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Involvement of Rho Family GTPases in p19Arf- and p53-Mediated Proliferation of Primary Mouse Embryonic Fibroblasts

Fukun Guo and Yi Zheng*

Division of Experimental Hematology, Children's Hospital Research Foundation, University of Cincinnati, Cincinnati, Ohio 45229

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The Rho family GTPases Rac1, RhoA, and Cdc42 function as molecular switches that transduce intracellular signals regulating gene expression and cell proliferation as well as cell migration. $p19^{Arf}$ and p53, on the other hand, are tumor suppressors that act both independently and sequentially to regulate cell proliferation. To investigate the functional interaction and cooperativeness of Rho GTPases with the $p19^{Arf}$-p53 pathway, we examined the contribution of Rho GTPases to the gene transcription and cell proliferation unleashed by deletion of *p19Arf* or *p53* in primary mouse embryo fibroblasts. We found that (i) $p19^{Arf}$ or p53 deficiency led to a significant increase in PI 3-kinase activity, which in turn upregulated RhoA and Rac1 activities; (ii) deletion of *p19Arf* or *p53* led to an increase in cell growth rate that was in part dependent on RhoA, Rac1, and Cdc42 activities; (iii) $p19^{Arf}$ or p53 deficiency caused an enhancement of the growth-related transcription factor NF-κB and cyclin D1 activities that are partly dependent on RhoA or Cdc42 but not on Rac1; (iv) forced expression of the activating mutants of Rac1, RhoA, or Cdc42 caused a hyperproliferative phenotype of the $p19Arf^{-/-}$ and $p53^{-/-}$ cells and promoted transformation of both cells; (v) RhoA appeared to contribute to p53-regulated cell proliferation by modulating cell cycle machinery, while hyperactivation of RhoA further suppressed a p53-independent apoptotic signal; and (vi) multiple pathways regulated by RhoA, including that of Rho-kinase, were required for RhoA to fully promote the transformation of $p53^{-/-}$ cells. Taken together, these results provide strong evidence indicating that signals through the Rho family GTPases can both contribute to cell growth regulation by *p19Arf* and *p53* and cooperate with *p19Arf* or *p53* deficiency to promote primary cell transformation.

Rho family small GTPases are molecular switches that transduce diverse intracellular signals leading to cell proliferation, gene induction, and survival as well as cytoskeleton remodeling (7, 46). Many mitogenic signals, including those from growth factor receptors and integrins, can promote the exchange of GDP for GTP on Rho GTPases (56), enabling them to interact with an array of effector targets to elicit specific cellular effects (4). Accumulating evidence has implicated Rho GTPases in many aspects of tumor development (5, 37). RhoA, Rac1, and Cdc42 are proto-oncogene products themselves that when hyperactivated can transform fibroblast cells (31–33). Activation of these Rho proteins can stimulate transcriptional activation of some of the critical genes involved in cell growth regulation, such as nuclear factor κB and cyclin D1 and leads to cell cycle progression (49–52). These Rho family members are required for Ras transformation (3, 17, 23, 58), and their deregulation correlates with poor cancer prognosis in some cases (37). Moreover, the Rho GTPases appear to be intimately associated with morphological changes of tumor cells and have been linked to tumor cell migration and invasion through their ability to regulate actin cytoskeleton, cellular-extracellular matrix adhesion, and cell-cell communication (7, 15, 41).

The p53 cell cycle inhibitor and its regulators, including $p19^{ARF}$, are well-established tumor suppressors that are components of a complex signaling network central to tumor suppression (13, 18, 43). Deletion or mutation in p53 or its regulators occurs in many tumor cases and correlates with the onset of a wide spectrum of cancers. p53 is a key transcription factor essential for the response to cellular stress from DNA damage, hypoxia, and oncogene activation. When activated, p53 can trigger cell cycle arrest or apoptosis (2, 18), whereas $p19^{ARF}$ may serve as a sensor to oncogenic insult to stabilize p53 by sequestering Mdm2, a negative regulator of p53 activity (43). The $p19^{ARF}$-p53 tumor suppressor pathway therefore is thought to be primarily involved in monitoring proliferation signals to prevent cells from uncontrolled growth (43). For example, it has been well documented that excess of mitogenic signals can turn on Ras, which in turn transiently stimulates p53 activity to induce cell cycle arrest, apoptosis, or senescence (8, 22, 42).

With appreciation of a central role of the p53 pathway in tumor suppression and the critical involvement of Rho GTPases in cell cycle progression, it seems logical to envision a functional connection and/or cooperation between the p53 pathway and Rho GTPase-mediated signaling processes in tumorigenesis. In particular, we are interested in determining the contribution of Rho family members to cell behaviors in a genetic background bearing defects of p53 or its regulators that might better represent that of tumor cells. Previously, we have shown that the $p19^{Arf}$-p53 pathway negatively modulates PI 3-kinase and Rho GTPase activities and regulates actin cytoskeleton and cell migration through the PI 3-kinase–Rac GTPase signaling module (12). To investigate the potential contribution of Rho GTPases to $p19^{Arf}$- and p53-mediated cell growth control, in the present study we have further charac-

* Corresponding author. Mailing address: Children's Hospital Research Foundation, Experimental Hematology, 3333 Burnet Ave., Cincinnati, OH 55229. Phone: (513) 636-0595. Fax: (513) 636-3768. E-mail: yi.zheng@chmcc.org.

terized the relationship between the p19^{Arf}-p53 tumor suppressor pathway and Rho proteins in regulating cell proliferation and gene expression. The possible cooperative nature of the p19^{Arf}-p53 pathway defect with hyperactive Rho GTPases in inducing cell hyperproliferation and transformation was examined in *p19Arf*- or *p53*-null mouse embryo fibroblasts (MEFs). Moreover, we have examined the contribution of distinct effector pathways emanating from active RhoA to the transformation of p53$^{-/-}$ cells. Our findings strongly indicate that the Rho family GTPases, Rac1, RhoA, and Cdc42, contribute to cell growth regulation through *p19Arf* and *p53* and that mitogenic activation of the Rho proteins may further cooperate with *p19Arf* or *p53* deficiency to promote cell transformation.

MATERIALS AND METHODS

DNA constructs. The Rac1, RhoA, and Cdc42 dominant negative mutants (Rac1N17, RhoAN19, and Cdc42N17), fast-cycling mutants (Rac1L28, RhoAL30, and Cdc42L28), and constitutively active mutants (Rac1L61, RhoAL63, and Cdc42L61) and the effector domain mutants of RhoA in the constitutively active backbone (RhoAL63-V39, RhoAL63-T40, RhoAL63-L40, and RhoAL63-C42) were generated by site-directed mutagenesis based on oligonucleotide-mediated PCR (19). For retroviral expression, cDNAs encoding the dominant negative, fast-cycling, and constitutively active forms of Rac1, RhoA, and Cdc42, the effector domain mutants of RhoA, and ROCKI were ligated into the *Bam*HI and *Eco*RI sites in frame with the nucleotides encoding a three-hemagglutinin (HA_3) tag at the 5′ end of the retroviral vector MIEG3 that expresses enhanced green fluorescent protein bicistronically. The constructs expressing *p19Arf* and *p53* were described previously (57).

Cell culture and retroviral induction. Primary wild type, *p53*$^{-/-}$ and *p19Arf*$^{-/-}$ MEFs were kind gifts from Martine Roussel (St. Jude, Memphis, Tenn.) that were derived from mouse embryos of the C57BL/6 × 129/sv genetic background (57) and were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 55 μM β-mercaptoethanol, and 10 μg of gentamicin/ml. Recombinant retroviruses were produced using the Phoenix cell packaging system (11, 12). Primary MEFs were infected with the respective retroviruses and harvested 48 to 72 h postinfection. The enhanced green fluorescent protein-positive cells were isolated by fluorescence-activated cell sorting (FACS).

Immunoblotting. Whole-cell lysates were prepared by extraction of the MEF cells by the lysis buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, and 0.5 mM dithiothreitol for 30 min. The nuclear proteins were purified by the method described before (14). Briefly, cells were washed in a hypotonic buffer (HB; 25 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$, 5 mM KCl) and lysed in HB containing 0.25% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml for 30 min. The lysates were centrifuged at 500 × *g* for 5 min. The nuclear pellet was washed with HB containing 2 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml, resuspended in a solution containing 20 mM Tris-HCl (pH 8.0), 0.42 M NaCl, 1.5 mM $MgCl_2$, and 25% glycerol, vortexed, and incubated at 4°C for 30 min. The extracts were centrifuged at 900 × *g* for 5 min, and the supernatants were taken as the nuclear protein lysates. Protein contents in the whole-cell lysates and nuclear lysates were normalized by the Bradford method. The lysates were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The ectopic expression of the dominant negative, the fast-cycling, or the constitutively active forms of Rac1, RhoA, and Cdc42 were probed by using an anti-HA antibody (Boehringer Mannheim). NF-κB and cyclin D1 from the nuclear extracts were probed by using the anti-NF-κB p65 and anti-cyclin D1 antibodies (Santa Cruz Biotechnology), respectively.

Endogenous Rho GTPase activity assay. Glutathione *S*-transferase (GST)–PAK1, GST-Rhotekin, and GST-WASP, which contain Rac1, RhoA, and Cdc42 interactive domains of PAK1, Rhotekin, and WASP, respectively, were used to probe the endogenous Rac1-GTP, RhoA-GTP, and Cdc42-GTP activities by the affinity precipitation method as previously described (20).

PI 3-kinase assay. The endogenous PI 3-kinase activities of *Arf*$^{-/-}$, *p53*$^{-/-}$, and *Arf*$^{-/-}$ or *p53*$^{-/-}$ cells reconstituted with *Arf*, *p53*, or Rho protein mutants, respectively, were assayed according to a described protocol (12). Briefly, the cells were lysed in buffer A, containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.3 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 0.1 mM sodium orthoranadate, and 25 mM NaF. After centrifugation at 4°C for 30 min at 14,000 rpm, the protein contents in the supernatant of cell lysates were measured by the Bradford method. Equal amounts of protein were incubated with anti-p85 polyclonal antibody coupled to protein A-agarose (Upstate Biotech., Inc.) overnight or subjected to anti-p85 Western blot analysis. The immunoprecipitates were washed twice with buffer C, containing 20 mM Tris-HCl (pH 8.0) 100 mM NaCl, and 10 mM $MgCl_2$ and washed once with a kinase assay buffer (20 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$). Five micrograms of sonicated phosphatidylinositol (PI) together with [γ-^{32}P]ATP (200 μCi/ml) in 45 μl of the kinase assay buffer was incubated with the washed beads at 25°C for 10 min. The reactions were terminated by the addition of 100 μl of 1 N HCl. The reaction products were extracted by 200 μl of $CHCl_3$–MeOH (1:1) and resolved on a thin-layer chromatographic silica plate coated with potassium oxalate in a solvent containing $CHCl_3$–MeOH–4 M NH_4OH (9:7:2). The PI kinase reactions were analyzed by autoradiography. The anti-p85 of PI 3-kinase antibody was obtained from Upstate Biotechnology, Inc., and the PI 3-kinase inhibitor wortmannin was obtained from Sigma.

Cell proliferation assay. Cell growth rates were measured by a [^{3}H]thymidine incorporation assay. Cells were cultured in a medium containing 2% serum for the assays. The cell cultures were assayed at 0, 1, 2, 3, and 4 days after the addition of 1 μCi of [^{3}H]-thymidine/ml to the medium followed by an incubation for 4 h at 37°C. The cells were harvested by trypsinization, and the [^{3}H]-thymidine incorporated into the cells was quantified by scintillation counting.

Luciferase reporter assay. To detect endogenous NF-κB and cyclin D1 gene expression, the luciferase reporter constructs fused with the promoter of NF-κB or cyclin D1 (Stratagene) that contain the promoter response elements of NF-κB and cyclin D1 were used to report transiently the relative activities of NF-κB and cyclin D1. Transient transfection of these reporter plasmids into primary MEFs was carried out using LipofectAMINE reagents (Invitrogen) according to the manufacturer's protocols. Twenty-four hours prior to harvesting, the cells were switched to a medium containing 0.5% serum. Analysis of luciferase and β-galactosidase activities of the transfected cells was performed by using a luciferase assay kit (Promega). Transfection efficiencies were routinely corrected by obtaining the ratio of the luciferase and the β-galactosidase activities observed in the same sample, as previously described (26).

Cell cycle progression and apoptosis analysis. Cell cycle progression of the p53$^{-/-}$ MEFs was monitored by cell cycle marker staining (propidium iodide labeling and Cytofix/Cytoperm fixation) followed by flow cytometry (phosphatidylethanolamine-conjugated anti-BrdU antibody) to examine whether dominant negative or constitutively active RhoA could affect the G_1/S phase and/or G_2/M phase transition in a p53-independent manner (11). The effects of RhoA mutants on p53-independent apoptotic response were tested by apoptosis staining of the p53$^{-/-}$ MEFs after DNA damage induction by gamma irradiation (20 Gy). The apoptotic cell population was revealed by 7AAD and allophycocyanin-conjugated Annexin V staining followed by flow cytometry analysis on a FACSCaliber machine (11).

Cell transformation assay. To determine the transforming activity of the *p53*$^{-/-}$ or *p19Arf*$^{-/-}$ MEF cells, 5,000 cells that stably express various fast-cycling or constitutively active mutants of Rac1, RhoA, or Cdc42 were combined with 5 × 10^4 parental *p53*$^{-/-}$ or *p19Arf*$^{-/-}$ cells. The cell cultures were fed every 2 days with fresh culture medium. Fourteen days postplating, foci were scored after fixation and staining of the cells on the plates.

RESULTS

p19Arf and p53 regulate PI 3-kinase, which in turn regulates Rho GTPase activities. Previously, we found that the endogenous PI 3-kinase, RhoA, and Rac1 activities were elevated in the *Arf*$^{-/-}$ and *p53*$^{-/-}$ primary MEF cells and that the PI 3-kinase and Rac1 activities were required for a fast-migration phenotype of the *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells. Reintroduction of the wild-type *Arf* or *p53* gene into *Arf*$^{-/-}$ or *p53*$^{-/-}$ cells reversed the PI 3-kinase and Rho GTPase activities as well as the migration phenotype, indicating that p19^{Arf} and p53 negatively regulate cell migration by suppression of PI 3-kinase and Rac1 activities (12). To further dissect the relationship between PI 3-kinase and Rho GTPases in the *p19Arf*$^{-/-}$ and

p53$^{-/-}$ cells, we measured the Rac1, RhoA, and Cdc42 activities in the *Arf*$^{-/-}$ and *p53*$^{-/-}$ MEFs with or without the PI 3-kinase inhibitor (wortmannin) treatment. As shown in Fig. 1A, wortmannin (50 nM) treatment of the *Arf*$^{-/-}$ and *p53*$^{-/-}$ MEFs resulted in markedly decreased Rac1-GTP and RhoA-GTP species, to a level similar to that in the *Arf*- or *p53*-reconstituted cells or the wild-type cells (Fig. 1A). As we have observed previously, the Cdc42-GTP level was not significantly affected by the deletion of the *Arf* or *p53* gene, nor was it changed upon wortmannin treatment or *Arf* or *p53* reconstitution (Fig. 1A). On the other hand, the elevated endogenous PI 3-kinase activity in the *p53*$^{-/-}$ cells (compared with that of the wild-type MEFs) was not significantly altered by the expression of dominant negative Rac1N17, RhoAN19, or Cdc42N17 or by the fast-cycling Rac1L28, RhoAL30, or Cdc42L28 mutant, contrary to the effect of the reconstitution of wild-type p53 (Fig. 1B). Similarly, the dominant negative Rho protein mutants had no effect on the PI 3-kinase activity of the *Arf*$^{-/-}$ cells (data not shown). These results indicate that PI 3-kinase acts downstream of *p19Arf* or *p53* but upstream of Rho proteins to regulate cell behaviors.

Rho GTPases contribute to the growth phenotype of p19Arf$^{-/-}$ and p53$^{-/-}$ cells. p19^{Arf} and p53 are checkpoint molecules that upon overexpression can induce cell cycle arrest or apoptosis (43). Deletion of *Arf* or *p53* resulted in a significant increase in cell growth rate and saturation density (Fig. 2). Since the *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells contained elevated levels of active Rho GTPase species (Fig. 1A), we examined whether Rac1, RhoA, or Cdc42 might contribute to the proliferative phenotype of these cells. As shown in Fig. 2, the increased cell growth rates due to *p19Arf* or *p53* deletion were partially inhibited by the dominant negative Rac1N17, RhoAN19, and Cdc42N17 mutants to various degrees under conditions at which the growth rate of wild-type MEFs was not significantly affected by these mutants. The expression levels of the dominant negative Rho mutants were comparable in these cells (12; data not shown), and the dominant negative inhibitory effects of Rac1N17, RhoAN19, and Cdc42N17 were specific toward the respective Rho proteins, as assayed by the effector domain pull-down assays (Fig. 2B). Although Cdc42 activity was not detectably upregulated by the *Arf* or *p53* deficiency (Fig. 1A), the fact that Cdc42N17 could partially inhibit the growth phenotype of the *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells suggests either that the effector probe (the p21-binding domain of WASP) used in the activity assay was not sensitive enough or that basal Cdc42 activity is required for the *Arf* or *p53* defect-mediated growth. These results indicate that part of the growth stimulatory signals unleashed by *p53* or *p19Arf* deletion are mediated through the Rho family members.

Involvement of Rho GTPases in NF-κB and cyclin D1 activation induced by p19Arf or p53 deficiency. Transcription factor NF-κB has been shown to be functionally interconnected with p53 (36, 48). Since NF-κB is regulated by the Rho family GTPases and could mediate cell growth regulation by the Rho proteins (14, 29, 52), we determined the effect of *p19Arf* or *p53* deletion on the activity and expression of NF-κB and examined the contribution of Rac1, RhoA, and Cdc42 to its modulation in the *Arf*- or *p53*-null background. The *p19Arf*$^{-/-}$ and *p53*$^{-/-}$ cells were transiently transfected with the NF-κB-luciferase reporter plasmid that contains the promoter response elements of NF-κB, and the relative luciferase activities were compared with that of wild-type MEFs and that of *Arf*$^{-/-}$ or *p53*$^{-/-}$ cells reconstituted with the respective tumor suppressor genes. The NF-κB transcriptional activity was upregulated by *p19Arf* and *p53* deletions by ~3.5- and ~5.5-fold, respectively, and the observed activity changes were completely reversed when the *Arf* or *p53* gene was reintroduced into the knockout cells (Fig. 3A). These results demonstrate that p19^{Arf} and p53 negatively regulate NF-κB activity. To address whether the Rho proteins contribute to NF-κB regulation in *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells, we transduced the dominant negative mutants of Rac1, RhoA, and Cdc42 (Rac1N17, RhoAN19, and Cdc42N17, respectively) as well as the GFP marker into the mutant cells and assayed the NF-κB reporter activities of these cells. Rac1N17 and Cdc42N17 had no detectable effect on the elevated reporter activities of NF-κB in *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells (Fig. 3B), nor did they show significant inhibition of the increased nuclear expression level of NF-κB in these cells (Fig. 3C). In comparison, RhoAN19 attenuated the NF-κB activity and its nuclear expression level in *p53*-deficient cells but not in *p19Arf*-deficient cells (Fig. 3B and C). The dominant negative Rac1N17, RhoAN19, and Cdc42 N17 mutants were expressed similarly in these MEFs (12) and displayed various degrees of inhibition on the respective Rho protein activity and cell growth (Fig. 2). Moreover, Rac1N17 reversed the migration phenotype of these cells (14), indicating that these dominant negative Rho mutants were functionally expressed. These results suggest that RhoA participates in NF-κB regulation by p53 but that Rac1 and Cdc42 do not contribute to the upregulation of its activity, due to *p19Arf* or *p53* defects.

Cyclin D1 has been suggested as one of the key downstream effectors that mediate cell growth control by the Rho GTPases (49, 50, 51, 58). Deletion of *p19Arf* and *p53* resulted in significant upregulation of cyclin D1 activity (~3- and ~11-fold, respectively) and nuclear expression (Fig. 4A and C). Reconstitution of *p19Arf* and *p53* into the respective mutant cells readily reversed the cyclin D1 activity (Fig. 4A), indicating that both p19^{Arf} and p53 negatively regulate its activity. Since Rac1, RhoA, and Cdc42 can each stimulate cyclin D1 activation and deletions of *p19Arf* and *p53* result in activation of the Rho protein activities, we examined the possible involvement of the Rho GTPases in cyclin D1 regulation by *Arf* and *p53*. Transduction of dominant negative RhoA and Cdc42 in *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells partially inhibited cyclin D1 at both the activity and the nuclear expression levels, whereas dominant negative Rac1 had no detectable effect (Fig. 4B and C). These results provide evidence that RhoA and Cdc42 but not Rac1 are involved in cyclin D1 regulation by p19^{Arf} and p53. It is therefore possible that RhoA and Cdc42 work through NF-κB and/or cyclin D1 modulation to contribute to the growth phenotype of the *p19Arf*$^{-/-}$ and *p53*$^{-/-}$ cells, while Rac1 adopts a distinct, NF-κB- and cyclin D1-independent pathway to influence *p19Arf*$^{-/-}$ and *p53*$^{-/-}$ cell growth.

Active Rho GTPases cooperate with p19Arf or p53 deletion to promote hyperproliferation and transformation. Since Rac1, RhoA, and Cdc42 appear to contribute to the growth regulation of *p19Arf*$^{-/-}$ and *p53*$^{-/-}$ cells, we next asked if hyperactive Rho proteins could further stimulate the proliferation of these cells. For this purpose, we introduced a set of

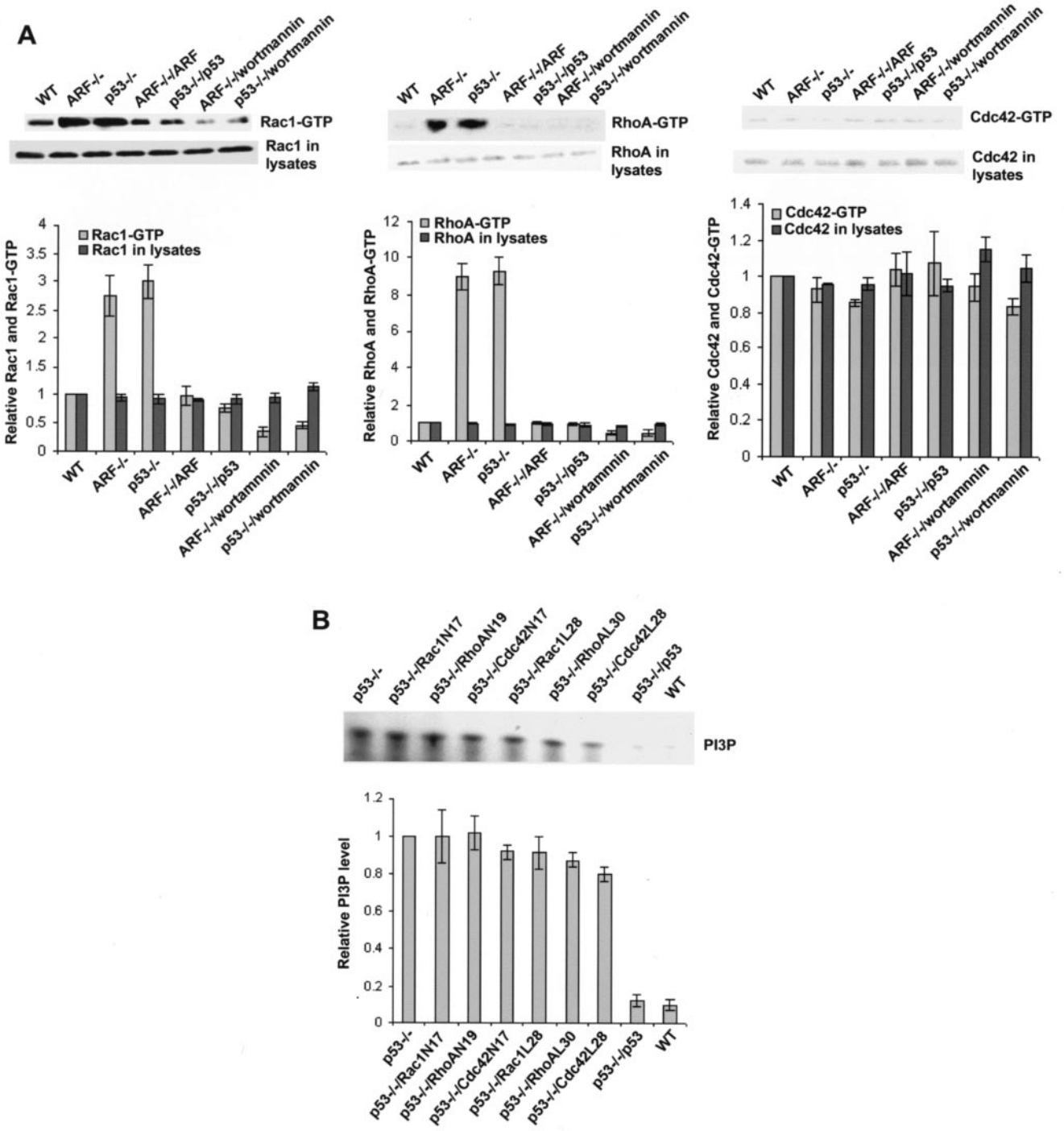

FIG. 1. Activation of Rac1 and RhoA by the p19^{Arf} or p53 defect is dependent on elevated PI 3-kinase activity. (A) The endogenous Rac1, RhoA, or Cdc42 activities in the *Arf* or *p53* knockout and reconstituted cells with or without wortmannin (50 nM) treatment were assayed by using log phase cells that were serum starved for 12 h. The lysates were subject to GST-PAK1, GST-Rhotekin, or GST-WASP pull-down analysis. The amount of Rac1-GTP, RhoA-GTP, or Cdc42-GTP was detected by Western blotting of the respective glutathione-agarose coprecipitates with anti-Rac1, anti-RhoA, or anti-Cdc42 antibody and was normalized to that of Rac1, RhoA, or Cdc42 in wild-type (WT) MEFs. The results are shown as the means ± the standard deviations of three experiments. (B) The PI 3-kinase activities of the *Arf* or *p53* knockout and reconstituted cells of the log phase cells were assayed. The cells were starved in a medium containing 0.5% serum for 12 h and harvested for anti-p85 immunoprecipitation. The PI 3-kinase activities in the immunoprecipitates were measured by an in vitro lipid kinase assay using exogenous PI as the substrate. The PI3P signals of various MEFs were normalized to those of the $p53^{-/-}$ MEFs in the quantification.

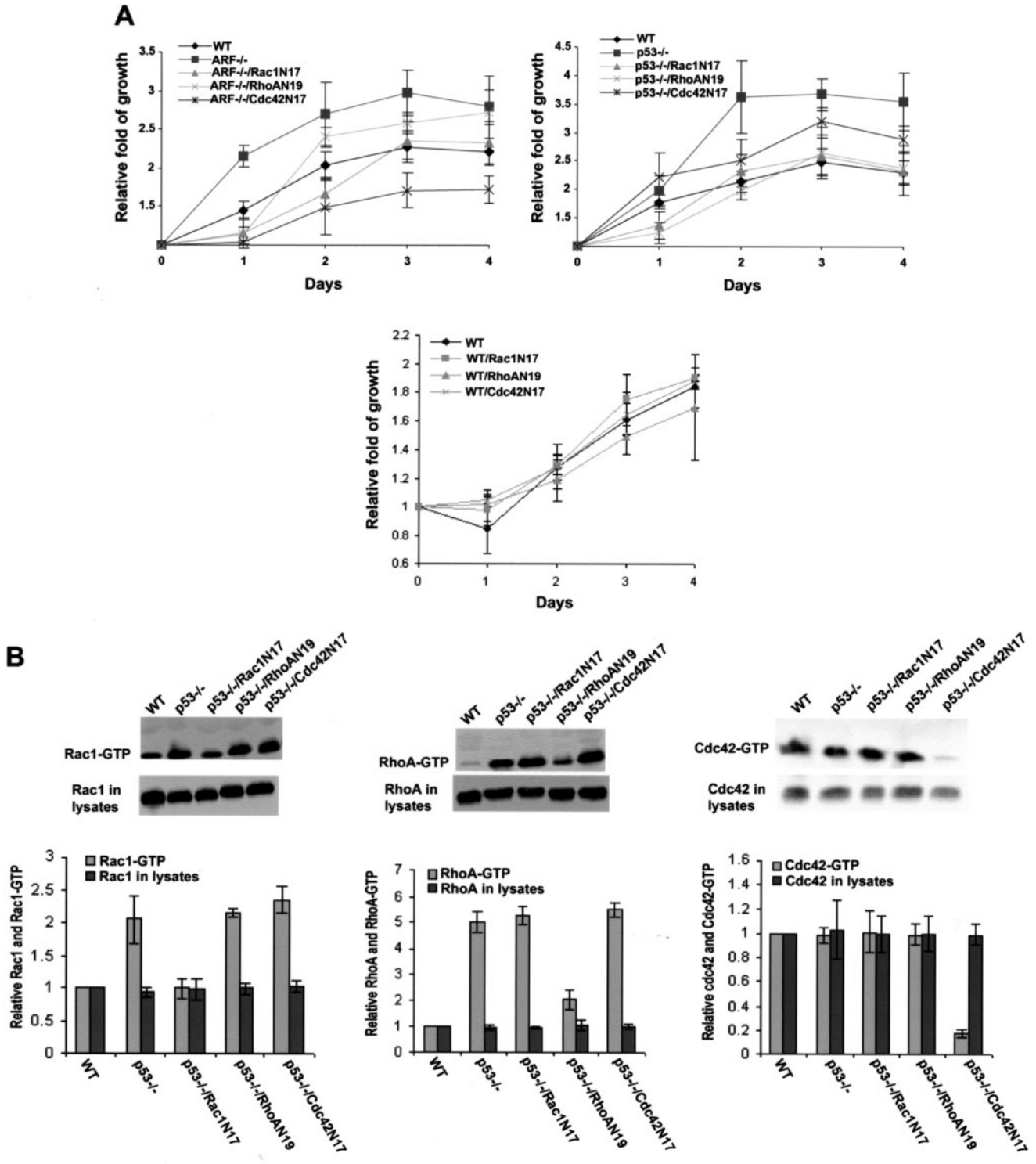

FIG. 2. Rac1, RhoA, and Cdc42 contribute to the growth regulation of *p19Arf*- or *p53*-null cells. The dominant negative mutants of Rho proteins were expressed in the $Arf^{-/-}$ or $p53^{-/-}$ MEFs by retroviral induction, and the Rho mutant-expressing cells were isolated by FACS. (A) Five thousand cells/well of the indicated cells were plated in 1-ml culture medium containing 5% fetal bovine serum on 24-well plates. At the time points of day 0, 1, 2, 3, and 4, incorporation of [^{3}H]thymidine into the cells was measured. Data are representative of three independent experiments and are expressed as the fold of growth relative to the respective value at day 0. Error bars represent the standard deviations of four repeats of one experiment. (B) Western blots of the endogenous Rac1, RhoA, and Cdc42 in the GTP-bound state were performed after the respective GST-effector domain pull-downs in the wild-type (WT) MEFs, p53-deficient MEFs, and p53-deficient MEFs expressing various dominant negative mutants of Rh proteins. The amounts of each Rho protein in the respective cell lysates and in the GTP-bound form were normalized to that of Rac1, RhoA, or Cdc42 in WT MEFs.

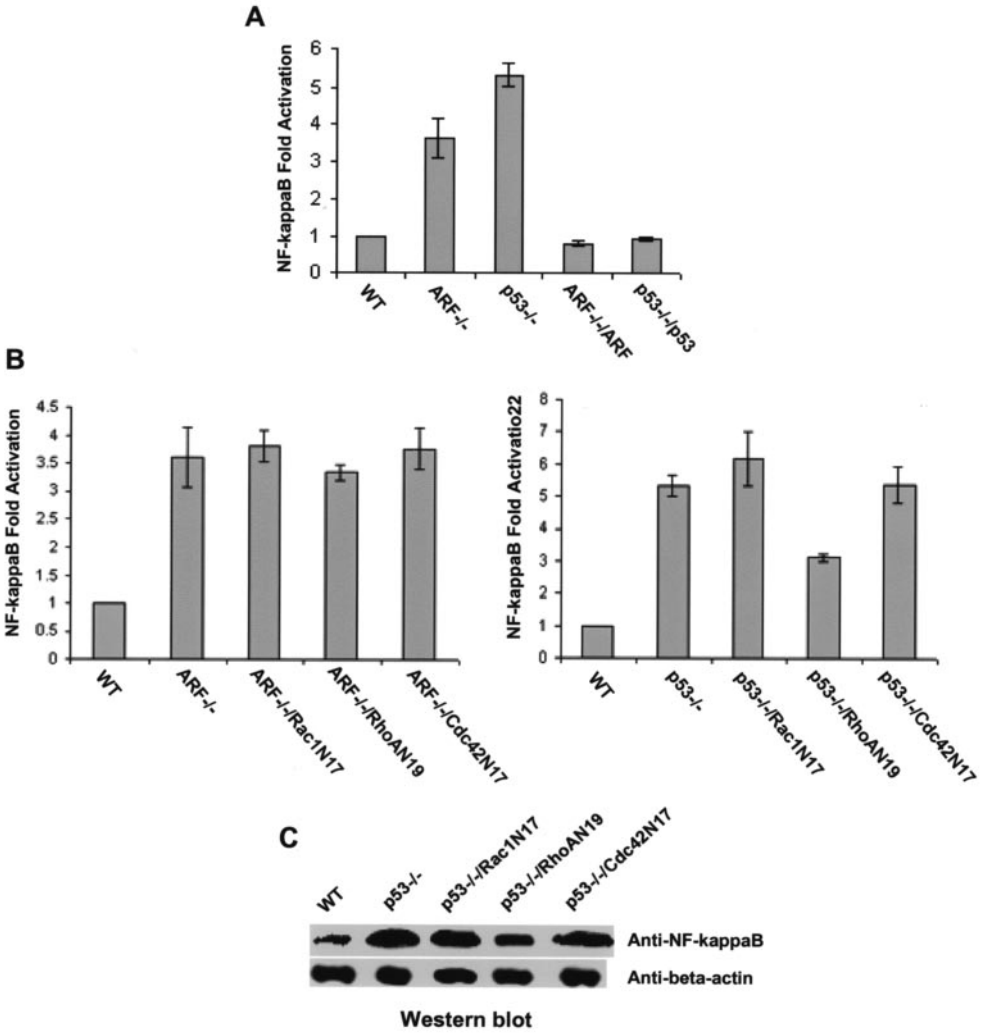

FIG. 3. Involvement of Rac1, RhoA, and Cdc42 in NF-κB activation induced by *p19Arf* or *p53* deletion. The NF-κB promoter-driven luciferase reporter was transiently expressed in the indicated MEF cells together with a vector expressing *Arf*, *p53*, or dominant negative mutants of Rho GTPases (A and B). After a 30-hour recovery followed by a 12-hour starvation, the luciferase activities in the cells were measured. The luciferase activities were expressed as the fold of activation relative to the activity induced by the empty vector alone in the wild-type (WT) cells and were normalized to an internal transfection control (β-galactosidase coexpressed with pCMV vector). To detect NF-κB protein levels (C), nuclear extracts containing 20 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and the amount of NF-κB present was probed by anti-NF-κB p65 antibody. Anti-beta actin blotting was carried out in parallel as a loading control.

fast-cycling mutants of Rac1, RhoA, and Cdc42 (Rac1L28, RhoAL30, and Cdc42L28, respectively), which possess an increased intrinsic rate of exchange of GDP by GTP-mimicking mitogenic stimulation (21, 53), into the *p19Arf*$^{-/-}$ or *p53*$^{-/-}$ cells. The expression of Rac1L28, RhoAL30, and Cdc42L28 were confirmed by Western blot analysis (Fig. 5A). As shown in Fig. 5B, these fast-cycling Rho GTPase mutants were able to further enhance the growth rates of *p19Arf*$^{-/-}$ and *p53*$^{-/-}$ cells to various extents under conditions in which they had only minor effects on wild-type MEF growth, suggesting that active Rho proteins can cooperate with p19^{Arf} and p53 defects to promote hyperproliferation of the cells.

Oncogenic Ras was able to cooperate with *p19Arf* or *p53* deletion to promote MEF transformation in vitro and in vivo (16). Because Rac1, RhoA, and Cdc42 are all important downstream mediators of Ras signaling (17, 31–33), we wondered if the hyperactive Rho proteins could be sufficient to promote *Arf*$^{-/-}$ and *p53*$^{-/-}$ cell transformation. For this purpose, in addition to the fast-cycling Rho protein mutants, we generated *Arf*$^{-/-}$ and *p53*$^{-/-}$ primary MEFs expressing the constitutively active forms of Rac1, RhoA, and Cdc42 (Rac1L61, RhoAL63, and Cdc42L61, respectively). These mutants expressed equally well in the *p53*$^{-/-}$ cells (Fig. 6A) and in the *p19Arf*$^{-/-}$ cells (data not shown). Both forms of the activating Rac1, RhoA, and Cdc42 mutants, i.e., the fast-cycling and the constitutively active forms, displayed various foci-forming activity in *p53*$^{-/-}$ MEFs as well as in *p19Arf*$^{-/-}$ MEFs (Fig. 6B). Some of these mutants, e.g., RhoAL63, displayed activities as potent as that of oncogenic Ras, while others, such as Rac1L61, was only weakly transforming (Fig. 6B). Thus, like oncogenic Ras, active Rac1, RhoA, or Cdc42 can cooperate with *p53* or *p19Arf* deletion to promote primary cell transformation.

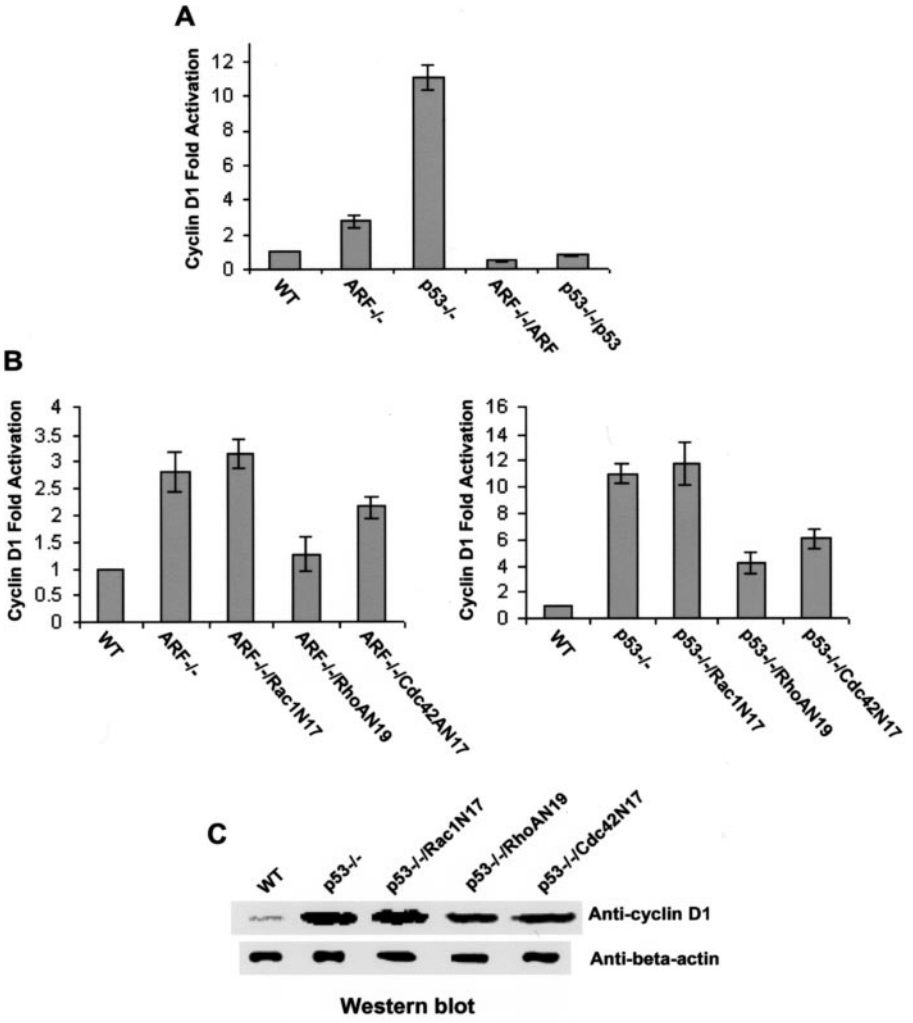

FIG. 4. Contribution of Rac1, RhoA, and Cdc42 to cyclin D1 regulation in *p19Arf*- or *p53*-deficient MEF cells. One microgram of cyclin D1-luciferase reporter plasmid was cotransfected with a vector expressing *Arf*, *p53*, or the dominant negative mutants of the Rho proteins into the indicated cells. The luciferase activities in the cell lysates were measured to determine the relative cyclin D1 transcriptional activities (A and B) and were normalized to those of a β-galactosidase transfection control. To directly compare the protein levels of cyclin D1, nuclear extracts containing 20 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and the amount of cyclin D1 was probed with an anti-cyclin D1 antibody (C). Anti-beta actin blotting was done in parallel. WT, wild-type.

RhoA signaling modulates cell cycle progression and apoptotic response of p53-null cells. To further address the role of Rho in p53-mediated cell proliferation, we carried out a set of experiments comparing the cell cycle and apoptotic properties of $p53^{-/-}$ MEFs expressing the fast-cycling active mutant of RhoA or dominant negative mutant of RhoA. As shown in Fig. 7A, when the cell cycle progression of wild-type and p53−/− MEFs was analyzed by PI staining followed by FACS, the dominant negative RhoA mutant was found to effectively extend the G_1 phase and suppress the G_2/M phase of $p53^{-/-}$ MEFs that were altered due to a p53 defect, whereas the active RhoA mutant did not significantly alter the relative phases of the cell cycle. Moreover, p53 deficiency led to a decrease in the cellular apoptotic response to gamma irradiation compared with that of wild-type cells, which could be further suppressed by the expression of the active RhoA mutant (Fig. 7B). These results indicate that RhoA may contribute to p53-regulated cell proliferation by modulating cell cycle progression and that RhoA activation may cause hyperproliferation of $p53^{-/-}$ cells by further suppressing a p53-independent apoptotic signal.

Multiple pathways regulated by RhoA are involved in promoting transformation of $p53^{-/-}$ MEFs. To begin to unveil the molecular pathways regulated by Rho GTPases that are important for promoting transformation of $p53^{-/-}$ cells, we next examined the involvement of a key effector of RhoA, ROCK, in RhoA-mediated transformation. As shown in Fig. 8A, treatment of the RhoAL63-expressing $p53^{-/-}$ MEFs with a ROCK inhibitor, Y27632, led to a partial inhibition of the foci-forming activity elicited by RhoAL63, and ectopic expression of ROCKI in $p53^{-/-}$ cells was able to only partially recapitulate the transforming activity. To confirm the contribution of ROCK to RhoA-mediated transformation and to assess the involvement of additional effector pathways downstream of RhoA, we further tested a set of effector-domain mutants of

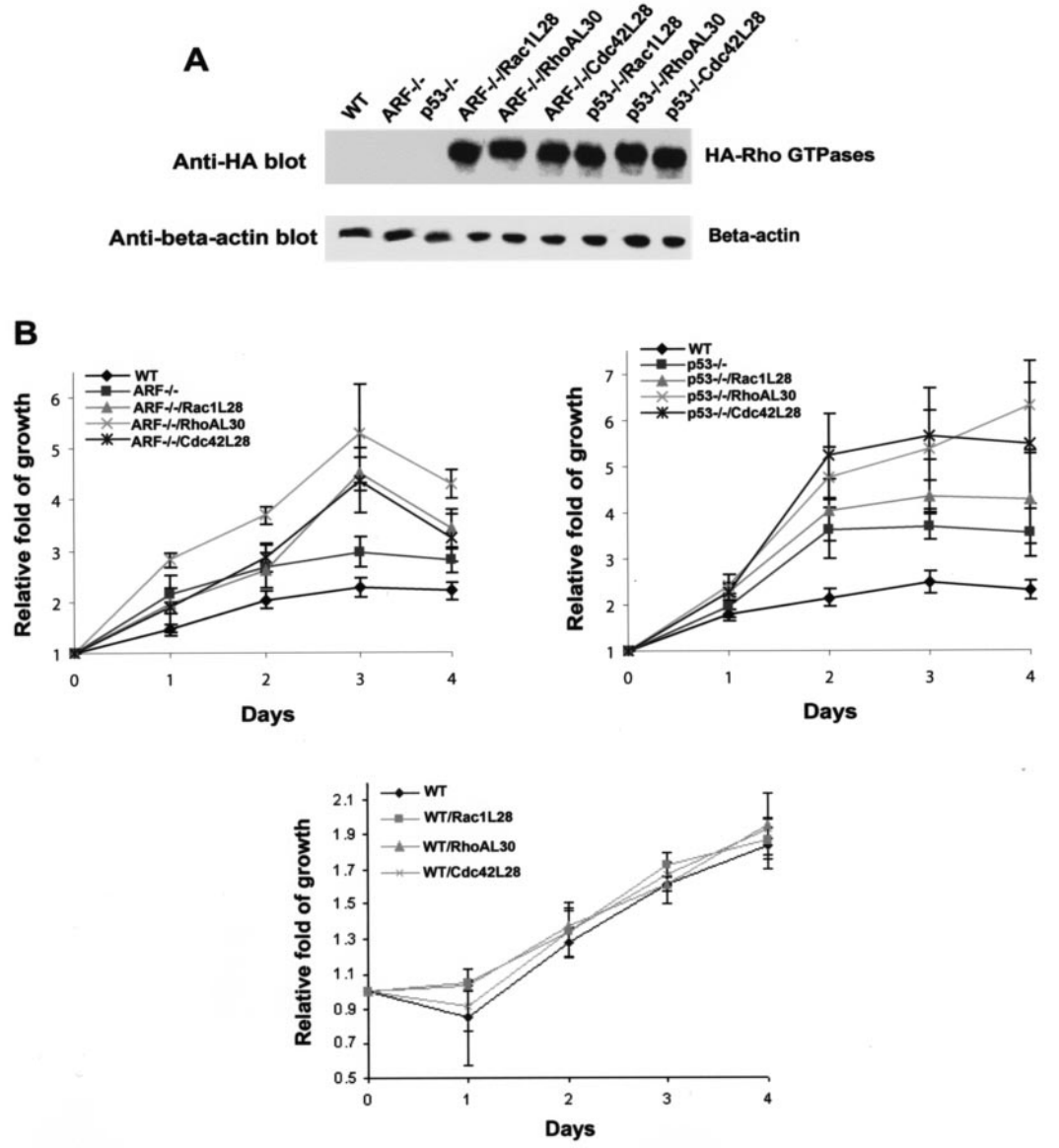

FIG. 5. The active Rac1, RhoA, and Cdc42 mutants can stimulate hyperproliferation of *p19Arf*- or *p53*-null cells. (A) Expression of the fast-cycling mutants of Rac1, RhoA, and Cdc42 that carry an N-terminal HA tag in *p19Arf*- or *p53*-null cells was probed by anti-HA Western blotting. (B) The indicated cells were plated in a culture medium containing 5% fetal bovine serum. At the indicated time points, the amount of incorporated [^{3}H]thymidine was quantified by scintillation counting. The data are representative of three independent experiments and are presented as the fold of growth relative to the respective cells at day 0. Error bars represent the standard deviations of four repeats. WT, wild type.

RhoA for their ability to induce transformation of $p53^{-/-}$ primary MEFs. As previously characterized in vitro (10, 38) and depicted in Fig. 8B, RhoA-F39V is defective in PKN binding but retains ROCK binding, RhoA-E40L is defective for ROCK recognition, RhoA-E40T retains ROCK and PKN binding but is defective in kinectin and mDia binding, and RhoA-Y42C is selectively defective in PKN binding. Consistent with the partial loss of transforming activity in the case of ROCK inhibitor-treated cells, RhoAL63-L40 that is defective in ROCK binding suffered partial loss of foci-forming activity (Fig. 8C). Since ROCK was found to be significantly upregulated in the mRNA level in the RhoA-transduced $p53^{-/-}$ cells in a gene array assay (unpublished data), these results strongly suggest that the RhoA-ROCK pathway is required for the RhoA-induced transformation of primary $p53^{-/-}$ MEFs, corroborating previous findings with NIH 3T3 cells (39). Interestingly, the RhoA mutant that retained ROCK and PKN binding but was defective for kinectin and mDia and possibly other effector pathways (RhoAL63-E40T) was partially active in transforming $p53^{-/-}$ cells, whereas the RhoA mutants that have lost PKN-binding ability (RhoA-F39V and RhoA-Y42C) remained partially or fully active (Fig. 8C). Therefore, additional effector pathways other than ROCK that emanated from active RhoA but not PKN appear to also be involved in the RhoA-mediated transformation of $p53^{-/-}$ cells. These results provide clues on the contribution of RhoA-regulated signaling

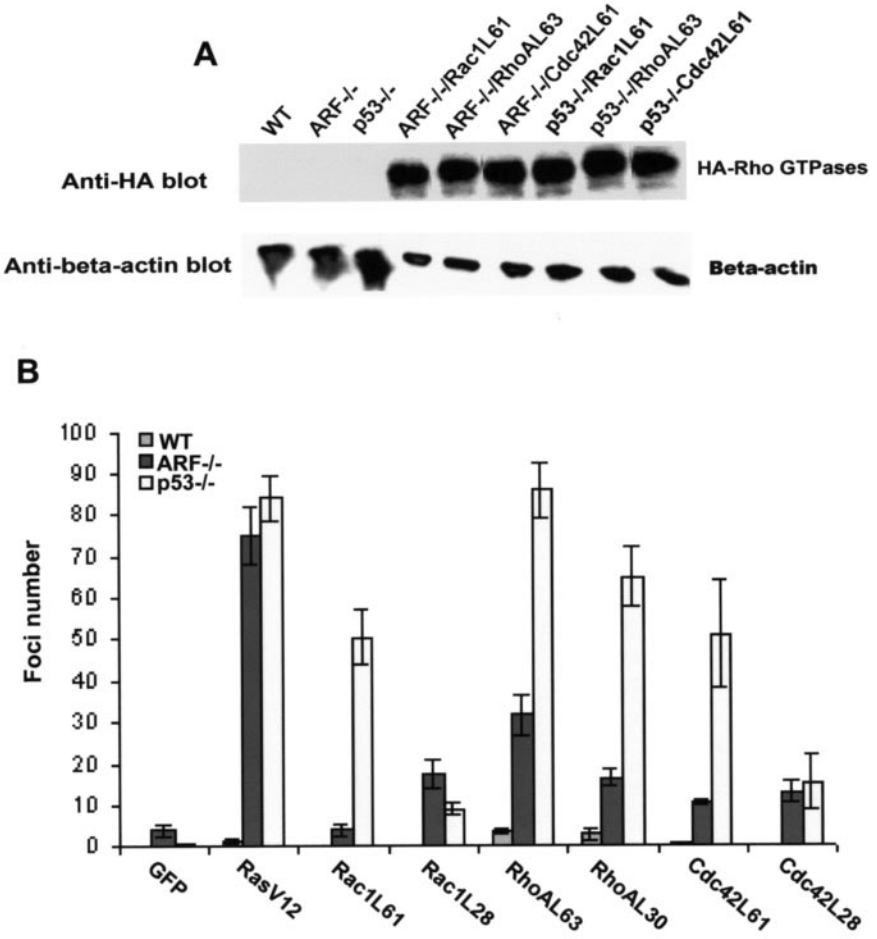

FIG. 6. The active Rac1, RhoA, and Cdc42 mutants cooperate with *p19Arf* or *p53* deletion to promote cell transformation. (A) Expression of the constitutively active mutants of Rac1, RhoA, and Cdc42 containing an N-terminal HA tag in *p19Arf*- or *p53*-null cells was probed by anti-HA Western blotting. (B) Five thousand MEF cells expressing the indicated proteins were mixed with 5×10^4 parental $p19Arf^{-/-}$ or $p53^{-/-}$ cells and cultured in 100-mm plates. The cell cultures were fed every 2 days with fresh culture medium. Fourteen days postplating, the foci were fixed, stained, and quantified under a microscope. The data are representative of two independent experiments. WT, wild type.

cascades to the transformation phenotype of a primary cell system.

DISCUSSION

In the present study, we demonstrate that Rho family GTPases Rac1, RhoA, and Cdc42 contribute to $p19^{Arf}$- and p53-regulated gene transcription and cell growth and that activation of these Rho GTPases can cooperate with p19Arf or p53 defects to promote hyperproliferation and transformation. The contributions of RhoA and Cdc42 to the growth phenotype of *p19Arf*- and *p53*-deficient cells may come in part through modulation of the transcriptional activities of a few key cell growth regulators, including the transcription factor NF-κB and the cell division kinase regulator cyclin D1, whereas Rac1 appears to be involved in p19Arf- p53 mediated cell growth independently of NF-κB and cyclin D1. Significantly, we show that RhoA is involved in p53-regulated cell proliferation by modulating the cell cycle and a p53-independent apoptotic signal and that multiple RhoA-regulated pathways, including that of ROCK, appear to be important for promoting transformation of $p53^{-/-}$ cells. Although more detailed mechanisms of the connection and cooperativeness between the Rho proteins and the $p19^{Arf}$-p53 tumor suppressor pathway remain to be explored, these results help establish an important functional relationship of Rho GTPases with the $p19^{Arf}$-p53 pathway, defects of which occur in many cases of human cancer (13, 43). The findings may have important implications for strategies that target Rho proteins in anticancer therapy.

Previous studies have shown by genetic disruption of the tumor suppressor genes that $p19^{ARF}$ and p53 but not $p27^{Kip1}$ or pRb have a profound negative effect on cell motility and migration (12). $p19^{ARF}$ likely depends on p53 for cell migration regulation, and a specific transcriptional activity or specific target genes controlled by p53 may serve as the link between p53 and actin cytoskeleton to promote the migration phenotype. In particular, we found that the endogenous PI 3-kinase and Rho GTPase activities were significantly elevated in $Arf^{-/-}$ and $p53^{-/-}$ cells and that both PI 3-kinase and Rac1 were required for the $p19^{Arf}$- and p53-regulated migration (12). In the present study, we further determined the relation-

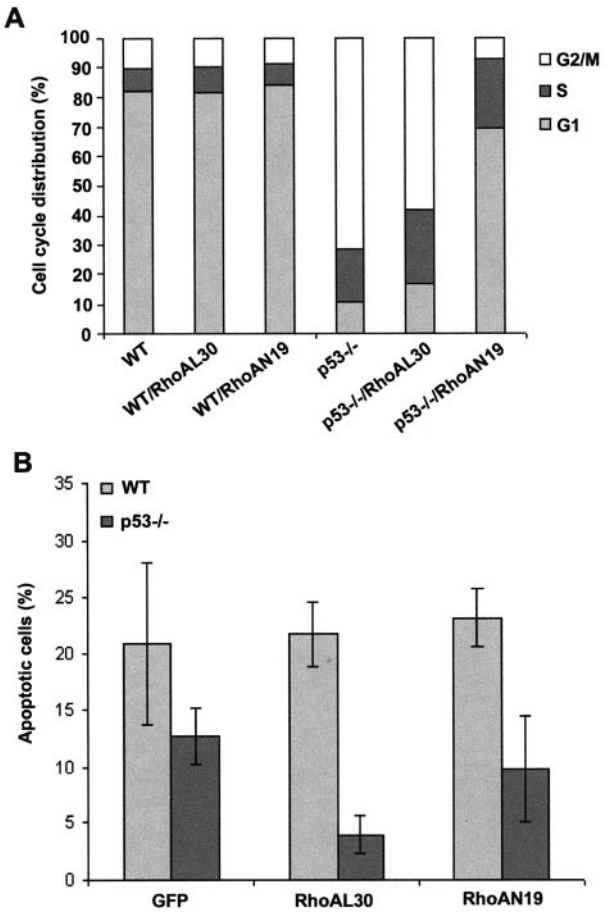

FIG. 7. Dominant negative RhoA affects cell cycle progression of $p53^{-/-}$ MEFs, while active RhoA suppresses gamma irradiation-induced apoptosis. (A) $p53^{-/-}$ cells transduced with GFP alone or with retrovirus expressing RhoAL30 or RhoAN19 were analyzed by FACS for cell cycle progression after PI staining in a culture medium containing 5% fetal bovine serum. The data are representative of the results of two independent experiments. (B) Various retroviral transduced MEF cells were analyzed by FACS for apoptotic cell population after gamma irradiation (20 Gy). Wild-type (WT) MEFs transduced with GFP alone were compared in parallel.

ship between PI 3-kinase and Rac1/RhoA activities in *p19Arf*- and *p53*-deficient MEFs and revealed that activation of Rac1/RhoA by a p19^{Arf} or p53 defect depended on the elevated PI 3-kinase activity and did not occur if PI 3-kinase activity was not elevated. Therefore, a pathway initiated from p19^{Arf} or p53 deficiency could lead to PI 3-kinase activation, which in turn activates Rac1 and RhoA (Fig. 9). We are currently examining the candidate genes whose expression profiles are altered by p19^{ARF} and p53 deficiencies in an effort to identify the molecule(s) responsible for PI 3-kinase and Rho GTPase activation. One possibility is that the gene(s) controlled by p53 (55) provides an autocrine mechanism that feeds back to stimulate the cells leading to PI 3-kinase activation and subsequently to Rho GTPase activation.

Aberrant activation of Rho GTPases can promote cell hyperproliferation and growth transformation (3, 5, 25, 58). The mechanism of Rho protein-stimulated cell growth leading to transformation appears to be at least twofold: activation of cell cycle promoting regulators such as cyclin D1 (49, 50) and inhibition of negative regulators of cell cycle progression such as p21^{Cip1} and p27^{Kip1} (28, 47). We found that *Arf* and *p53* deletion caused a markedly increased cyclin D1 activity which is in part dependent on RhoA and Cdc42, suggesting that these Rho proteins contribute to the growth phenotype of the $Arf^{-/-}$ and $p53^{-/-}$ cells by transducing signals to stimulate cyclin D1 activity. Surprisingly, Rac1 does not appear to be involved in the cyclin D1 regulation by p19^{Arf} or p53, but it must employ another mechanism to affect the proliferation of these cells, because Rac1 is required for the increased proliferation of the $Arf^{-/-}$ and $p53^{-/-}$ cells.

A number of mechanisms have been proposed for cyclin D1 activation and growth regulation by Rho GTPases. Rac1 and RhoA can activate NF-κB, which in turn activates cyclin D1 (14, 29). Rac1 and Cdc42 might promote extracellular signal-regulated kinase 1 and 2 activation by means of PAKs, which can directly phosphorylate and activate Raf and MEK (51). In addition, Rac1-mediated activation of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase cascades can lead to increased phosphorylation and activity of the AP-1 components Jun and ATF (58), and the recently identified Cdc42-Par6 atypical protein kinase C pathway may also stimulate NF-κB, causing cyclin D1 activation (7). We have observed that RhoA but not Rac1 or Cdc42 plays a role in NF-κB upregulation by *Arf* or *p53* deletion, raising the possibility that the contribution of RhoA to the growth phenotype of the cells may be due partly to activation of NF-κB, which in turn stimulates cyclin D1. The differential regulation of NF-κB and cyclin D1 activities by RhoA, Rac1, and Cdc42 in the $p19Arf^{-/-}$ and $p53^{-/-}$ cells suggests that each member of Rho family GTPases may utilize a distinct mechanism to contribute to the growth phenotype. The finding that Rac1 does not appear to contribute to the p19^{Arf}- or p53-mediated NF-κB or cyclin D1 regulation is somewhat surprising, since previous work by a number of laboratories using cloned fibroblast cell lines have implicated Rac1 as one important regulator of NF-κB and cyclin D1 activity (29, 50, 52). It is likely that the involvement of Rac1 in these transcription processes is cell context and pathway specific. Furthermore, our observations that individual Rho proteins may be involved differentially in p19Arf- and p53-regulated gene induction and cell proliferation add further evidence that p19^{Arf} and p53 have overlapping and interdependent as well as independent functions in the regulation of cell growth (24, 34, 43).

Whether the Rho proteins, Rac1 and Cdc42 in particular, can further regulate cell growth through suppression of cell cycle inhibitors such as p21^{Cip1} or p27^{Kip1} in the $Arf^{-/-}$ and $p53^{-/-}$ background remains to be examined further. It is clear, however, that RhoA could have an impact on cell proliferation in both a p53-dependent and a p53-independent manner through modulation of cell cycle and apoptotic machineries, given our observation that the dominant negative RhoA mutant could effectively extend the G_1 phase and suppress the G_2/M phase of $p53^{-/-}$ MEFs, while the active RhoA mutant could suppress the gamma irradiation-induced apoptosis independently of p53.

Constitutively active or fast-cycling Rho GTPase mutants, as well as many of their GEFs, can induce foci formation or

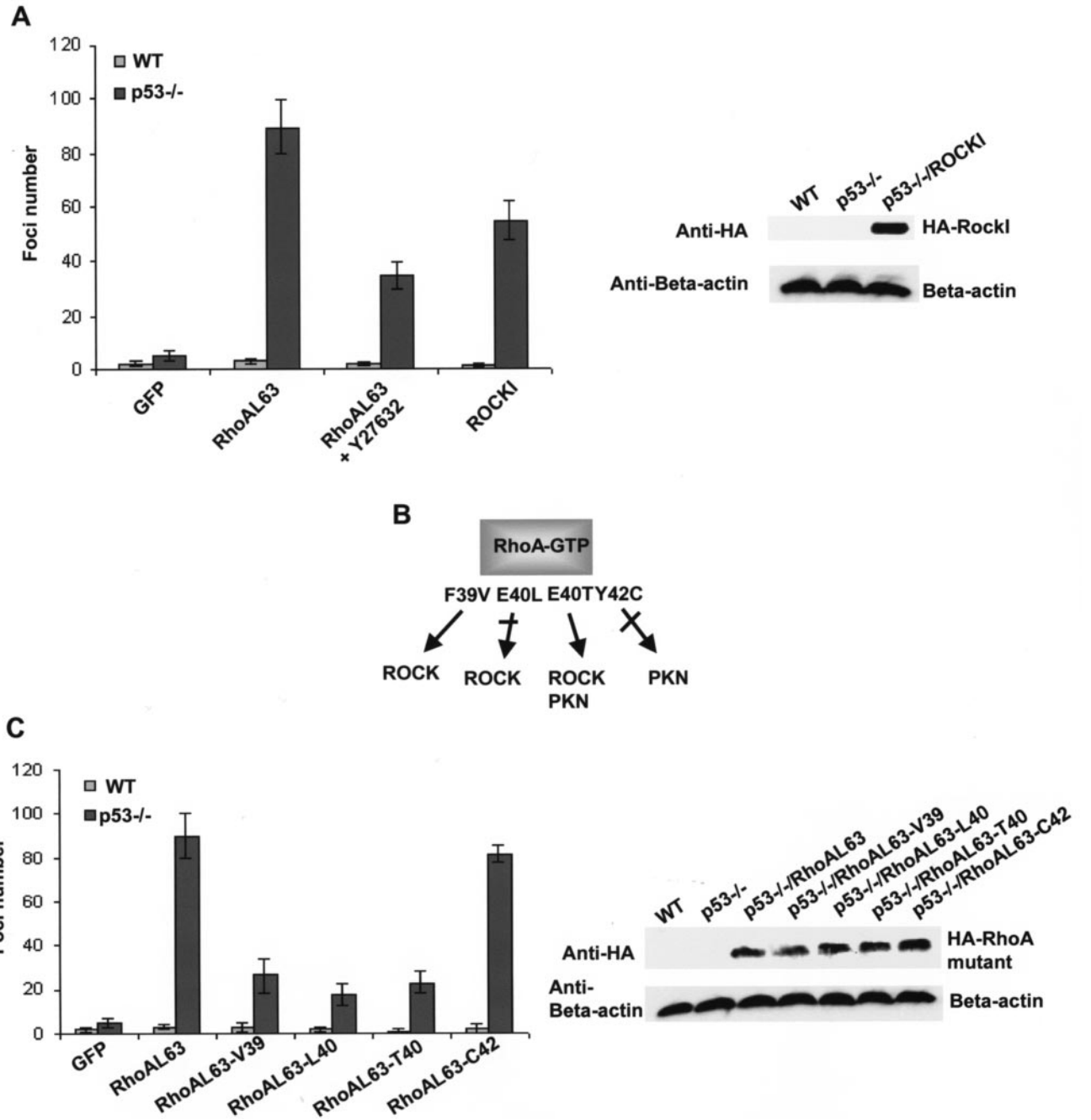

FIG. 8. Multiple effector pathways regulated by RhoA contribute to the transformation phenotype of $p53^{-/-}$ MEF cells. (A) Involvement of ROCK in the RhoAL63-elicited transformation of $p53^{-/-}$ MEFs. Colony-forming activities of $p53^{-/-}$ MEFs transduced with the RhoAL63 mutant or ROCK1 in the presence or absence of Y27632 (20 μM) were determined 14 days postplating. (B) Effector domain mutants of RhoA allow selective uncoupling of RhoA with ROCK, PKN, or other effectors. Single-point mutants made in the switch I domain of RhoA impair or retain specific effector binding as depicted. (C) The effect of effector domain mutations on active RhoA-induced transformation of $p53^{-/-}$ cells. Expression of the respective RhoAL63 mutants in the $p53^{-/-}$ cells was detected by anti-HA Western blot. The data shown are representative of two independent experiments. WT, wild type.

anchorage-independent growth of NIH 3T3 cells (21, 35, 52, 56, 58). We found that the activating mutants of Rac1, RhoA, and Cdc42 can induce a hyperproliferative and transforming phenotype in both the $Arf^{-/-}$ and $p53^{-/-}$ primary MEFs to an extent similar to that caused by oncogenic Ras in certain cases (e.g., active RhoA) (Fig. 6). Since a number of downstream effectors of Ras other than the Rho proteins, including PI 3-kinase and Raf, combine to contribute to the transforming phenotype of oncogenic Ras (3), the potency of some of the Rho members suggests that they may turn on distinct signaling components from Ras or may be more efficient in turning on the same set of growth-promoting factors as Ras in inducing

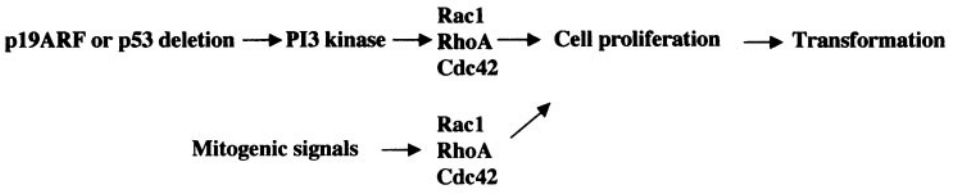

FIG. 9. A model depicting the relationship between the p19^{Arf}-p53 tumor suppressor pathway and the Rho GTPase signaling module in the regulation of cell proliferation and transformation. In addition to malfunction in checkpoint and apoptosis control, deficiency of p19^{Arf}and/or p53 may alter transcriptional balance and promote cell growth by upregulating PI 3-kinase and Rho GTPase activities. Mitogenic signals that cause activation of RhoA, Rac1, or Cdc42 could further modulate cell cycle and apoptotic machineries and cooperate with p53 deficiency to promote cell hyperproliferation and transformation.

cell transformation. In this regard, we have tested the latter possibility, that further enhancement of NF-κB and cyclin D1 activities by the active Rho mutants could be responsible for the efficient induction of transformation in the *Arf*$^{-/-}$ and *p53*$^{-/-}$ cells. We found that both the luciferase reporter activities and the nuclear expression levels of NF-κB and cyclin D1 remain unchanged with or without the expression of the active Rho protein mutants (data not shown). It is therefore possible that the active Rho GTPases stimulate additional factors that cooperate with the p53 pathway defects to promote cell transformation. It is also worth noting that the two different forms of active Rho GTPases, i.e., the fast-cycling and constitutively GTP-bound forms, may have distinct transcription profiles, as we have observed in a gene array study (unpublished data), which could help explain the quantitative differences of these mutants in promoting p19Arf$^{-/-}$ or p53$^{-/-}$ MEF transformation.

In an attempt to further dissect the requirement of RhoA downstream effectors for the transforming phenotype of active RhoA, we utilized ROCKI and the ROCK inhibitor, Y27632, as well as a set of the effector domain mutants of RhoA that impair or retain coupling with specific effectors in the transformation assay. Our results confirm an important role of the ROCK pathway in RhoA-mediated transformation of *p53*$^{-/-}$ cells, while also implying that multiple effectors downstream of RhoA may collaborate to allow the optimal effect of RhoA.

Our results present a seemingly paradoxical relationship between Rho GTPases and the p53 tumor suppressor pathway. On the one hand, basal activities of RhoA, Rac1, and Cdc42 are all part of the required proliferative signals of the p19Arf- or p53-regulated networks. On the other hand, hyperactive Rho proteins can cooperate with *p19Arf* or *p53* deletion or mutation to promote transformation, suggesting that events leading to the activation of Rho family GTPases may constitute second hits in tumor induction (Fig. 9). The later synergism between the Rho GTPases and the p53 pathway further indicates that signaling components of the Rho GTPases are capable of delivering quantitatively different and/or additional inputs to cell proliferation regulation by the p53 pathway. Given the intertwining, complex nature of cell growth regulatory mechanisms of the p53 pathway and Rho proteins, one remaining challenge would be to delineate which of the known or unknown pathways controlled by p19^{Arf}-p53 and each Rho GTPase might cooperate to promote cell hyperproliferation and transformation. Rho family GTPases have been implicated in many aspects of tumor development. Overexpression, upregulation, or rearrangement of RhoA, RhoC, Rac1, Rac2, Rac3, Cdc42, and RhoH have been detected in human tumors ranging from colon, breast, lung, and myeloma to head and neck squamous-cell carcinoma (6, 9, 26, 30, 40, 45). Due to their recognized roles in Ras transformation (58), growth factor and integrin signaling (41), cell cycle control (27), apoptosis (1, 44), and invasion and metastasis (54), Rho GTPases have been proposed as potential anticancer therapeutic targets (37). Interestingly, to date no activating mutations like those found in oncogenic *ras* have been discovered in Rho family members, suggesting that Rho proteins might primarily serve as signaling links from upstream mitogenic signals to play a modifier role in tumor induction. Our studies demonstrating a role of Rho proteins in p19^{Arf}- and p53-controlled cell growth and migration and a synergistic effect of active RhoA, Rac1, and Cdc42 with *p19Arf* or *p53* deletion on cell transformation further strengthen the view that these Rho GTPases may contribute to and/or cooperate with p53 deficiency in tumorigenesis and tumor progression. It remains to be seen if upregulation of Rho GTPase activities in the *p19Arf*$^{-/-}$ or *p53*$^{-/-}$ genetic background could result in a shortened latency in tumorigenesis in an animal model. Should that be the case, targeting individual Rho GTPases would be a worthy endeavor among future anticancer strategies.

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