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The Role of Mg$^{2+}$ Cofactor in the Guanine Nucleotide Exchange and GTP Hydrolysis Reactions of Rho Family GTP-binding Proteins*

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The biological activities of Rho family GTPases are controlled by their guanine nucleotide binding states in cells. Here we have investigated the role of Mg$^{2+}$ cofactor in the guanine nucleotide binding and hydrolysis processes of the Rho family members, Cdc42, Rac1, and RhoA. Differing from Ras and Rab proteins, which require Mg$^{2+}$ for GDP and GTP binding, the Rho GTPases bind the nucleotides in the presence or absence of Mg$^{2+}$ similarly, with dissociation constants in the submicromolar concentration. The presence of Mg$^{2+}$, however, resulted in a marked decrease in the intrinsic dissociation rates of the nucleotides. The catalytic activity of the guanine nucleotide exchange factors (GEFs) appeared to be negatively regulated by free Mg$^{2+}$, and GEF binding to Rho GTPase resulted in a 10-fold decrease in affinity for Mg$^{2+}$, suggesting that one role of GEF is to displace bound Mg$^{2+}$ from the Rho proteins. The GDP dissociation rates of the GTPases could be further stimulated by GEF upon removal of bound Mg$^{2+}$, indicating that the GEF-catalyzed nucleotide exchange involves a Mg$^{2+}$-independent as well as a Mg$^{2+}$-dependent mechanism. Although Mg$^{2+}$ is not absolutely required for GTP hydrolysis by the Rho GTPases, the divalent ion apparently participates in the GTPase reaction, since the intrinsic GTP hydrolysis rates were enhanced 4–10-fold upon binding to Mg$^{2+}$, and $k_{cat}$ values of the Rho GTPase-activating protein (RhoGAP)-catalyzed reactions were significantly increased when Mg$^{2+}$ was present. Furthermore, the p50RhoGAP specificity for Cdc42 was lost in the absence of Mg$^{2+}$ cofactor. These studies directly demonstrate a role of Mg$^{2+}$ in regulating the kinetics of nucleotide binding and hydrolysis and in the GEF- and GAP-catalyzed reactions of Rho family GTPases. The results suggest that GEF facilitates nucleotide exchange by destabilizing both bound nucleotide and Mg$^{2+}$, whereas RhoGAP utilizes the Mg$^{2+}$ cofactor to achieve high catalytic efficiency and specificity.

The Rho family GTP-binding proteins Cdc42, Rac1, and RhoA belong to the Ras superfamily and are important regulators of diverse cell functions (1–3). These small GTPases have been implicated in cellular processes including cell polarization (4, 5) and morphogenic changes (6), endocytosis and exocytosis (7, 8), neutrophil NADPH oxidase activation (9), oncogenic transformation (10), cell to cell and cell to extracellular matrix adhesion (11, 12), and cell movement (13). A well-established biochemical model depicts them as molecular switches linking extracellular stimuli to the intracellular signaling pathways by cycling between the inactive, GDP-bound state and the active, GTP-bound state (14). The GTP binding/GTP hydrolysis cycle of these GTPases serves to turn on and off the incoming signals and appears to be under tight regulation by multiple regulatory proteins, among which the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs) are responsible for the activation and the deactivation of the Rho GTPases, respectively (15, 16).

Mg$^{2+}$ has been established as an essential cofactor for GTP-binding protein functions (17). For many Ras superfamily small GTPases, Mg$^{2+}$ has been shown to be necessary for both guanine nucleotide binding and GTP-hydrolysis. In the presence of Mg$^{2+}$, Ras exhibits an extremely high binding affinity to the guanine nucleotides with a dissociation constant on the order of subnanomolar concentration (18, 19, 40). In the cases of Rab3A and related Sec4 GTPases, removal of Mg$^{2+}$ by EDTA treatment drastically increases the off rate of bound nucleotides and completely abolishes GTPase binding capability (20, 21). The intrinsic GTPase activity of Ras and Rab3A became undetectable when Mg$^{2+}$ cofactor was removed (19, 20), indicating that the bivalent ion is absolutely required for GTP hydrolysis in the respective GTPase reactions. In the guanine nucleotide exchange reactions of Ras and ARF, the specific GEFs Sos and ARNO appear to promote the dissociation of GDP in part through destabilizing bound Mg$^{2+}$ from the respective GTPases (22–24). The role of the GEFs in these cases was proposed to stimulate GDP dissociation and to facilitate the formation of a reaction transition state in which the GEFs are tightly bound to the nucleotide-depleted form of the small GTPases (25).

The three-dimensional structures of the Rho family GTPases, RhoA, Cdc42, and Rac1, in complex with Mg$^{2+}$ and guanine nucleotides have been resolved recently (26–29). Not unlike other small GTP-binding proteins, they utilize the conserved residues in the guanine nucleotide binding core to chelate GDP or GTP, but distinct switch I and switch II residues in the surrounding area of the chelating center are also involved in maintaining the overall conformation. The critical Mg$^{2+}$-interacting sites appear similar to that of Ras with some unique distinctions. For example, the coordination of Mg$^{2+}$ in RhoA-GTPγS was found to be identical to that in Ras bound to

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The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GST, glutathione S-transferase; mantGDP, 2’/3’-(N-methylanthraniloyl)GDP; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; GTPγS, guanosine 5’-3-O-thio-ribose phosphate; ARF, ADP-ribosylation factor; DTT, dithiothreitol; GMP-PNP, β,γ-imidoguanosine 5’-triphosphate.

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GMP-PNP (27), whereas Rho-GDP seems to employ three water molecules in contrast to four in Ras and an additional hydroxyl of Thr$^{19}$, carbonyl oxygen of Thr$^{37}$, and the $\beta$-phosphorus oxygen for $Mg^{2+}$ coordination (26), due to the conformational differences in the switch I domain. In the Rac1-GMP-PNP structure, residues in the switch I region are disordered (29), suggesting that the nucleotide binding core of Rac1 may adopt a more flexible conformation. The regulatory molecules of Rho GTPases such as RhoGEFs and RhoGAPs, on the other hand, are structurally divergent from other Ras regulator families (14). Although certain key features of action may appear similar (e.g. a conserved arginine residue of RhoGAP is critical for its catalytic function like that in RasGAP (30)), the uniqueness of these regulatory proteins suggests that distinct features for the GTPase-activating and guanine nucleotide exchange reactions are probably involved in the deactivation and activation processes, respectively, for the Rho GTPases.

The biochemical properties of guanine nucleotide binding by the Rho family GTPases have not been investigated in detail. Moreover, much remains unknown about the role of $Mg^{2+}$ in the regulatory interactions of the Rho proteins with their unique sets of GEFs and GAPs. In this study, we provide the first quantitative analysis of the kinetic and equilibrium nucleotide binding properties of three Rho GTPases, Cdc42, Rac1, and RhoA. Our results reveal that both the nucleotide binding kinetics and binding affinity of Rho GTPases differ significantly from that of other subfamily members of the Ras superfamily. The $Mg^{2+}$ cofactor does not affect the nucleotide binding affinity of the Rho proteins per se; rather, it acts solely as a stabilizer for the bound nucleotides by slowing down the off and on rates. We found that RhoGEF plays dual roles in the Rho GTPase activation reaction via a $Mg^{2+}$-dependent as well as a $Mg^{2+}$-independent mechanism and that although $Mg^{2+}$ is not absolutely required for the intrinsic and GAP-stimulated GTP-hydrolysis, it is directly involved in the regulation of the basal GTPase and the GTPase-activating activities. Finally, our studies provide evidence that RhoGEF facilitates guanine nucleotide exchange by destabilizing both bound nucleotide and Mg$^{2+}$, whereas RhoGAP utilizes the Mg$^{2+}$ cofactor to achieve high catalytic efficiency and specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The radioactive nucleotides $[^{3}H]GDP$, $[^{32}P]GTP$, and $[^{35}S]GTP$-S were obtained from NEN Life Science Products. GDP, GTP, and GTP-S were purchased from Sigma, mantGDP and 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) were synthesized as described previously (31). All chemicals were of the highest quality available, and the water used for buffer preparations were generated by the Milli-Q Academic purification system (Millipore Corp.) with a conductivity resistance of 18.2 megohms/cm. In order to focus on the G-protein binding core without the complications brought by the flexible carboxyl-terminal ends, recombinant human Cdc42, Rac1, and RhoA proteins containing carboxyl-terminal truncation of seven, eight, and eight residues, respectively, were used in the studies as described previously (31, 32). The Rho family proteins were expressed in *Escherichia coli* as amino-terminal His$_{10}$-tagged fusions by using the PET expression system (Novagen). The GAP domain of p50RhoGAP (also known as Cdc42GAP) contains amino acids 205–430 and was expressed in *E. coli* as a glutathione S-transferase (GST) fusion using the pGEX-KG vector (33). The GST-Dbl protein and GST-Ha-Ras were generated in a baculovirus system or in *E. coli* as described previously (34). The Trio N-terminal fragment contains residues 1225–1537, including the intact Dbl homology and Pleckstrin homology domains, and was expressed as a His$_{10}$-tagged fusion protein in DE52 strain cells. The N-terminal tagged proteins were purified by glutathione or Ni$^{2+}$-agarose affinity chromatography, and the final purity of all proteins used in the assays was >90% as judged by Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis. In general, the proteins stored in 30% glycerol at −20 °C are stable for up to 2 weeks without activity loss. The protein concentrations were determined by using the BCA assay kit (Pierce), and the effective concentrations of GTPases were measured by using the MESG/phosphorylase system assaying the amount of $\gamma$P release after one round of single turnover GTP hydrolysis reaction as described (33).

**Preparation of Metal Ion- and Nucleotide-free Apo-GTPases**—To remove Mg$^{2+}$ and nucleotides from the RhoA and RhoGAP samples (−100 μM) in buffer A containing 50 mM HEPES, pH 7.6, and 2 mM EDTA, were incubated for 20 min at 25 °C, followed by centrifugation in a Centricon 10 concentrator (Amicon) at 4000 × g for 1 h. This typically results in over 10-fold enrichment of the proteins. The treatments of the samples with buffer A and Centricon 10 were repeated three more times, and the samples were subsequently exchanged into buffer B containing 50 mM HEPES, pH 7.6, 100 mM NaCl, and 1 mM DTt at ~400 μM concentration. To examine the content of the nucleotide remaining in the protein samples, an aliquot (0.5 ml) of the sample was prepared in a D$_{2}$O buffer containing 50 mM HEPES, pH 7.4, 10 mM MgCl$_{2}$, and 0.1 mM DTt at 1 mM concentration, and the phosphorous contents were determined by $^{31}$P NMR measurement as described by Geyer et al. (35) on a Varian INOVA 600-MHz spectrometer. The $^{31}$P spectra were recorded at 5 °C at a phosphorous resonance frequency of 202 MHz and were referenced to external 85% H$_{3}$PO$_{4}$. Over 15,000 free induction decays were summed after excitation with a 65° pulse using a repetition time of 3 s. In addition, the UV absorption spectra of the EDTA-treated samples were measured at wavelengths between 240 and 300 nm and were compared with that of untreated GTPase samples.

**Guanine Nucleotide Binding Assay**—To determine the nucleotide binding affinities to Rho GTPases, $[^{3}H]$GDP or $[^{35}S]$GTP-S at a specific activity of 6000 cpm/μM was incubated with the respective apo-GTPases at 25 °C for 6 h in buffer B containing 50 mM HEPES, pH 7.6, 100 mM NaCl, and 1 mM DTt with or without supplement of MgCl$_{2}$ to the indicated concentrations. The binding reactions were stopped by filtration of the mixtures through nitrocellulose filters, and the radiomucleotides remaining bound to the Rho GTPases were quantified by scintillation counting (36). The dissociation binding constants ($K_{d}$) of the nucleotide-G protein complex were derived by best fitting the data into a bimolecular binding model.

To measure the dissociation rate of guanine nucleotides from Rho GTPases, the apo-GTPases (250 nM) were first complexed with $[^{3}H]$GDP or $[^{35}S]$GTP-S in buffer B with or without the supplement of 10 mM MgCl$_{2}$. After a binding equilibrium was reached (~60 min), the dissociation reactions were initiated by the addition of 500 μM GDP or GTP-S to the incubation mixtures. At the indicated time intervals, aliquots of the mixtures were withdrawn, and the remaining G-protein-bound radionucleotides were quantified by nitrocellulose filtration. The data were fitted into a single exponential equation to arrive at an apparent dissociation rate constant, $k_{off}$.

**Fluorescence Measurements**—The fluorescence measurements were carried out using an SLM-Amino Series 2 Luminescence Spectrometer (31). To monitor the mantGDP fluorescence, the excitation wavelength was set at 360 nm and the emission wavelength at 440 nm. All measurements were performed at 25 °C in buffer B with or without the supplement of 10 mM MgCl$_{2}$ to the reaction mixtures. The treatments of the samples with buffer A and Centricon 10 were repeated three more times, and the samples were subsequently exchanged into buffer B containing 50 mM HEPES, pH 7.6, 100 mM NaCl, and 1 mM DTt at ~400 μM concentration. To examine the content of the nucleotide remaining in the protein samples, an aliquot (0.5 ml) of the sample was prepared in a D$_{2}$O buffer containing 50 mM HEPES, pH 7.4, 10 mM MgCl$_{2}$, and 0.1 mM DTt at 1 mM concentration, and the phosphorous contents were determined by $^{31}$P NMR measurement as described by Geyer et al. (35) on a Varian INOVA 600 MHz spectrometer. The $^{31}$P spectra were recorded at 5 °C at a phosphorous resonance frequency of 202 MHz and were referenced to external 85% H$_{3}$PO$_{4}$. Over 15,000 free induction decays were summed after excitation with a 65° pulse using a repetition time of 3 s. In addition, the UV absorption spectra of the EDTA-treated samples were measured at wavelengths between 240 and 300 nm and were compared with that of untreated GTPase samples.

**GTpase Activity Assay**—GTP-hydrolysis by the Rho GTPases were measured by the MESG/phosphorylase system, which monitors free $\gamma$P release from the bound GTP as described previously (33). Briefly, a 0.8 ml solution containing 50 mM HEPES, pH 7.6, 0.2 mM MEGS, 10 units of purine nucleotide phosphorylase, 200 μM GTP, and the indicated amount of Rho protein was mixed in a 4-mm width, 10-mm path length cuvette. The time courses of absorbance change at 360 nm resulting from the P-phosphorylase coupling reaction were recorded on an Amersham Pharmacia Biotech Ultraspec III spectrometer. In the cases of GAP-catalyzed reactions, an indicated catalytic amount of p50RhoGAP was included in the reaction buffer. Kinetic data were analyzed by nonlinear regression using equations derived for Cdc42, Rac1, and RhoA-GAP interactions (33, 37, 38) with the program Enzfitter (Elsevier Biosoft). A modified Michaelis-Menten equation was used to derive kinetic parameters for GAP-catalyzed GTP hydrolysis assuming GAP acting as the enzyme catalyst, GTpase-GTP as the substrate, and the GDP-bound GTpase and P, as the products as described previously (33).

**Guanine Nucleotide Exchange Assay**—The $[^{3}H]$GDP/GTP exchange of Cdc42 was measured at 25 or 4 °C as described previously (34). The exchange reactions were carried out in buffer B with or without the supplement of 5 mM MgCl$_{2}$ in the presence or absence of 20 μM purified GST-Dbl. The reactions were terminated at the 5-min time point by nitrocellulose filtration, and the amounts of $[^{3}H]$GDP remaining bound to Cdc42 were normalized as the percentage of $[^{3}H]$GDP bound at time 0. For the measurement of the Rac1 exchange reaction, fluorescence...
emissions at 440 nm of the mantGDP-bound Rac1 were monitored in buffer B with time at various Mg\(^{2+}\) concentrations in the presence or absence of the indicated amount of Trio. The reactions were initiated by the addition of excess free GTP (400 \(\mu\)M) into the reaction mixture. To extract kinetics parameters of the Trio-catalyzed exchange, the initial reaction rates (\(V_0\)) were determined at increasing concentrations of Rac1-mantGDP in the presence or absence of Mg\(^{2+}\). The resulting hyperbolic curves were best fitted into a modified Michaelis-Menten equation with a correction term of the intrinsic nucleotide exchange included, 

\[
V_0 = V_{\text{max}} \frac{[\text{Rac1-mantGDP}]_0}{K_m + [\text{Rac1-mantGDP}]_0} + k_{\text{exchange}}[\text{Rac1-mantGDP}]_0
\]

where \(V_0\) is the initial reaction rate, \([\text{Rac1-mantGDP}]_0\) is the total reaction substrate concentration, and \(k_{\text{exchange}}\) is the basal exchange rate of Rac1. \(K_m\) is derived by \(V_{\text{max}}/[\text{Trio}]\), with [Trio] representing the GEF concentration present in the reaction.

RESULTS

Mg\(^{2+}\)-independent Guanine Nucleotide Binding of Rho GTPases—In order to examine the role of Mg\(^{2+}\) in the guanine nucleotide binding process of Rho GTPases, we attempted to generate the metal ion- and nucleotide-free form (apo form) of Rho proteins by using a Centricon-based centrifugation protocol combined with EDTA treatment. This method is similar to what John et al. (19) have described for apo-Ras generation, in which treatment with 5 mM EDTA followed by a high pressure liquid chromatography isolation step led to the nucleotide- and metal ion-free GTPases while retaining >90% of the GDP binding activities. We found that the Rho proteins, Cdc42, RhoA, and Rac1, after repeated treatments by a 60-min centrifugation through Centricon 10 cells following incubations with EDTA in buffer A (50 mM HEPES, pH 7.6, 20 mM EDTA, and 1 mM DTT), were mostly devoid of bound guanine nucleotide. The removal of bound nucleotide was verified by the measurement of \(^{31}\)P NMR spectra of the resulting protein products and by a comparison of the UV absorption spectra of the treated GTPases with the original protein samples. Even after 15,000 free induction decays, little \(^{31}\)P signal was detectable for the EDTA-treated GTPase samples at the phosphorus resonance frequency of 202 MHz, whereas the untreated GTPases showed two resonances corresponding to the \(\alpha\) - and \(\beta\)-groups of the bound GDP (data not shown). Furthermore, the UV absorption spectra of the treated proteins displayed a single absorption maximum at 276.0 nm, compared with a 274.1-nm absorption peak and a secondary maximum at 253.0 nm for the original Rho GTPase-GDP complex (data not shown). The Mg\(^{2+}\) content was estimated to be less than 0.01 mol/mol of the treated proteins, assuming that the Mg\(^{2+}\)-EDTA complex has a dissociation constant of 1.7 \(\mu\)M at pH 7.6 (19). The proteins generated in this manner were thus regarded as apo-GTPases and were employed in the following nucleotide binding studies.

The nucleotide binding properties of the Rho family GTPases were first examined by incubation of the apo-GTPases with \(^{[3]H}\)GDP or \(^{[35]}\)S\(\text{GTP}\)\(\gamma\)S at 25 \(^\circ\)C in buffer B (50 mM HEPES, pH 7.6, 100 mM NaCl, and 1 mM DTT), which is free of Mg\(^{2+}\). The amounts of nucleotides bound to the apo-GTPases were assessed after 60 min. Interestingly, we observed that in the absence of Mg\(^{2+}\), both \(^{[3]H}\)GDP and \(^{[35]}\)S\(\text{GTP}\)\(\gamma\)S were able to bind to apo-Rac1 in a saturable fashion with the molar stoichiometry of Rac1/nucleotide at \(~1:0.95\) (Fig. 1). A wide range of Mg\(^{2+}\) concentrations in the incubation buffer did not affect the equilibrium binding stoichiometry. Cdc42 and RhoA displayed similar properties in binding to \(^{[3]H}\)GDP or \(^{[35]}\)S\(\text{GTP}\)\(\gamma\)S in the absence of Mg\(^{2+}\) (data not shown).

To confirm the Mg\(^{2+}\)-independent nucleotide binding property of the Rho proteins, we carried out a different assay using the fluorescent GDP analog, mantGDP, as a ligand. Fig. 2A shows that the addition of apo-Rac1 to mantGDP in buffer B led to a dose-dependent increase of fluorescence emission, indicating the formation of a Rac1-mantGDP complex under the
assay conditions. The mantGDP binding to Rac1 was saturable in the absence of Mg\(^{2+}\) (Fig. 2B), and the presence of excess EDTA (−1 mM) in the binding assay had no effect on the result (data not shown), again suggesting that Mg\(^{2+}\) cofactor is not required for the nucleotide binding to Rac1. This is in contrast to the previous observations made for other members of Ras superfamily GTPases such as Ras (19), Rab3A (20), Sec4p (21), and RalA (39), in which cases removal of bound Mg\(^{2+}\) by EDTA treatment was found to completely abolish their nucleotide binding capabilities. We conclude that the Rho family GTPases behave differently from other Ras-related molecules in this aspect.

**Guanine Nucleotide Binding and Dissociation Properties of Rho GTPases**—To understand the role of Mg\(^{2+}\) in the guanine nucleotide exchange and GTPase-activating reactions of the Rho proteins, we first sought to obtain the binding affinities of GDP and GTP\(^{\alpha}\)S to Cdc42, RhoA, and Rac1 in the absence or presence of MgCl\(_2\). Fig. 3A shows that the equilibrium binding isotherm of \[^{3}H\]GDP to Rac1 differed significantly from that of Ha-Ras at the similar assay conditions with 50 nM small G-proteins and 10 mM Mg\(^{2+}\), yielding a dissociation constant (K\(_{d}\)) of 0.65 μM ± 0.07 compared with that of Ha-Ras at ≤10 nM by fitting the equilibrium binding data into a bimolecular binding model. The much tighter binding of Ras to \[^{3}H\]GDP does not allow us to accurately determine the affinity under the assay conditions. Fig. 3, B and C, illustrate the equilibrium binding experiments in which 500 nM apo-Rac1 was incubated with increasing concentrations of \[^{3}H\]GDP or \[^{35}S\]GTP\(^{\alpha}\)S in the presence or absence of 10 mM Mg\(^{2+}\). The derived K\(_{d}\) values of GDP and GTP\(^{\alpha}\)S binding to Rac1, Cdc42, and RhoA are presented in Tables I and II. These values ranged from 0.62 μM (Rac1), 0.59 μM (Cdc42), and 0.48 μM (RhoA) for GDP and 0.24 μM (Rac1), 0.17 μM (Cdc42), and 0.16 μM (RhoA) for GTP\(^{\alpha}\)S in the presence of Mg\(^{2+}\) to 0.81 μM (Rac1), 0.73 μM (Cdc42), and 0.65 μM (RhoA) for GDP and 0.77 μM (Rac1), 0.57 μM (Cdc42), and 0.51 μM (RhoA) for GTP\(^{\alpha}\)S in the absence of Mg\(^{2+}\). When the data of mantGDP binding to Rac1 collected by monitoring the Rac1-induced fluorescence change of mantGDP in buffer B were fitted into an equilibrium binding equation (Fig. 2B), an apparent binding constant (K\(_{a}\)) value of 0.24 ± 0.04 μM was obtained. Similar titration of apo-Cdc42 and apo-RhoA to mantGDP yielded K\(_{a}\) values of 0.22 ± 0.06 μM and 0.11 ± 0.03 μM, respectively. The K\(_{a}\) values determined by the fluorescence measurement are consistently 4–6-fold lower than the K\(_{a}\) values obtained by the radiolabelled nucleotide binding method, reflecting the difference between the fluorescent GDP analog (mantGDP) and GDP as previously suggested (47, 48). Overall, Cdc42, RhoA, and Rac1 displayed a 2–4-fold higher affinity for GTP\(^{\alpha}\)S than for GDP, and the apparent binding constants are in the submicromolar concentration range as compared with the subnanomolar concentration for Ras (40). It is remarkable that in the absence of Mg\(^{2+}\) cofactor, the Rho proteins were still capable of binding to GDP or GTP\(^{\alpha}\)S with equilibrium affinities similar to those in the presence of Mg\(^{2+}\). These results further indicate that Mg\(^{2+}\) is not required for the nucleotide binding of Rho GTPases per se.

We next examined the effect of Mg\(^{2+}\) on the dissociation rate of nucleotides from the Rho GTPases. Fig. 4A shows a Mg\(^{2+}\) concentration jump experiment in which the decrease of Mg\(^{2+}\) from 1 mM to less than 50 nM by the addition of EDTA led to a drastic increase of \[^{3}H\]GDP dissociation rate, similar to the effect on Ha-Ras or Rab3A (19, 20). The apparent dissociation rate constants of \[^{3}H\]GDP and \[^{35}S\]GTP\(^{\alpha}\)S bound to the Rho proteins, k\(_{off}\), were measured as shown in Fig. 4, B and C, by the addition of excess GDP or GTP\(^{\alpha}\)S in the exchange mixtures. We found that in the absence of Mg\(^{2+}\), k\(_{off}\) of GDP dissociation from Cdc42, RhoA, and Rac1 were at 2.54, 1.40, and 8.74 min\(^{-1}\), respectively, whereas k\(_{off}\) of GTP\(^{\alpha}\)S dissociation from the GTPases were significantly slower at 0.06, 0.042, and 0.11 min\(^{-1}\), respectively (Tables I and II). The apparent dissociation rates of mantGDP for Cdc42, RhoA, and Rac1 in the absence of Mg\(^{2+}\) determined by using mantGDP (k\(_{off}\)) were found to be ~3-fold slower than the k\(_{off}\) values determined by \[^{3}H\]GDP binding at 0.69 ± 0.20, 0.40 ± 0.12, and 2.52 ± 0.34, respectively, which again may be attributed to the differences between mantGDP and GDP (47, 48). The presence of Mg\(^{2+}\) resulted in a drastic decrease of k\(_{off}\) of GDP and, to a lesser extent, of GTP\(^{\alpha}\)S (Fig. 4). In the presence of 10 mM Mg\(^{2+}\), the off rate of \[^{3}H\]GDP was reduced by 150-fold for Cdc42, 10-fold for RhoA, and 300-fold for Rac1, whereas the rates of
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The dissociation constants were obtained by nonlinear regression analysis of the binding of radiolabeled \(^{3}H\)GDP to the apo form of Rho proteins in the presence or absence of \(10 \text{ mM Mg}^{2+}\) as shown for Rac1 in Fig. 3B (\(K_d\)). The apo-Rho GTPases were incubated at 25 °C with 0.2–10 \(\mu\)M \(^{3}H\)GDP. At the 6-h time point, duplicate 20-\(\mu\)l aliquots were removed from the samples, and bound-nucleotides were quantified as outlined under “Experimental Procedures.” To determine the \(k_{\text{on}}\) (apparent dissociation rate constant) values, 250 \(\mu\)M Rho proteins preloaded with \(^{3}H\)GDP were incubated at 25 °C in buffer B or buffer B supplemented with 10 \(\text{mM MgCl}_2\) in the presence of 500 \(\mu\)M GTP (as shown for Rac1 in Fig. 4B). At the designated times, duplicate 20-\(\mu\)l aliquots were taken for the measurement of protein-bound radioactivity. The data were best fitted into a single exponential equation to arrive at the \(k_{\text{on}}\) values.

### Table I

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### Table II

The equilibrium dissociation constants (\(K_d\)) between the indicated Rho GTPases and GTP\(^{\gamma}\)S were determined by filter binding assays using \(^{35}S\)GTP\(^{\gamma}\)S as the radiolabeled ligand as shown for Rac1 in Fig. 3C. \(K_d\) represents the first-order \(^{35}S\)GTP\(^{\gamma}\)S dissociation rate constant as determined for the case of Rac1 in Fig. 4C. Assay conditions and data treatments were similar to those in Table I. Each value represents the results of two separate experiments.

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<th>Constants</th>
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\[^{35}S\]GTP\(^{\gamma}\)S dissociation were slowed by ~4-fold for all three proteins (Tables I and II). Similarly, when a set of apparent on-rate constants of the nucleotides (\(k_{\text{on}}\)) are derived from \(k_{\text{off}}\)/\(K_d\), significant inhibition by the presence of \(\text{Mg}^{2+}\) was also evident (data not shown). When the nucleotide dissociation was examined in the presence of various concentrations of \(\text{MgCl}_2\), it became clear that the dissociation rate decreased with increasing concentrations of \(\text{Mg}^{2+}\). As shown in Fig. 5, the changes in the initial rates of both GDP and GTP\(^{\gamma}\)S dissociation from Cdc42 underwent a major transition at a concentration range of \(\text{Mg}^{2+}\) between 5 and 20 \(\mu\)M. From these data, we can arrive at apparent dissociation constants of 17 and 8 \(\mu\)M for \(\text{Mg}^{2+}\) binding to the GDP- and the GTP\(^{\gamma}\)S-bound Cdc42, respectively. These results establish that \(\text{Mg}^{2+}\) is an important regulator of the kinetics of guanine nucleotide binding to the Rho GTPases, functioning as a stabilizer for the bound nucleotides. It is intriguing that the kinetics of nucleotide binding to the Rho proteins appear to be orders of magnitude slower than that of Ras or Rab (19, 20, 40), further suggesting mechanistic differences among the small G-protein subfamilies.

**Effect of \(\text{Mg}^{2+}\) on the GEF-catalyzed Guanine Nucleotide Exchange of Rho GTPases—** Guanine nucleotide exchange reaction of Ras is limited by the off rate of the bound GDP. The role of GEFs in the nucleotide exchange reaction has been postulated, in the cases of Ras and ARF, as to displace \(\text{GDP}^{\gamma}\) and thereby to allow GDP dissociation and GTP binding to occur (25). The difference between the nucleotide dissociation rates of the Rho GTPases in the presence and absence of \(\text{Mg}^{2+}\) (Tables I and II) suggests that GEF for Rho GTPases may also function by mediating the GDP dissociation step through an effect on \(\text{GDP}^{\gamma}\) ion. To directly examine the effect of \(\text{Mg}^{2+}\) on GEF action, we assayed the GEF activity of the N-terminal fragment of Trio, a Rac-specific GEF, toward Rac1 at increasing concentrations of \(\text{Mg}^{2+}\). \(\text{Mg}^{2+}\) inhibited the Trio-stimulated mantGDP dissociation at increasing concentrations (10 \(\mu\)M to 5 mM) (Fig. 6A), and the inhibitory effect was not due to the change of ionic strength brought by the increased \(\text{Mg}^{2+}\) concentrations in the assay mixture (data not shown). A similar inhibitory effect by \(\text{Mg}^{2+}\) was also observed for the Dbl-catalyzed GDP/GTP exchange on Cdc42 (data not shown). When the initial rates of mantGDP dissociation from Rac1 with or without Trio catalysis were analyzed as a function of \(\text{Mg}^{2+}\) concentration, it appeared that Trio had effectively caused a lower shift of \(\text{Mg}^{2+}\) binding affinity to Rac1 from ~15 to ~150 \(\mu\)M (Fig. 6B). A detailed kinetic analysis of the GEF reaction of the Trio-Rac1 interaction by treating the nucleotide-bound Rac1 as substrate and Trio as enzyme revealed that in the absence of \(\text{Mg}^{2+}\), the intrinsic mantGDP dissociation rate was 4.7 \(\times 10^{-3}\) s\(^{-1}\) with a \(K_m\) value of 1.46 ± 0.60 \(\mu\)M and a \(k_{\text{on}}\) of 0.12 \(s^{-1}\). In the presence of 1 \(\mu\)M \(\text{Mg}^{2+}\), on the other hand, the intrinsic mantGDP dissociation rate was 3.0 \(\times 10^{-4}\) s\(^{-1}\) with a \(K_m\) of 24.5 ± 5.9 \(\mu\)M and a \(k_{\text{on}}\) of 0.094 \(s^{-1}\) (Fig. 6C). \(\text{Mg}^{2+}\) seems to affect only the \(K_m\) parameter of Trio catalysis without much effect on \(k_{\text{on}}\). Taken together, these results indicate that one role of GEF is to destabilize bound GDP through the displacement of bound \(\text{GDP}^{\gamma}\) and that \(\text{Mg}^{2+}\) is mostly involved in the regulation of the binding interaction of GEF with Rho GTPase substrate.

A further examination of the GDP/GTP exchange reaction revealed that both Dbl and Trio were able to stimulate GDP dissociation from their respective substrates, Cdc42 and Rac1, in the absence of \(\text{Mg}^{2+}\) (Fig. 7). Dbl was active in stimulating the GDP dissociation from Cdc42 that was deprived of bound \(\text{GDP}^{\gamma}\) and was undergoing a fast spontaneous nucleotide exchange, but it suffered approximately 50% loss of the ability compared with the extent of stimulation in the presence of \(\text{GDP}^{\gamma}\) (Fig. 7A). Similarly, Trio displayed a dose-dependent GEF activity toward Rac1 in the absence of \(\text{Mg}^{2+}\) and in the presence of 10 mM EDTA (Fig. 7B). These results indicate that GEF also contributes to the destabilization of Rho GTPase-bound nucleotide through a \(\text{Mg}^{2+}\)-independent mechanism in the exchange reaction.

**Effect of \(\text{Mg}^{2+}\) on the Intrinsic and GAP-catalyzed GTPase Activities of Rho GTPases—** The intrinsic GTPase activity of the Rho proteins was measured by monitoring \(\gamma\)P release from the respective GTPases incubated with an excess amount of free GTP. In the absence of \(\text{Mg}^{2+}\), GTP hydrolysis by the Rho GTPases still occurred, albeit at a very slow rate of 0.002 min\(^{-1}\) (Fig. 8), approximately 10-fold slower than that in the presence of \(\text{Mg}^{2+}\) (31, 32). The addition of excess EDTA (~1 mM) to the reaction mixture that ensured the free \(\text{Mg}^{2+}\) concentration was less than 10 nM did not alter this rate (data not shown). Replenishing \(\text{MgCl}_2\) in the reaction mixture to 5 mM caused an increase in \(\gamma\)P release by over 5-fold (Fig. 8), indicating that

\[\text{Mg}^{2+}\text{ binds to Rho proteins, stabilizing their GTP-bound conformation.} \]
although Mg\textsuperscript{2+} is not essential for the intrinsic GTPase activity of the Rho proteins, the divalent ion is involved in maintaining a basal GTPase activity of the Rho proteins at the physiological concentration.

To examine the effect of Mg\textsuperscript{2+} on the GAP-catalyzed GTP hydrolysis, p50RhoGAP was included in the GTPase reaction mixtures in the presence or absence of 5 mM MgCl\textsubscript{2}. At the indicated time, 10 mM EDTA was added to the sample containing Mg\textsuperscript{2+} to lower the free Mg\textsuperscript{2+} concentration to \( \sim \)10 nM. B and C, apo-Rac1 (250 nM) preloaded with \([3H]\)GDP or \([35S]\)GTP\_S was incubated at 25 °C in buffer B or buffer B supplemented with 10 mM MgCl\textsubscript{2} in the presence of 500 \muM GDP (B) or GTP\_S (C). At the designated times, duplicate 20-\mu l aliquots were removed from each sample, and the Rac1-bound nucleotides were quantified by the filter binding method. Data were fitted into a single exponential to derive the apparent dissociation rate constants \( k_{off} \) shown in Tables I and II. Results are representative of three independent experiments.

**FIG. 4.** Determination of the dissociation kinetics of GDP and GTP\_S from Rac1. A, Mg\textsuperscript{2+} jump experiment of Rac1. 500 nM apo-Rac1 was preloaded with \([3H]\)GDP and incubated with buffer B supplemented with 10 mM EDTA or 1 mM Mg\textsuperscript{2+}. At the indicated time, 10 mM EDTA was added to the sample containing Mg\textsuperscript{2+} to lower the free Mg\textsuperscript{2+} concentration to \( \sim \)10 nM. B and C, apo-Rac1 (250 nM) preloaded with \[3H\]GDP or \[35S\]GTP\_S was incubated at 25 °C in buffer B or buffer B supplemented with 10 mM MgCl\textsubscript{2} in the presence of 500 \muM GDP (B) or GTP\_S (C). At the designated times, duplicate 20-\mu l aliquots were removed from each sample, and the Rac1-bound nucleotides were quantified by the filter binding method. Data were fitted into a single exponential to derive the apparent dissociation rate constants \( k_{off} \) shown in Tables I and II. Results are representative of three independent experiments.

**FIG. 5.** Mg\textsuperscript{2+}-dependent nucleotide dissociation from Cdc42. The initial rates of \([3H]\)GDP or \([35S]\)GTP\_S dissociation from Cdc42 were determined in buffer B supplemented with various concentrations of Mg\textsuperscript{2+}. The difference between the initial rates of the nucleotide dissociation in the absence of Mg\textsuperscript{2+} and in the presence of 10 mM Mg\textsuperscript{2+} was taken as 100%. The assay conditions were similar to those for Fig. 4.

**FIG. 6.** Effect of Mg\textsuperscript{2+} on the Trio-catalyzed GEF reaction of Rac1. A, 0.5 \muM Rac1-mantGDP was incubated in buffer B supplemented with various concentrations of MgCl\textsubscript{2} and 500 nM Trio. At the indicated time point (arrow), 400 \muM GTP was added to the mixture to initiate the exchange reaction. B, the initial mantGDP dissociation rates from Rac1 (\( V_0 \)) in the presence (filled circles) or absence (open circles) of 500 nM Trio were analyzed as a function of Mg\textsuperscript{2+} concentrations. C, the kinetics of Trio-catalyzed mantGDP dissociation of Rac1 in the presence or absence of 1.0 mM MgCl\textsubscript{2} were measured at increasing concentrations of Rac1-mantGDP and a constant amount of GTP (400 \muM). Trio was at 200 nM (in the absence of Mg\textsuperscript{2+}) or 500 nM (in the presence of Mg\textsuperscript{2+}) concentration. The data were fitted to a hyperbolic equation as described under “Experimental Procedures” to derive a set of reaction kinetic parameters.

The Role of Mg\textsuperscript{2+} Cofactor of Rho Family GTPases

The initial rates of \([3H]\)GDP or \([35S]\)GTP\_S dissociation from Cdc42 were determined in buffer B supplemented with various concentrations of Mg\textsuperscript{2+}. The difference between the initial rates of the nucleotide dissociation in the absence of Mg\textsuperscript{2+} and in the presence of 10 mM Mg\textsuperscript{2+} was taken as 100%. The assay conditions were similar to those for Fig. 4.

**FIG. 6.** Effect of Mg\textsuperscript{2+} on the Trio-catalyzed GEF reaction of Rac1. A, 0.5 \muM Rac1-mantGDP was incubated in buffer B supplemented with various concentrations of MgCl\textsubscript{2} and 500 nM Trio. At the indicated time point (arrow), 400 \muM GTP was added to the mixture to initiate the exchange reaction. B, the initial mantGDP dissociation rates from Rac1 (\( V_0 \)) in the presence (filled circles) or absence (open circles) of 500 nM Trio were analyzed as a function of Mg\textsuperscript{2+} concentrations. C, the kinetics of Trio-catalyzed mantGDP dissociation of Rac1 in the presence or absence of 1.0 mM MgCl\textsubscript{2} were measured at increasing concentrations of Rac1-mantGDP and a constant amount of GTP (400 \muM). Trio was at 200 nM (in the absence of Mg\textsuperscript{2+}) or 500 nM (in the presence of Mg\textsuperscript{2+}) concentration. The data were fitted to a hyperbolic equation as described under “Experimental Procedures” to derive a set of reaction kinetic parameters.
three Rho GTPases, whereas the reaction $K_{m}$ values remained constant and were similar at $3 \mu M$ for Cdc42, Rac1, and RhoA with or without 5 mM Mg$^{2+}$ in the reaction buffer. Remarkably, the superior catalytic efficiency of p50RhoGAP toward Cdc42 diminished completely in the absence of Mg$^{2+}$ as indicated by a $>40$-fold decrease in $k_{cat}$ and $k_{cat}/K_{m}$ values (Table III, Fig. 9C), suggesting that p50RhoGAP utilizes the Mg$^{2+}$ cofactor to elicit catalytic specificity for Cdc42. We conclude that Mg$^{2+}$ is not absolutely required for RhoGAP action but is important for the optimal GAP catalytic efficiency and specificity.

DISCUSSION

Mg$^{2+}$ is an essential cofactor for small G-protein functions. To date, the effect of Mg$^{2+}$ on the interaction of members of Rho family GTPases with guanine nucleotides has not been characterized. Moreover, it remains unclear what role Mg$^{2+}$ plays in the regulatory reactions of these small GTPases. In the present study, we have prepared the metal ion- and nucleotide-free forms of Cdc42, RhoA, and Rac1 (apoproteins) and quantitatively analyzed the Mg$^{2+}$ effect on the intrinsic guanine nucleotide binding and GTP hydrolysis processes of the GTPases and on the GEF- and GAP-catalyzed reactions. Our results demonstrate that, unique to the Rho family proteins, Mg$^{2+}$ cofactor is not required for the high affinity nucleotide binding per se; rather, it acts as an effective gatekeeper to regulate the nucleotide binding kinetics. In the guanine nucleotide exchange reaction, RhoGEF plays dual roles in stimulating GDP dissociation, to displace bound Mg$^{2+}$ and to destabilize bound nucleotide, whereas in the GTPase-activating reaction, RhoGAP depends upon the Mg$^{2+}$ to elicit high catalytic efficiency and specificity.

The basic structure of the nucleotide binding core of the Rho family GTPases is similar to that of other Ras-like proteins (17, 25): the switch I loop provides an essential serine or threonine residue (Thr$^{35}$ in Ras, Rac1, and Cdc42 and Thr$^{37}$ in RhoA) ligated to the Mg$^{2+}$ ion. The loop region of switch II contains a
conserved aspartic acid residue (Asp$^{57}$ in Ras, Rac1, and Cdc42 and Asp$^{69}$ in RhoA) that serves as a water-mediated Mg$^{2+}$ ligand. The P-loop preceding the first α-helix wraps around the α- and β-phosphates of the nucleotide with a lysine residue (Lys$^{16}$) and a set of main chain amides, and in addition, offers a conserved serine or threonine (Ser$^{17}$ in Ras, Thr$^{17}$ in Rac1 and Cdc42) to coordinate the Mg$^{2+}$ ion. Even with these shared features, it is becoming clear that substantial differences exist in the nucleotide binding properties among the Ras-like small G-proteins. Previous studies have reported that the nucleotide binding affinities of Ras, Rab3A, RalA, and Sec4 ranged from 10 pM to 70 nM, and the binding preference for GTP over GDP binding affinities of Ras, Rab3A, RalA, and Sec4 ranged from (19, 20). However, the nucleotide dissociation constant (K$\text{d}$) is in part dependent on Mg$^{2+}$, which may result in a significant increase of the nucleotide dissociation constant (K$\text{d}$) in the absence of bound Mg$^{2+}$, leading to a significant increase in the catalytic activity of the Rho GTPases in response to the presence of Mg$^{2+}$ in the GDP dissociation reaction, whereas the Mg$^{2+}$-cofactor induced significant conformational changes in the switch I region of RhoA by opening up the nucleotide binding site, similar to what has been observed in the switch I region of Ha-Ras in complex with Sos. This structure is consistent with the current finding that RhoGEF’s may serve to weaken the Mg$^{2+}$ to produce an open conformation in the exchange reactions. We show that both Trio and Dbl were able to further stimulate GDP dissociation from their substrates in the absence of Mg$^{2+}$, suggesting that the displacement of bound Mg$^{2+}$ is only part of the effect imposed on Rho GTPases by GEFs. It is likely that RhoGEF’s contain dual biochemical activities in the GEF reaction: on one hand disrupting bound Mg$^{2+}$ to enhance the GDP dissociation rate, and on the other hand loosening the binding of the bound GDP to the nucleotide binding core of the GTPases.

The role of Mg$^{2+}$ in the GTP hydrolysis of Rho GTPase is thought to stabilize the switch I effector loop at Tyr$^{34}$, which is in turn position the Mg$^{2+}$ and γ-phosphate optimally for hydrolytic attack (45). We have shown here that the intrinsic and the GAP-catalyzed GTP hydrolysis of Rho GTPases does occur in the absence of bound Mg$^{2+}$, albeit at much slower rates. This result indicates that the Mg$^{2+}$ cofactor is not absolutely required for GTPase activity of the Rho proteins, which deviates from the cases reported for Ras and Rab3A (19, 20). However, the reaction kinetics of the GTPase and GTPase-activating reactions were immensely affected by the presence of Mg$^{2+}$. In particular, the presence of Mg$^{2+}$ led to a significant increase in the catalytic potency of the GTPases as reflected by the enhancement of K$\text{cat}$ values, whereas the K$\text{m}$ values of GAP reactions that are closely correlated with the binding affinity to the active Rho GTPases remained unchanged. Furthermore, the specificity of p50RhoGAP toward Cdc42 was solely dependent on the Mg$^{2+}$ cofactor. These biochemical data demonstrate that Mg$^{2+}$ is intimately involved in the intrinsic as well as the catalytic activity of the Rho GTPases.

TABLE III $^2$

<table>
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$^2$Results are representative of at least two independent measurements.
GAP-catalyzed GTP hydrolysis reactions and may act as the major factor dictating the GAP specificity. By a dissociative transition state model for GTP-hydrolysis (46), the chemical nature of the observed Mg$^{2+}$ effect on the GTPase and GAP reactions can be interpreted as stabilization of charge buildup on the γ-phosphoryl oxygen or increasing in negative charge on the nonbridging β-phosphoryl oxygens in the transition state, which allows electrostatic interactions to be catalytic.

In summary, our studies directly demonstrate a role of Mg$^{2+}$ in regulating the kinetics of guanine nucleotide binding and GTP hydrolysis of Rho family GTPases. The observations that GEF facilitates GDP/GTP exchange by acting to displace bound Mg$^{2+}$ and to destabilize bound nucleotide and that RhoGAP utilizes the Mg$^{2+}$ cofactor to elicit catalytic efficiency and specificity in GTP-hydrolysis provide important insight into the GEF and GAP reaction mechanisms of the Rho proteins.

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REFERENCES