New Insights into How the Rho Guanine Nucleotide Dissociation Inhibitor Regulates the Interaction of Cdc42 with Membranes

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The subcellular localization of the Rho family GTPases is of fundamental importance to their proper functioning in cells. The Rho guanine nucleotide dissociation inhibitor (RhoGDI) plays a key regulatory role by influencing the cellular localization of Rho GTPases and is essential for the transforming activity of oncogenic forms of Cdc42. However, the mechanism by which RhoGDI helps Cdc42 to undergo the transition between a membrane-associated protein and a soluble (cytosolic) species has been poorly understood. Here, we examine how RhoGDI influences the binding of Cdc42 to lipid bilayers. Despite having similar affinities for the signaling-inactive (GDP-bound) and signaling-active (GTP-bound) forms of Cdc42 in solution, we show that when RhoGDI interacts with Cdc42 along the membrane surface, it has a much higher affinity for GDP-bound Cdc42 compared with its GTP-bound counterpart. Interestingly, the rate for the dissociation of Cdc42-RhoGDI complexes from membranes is unaffected by the nucleotide-bound state of Cdc42. Moreover, the membrane release of Cdc42-RhoGDI complexes occurs at a similar rate as the release of Cdc42 alone, with the major effect of RhoGDI being to impede the re-association of Cdc42 with membranes. These findings lead us to propose a new model for how RhoGDI influences the ability of Cdc42 to move between membranes and the cytosol, which highlights the role of the membrane in helping RhoGDI to distinguish between the GDP- and GTP-bound forms of Cdc42 and holds important implications for how it functions as a key regulator of the cellular localization and signaling activities of this GTPase.

The Rho family GTPases are a tightly regulated class of signaling proteins that controls a number of important cellular processes. Known most prominently for their ability to remodel the actin cytoskeleton in mammalian cells (1–3), members of this GTPase family have been shown to play essential roles in cell migration, epithelial cell polarization, phagocytosis, and cell cycle progression (4–11). The Rho family member Cdc42 was discovered for its essential role in bud formation in Saccharomyces cerevisiae (12). However, after its identification in higher organisms (13), Cdc42 has been implicated in a diverse array of signaling pathways including those involved in the regulation of cell growth and in the induction of malignant transformation (14). Indeed, point mutations which enable Cdc42 to undergo the spontaneous exchange of GDP for GTP cause NIH3T3 cells to form colonies in soft agar and grow in low serum, two hallmarks of cellular transformation (15). The introduction of activated Cdc42 mutants into nude mice gives rise to tumor formation (16). Moreover, cellular transformation by oncogenic Ras, one of the most commonly mutated proteins in human cancers, requires the activation of Cdc42 (17).

At the molecular level, there are a number of mechanisms that possibly contribute to the roles played by Cdc42 in cell growth control and cellular transformation. These include the ability of Cdc42 to activate the c-Jun NH2-terminal kinase and p38/Mpk2 signaling pathways (18–20) as well as spatially regulate proteins implicated in the establishment of microtubule-dependent cell polarity including glycan synthase kinase-3β and adenomatous polyposis coli (21), extend the lifetime of epidermal growth factor receptor-signaling activities by sequestering Cbl, a ubiquitin E3 ligase (22), and influence intracellular trafficking events (23, 24). To mediate such a wide range of cellular responses, two parameters must be properly regulated; that is, the activation state of Cdc42 and its subcellular localization. As is the case with other GTPases, the activation of Cdc42 occurs as an outcome of GDP-GTP exchange, which then enables it to undergo high affinity interactions with effector proteins (25–27). Upon the hydrolysis of GTP to GDP, Cdc42 is converted back to a signaling-inactive state. Two families of proteins work in opposing fashion to regulate the GTP-binding/GTPase cycle of Cdc42. GTPase-activating proteins recognize the GTP-bound form of Cdc42 and accelerate the hydrolysis of GTP to GDP, rendering Cdc42 inactive (28, 29). Guanine nucleotide exchange factors (GEFs) stimulate the dissociation of GDP from Cdc42, thereby promoting the formation of its signaling-active, GTP-bound state (29, 30).

The subcellular localization of the Rho family GTPases is of fundamental importance to their proper functioning in cells. The Rho guanine nucleotide dissociation inhibitor (RhoGDI) plays a key regulatory role by influencing the cellular localization of Rho GTPases and is essential for the transforming activity of oncogenic forms of Cdc42. However, the mechanism by which RhoGDI helps Cdc42 to undergo the transition between a membrane-associated protein and a soluble (cytosolic) species has been poorly understood. Here, we examine how RhoGDI influences the binding of Cdc42 to lipid bilayers. Despite having similar affinities for the signaling-inactive (GDP-bound) and signaling-active (GTP-bound) forms of Cdc42 in solution, we show that when RhoGDI interacts with Cdc42 along the membrane surface, it has a much higher affinity for GDP-bound Cdc42 compared with its GTP-bound counterpart. Interestingly, the rate for the dissociation of Cdc42-RhoGDI complexes from membranes is unaffected by the nucleotide-bound state of Cdc42. Moreover, the membrane release of Cdc42-RhoGDI complexes occurs at a similar rate as the release of Cdc42 alone, with the major effect of RhoGDI being to impede the re-association of Cdc42 with membranes. These findings lead us to propose a new model for how RhoGDI influences the ability of Cdc42 to move between membranes and the cytosol, which highlights the role of the membrane in helping RhoGDI to distinguish between the GDP- and GTP-bound forms of Cdc42 and holds important implications for how it functions as a key regulator of the cellular localization and signaling activities of this GTPase.

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Of equal importance to its activation status is the spatial regulation of Cdc42. This is highly contingent on the particular cellular membranes that serve as sites of binding and/or recruitment of Cdc42 (31–33). The vast majority of in vitro studies performed on Cdc42 have been carried out in the absence of lipids, which is an important omission considering that virtually all of the physiological functions of Cdc42 occur on a membrane surface (34). Cdc42, along with most other Rho family GTPases, undergoes a series of carboxyl-terminal modifications which result in the covalent attachment of a 20-carbon geranylgeranyl lipid anchor (35–37). Directly preceding this lipid tail is a sequence of basic residues that further stabilizes the association of Cdc42 with the membrane surface (31, 33, 38). A ubiquitously expressed 22-kDa protein called Rho guanine nucleotide dissociation inhibitor (RhoGDI) was found to form a soluble (cytosolic) complex with Cdc42 and other Rho GTPases and to apparently promote their release from membranes (39, 40). RhoGDI was originally discovered and named for its ability to block the GEF- and EDTA-stimulated nucleotide exchange activity of Rho family GTPases (39, 41, 42) and then subsequently shown to inhibit the GTP-hydrolytic activity of Cdc42 (43) and to be capable of interacting with the GDP- and GTP-bound forms of Cdc42 in solution with equal affinity (44). The x-ray crystal structure of a complex between RhoGDI and Cdc42-GDP revealed two types of binding interactions (45). An amino-terminal regulatory arm of RhoGDI was shown to form a helix-loop-helix motif that binds to both of the switch domains of Cdc42, leading to the inhibition of GTP hydrolysis and GDP dissociation (45, 46). The carboxyl-terminal two-thirds of RhoGDI assumes an immunoglobulin-like domain, forming a hydrophobic pocket that in effect provides a membrane substitute for the geranylgeranyl moiety of Cdc42. After release from membranes, the lipid anchor of Cdc42 binds in the hydrophobic pocket of RhoGDI, thereby helping to maintain Cdc42 in solution (45–47).

Prior work from our laboratory has demonstrated an essential role for RhoGDI in Cdc42-mediated cellular transformation. Based on the x-ray crystal structure for the Cdc42-RhoGDI complex, Arg-66 of Cdc42 makes multiple contacts with RhoGDI. When this residue was changed to alanine, Cdc42 was unable to bind to RhoGDI but was still capable of interacting with its other regulatory and effector proteins. Interestingly, when the R66A mutant of Cdc42 was examined in the constitutively active Cdc42(F28L) background, the resulting Cdc42 was unable to bind to RhoGDI but was still capable of interacting with both of the switch domains of Cdc42, leading to the inhibition of GTP hydrolysis and GDP dissociation (45, 46). The carboxyl-terminal two-thirds of RhoGDI assumes an immunoglobulin-like domain, forming a hydrophobic pocket that in effect provides a membrane substitute for the geranylgeranyl moiety of Cdc42. After release from membranes, the lipid anchor of Cdc42 binds in the hydrophobic pocket of RhoGDI, thereby helping to maintain Cdc42 in solution (45–47).

In the present study we have set out to better understand how RhoGDI regulates the signaling functions of Cdc42 and, in particular, how RhoGDI affects the association of Cdc42 with membranes. We show how the membrane plays a previously unappreciated role in allowing RhoGDI to distinguish between the signaling-inactive (GDP-bound) and signaling-active (GTP-bound) forms of Cdc42. By assaying the binding of Cdc42 to insect cell membranes and compositionally defined liposomes through different approaches including a sensitive, real-time fluorescence resonance energy transfer (FRET) readout, we have been able to establish how RhoGDI influences the ability of Cdc42 to transition between a membrane-bound and soluble species. This has led us to propose a new mechanism describing how RhoGDI performs its important regulatory function.

**EXPERIMENTAL PROCEDURES**

**Preparation of Insect Cell-expressed Cdc42**—Cdc42 was purified as a His$_{6}$-tagged protein by baculovirus-mediated expression in Spodoptera frugiperda (SF21) insect cells. All purification steps were performed at 4 °C. One-liter stirred cultures of SF21 cells underwent baculoviral infection for 48 h as carried out at Kinnakeet Biotechnology (Midlothian, VA). Cell pellets were resuspended in 50 ml of hypotonic buffer (20 mM sodium borate, pH 10.2, 5 mM MgCl$_2$, 200 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and leupeptin) and disrupted by Dounce homogenization. The membrane-containing components of the lysate were spun down at 150,000 g for 20 min at 4 °C and discarded. The supernatant containing non-prenylated Cdc42 was discarded, and the pellet was resuspended in 50 ml of TBS-containing magnesium (TBSM; 50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM MgCl$_2$). The procedure was repeated twice, and the resulting pellet was resuspended in TBSM that contained 1% Triton X-100. The lysate was further homogenized and agitated for 30 min on a rotisserie, resulting in the solubilization of the geranylgeranylated Cdc42. The remaining insoluble fraction was pelleted in a tabletop centrifuge at 9000 × g for 20 min at 4 °C and discarded.

**Preparation of Esherichia coli-expressed Cdc42 and RhoGDI**—Bacterial cells harboring plasmids encoding His$_{6}$-Cdc42 or glutathione S-transferase-RhoGDI were grown at 37 °C until an absorbance of 0.8 was reached. Induction was initiated by the addition of isopropyl-β-D-galactopyranoside (1 mM), and the cells were allowed to grow for another 3 h before pelleting at 6000 × g for 10 min. Cell pellets were homogenized in TBSM and lysed by sonication. Cell debris was centrifuged at 20,000 × g for 30 min, and the supernatant was used for purification. His$_{6}$-tagged Cdc42 was purified using Ni$^{2+}$-charged Sepharose beads as described above. Supernatants containing glutathione S-transferase-tagged RhoGDI were incubated with glutathione beads (Amersham Biosciences) and equilibrated with TEDA buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM sodium azide) for 30 min at 4 °C. The beads were then washed with several column volumes of TEDA-containing 500 mM NaCl. After a final rinse with TBSM, the protein was eluted with 10 mM glutathione in TBSM. All eluents were concentrated in a 10 MWC Amicon Ultra concen-
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Preparation of Membrane Vesicles from Insect Cells—Membranes were prepared from uninfected SF21 cells using a modification of the Thom procedure (49). Briefly, 10 ml of stationary phase cells were incubated in 1 ml of hypotonic buffer (20 mM sodium borate, pH 10.2) at 4 °C and then subjected to Dounce homogenization. The cellular debris was pelleted by centrifugation at 900 $\times g$ for 10 min, and the supernatant was transferred to a new tube and centrifuged at 16,000 $\times g$ for 20 min, separating the membranes from the soluble components. The membrane-rich pellet was resuspended in 1 ml of TBSM.

Preparation of Liposomes—Two approaches were used to prepare liposomes. For large liposomes (i.e. several microns in diameter) that can be pelleted by low speed centrifugation, rapid solvent exchange was utilized (50). For fluorescence spectroscopy experiments, smaller lipid vesicles were prepared by extrusion (Avanti mini-extruder). All lipids used in these experiments were obtained from Avanti Polar Lipids unless stated otherwise. The standard lipid composition in molar percentages was 35% phosphatidylethanolamine, 25% phosphatidylinositol, 5% phosphatidylserine, and 35% cholesterol (Nu Chek Preps).

Liposome/Insect Cell Membrane Centrifugation Assays—To assay the binding of Cdc42 or RhoGDI to liposomes, 1 μg of E. coli-expressed Cdc42, insect cell-expressed Cdc42, or E. coli-expressed RhoGDI was incubated in 200 μl of 1 mg/ml lipids, prepared by rapid solvent exchange, for 10 min at room temperature and centrifuged at maximum speed for 20 min. Supernatants and lipid pellets were examined by SDS-PAGE. For radioactive assays measuring the dissociation of Cdc42 from liposomes or insect cell membranes, Cdc42 (100 pmol) was pre-loaded with $[^{35}S]$GTPγS (1400 cpm/pmol) or [α-32P]GTP (1500 cpm/pmol) by EDTA-stimulated nucleotide exchange. The [α-32P]GTP bound to Cdc42 was allowed to hydrolyze to [α-32P]GDP by performing a 30-min incubation on ice in the presence of excess magnesium. The protein was then mixed with 500 μl of insect cell membranes (or with liposomes prepared from 1 mg/ml lipids by rapid solvent exchange) for 10 min and pelleted for 20 min at 16,000 $\times g$ in a microcentrifuge. The lipids were resuspended in TBSM buffer containing different concentrations of RhoGDI and incubated for 10 min and subjected to a final centrifugation, and then radioactivity was measured in the supernatant and lipid pellet. The degree of release of the Cdc42-RhoGDI complex from the liposome/membrane preparations as a function of RhoGDI concentration was fit to the equation,

$$[\text{Cdc42-GDI}] = \frac{[\text{Cdc42}]_{\text{total}}[\text{GDI}]}{K_0 + [\text{GDI}]} \quad (\text{Eq. 1})$$

where $K_0$ is the dissociation constant describing the interaction between Cdc42 and RhoGDI, as reflected by the transition between the membrane and soluble forms of Cdc42, and [GDI] is the concentration of free RhoGDI where [GDI] $\sim$ [GDI]$_{\text{total}}$.

Fluorescence Assays for the Interaction of Cdc42 with Liposomes—Fluorescence measurements were made using a Varian Cary Eclipse fluorimeter in the counting mode. Excitation and emission wavelengths were 365 and 440 nm, respectively. Samples were stirred continuously at 25 °C in TBSM. To prepare hexadecanoylaminofluorescein (HAF)-labeled lipids for FRET assays, 1.25 nmol of HAF (Molecular Probes) was vortexed in 50 μl of lipids (1 mg/ml).

The association of insect cell Cdc42 with liposomes was assayed as follows. A Cdc42-Mant-nucleotide complex (50 nm) was mixed with liposomes prepared by extrusion that contained different concentrations of HAF-labeled lipids, resulting in the quenching of Mant fluorescence. For comparison, the association curves were fit to a single exponential equation of the form,

$$F(t) = F_o (1 - e^{-k_{\text{obs}}t}) \quad (\text{Eq. 2})$$

or, in the case of increasing fluorescence (i.e. when monitoring the release of Cdc42-Mant-nucleotide complexes from liposomes containing HAF),

$$F(t) = F_o e^{-k_{\text{obs}}t} \quad (\text{Eq. 3})$$

where $F(t)$ represents the relative fluorescence as a function of time, $F_o$ represents the initial fluorescence, and $k_{\text{obs}}$ is the rate constant describing the fluorescence change. A rate constant ($k_{\text{off}}$) characterizing the dissociation of Cdc42 from liposomes was estimated by applying the equation (also, see supplemental Equations S1),

$$k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[\text{lipids}] \quad (\text{Eq. 4})$$

To monitor the release of Cdc42 from liposomes, Cdc42 was preloaded with Mant-nucleotide (GDP or GMP-PNP) and incubated with 30 μl of HAF-containing liposomes at room temperature for 5 min. The mixture was added to the cuvette, and at the designated time point RhoGDI was added with stirring. Typically, the initial 2.5 min were recorded, generating traces that monitored the changes in Mant fluorescence due to changes in FRET between Mant-nucleotide-bound Cdc42 and liposomes containing HAF.

The FRET assay used in these studies provides a real-time readout of the formation of the Cdc42-RhoGDI complex as a function of time. The interaction between Cdc42 and RhoGDI is proposed to consist of two steps, an initial binding step and a rate-limiting membrane dissociation step, with $k_1$ and $k_{-1}$ representing the forward and reverse rate constants, respectively, for step 1, whereas $k_2$ and $k_{-2}$ are the forward and reverse rate constants, respectively, for step 2.

$$\text{Cdc42} + \text{RhoGDI} \quad \xrightarrow{k_1} \text{Cdc42} \cdot \text{RhoGDI}$$

(membrane-associated complex)

$$\text{Cdc42} \cdot \text{RhoGDI} \quad \xrightarrow{k_{-1}} \text{Cdc42} + \text{RhoGDI}$$

(soluble complex)

By assuming the initial binding step is in rapid equilibrium and that the second step is rate-limiting, we arrive at the following...
rate equation for the association of Cdc42 with RhoGDI (also, see supplemental Equations S2),

$$\frac{[\text{Cdc42-GDI}]}{[\text{Cdc42-GDI}]_{\text{max}}} = 1 - e^{-k_{\text{obs}}t} \quad (\text{Eq. 6})$$

where

$$k_{\text{obs}} = \left[ \frac{k_2[\text{GDI}]}{K_{\text{D1}} + [\text{GDI}]} \right] + k_{-2} \quad (\text{Eq. 7})$$

The plots of $k_{\text{obs}}$ versus [GDI] were fit with Equation 7 where $K_{\text{D1}} = k_{-1}/k_1$.

RESULTS

RhoGDI Distinguishes between Membrane-associated Forms of GDP- and GTP-bound Cdc42—As an initial step toward determining how RhoGDI helps to increase the amount of soluble (non-membrane-associated) Cdc42 as well as examining whether RhoGDI interacts preferentially with a specific nucleotide-bound state of the membrane-associated GTPase, we set out to establish experimental systems for monitoring the binding of Cdc42 to membranes. We first verified that insect cell (Sf21)-expressed recombinant Cdc42, by virtue of its carboxyl-terminal geranylgeranyl moiety, was capable of associating with lipid bilayers. The results presented in Fig. 1A show that when the insect cell-expressed Cdc42 protein was briefly incubated with liposomes prepared by rapid solvent exchange (50), pelleted by centrifugation, and analyzed by SDS-PAGE, virtually all of the Cdc42 was recovered in the membrane pellet. In contrast, when E. coli-expressed recombinant Cdc42, which lacks the carboxyl-terminal geranylgeranyl tail, was used, the entire pool of Cdc42 was detected in the soluble fraction.

We then confirmed these results by examining the ability of the different recombinant Cdc42 proteins bound to radiolabeled guanine nucleotides to associate with membranes derived from insect cells. Recombinant Cdc42 purified from insect cells, when bound to either $[\alpha-^{32}\text{P}]\text{GDP}$ or $[^{35}\text{S}]\text{GTPyS}$, was able to associate with insect cell membranes. An example for $[^{35}\text{S}]\text{GTPyS}$-bound Cdc42 is shown in Fig. 1B, where the majority of the insect cell Cdc42 protein was present in the membrane pellet. Again the converse was true for E. coli recombinant Cdc42, as the $[^{35}\text{S}]\text{GTPyS}$-bound protein was mainly present in the soluble (supernatant) fraction.

We then examined the ability of RhoGDI to increase the amount of insect cell Cdc42 in the soluble fraction and, in particular, set out to determine whether there were differences in the effectiveness of RhoGDI depending upon whether Cdc42 was bound to GDP or GTP. Fig. 2A shows that the E. coli recombinant RhoGDI, in a dose-dependent manner, was able to increase the amount of GTPyS-bound, His$_6$-Cdc42 in the soluble (supernatant) fraction relative to the membrane fraction. We then compared the effects of RhoGDI on the dissociation of GDP- versus GTPyS-bound Cdc42 from insect cell membranes. Fig. 2B shows that the decrease in the amount of $[\alpha-^{32}\text{P}]\text{GDP}$-bound Cdc42 associated with membrane pellets, as a function of RhoGDI concentration, closely mirrored the appearance of GDP-bound Cdc42 in the soluble supernatant fraction. The apparent $K_D$ values (Equation 1) estimated for the interaction of $[\alpha-^{32}\text{P}]\text{GDP}$-Cdc42 with RhoGDI from these two dose-response curves were similar (i.e. ranging from ~60–70 nM).

Fig. 2C shows the corresponding set of assays for the effects of RhoGDI on membrane-associated, $[^{35}\text{S}]\text{GTPyS}$-bound Cdc42. A significantly greater amount of RhoGDI was necessary to achieve similar effects with GTPyS-bound Cdc42 compared with the GDP-bound form of the protein. In particular, the apparent $K_D$ values ranging from ~440 to 465 nM were obtained for the ability of RhoGDI to bind and increase the amount of GTPyS-bound Cdc42 detected in the soluble fraction. Thus, these results provided us with our first indication that RhoGDI exhibited a binding preference for membrane-

![Image](https://example.com/image.png)
associated GDP-bound Cdc42 compared with the GTPγS-bound form of the protein.

We went on to further verify these findings by using compositionally defined liposomes, as we were ultimately interested in applying a real-time spectroscopic assay to model membrane systems to more closely analyze the kinetics of the interactions of Cdc42 with membranes and the effects of RhoGDI (see below). The results presented in Figs. 3, A and B, show that the ability of RhoGDI to distinguish between membrane-associated GDP- versus GTPγS-bound Cdc42 was also observed in liposomes, although there was a general shift in the dose-response profiles for the effects of RhoGDI. In the case of lipos-
some-associated, [α-32P]GDP-bound Cdc42, we determined the apparent $K_D$ values ranging from ~100 to 180 nM for its interaction with RhoGDI compared with values of ~2.8–6 μM for the corresponding interaction of [35S]GTPγS-bound Cdc42 with RhoGDI. The shift in the dose-response profiles for the effects of RhoGDI on liposome-associated Cdc42 compared with those obtained with insect cell membranes is likely because of the large excess of synthetic liposomes relative to the concentrations of Cdc42 and RhoGDI used in these experiments. Because both Cdc42 and RhoGDI are able to bind independently to lipid bilayers (see supplemental Fig. S3), this results in higher concentrations of RhoGDI being required to achieve the same degree of Cdc42-RhoGDI complex formation on the surfaces of liposomes compared with the case for insect cell membranes.

A Real-time Spectroscopic Readout for the Association of Cdc42 with Lipid Bilayers—Fig. 4A depicts the fluorescence readout that we used to examine the interactions of Cdc42 with liposomes in real time. This assay takes advantage of the FRET that occurs between Mant-nucleotide-bound Cdc42 (designated Mant-Cdc42 in Fig. 4A) and liposomes containing the lipid molecule HAF, as an outcome of the membrane association of Cdc42 (47). Mant-labeled guanine nucleotides, when bound to the nucleotide-binding site of Cdc42, exhibit an increased fluorescence emission at 440 nm. Because the emission spectrum for Mant partially overlaps the excitation spec-
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Titration experiments were performed where we varied the bulk lipid concentrations in preparing the liposomes and then examined the association of these lipid vesicles with either Cdc42-Mant-GDP (not shown) or Cdc42-Mant-GMP-PNP (Fig. 4B). The fluorescence quenching curves that were obtained as an outcome of the association of Mant-nucleotide-bound Cdc42 with liposomes containing HAF (Fig. 4B, inset) were fit with Equation 2 to indicate apparent rate constants ($k_{obs}$). When the observed rate constants were plotted against lipid concentration, a linear relationship was obtained. Interestingly, the rate of dissociation for both Mant-GMP-PNP-bound Cdc42 and Mant-GDP-bound Cdc42 from liposomes ($k_{off}$), obtained by fitting the data to Equation 4 (“Experimental Procedures”), was $\sim$0.1 s$^{-1}$. This indicates that Cdc42 is undergoing rapid translocation between the membrane surface and solution even in the absence of RhoGDI and that the GDP- and GTP-bound forms of Cdc42 dissociate from liposomes at essentially the same rate.

Further verification for the rapid equilibration of Cdc42 on and off the membrane surface was obtained by assaying the exchange of Cdc42 between different populations of liposomes. A depiction of how this liposome exchange assay was carried out is shown in Fig. 5A. In these experiments Mant-GMP-PNP-bound Cdc42 was initially incubated with one population of liposomes that lacked HAF. A second population of liposomes containing HAF was then added to the mixture. The exchange of Cdc42 between the initial vesicle population and the vesicles containing HAF was monitored in real time by the changes in FRET that accompany the association of Mant-GMP-PNP-bound Cdc42 with the HAF-containing liposomes. These liposome exchange assays were performed with varying amounts of

**FIGURE 5.** Cdc42 translocates between liposomes in the absence of RhoGDI. A, schematic of the inter-vesicle transfer of Cdc42 between the surfaces of unlabeled liposomes or between unlabeled liposomes and liposomes containing HAF. B, insect cell recombinant Cdc42-Mant-GMP-PNP (50 nM) was bound to unlabeled liposomes at a lipid concentration of 20 μM and mixed with increasing amounts of HAF-containing liposomes, and the Mant fluorescence was monitored. The plotted values for $k_{obs}$ were determined by measuring the time at which the quenching of Mant fluorescence was halfway completed. The raw data obtained are shown with the indicated concentrations of labeled lipids. C, the $k_{obs}$ values obtained from the fluorescence data in B were plotted as a function of the labeled lipid concentration. The dashed line shows that the $k_{obs}$ values reach a value that is similar to the rate constant for the dissociation of Cdc42 from liposomes, obtained from the data in Fig. 4B.
labeled lipids (Fig. 5B), yielding the plot that shows the half-time for the re-distribution of Mant-GMP-PNP-bound Cdc42 between the different liposome populations \( (k_{\text{obs}}) \) as a function of the concentration of labeled lipids (Fig. 5C). Note that the maximum value for the rate constant describing the exchange of Mant-GMP-PNP-bound Cdc42 between the two liposome populations (dashed line in Fig. 5C) approaches the value of the rate constant measured for the dissociation of Cdc42 from liposomes, as obtained from the experiments described in Figs. 4, A and B, above.

Spectroscopic Assay for the Release of Cdc42 from Lipid Bilayers in the Presence of RhoGDI—We then used the FRET assay to examine the nucleotide-dependent kinetics for the release of Cdc42 from liposomes in the presence of RhoGDI. Fig. 6A shows the results of an experiment where insect cell-expressed Cdc42 was bound with Mant-GDP, as an outcome of the EDTA-catalyzed dissociation of GDP and its exchange with the Mant-nucleotide, and then mixed with liposomes containing HAF at the indicated time point. A partial quenching of the Mant fluorescence was observed over a time period of seconds. Upon the addition of RhoGDI, there was a complete recovery of the Mant fluorescence (i.e., the component of the fluorescence emission that was originally quenched by the labeled lipids). These results are consistent with the interpretation that the initial quenching of the Mant-nucleotide fluorescence is because of the FRET that occurs between the Mant-nucleotide-bound Cdc42 and HAF upon the association of Cdc42 with the HAF-containing liposomes and that the subsequent recovery of fluorescence is because of the release of Cdc42 from the liposome surface that occurs in the presence of an excess of RhoGDI.

The insets to Figs. 6, B and C, show the results of FRET experiments in which we monitored the rates of dissociation of Mant-GDP-bound Cdc42 and Mant-GMP-PNP-bound Cdc42,
respectively, from liposomes containing HAF in the presence of different concentrations of RhoGDI. In these experiments the release of the different guanine nucleotide-bound forms of Cdc42 was monitored in real time by the increase in Mant emission. Significantly higher concentrations of RhoGDI were required to increase the amount of Mant-GMP-PNP-bound Cdc42 in the soluble fraction (i.e. released from HAF-containing liposomes) compared with the amount of RhoGDI necessary to cause a similar increase in soluble Mant-GDP-bound Cdc42. This is consistent with what we had observed when using radiolabeled guanine nucleotides bound to Cdc42 (Figs. 3, A and B). The recovery of Mant fluorescence that accompanied the dissociation of Mant-nucleotide-bound Cdc42 from the HAF-containing liposomes at each level of RhoGDI could be described by a single exponential equation. Figs. 6, B and C, shows plots of the rate constants \( k_{\text{obs}} \) for the release of Mant-GDP- and Mant-GMP-PNP-bound Cdc42 from liposomes as a function of RhoGDI concentration. When these plots were fit to Equation 7, they yielded the same maximum value for \( k_{\text{obs}} \). These findings suggested that despite RhoGDI being able to distinguish between the GDP- and GTP-bound forms of Cdc42 when they are associated with membranes, a common rate-limiting step exists for the membrane release of both nucleotide-bound forms of Cdc42.

The Mechanism by Which RhoGDI Promotes the Transition of Cdc42 from a Membrane-associated State to a Soluble Species—The findings described above are consistent with the model presented in Fig. 7A, which depicts how RhoGDI influences the transition of Cdc42 between a membrane-associated state and a soluble species. The first step represents the initial binding of RhoGDI to Cdc42 along the surface of the membrane and is assumed to be in rapid equilibrium. The large differences in the apparent affinities of RhoGDI for the GDP- and GTP-analog-bound forms of Cdc42 are reflected in this first step; for example, the apparent \( K_D \) value for Mant-GDP-bound Cdc42 is ~8-fold lower than that for Mant-GMP-PNP-bound Cdc42 (i.e. 180 versus 1400 nM; see Fig. 7B). RhoGDI is depicted as being at the membrane surface during its initial binding to Cdc42, based on experiments that show it associates with liposomes (see supplemental Fig. S3). This is also consistent with the findings that Rho GTPase binding-defective mutants of RhoGDI localize predominantly at the plasma membrane (51).
The addition of increasing amounts of Cdc42 to mixtures of RhoGDI and a fixed concentration of lipids resulted in shifting RhoGDI from the membrane-associated pellet fraction to the soluble fraction (see supplemental Fig. S3). This indicates that both Cdc42 and RhoGDI have the capability of increasing the amount of their respective binding partner in the soluble fraction, presumably as an outcome of their forming a complex that is subsequently released from the membrane. The rate-limiting step that represents the actual dissociation of the Cdc42-RhoGDI complex from the membrane is reflected experimentally by the changes in Mant fluorescence shown in Figs. 6, A–C. The measured rate constants for this step ($k_2$ and $k_{-2}$ in Figs. 7, A and B) are virtually the same for the two nucleotide-bound forms of Cdc42. Thus, as alluded to in the previous section, the release of Cdc42 from the membrane is not influenced by its nucleotide-bound state. Importantly, the release of Cdc42 from liposomes in the presence of RhoGDI is described by a rate constant that is very close in value to the rate constant measured for the RhoGDI-independent dissociation of Cdc42 from membranes. Therefore, RhoGDI is not actively extracting Cdc42 from the membrane. However, when Cdc42 dissociates from membranes in a complex with RhoGDI, the geranylgeranyl moiety of Cdc42 binds within the carboxyl-terminal, isoprenoid binding pocket of RhoGDI, thus stabilizing the Cdc42-RhoGDI complex in solution and reducing the rate at which Cdc42 re-associates with membranes. This could then account for previous observations that RhoGDI appears to promote the release of Cdc42 from membranes (39, 42).

**DISCUSSION**

*RhoGDI Discriminates between the GDP- and GTP-bound Forms of Cdc42 in Membranes*—Previous work by our group and others has shown that RhoGDI can bind to both the GDP- and GTP-bound forms of Cdc42 in solution. Using Mant-labeled guanine nucleotides bound to Cdc42 as fluorescent reporter groups, we found that RhoGDI was able to interact with the GDP- and GTP-bound forms of the GTPase with essentially equal affinities (44). Likewise, Hancock and Hall (52) showed that equivalent amounts of RhoGDI were precipitated with glutathione S-transferase-Cdc42 bound to either GDP or GTPyS. Moreover, we demonstrated that the binding of RhoGDI to GTP-bound forms of Cdc42 had functional consequences, as RhoGDI inhibited both the intrinsic and GTPase-activating protein-stimulated GTP hydrolytic activity of Cdc42 (43). Taken together, these findings seemed to be consistent with recent structure-function studies where through a combination of x-ray crystallography, NMR experiments, and fluorescence spectroscopy, we concluded that the GDP- and GTP-analog-bound forms of Cdc42, when analyzed in the absence of effector proteins, showed little if any differences in the conformations of their switch I and switch II domains (53). Thus, the inability of RhoGDI to distinguish between the GDP- and GTP-bound forms of Cdc42 in solution (44) seemed to be explained by the idea that the overall orientation and/or arrangement of the primary interaction sites for RhoGDI on these two guanine nucleotide-bound forms of Cdc42 were virtually identical.

However, in the present study we now show that when Cdc42 is docked onto a membrane surface, its GDP- and GTP-bound states become distinguishable to RhoGDI to an extent where their respective dissociation constants differ by approximately one order of magnitude. These findings hold some potentially important implications regarding the role of the membrane in Cdc42-signaling activities. They offer a possible mechanism by which GTP-bound (activated) forms of Cdc42 accumulate in membranes given that RhoGDI works in a preferential manner to increase the amount of soluble GDP-bound Cdc42. These findings also raise some interesting possibilities regarding whether the ability of RhoGDI to distinguish between the GDP- and GTP-bound states of Cdc42 and/or the ability of Cdc42 to dissociate from membranes might vary for different intracellular membranes depending on their lipid composition. Indeed, we have found that when Cdc42 is associated with liposomes containing PIP$_2$, it shows a significantly weaker interaction with RhoGDI such that there is very little release of GTP-bound Cdc42 from these membranes, consistent with the idea that activated Cdc42 molecules preferentially accumulate and/or signal within specific membrane locations (e.g. the plasma membrane) in the cell.

Another potentially important implication has to do with the fundamental role of the membrane in facilitating structural changes in Cdc42 that accompany GDP-GTP exchange. The cell membrane, rather than simply serving as an inert docking platform for Cdc42 to initiate its signaling activities, may interact with Cdc42 in a manner that significantly influences its ability to assume an activated conformational state. Based on our inability to detect significant differences in the switch I and switch II conformations when comparing Cdc42 molecules bound to GDP versus GTP both in solution and in x-ray crystal structures, we proposed that effector proteins were able to lock GTP-bound Cdc42 into a signaling-competent conformational state, whereas GDP-bound Cdc42 was not susceptible to such effector-induced changes (53). In light of our findings with RhoGDI in the present study, we can now revise that working model by adding the provision that the membrane may help GTP-bound Cdc42 to assume a conformational state that is more readily distinguishable from the GDP-bound GTPase and, thus, more receptive to binding target/effector proteins. It will be interesting to see whether this is also the case for other Rho family GTPases that respond to RhoGDI. We have in fact found that Rac1 behaves in a very similar manner to Cdc42 such that membrane-associated GDP-bound Rac1 shows a much stronger affinity (~10-fold greater) for RhoGDI compared with its GTP-bound counterpart either when assaying membrane association by FRET (data not shown) or using radiolabeled nucleotides (supplemental Fig. S4, A and B). However, interestingly, we have not observed the same marked differences when comparing the ability of RhoGDI to associate with GDP- versus GTP-bound forms of RhoA (data not shown). This would appear to be consistent with studies performed in cells which showed that the overexpression of RhoGDI had little effect on the localization of GFP-tagged RhoA while strongly affecting the localization of Cdc42 and Rac1 (40). Thus, it will be interesting in the future to better understand what might be the

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molecular basis for these differences between Cdc42/Rac1 and RhoA and what consequences this might hold for their respective signaling capabilities.

Consequences of the Mechanism by Which RhoGDI Influences the Release of Cdc42 from Membranes—Using real-time FRET assays to monitor the binding of Cdc42 to liposomes, we made the surprising finding that Cdc42 dissociated from these lipid vesicles on a time scale of ∼5–10 s, which appeared to correspond to the rate for the release of Cdc42 from liposomes in the presence of RhoGDI. This, rather than playing an active role in stimulating the release of Cdc42 from lipid bilayers as originally assumed, RhoGDI instead takes advantage of the intrinsic ability of Cdc42 to dissociate from membranes on a time scale of seconds and then, by providing a hydrophobic pocket for the isoprenoid moiety of the GTPase, ensures that Cdc42 remains in the soluble fraction. This idea is consistent with studies examining the interactions of the small GTPase Rac with the plasma membrane in cells, where it was suggested that RhoGDI surprisingly had little effect on the rate at which Rac dissociated from the membrane surface (54).

Therefore, these findings indicate that the principle regulatory role played by RhoGDI is to significantly reduce the rate at which soluble (cytosolic) Cdc42 is able to re-bind to lipid bilayers. What might be the physiological relevance for lowering the rate at which Cdc42 associates with membranes? One attractive possibility comes from a recent study examining how Cdc42 is able to concentrate in small, local regions on the plasma membrane, as occurs during bud-site formation in yeast (55). Indeed, the recruitment and local concentration of Cdc42 at membrane sites is required for many of its biological functions (56, 57). It has further been suggested that Cdc42-signaling responses may sometimes involve a positive feedback loop in which membrane-bound Cdc42 stimulates the recruitment and co-localization of additional Cdc42 molecules. Altschuler et al. (55) put forward a model based on this idea consisting of three distinct events: membrane-association, membrane-release, and membrane-recruitment. It was initially shown through computational simulations and subsequently verified by experiments that Cdc42 could effectively accumulate at small regions on the membrane surface provided that its rate of association with membranes in general was low relative to the rate at which it was recruited to specific membrane-signaling sites. RhoGDI might indirectly contribute to the recruitment of Cdc42 to specific signaling sites by reducing its ability to bind indiscriminately to membrane surfaces throughout the cell. Consistent with this idea, RhoGDI-deletion experiments in Candida albicans resulted in reduced polarized growth (58).

In light of these findings it is also interesting to consider that the recruitment of the Cdc42(F28L) mutant, which is capable of constitutive GDP-GTP exchange, to specific sites at the plasma membrane may be required for its ability to transform cells. This could then explain the essential role played by RhoGDI in Cdc42(F28L)-induced cellular transformation (48). For example, RhoGDI might prevent GDP-bound Cdc42(F28L) in the cytosol from indiscriminately associating with membranes that lack its important signaling effectors. After GDP-GTP exchange, the activated Cdc42(F28L) molecules might then be able to “escape” from RhoGDI by binding to specific membrane-associated effector proteins and thereby accumulate at those membrane sites from which the necessary signals for cellular transformation are propagated.

This then raises the question of how wild-type, GDP-bound Cdc42 disengages from RhoGDI to become activated through GDP-GTP exchange so as to initiate its signaling activities. The phosphorylation of RhoGDI has been suggested as one mechanism by which GTPases might be released from RhoGDI at the membrane so that they can undergo GEF-catalyzed nucleotide exchange (59). However, in some circumstances or cellular contexts, a membrane-localized GEF may be all that is required to effectively free Cdc42 from the actions of RhoGDI. Upon GEF-stimulated nucleotide exchange, the membrane-bound Cdc42-GTP species would have a reduced affinity for RhoGDI and an increased affinity for effector proteins, most of which are also membrane-bound. This would have the effect of slowing the release of Cdc42 from membranes and shifting the population of Cdc42 from the cytosol to the designated membrane surface. Support for this idea was provided by a recent study of the interaction between the small GTPase Rab1 and RabGDI (60). Here, the authors studied the Legionella pneumophilia protein, SidM, and showed that it can act as a RabGDI displacement factor for Rab1. Interestingly, SidM was also found to catalyze nucleotide exchange on Rab1, with the region responsible for this activity overlapping the site on SidM that was shown to be necessary for RabGDI displacement activity. The authors hypothesized that eukaryotic RabGEFs may be sufficient to dissociate Rab GTPases from RabGDI and recruit them to their target membrane sites. Thus, it is attractive to consider that functional parallels exist between the Rho and Rab families, as Rho GTPases may also depend on GEFs for their recruitment to membrane-signaling sites.

Given that we now have a new appreciation of the role of the membrane surface in helping Cdc42 to assume a signaling-active conformational state as well as a greater understanding of how RhoGDI influences the dynamics of the membrane-cytosol partitioning of this GTPase, we should be able to better evaluate what regulatory mechanisms the cell may utilize to properly localize Cdc42 and how this ultimately influences its signaling activities and transforming potential.

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