Antiapoptotic Cdc42 Mutants Are Potent Activators of Cellular Transformation†

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Received May 21, 2002; Revised Manuscript Received August 19, 2002

ABSTRACT: Cdc42 is a small GTP-binding protein which has been implicated in a number of cellular activities, including cell morphology, motility, cell-cycle progression, and malignant transformation. While GTPase-defective forms of Cdc42 inhibit cell growth, a mutation [Cdc42(F28L)] that allows the constitutive exchange of GDP for GTP and is GTPase-competent induces cellular transformation. These results suggest that Cdc42 must cycle between its GTP- and GDP-bound states to stimulate cell growth. In attempting to design Cdc42 molecules with more potent transforming activity, we set out to generate other types of Cdc42 mutants capable of constitutive GDP−GTP exchange. Here, we describe one such mutant, generated by changing a conserved aspartic acid residue at position 118 to an asparagine. The Cdc42(D118N) protein exchanges GDP for GTP more rapidly than wild-type Cdc42, but significantly more slowly than the Cdc42-(F28L) mutant. Despite its slower rate of activation, the Cdc42(D118N) mutant is more potent at inducing cellular transformation than the Cdc42(F28L) protein, and causes a significant loss in actin stress fibers, reminiscent of what is observed with fibroblasts transformed by oncogenic Ras mutants. Effector-loop mutations made within the D118N background inhibit Cdc42-induced transformation and Cdc42-mediated antiapoptotic (survival) activity to similar extents. In addition, mutating aspartic acid 121 (to asparagine), which forms part of a caspase cleavage site (DLRD, residues 118−121 of Cdc42), in combination with the F28L mutation generates a Cdc42 molecule [Cdc42(F28L/D121N)] with transforming activity significantly stronger than that of Cdc42(F28L). Thus, mutations that combine some capacity for cycling between the GTP- and GDP-bound states with increased survival against apoptotic signals yield Cdc42 molecules with the maximum capability for inducing cellular transformation.

The GTP-binding protein Cdc42 has been implicated in a wide variety of cellular activities ranging from polarity-dependent processes affecting cell morphology and motility to the regulation of cell-cycle progression (*1*−*5*). Upstream activators of Cdc42, for which the Dbl protein is a prototype, work by stimulating the GDP−GTP exchange reaction (*6*). The Dbl protein, and many other members of the Dbl family that are capable of activating Cdc42, give rise to cellular transformation when important regulatory regions are deleted or altered by mutation. This implied that hyperactivated forms of Cdc42 would also be oncogenic. However, we have found it difficult to produce stable NIH 3T3 cell lines expressing dominant-active, GTPase-defective forms of Cdc42 such as Cdc42(G12V) or Cdc42(Q61L), and when such lines were generated, their growth rates were significantly inhibited relative to that of control (parental) fibroblasts.

These findings led us to suspect that the ability of Cdc42 to cycle between its GDP- and GTP-bound states was necessary for Cdc42 to stimulate (rather than inhibit) cell growth. Thus, we have searched for mutations in Cdc42 which more accurately mimic the functional effects of Dbl and related proteins, causing Cdc42 to constitutively exchange GDP for GTP while maintaining a normal rate of GTP hydrolysis. Indeed, the substitution of a leucine residue for a conserved phenylalanine at position 28 yielded a Cdc42 protein that exhibited a greatly increased rate of GDP dissociation, while still being capable of binding GTP at concentrations similar to the normal cellular (micromolar) levels. Structural studies show that phenylalanine 28 is positioned at an approximately right angle to the guanine ring of GDP, and stabilizes it through $\pi-\pi$ orbital interactions (*7*−*9*). This stabilizing effect is lost when leucine is substituted for phenylalanine. The Cdc42(F28L) mutant hydrolyzed GTP with the same turnover number as the wild-type protein, and was capable of transforming NIH 3T3 cells (*5*, *10*). Because this mutant undergoes an accelerated GDP−GTP exchange reaction, we have referred to it and related types of molecules as "fast-cycling" mutants.

To examine whether the rate of cycling between the GDP- and GTP-bound states influenced Cdc42-mediated cell growth regulation, as well as to identify Cdc42 molecules with more potent transforming activity, we have been interested in generating other types of fast-cycling Cdc42

† This work was supported by a predoctoral fellowship from the Pharmaceutical Research and Manufacturers of America Foundation (39337) to S.S.T. and by grants from the National Institutes of Health (GM47458 and GM61762) to R.A.C. K.H. was supported by Grant RO1 GM-57464.

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10.1021/bi026167h CCC: $22.00
Published on Web 09/20/2002

mutants. This has caused us to examine the TQID motif that corresponds to the NKXD motif found in Ras and virtually all other GTP-binding proteins. This motif is also involved in stabilizing the guanine ring moiety, mainly through hydrogen bond interactions between the conserved aspartic acid residue and the ring nitrogen (N7). It had earlier been shown that mutating this aspartic acid residue in Ras could give rise to an oncogenic protein (*11*). However, studies in *Saccharomyces cerevisiae* suggested that the Cdc42(D118A) protein behaved like a dominant-negative mutant (*12*, *13*). Moreover, we found that the D118A substitution in the human Cdc42 protein significantly inhibited its ability to bind either GDP or GTP (Y. Zheng et al., unpublished data). Interestingly, this is not the case when the aspartic acid at position 118 in Cdc42 is changed to an asparagine residue.

Here we show that the Cdc42(D118N) mutant is capable of undergoing an accelerated rate of GDP−GTP exchange compared to wild-type Cdc42. Surprisingly, while the Cdc42-(D118N) mutant exhibits a slower rate of activation than the Cdc42(F28L) mutant, the Cdc42(D118N) mutant is significantly more effective at causing cellular transformation. This enhanced capability for inducing transformation cannot be attributed to any differences in target/effector-binding capability, nor can it be explained by a difference in the lifetime of the activated state. Rather, we show that the ability of Cdc42(D118N) to resist caspase cleavage (*14*) and to survive under apoptotic conditions (serum starvation and anoikis) accounts for its superior transforming activity compared to other activated versions of Cdc42, including the Cdc42-(F28L) mutant.

EXPERIMENTAL PROCEDURES

Cell Culture. Cos-7 cells were maintained in a humidified 7% CO_2 environment in Dulbecco's modified medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc.). Medium for Cos-7 cells was supplemented with 10% fetal bovine serum. NIH 3T3 cells were maintained in a humidified 7% CO_2 environment in Dulbecco's modified medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc.). Medium for NIH 3T3 cells was supplemented with 10% calf serum.

Preparation of Cell Extracts for Western Blot Analyses. Cos-7 or NIH 3T3 cells were washed with ice-cold PBS and then resuspended in lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. Lysates were incubated at room temperature for 15 min, separated on a 10% SDS−polyacrylamide gel, and immunoblotted with an anti-hemaglutinin (HA) monoclonal antibody from Covance.

Molecular Constructs. Cdc42 mutants were created from cDNAs encoding Cdc42 which were subsequently subcloned into the *Bam*HI−*Eco*RI site of pcDNA3. Using the resulting plasmid, we constructed single or double mutations by the polymerase chain reaction. For transient expression in Cos-7 cells, the cDNAs encoding the GTP-binding proteins were subcloned into the HA-tagged pcDNA3 vector, using the *Bam*HI−*Eco*RI restriction sites. For stable expression in NIH 3T3 cells, constructs were subcloned into the (HA-tagged) pJ4H vector using the same restriction sites.

PBD[1] *Assay for the Activation of Cdc42 in Cells.* This activation assay using the PBD of the Cdc42/Rac-target Pak has been previously described in detail (*15*, *16*). Cos-7 cells were transiently transfected with the cDNA for the indicated GTP-binding protein in the pcDNA3 vector. Cells were allowed to grow in the presence of 10% fetal bovine serum for 24 h. The cells were lysed in 20 mM Hepes (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 20 mM β-glycerol phosphate, 20 μM GTP, 1 mM sodium vanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotonin and incubated with 50 μg of the GST−PBD fusion peptide. Cell lysates were then rocked at 4 °C for 3 h. The GST−PBD fusion peptide was precipitated with glutathione−agarose beads, washed three times with lysis buffer, and subjected to SDS−polyacrylamide gel electrophoresis and immunoblotting using the indicated antibodies.

Assessing Cellular Apoptosis. Stable cell lines were cultured on dual-chamber microscope slides (Nunc) for 1 day in normal medium, and then serum-starved for 8 h. Cells were then treated with 100 ng/mL Fas ligand as previously described (*14*). The medium was removed, and the cells were kept at 4 °C and gently washed with 10 mL of PBS. Trypsin (1 mL) was then added to remove adherent cells. The cells were assayed for apoptosis using the Annexin-V−FITC apoptosis detection kit (Oncogene Research Products) according to the manufacturer's procedures. Annexin-V and propidium iodine (Molecular Probes) staining were used to test for membrane blebbing as described previously (*17*).

Growth Properties of Cdc42 Mutants. Saturation densities were measured by growing cells in 10% calf serum (CS). Cells (1×10^5) of each cell line were seeded in six-well plates in DMEM and 10% CS. Cells were trypsinized at days 1, 3, 5, and 7, and then counted with a hemacytometer under a phase-contrast microscope. Growth in low serum was assessed by growing cells in 1% CS. Cells (1×10^5) of each cell line were seeded in six-well plates in the presence of DMEM and 10% CS. After 6 h, the medium was removed and cells were placed in DMEM supplemented with 1% CS. The cells were maintained in this condition and counted as described above.

Growth in Soft Agar. Cells (2×10^3) of each stable cell line were plated in six-well plates embedded between 0.5% agar (bottom) and 0.3% agar (top). Cells were fed every week by adding a new layer of top agar. Colonies larger than 50 μm were scored under a microscope after growth for 2−4 weeks.

Expression and Assays of Recombinant Proteins. Glutathione *S*-transferase (GST)-tagged Cdc42 and its mutants were produced in *Escherichia coli* and purified using GST affinity chromatography followed by dialysis and thrombin proteolysis of the GST tag. *E. coli* cells were transformed with pGEX-Cdc42 and induced to express the protein upon addition of 400 μM IPTG (Sigma Chemicals). Cells were pelleted by low-speed centrifugation (10 min at 1500 rpm, Beckman JA10.5 rotor) to remove cell debris and then lysed in 20 mM Hepes (pH 7.4), 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/mL leupeptin,

[1] Abbreviations: Ack-2, activated Cdc42-associated kinase; GST, glutathione *S*-transferase; MRCK, myotonic dystrophy-related Cdc42-associated kinase; Pak, p21-activated kinase; PBD, p21-binding domain; WASP, Wiscott-Aldriche syndrome protein.

and 10 μg/mL aprotinin. The membrane fraction was pelleted (15 000 rpm, Beckman JA20 rotor, 30 min), washed twice in resuspension buffer [20 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 3.75 mM $MgCl_2$, 10 μM GDP, 1 μg/mL leupeptin, and 1 μg/mL aprotinin], and solubilized in the same buffer supplemented with 0.4% (w/v) CHAPS. The supernatant was then loaded onto a GST column equilibrated with 20 mM Tris (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 1 μg/mL aprotinin, and 1 μg/mL leupeptin. After the cells had been extensively washed with resuspension buffer, the protein was eluted using the same buffer supplemented with 20 mM glutathione.

When [^{35}S]GTPγS−GDP exchange activity was being measured, the recombinant GST−Cdc42 fusion proteins were eluted from the glutathione−Sepharose beads and then dialyzed overnight against 20 μM Hepes (pH 7.4), 2 mM $MgCl_2$, and 10 mM sodium azide at 4°C. Following dialysis, the GST−Cdc42 proteins were stored in the dialysis buffer together with 1 mM GDP. Nucleotide exchange assays were performed by diluting the recombinant Cdc42 proteins into an assay solution containing excess (200 μM) [^{35}S]GTPγS. The [^{35}S]GTPγS binding and [γ-^{32}P]GTP hydrolysis assays were performed as previously described (*18*).

RESULTS AND DISCUSSION

Cdc42(D118N) Cycles between the GDP- and GTP-Bound States. We have been interested in generating Cdc42 mutants that show varying capabilities for cycling between the off (GDP-bound) and on (GTP-bound) states. The first such mutant that we characterized resulted from a change at position 28 [Cdc42(F28L)], which disrupted a π-orbital stabilization of the guanine ring and yielded a Cdc42 molecule capable of spontaneous GDP−GTP exchange and cellular transformation (*5*). We examined other mutations that would be expected to disrupt the stability of the guanine ring moiety, ultimately turning our attention to the TQID motif of Cdc42. This motif has been shown to be involved in hydrogen bond interactions with the nitrogen groups in the guanine base. In vitro guanine nucleotide binding assays have suggested that the Cdc42(D118A) mutant has a severely weakened affinity for both GDP and GTP, making it likely to exist in a nucleotide-depleted state in most cell types, and thus probably most often act as a dominant-negative mutant.

We then examined the consequences of changing Asp118 in Cdc42 to an asparagine residue. Unlike the Cdc42(D118A) mutant, the Cdc42(D118N) mutant should retain some capability for hydrogen bonding to the guanine ring, and thus potentially maintain the ability to bind GTP. The Cdc42-(D118N) mutant was expressed as a GST fusion protein in *E. coli* and then purified to apparent homogeneity using previously described procedures (*19*). We first compared the ability of this mutant, relative to those of Cdc42(F28L) and wild-type Cdc42, to bind [^{35}S]GTPγS (Figure 1A). All GST−Cdc42 proteins have been purified in the presence of excess GDP, and therefore are initially bound to GDP. Thus, in Figure 1A, we examine the ability of these Cdc42 mutants to spontaneously exchange GDP for GTPγS. In the presence of physiological concentrations of Mg^{2+} (2 mM), wild-type Cdc42 showed little spontaneous loading of [^{35}S]GTPγS. This is due to the fact that the wild-type protein contains a

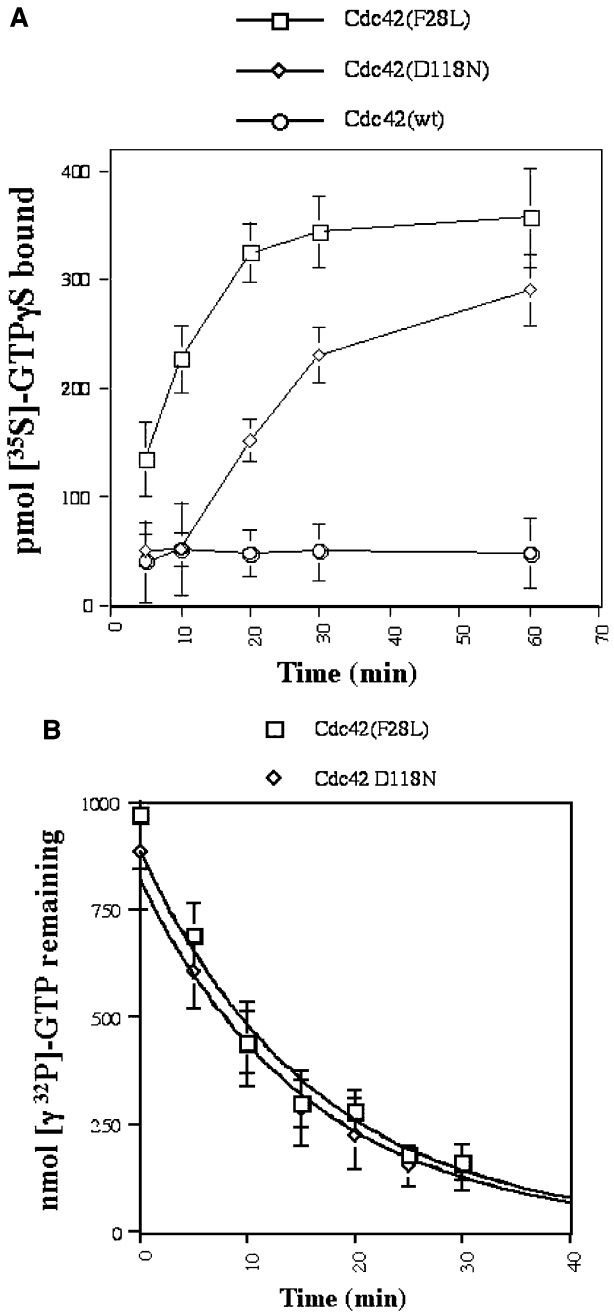

FIGURE 1: Cdc42(D118N) is capable of cycling between the GDP- and GTP-bound states. (A) Cdc42(D118N) binds [^{35}S]GTPγS at an accelerated rate compared to wild-type Cdc42. *E. coli* recombinant, wild-type Cdc42, Cdc42(F28L), or Cdc42(D118N) proteins were purified as GST fusion proteins and incubated with 200 μM [^{35}S]GTPγS (~90 Ci/mmol). The amount of protein-bound radioactivity was measured as described in ref *18*. Values that are shown are the average of three independent experiments. (B) Measurements of the GTP hydrolytic activities of the recombinant Cdc42(F28L) and Cdc42(D118N) proteins. GTP hydrolysis assays were performed as described in ref *18*.

stoichiometric amount of tightly bound GDP, and that the rate-limiting step for GDP−GTPγS exchange is the dissociation of GDP. This can be accelerated either by adding excess EDTA to chelate any residual Mg^{2+} or by adding a guanine nucleotide exchange factor (e.g., Dbl) which perturbs the Mg^{2+} coordination on Cdc42 and thus loosens the binding of GDP. In the absence of these treatments, the intrinsic rate of dissociation of GDP from wild-type Cdc42 in the presence of millimolar levels of Mg^{2+} is extremely slow.

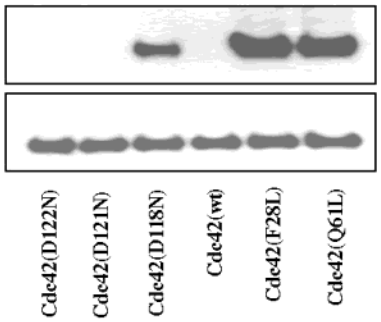

FIGURE 2: Cdc42(D118N) is activated in cells. Hemaglutinin (HA)-tagged Cdc42 constructs were transiently expressed in Cos-7 cells (*16*), and then cell lysates were incubated with the GST−PBD protein. The top panel shows the results obtained when the GST−PBD protein was precipitated with glutathione−agarose beads, and the precipitates were analyzed by Western blotting with anti-HA (Covance). The bottom panel shows the relative levels of expression of the different HA-tagged Cdc42 proteins.

Both the Cdc42(F28L) and Cdc42(D118N) mutants were able to bind [^{35}S]GTPγS more rapidly than the wild-type protein. Half-maximal binding of [^{35}S]GTPγS to Cdc42-(F28L) typically occurred within 5 min, whereas nucleotide binding to the Cdc42(D118N) mutant, while occurring at an increased rate relative to that for wild-type Cdc42, was clearly slower than that of the Cdc42(F28L) protein with a half-time of ~20 min.

On the other hand, both Cdc42(F28L) and Cdc42(D118N) were capable of essentially identical GTP hydrolytic activities under single-turnover conditions (Figure 1B); these activities were indistinguishable from the GTP hydrolytic activity of wild-type Cdc42 (data not shown). Thus, like the Cdc42-(F28L) mutant, the Cdc42(D118N) mutant showed the ability to cycle between the GDP- and GTP-bound states.

Cdc42(D118N) Shows Constitutive GTP Binding Activity in Cells. We next examined whether the Cdc42(D118N) mutant showed a greater extent of activation (GDP−GTP exchange) than wild-type Cdc42 in cells. Previously, we developed an assay for assessing the cellular activation of Cdc42 by taking advantage of the fact that the activated form of this GTP-binding protein binds with high affinity to its target/effector, Pak (*16*). Specifically, cells transfected with different Cdc42 constructs can be treated with various factors or cotransfected with putative guanine nucleotide exchange factors, and then the extent of activation of the Cdc42 proteins can be monitored by their relative abilities to be precipitated by the limit-Cdc42-binding domain of Pak, called the PBD (for the p21-binding domain). Thus, we transiently transfected Cos-7 cells with HA-tagged Cdc42(F28L) and Cdc42(D118N). Two other Cdc42 mutants that contained single substitutions in the vicinity of position 118, Cdc42-(D121N) and Cdc42(D122N), together with wild-type Cdc42 served as negative controls, whereas GTPase-defective Cdc42(Q61L) was a positive control. Lysates from the different transfectants were incubated with GST fused to the PBD. The relative levels of expression of the HA-tagged Cdc42 proteins were essentially identical, as shown in the bottom panel of Figure 2. The relative amounts of Cdc42 complexed to the PBD were assessed by precipitation of the GST−PBD protein with glutathione−agarose beads followed by SDS−PAGE and Western blot analysis to detect the associated Cdc42 proteins. The top panel of Figure 2 shows the results of such an experiment. Under conditions where wild-type Cdc42, Cdc42(D121N), and Cdc42(D122N) were unable to associate with the GST−PBD protein, consistent with the inability of these Cdc42 proteins to constitutively exchange GDP for GTP, we observed a detectable association of the Cdc42(D118N) mutant with PBD. However, we have consistently found that the amount of Cdc42(D118N) bound to this target is 10−30% of the amount of either Cdc42-(F28L) or the dominant-active, GTPase-defective Cdc42-(Q61L). These results support the in vitro GTP binding data presented in Figure 1A and are consistent with the idea that at any particular moment within cells, a greater percentage of the total pool of Cdc42(F28L) will be in the GTP-bound state compared to the Cdc42(D118N) mutant.

Morphological Changes Associated with Cdc42(D118N). It was previously shown that the microinjection of dominant-active, GTPase-defective forms of Cdc42 [i.e., Cdc42-(G12V)] into Swiss 3T3 cells resulted in the appearance of microspikes (*1*). We have found that this is also the case for NIH 3T3 cells transiently transfected with GFP constructs encoding the GTPase-defective Cdc42(Q61L) protein, as well as for cells expressing the fast-cycling Cdc42(F28L) mutant (*5*), and the Cdc42(D118N) mutant, whereas the dominant negative Cdc42(T17N) mutant is ineffective at stimulating the formation of microspikes (Figure 3A,B). It is interesting that even though a smaller percentage of Cdc42(D118N) molecules is activated at any particular time, relative to either the Cdc42(Q61L) or Cdc42(F28L) mutant, cells expressing the Cdc42(D118N) mutant produced a maximum response.

We also examined the effects of these different Cdc42 proteins on the actin cytoskeleton by phalloidin staining, after their stable expression in NIH 3T3 fibroblasts (Figure 3C). Each stable cell line expresses similar levels of the HA-tagged Cdc42 protein (see the inset of Figure 7A below). Control cells, as well as cells expressing either wild-type Cdc42 (not shown) or the Cdc42(D121N) mutant (Figure 3C), exhibited actin stress fibers. Cells expressing the Cdc42-(F28L) protein also showed actin stress fibers, as reported previously (*5*; also Figure 3C). Although microspikes are sometimes detected in cells that stably express fast-cycling Cdc42 mutants (*5*), this phenotype is more conspicuous in cells which transiently express the fast-cycling Cdc42 mutants (Figure 3A), probably because of the higher levels of Cdc42 expression achieved with transient transfection approaches compared to those achieved with stable transfection. It is interesting that the expression of the Cdc42-(D118N) mutant consistently showed a significant reduction in the level of actin stress fibers. Similar results were obtained when the Cdc42(D118N) mutant was expressed in Swiss 3T3 cells and MDCK cells (data not shown). A similar phenotype accompanies cellular transformation by Ras (*20*). While at the present time we do not know the molecular basis of the Cdc42(D118N)-induced dissolution of actin stress fibers, it led us to suspect that this Cdc42 mutant may have potent transforming capability.

Growth Characteristics of Cdc42(D118N)-Expressing Cells. Figure 4A compares the growth rates in low levels of serum for cells expressing a neomycin-resistant control vector, or for cells expressing either the Cdc42(F28L) or Cdc42(D118N) mutant. Parental NIH 3T3 fibroblasts, as well as fibroblasts transfected with the control vector, show little ability to grow under low-serum conditions. The overex-

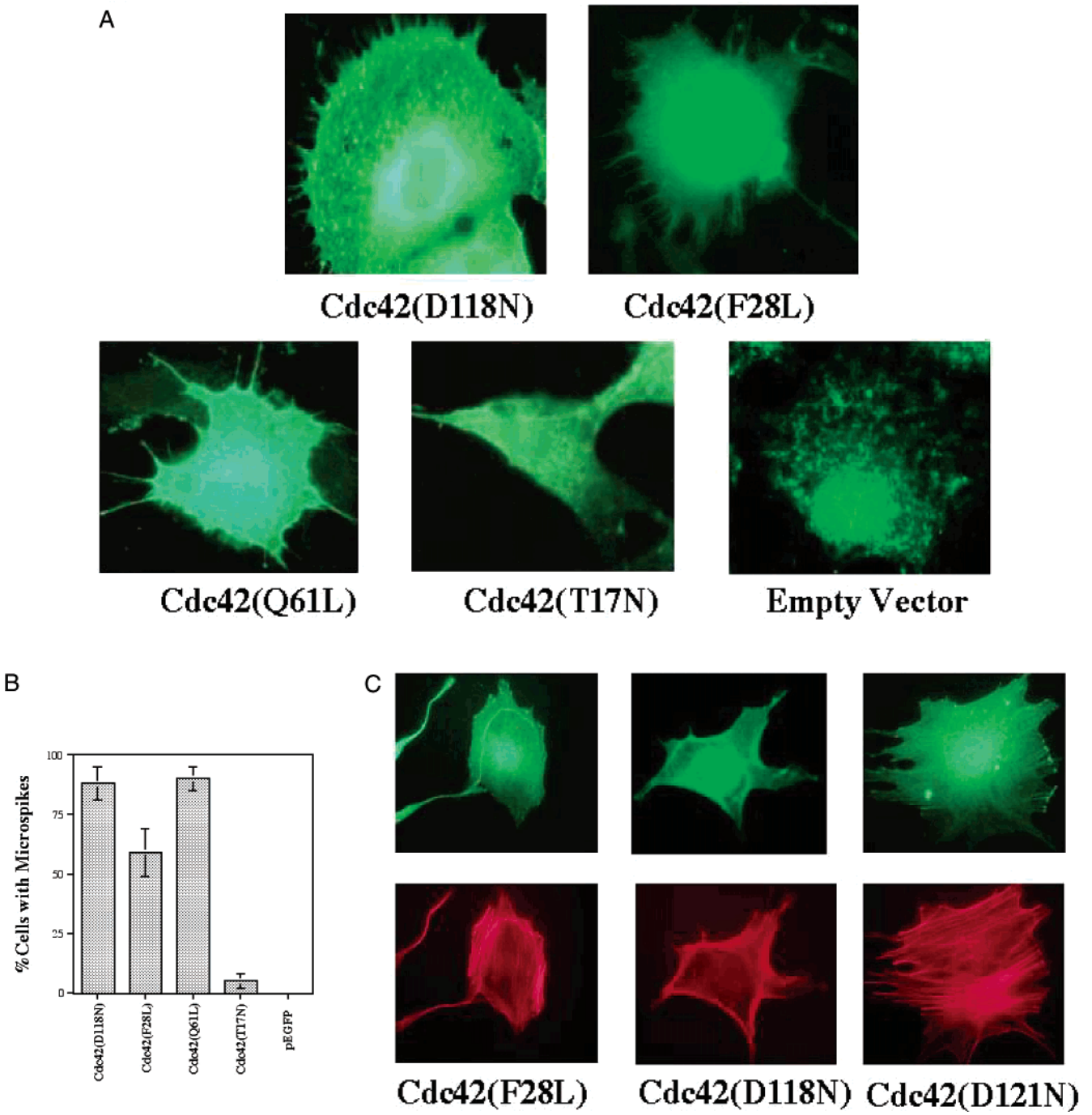

FIGURE 3: Morphological characteristics of Cdc42(D118N)-expressing fibroblasts. (A) Cdc42(D118N)-expressing cells exhibit microspikes. NIH 3T3 cells were transiently transfected with pEGFP Cdc42(D118N), Cdc42(F28L), Cdc42(T17N), Cdc42(Q61L), or empty vector. (B) Quantitation of microspikes for cells expressing different Cdc42 constructs. In each case, at least 200 cells were examined. Cells that expressed microspikes greater than 1 mm in length and covering at least 50% of the cell were counted as positive for microspike formation. The data that are shown are representative of three independent experiments. (C) Cdc42(D118N)-expressing cells show a reduction in the level of actin stress fibers. NIH 3T3 cells stably transfected with pcDNA3-Neo, Cdc42(D118N), Cdc42(D121N), or Cdc42(F28L) were fixed and stained with HA antibody and then stained specifically for F-actin using Texas red-conjugated phalloidin. The results are representative of 200 cells that were examined for each condition (400× magnification).

pression of wild-type Cdc42 did not cause any obvious change in the growth properties (data not shown; also, see ref *5*), whereas the expression of the Cdc42(F28L) mutant enabled fibroblasts to grow under low-serum conditions. However, cells which at the outset stably expressed the Cdc42(D118N) mutant at levels similar to the level of expression of the Cdc42(F28L) mutant (e.g., see the inset of Figure 7A below) exhibited an even greater proliferative response.

The same pattern was observed when assaying the ability of fibroblasts to grow in the absence of a substratum (i.e., colony formation in soft agar) (Figure 4B,C). As expected, control cells (stably transfected with vector alone), or cells expressing wild-type Cdc42 (not shown), were incapable of forming colonies in soft agar. The Cdc42(F28L) mutant, on the other hand, was effective at stimulating growth in soft agar. However, the Cdc42(D118N) mutant was significantly more potent in stimulating colony formation. The abilities

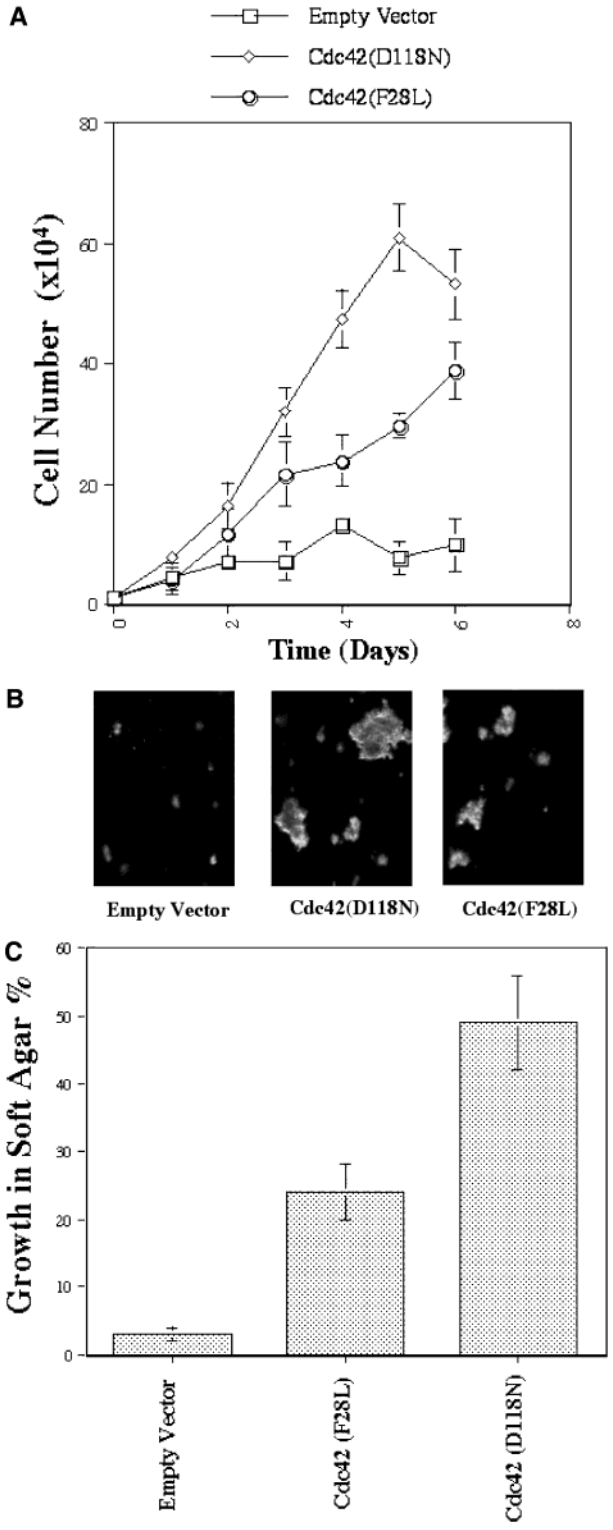

FIGURE 4: Cdc42(D118N) is capable of inducing cellular transformation. (A) Fibroblasts stably expressing Cdc42(D118N) exhibit diminished serum dependence. Cell lines were cultured in the presence of 1% serum and at the indicated times trypsinized and counted. Data are representative of three experiments. (B) Fibroblasts stably expressing Cdc42(D118N) exhibit anchorage-independent growth. NIH 3T3 cells that stably express Cdc42(D118N), Cdc42(F28L), or a control (neomycin) vector were mixed with medium supplemented with 0.3% agar and 10% fetal calf serum and plated on top of a 0.5% agarose layer (40× magnification). (C) Quantitation of colony formation in soft agar. Growing colonies were scored after 14 days for the various cell lines. Values that are shown are the average of three independent experiments; 100% represents 120−180 cells counted.

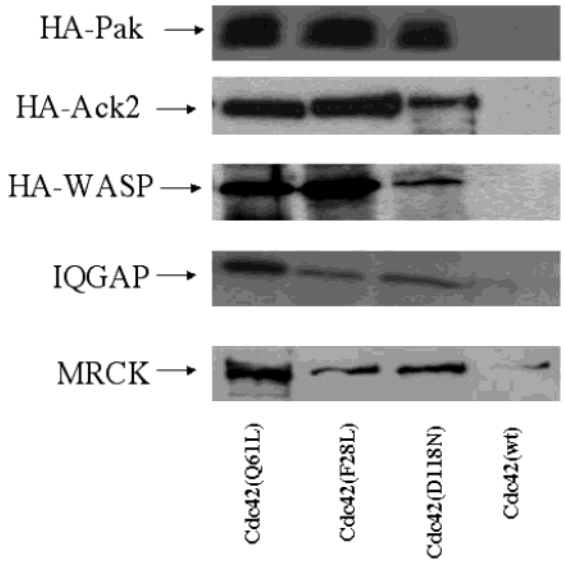

FIGURE 5: Comparison of the abilities of Cdc42(Q61L), Cdc42-(F28L), and Cdc42(D118N) to bind known targets. Either hemaglutinin (HA)-tagged Pak, Ack-2, or WASP was transiently expressed in NIH 3T3 cells, and then the lysates were incubated with the indicated recombinant GST−Cdc42 mutants which contained bound GTPγS. The GST−Cdc42 proteins were precipitated with glutathione−agarose beads, and the precipitates were analyzed with either anti-HA antibody (Covance) or anti-IQGAP (Upstate Biotechnology) to detect endogenous IQGAP, or anti-MRCK (BD PharMingen) to detect endogenous MRCK.

of cells to grow under low-serum conditions and to form colonies in soft agar have been hallmarks for cellular transformation, so these results would argue that despite the fact that Cdc42(D118N) undergoes a slower rate of GDP−GTP exchange than Cdc42(F28L), it has an enhanced capability for inducing an oncogenic or transformed phenotype.

Link between Protection against Apoptosis and Cellular Transformation. We were interested in understanding how the Cdc42(D118N) mutant was able to cause cellular transformation more effectively than the Cdc42(F28L) mutant. We have not found that the enhanced transforming signal induced by Cdc42(D118N) can be attributed to an improved ability to bind to a known target/effector for Cdc42. Rather, we have found that the Cdc42(D118N) mutant binds targets as effectively as Cdc42(F28L), or in some cases less well, but not with a significantly higher affinity. Figure 5 illustrates this by comparing the abilities of dominant-active, GTPase-defective Cdc42(Q61L) (positive control), Cdc42-(F28L), Cdc42(D118N), and wild-type Cdc42 (negative control) to bind to a number of different Cdc42-target/effector proteins, including the serine/threonine kinase Pak, the nonreceptor tyrosine kinase Ack-2, WASP, IQGAP, and MRCK.

We recently have found that Cdc42 is a substrate for caspases and that the Cdc42(D118N) mutant is insensitive to caspase-catalyzed proteolysis (*14*). Given that conditions used to assay cellular transformation such as growth under low-serum or colony formation in soft agar will promote the death of normal cells, it therefore seemed possible that the enhanced oncogenic capability of the Cdc42(D118N) mutant may be due to its enhanced survival (antiapoptotic) activity. NIH 3T3 cell lines stably expressing a neomycin control vector undergo extensive apoptosis (Annexin-V-

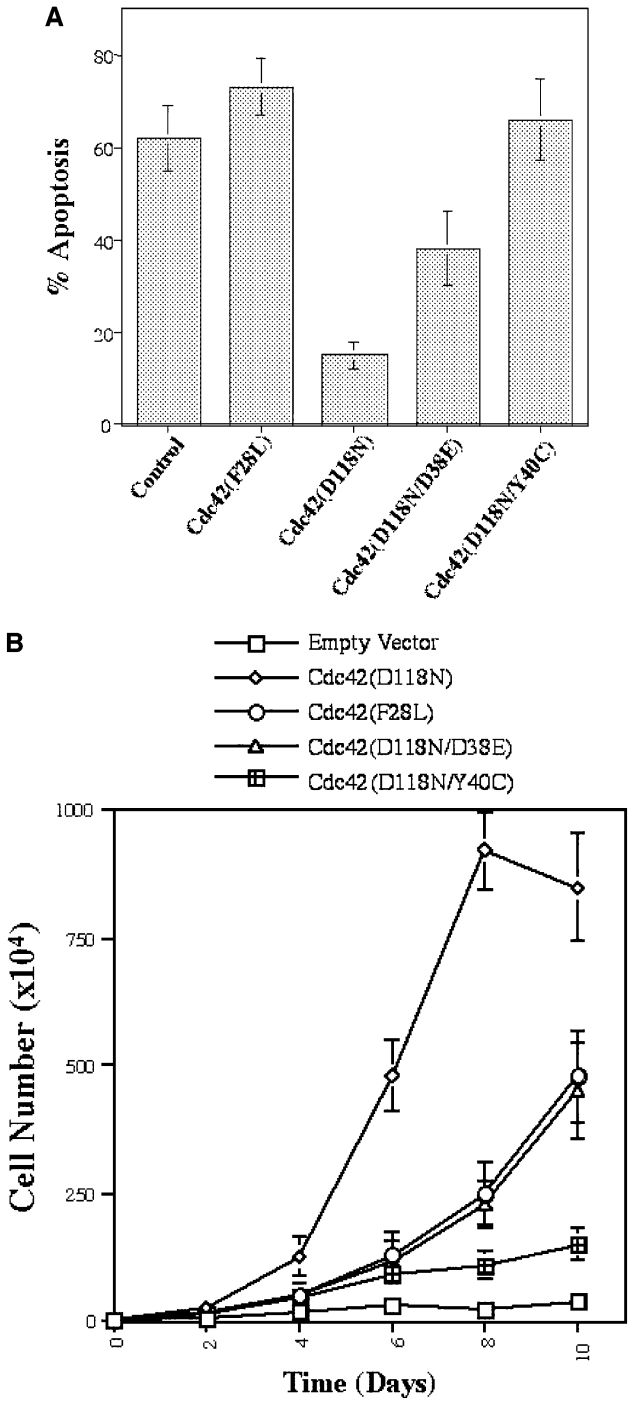

FIGURE 6: Effects of effector-loop mutations on Cdc42-mediated survival and transformation. (A) Effects of Cdc42 double mutants on the ability of cells to undergo Fas ligand-induced apoptosis. Fibroblasts stably expressing Cdc42(F28L), Cdc42(D118N), Cdc42-(D118N/D38E), Cdc42(D118N/Y40C), or neomycin control vector were serum-starved in DMEM with 1% calf serum and then treated with 100 ng/mL Fas ligand for 4 h. Apoptosis was assayed by staining with Annexin-V and propidium iodine. The results represent the analysis of 500 cells ($n = 6$). (B) Effects of Cdc42 double mutants on the ability of cells to grow under low-serum conditions. Cell lines were cultured in the presence of 1% serum and at the indicated times trypsinized and counted. Data are representative of three experiments.

positive) when treated with Fas ligand (Figure 6A). This is also the case for cells expressing the constitutively active Cdc42(F28L) mutant, despite the ability of this mutant to transform fibroblasts. However, in contrast, cells stably transfected with Cdc42(D118N) have a significantly reduced Annexin-V population when treated with Fas ligand.

We then set out to examine whether a correlation could be established between the antiapoptotic activity of Cdc42-(D118N) and its ability to stimulate an enhanced transformation signal. One Cdc42 target that was a particularly attractive candidate for mediating an antiapoptotic signal was Pak, given that this serine/threonine kinase has been implicated in mediating cell survival, in some cases through its activation of NFκB (*21*). Thus, we examined the effects of an Asp to Glu mutation at position 38 in Cdc42, as this mutation was previously shown to block the binding of Pak (*22*). In addition, we examined the effects of a Tyr to Cys change at position 40 which is thought to inhibit all targets (including Pak) that contain a CRIB (Cdc42/Rac-interactive-binding) motif (*23*). NIH 3T3 cells stably transfected with either the Cdc42(D118N/D38E) or Cdc42(D118N/Y40C) double mutant showed significantly greater extents of apoptosis than cells expressing the Cdc42(D118N) mutant (Figure 6A). However, whereas the Cdc42(D118N/D38E) mutant consistently showed a minimal protective effect, the Cdc42(D118N/Y40C) mutant lost all survival capabilities (Figure 6A).

We examined the effects of these same mutations on Cdc42(D118N)-mediated cellular transformation. Cells stably transfected with Cdc42(D118N/D38E) showed a reduced ability, relative to cells expressing Cdc42(D118N), to undergo cellular transformation as read out either by growth under low-serum conditions (Figure 6B) or by colony formation in soft agar (data not shown). The transformation capability of the Cdc42(D118N/D38E) mutant was comparable to that of the Cdc42(F28L) mutant, whereas Cdc42-(D118N/Y40C) was severely compromised in its ability to stimulate transformation as assessed by growth under low-serum conditions (Figure 6B). The fact that the D38E and Y40C mutations, when made in a D118N background, show the same relative inhibitory effects when assaying either Cdc42(D118N)-mediated protection against apoptosis or Cdc42(D118N)-induced cellular transformation suggests that the ability of the Cdc42(D118N) protein to provide a survival advantage against apoptotic stimuli is coupled to its enhanced transformation activity.

To further establish this link, we examined whether mutating the terminal residue of the DLRD sequence, aspartic acid 121, to an asparagine, within an F28L background resulted in an enhanced transformation activity relative to that for the Cdc42(F28L) mutant. The results presented in Figure 7 show that this was in fact the case. The Cdc42-(F28L/D121N) double mutant has the same fast-cycling capability as the Cdc42(F28L) protein, but we had earlier shown that unlike Cdc42(F28L), the Cdc42(F28L/D121N) protein has the ability to protect cells against apoptotic stimuli (*14*). Moreover, as shown in Figure 7A, the Cdc42(F28L/D121N) double mutant showed an enhanced ability to grow under low-serum conditions compared to Cdc42(F28L)-expressing cells, and was similar to Cdc42(D118N). Similar results were obtained when assaying growth in soft agar (Figure 7B). These results confirm the idea that maximal transformation activity occurs in Cdc42 mutants that are resistant to caspase-catalyzed degradation and thus are able to elicit protection against apoptotic signals.

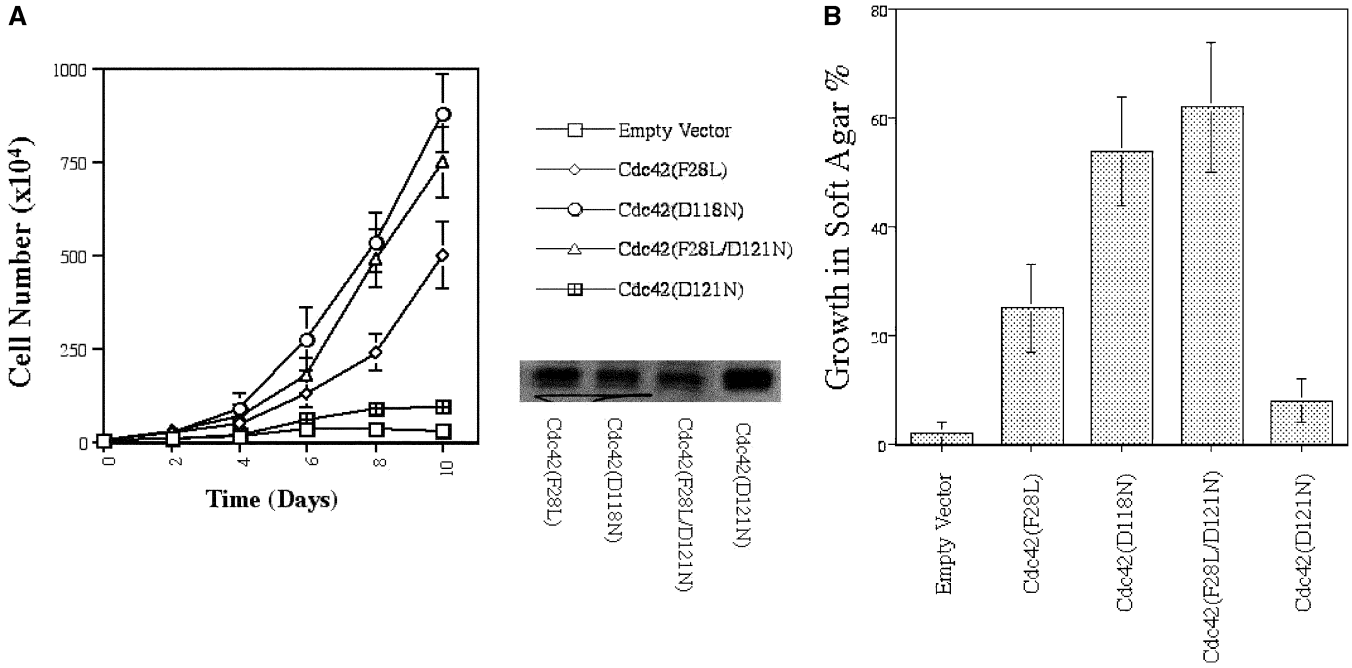

FIGURE 7: Fast-cycling and caspase-resistant mutants of Cdc42 enhance cellular transformation. (A) Effects of different Cdc42 double mutants on the ability of cells to grow under low-serum conditions. Fibroblasts stably expressing HA-tagged Cdc42(F28L), Cdc42(D118N), Cdc42(F28L/D121N), Cdc42(D121N), or neomycin control vector were cultured in the presence of 1% serum and at the indicated times trypsinized and counted. Data are representative of three experiments. The inset shows relative levels of expression of the different HA-tagged Cdc42 proteins at the beginning of the experiment as assessed by Western blot analysis using an anti-HA antibody. (B) Quantitation of colony formation in soft agar. Growing colonies were scored after 14 days for the various cell lines. Values that are shown are the average of three independent experiments; 100% represents 120−180 cells counted.

CONCLUSIONS

Unlike Ras, where persistent activation leads to a strong proliferative response, the Cdc42 protein needs to cycle between its GDP- and GTP-bound states to stimulate cell growth and induce cellular transformation. This appears most likely to be due to an involvement of Cdc42 in the trafficking of a growth regulatory protein(s), as only activated Cdc42 mutants that are competent to hydrolyze GTP are able to stimulate cellular trafficking activities and give rise to cellular transformation whereas GTPase-defective Cdc42 mutants inhibit both cellular trafficking and the transformation of NIH 3T3 cells (*24*). Thus, we have been interested in generating mutants which reach the GTP-bound state more rapidly than the wild-type Cdc42 protein while still maintaining the ability to hydrolyze GTP to GDP, thus mimicking the functional outcome of activated guanine nucleotide exchange factors such as the Dbl oncoprotein. Here, we describe the Cdc42-(D118N) mutant, which exhibits a faster rate of intrinsic GDP−GTP exchange than wild-type Cdc42 but is significantly slower than the rapidly cycling Cdc42(F28L) protein. Unexpectedly, the Cdc42(D118N) mutant is much more effective than Cdc42(F28L) in inducing cellular transformation. This difference in transforming activity cannot be explained by differences in target/effector binding, as the Cdc42(D118N) protein does not show an enhanced ability relative to other activated Cdc42 mutants [e.g., Cdc42(Q61L) and Cdc42(F28L)] to bind known Cdc42-target/effectors. Rather, there is an increased survival capability that is exhibited by the Cdc42(D118N) mutant which is not observed with the Cdc42(F28L) mutant.

Previously, we proposed that apoptotic stimuli may activate Cdc42 to ensure the proper timing for cellular apoptosis (*14*). Cdc42 is a caspase substrate, and thus, it was attractive to envisage a scheme where the eventual caspase-catalyzed degradation of Cdc42 would allow apoptosis to proceed at a maximal rate. Cdc42 mutants that are not susceptible to caspase degradation (i.e., substitutions for aspartic acid 118 and/or 121) have the potential to provide protection against apoptotic factors (*14*). Thus, cells expressing caspase-resistant Cdc42(D118N) are able to grow effectively under the apoptotic conditions that accompany serum starvation or in the absence of a substratum. Mutations that eliminate the binding of Pak and potentially other CRIB motif-containing targets yield corresponding reductions in the ability of the Cdc42(D118N) mutant to protect against apoptosis and induce cellular transformation, suggesting that the coupling of these two events accounts for the significantly enhanced transforming activity of the D118N mutant. In addition, a Cdc42(F28L/D121N) double mutant, which is also able to protect cells against apoptotic signals, is capable of an enhanced transforming activity compared to Cdc42-(F28L) and behaves like the Cdc42(D118N) mutant. Thus, we find that the Cdc42-induced transformation signal is most potent when the GTP-binding protein both is capable of an accelerated cycling between the GTP- and GDP-bound states, compared to the exceedingly slow intrinsic nucleotide exchange activity of wild-type Cdc42, and is resistant to caspase cleavage and therefore able to protect cells against apoptotic stimuli. It is interesting that the Cdc42(D118N) mutant has effects on the actin cytoskeleton markedly different from those of the Cdc42(F28L) protein, giving rise to a dissolution (rather than a maintenance) of actin stress fibers. We do not know if these effects on the actin cytoskeleton are in some way connected to the antiapoptotic/survival activity of the Cdc42(D118N) protein, although the Cdc42(D118N)-induced dissolution of actin stress fibers is

similar to the transformation phenotype caused by oncogenic Ras (*20*), which is a known survival factor (*25*, *26*). Thus, future studies will be directed at exploring the potential link between actin cytoskeletal changes and effects on apoptotic signaling activities, as well as at further examining the specific roles of targets such as Pak in Cdc42-mediated survival and transformation.

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BI026167H