# Influencing Cellular Transformation by Modulating the Rates of GTP Hydrolysis by Cdc42[†]

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ABSTRACT: The small GTPase Cdc42 has been implicated in a number of cellular responses ranging from the regulation of the actin cytoskeletal architecture to intracellular trafficking and cell cycle progression. Cdc42 mutants that constitutively exchange GDP for GTP but still hydrolyze GTP (called 'fast-cycling' mutants) promote cellular transformation, whereas Cdc42 mutants that are unable to hydrolyze GTP and are irreversibly trapped in the GTP-bound state often inhibit cell growth. In this work, we have set out to further establish that Cdc42 needs to cycle between its 'on' and 'off' states to stimulate cell growth, by examining the consequences of manipulating its GTP-binding/GTP hydrolytic cycle in two different ways. One approach was to examine whether substitutions that act in a manner opposite to the 'fast cyclers', and extend the lifetime of the activated GTP-bound state by slowing the GTP hydrolytic reaction (i.e., 'slow-cycling' mutations), positively influence cell growth. Indeed we show that one such slow-cycling mutant, Cdc42[Y32A], which is insensitive to Cdc42GAP but still exhibits a measurable intrinsic GTP hydrolytic activity, gives rise to increased levels of activated Cdc42 in NIH 3T3 cells. We go on to show that the Y32A mutant stimulates the actin cytoskeletal changes that lead to filopodia formation, confer growth advantages to fibroblasts under low serum conditions, and enable cells to grow to high densities when exposed to normal levels of serum. The second approach was to determine whether the transforming activity of the fast-cycling Cdc42[F28L] mutant can be reversed by compensating for its accelerated nucleotide exchange reaction through the expression of the GTPase-activating protein (Cdc42GAP) and the ensuing stimulation of GTP hydrolytic activity. We showed that expression of the limit functional domain of Cdc42GAP inhibited Cdc42[F28L]-induced transformation, as well as selectively reversed the transformed phenotypes caused by the hyperactivation of wild-type Cdc42 in cells expressing the oncogenic version of Dbl (for Diffuse B cell lymphoma), a guanine nucleotide exchange factor for Cdc42 and the related Rac and Rho GTPases. Overall, the results reported here establish the requirement for Cdc42 to cycle between its signaling-on and -off states in order to positively influence cell growth and highlight how the Cdc42GAP can play an important role in regulating cell proliferation.

Small, monomeric GTPases[1] act as molecular switches in cellular signaling pathways, alternating between an active, GTP-bound state and an inactive, GDP-bound state. The ability of GTPases to properly bind and hydrolyze GTP is of utmost importance for the maintenance of normal cellular function. This is particularly true for cell growth control, as demonstrated by the role played by Ras in a variety of growth factor-coupled signaling pathways that stimulate cell-cycle progression. Mutations in Ras that block GTP hydrolysis and irreversibly lock it in the 'on' (GTP-bound) state can transform several cell types and have been found in more than 30% of all human tumors (*1*). A number of other Ras-related GTPases have been implicated in cell growth control, including members of the Rho subfamily. One in particular that has received a good deal of attention is Cdc42.

Like other members of the Rho subfamily, Cdc42 was originally suspected to be primarily responsible for the proper maintenance of the actin cytoskeleton and for processes related to cell polarity (*2*−*4*). However, it has become increasingly appreciated that Cdc42 participates in a variety of additional cellular activities including apoptosis (*5*), transcriptional activation (*6*−*10*), endocytosis (*11*), and intracellular trafficking (*12*−*17*). Given the wide scope of cellular responses influenced by Cdc42, it is essential that its GTP-binding/GTP-hydrolytic cycle is carefully regulated. In fact, a number of regulatory proteins have been identified for Cdc42 and related members of the Rho subfamily of GTPases. These include guanine nucleotide exchange factors (GEFs), which stimulate the dissociation of GDP and catalyze GDP−GTP exchange (*18*−*20*), and guanine nucleotide

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[1] Abbreviations: GTPase, guanosine 5′-3′-*O*-triphosphate (GTP) binding protein; GAP, GTPase-activating protein for Cdc42; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GDP, guanosine 3′-*O*-diphosphate; GppCp, guanosine-5′-(*β*,*γ*-methylene)triphosphate; HA, hemaglutinin; Mant-GDP, 2′-*O*-(*N*-methylanthraniloyl) guanosine 5′-diphosphate; PBD, p21-binding domain.

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dissociation inhibitors (GDIs), which significantly slow the rate of dissociation of GDP and consequently antagonize the actions of GEFs (*21*). A third family of regulatory proteins, designated as GTPase-activating proteins (GAPs), stimulate GTP hydrolysis and return the GTPase to its inactive state, thus, terminating its interactions with downstream effectors (*22*).

Various lines of evidence have shown that conditions resulting in the hyperactivation of Cdc42 lead to cellular transformation. One early example came from studies with the oncogenic Dbl protein, the founding member of a family of GEFs for Rho-related GTPases. Deletion of the amino-terminal 498 amino acids from proto-Dbl yields an oncogenic variant that is strongly transforming, as reflected by its ability to stimulate the formation of foci and support cell growth in low serum or in soft agar, as well as promote the loss of contact inhibition. The transforming activity of oncogenic Dbl has been directly attributed to its ability to excessively activate its Rho GTPase substrates, including Cdc42 (*23*, *24*).

However, perhaps the strongest piece of evidence linking the hyperactivation of Cdc42 to oncogenic transformation came from studies performed with the so-called 'fast-cycling' Cdc42 mutants which are capable of constitutive nucleotide exchange, but still exhibit full GTP hydrolytic activity (*23*, *25*). One example is the Cdc42[F28L] mutant which is unable to stabilize the binding of the guanine ring of GDP through the usual $\pi-\pi$ orbital interactions, and so the rate of GDP dissociation from this mutant is increased by at least 50-fold. Because of the significantly higher cellular levels of GTP, compared to GDP, the Cdc42[F28L] mutant is capable of exchanging GDP for GTP in the absence of a GEF, thus, significantly increasing the cellular pool of activated Cdc42. Consequently, NIH 3T3 cells expressing the Cdc42[F28L] mutant show the hallmarks of cellular transformation, including the ability to form colonies in soft agar as well as grow in low serum.

Whereas Cdc42[F28L] and other fast-cycling mutants exhibit transforming activity, dominant-active Cdc42 mutants that are incapable of GTP hydrolysis are often toxic to cells. This differs from the case for Ras where GTP hydrolysis-defective mutants show potent oncogenic capability (*1*) and therefore suggests that it is not sufficient for Cdc42 to reach the GTP-bound state to trigger signals essential for cell growth. Rather, it appears that Cdc42 needs to complete a full GTP-binding/GTP hydrolytic cycle in order to propagate such signals. To further establish that this indeed is the case, we have pursued two lines of approach. First, we set out to see whether other mutations that alter the rate of cycling between the GTP- and GDP-bound states will also give rise to oncogenic transformation. Thus far, we have exclusively examined 'fast-cycling' mutations that accelerate the conversion between the GDP- and GTP-bound states. However, one can also envisage transformation occurring with what might be referred to as 'slow-cycling' mutations that decrease (but do not irreversibly block) the return of GTP-bound Cdc42 back to the GDP-bound state. We felt that one interesting possibility was the Cdc42[Y32A] mutant. The structure of Cdc42 bound to Cdc42GAP (*26*) implicated Tyr32 of Cdc42 in GAP-stimulated GTP hydrolysis. The side chain of Tyr32 appears to stabilize the "arginine finger", a key catalytic residue in the GAP-catalyzed GTP hydrolytic reaction. Mutational and functional analyses of Cdc42 established that changing Tyr32 to an alanine yielded a Cdc42 molecule that exhibited a slower rate of intrinsic GTP hydrolysis compared to wild-type Cdc42, as well as conferred an insensitivity to Cdc42GAP (*27*). The fact that this GAP-insensitive, Cdc42 mutant was capable of hydrolyzing GTP, but at a decreased rate, suggested that it would allow Cdc42 to remain in the GTP-bound state for an extended (but not infinite) period of time and that its expression in NIH 3T3 cells might lead to increased levels of activated Cdc42. Thus, we examined the cellular consequences of expressing the GAP-insensitive Cdc42[Y32A] mutant in NIH 3T3 fibroblasts. We have also taken the reciprocal approach and examined whether the expression of the limit functional domain of Cdc42GAP, by accelerating the conversion of GTP-bound Cdc42 back to the GDP-bound state, might compensate for the enhanced rate of GDP−GTP exchange exhibited by the Cdc42[F28L] mutant and thereby inhibit its transforming activity. Moreover, we have taken this one step further and examined whether Cdc42GAP might also be capable of reversing the effects of oncogenic Dbl, and if so, possibly provide us with insights into the specific characteristics of Dbl-transformed cells that can be attributed to the hyperactivation of Cdc42.

## MATERIALS AND METHODS

*Molecular Constructs.* GST-Cdc42 was made using pGEX constructs for bacterial expression. The Cdc42[Y32A] mutant was generated by point mutating pGEX Cdc42 (wild-type) with the Stratagene Quick-change mutagenesis kit and confirmed by nucleotide sequencing. The construct was also subcloned into pJ4H and pcDNA vectors for stable and transient expression in mammalian cells. The mammalian expression vector for ACK2 was generously provided by Dr. Wannian Yang (Geisinger Research Institute, Danville, PA). The construct encoding the limit functional domain for the Cdc42GAP (residues 234−439), designated from here on simply as GAP, was initially cloned into the pGEX expression vector system (Novagen), and then subcloned into an HA-tagged pJ4H mammalian expression vector using the BamHI/EcoRI restriction enzyme sites.

*Assaying the Binding of Cdc42 to ACK2.* Expression of Myc-tagged ACK2 was achieved by transiently transfecting COS-7 cells using the Effectein transfection kit (Invitrogen). After 48 h, the cells were incubated with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 5 mM $MgCl_2$) for 20 min followed by a 10 min centrifugation (4 °C, 16 000*g*). ACK2-containing supernatants were then incubated with glutathione-agarose beads conjugated with either GST-Cdc42 (wild-type) or GST-Cdc42[Y32A], which were pre-loaded with either GDP or GMP-PCP (Sigma). Complex formation was achieved at 4 °C while mixing end-over-end for 1 h. Agarose-anchored complexes were pelleted (4 °C, 5000*g*) and washed in lysis buffer (2×). Complexes were resolved using 12% SDS−PAGE and then transferred onto PVDF. Myc-tagged ACK2 was detected using a monoclonal anti-Myc antibody (Covance).

*Measuring Guanine Nucleotide Exchange.* Mant-labeled deoxy-guanine nucleotides were generated, and nucleotide exchange assays were performed using previously described procedures (*28*, *29*). Cdc42 (1 $\mu$M) was first incubated in

HMN buffer (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 100 mM NaCl), and then either Mant-dGDP (1 mM) or Mant-dGDP, together with the Cdc42-specific GEF, IntersectinC (38 nM), was added to the reaction mixture. IntersectinC is a truncated version of the full-length Intersectin-L protein, which includes 3 of its 5 SH3 domains (designated SH3C, D, and E) as well as its Dbl- and Pleckstrin-homology domains (*30*). Changes in Mant fluorescence (excitation = 350 nm, emission = 440 nm), indicative of the exchange of GDP for the Mant-nucleotide on Cdc42, were measured in an SLM 8000c spectrofluorimeter. The data were fit to a single exponential using the Igor Wavemetrics software package.

*Cell Culture.* NIH 3T3 cells were cultured in a 37 °C incubator while maintaining a 5% $CO_2$, humid environment. Media consisted of Dulbecco's modified medium supplemented with 10% calf serum, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (Gibco). COS-7 cells were also cultured in a 37 °C incubator while maintaining a 5% $CO_2$, humid environment; however, their media consisted of Dulbecco's modified medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin.

*Immunofluorescence.* NIH 3T3 cells were transiently transfected with either pcDNA Cdc42[Q61L], Cdc42[Y32A], or wild-type Cdc42 using the Effectein transfection kit (Invitrogen). The following day, the transfected cells were trypsinized and counted, and then $2 \times 10^3$ cells were plated onto dual chamber cell culture slides. The cells were allowed to attach to the slides and then were incubated in serum-free media. The cells were fixed and permeabilized with 3.7% formaldehyde and 0.2% Triton X-100 in PBS, and then were incubated with PBS containing 5% BSA and monoclonal anti-hemaglutinin (HA) antibody for 1 h at room temperature. After thorough washing with PBS, cells were incubated with either a mixture of rhodamine-phalloidin and goat anti-mouse antibody conjugated to Oregon green (Molecular Probes), or a mixture of Oregon green-phalloidin and goat anti-mouse antibody conjugated to Texas Red (Molecular Probes) in PBS, for visualization with a fluorescent microscope (Olympus).

*Assays for the Cellular Activation of Cdc42.* NIH 3T3 cells expressing HA-tagged Cdc42 proteins were incubated in lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 5 mM $MgCl_2$) for 20 min followed by a 10 min centrifugation (4 °C, 16 000*g*). Supernatants were then incubated with glutathione-agarose beads conjugated with GST fused to the Cdc42-interaction domain of PAK (called PBD for p21-binding domain) and incubated at 4 °C while mixing end-over-end for 2 h. Agarose-anchored complexes were pelleted (4 °C, 5000*g*) and washed in lysis buffer (2×). Complexes were resolved using 12% SDS−PAGE and then transferred onto PVDF. HA-tagged Cdc42 was detected using a monoclonal anti-HA antibody (Covance).

*Stable Cell Lines.* Stable cell lines expressing HA-tagged Cdc42[Y32A] were generated by cotransfecting the pJ4H Cdc42[Y32A] construct and pcDNA3 into NIH 3T3 fibroblasts using Lipofectamine (Invitrogen), at a 20:1 ratio. Twenty-four hours post-transfection, the media was replaced with culturing media supplemented with G418 (Sigma). Individual colonies were selected, cultured, and screened for expression of HA-tagged Cdc42[Y32A] by Western blot analysis.

Stable cell lines coexpressing either HA-Cdc42[F28L] or oncogenic Dbl with HA-tagged GAP were generated using previously described procedures (*23*, *24*). The puromycin-resistance vector (Puro) was a gift from Dr. Jun-Lin Guan's laboratory (Cornell). The pJ4H GAP and Puro vectors were introduced into the Cdc42[F28L]- and oncogenic Dbl-expressing cell lines using Lipofectamine. Twenty-four hours post-transfection, the media was replaced with culturing media supplemented with 5 $\mu$g/mL puromycin (Sigma). Following colony formation, cells lines were transferred to individual plates and tested for expression by Western blotting with anti-HA antibody.

*Transformation Assays.* Three assays for cellular transformation were used: growth in low serum, contact inhibition/saturation density, and colony formation in soft agar. When assaying growth in low serum, $10^5$ cells of each indicated cell line were seeded in six-well plates using DMEM supplemented with 10% calf serum. Six hours later, the media was replaced with DMEM supplemented with 1% calf serum. The cells were trypsinized and counted on days 2, 4, and 6.

For the experiments assaying saturation density, $10^5$ cells from each cell line were seeded using DMEM supplemented with 10% calf serum. The cells were fed every 2 days until day 6 when they were trypsinized and counted.

Soft agar assays were performed by plating the cells ($3 \times 10^3$) in 1.5 mL of 0.3% agarose (Invitrogen) in DMEM supplemented with 10% calf serum. A 0.5% agarose underlayer was first poured in order to prevent attachment of cells to the tissue culture plate. Cells were fed 7 days after plating with a fresh layer of 0.3% agarose in DMEM supplemented with 10% calf serum. The colonies were counted after 14 days of growth.

*EGF-Stimulated ERK Phosphorylation.* To examine EGF-stimulated ERK phosphorylation in cells expressing Cdc42[F28L], in the presence or absence of HA-GAP, the cell lines were seeded in six-well plates, allowed to adhere, and then were serum-starved for 24 h. The cells were then treated with EGF (100 ng/mL) for varying periods of time up to 6 h, and then lysed in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 20 mM $\beta$-glycerol phosphate, 1 mM sodium vanadate, 10 $\mu$g/mL leupeptin, and 10 $\mu$g/mL aprotonin (Sigma). Total lysate protein was determined using the Bio-Rad protein assay, and then equivalent amounts of lysate (40 $\mu$g of protein) were resolved by SDS−PAGE (using 10% gels). The samples were transferred to PVDF and immunoblotted with an anti-phospho-ERK1/2 (p44/p42) antibody (Transduction). Densitometry was conducted on the immunoblots using an Amersham Pharmacia Biotech Image Scanner and Total lab version 1.00 software.

## RESULTS

*The Cdc42[Y32A] Mutant Is Activated by GEFs and Retains Target/Effector-Binding Activity.* Thus far, we have used Cdc42 mutants that exhibited an accelerated ability to exchange GDP for GTP ('fast-cycling' mutants) to examine the role of this GTPase in cell growth control (*23*−*25*). These mutants show strong transforming capabilities, presumably

by virtue of a significant increase in the cellular pool of the GTP-bound form of the protein. This raises the question of whether, instead of accelerating the 'forward' half of the GTP-binding/GTP hydrolytic cycle (i.e., GDP−GTP exchange), we can achieve the same outcome by slowing the 'back' half of the cycle (i.e., the hydrolysis of GTP to GDP). We set out to address this question using a Cdc42 mutant, Cdc42[Y32A], that we had previously found to be unresponsive to Cdc42GAP, while its intrinsic GTP hydrolytic activity was decreased by about 3-fold compared to that of wild-type Cdc42 (*27*).

Tyrosine 32 is located within the Switch I domain of Cdc42. This region not only contains residues that are important for the GTP hydrolytic reaction, but also represents the binding site for target proteins and GEFs. Thus, we wanted to be certain that substituting an alanine for tyrosine at position 32 in Switch I did not compromise the ability of Cdc42 to undergo GEF-mediated GDP−GTP exchange, or upon activation, to engage target/effectors. We first examined the ability of the Cdc42[Y32A] mutant to respond to a form of Intersectin-L, a specific activator of Cdc42 (*30*, *31*), that includes its functional GEF (i.e., Dbl-homology) domain. Guanine nucleotide exchange activity was assayed in real-time using Mant-dGDP, as this GDP derivative undergoes a significant enhancement in fluorescence upon binding to GTPases such as Ras and Cdc42 (*29*, *32*, *33*). The results presented in Figure 1A demonstrate that the fluorescence changes accompanying the binding of Mant-dGDP to the Cdc42[Y32A] mutant, due to the GEF-stimulated exchange of the fluorescent nucleotide for GDP at the nucleotide-binding site, were indistinguishable from those observed with wild-type Cdc42. Figure 1B verifies that the rates of nucleotide exchange for the mutant and wild-type Cdc42 protein were also essentially identical.

The functional similarities between Cdc42[Y32A] and wild-type Cdc42 also extended to target/effector-binding interactions. It has been well-documented that Cdc42-effectors containing a CRIB (for Cdc42/Rac-interactive-binding) motif (*34*) bind to Cdc42 through the Switch I region. The Cdc42[Y32A] mutant is able to bind to CRIB-motif-containing targets in a manner similar to the wild-type GTPase. One example is the nonreceptor tyrosine kinase ACK2, a member of the ACK-family of specific targets for Cdc42 that was originally isolated from bovine brain (*35*). Figure 1C shows the results of a pull-down experiment where equivalent amounts of GST-Cdc42[Y32A] and GST-Cdc42 (wild-type), either in the GDP-bound state or loaded with the GTP analogue GMP-PCP, were incubated with lysates from COS-7 cells expressing the Myc-tagged ACK2 protein and then precipitated. It is clear that the Y32A mutation does not impede the ability of Cdc42 to bind to ACK2 in a GTP-dependent manner.

*Cdc42[Y32A] Is Active in Cells.* We next examined whether the Cdc42[Y32A] mutant, when expressed in cells, showed the characteristics of an activated GTPase. This was assessed by assaying the ability of Cdc42[Y32A] to bind GST-PBD which contains the limit-binding domain from the Cdc42/Rac-effector PAK. Only the GTP-bound form of Cdc42 is able to undergo a high-affinity interaction with the PBD, and so this assay provides a read-out for the relative levels of the activated GTPase in cells (*36*). Extracts from cultured NIH 3T3 cells stably expressing HA-tagged Cdc42-

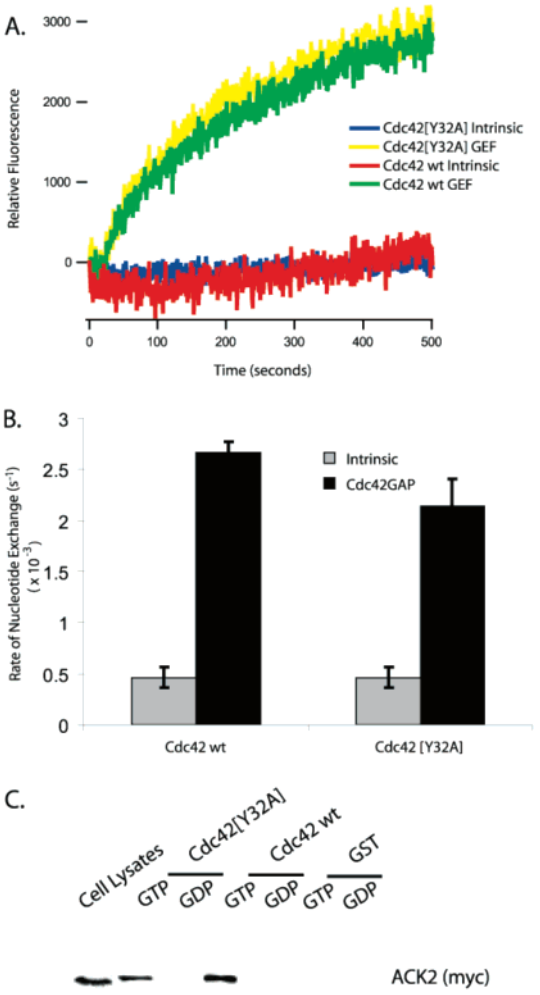


FIGURE 1: Biochemical characterization of the GAP-insensitive Cdc42[Y32A] mutant. (A and B) To ensure that the Switch I mutant (Cdc42[Y32A]) was still capable of undergoing GEF-mediated nucleotide exchange, the binding of Mant-dGDP to wild-type Cdc42 or the Cdc42[Y32A] mutant, in the presence and absence of the Intersectin-L construct, IntersectinC (see Materials and Methods), was assayed. Nucleotide exchange was measured by monitoring the enhancement of Mant fluorescence. The data are representative of three independent experiments. (C) To determine if the Switch I domain of the Cdc42[Y32A] mutant was still capable of interacting with effectors, the ability of this mutant to bind to ACK2 was tested. Equivalent amounts of GST-Cdc42 (wild-type) and GST-Cdc42-[Y32A] were preloaded with GMP-PCP or GDP and then incubated in cell lysates transiently expressing Myc-tagged ACK2. Precipitated protein complexes were resolved by 12% SDS−PAGE, and Myc-tagged ACK2 was detected with an anti-Myc antibody.

[Y32A] were incubated with GST-PBD and then precipitated, with the associated Cdc42 being detected by Western blot analysis using an anti-HA antibody. Figure 2A (top panel) shows that in cells there is a significantly greater percentage of the Cdc42[Y32A] mutant in the GTP-bound state, compared to wild-type HA-tagged Cdc42, with the extent of activation for the Y32A mutant approaching that obtained with comparable levels of the GTPase-defective HA-Cdc42-[Q61L] mutant.

We next asked whether the Cdc42[Y32A] mutant was able to send downstream signals in cells that mirrored those seen with other activated forms of Cdc42. Perhaps the best known cellular read-out for Cdc42 is the formation of filopodia or microspikes (*37*, *38*). It has been previously demonstrated

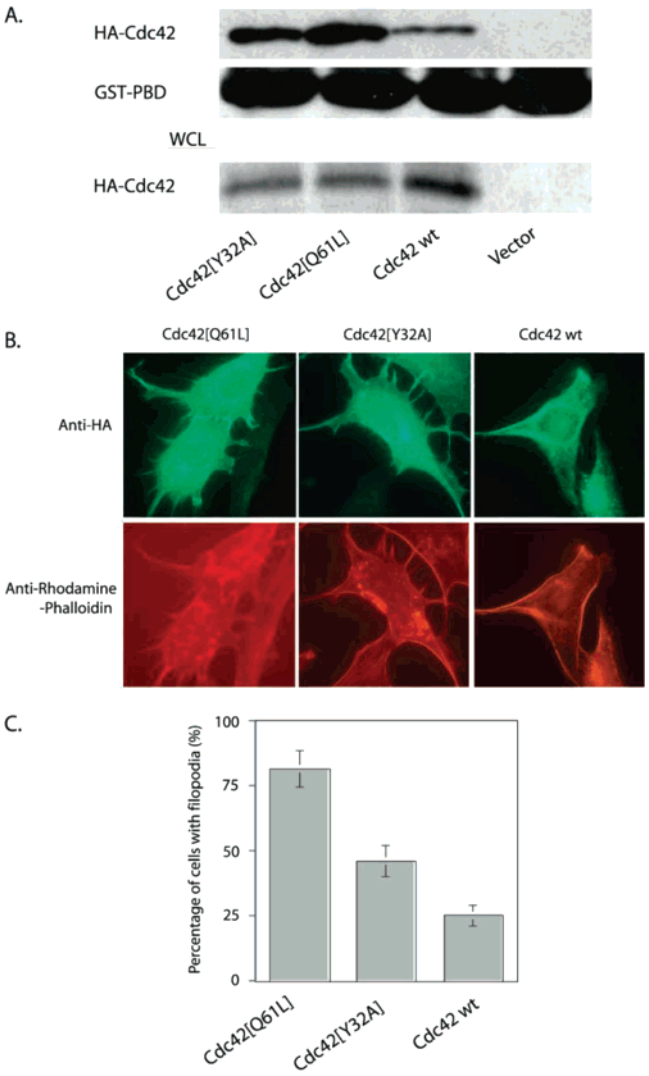


FIGURE 2: The Cdc42[Y32A] mutant is activated in cells. (A) A GST-fusion protein containing the p21-binding domain of PAK (PBD) was incubated with cell lysates expressing HA-tagged Cdc42[Q61L], HA-Cdc42[Y32A], or HA-Cdc42(wild-type). Complexes were resolved using SDS−PAGE, and the HA-Cdc42 constructs were detected using an anti-HA antibody (top panel). The middle panel shows the relative amounts of GST-PBD used in the pull-down experiments, and the bottom panel compares the relative expression levels of the HA-Cdc42 constructs in whole cell lysates. (B) NIH 3T3 cells were transiently transfected with either mammalian constructs encoding HA-tagged Cdc42[Q61L], Cdc42-[Y32A], or wild-type Cdc42, fixed, and stained with anti-HA to detect cells expressing the various Cdc42 constructs, and with rhodamine-phalloidin to stain the actin cytoskeleton. (C) Quantification of cells bearing filopodia when overexpressing the different forms of HA-tagged Cdc42 shown in panel B.

that the transient expression of either the constitutively active Cdc42[F28L] mutant or the GTPase-defective Cdc42[Q61L] protein enhances the formation of filopodia in fibroblasts (*23*, *24*). An example is shown in Figure 2B for the HA-tagged Cdc42[Q61L] mutant, where following its transient expression in NIH 3T3 cells and then staining the cells with phalloidin, microspikes were clearly visible. The HA-Cdc42-[Y32A] mutant was also able to increase microspike formation, compared to what was observed for cells expressing wild-type HA-Cdc42 (Figure 2B,C). These results were consistent with the Cdc42[Y32A] mutant exhibiting an extended lifetime in the GTP-bound, activated state.

*The Cdc42[Y32A] Mutant Exhibits Transforming Capability.* We next asked whether the stable expression of the Cdc42[Y32A] mutant, like the constitutively active Cdc42-[F28L], can change the growth properties of NIH 3T3 cells and cause them to undergo transformation. To address this question, three independent clones that stably expressed HA-tagged Cdc42[Y32A] were chosen (Figure 3A).

One hallmark of cells that have undergone transformation is their ability to survive and grow under stressful conditions, for example, when the levels of serum and growth factors are low. The data presented in Figure 3B shows that, as previously reported (*23*, *24*), cells that highly expressed the Cdc42[F28L] mutant grew well under low-serum conditions (i.e., 1% FBS), whereas control NIH 3T3 cells were not able to grow and underwent cell death over a 2−6 day period. While we have not been able to generate stable cell lines that expressed HA-Cdc42[Y32A] to the same extent as Cdc42[F28L], we nonetheless found that cells expressing the Y32A mutant remained viable through 6 days, under low-serum conditions. Figure 3C shows a comparison of the morphology of the control cells versus cells expressing HA-Cdc42[F28L] and HA-Cdc42[Y32A], and again, it is clear that there were striking differences in the ability of cells expressing the Cdc42[Y32A] mutant to survive in low serum, compared to control fibroblasts.

Another indicator that cells have undergone transformation is their loss of contact inhibition. When normal NIH 3T3 cells are cultured in 10% FBS, they grow until a monolayer has formed and then undergo contact inhibition. However, when transformed cells reach 100% confluence, they continue to grow, forming multiple layers and displaying a higher cell density. Figure 3D compares the ability of vector-control cells and cells stably expressing HA-Cdc42[F28L], as well as different cell lines stably expressing HA-Cdc42-[Y32A], to grow to high density. NIH 3T3 cells stably expressing the Cdc42[Y32A] mutant were able to grow to higher saturation densities than control cells and approached densities comparable to cells expressing Cdc42[F28L].

*Can Cdc42GAP Negatively Regulate the Transforming Activity of Cdc42[F28L]?* The results presented in the preceding section showed that the Y32A mutation, by slowing the rate at which Cdc42 returns from the GTP-bound (active) state to the GDP-bound (inactive) state, conferred transforming properties to this GTPase. We then wanted to see whether we could modulate the transforming activity of the fast-cycling Cdc42[F28L] mutant by accelerating its ability to undergo GTP hydrolysis.

The limit functional domain of Cdc42GAP, referred to simply as GAP, encompasses the C-terminal half of the 50 kDa protein, beginning at residue 234 (*26*, *39*). We previously demonstrated that the Cdc42[F28L] mutant was fully responsive to GAP, yielding the same rate of GTP hydrolysis as that measured for the GAP-stimulated activity of wild-type Cdc42 (*23*). Thus, two cell-lines stably expressing HA-Cdc42[F28L] and different levels of HA-tagged GAP were generated (designated as clones 1 and 2 in Figure 4A). A control cell line expressing HA-GAP alone (i.e., in the absence of Cdc42[F28L]) was used to establish whether any toxic side effects occurred because of excessive stimulation of the GTP hydrolytic activity by Cdc42, whereas cells expressing only HA-Cdc42[F28L] were used as a positive control for transformation.

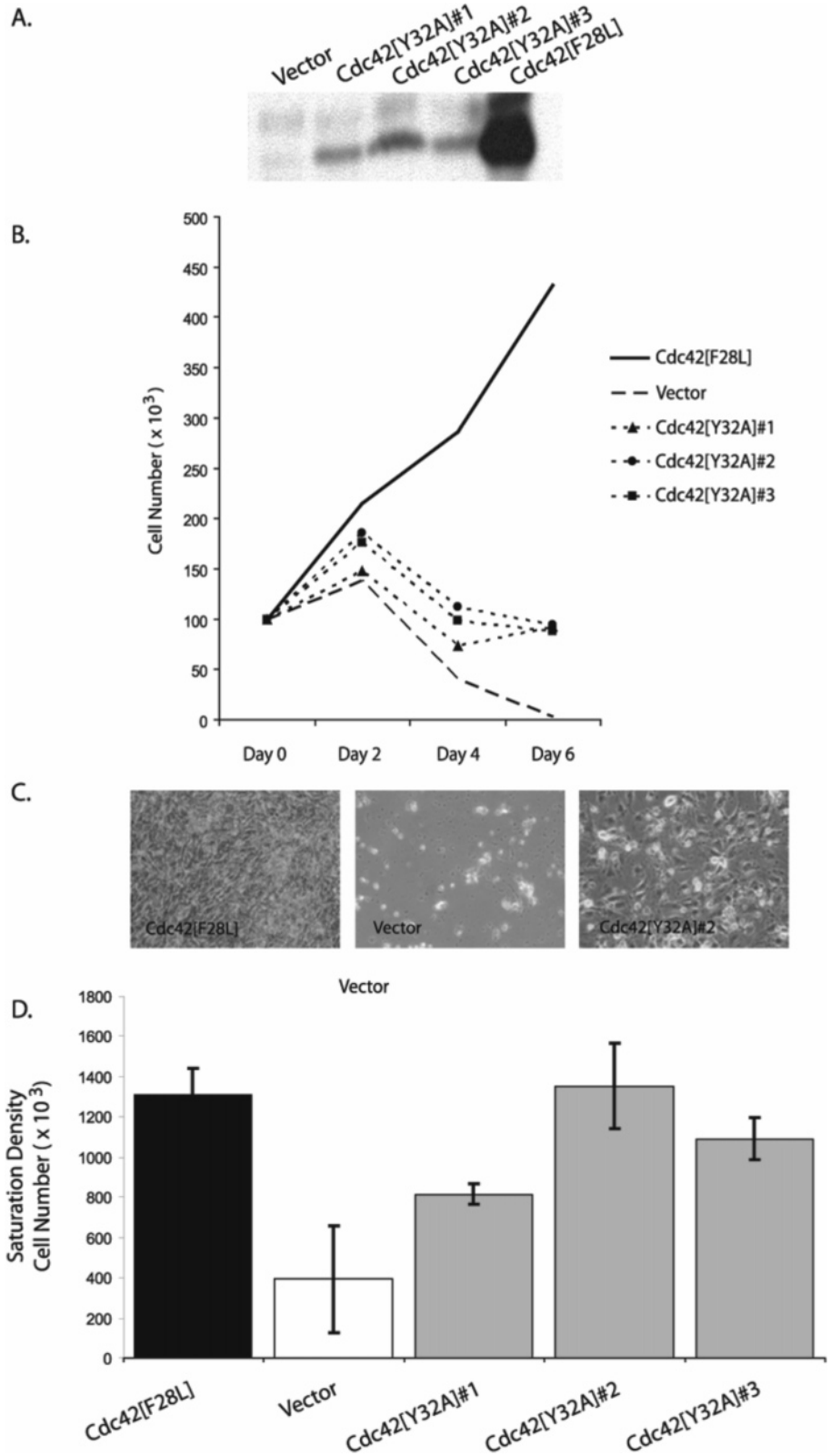


FIGURE 3: The Cdc42[Y32A] mutant and cellular transformation. (A) Western blot analysis of NIH 3T3 cells stably expressing Cdc42-[Y32A] or Cdc42[F28L]. The expression levels of the Cdc42 constructs were detected using an anti-HA antibody. The numbers represent specific clonal cell lines expressing HA-Cdc42[Y32A]. (B) NIH 3T3 cells stably expressing the various Cdc42 constructs were tested for their ability to grow in low-serum conditions. Stable cell lines expressing HA-Cdc42[F28L], HA-Cdc42[Y32A], or vector alone were seeded at $10^5$ cells per well and fed every 2 days with DMEM supplemented with 1% FBS. On the indicated days, the samples were trypsinized and counted in order to monitor their growth. The numbers represent different cell lines stably expressing Cdc42[Y32A], at the relative levels shown in Figure 2A. The data are representative of three to four independent experiments. (C) After 6 days of growth, pictures were taken using an Olympus digital camera and an Olympus light microscope. (D) Cell lines were tested for their ability to grow to high-cell densities. Stable cell lines expressing HA-Cdc42[F28L], HA-Cdc42[Y32A], or vector alone were seeded at $10^5$ cells per well and fed every 2 days with DMEM supplemented with 10% FBS. After 6 days of growth, cells were trypsinized and counted. The data are representative of three to four independent experiments.

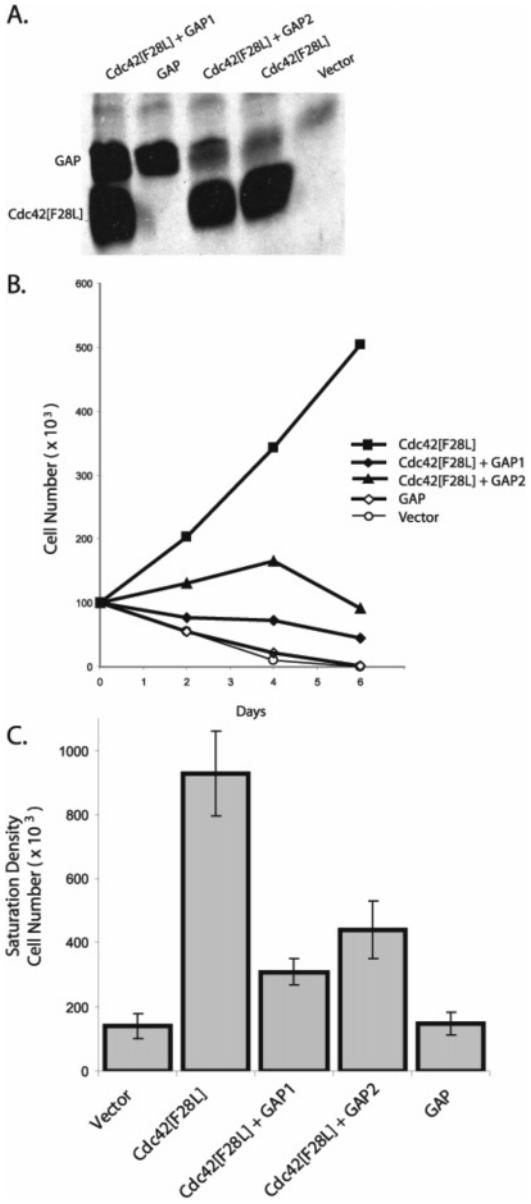


FIGURE 4: Examining the effects of GAP on Cdc42[F28L]-stimulated cell growth. (A) Western blot analysis of NIH 3T3 cells stably expressing HA-tagged Cdc42[F28L], alone, or in combination with HA-GAP. The effects of GAP on Cdc42[F28L]-induced cellular transformation were examined using two different cell lines expressing HA-tagged Cdc42[F28L] and different levels of HA-tagged GAP (denoted by the numbers 1 and 2), as well as cell lines expressing either HA-Cdc42[F28L] or GAP, alone, and a vector-control. The expression levels of the various constructs were detected with an anti-HA antibody. The upper band detected in the lane for cells expressing HA-Cdc42[F28L], as well as the band seen in the lane for vector-control cells, represents a nonspecific reaction and is not consistently observed. (B) The cell lines were tested for their ability to grow in low-serum conditions. Stable cell lines expressing HA-Cdc42[F28L] (■), HA-Cdc42[F28L] with a higher level of GAP (#1) (♦), HA-Cdc42[F28L] plus a lower level of GAP (#2) (▲), GAP alone (◊), and the vector-control (○) were grown in 1% FBS, as described in Figure 3B. On the indicated days, the samples were trypsinized and counted. The data are representative of three independent experiments. (C) NIH 3T3 cell lines were tested for their ability to grow to high-cell densities. Stable cell lines expressing HA-Cdc42[F28L], HA-Cdc42[F28L] with a higher level of GAP (#1), HA-Cdc42[F28L] plus a lower level of GAP (#2), GAP alone, and the vector-control were grown in 10% FBS as described in Figure 3D. After 6 days of growth, cells were trypsinized and counted. The data are representative of three independent experiments.

The ability of cells to grow in low serum was tested to determine if the coexpression of HA-GAP with HA-Cdc42-[F28L] affected the transforming potency of this constitutively active Cdc42 mutant. The results in Figure 4B demonstrate that the presence of GAP markedly decreased the ability of Cdc42[F28L] to stimulate cell growth under low-serum conditions, with the greater degree of growth inhibition being observed in fibroblasts that expressed the higher level of GAP. NIH 3T3 cells stably expressing GAP alone behaved similarly to cells expressing the control vector, undergoing cell death by day 6 of the experiment.

The two cell lines coexpressing Cdc42[F28L] and GAP were also compared with Cdc42[F28L]-transformed cells, as well as with vector-control cells, for their ability to grow to a higher density in media supplemented with 10% serum. After 6 days of growth, the cells were trypsinized and counted. The data presented in Figure 4C shows that GAP reversed the loss of contact inhibition exhibited by Cdc42-[F28L]-transformed NIH 3T3 cells. Both cell lines expressing GAP together with Cdc42[F28L] grew to lower densities compared to cells expressing Cdc42[F28L] alone, with cells expressing the higher level of GAP exhibiting less density compared to cells expressing lower levels of GAP. Again, we found that expression of GAP alone did not inhibit the growth or change the normal behavior of the cells.

Another characteristic of neoplastic cell growth is the ability of transformed cells to grow without adhering to a substrate. Transformed cells are able to grow and form colonies within an agarose matrix, whereas nontransformed cells will not grow but instead appear as single cell entities within the matrix even after a 2-week period of time. Cell lines expressing different combinations of HA-Cdc42[F28L] and HA-GAP were tested for their ability to form colonies in soft agar. As reported earlier (*23*), NIH 3T3 cells that stably expressed Cdc42[F28L] were able to form colonies with an incidence of ~30%, while vector-control cells did not form colonies under these conditions (Figure 5A). In agreement with the low serum and saturation density data, cells that stably expressed HA-GAP alone behaved like control cells and did not grow in soft agar. Coexpression of HA-GAP with HA-Cdc42[F28L] reduced the incidence of colony formation by nearly 50% (Figure 5A). In addition, the colonies that did form from the cell lines expressing both Cdc42[F28L] and GAP appeared much smaller than those from cells expressing Cdc42[F28L] alone (Figure 5B).

*Effects of GAP on the Interplay between Cdc42 and EGF-Coupled Signaling Activities*. We previously showed that an important part of the transforming capability of the Cdc42-[F28L] mutant was its ability to inhibit the Cbl-catalyzed ubiquitination of EGFRs, through the formation of a ternary complex between GTP-bound Cdc42[F28L], Cool-1 (for Cloned-out of library-1)/$\beta$-Pix (for PAK-interactive exchange factor), and Cbl which effectively sequestered the E3 ubiquitin ligase Cbl away from the EGFR (*40*). Consequently, the amplitude and lifetime of EGF-coupled signaling through Ras to ERK1/2 was significantly increased (e.g., compare the intensity of the phospho-ERK1/2 doublet as a function of time in the top two panels on the left in Figure 6A). Figure 6 also shows the effects of expressing GAP together with Cdc42[F28L] on EGF-stimulated ERK activation. In cells expressing both GAP and Cdc42[F28L], there was a consistent reduction in the relative strength of the upper

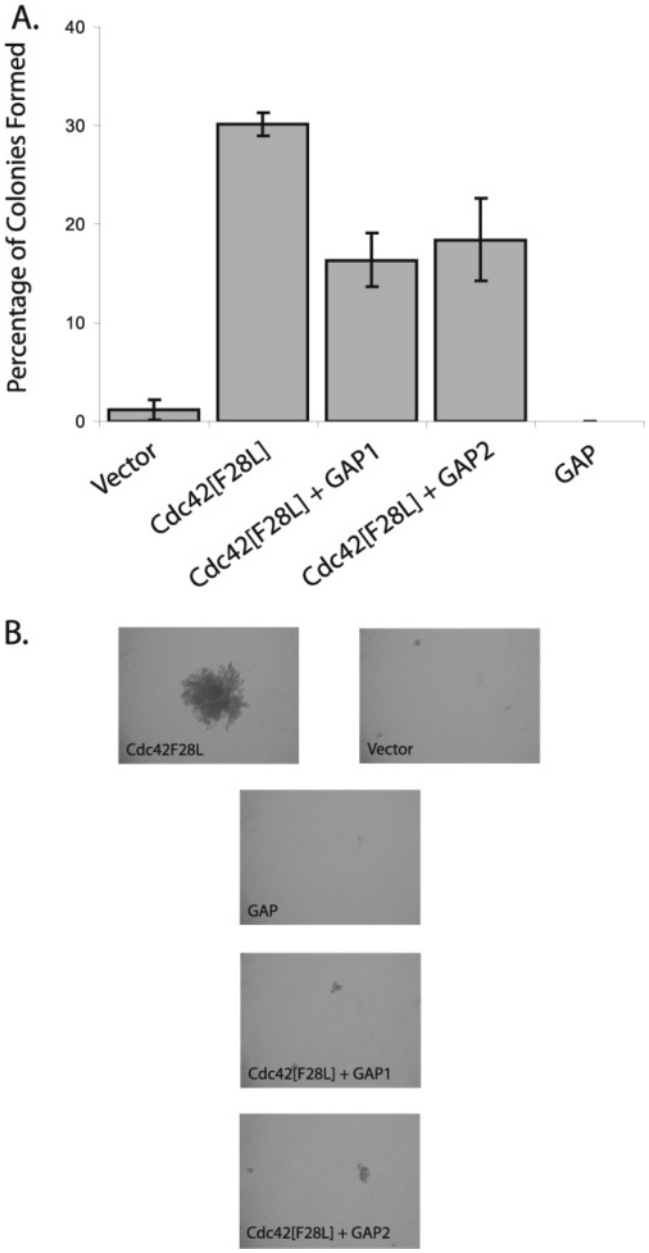


FIGURE 5: Effects of GAP on Cdc42[F28L]-stimulated colony formation in soft agar. (A) Stable cell lines expressing HA-tagged Cdc42[F28L], HA-Cdc42[F28L] with a higher level of GAP (Clone 1), HA-Cdc42[F28L] plus a lower level of GAP (Clone 2), GAP alone, and vector alone were seeded at $3 \times 10^3$ cells per well and fed every 7 days with DMEM supplemented with 1% FBS and 0.3% agarose. After 14 days of growth, the samples were counted in order to monitor the number of colonies formed. The data are representative of three independent experiments. (B) Photomicrographs of the colony formation of cells in soft agar, as described in panel A.

band of the doublet representing phospho-ERK1/2 (e.g., compare the two lower panels with the second panel from the top in Figure 6A), as well as a diminished lifetime for ERK1/2 activation (Figure 6B).

*Coexpression of Dbl and GAP in NIH 3T3 Cells.* The data from Figures 4 and 5 indicated that overexpressing GAP inhibited the transforming activity of Cdc42[F28L]. We then wanted to see whether GAP expression might also affect cellular transformation by the oncogenic Dbl protein, an upstream activator of Cdc42. Cell lines stably expressing oncogenic Dbl, alone, or together with HA-GAP were isolated and characterized by Western blot analysis (Figure 7A). These cells were then examined for their abilities to grow in low serum (Figure 7B), as well as to grow to high density (Figure 7C) and form colonies in soft agar (Figure 8).

We found that when assaying cell growth, either under conditions of low serum or under normal serum conditions (but to high density), the expression of HA-GAP exerted little or no change on the growth-promoting properties of oncogenic Dbl (Figure 7B,C). However, when comparing the abilities of the different cell lines to form colonies in soft agar, expression of HA-GAP caused obvious effects (Figure 8). NIH 3T3 cells stably expressing oncogenic Dbl exhibited colony formation with a frequency of ~35%, whereas cells expressing oncogenic Dbl together with GAP showed a significant reduction in the incidence of colony growth (i.e., to a frequency of ~15%, Figure 8A). The reduced incidence of Dbl-induced colony formation in the presence of GAP was also accompanied by markedly smaller colonies (Figure 8B). Overall, these results confirm earlier suggestions that the ability of oncogenic Dbl to promote growth in soft agar is primarily mediated through the activation of Cdc42 (*24*) and now shows that this cellular phenotype can be selectively inhibited by attenuating Cdc42 activation via its GAP.

*The GAP[R305A] Mutant Is Ineffective in Blocking Cdc42-[F28L]- or Dbl-Induced Transformation.* We wanted to be certain that the effects of GAP on the abilities of the constitutively active Cdc42[F28L] mutant or oncogenic Dbl to transform NIH 3T3 cells were indeed an outcome of accelerated GTP hydrolysis by Cdc42, rather than due to the GAP simply binding to activated Cdc42 and competitively inhibiting its interactions with essential target/effectors. Thus, we examined whether the expression of a GAP mutant (GAP[R305A]) in which a highly conserved arginine residue (often referred to as the arginine finger) was changed to an alanine, such that its ability to stimulate GTP hydrolysis was significantly compromised (*26*, *27*, *39*), had any effect on either Cdc42[F28L]- or Dbl-induced transformation. Figure 9 shows that the HA-tagged GAP[R305A] mutant had no effect on the ability of either HA-tagged Cdc42[F28L] or oncogenic Dbl to stimulate the growth of NIH 3T3 cells in low serum, or to high density at normal serum levels, and was also ineffective at blocking colony formation in soft agar. These results verify that the inhibitory effects of GAP on the transforming activity of constitutively active Cdc42 and Dbl were in fact an outcome of its ability to accelerate the GTP hydrolytic activity of Cdc42.

## DISCUSSION

GTPases function as molecular switches in mediating a host of cellular signaling activities. This function relies heavily on the tight regulation of their GTP-binding/GTP hydrolytic cycles. The rate at which a GTPase cycles between the signaling-competent, GTP-bound state and the signaling-defective, GDP-bound state sets the timing and overall lifetime of the particular cellular response that is being mediated. This is especially true in the case of the Ras-related protein, Cdc42, as its ability to cycle with the proper frequency between its GTP-bound and GDP-bound state is essential for ensuring normal cell growth and survival. Thus,

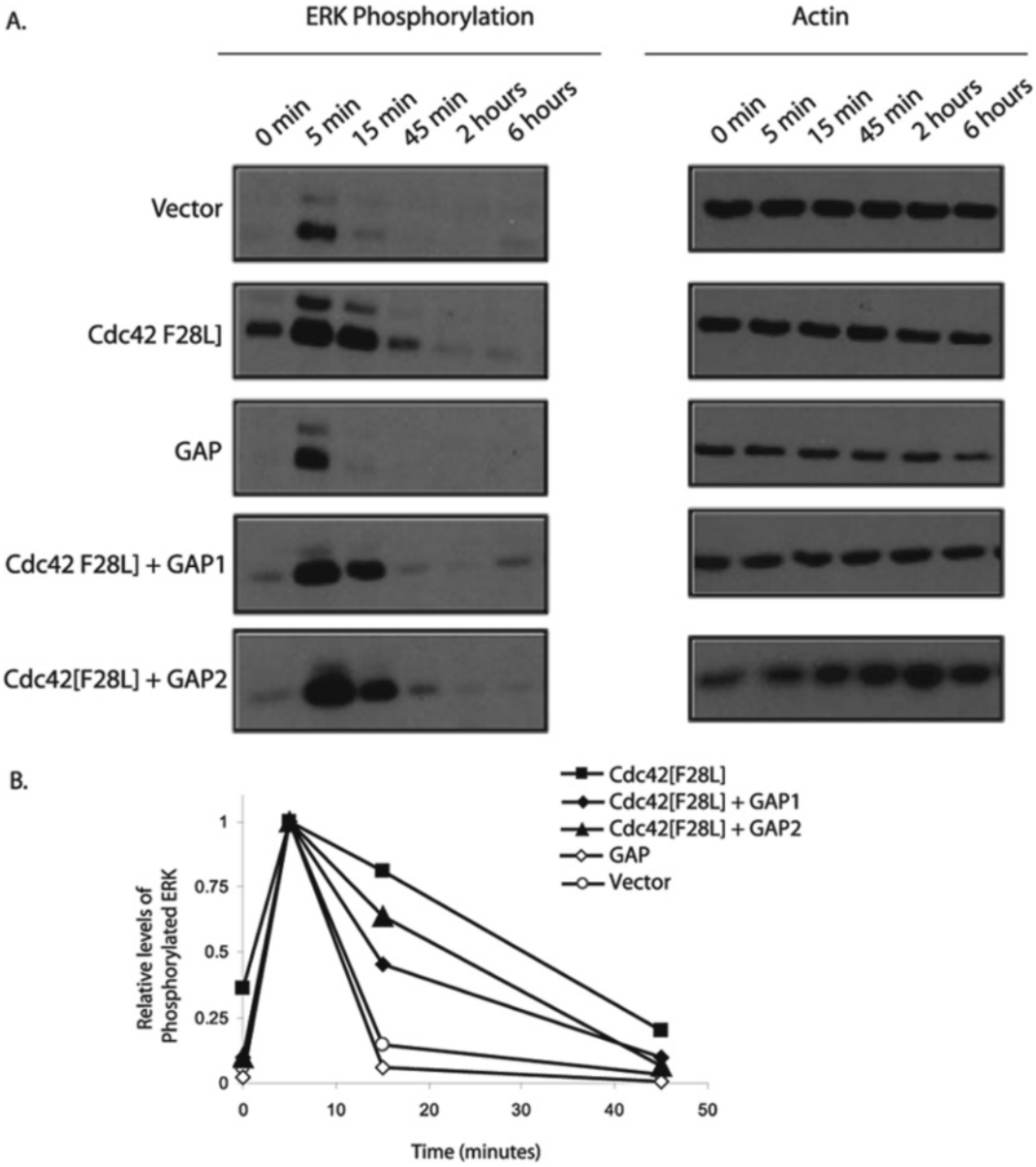


FIGURE 6: GAP affects EGF-coupled signaling to ERK1/2 in Cdc42[F28L]-transformed cells. (A) Time course for the EGF-dependent activation of ERK1/2 in cells expressing HA-tagged Cdc42[F28L], HA-Cdc42[F28L] with different levels of GAP (higher expression is Clone 1 and lower expression is Clone 2), or vector-control. Anti-phospho-p44/42 ERK was used to detect activated ERK1/2 (left panels). Loading controls using actin are shown in the right panels. The results presented here are representative of three experiments. (B) Relative levels of ERK1/2 phosphorylation for the different conditions shown in panel A were determined by densitometry as described in Materials and Methods.

mutations that prevent Cdc42 from undergoing activation through GDP−GTP exchange, or from being deactivated as an outcome of GTP hydrolysis, severely inhibit the growth of fibroblasts. Consequently, such Cdc42 mutants are extremely difficult to stably express in cells.

Given the importance of maintaining the careful regulation of Cdc42's GTP-binding/GTP hydrolytic cycle, we have been interested in seeing how different ways of manipulating this cycle might impact cell proliferation and cellular transformation. Indeed, we have found that mutations accelerating GDP−GTP exchange without impairing GTP hydrolysis, which we have referred to as fast-cycling mutations, are strongly transforming (*23*−*25*). One example is the F28L mutation that yields a Cdc42 molecule with a 50-fold enhanced rate of GDP dissociation and, consequently, exhibits a marked increase in its ability to exchange GDP for GTP, such that the F28L mutant does not require a GEF. Unlike the more traditional dominant-active forms of Cdc42 that are unable to hydrolyze GTP and are growth-inhibitory, expression of the fast-cycling Cdc42[F28L] mutant stimulates cell growth under low-serum conditions and in the absence of a substratum.

In light of the ability of fast-cycling Cdc42 mutants to enhance cell growth and promote cellular transformation, we then questioned whether mutations that slow the GTP-binding/GTP hydrolysis cycle, by decreasing the GTP hydrolytic activity of Cdc42, might achieve similar outcomes. Here, the idea was that by reducing the rate at which Cdc42 hydrolyzes GTP and extending its activation lifetime, we would mirror the effects obtained with fast-cycling mutants. This idea in turn raised an additional question, namely, whether stimulating the GTP hydrolytic reaction of a fast-

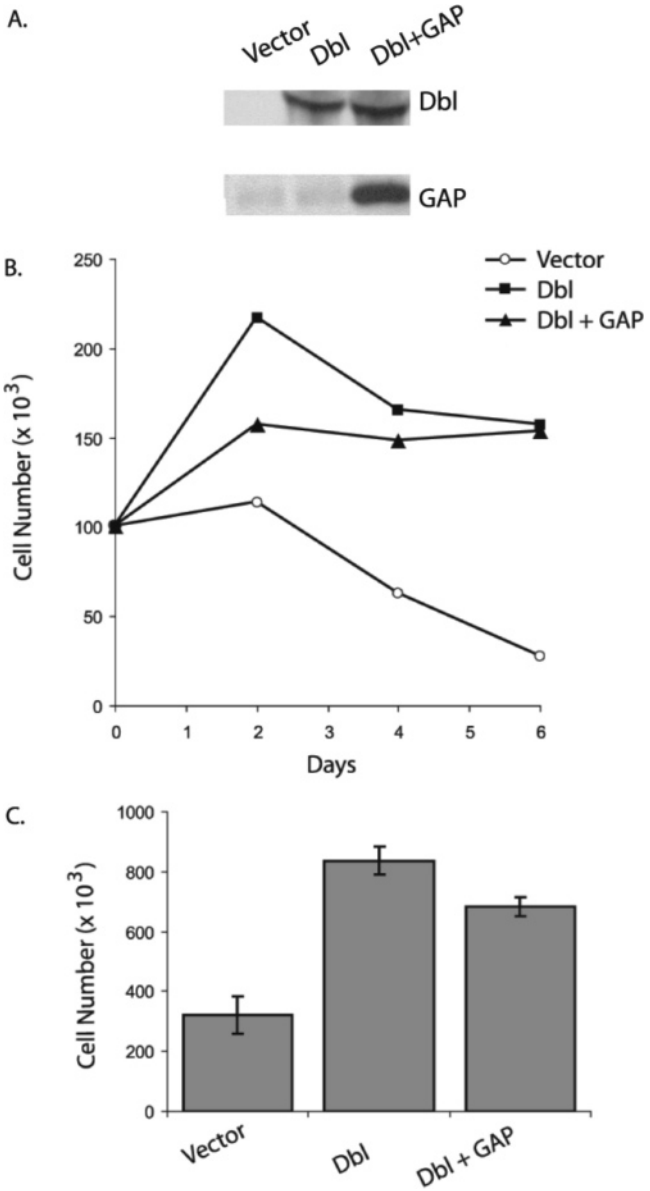


FIGURE 7: The effect of GAP on onco-Dbl-stimulated cell growth. (A) NIH 3T3 cells stably expressing oncogenic Dbl were used to introduce GAP under puromycin selection. The top panel compares the relative expression of oncogenic Dbl in the different cell lines, as assessed using an anti-Dbl antibody. The bottom panel compares the relative expression of HA-tagged GAP based on Western blotting with an anti-HA antibody. (B) NIH 3T3 cell lines stably expressing oncogenic Dbl and Dbl with GAP were grown in 1% FBS, as described in Figure 3B. On the indicated days, the samples were trypsinized and counted in order to monitor their growth. (C) To test for the loss of contact inhibition, the stable cell lines were grown in 10% FBS, as described in Figure 3D. After 6 days of growth, cells were trypsinized and counted. The data are representative of two or three independent experiments.

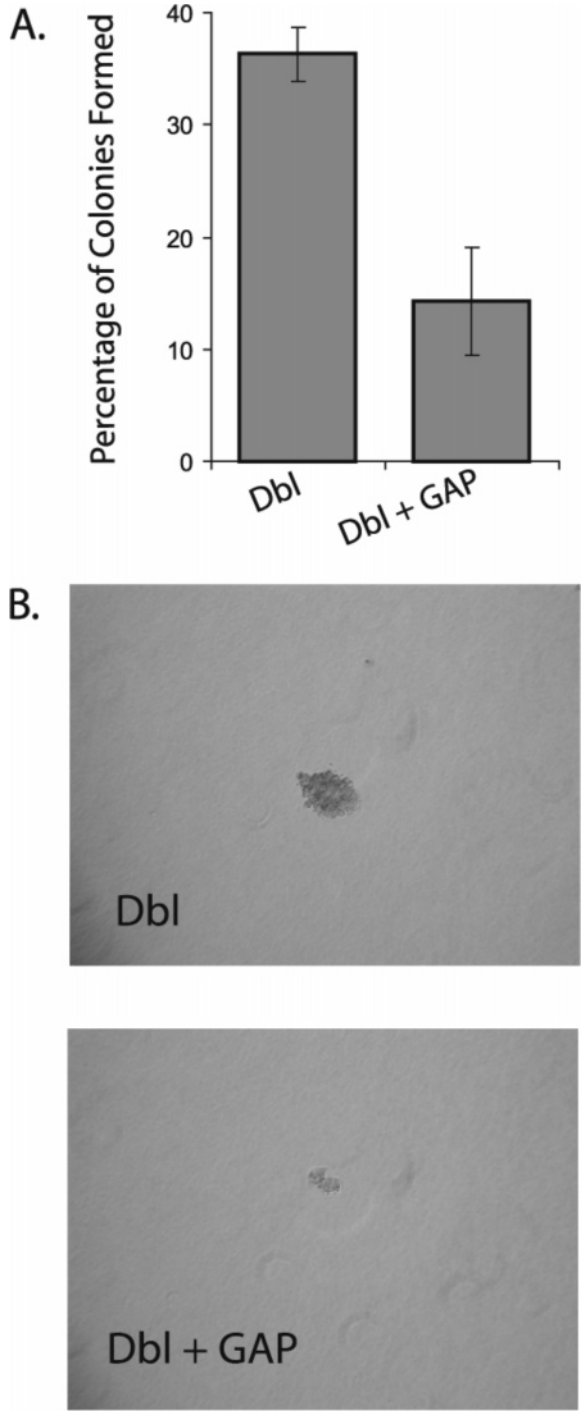


FIGURE 8: Effects of GAP on onco-Dbl-stimulated colony formation in soft agar. (A) Stable cell lines expressing oncogenic Dbl and Dbl with GAP were seeded at $3 \times 10^3$ cells per well and fed every 7 days with DMEM supplemented with 1% FBS and 0.3% agarose. After 14 days of growth, the samples were counted in order to monitor the number of colonies formed. These data are representative of three independent experiments. (B) Photomicrographs of the colony formation of cells in soft agar, as described in panel A.

cycling mutant like Cdc42[F28L], through the introduction of Cdc42GAP, would compensate for the accelerating effects on GDP−GTP exchange and inhibit its transforming capability.

We set out to address these questions by first examining whether the Cdc42[Y32A] mutant, which was previously shown to be compromised in its ability to undergo Cdc42GAP-stimulated GTP hydrolysis (*27*), existed for an extended lifetime in the GTP-bound state in cells, and if so, whether it might stimulate cell growth and/or transformation. While the Cdc42[Y32A] mutant is able to bind Cdc42GAP, the substitution of Tyr32 within the Switch I region of Cdc42 prevents the proper orientation of the essential arginine residue provided by the GAP (i.e., Arg305). Consequently, the Cdc42GAP is unable to stimulate the GTP hydrolytic activity of the Cdc42[Y32A] mutant (*27*). However, unlike the Cdc42[Q61L] mutant which is completely defective for hydrolyzing GTP, Cdc42[Y32A] retains some intrinsic ability

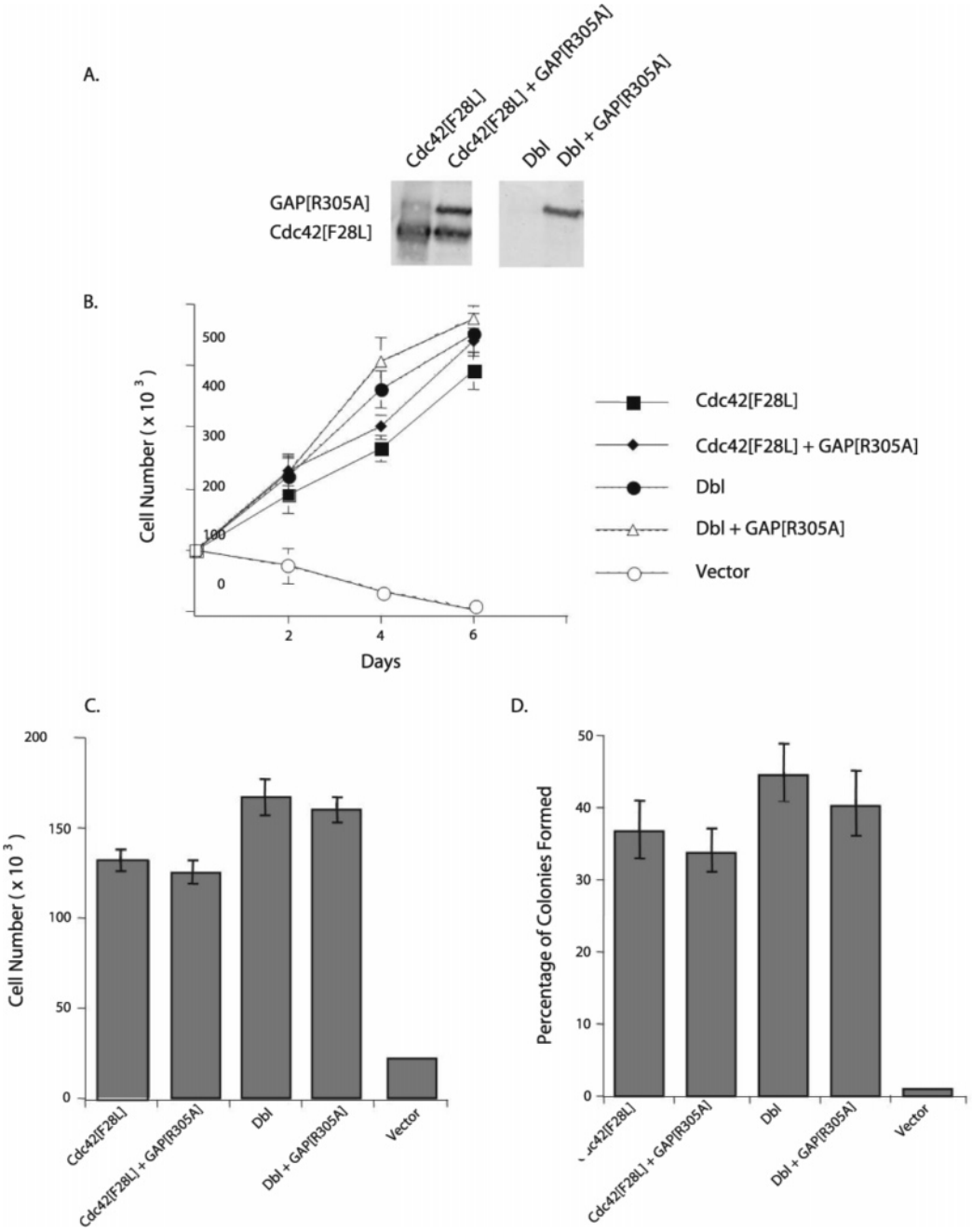


FIGURE 9: Effects of the GAP(R305A) mutant on the abilities of Cdc42[F28L] and onco-Dbl to stimulate cell growth and colony formation in soft agar. (A) Western blot analysis of NIH 3T3 cells stably expressing HA-tagged Cdc42[F28L] or oncogenic Dbl, alone, or in combination with the HA-tagged GAP(R305A). The expression levels of Cdc42[F28L] and GAP(R305A) were detected with an anti-HA antibody. The cells were then assayed for their ability to grow in 1% FBS (B), to grow to high density in the presence of 10% FBS (C), or to form colonies in soft agar (D), as described in Figures 3B, 3D, and 5A, respectively.

to hydrolyze GTP to GDP and is not irreversibly trapped in the GTP-bound state. Thus, Cdc42[Y32A] exhibited the properties of what might be referred to as a slow-cycling mutant.

Consistent with Cdc42[Y32A] having an extended lifetime in the activated state, we found that an increased percentage of its total cellular pool was GTP-bound, compared to wild-type Cdc42, as determined through PBD assays. If the GAP-insensitive Cdc42[Y32A] mutant existed for an extended period of time in the active conformation, then we might expect that, when expressed transiently in NIH 3T3 fibroblasts, it would stimulate morphological changes similar to those induced by activated forms of Cdc42, namely, an enhanced formation of filopodia (actin microspikes). This in fact was the case, as the expression of both Cdc42[Y32A] and Cdc42[Q61L] stimulated the formation of microspikes in NIH 3T3 cells.

We then went on to examine whether Cdc42[Y32A] was capable of transforming activity, by generating NIH 3T3 cell lines that stably expressed this mutant. Experiments assaying the saturation density of NIH 3T3 cells under different conditions showed that, upon stable expression of the Cdc42-[Y32A] mutant, these fibroblasts were able to grow to high-cell densitities, similar to what was observed upon the expression of the transforming Cdc42[F28L] mutant. However, when examining growth in low serum, a less dramatic effect was observed upon expression of the Cdc42[Y32A] mutant, such that the ability of these cells to grow was not

nearly as effective as cells expressing the Cdc42[F28L] mutant. It is possible that the differences observed between the Cdc42[Y32A] and Cdc42[F28L] mutants, when assaying growth in low serum, reflected a combination of factors including a more stringent dependence of this assay on the relative levels of Cdc42 expression. We routinely achieve significantly higher levels of expression of the F28L mutant compared to the Y32A mutant. Still, fibroblasts expressing Cdc42[Y32A] remained alive through 6 days under low-serum conditions, whereas the vector-control cells were completely dead.

Given these observations, we then addressed the question of whether accelerating the GTP hydrolytic reaction of the Cdc42[F28L] mutant, through its coexpression with GAP, might compensate for its constitutive GDP−GTP exchange activity and reduce the amount of this mutant that exists in the activated GTP-bound state in cells. If so, we would then expect that introduction of GAP would inhibit Cdc42[F28L]-induced cellular transformation. However, it was first important to determine that GAP did not have adverse effects on the growth of normal cells. For example, it was conceivable that overexpression of GAP, by blocking the activation of Cdc42 by serum factors, could have deleterious effects. This turned out not to be the case, at least for the levels of stable GAP expression that we were able to achieve in NIH 3T3 cells. In fact, both cell growth data and growth factor-stimulated signaling assays (e.g., ERK activation) demonstrated that overexpression of GAP was without adverse effects.

To determine if the expression of GAP affected the transforming properties of Cdc42[F28L], stable cell lines coexpressing the fast-cycling GTPase and GAP were generated. We found that GAP can inhibit the transformation phenotypes induced by the Cdc42[F28L] mutant. Two independent clonal lines expressing Cdc42[F28L] and different amounts of GAP exhibited a dose-dependent inhibition of growth in low serum, as well as an increase in contact inhibition. When tested for colony growth in soft agar, not only did the cell lines expressing Cdc42[F28L] and GAP exhibit less colony formation, but the colonies were also much smaller in size.

Previous studies from our laboratory have shown that the fast-cycling Cdc42[F28L] can enhance EGF-coupled signaling to ERK (*40*). This was shown to be the outcome of activated, GTP-bound Cdc42[F28L] forming a stable complex with p85-Cool-1/$\beta$-Pix and the E3 ubiquitin ligase, c-Cbl, as this complex sequesters c-Cbl away from the EGFR, preventing Cbl from catalyzing receptor ubiquitination and degradation. Coexpressing GAP with Cdc42[F28L] affected the EGF-stimulated ERK1/2 activation profile, supporting the idea that the GAP effectively shortens the lifetime of activation for the Cdc42[F28L] mutant by promoting its ability to hydrolyze GTP, thereby increasing the amount of Cbl that is free to bind to EGFRs and mark them for degradation.

We then went on to examine whether GAP might reverse the transforming activity of a deregulated Cdc42-GEF. Of the various Rho family GEFs that have been assayed for cellular transformation activity, oncogenic Dbl exhibits the most potent transforming capability. Dbl has been shown to be an effective GEF for Cdc42 and RhoA, based on in vitro [$^3$H]GDP-GTP$\gamma$S exchange assays (*41*), as well as to be capable of activating Rac in cells (presumably downstream from Dbl-induced Cdc42 activation) (*36*). Lin et al. then showed that Cdc42, Rac, and RhoA all contributed to the cellular phenotypes induced by Dbl, with Cdc42 being responsible for colony formation in soft-agar, Rac for stimulating lamellipodia formation, and RhoA for inducing actin stress fibers and the abilities of cells to grow to high densities and form foci (*24*). Given that Cdc42GAP is significantly more effective at stimulating the GTP hydrolytic activity of Cdc42, compared to either Rac or RhoA (*42*), we were particularly interested in seeing whether the coexpression of oncogenic Dbl with GAP led to the specific inhibition of those aspects of Dbl-induced transformation attributable to activated Cdc42. Indeed, we found that expression of GAP inhibited the growth of Dbl-expressing cells in soft agar, without significantly affecting their ability to grow in low serum or to high density.

Thus, in conclusion, the results presented here verify the importance of the cycling of Cdc42 between its GTP- and GDP-bound states for the ability of this GTPase to propagate cell growth-promoting signals. They also lend support to the potential roles played by GAPs in ensuring normal cellular homeostasis. Thus far, there has been little direct evidence demonstrating such roles. However, DLC (for Deleted in Liver Cancer), which is underexpressed in tumorigenic liver tissue and exhibits GAP activity toward Cdc42 and Rho, was shown to block foci formation when transfected into Ras-transformed cells (*43*). While the relationship between Cdc42 and human cancer is still not completely understood, high levels of Cdc42 expression have been observed in a number of different cancers including testicular cancer (*44*) and breast cancer (*45*). In testicular cancer, the highest Cdc42 expression levels correlate with higher stage, more progressive tumors (*44*), and the levels of activated, GTP-bound Cdc42 were found to be aberrantly high in human rhadomyosarcomas (*46*). In those cases where increased levels of GTP-bound Cdc42 have been noted and not attributed to changes in protein expression, it is likely that the excessive activation of Cdc42 is due to the faulty regulation of a GEF or GAP. Therefore, as further links between Cdc42 and malignant transformation are established, the importance of maintaining the proper cycling of Cdc42, as well as the function of Cdc42GAP and the regulation that it imparts, is likely to gain increasing attention in the design of future therapeutic strategies.

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