examine the mechanism of Rho family GTPase interaction with GAPs and effectors in detail and to make direct comparison of these interactions among Rac, Cdc42, and Rho, we have set out to determine the kinetic parameters and binding affinities of a panel of mammalian RhGAPs, i.e. p190, p50RhoGAP, Bcr, and 3BP-1, and a few putative effectors for Rac1 and Cdc42, i.e. PAK1, PAK2, and IQGAP1, to the small GTPases. Previous studies of Cdc42 and RhoA suggest that there exists a unique mechanism for each specific interaction between a Rho protein and a RhoGAP, and that relative tight binding to the small G-proteins may be necessary to constitute an effective effector target (32, 43). In the present work, we have determined the intrinsic and GAP-stimulated rates of GTP hydrolysis by Rac1, obtained the apparent binding affinities of the RhoGAP domains to both GTP- and GDP-bound forms of Rac1, and made direct comparisons with similar interactions of Cdc42 and RhoA. In addition, we have also determined the affinities of Rac1-GTP and Cdc42-GTP to the p21-binding domains (PBDs) of PAK1, PAK2, and IQGAP1 based on the property of the effectors to inhibit the intrinsic rate of GTP hydrolysis of Rac1. Our results suggest that p50RhoGAP, p190, Bcr, and 3BP-1 are all capable of acting as a negative regulator for Rac1-mediated signaling and that, although PAK1 and IQGAP1 can couple tightly with both Rac1 and Cdc42, PAK2 is likely to be a specific effector for Rac1 instead of Cdc42.

**EXPERIMENTAL PROCEDURES**

**Materials—**GDP, GTP, and bacterial purine nucleoside phosphorylase were purchased from Sigma. GMP-PNP was obtained from Boehringer Mannheim. The phosphorylase substrate, 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), was synthesized as described (33), and the linearity of its absorbance response at 360 nm for measuring a range of P1 concentrations from 1 μM to 70 μM was ensured by using the coupling reaction of purine nucleoside phosphorylase in a bovine serum albumin (BSA) as a standard, and the effective concentrations of the small G-proteins were determined using the MESG/phosphorylase system under single turnover conditions as described below.

**Spectroscopic Measurements of GTPase Activity—**The rates of GTP hydrolysis of the small GTPases were measured by the MESG/phosphorylase system monitoring the absorbance increase of the reaction mixture at 360 nm as described for the cases of Rac and Cdc42 (32, 35). Briefly, a 0.8-ml solution containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 0.2 mM MESG, 10 units of purine nucleoside phosphorylase, 200 μM GTP, and the indicated amount of recombinant G-proteins was mixed in a 4-mm window, 10-mm pathlength cuvette, and the time courses of absorbance change at 360 nm, which reflect the rate of single turnover of the G-proteins, were recorded. Single turnover GTPase reactions were initiated by the addition of MgCl2 to a final concentration of 5 mM. For measurements of GAP-catalyzed reactions, 5–50 μl of stock solution containing the indicated amount of GAP domains were added together with MgCl2 to the reaction mixtures. A control experiment in which the GTPases were omitted was carried out in each independent measurement to provide a background of absorbance to be subtracted from the sample signals. The concentration of Pi in the reaction solution was calculated by a factor of extinction coefficient ε360 nm = 11,000 M⁻¹ cm⁻¹ at pH 7.6 from the absorbance change (33), and was used to determine the effective concentrations of the small G-proteins after one round of single turnover reaction. Because the phosphorylase coupling reaction is extremely fast with a rate constant of 10⁶ M⁻¹ s⁻¹ (33), the slope of the absorbance in the time course is treated as proportional to the rate of the GTPase activity.

**Competition Binding Assay—**Recombinant Rac1 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 mg/ml BSA, and 0.5 mM EDTA for 10 min at ambient temperature before the addition of MgCl2 to a final concentration of 5 mM. An aliquot of the [γ-32P]GTP-loaded Rac1 (~ 20 nM) 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 domains and Rac1 preloaded with GMP-PNP or GTP at indicated concentrations. At the 5-min time point, the reactions were terminated by filtering the reaction mixture through nitrocellulose filters followed by washing with 10 ml of ice-cold buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, and 10 mM MgCl2. The radioactivity retained on the filters was then subjected to quantitation by scintillation counting (16).

**Data Analysis—**Kinetic data were analyzed by nonlinear regression using equations derived before for Cdc42-GAP interactions (32) with the program Enzfitter (Elsevier Biosoft). The apparent rate constants (Kc) of intrinsic GTP hydrolysis by the small GTPases were determined by fitting data to a single exponential function for single turnover reactions or to a linear equation for EDTA-induced multiple turnover reactions. A modified Michaelis-Menten equation was used to derive kinetic parameters for GAP-catalyzed GTP hydrolysis assuming GAP acting as the enzyme catalyst, Rac1-GTP as the substrate, and the Rac1-GDP and Pi as the products.

\[
V_o = \frac{V_{max[Rac1]}}{K_{c[Rac1]} + [Rac1]} + K_{GAP[Rac1]}, 
\]

\(V_o\) is the initial rate of hydrolysis, \([Rac1]\) is the total Rac1 concentration, \(K_{c[Rac1]}\) is the apparent rate constant of intrinsic GTPase activity, and the term \(K_{GAP[Rac1]}\) is a correction for the rate of intrinsic GTP hydrolysis by Rac1. \(K_{GAP[Rac1]}\) is derived by \(V_{max[GAP][Rac1]}\) with \([GAP]\) representing total GAP concentration.

The dissociation constants (\(K_d\)) of GAP binding to Rac1 were derived by fitting data of the competition assay to the following derived equation under conditions that the total concentration of competitor \([C]\) (C representing GMP-PNP- or GDP-bound Rac1) and reaction \(K_{d[GAP]}\) values are of magnitude larger than the concentration of Rac1-\(\gamma\)32P]GTP.

Inhibition of GAP-catalyzed GTP hydrolysis %

\[1 - \frac{1}{2K_{d[GAP]} + [C]}\] (Eq. 2)

The binding constants of the PBD of effectors to Rac1-GTP and Cdc42-GTP were extracted by fitting data of the initial rate of GTP hydrolysis of the GTPases at various concentrations of the effector domains to the following derived equation, assuming that the rate of hydrolysis of the small GTPase in complex with effector is much slower than Rac1-GTP or Cdc42-GTP alone.

\[V_o = \frac{1}{2K_{d[Rac1]} + K_{d[GAP]} + [GAP]} + \frac{1}{2K_{d[GAP]} + [GAP]}\] (Eq. 3)

\(-4[E][Rac1][GAP]=\)
Fig. 1. Comparison of kinetics of intrinsic GTP hydrolysis of Rac1 with that of Cdc42 and RhoA. γP, released from GTP-bound small G-proteins were measured by the continuous spectroscopic assay of MESG/phosphorylase system as described under "Experimental Procedures". The absorptions at 360 nm of 5.0 μM solution of small GTPases were monitored during the time course in a buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 200 μM GTP, and 0.2 mM MESG with 10 units coupling phosphorylase at 20 °C which provides a condition for the G-proteins to undergo multiple turnovers. Arrow indicates the time point at which a final concentration of 5 mM MgCl₂ was added to initiate single turnover reactions. The dotted lines represent the periods of addition and mixing.

\[ V_0 \text{ and } [E]_0 \text{ are the initial rate of GTP hydrolysis and the total concentration of PBD added to the assay, respectively, and } K_r \text{ represents the binding affinity (dissociation constant) of the PBD to GTP-bound G-proteins.} \]

**RESULTS**

The rate of GTP hydrolysis by Rac1 directly affects the time span in which Rac1 remains active, and therefore may have a major influence on the duration of signaling output from the small G-protein. To determine the rate of intrinsic GTP hydrolysis, the time courses of GTPase reaction of Rac1 under both multiple and single turnover conditions were monitored by the absorbance change at 360 nm wavelength reflects γP release from Rac1-GTP detected by the phosphorylase coupling reaction with MESG as a substrate, and the initial presence of 0.1 mM EDTA and 200 μM GTP and the absence of Mg²⁺ provide a condition at which the G-protein undergoes rapid GDP/GTP exchange. Interestingly, a very slow phase of GTP hydrolysis by Rac1 at 5 μM concentration was observed at this condition, with an apparent rate of 0.054 × 10⁻² min⁻¹, in contrast with the over 60- and 8-fold faster rates displayed by Cdc42 and RhoA, respectively (Fig. 1; Table I). The differences in the rate of EDTA-induced multiple turnover of the small GTPases may be attributed to their distinction in Mg²⁺ binding affinity and the subsequent GTP binding ability, since Rac1 seems to bind most weakly to Mg²⁺ among the three GTPases, whereas their rates of GDP dissociation under these conditions are similar. When 5 mM MgCl₂ was added to the mixture to initiate single turnover reaction (Fig. 1), an apparent rate constant of 0.030 min⁻¹ by Rac1 was obtained by fitting the data to a single exponential function (Table I). This falls between that of Cdc42 (0.064 min⁻¹) and RhoA (0.015 min⁻¹) under similar conditions (Table I). Thus, Rac1 behaves similarly to other members of the Rho family GTPases in its intrinsic ability to hydrolyze GTP which is ~10-fold faster than that of Ras, and seems to possess a unique property in Mg²⁺ and/or GTP binding, which affects its rate of EDTA-induced multiple turnover.

When p50RhoGAP was added together with Mg²⁺ to the single turnover reactions of the small GTPases, increases in the rate of GTP hydrolysis were observed (Fig. 2A). The scale of response to p50RhoGAP by Rac1 was apparently different from that of Cdc42 and RhoA, reflecting differences in kinetics for the respective GAP-reactions. To determine the kinetic parameters of GAP-stimulated GTP hydrolysis by Rac1, the initial rate of γP release by Rac1-GTP was measured as a function of Rac1 concentration at a fixed concentration of GAP, as shown for the case of p50RhoGAP catalysis in Fig. 2B. Since the amount of the small G-protein (treated as a substrate) was in large excess of GAPs (treated as enzymes), the reactions can be treated by Michaelis-Menten kinetics with the adjustment of the intrinsic rate of GTP hydrolysis by Rac1 (see Equation 1 under "Experimental Procedures"). Fitting of the data obtained for p50RhoGAP, p190, Bcr, and 3BP-1 (Fig. 2C) gave K_m values at 18.7–49.3 μM range (Table II). The K_cat values were further derived from V_max (Table II) assuming that all GAPs present were active, and ranged from 23.4 to 72.3 min⁻¹ under these conditions. When the catalytic efficiencies (K_cat/K_m) of these GAPs for Rac1 were compared, the differences were found to be less than 3-fold (ranging from 0.9 to 2.6 min⁻¹ μM⁻¹). These catalytic properties of GAPs on Rac1 apparently differ from those on Cdc42 for which p50RhoGAP demonstrated greater than 50-fold preference over the other GAPs (32), and from RhoA for which both p190 and p50RhoGAP were 20-fold more active while Bcr and 3BP-1 showed only marginal activities (43).

To determine the affinity of Rac1 binding to GAPs, the ability of GMP-PNP (a nonhydrolyzable GTP analog)-bound Rac1 to inhibit competitively the GAP-stimulated hydrolysis of Rac1-γ³²P/GTP was measured (Fig. 3). The concentration range in which Rac1-GMP-PNP inhibits GAP-mediated GTP hydrolysis is an indication of its affinity (K_D) for GAP. By fitting data to Equation 2 as described under "Experimental Procedures," we derived that p50RhoGAP bound to Rac1-GMP-PNP with an K_D of 26.4 μM, p190 bound with an K_D of 40.5 μM, Bcr bound with 10.5 μM, and 3BP-1 bound with 27.7 μM (Table III). Similar to the cases for Cdc42 and RhoA (32, 43), the K_D values of these GAPs on Rac1 show a general correlation with the K_m values (Tables II and III), suggesting rapid equilibrium binding of the GAPs to Rac1-GTP, which is not a rate-limiting step in the GAP-catalyzed reactions. However, distinct from Cdc42 or RhoA, Rac1 does not seem to recognize any of the GAPs with micromolar affinity (the tightest binding occurs for Bcr with a K_D value of 10.5 μM), whereas the Cdc42-3BP50RhoGAP, RhoA-p50RhoGAP, and RhoA-p190 interactions were found to be at over 5-fold higher affinity (Table III). To see if there is an effect of product inhibition in the time courses of GAP-stimulated GTP hydrolysis as has been observed for the RhoA-p50RhoGAP

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2 B. Zhang and Y. Zheng, unpublished observation.
reaction (43), we measured the affinity of Rac1-GDP to various GAPs by a similar assay, and found that both p50RhoGAP and 3BP-1 bound to Rac1-GDP with negligible affinity (160 μM and 97.6 μM, respectively), while Bcr and p190 recognize Rac1-GDP with affinities at 21.7 μM and 59.3 μM, respectively. We conclude that there were minimal, if any, Rac1-GDP inhibitory effect on the GAP reactions of Rac1 at up to 20 μM concentration.

A few Rho GAPs have been speculated to act as effectors for the G-protein functions (30, 31), and Rac1 and Cdc42 seem to share additional downstream effectors based upon the binding profiles of certain PBD of putative effectors (2). We sought to quantify the interactions of Rac1 with some of the putative effectors and to make comparisons with that of the GAPs, and with Cdc42. To determine the direct binding affinity of the effector domains to Rac1-GTP, we conducted GTPase assays of Rac1 in the presence of various doses of purified PBDs of PAK1, catalysis as a function of Rac1 concentration. The initial rates of GTP hydrolysis monitored by γ^32P release were measured in the presence of a constant amount of the GAP domain of p50RhoGAP (18.8 μM, filled circles), p190 (66.0 μM, filled triangles), Bcr (37.2 μM, open circles), or 3BP-1 (137 μM, open triangles) and increasing concentrations of Rac1-GTP. The GAP-catalyzed GTP hydrolysis rates were fitted into the modified Michaelis-Menten equation (Equation 1 under “Experimental Procedures”) to yield $K_m$ and $V_{max}$ values of the reactions.

### Table II

| GAP          | $V_{max}$ (μM/min) | $K_m$ (μM) | $K_{cat}$ (min⁻¹) | $K_{cat}/K_m$ (μM⁻¹) |
|--------------|--------------------|-----------|------------------|---------------------|
| p50RhoGAP    | 1.36 ± 0.21        | 27.5 ± 3.8| 72.3 ± 10.6      | 2.6 ± 0.4           |
| p190         | 3.82 ± 0.11        | 49.3 ± 2.6| 57.8 ± 1.7       | 1.2 ± 0.1           |
| Bcr          | 0.87 ± 0.19        | 18.7 ± 2.9| 23.4 ± 2.8       | 1.3 ± 0.2           |
| 3BP-1        | 4.37 ± 0.54        | 35.2 ± 4.5| 31.9 ± 4.0       | 0.9 ± 0.1           |

**FIG. 2.** Determination of the kinetic parameters of GAP-stimulated Rac1-GTP hydrolysis.

**A**, time courses of γ^32P release from Rac1, Cdc42, and RhoA under stimulation by p50RhoGAP. Reaction conditions were similar to Fig. 1, and GAP reactions were initiated by adding p50RhoGAP at 5 nM final concentration together with MgCl₂ (arrow) to 5.0 μM small GTPases. **B**, time courses of GTP hydrolysis by Rac1 in the presence of 18.8 nM p50RhoGAP at four different Rac1 doses as indicated. **C**, initial rates of GTP hydrolysis by Rac1 under GAP catalysis as a function of Rac1 concentration. The initial rates of GTP hydrolysis monitored by γ^32P release were measured in the presence of a constant amount of the GAP domain of p50RhoGAP (18.8 nM, filled circles), p190 (66.0 nM, filled triangles), Bcr (37.2 nM, open circles), or 3BP-1 (137 nM, open triangles) and increasing concentrations of Rac1-GTP. The GAP-catalyzed GTP hydrolysis rates were fitted into the modified Michaelis-Menten equation (Equation 1 under “Experimental Procedures”) to yield $K_m$ and $V_{max}$ values of the reactions.
PAK2, and IQGAP1. As shown in Fig. 4A, the intrinsic GTPase activity of Rac1 is inhibited by the addition of PAK1 PBD, and this inhibitory effect is dependent on the concentrations of the PBD. The initial rates of GTP hydrolysis as a function of the concentrations of the inhibitory PBD were fitted by a nonlinear regression (Equation 3) to extract the binding constants (K_i) to Rac1-GTP and Cdc42-GTP (Fig. 4B), and K_i values of 0.71, 0.26, and 2.13 μM for PAK1, PAK2, and IQGAP1 binding to Rac1-GTP, and 2.9, 20.5 and 0.39 μM for binding to Cdc42-GTP, respectively, were obtained (Table IV). Both of the PBDs of PAK1 and IQGAP1 were able to bind to Rac1-GTP and Cdc42-GTP with affinities at micromolar range, whereas the PBD of PAK2 demonstrated an apparent preference to Rac1-GTP over Cdc42-GTP. When the interactions of Rac1 with the effector domains were compared with that of the GAP domains, it appears that Rac1-GTP binds to GAP domains with significantly lower affinity than to the effector PBDs, suggesting that the GAPs may function as negative regulators rather than effectors for Rac1 signaling.

### DISCUSSION

In the present study, we have investigated the kinetic properties of the GTPase reaction of Rac1 under catalysis of a panel of mammalian GAPs, i.e. p50RhoGAP, p190, Bcr, and 3BP-1, and made comparisons with similar reactions of two closely related members of Rho family GTPases, Cdc42 and RhoA, by using a quantitative assay measuring γP release from Rac1-GTP in real time. The data obtained show that there is no significant difference in kinetic mechanism between the GAP reactions of Rac1 involving these GAP molecules and that the four GAPs examined are approximately equally capable of acting as a negative regulator of Rac1. There are, however, some significant differences between these GAP-catalyzed reactions of Rac1 and those of Cdc42 and RhoA. For example, the fact that all four GAPs can function efficiently to stimulate the GTPase activity of Rac1 is in contrast with the case for Cdc42, in which p50RhoGAP contains over 50-fold supreme GAP activity over the other GAPs (32) and with the case for RhoA, in which both p50RhoGAP and p190 are orders of magnitude more potent than Bcr and 3BP-1 (43), reflecting distinct specificities of these GAPs toward individual small G-proteins. In addition, by comparing the affinities of the PBD of putative effectors, i.e. PAK1, PAK2, and IQGAP1, to the active forms of Rac1 and Cdc42, we found that while all three effectors can couple to Rac1-GTP with dissociation constants at close to micromolar, PAK2 binds to Cdc42-GTP with ~100-fold lower affinity than binding to Rac1-GTP. These results suggest that while PAK1 and IQGAP1 may function as potential targets to transduce signals from both activated Rac1 and Cdc42, PAK2 is likely to be a specific effector for Rac1 instead of Cdc42.

The similarities in kinetic parameters of the interactions of the four GAPs with Rac1-GTP suggest that the basic reaction mechanisms involved in activating Rac1 GTPase are analogous to GTPase-inhibitory reactions of the PBDs were carried out as shown in Fig. 4A. The initial rate of GTP-hydrolysis as a function of the concentrations of the PBDs was treated by nonlinear regression fitting to Equation 3 under "Experimental Procedures" as shown in Fig. 4B. Comparison of binding affinities of Rac1-GTP to effector domains with that of Cdc42-GTP. The initial rates of GTP hydrolysis by Rac1 and Cdc42 as a function of the concentrations of PBD of PAK1 and PAK2 are shown. 15.0 μM Rac1 (triangles) or Cdc42 (circles) and increasing concentrations of PBD of PAK1 (filled symbols) or PAK2 (open symbols) were present in the reactions. Data were fitted to Equation 3 described under "Experimental Procedures" to derive the binding constants K_i in Table IV.

### Table III

**Affinity of GAPs for Rac1 compared with that for Cdc42 and RhoA**

The competition reactions were carried out under conditions described in Fig. 3. The dissociation constants (K_i) were derived by fitting data shown in Fig. 3 to Equation 2 under "Experimental Procedures."

| GAP     | Rac1-GMP-PNP K_i (μM) | Cdc42-GMP-PNP K_i (μM) | RhoA-GMP-PNP K_i (μM) |
|---------|-----------------------|------------------------|-----------------------|
| p50RhoGAP | 26.4 ± 2.0           | 2.78 ± 0.06            | 2.47 ± 0.41           |
| p190    | 40.5 ± 3.5           | 55.2 ± 1.1             | 2.18 ± 0.08           |
| Bcr     | 10.5 ± 0.7           | 24.05 ± 0.04           | 21.95 ± 0.51          |
| 3BP-1   | 27.7 ± 1.0           | 46.6 ± 1.5             | 16.3 ± 1.5            |

* Data obtained under similar conditions in Refs. 43 and 32.

### Table IV

**Affinity of the PBD of effectors for Rac1 and Cdc42**

The GTPase-inhibitory reactions of the PBDs were carried out as shown in Fig. 4A. The initial rate of GTP-hydrolysis as a function of the concentrations of the PBD was treated by nonlinear regression fitting to Equation 3 under "Experimental Procedures" as shown in Fig. 4B to derive the dissociation constants (K_i) for the interaction between Rac1-GTP and Cdc42-GTP and respective effector domains.

| p21-binding domain | Rac1-GTP K_i (μM) | Cdc42-GTP K_i (μM) |
|--------------------|-------------------|-------------------|
| PAK1               | 0.71 ± 0.02       | 2.90 ± 0.34       |
| PAK2               | 0.26 ± 0.04       | 20.5 ± 2.1        |
| IQGAP1             | 2.13 ± 0.58       | 0.39 ± 0.08       |
in these cases. The $K_m$ values of the GAP reactions show good agreement with the $K_m$ values of GAP binding to Rac1-GMP-PNP, which is reminiscent of the Cdc42-GAP interactions (32) and the RhoA-p190GAP and RhoA-p50RhoGAP interactions (43) and is consistent with a mechanism of GAP-catalyzed GTP hydrolysis, which involves fast equilibrium binding to Rac1 followed by a rate-limiting step of $\gamma$P cleavage of bound GTP. This is also similar to the well characterized Ras-RasGAP interaction, for which it has been shown that a fast equilibrium between Ras and RasGAP proceeds the GAP-catalyzed bond cleavage reaction (36). Recently available x-ray crystal structure of RhoA in a transition state complex with p50RhoGAP, GDP, and AIF$_2$ has highlighted the role of a conserved arginine residue (Arg-85) in the GAP domains contributing to the catalytic reactions of Rho GTPases (37). Indeed, in three distinct GTPase-catalyzing machines, i.e. RhoGAP, RasGAP, and a built-in domain of Go$_i$, the arginine fingers are found to converge at the same point of the GTPase stabilizing $\gamma$-phosphate oxygens and the leaving group, albeit from different directions (38). It seems highly likely that Rac1 and the GAPs adopt a similar mechanism of intermolecular interaction involving an arginine finger in the catalytic core of the small GTPase.

The difference between the seemingly non-selective GAP activity of the GAPs toward Rac1 and the extraordinary specificity demonstrated by p50RhoGAP toward Cdc42 (32) and the preference of p190 for RhoA (43) is likely to reside in the structural differences between Rho proteins. We have shown in a recent study of the p190-RhoA interaction that the specificity of this coupling involves unique structural determinants outside of the switch I domain of the GTPase which is required for the GAP reaction (20). The charged residue Asp-90 of RhoA, in particular, makes over 10-fold contribution to the binding affinity and thereby specificity of p190 recognition. Comparison of the three dimensional structures of Rac1 and RhoA reveals that although they adopt highly conserved tertiary and secondary folding patterns, Rac1 displays a relatively neutral surface, whereas RhoA shows a predominantly electronegative surface mostly due to aspartates at residues 13, 87, 90, and 124 (39, 40). The lack of unique salt bridges between Rac1 and the GAPs, therefore, may partly account for the mediocre and similar catalytic efficiencies and binding affinities of these GAPs toward Rac1.

The in vivo functions of Rho GAPs remain to be determined, but the kinetic parameters of Bcr and 3BP-1 are in agreement with previously observed phenotype in fibroblast cells (41, 42) and are consistent with Bcr being a negative regulator of Rac function in in vivo situations (21). Microinjections performed using RhoGAP domains suggest that, whereas p50RhoGAP and p190 seem to be able to inhibit the RhoA regulated actin-stress fiber formation in fibroblast cells, both Bcr and 3BP-1 can down-regulate Rac-mediated membrane ruffling (41, 42). It is not clear why p50RhoGAP or p190 was not observed to inhibit Rac1 function under the microinjection conditions given their similar in vitro behaviors toward Rac1 as Bcr and 3BP-1. One possible explanation is that the 2–3 $\mu$M binding affinity and high catalytic efficiency of p190GAP and p50RhoGAP for RhoA (43) compared with the over 10 $\mu$M affinity for Rac1 have caused a preferential recognition and catalysis of RhoA GTPase by these GAPs, whereas the down-regulation of Rac1 would require a higher threshold of the GAP concentrations to be detected in cellular context.

The effector molecules responsible for the cellular functions of Rac1, which include inductions of membrane ruffling and lamellipodia, focal complex formation, and gene activation, remain unknown. However, the two human p21-activated kinases, PAK1 and PAK2, and the RasGAP domain containing actin-associated molecule IQGAP1 are likely candidates for some of these effects of Rac1, given the recent findings that PAK can induce both actin reorganization and the activation of a mitogen-activated protein kinase cascade involving the Jun N-terminal kinase (24–26) and that IQGAP1 can serve to induce bundling of filamentous actins in cells (28). To quantify these potentially important interactions involving Rac1 and to compare with that of Cdc42, with which these Rac1 effector candidates share similar binding profiles, we have measured the binding affinities of the PBDs of these putative targets to Rac1-GTP and Cdc42-GTP under similar conditions. Based on their ability to inhibit the intrinsic rates of GTP hydrolysis upon binding to the small GTPase, we derived that PAK2 binds to Rac1 with a dissociation constant of 0.26 $\mu$M, PAK1 binds with 1-fold weaker affinity, and IQGAP1 binds with an ~8-fold weaker affinity. These binding profiles fall within an affinity of the micromolar range and are likely to bear physiological significance comparing to other well characterized protein-protein interactions of the Ras signaling pathway (29). By comparison, a marked difference of binding profiles of these effector domains to Cdc42-GTP was observed. IQGAP1 demonstrates the tightest binding with an affinity at 0.39 $\mu$M, PAK1 binds with an 2.9 $\mu$M affinity, while PAK2 shows a much reduced affinity at 20.5 $\mu$M. Therefore, in vitro, IQGAP1 and PAK1 may constitute as potential effectors for both Rac1 and Cdc42, whereas PAK2 is likely to be a specific effector for Rac1 instead of Cdc42. One important question that must be addressed in the future is how small GTPases like Rac1 differentially recognize these potential effectors, which differ by up to 8-fold binding affinity under physiological conditions. It is possible that distinct patterns of tissue distribution and/or cellular localization of these effectors contribute to the occurrence of some of the interactions leading to defined physiological effects and/or that the interactions involving the effector domains are highly regulated in the context of the full-length molecules in response to specific stimulatory signals.

One of the criteria for being a candidate effector of small GTPases is the relative tight binding capability to the activated form of GTPases. The four Rho GAPs characterized in this study all contain a binding affinity at above the 10 $\mu$M $K_m$ value to Rac1, comparing to the typical micromolar range of affinities of PAKs and IQGAP1, suggesting that they may not be recognized by Rac1-GTP as effectors. However, given the observed effect by one of the Rac1-specific GAPs, n-chimaerin, which is able to act synergistically with Rac1 to induce lamellipodia formation in fibroblast cells (31), and the conditional effector functions demonstrated by RasGAP in certain biological systems, which also possesses an over 10 $\mu$M binding affinity toward Rac-GTP (29), it remains to be seen if any of the Rho GAPs, especially in its full-length environment, can play an effector role in Rac1 signaling. How the interaction of Rac1 with multiple regulators and effectors, which may bear opposite effects on its guanine-nucleotide binding state, influences the outcome of Rac1 signaling represents another important area of future work.

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