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RhoGDI Is Required for Cdc42-Mediated Cellular Transformation

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Summary

Background: Cdc42, a Rho-related small GTP binding protein, plays pivotal roles in actin cytoskeletal organization, Golgi vesicular trafficking, receptor endocytosis, and cell cycle progression. However, the target/effectors mediating these cellular activities and, in particular, those responsible for Cdc42-mediated cell growth regulation and transformation are still being determined. In this study, we set out to examine how the regulatory protein RhoGDI influences the cellular responses elicited by activated Cdc42.

Results: X-ray crystallographic analysis of the Cdc42-RhoGDI complex suggested that arginine 66 of Cdc42 is essential for its interaction with RhoGDI. Here we show that mutation of either arginine 66 or arginine 68 within the Switch II domain of Cdc42 completely abolished the binding of Cdc42 to RhoGDI without affecting the binding of other known regulators or target/effectors of this GTP binding protein. Introduction of the RhoGDI binding-defective mutation R66A within a constitutively active Cdc42(F28L) background was accompanied by changes in cell shape and an accumulation of Cdc42 in the Golgi when these cells were compared to those expressing Cdc42(F28L). However, the most striking change was that unlike Cdc42(F28L), which was able to induce the transformation of NIH 3T3 fibroblasts as assayed by their growth in low serum or their ability to form colonies in soft-agar, the Cdc42(F28L,R66A) mutant was transformation-defective. Likewise, the introduction of RhoGDI siRNA into Cdc42(F28L)-transfected cells inhibited their transformation.

Conclusions: Taken together, the results reported here indicate that despite being a negative regulator of Cdc42 activation and GTP hydrolysis, RhoGDI plays an essential role in Cdc42-mediated cellular transformation.

Introduction

Cdc42 is a member of the Rho family of GTP binding proteins, which serve as molecular switches transducing external signals to multiple downstream targets and giving rise to a variety of cellular responses, including the organization of the actin cytoskeleton, cell cycle progression, cell polarity, and morphology [1]. As is the case for all members of the Rho family, the activity and cellular localization of Cdc42 are regulated by three classes of proteins: (1) Guanine nucleotide exchange factors (GEFs), which facilitate the release of GDP from Cdc42 and thereby catalyze the GDP-GTP exchange (activation) reaction; (2) GTPase-activating proteins (GAPs), which stimulate the GTP hydrolytic activity of the GTP binding protein; and (3) Guanine nucleotide dissociation inhibitors (GDIs), which inhibit the dissociation of GDP from Cdc42. The GDIs are typically depicted as negative regulators of Rho-related GTP binding proteins because of their ability to inhibit GDP dissociation and thus GDP-GTP exchange [2]. However, RhoGDI-1 (from here on simply referred to as RhoGDI) is capable of two other biochemical activities, namely the inhibition of GTP hydrolysis and the release of Rho proteins from membranes [3, 4]. These two additional activities thus raise the interesting possibility that RhoGDI may play positive roles in signaling by maintaining the GTP binding proteins in an activated (GTP bound) state and by mediating their movement between different intracellular membrane locations.

Fluorescence-spectroscopic studies coupled with three-dimensional structural information have suggested that the interaction of Cdc42 with RhoGDI is a two-step process [5, 6]. The first step represents a rapid binding between the Switch II region of Cdc42 and an amino-terminal helical domain of RhoGDI. The second step reflects the removal of Cdc42 from membranes after the insertion of the geranyl-geranyl moiety of Cdc42 into a hydrophobic pocket present within an antiparallel β-pleated sheet in the carboxyl terminal portion of RhoGDI [6]. An examination of the structure of the Cdc42-RhoGDI complex indicated that arginine residues at positions 66 and 68 within the Switch II domain were essential components of the Cdc42-RhoGDI binding interface [6]. We mutated these residues in Cdc42 to examine the biological consequences of disrupting Cdc42-RhoGDI interactions in cells and made the somewhat unexpected discovery that RhoGDI is required for Cdc42-stimulated cellular transformation.

Results

Mutation of Either Arginine 66 or Arginine 68 of Cdc42 Selectively Disrupts Its Interaction with Rho-GDI

A close look at the three dimensional structure of the Cdc42-RhoGDI complex [6], as well as the structures for Cdc42 bound to its GAP and various target/effectors [7–10], suggested that mutating arginine 66 to an alanine within the canonical Switch II region would allow us to specifically assess the importance of Cdc42-RhoGDI interactions in the cellular responses mediated by Cdc42. We first verified that an R66A mutation, when made within a wild-type Cdc42 background, blocked the binding of RhoGDI. As shown in Figure 1A, GFP-tagged (wild-type) Cdc42 could be precipitated with a

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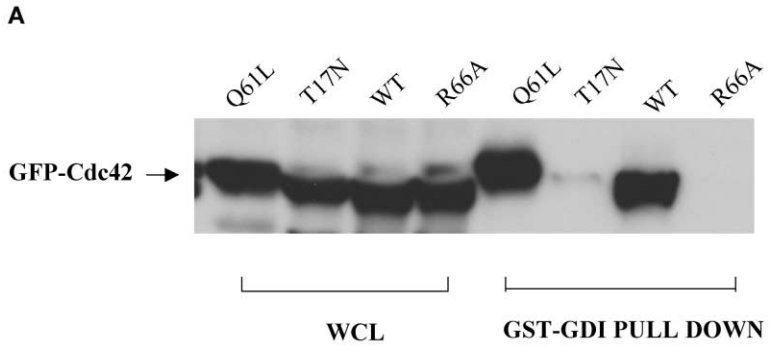
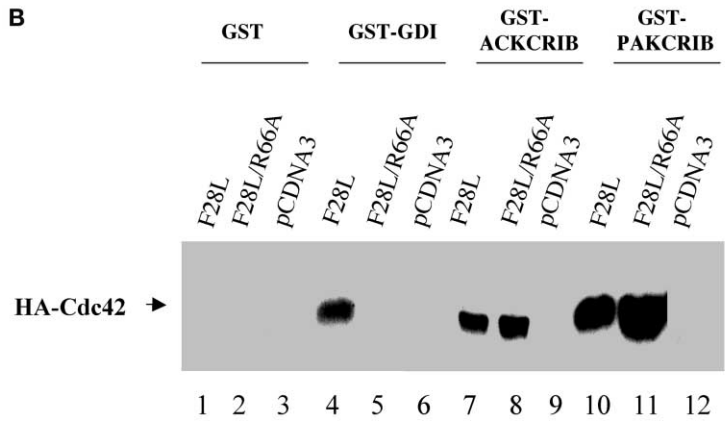


Figure 1. The Cdc42(R66A) Mutant Is Specifically Defective in Binding to RhoGDI

(A) Binding assays for GFP-tagged Cdc42 mutants with GST-RhoGDI. COS-7 cells were transiently transfected with GFP-tagged Cdc42 or its mutants for 48 hr. The cells were lysed and incubated with GST-RhoGDI protein purified from *E. coli*. The protein was detected by Western blotting with an anti-GFP monoclonal antibody.

(B) Binding assays for Myc-tagged Cdc42 (F28L) and other Cdc42 mutants with GST-RhoGDI, GST-ACKCRIB and GST-PAKCRIB. The GST fusion proteins, immobilized on glutathion-agarose beads, were incubated with the lysates from COS-7 cells expressing Cdc42. The bound Cdc42 proteins were detected by Western blotting with an anti-Myc antibody (9E10).



GST-RhoGDI fusion protein after the addition of glutathione-agarose beads to COS-7 cells that transiently expressed wild-type Cdc42, whereas the GFP-Cdc42 (R66A) mutant was not able to bind to GST-RhoGDI. As previously reported, we also found that an activated form of Cdc42 (the GTPase-defective Cdc42(Q61L) mutant) was able to bind to GST-RhoGDI [3, 11], whereas the nucleotide-depleted Cdc42(T17N) mutant, which has a high affinity for Dbl and other guanine nucleotide exchange factors (GEFs) for Cdc42 [12], showed little detectable binding to RhoGDI.

We then asked whether the R66A mutation, when made within the background of a constitutively active form of Cdc42, Cdc42(F28L), which allows the spontaneous exchange of GTP for GDP [13], also blocked RhoGDI interactions and/or the binding of activated Cdc42 to its other known target/effectors. We were especially interested in these questions because we have shown that Cdc42(F28L) is an extremely valuable mutant for studying the cellular responses elicited by activated forms of Cdc42. Unlike GTPase-defective Cdc42 mutants, which are toxic to NIH 3T3 cells, the Cdc42(F28L) mutant can be stably expressed and gives rise to cellular transformation [13]. The results presented in Figure 1B (lane 4) show that when HA-tagged Cdc42(F28L) was transiently expressed in COS-7 cells, it was able to bind to a GST-RhoGDI fusion protein, whereas the Cdc42 (F28L,R66A) mutant was completely ineffective in binding to RhoGDI (lane 5). However, the Cdc42(F28L,R66A) protein was as effective as Cdc42(F28L) in binding to Cdc42 targets that contain CRIB (for Cdc42/Rac-interactive binding) domains. An example is shown for the limit Cdc42/Rac binding domain from PAK (GST-PAK-CRIB), a serine/threonine kinase-target of Cdc42 and Rac (compare lanes 10 and 11 in Figure 1B). The same was true when we compared the binding of the Cdc42(F28L) and Cdc42(F28L,R66A) proteins to the limit binding domain of ACK (GST-ACKCRIB), a non-receptor tyrosine kinase that is a specific target for Cdc42 (see lanes 7 and 8 in Figure 1B). We found that, similar to the case for arginine 66 of Cdc42, changing the arginine residue at position 68 to an alanine eliminated the ability of an activated Cdc42 protein to bind RhoGDI without affecting its binding to CRIB-motif containing targets such as PAK and ACK (data not shown).

We next set out to make certain that the RhoGDI binding-defective Cdc42 mutants were able to bind effectively to full-length versions of these targets. Figure 2A shows that the Cdc42(F28L,R66A) double mutant, like the Cdc42(F28L) protein, is able to bind to full-length ACK in cells, whereas wild-type (GDP-bound) Cdc42 showed no detectable binding. We have also verified that the tyrosine kinase activity of ACK2 can be fully stimulated by Cdc42(F28L,R66A), as can the serine/thre-

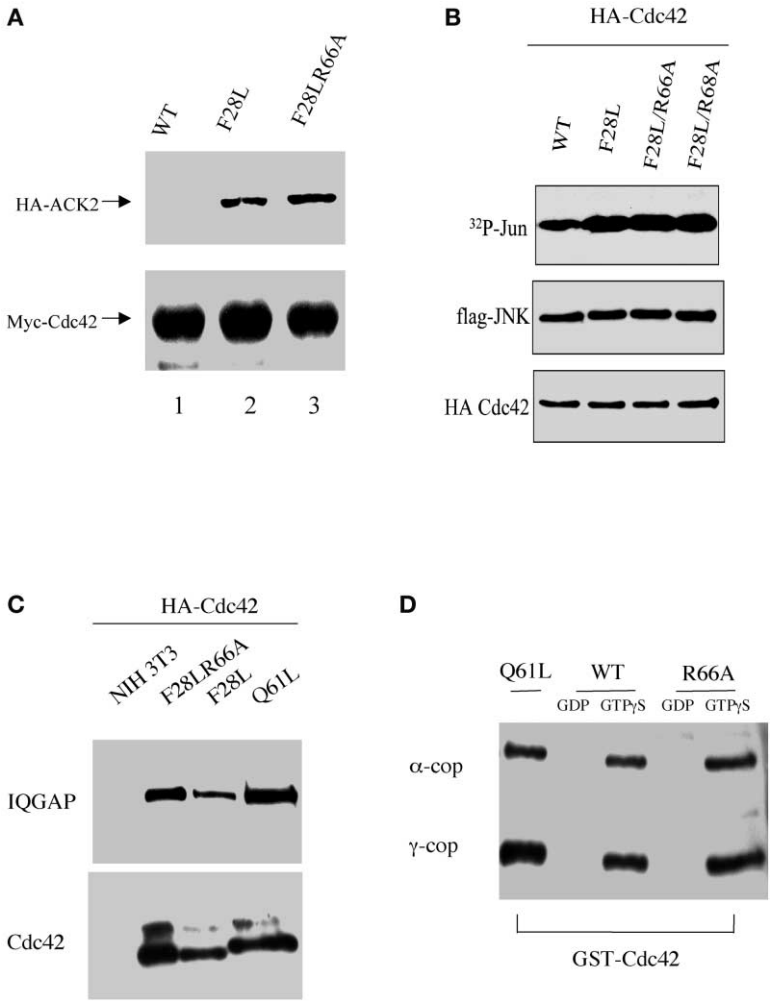


Figure 2. Examination of How the R66A Mutation Affects Cdc42-Target/Effector Interactions

(A) The interaction of full-length ACK2 with Cdc42(F28L) and Cdc42(F28L,R66A). Myc-tagged Cdc42 and HA-tagged ACK2 were co-transfected in COS-7 cells, and then the cells were lysed and immunoprecipitated with an anti-Myc antibody. Coimmunoprecipitated-ACK2 was detected with anti-HA antibody.

(B) Assays of Cdc42-mediated activation of the c-Jun kinase (JNK1). COS-7 cells were transiently transfected with HA-tagged Cdc42 or its mutants, together with flag-tagged JNK. JNK then was immunoprecipitated from the cell lysates, and its kinase activity was assayed using GST-c-Jun as a substrate as described previously [13, 14].

(C) Assays of the binding of Cdc42 to IQGAP. HA-tagged Cdc42(F28L) or Cdc42(F28L,R66A) was transfected and expressed in NIH 3T3 cells and immunoprecipitated via an anti-HA antibody. Coimmunoprecipitated IQGAP was detected with an anti-IQGAP antibody [16].

(D) Assays of the binding of Cdc42 to the γ-coatomer subunit. The Cdc42 GST fusion proteins, containing bound GTP in the case of the Q61L mutant or loaded with GDP or GTPγS as indicated, were immobilized on glutathione-agarose beads and incubated with COS-7 cell lysates. Bound coatomer subunits were blotted with an antibody that recognizes both the α- and γ-coatomer subunits [17].

onine kinase activity of full-length PAK3 (data not shown). Moreover, as shown in Figure 2B, the Cdc42 (F28L)-stimulated activation of the c-Jun kinase (JNK), which results in a 2- to 3-fold increase in JNK activity in COS-7 cells [13] and requires PAK activation [14], is unaffected by either the R66A or R68A mutation.

Thus far, we have found the same to be true for other CRIB-motif-containing Cdc42 targets (e.g., full-length WASP)—that is, neither the R66A nor the R68A mutation interfered with their binding to activated Cdc42. In addition, this holds for other Cdc42-targets that lack a CRIB motif. The two presently known examples are IQGAP and the γ-coatomer subunit of the COPI complex [15–17]. The former has been implicated in interfacing Cdc42 with the actin cytoskeleton [15, 16], and recent studies performed in *S. cerevisiae* have suggested that IQGAP plays a key role in linking actin cytoskeletal rearrangements to cellular trafficking events and cytokinesis [18]. As shown in Figure 2C (upper panel), the GDI binding-defective Cdc42(F28L,R66A) double mutant can associate with IQGAP as read out by immunoprecipitation of HA-tagged Cdc42 with anti-HA antibody and subsequent Western blotting with a specific anti-IQGAP antibody (the lower panel in Figure 2C shows the relative amounts of the different HA-tagged Cdc42 proteins that were immunoprecipitated). Similar results were obtained from assays of the binding of Cdc42 to the γ-coatomer subunit. Figure 2D shows that a GST fusion protein encoding the GTP bound, GTPase-defective Cdc42 [Cdc42(Q61L)] as well as the GTPγS bound forms of GST-wild-type Cdc42 and the GST-Cdc42(R66A) mutant all exhibited comparable abilities to bind to the γ-coatomer subunit. Other subunits of the COPI complex (e.g., α-coatomer) were also coprecipitated with each of these versions of activated Cdc42, through their association with the γ-coatomer subunit [13].

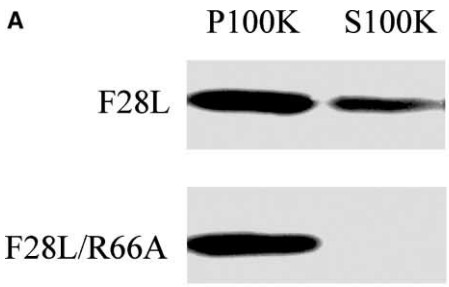
Taken together, the results presented in Figures 1 and 2 indicate that Switch II point mutations that interfere with the binding of Cdc42 to RhoGDI do not compromise the interactions between Cdc42 and its target/effectors. The fact that Cdc42 Switch II mutations that prevent the binding of RhoGDI do not affect the binding of other targets may be related to the indication from the X-ray crystallographic structures of different Cdc42 species [6] that the Switch II domain is not acting like a bona fide conformationally sensitive region in Cdc42 and thus does not function as a target binding domain. We and others have also found that mutating arginine 66 of Cdc42 does not alter the functional regulation of guanine nucleotide exchange activity by Dbl-family members

(data not shown; also [19, 20]). The same holds true for the GAP-stimulated GTP hydrolytic activity of Cdc42 [21]. Overall, these results suggest that mutating arginine 66 in Cdc42 uniquely interrupts its interaction with RhoGDI and does not interfere with the binding or function of other regulatory or target/effector proteins. Given these findings, we further examined the consequences of mutating arginine 66 to alanine on the cellular activities normally triggered by activated Cdc42.

Disruption of Cdc42(F28L)-RhoGDI Interactions Affects the Cellular Localization of Activated Cdc42 and Cell Morphology

One of the primary biochemical activities of RhoGDI is its ability to stimulate the release of Cdc42 and related Rho GTP binding proteins from membranes. Thus, it would be expected that by blocking the interactions of Cdc42 with RhoGDI, one could reduce the cytosolic levels of Cdc42. Figure 3A shows that this was indeed the case when the R66A mutation was introduced in an activated F28L background. The particulate (microsomal membrane) and cytosolic fractions from NIH 3T3 cells stably expressing HA-tagged Cdc42(F28L) and HA-Cdc42(F28L,R66A) were resolved by high-speed centrifugation. As shown in Figure 3A, the Cdc42(F28L,R66A) protein was completely absent from the cytosolic (S100K) fraction, as opposed to the case for the Cdc42(F28L) mutant, suggesting that in the absence of RhoGDI interactions, activated Cdc42 is associated exclusively with cellular membranes. A careful examination of the immunofluorescence profiles for COS-7 cells that transiently expressed GFP-labeled Cdc42(F28L) and GFP-Cdc42(F28L,R66A) also suggested that there was a shift in the overall membrane distribution of Cdc42, such that the majority of the RhoGDI binding-defective Cdc42(F28L,R66A) double mutant was perinuclear (Figure 3B, lower panel, arrow), whereas the Cdc42(F28L) protein was perinuclear and was also detected along the outer surface of the cells (Figure 3B, upper panel, see arrows).

We also have observed cell morphology changes that were most apparent when we compared NIH 3T3 fibroblasts expressing Cdc42(F28L) versus the Cdc42 (F28L,R66A) double mutant. In these cases, a Tet-off eukaryotic expression system was used to establish tetracycline-inducible NIH 3T3 cell lines that were transfected with plasmids encoding wild-type Cdc42, as well as the Cdc42(R66A), Cdc42(F28L), and Cdc42(F28L,R66A) mutants. We induced the expression of these different proteins by removing tetracycline from the culture medium (see Figure 4B). Although neither the inducible expression of wild-type Cdc42 nor that of the Cdc42(R66A) mutant in NIH 3T3 cells elicited any obvious effects on cell morphology (data not shown), cells expressing Cdc42(F28L) exhibited elongated cell bodies, whereas cells expressing the Cdc42(F28L,R66A) double mutant were flat and often appeared larger than either control or Cdc42(F28L)-expressing fibroblasts (Figure 4A). Some good examples of this are shown in Figure 4C, where Cdc42(F28L)- and Cdc42(F28L,R66A)-expressing cells were stained for F-actin with rhodamine-labeled phalloidin (top panels) and for focal adhesion complexes with anti-paxillin antibody (bottom panels), respectively. In fact, one of the cells expressing Cdc42(F28L,R66A) contained two nuclei (Figure 4C). We had earlier reported that expression of the constitutively



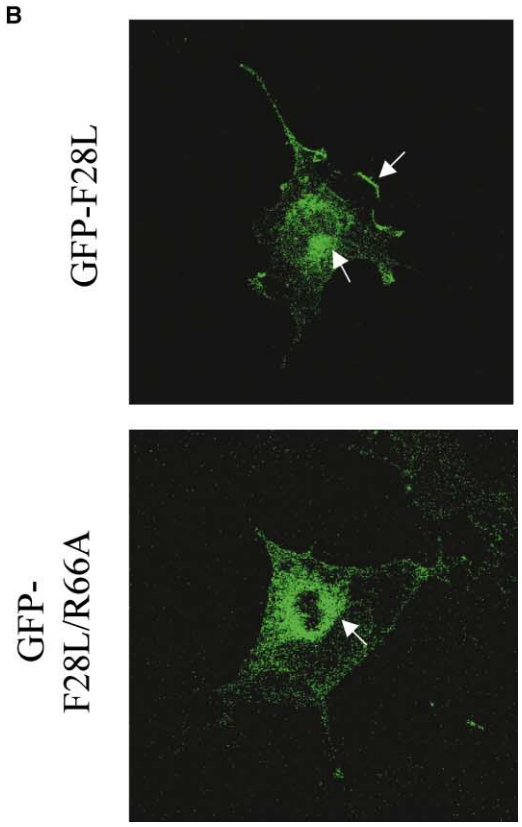


Figure 3. Effects of the R66A Mutation on the Cellular Localization of Activated Cdc42

(A) NIH 3T3 cells that stably expressed Cdc42(F28L) or Cdc42(F28L,R66A) were collected, homogenized, and fractionated as described in the Experimental Procedures. The particulate (P) and the cytosolic fractions (40 μg protein/lane) were separated by centrifugation. Cdc42 protein was detected by immunoblotting with an anti-HA antibody (Babco).

(B) COS-7 cells were transiently transfected with GFP-tagged Cdc42(F28L) or Cdc42(F28L,R66A). The localization of GFP-tagged Cdc42(F28L) or Cdc42(F28L,R66A) was examined with a Leica confocal microscope.

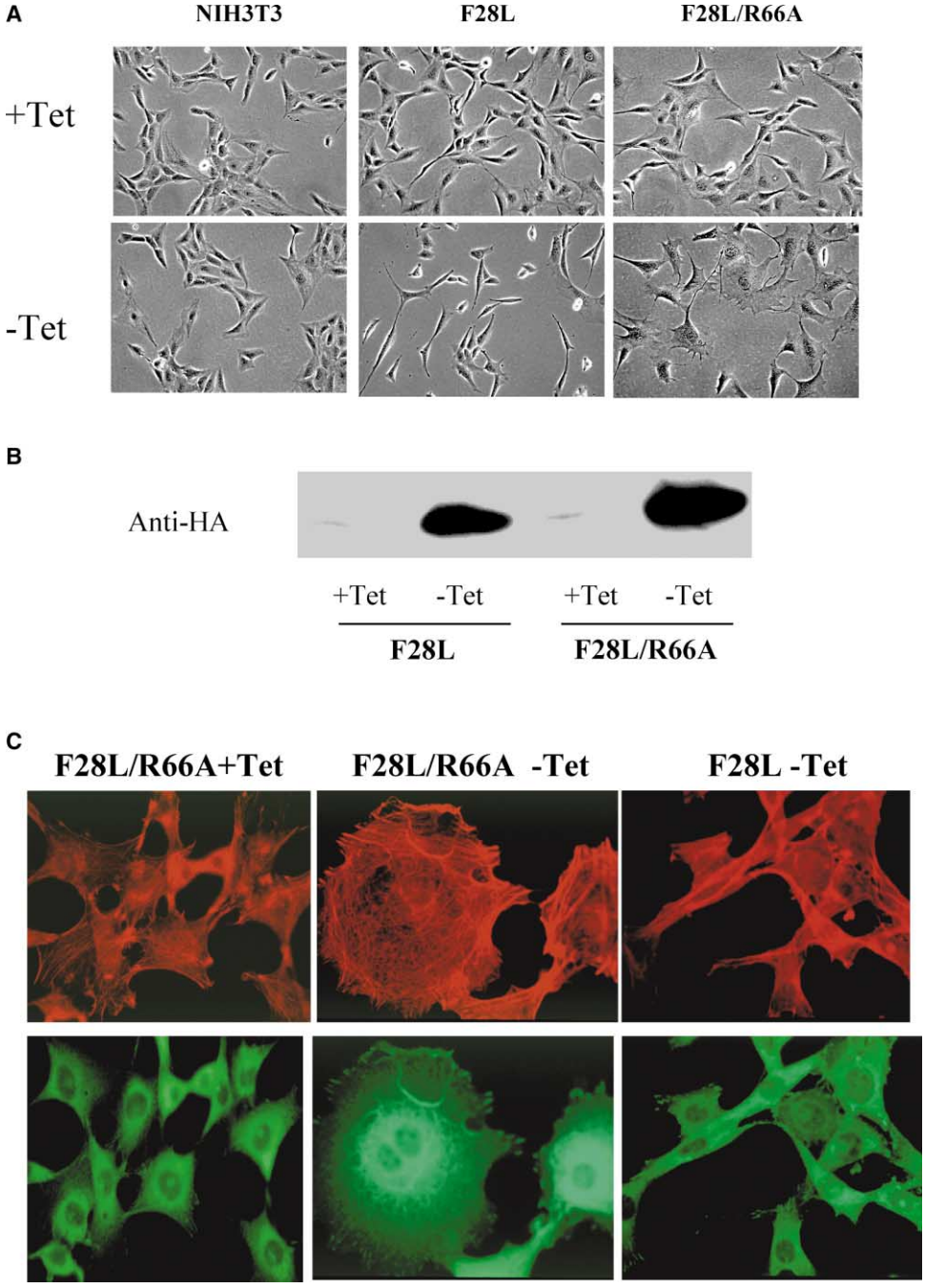


Figure 4. Cdc42(F28L,R66A) Induces Morphological Changes in NIH 3T3 Cells

(A) NIH 3T3 cells stably transfected with pTet-Cdc42(F28L) and Cdc42(F28L,R66A) were grown in normal serum with or without tetracycline for 5 days, and the morphology of the cells was visualized with a Zeiss phase-contrast microscope. +Tet, uninduced; -Tet, induced.

(B) Expression of HA-tagged Cdc42(F28L) and Cdc42(F28L,R66A) shown in Figure 4A. The proteins were detected via immunoblotting with an anti-HA antibody. +Tet, uninduced; -Tet, induced.

(C) NIH 3T3 cells that expressed Cdc42(F28L,R66A) or Cdc42(F28L) were fixed and immunostained with Texas-red-conjugated phalloidin (top panels) or with anti-paxillin (bottom panels).

active Cdc42(F28L) mutant resulted in some cells (typically approsimately 5%) having multiple nuclei, similar to what had earlier been described for cells expressing oncogenic Dbl, a GEF for Cdc42 [13] and as opposed to control NIH 3T3 cells, which typically do not show this phenotype (<1% of the cells). We find that as many as 10% of the cells expressing Cdc42(F28L,R66A) are multi-nucleate in appearance, and we suspect that by blocking Cdc42-RhoGDI interactions, this phenotype, which is associated with the expression of hyper-activated forms of Cdc42, is somehow exaggerated. Although the Cdc42(F28L,R66A) double mutant, similar to the

Cdc42(F28L) mutant, is able to stimulate the formation of focal complexes (Figure 4C, bottom panels), comparisons of Cdc42(F28L)- and Cdc42(F28L,R66A)-expressing cells in Figure 4C (top panels) also show what appears to be a general disorganization of actin stress fibers upon expression of the RhoGDI binding-defective Cdc42 mutant. These changes in cell shape and cytoskeletal arrangement that accompanied the inhibition of the interactions between activated Cdc42 and RhoGDI led us to question whether blocking Cdc42-RhoGDI interactions might also impact Cdc42(F28L)-induced cellular transformation.

Cdc42-RhoGDI Interactions Are Required for Cdc42-Induced Transformation

Our previous studies have shown that expression of the constitutively active Cdc42(F28L) mutant in NIH 3T3 cells induced cellular transformation and caused tumor formation in nude mice [13]. To test the importance of RhoGDI in Cdc42-mediated cellular transformation, we utilized the inducible cell lines expressing the Cdc42(F28L) and Cdc42(F28L,R66A) mutants that were described in the preceding section. When we used normal (5%) concentrations of serum in the culture medium, the growth of cells that expressed the activated Cdc42(F28L) mutant was about 2-fold faster than either control cells or cells expressing the Cdc42(F28L,R66A) double mutant (Figure 5A). However, when we measured cell growth in low (1%) concentrations of serum, both control NIH 3T3 cells and cells expressing Cdc42 (F28L,R66A) had arrested growth during the first 24 hr, followed by a progressive cell death, whereas cells expressing Cdc42(F28L) were able to steadily proliferate, which is one hallmark of cellular transformation (Figure 5B).

We also examined the abilities of these different cell lines to grow in soft agar because this has often been considered to be the most reliable indicator of cellular transformation. As shown in Figures 5C and 5D, NIH 3T3 cells that expressed the constitutively active Cdc42(F28L) protein exhibited anchorage-independent growth, consistent with what we had earlier reported [13], whereas, similar to the control cells, cells expressing the Cdc42(F28L,R66A) double mutant were unable to form colonies in soft agar. Taken together, the results presented in Figure 5 strongly suggested that Cdc42-RhoGDI interactions were in fact essential for Cdc42-mediated cellular transformation.

However, in order to rule out the possibility that the transformation defects exhibited by the Cdc42(F28L,R66A) double mutant were due to an inability of this activated Cdc42 protein to bind to an as-yet-unidentified target (which would presumably be essential for transformation), we set out to reduce the levels of endogenous RhoGDI by using small inhibitory RNA (siRNA) approaches [22]. As shown in Figure 6A, we have been able to reduce the levels of endogenous RhoGDI by at least 50% (ranging from approximately 50%–75%) upon transfecting fibroblasts with RhoGDI siRNA. This reduction in the levels of endogenous RhoGDI was accompanied by detectable changes in the morphology of cells expressing the Cdc42(F28L) mutant (Figure 6B); these changes were similar to those observed in cells that expressed the RhoGDI binding-defective Cdc42 (F28L,R66A) protein (Figure 4A). Changes in the actin cytoskeletal organization that were qualitatively similar to those described for Cdc42(F28L,R66A)-expressing cells accompanied expression of the siRNA for RhoGDI (data not shown), although in general these changes were less pronounced in the siRNA experiments, perhaps because we did not achieve a complete elimination of RhoGDI expression. However, we were able to clearly see that the transformation capability of the Cdc42 (F28L) protein in cells expressing reduced levels of RhoGDI was significantly compromised, as assayed by colony formation in soft agar (Figures 6C and 6D). Thus, these results verify the importance of RhoGDI for Cdc42-induced cellular transformation.

Discussion

RhoGDI was originally named for its ability to block the dissociation of GDP from Rho and related GTP binding proteins [2, 4, 23]. Thus, this protein has often been assumed to act as a negative regulator by inhibiting the activation (i.e., GDP-GTP exchange activity) of Rho-family GTP binding proteins. However, RhoGDI exhibits two other biochemical activities that are likely to contribute to its overall regulatory functions. One of these is the stimulated release of Rho-related GTP binding proteins from membranes [4, 24]. This function has been traditionally associated with the early finding that the ability of RhoGDI to bind to its target GTP binding proteins with high affinity requires their isoprenylation [4, 25], and in fact the X-ray crystallographic structure of the Cdc42-RhoGDI complex shows that the GDI contains a substantial hydrophobic binding pocket for the geranyl-geranyl moiety of Cdc42 [6]. A second activity is that unlike the RabGDIs, which also extract Rab proteins from membranes but bind with high selectivity to the GDP bound form of the Rabs, the RhoGDI can bind with essentially equal affinity to the GDP and GTP bound forms of Cdc42 [11] and is capable of inhibiting the GTP hydrolytic activity of Cdc42 [3]. Thus, these multiple biochemical activities exhibited by RhoGDI expand its potential repertoire of regulatory roles in cells.

In the present study, we describe the surprising finding that the interaction of activated Cdc42 with RhoGDI is essential for Cdc42-mediated cellular transformation. To our knowledge, this is the first demonstration of a positive (rather than negative) regulatory role for the GDI in cell growth control. In making these findings, we first took advantage of the X-ray-crystallographic structure for the Cdc42-RhoGDI complex to generate Cdc42 mutants that were specifically defective in their ability to bind the GDI while maintaining their binding affinity for other known targets and regulatory proteins. In particular, we changed Cdc42 Switch II domain residues (i.e., arginine 66 or 68) that engage an amino terminal helical arm of RhoGDI that is essential for the first step in the Cdc42-RhoGDI interaction. We found that when we directly compared the abilities of the constitutively active Cdc42(F28L) mutant and the constitutively active but RhoGDI binding-defective Cdc42(F28L,R66A) to trans-

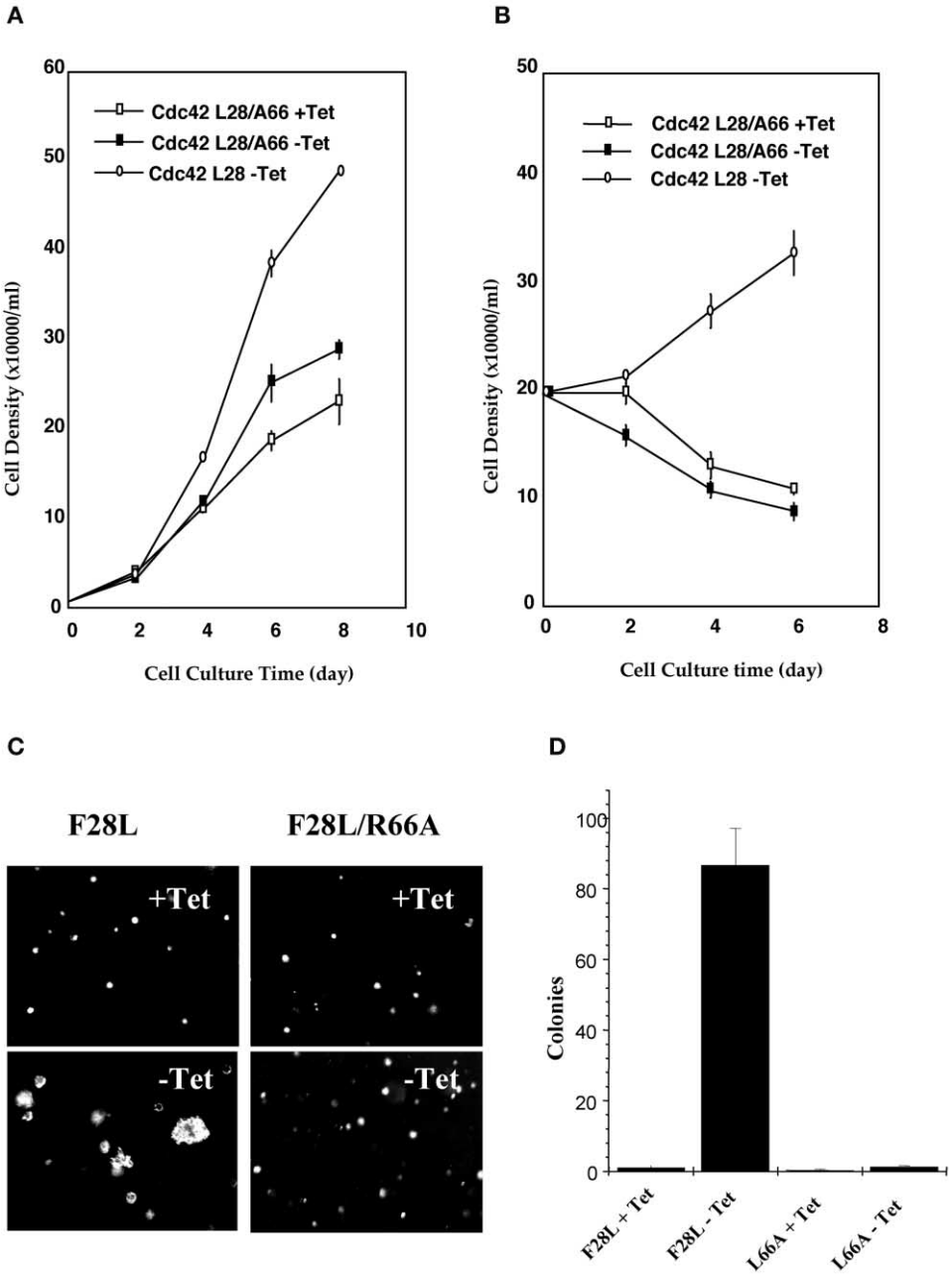


Figure 5. Binding to RhoGDI Is Required for Cdc42-Stimulated Cell Growth and Transformation

(A) The growth rate of NIH 3T3 cells stably transfected with Cdc42(F28L) or Cdc42(F28L,R66A) and cultured at normal concentrations (5%) of calf serum. +Tet, uninduced; -Tet, induced.

(B) The growth rate of the indicated cell lines that were cultured at a low concentration (1%) of calf serum. +Tet, uninduced; -Tet, induced.

(C) Anchorage-independent growth of Cdc42(F28L)- and Cdc42(F28L,R66A)-expressing cells. Anchorage-independent growth was determined by an assay of colony formation in soft agar. The cells were cultured in the medium with or without tetracycline for 24 hr before being plated on soft agar. +Tet, uninduced; -Tet, induced.

(D) Quantification of colony formation on soft agar. Colonies were scored after 14 days of growth on soft agar as shown in Figure 5C. In each experiment, three duplicate 35 mm plates were counted at three randomly chosen areas.

form NIH 3T3 cells under conditions where these proteins were expressed to identical levels, only the Cdc42(F28L) protein was capable of inducing a transformed phenotype. As further support for the essential role of RhoGDI in Cdc42-mediated cellular transformation, we used siRNA approaches to show that reducing the expression of the GDI gave rise to a corresponding reduction in transformation activity.

How might RhoGDI contribute to the signaling function of Cdc42 such that Cdc42-RhoGDI interactions are essential for Cdc42-mediated transformation? Clearly, the levels and functional activities of this regulatory pro-

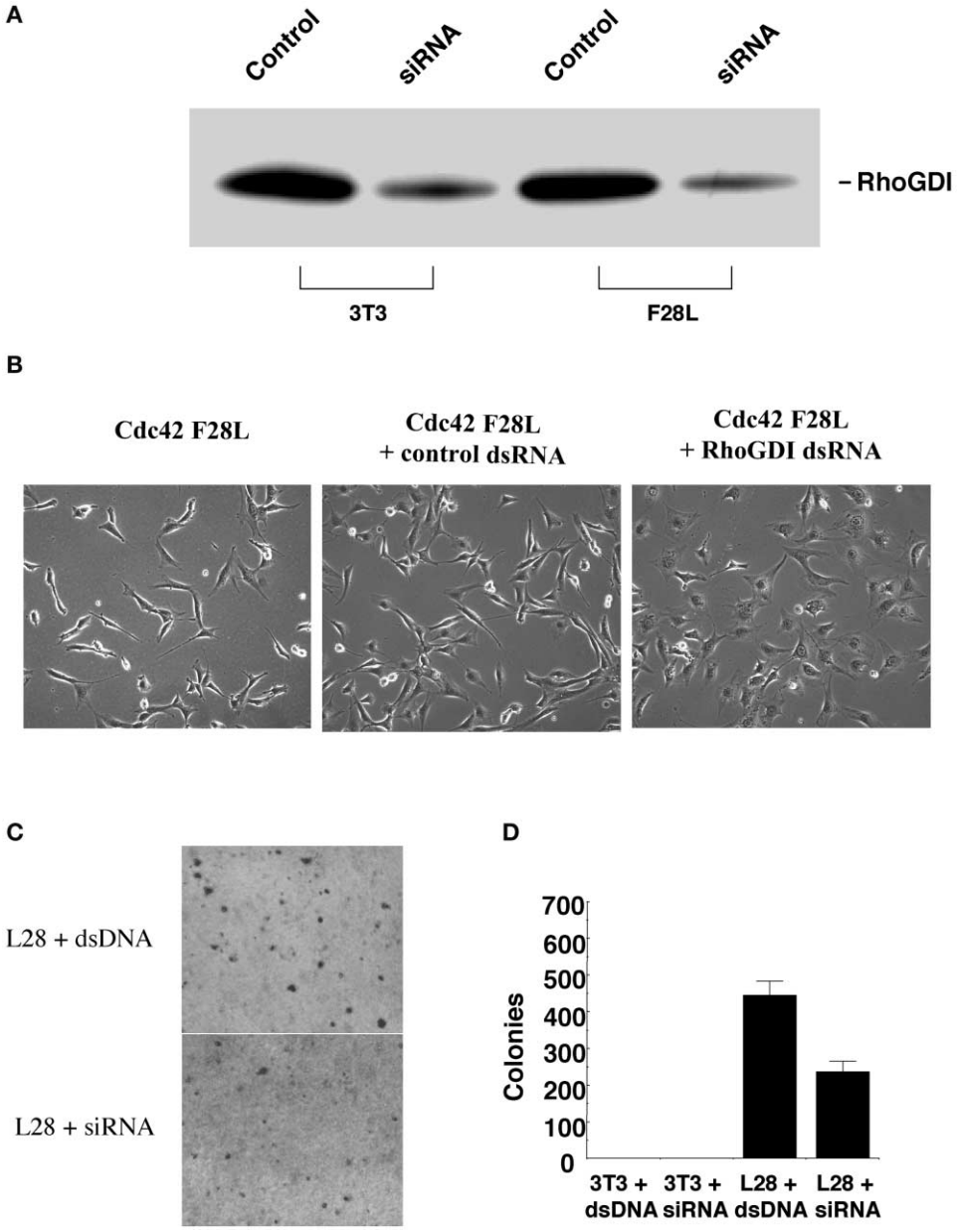


Figure 6. Reduction in the Levels of Endogenous RhoGDI Influences Cdc42(F28L)-Mediated Effects on Cell Morphology and Cellular Transformation

After 48 hr of transfection with control oligomer or RhoGDI siRNA, NIH 3T3 cells were examined for morphology changes and were plated on soft agar to assay for anchorage-independent growth. Colonies were scored after 6–7 days incubation in soft agar. A 21-oligomer of DNA generated from the coding region of the tyrosine kinase domain of ACK2, or a siRNA that has no complete sequence match in the GenBank database, was used as a control for the RhoGDI siRNA transfections. Both controls gave no phenotype when expressed in NIH 3T3 cells.

(A) The expression of endogenous RhoGDI in control NIH 3T3 cells and in Cdc42(F28L)-expressed cells that were transfected 48 hr with either the 21 bp DNA oligomer or RhoGDI siRNA was assessed with an anti-RhoGDI antibody from Transduction Laboratories.

(B) Cell morphology of NIH 3T3 cells expressing Cdc42(F28L) transfected with control dsRNA and RhoGDI siRNA.

(C) Top panel: colonies formed after 6–7 days of culturing Cdc42(F28L) + control 21 bp DNA oligomer-transfected cells in soft-agar medium. Bottom panel: colonies formed after 6–7 days of culturing Cdc42(F28L) + RhoGDI siRNA-transfected cells in soft-agar medium.

(D) Quantification of colony formation in soft agar for the experiments shown in Figure 6C.

tein need to be carefully regulated and balanced because the excessive expression of RhoGDI would be expected to inhibit cell growth through its ability to inhibit the dissociation of GDP from Cdc42 and other Rho-related GTP binding proteins and thus antagonize their activation. Moreover, it appears blocking Cdc42-RhoGDI interactions accentuates a cellular phenotype associated with the hyperactivation of Cdc42 (multinucleate cells). However, despite the potential role for RhoGDI in negatively regulating the activation of Rho

proteins, the ability of this regulatory protein to confer a positive contribution to signaling responses is not without precedent; the Rac-RhoGDI complex was initially identified to be essential for the stimulatory regulation of the NADPH oxidase system in neutrophils [26–28], implying that RhoGDI somehow properly presents Rac to the NADPH oxidase in membranes to enable optimal Rac-stimulated oxidase activity. For membrane transport systems involving the Rab proteins, it has also been established that RabGDI has an important role in shuttling the Rab proteins between intracellular membrane sites in order to ensure vectorial transport activity [29, 30]. Thus, it seems reasonable to envisage a similar type of role for RhoGDI in "presenting" Cdc42 to important membrane-associated target/effectors, particularly because we and others have shown that Cdc42 is present in and signals from both the plasma membrane and the Golgi [17]. We find that the GDI binding-defective Cdc42(F28L,R66A) mutant is exclusively membrane-associated and not detected in the cytosol. It also was recently shown that another GDI binding-defective mutant [Cdc42(R66E)], similar to the mutant described in this study, was not able to be translocated from the Golgi apparatus to the cytosol, as is typically observed with wild-type Cdc42 [21]. Moreover, we have observed what appears to be a shift in the membrane distribution of Cdc42(F28L,R66A) relative to Cdc42(F28L), such that the GDI binding-defective Cdc42 mutant accumulated in the Golgi and was less detectable along the cell surface compared to its Cdc42(F28L) counterpart.

Thus, when taken together with the known role for RabGDI in directing the proper membrane targeting of Rab GTP binding proteins, these observations raise the intriguing possibility that in a similar fashion, RhoGDI needs to shuttle Cdc42 between the Golgi and other cellular (membrane) sites in order to mediate Cdc42-signaling activities that impact cell growth and transformation. There are a number of attractive aspects to this model. One stems from the fact that RhoGDI can bind to and help maintain the activated state of Cdc42. It seems likely that it is the rather unusual nature of the Switch II domain of Cdc42 (and presumably other Rho-related proteins) that underlies the ability of RhoGDI to bind activated forms of Cdc42. Although the Switch II domain of Ras was originally identified because it is conformationally sensitive to guanine nucleotides (i.e., it undergoes a structural change upon the exchange of bound GDP for GTP [31]) and has been shown to be especially important for the recognition of upstream activators (GEFs), the corresponding region in Cdc42 appears to exhibit little change between different guanine nucleotide bound states [6, 7, 32]. We had earlier proposed that the minimal changes associated with Switch II may account for the similar affinities of the two guanine nucleotide bound states of Cdc42 for the GDI [6]. The ability of RhoGDI to recognize the activated state of Cdc42, combined with its ability to remove activated forms of Cdc42 from the membrane, may provide a means for coordinating Cdc42-dependent activation of target/effectors at different membrane locations. When one considers such a role for RhoGDI, it is important to realize that the persistent activation of Cdc42 (for example, as exhibited by GTPase-defective forms of the protein) can be toxic to NIH 3T3 cells. The interactions of RhoGDI with activated forms of Cdc42, which stabilize the active state by blocking GTP hydrolysis, must therefore be of a finite duration in cells if the GDI is to have a positive impact on Cdc42-mediated growth and transformation. However, this would be consistent with the idea that RhoGDI shuttles activated Cdc42 to specific membrane sites, disengages, and thus allows Cdc42 to bind to target/effectors and ultimately to GAPs. By cycling between different membrane locations and directing the movement of Cdc42 molecules to distinct cellular sites, a single GDI molecule might then be capable of mediating the proper localization of multiple Cdc42 molecules. Finally, perhaps the most attractive feature of this general model stems from the earlier finding that Cdc42-γ-coatomer interactions are essential for the transforming signal [17]. This led to the conclusion that the participation of Cdc42 in some type of cellular transport activity was linked to its ability to transform cells. Thus, RhoGDI, by acting in a manner analogous to RabGDI, may play an important role in a transport function and in this way serve as an essential regulator of the transforming activity of Cdc42.

Experimental Procedures

Plasmid Construction and Mutagenesis

Cdc42 mutants Cdc42(R66A), Cdc42(F28L,R66A), Cdc42(R68A), and Cdc42(F28L,R68A) were prepared by PCR with the Rapid-site mutagenesis kit (Strategene). The pTet wild-type Cdc42 and pTet Cdc42(F28L) constructs were generated by subcloning of the cDNA fragment that encodes HA-tagged wild-type Cdc42 or the Cdc42(F28L) mutant into the Hind III and EcoR V sites of the pTet-Splice vector (Life Technologies). Other plasmids and mutants were made as described previously [13].

Cell Culture and Transfection

NIH 3T3 cells were cultured in DMEM plus 10% calf serum (CS) at 37°C with 5% CO_2. COS-7 cells were cultured in DMEM plus 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. For transient transfections, the cells were split at 2×10^5 in a 60 mm dish, 18 hr before transfection. The transfections were performed with the Superfect transfection kit (Qiagen) according to the manufacturer's protocol. We prepared cell lysates 48 hr after transfection by lysing the cells with a buffer that contains 40 mM HEPES (pH 7.4), 100 mM NaCl, 1% Triton X-100, 25 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 mM EDTA, and 10 μg/ml each of leupeptin and aprotinin.

Tetracycline-Inducible Cell Lines

The tetracycline-inducible cell lines were generated by cotransfection of pTet constructs with the activator plasmid (pTAK) and puromycin-resistant plasmid into NIH 3T3 cells. After transfection, the cells were cultured in DMEM plus 10% CS and 1 μg/ml tetracycline. The selection of puromycin-resistant cell colonies was carried out 48 hr after transfection by addition of 5 μg/ml puromycin in the culture medium. The cell colonies resistant to puromycin were selected and subcultured in DMEM plus 10% CS, 1 μg/ml tetracycline and 5 μg/ml puromycin. The expression of proteins was induced by removal of tetracycline from the culture medium for 18–48 hr.

Expression and Purification of GST Fusion Proteins and GST Fusion Protein Precipitation Assays

Expression and purification of GST fusion proteins were performed as described previously [13, 17]. For binding assays using GST fusion proteins, the immobilized GST fusion proteins (40 μg) on glutathione-agarose beads were incubated with mammalian cell lysates for 2 hr at 4°C with rotation, then the beads were washed (3×) with bacterial lysis buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA. The proteins

precipitated from the lysates were visualized by Western blot analysis.

The JNK assays and soft-agar colony formation were performed as described previously [13, 17].

Inhibition of Expression of Endogenous RhoGDI by Transfection with RhoGDI siRNA

The 21-nucleotide siRNA sequence corresponding to the coding region between nucleotides 96 and 117 of the mouse RhoGDI was chemically synthesized (Dharmacon). Transfection of the siRNA (final concentration: 40 nM) for targeting RhoGDI was carried out with LipofectAmine (BRL). Transfection efficiencies were 70%–90%, as determined by Western blot analysis. Control oligomers represented either a 21-oligomer of DNA generated from the coding region of the tyrosine kinase domain of ACK2 or a siRNA that lacks a complete sequence match in the GenBank database (with the closest match being a two base mismatch to the bovine ACK2 sequence).

Immunofluorescent Staining

The lipofectamine transfection kit (Invitrogene) was used for transiently transfecting COS-7 cells with pEGFP-Cdc42 mutants. Cells were plated on chamber slides (Falcon) 24 hr after transfection. After subculture for 24 hr, the cells were fixed and then immunostained [13, 17]. The GFP fluorescence of the cells was visualized with a Leica DM1RE2 inverted confocal microscope, and the images were captured and analyzed with Leica confocal Software. Immunofluorescence experiments were performed on NIH 3T3 cells stably expressing different Cdc42 constructs as previously described [33].

Membrane Fractionation Analysis

NIH 3T3 cells stably expressing HA-tagged Cdc42(F28L) and HA-Cdc42(F28L,R66A) were washed with ice-cold PBS and collected via scrapping in ice-cold HYPO buffer (10 mM Tris [pH 7.4], 1 mM $MgCl_2$, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). The cells were homogenized with an eppendorf tube homogenizer, and the homogenates were fractionated by centrifugation. Nuclei and unlysed cells were separated from the remainder of the homogenate by centrifugation at 2000 $\times$ *g* for 5 min at 4°C. The cytosol and microsomal membrane fractions were separated by high-speed centrifugation (100,000 $\times$ *g* for 30 min at 4°C). The pellets were resuspended in SDS-PAGE sample buffer, and the Cdc42 proteins were detected by Western blot with an anti-HA antibody.

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