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Rho Activity Can Alter the Translation of p27 mRNA and Is Important for Ras^{V12}-induced Transformation in a Manner Dependent on p27 Status*

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The amount of $p27^{Kip1}$ establishes a threshold to which G_1 cyclin-cyclin-dependent kinase complexes must surpass prior to cells progressing into S-phase. The amount of p27 is greatest in G_0 cells, intermediate in G_1 cells, and lowest in S-phase cells. However, there is little known regarding the pathways and mechanisms controlling p27 accumulation in G_0 cells. We report that inhibition of Rho, by either lovastatin or C3 exoenzyme, can increase the translational efficiency of p27 mRNA. Similar pharmacologic inhibition of the phosphatidylinositol 3-kinase, the S6 kinase, and the Mek1 kinase pathways all fail to increase translational efficiency in MDA468 cells. This Rho-responsive element lies within a 300-nucleotide region at the 3′-end of the mRNA. By supporting the significance of this signaling pathway to Rho function, we showed that the suppression of Ras^{V12} transformation by $RhoA^{N19}$ is blocked in *p27−/−* cells. In contrast this activity is not blocked in *Rb−/−* or *p16−/−* cells. The resistance of *p27−/−* cells to $RhoA^{N19}$ is not associated with a failure of $RhoA^{N19}$ to accumulate to amounts sufficient to block Rho activity as measured by the organization of actin stress fibers. Together these results indicate a link between Rho and p27.

p27 is a member of the Kip family of G_1 cyclin-cdk[1] inhibitors (reviewed in Refs. 1 and 2). In cycling cells, p27 targets cyclin-cdk2 complexes for inactivation, is associated with cyclin D2/3-cdk4/6 complexes in a non-inhibitory fashion (3, 4), and is an assembly factor for cyclin D1-cdk4 complexes (5). Together the assembly functions and sequestration of p27 in cyclin D-cdk4 complexes can titrate the amount of p27 from cyclin E-cdk2, allowing cells to progress through G_1-phase and enter S-phase (6). However, because accumulation of p27 is biphasic with a maximal amount in quiescent non-cycling cells and a lower intermediate amount in G_1 cells, the initial reduction of p27 might be essential for progression from G_0 to G_1.

Although multiple processes conspire to determine the threshold amount of p27 at each stage of the cell cycle, including regulation at the level of transcription (7–12), protein synthesis (13–15), protein degradation (16, 17), sequestration (4), and cellular localization (18), the major contributions to the threshold in G_0/G_1 cells come from a combination of cdk2-independent proteolysis (16) and synthesis rate (14). Following entry into S-phase, p27 levels reach a nadir because of cdk2-dependent ubiquitin-dependent proteolysis (14). Because p27 abundance is important to the decision to enter and exit the cell cycle, we expected that the rate of p27 synthesis as cells transit the G_0-G_1 period might be a target of mitogenic signals, so we set out to determine the signaling pathways that control translation of p27 mRNA.

Here we report that the amount of p27 mRNA translation was sensitive to the activity of GTPases of the Rho family, being maximally induced when Rho is inactivated in a G_0 cell. Cell cycle arrest induced by inhibition of either the PI3 or p70S6 kinase pathways or simply reducing the amount of activated Rho or activated Erk without inducing cell cycle arrest failed to affect the amount of p27 mRNA translation. We have shown that this aspect of Rho-dependent regulation required an element within the last 300 nucleotides of the mRNA. We also established that $RhoA^{N19}$, a dominant negative allele of Rho, could inhibit Ras^{V12}-induced foci formation in wild-type cells and cells lacking either Rb or p16 but not those lacking p27. However, p27 status did not affect the ability of $RhoA^{N19}$ to interfere with stress fiber formation. Combining these observations we suggest a model wherein Rho-dependent changes in p27 synthesis rates may impact progression during the G_0/G_1 interval of the cell cycle.

EXPERIMENTAL PROCEDURES

Plasmids—The series of SvL vectors have been described (19). The expression vectors pCMV-p27 (20), pCEFL-$RhoA^{Q63L}$ and pCEFL-$RhoA^{N19}$ (21, 22), and pBabe-Ras^{V12} (23) have been described.

To generate retroviruses expressing $RhoA^{N19}$, an N-terminal hemagglutinin tag was added by PCR to the cDNA of $RhoA^{N19}$ from a pCEFL derivative, and the product was subcloned into pWZL-hygro (23).

Cell Culture, Transfection, and Drug Treatments—MDA468 cells were grown in Dulbecco's modified Eagle's high glucose:F-12 supplemented with non-essential amino acids, 10% fetal bovine serum, and 2 mM glutamine. Transient transfections were carried out for MDA468 cells essentially as described (19). Cells were plated at 10^6 cells per 10-cm dish, grown overnight, and then transfected using the calcium phosphate method with 5 μg of luciferase reporter plasmid, 2.5 μg of pCMV-β (CLONTECH), and carrier DNA to a total of 20 μg per dish, unless otherwise stated. Sixteen hours after transfection precipitates

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[1] The abbreviations used are: cdk, cyclin-dependent kinase; UTR, untranslated region; PBS, phosphate-buffered saline; MEF, mouse embryo fibroblasts; FITC, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3 kinase, phosphatidylinositol 3-kinase; Rb, retinoblastoma.

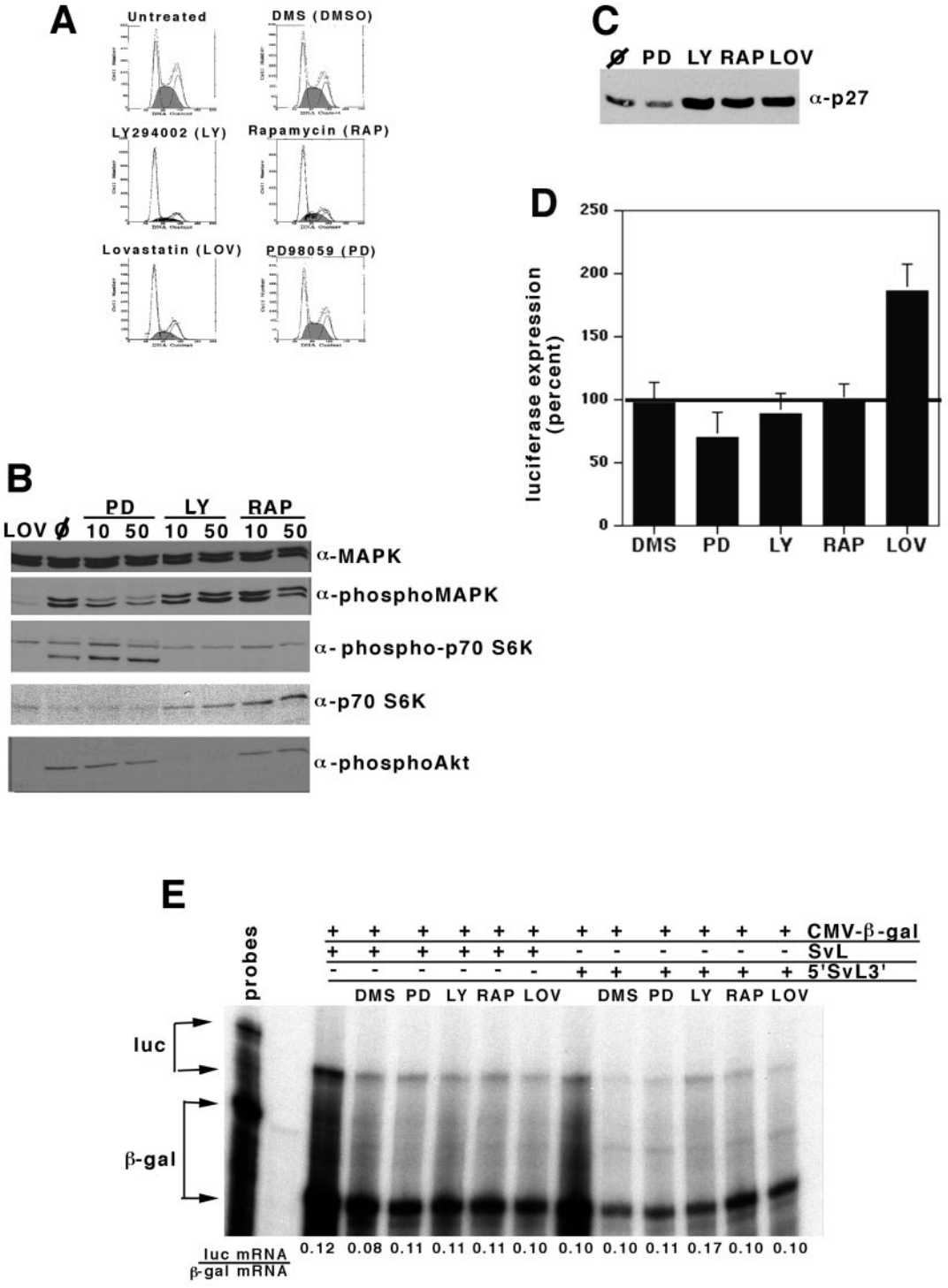


FIG. 1. **Lovastatin induces the translation of p27 mRNA.** MDA468 cells were transfected with reporter constructs, and 24 h later they were treated with either 10 or 50 μM PD98059 (*PD*), LY294002 (*LY*), 10 or 50 nM rapamycin (*RAP*), or 30 μM lovastatin (*LOV*) for additional 24 h prior to analysis. *DMS* (*DMSO*), Me2SO. Replicate dishes were used to assess cell cycle distribution (*A*), efficiency of the treatment on their respective targets (*B*), p27 protein accumulation (*C*), and expression from the reporter plasmids (*D* and *E*). *A*, cell cycle distribution. Samples were analyzed for DNA content by flow cytometry. The samples are indicated *above* each panel, and the percent of cells in each phase of the cell cycle for a representative experiment is shown. The experiment was repeated at least three times for each drug. *B*, extracts were prepared as described under "Experimental Procedures" and 40 μg resolved by electrophoresis on SDS-polyacrylamide gels (8% for p70S6k and 10% for MAPK and Akt), transferred to PVDF, and the amount of MAPK, phosphorylated MAPK (phospho-MAPK), p70S6kinase (p70S6K), phosphorylated p70S6kinase (phospho-p70S6K), and the phosphorylated form of Akt (phospho-Akt) was determined. The treatments are indicated *above* each lane, and the respective antibodies used for immunoblotting are to the *right* of each panel. This experiment was repeated at least three times for each drug. *C*, p27 accumulation. Extract was prepared in RIPA buffer and 50 μg was resolved by electrophoresis on 12% SDS-polyacrylamide gels, transferred to PVDF, and probed with a p27-specific antibody. The drug treatment is indicated *above* each lane. The experiment was repeated at least three times. *D*, only lovastatin induces luciferase accumulation from the reporters. Lysates were prepared from transfected cells, and luciferase and β-galactosidase activity was measured as described (19). The experiment was repeated at least three times. *E*, RNase protection assay analysis. 5 μg of total RNA prepared from transfected and drug-treated cells was subjected to RNase protection assay using the probes in the *left-hand lane*. *Below* each lane, we indicate the ratio of luciferase (*luc*) mRNA to β-galactosidase (β-*gal*) mRNA determined by PhosphorImager quantitation. The experiment was repeated at least three times, and RNA was used at both 5 and 10 μg in each case to ensure linearity of the assay.

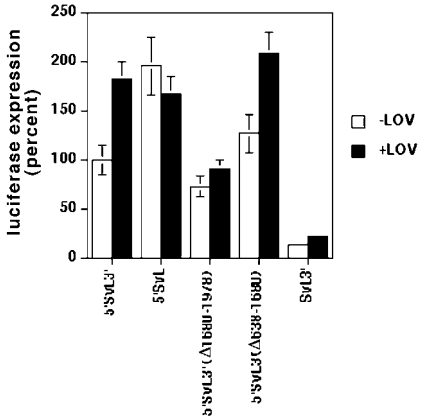


FIG. 2. **The lovastatin response element is in the last 300 nucleotides of the 3′-UTR.** Cells were transfected with the reporters indicated *below* each bar and the accumulation of luciferase, normalized for β-galactosidase, was measured in non-treated (*white bars*) and lovastatin-treated cells (*black bars*). This experiment was repeated five times, with three independent preparations of plasmid DNA. *LOV,* lovastatin.

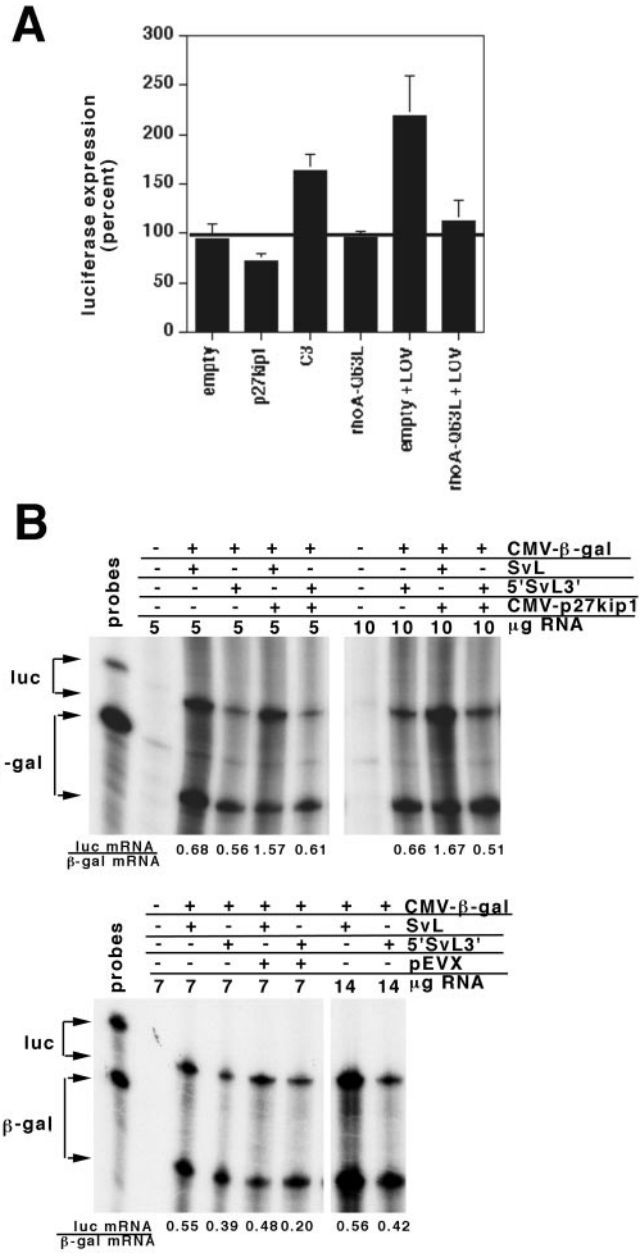


FIG. 3. **Rho activity is a determinant of translation of p27 mRNA as measured by the reporters.** *A*, the accumulation of luciferase from the reporter correlates with changes in Rho activity. The experiment is similar to that described in the legend to Fig. 1*D*. Samples are indicated *below* each bar. The experiment was performed at least four times. *B*, RNase protection assay analysis. RNA was prepared from cells transfected with either p27 ^{Kip1} or C3 exoenzyme (*pEVX*) as indicated *above* each lane. The amount of total RNA hybridized to the probes (in the *left-hand lane*) is indicated *above* each lane. *Below* each lane, we indicate the ratio of luciferase (*luc*) mRNA to β-galactosidase (*β-gal*) mRNA as determined by PhosphorImager quantitation. RPA was repeated at least twice for each sample.

were washed out with fresh medium. Where indicated, replicate dishes, transfected with the same DNA mixtures, were drug-treated 8–10 h after the washes. Luciferase and β-galactosidase activities were assayed 24 or 48 h afterward as described (19) Luciferase activity was normalized for efficiency (luciferase/β-galactosidase ratio) in each transfection. To calculate the change in expression by each treatment and construct, we divided the normalized activity in treated cells by the value obtained in untreated cells, and we expressed this relative to the change seen in expression from constructs lacking any UTR sequences.

We measured the amount of RNA expressed from each reporter construct by RNase protection assay as described (19). Quantitation was performed using an analyzer Fuji Film Bas 2500 and the MacBas version 2.5 software.

Lovastatin (Merck) was activated as described previously (24). We obtained PD98059 from Biomol and LY294002 and rapamycin from Calbiochem. The amount and duration of treatment are indicated in the legends to the figures.

Cell Cycle Analysis—Cell cycle phase distribution was determined by flow cytometry of propidium iodide-stained cells. Whole cell suspensions were washed in PBS, fixed in 70% ethanol, stained in 50 μg/ml propidium iodide, 1 mg/ml RNase, 0.1% Triton X-100, and analyzed using the Multicycle software.

For analysis of transfected populations, an expression vector of the CD19 surface marker was included in the DNA transfection mixture. CD19-positive transfected cells were stained as described (25); cells were detached in PBS, 0.1% EDTA, washed, and stained with a FITC-conjugated anti-CD19 antibody (BD PharMingen). After fixation in 75% ethanol, cell pellets were washed in PBS, 1% bovine serum albumin, 0.5% Tween 20 and stained in 5 μg/ml propidium iodide, 200 μg/ml RNase, 0.1% Triton X-100. DNA profiles were obtained from the FITC-positive population.

Immunoblotting—For immunodetection of activated forms of signaling molecules after drug treatments (Fig. 1), cells in the dishes were washed with ice-cold PBS prior to addition of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 1.5 mM $MgCl_2$) containing phosphatase and protease inhibitors (20 mM Na_3VO_4, 1 mM NaF, 4 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin). Cells were scraped out of the dishes and incubated for 15 min on ice. Otherwise, cell pellets were lysed in RIPA buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.1% deoxycholate) containing protease inhibitors (4 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin and leupeptin) for 15 min on ice and then sonicated. All lysates were clarified by centrifugation at 13,000 × *g* for 15 min. Protein concentration was determined by Bradford assay (Bio-Rad). After SDS-PAGE, proteins were transferred onto PVDF membranes and detected with antibodies using ECL detection system. The affinity-purified anti-p27 antibody has been described (4). Antibodies are as follows: to MAPK (06-182, Upstate Biotechnology, Inc.), p70S6k (9202, New England Biolabs), phospho-MAPK (9101, New England Biolabs), phospho-Akt (9271, New England Biolabs), phospho-p70S6k (9204, New England Biolabs), anti-hemagglutinin epitope (clone 12CA5, Roche Molecular Biochemicals), and α-tubulin (T9026, Sigma).

Foci Formation Assay—Mouse embryo fibroblasts were isolated from 13.5-day post-coitum embryos generated in intercrosses of p27+/− (26), Rb+/− (27), or Ink4a−/− (28) mice. After harvesting the embryos, heads and red organs were removed and used for genotyping by both PCR and Western blot. Carcasses were cut into pieces and then digested in tyrosine at 37 °C for 30 min. Cell pellets from single embryos were resuspended in Dulbecco's modified Eagle's high glucose medium supplemented with 2 mM glutamine, 10% fetal bovine serum, gentamicin, penicillin, and streptomycin, plated in a 10-cm dish, and considered as passage 0. Immortal derivatives were established following a 3T3 protocol.

3T3 fibroblasts of different genotypes were plated at 10^6 cells per 10-cm dish and transfected by the calcium phosphate method with 2 μg of either pBabe-puro or pBabe-Ras^{V12} and 18 μg of either pWZL-hygro or pWZL-RhoN19 per dish. After an overnight incubation, the precipitates were washed out. Cells were grown in complete medium for 24 h and then selected in the presence of 75 μg/ml hygromycin for 4–5 days. Selected cells were counted, mixed with wild-type primary MEFs (3×10^3 drug-resistant cells plus 3×10^5 wild-type cells), and plated at 3×10^5 cells per 60-mm dish. The medium was changed every other day. After 14 days, foci were stained with Giemsa. Foci were counted on a Bio-Rad Image Analyzer using the Bio-Rad Quantity One Analysis Software and confirmed by visual counting under microscope.

RhoN19-expressing MEFs—Retroviral supernatants were generated by transfection of 293T cells (5×10^6 cells/10 cm dish) with 15 μg of retroviral vector and 15 μg of ecotropic helper virus. Supernatants were collected 48 h after transfection, pooled from replicate dishes, and filtered through a 0.45-μm filter.

MEFs were plated at 8×10^5 cells/10-cm dish the night before infection and exposed to viral supernatants (4 ml per dish) containing 4 μg/ml Polybrene for 24 h. Cells were grown in complete medium for 24 h and then selected in the presence of 75 μg/ml hygromycin for 4 days.

Infected MEFs plated in coverslips were fixed in 4% paraformaldehyde for 30 min, permeabilized in 1 M Hepes, pH 7.5, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100 for 4 min on ice, and then stained with 2 μg/ml FITC-phalloidin (Sigma). Counts were performed on captured images.

RESULTS

A Lovastatin-induced Translational Element Is Contained within a 300-Nucleotide Sequence at the 3′-End of p27 mRNA—To determine whether specific signaling pathways affected the translation of p27 mRNA, we used an assay developed to measure the translational efficiency of p27 mRNA (19). This assay takes advantage of a reporter plasmid that expresses the luciferase gene product under the control of the SV40 promoter, SV40 polyadenylation signal, and the untranslated regions of the p27 mRNA. Following co-transfection of the reporter vector with a vector expressing β-galactosidase from a cytomegalovirus promoter into MDA468 cells, we treated them with an assortment of drugs that affected different signaling pathways. We used an inhibitor of Mek1 (PD98059), an inhibitor of PI3 kinase (LY294002) which affects both the activity of p70S6 kinase and Akt, an inhibitor of p70S6 kinase (rapamycin), or an inhibitor of protein prenylation (lovastatin). As measured by trypan blue staining the toxicity of these treatments was insignificant (data not shown).

PD98059 had only a modest effect on cell cycle progression, whereas cells treated with the other drugs accumulated with a G_0/G_1 content of DNA (Fig. 1*A*). We then prepared extracts from these cells and blotted for Erk1/2, phosphorylated Erk1/2, p70S6k, phosphorylated p70S6k, phosphorylated Akt, and p27 (Fig. 1*B*). The amount of phosphorylated Erk1/2 was decreased in PD98059-treated and lovastatin-treated cells but not in LY294002- or rapamycin-treated cells. The amount of phosphorylated S6 kinase was decreased in rapamycin-, LY294002-, and lovastatin-treated cells but not in those treated with PD98059. The amount of phosphorylated Akt was decreased in LY294002- and lovastatin-treated cells but not in PD98059- or rapamycin-treated cells. The p27 protein accumulated in cells treated with lovastatin, rapamycin, or LY294002 but not PD98059 (Fig. 1*C*).

Similar to the increase of endogenous p27, lovastatin induced luciferase accumulation (Fig. 1*D*). Lovastatin is known to increase the synthesis of p27 (15) and decrease the turnover of p27 (29). Increased synthesis doubles the available p27, and the reduction in proteolysis must make up the rest to achieve the 3–4-fold increase in steady state observed in the treated cells. Rapamycin and LY294002 did not increase reporter expression consistent with previous reports (30, 31) that these agents affect the rate of p27 turnover. The steady state amounts of luciferase and β-galactosidase mRNA were not differentially affected by the drugs used (Fig. 1*D*). This suggested that increased translation of p27 would not be a general effect of G_1 arrest, although an increase in p27 protein might be.

To map the sequences responsible for the lovastatin-dependent induction of p27 synthesis, we examined the effect of lovastatin treatment on different mutant constructs. Constructs containing only the 3′-UTR alone had negligible activity, consistent with our previous report (19) that the 5′-UTR contains sequences required for both basal and induced translation of the p27 mRNA. The reporters containing only the 5′-UTR were not affected by lovastatin, suggesting that the 3′-UTR was required for the lovastatin-dependent induction of p27 (Fig. 2). We found that deleting nucleotides 1680–1978 of the 3′-UTR eliminated the lovastatin-inducible element. This loss was not due to the length of the 3′-UTR because expression from an even shorter construct lacking two-thirds of the 3′-UTR, but including this 300-nucleotide region (Δ638–1680), was induced by lovastatin treatment (Fig. 2). The yield of mRNA was equivalent in all the mutants (data not shown). We conclude that the ability of lovastatin to enhance p27 translation lies in an element within the most distal 300 nucleotides of the 3′-UTR.

The Lovastatin Effect Is Mediated by Inactivating Rho—Lovastatin blocks the metabolic pathway leading to protein prenylation by inhibiting hydroxymethylglutaryl-CoA reductase. This causes mislocalization of several membrane-anchored proteins and can have effect on the activity of Erk, Akt, and p70S6 kinases as well as other proteins. As the inactivation of Erk, Akt, and p70S6 kinase did not enhance synthesis individually (Fig. 1*D*), we began to look at the Rho family of GTPases. Because both the Rho proteins, by C-terminal geranylgeranylation, and their GTP exchange factors, by lipid interactions with the pleckstrin homology domain (32), are membrane-localized, lovastatin will block the prenylation of Rho required for its membrane localization and its subsequent activation. Consistent with this, cells treated with lovastatin showed typical changes in morphology as well as redistribution of Rho from membranes to the cytosolic fraction (data not shown).

We examined whether direct inhibition of Rho would enhance synthesis from the reporters, similar to the lovastatin-dependent localization failure. The toxin C3 exoenzyme is a highly specific inhibitor of RhoA, RhoB, and RhoC that only weakly affects other GTPases (33). C3 catalyzes ADP-ribosylation at asparagine 41, preventing interactions of these Rho family members with their effectors (33). Cells co-transfected with vectors expressing C3 toxin, a CD19+ cell surface marker expression vector, and the reporters showed increased luciferase activity similar to lovastatin-treated cells (Fig. 3*A*). Co-expression of p27 in place of C3 toxin did not increase reporter expression (Fig. 3*A*), despite the fact that both the toxin and p27 caused growth arrest in the CD19+ population of cells (data not shown). Co-transfection of either C3 or p27 did not affect the relative amounts of luciferase and β-galactosidase mRNA produced in a manner consistent with the change in activity (Fig. 3). Together these data indicate that inactivation of Rho enhances the synthesis of p27.

Next we asked if we could overcome the effect of lovastatin on reporter expression by using a GTPase-deficient, constitutively active mutant of RhoA, RhoA^{Q63L}. There is a disagreement in the literature as to the specific requirements of membrane localization for activity; however, many groups (34–37) have shown that constitutively active alleles of Rho act in the absence of proper membrane localization. This may be attributed to the overexpression of the mutant, and high levels might allow it to fulfill some of the activated Rho functions simply by mass action. We co-transfected RhoA^{Q63L} together with the

reporter vectors prior to addition of lovastatin. The active form of Rho prevented the increase in translation caused by treatment with lovastatin (Fig. 3), suggesting that inactivation of Rho is a required event. This allele does not abrogate the G_1 arrest induced by lovastatin (data not shown), indicating that Rho inactivation is only part of the cellular response to lovastatin. Additionally, *p27*−/− mouse fibroblasts arrest in response to lovastatin, arguing that changes in p27 are only part of the cellular response to the drug (data not shown). We did not attempt to ask if the $RhoA^{Q63L}$ allele would reverse the effect of C3 exoenzyme because ADP-ribosylation would interfere with the ability of the Rho^{Q63L} to associate with effector proteins. Thus, we concluded that the lovastatin induced increase in reporter synthesis, and the ability of the activated mutant of Rho to suppress this is consistent with the notion that Rho could affect p27 accumulation directly, independent of cyclin E-cdk2-initiated proteolysis as shown previously (38) in IIC9 cells.

Finally we asked if inhibiting Rho activity would be sufficient to induce p27 synthesis. We were unable to induce synthesis from the reporters when co-transfected with a vector expressing $RhoA^{N19}$, a dominant negative mutant of Rho into MDA468 cells (data not shown). On the other hand, we could not document that the amount of $RhoA^{N19}$ expressed was sufficient to inhibit endogenous Rho activity. We next tried NIH3T3 cells. These cells, unlike MDA468 cells, have a well organized cytoskeleton with stress fibers easily detected by phalloidin staining. Stress fiber formation is a reflection of Rho activity (32). NIH3T3 cells were transfected with $RhoA^{N19}$ and reporter activity scored and correlated to the disruption of the cytoskeleton. Although the cytoskeleton was disrupted, cells failed to arrest growth, and we failed to observe a change in reporter activity (data not shown). Consequently, from this and the data above, we conclude that Rho activity is necessary but not sufficient in cycling cells to induce changes in p27 synthesis rate.

Inhibition of Rho Activity Suppresses Ras^{V12}-induced Transformation in a p27-dependent Manner—Rho function had been shown to be necessary for the transforming activity of Ras^{V12} (39–42). Thus we tested if $RhoA^{N19}$ would interfere with Ras^{V12} transformation in a p27-dependent manner.

To overcome the Arf/p53-dependent growth arrest that occurs when introducing Ras^{V12} into primary mouse fibroblasts (28, 43), we immortalized p27-deficient and Rb-deficient cells by continued passage on a strict 3T3 protocol.[2] Additionally, we used *Ink4a*−/− MEFs, deficient for $p16^{Ink4a}$ (and $p19^{Arf1}$) (28). The Arf co-deletion confers immortality. We also used NIH3T3 cells as a prototype model cell line. Most of the 3T3 clones were still dependent on serum for growth and became quiescent when grown to confluence (data not shown). There was an increase in the spontaneous transformation rate of *Rb*−/− cells (Footnote 2 see foci formation in Fig. 4*A* as an example).

These four lines were co-transfected with retroviral constructs encoding Ras^{V12}, $RhoA^{N19}$, both, or the respective empty vectors, and cells were selected for drug resistance. For each line, 3,000 resistant cells were pooled and combined with 300,000 wild-type primary mouse fibroblasts and plated into 60-mm dishes. Foci were scored 2 weeks later after Giemsa staining. The number of foci is representative of the number of cells transformed by Ras^{V12}; they are no longer arrested by growth to confluence. A representative experiment is shown in Fig. 4*A*. In determining the number of foci in *Rb*−/− cells, we have subtracted the number of colonies arising spontaneously and suppressed by $RhoA^{N19}$ co-expression. The extent of transformation by Ras^{V12} was nearly the same in all genotypes, but colony size was different, with Rb-deficient cells producing fairly large colonies relative to the *Ink4*−/−, *p27*−/−, and NIH3T3 cells (Fig. 4, *A* and *B*). Overall, in two independent experiments with two independent clones of each genotype, we find that the steady state amount of Ras^{V12} was always lower in Rb-null cells (Fig. 4*C*). We do not know why.

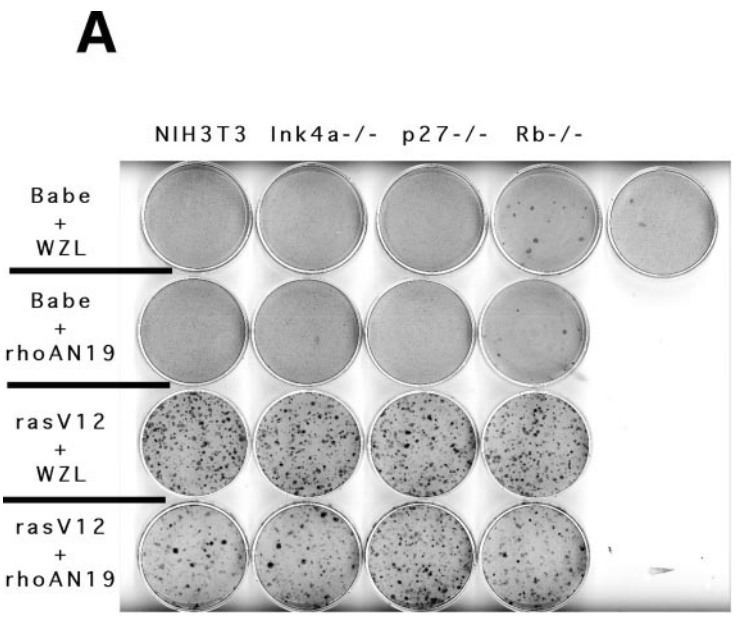
Co-expression of $RhoA^{N19}$ with Ras^{V12} could reduce foci formation in all except for the *p27*−/− cells, where it had no effect (Fig. 4, *A* and *B*). The amount of Rho expressed was equivalent in all four genotypes (Fig. 4*C*). Similar Rho-dependent repression effects on Ras^{V12} were observed in passage 2 fibroblasts from wild-type and *p27*−/− cells co-transfected with Ras^{V12} and SV40 large T antigen which eliminates both Rb and p53 functions (data not shown). Taken together, these results indicate that the ability of $RhoA^{N19}$ to interfere with the transforming activity of oncogenic Ras is dependent on the presence of p27.

Inhibition of Rho Induces Cytoskeletal Changes Independently of p27 Status—The transformation studies above indicate that p27, but not Rb or p16, were targets of Rho activity associated with transformation by Ras^{V12}. The ability of p27 deficiency to alter the requirement for Rho signaling might have reflected a general change in the activity of Rho in these cells. Stress fibers are a measure of the activity of Rho (32). We examined the appearance of stress fibers by phalloidin staining to assess the basal activity of Rho in *p27*−/− cells and cells expressing the dominant negative mutant. We transduced both wild-type and p27-deficient passage 2 primary mouse embryo fibroblasts (MEF) with a retroviral construct expressing $RhoA^{N19}$ and a hygromycin resistance gene from a bicistronic message. Pools of cells were isolated following drug selection. Because of the selection, cells in these pools were expected to contain $RhoA^{N19}$, at levels that would not prevent their proliferation. We confirmed this by measuring thymidine incorporation during a short pulse and by examining DNA content by flow cytometry in passage 4 cells (data not shown). Moreover, we did not detect a significant increase in the steady state amount of p27, the related cdk inhibitor p21, or cyclin E at passage four (Fig. 5*A*). In wild-type cells at passage 4, there was a small increase (30%) in the synthesis of p27 as measured by immunoprecipitable material following a 30-min pulse with [^{35}S]methionine in the presence of LLnL, an inhibitor of the proteasome (data not shown). The amount of $RhoA^{N19}$ expressed was nearly equivalent in the wild-type and *p27*−/− cells, perhaps being a bit higher in the *p27*−/− cells (Fig. 5*A*). Stress fibers were readily observed in the control-infected *p27*−/− cells and clearly disrupted in p27-deficient cells expressing $RhoA^{N19}$ (Fig. 5*B*). These results were quantitated for 500 cells in three independent transductions where control wild-type fibroblasts expressing $RhoA^{N19}$ were also examined (Fig. 5*B*). As the disruption of actin stress fibers was quantitatively similar, the data suggest that p27-deficient cells do not have a significantly higher level of basal Rho activity and that the dominant negative Rho protein can effectively function, at least with regards to its role in cytoskeleton organization.

DISCUSSION

There is accumulating evidence that G_1 progression is impacted by the activation status of Rho family GTPases. Rho has been linked to transcription of p21 (44), the transcription of cyclin D1 (45), and perhaps directly to the activation of cyclin E-cdk2 and the ubiquitin-dependent degradation of p27 (38). Furthermore, transformation by Ras^{V12} can be suppressed by dominant negative Rho (40–42), and this at least partially involves the activation of ROCK (46). Here we show that p27 synthesis can be modulated in a Rho-dependent fashion in

[2] A. Vidal, S. S. Millard, J. P. Miller, and A. Koff, unpublished data.



B

		Babe+WZL	Babe+RhoN19	RasV12+WZL	RasV12+RhoN19
NIH3T3	1	0	0	62	34
	2	ND	ND	76	33
Ink4a-/-	1	0	0	110	33
	2	ND	ND	88	38
	3	0	0	72	33
p27-/-	1	0	0	45	46
	2	0	0	61	55
	3	ND	ND	44	41
Rb-/-	1	15	11	64	40
	2	ND	ND	58	36

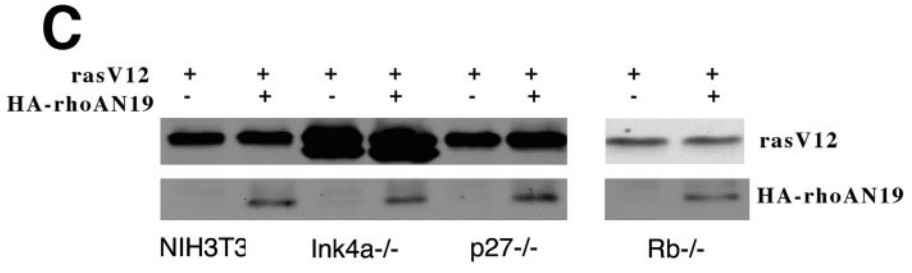


FIG. 4. **Rho^{N19} inhibits transformation by Ras^{V12} in a p27-dependent manner.** *A*, representative experiment showing foci formation by Giemsa staining 2 weeks after plating. Populations of wild-type, Rb null, p16/p19 null, and p27 null mouse embryo fibroblasts were transfected with the vectors indicated to the *left*, selected, and 3,000 of these cells were plated with 300,000 wild-type MEFs and grown 14 days prior to staining and counting of foci. Foci were counted on a Bio-Rad Image Analyzer using the Bio-Rad Quantity One Analysis software. *B*, the average values of duplicate plates from at two to three independent experiments are shown in tabular format. *C*, the amounts of Ras^{V12} and RhoA^{N19} were determined by direct immunoblotting of the selected cells or immunoprecipitation/immunoblotting with hemagglutinin antibodies, respectively. This was repeated twice for each clone.

growth-arrested cells, higher in cells with inactive Rho and lower in cells with active Rho. Additionally, we show that the suppression of Ras^{V12} transformation by dominant negative Rho is dependent on p27 but not on either Rb or p16.

Rho and Control of p27 Synthesis—Here we establish that Rho activity can affect synthesis of p27 in non-cycling cells through a cis-element contained in the last 300 nucleotides of the 3′-UTR of the mRNA. In breast epithelial cells (above) and NIH3T3 cells (data not shown), we have shown that inactivation of the Rho signaling pathway by a generic inhibitor of protein prenylation (lovastatin) and by a specific inhibitor (C3 exoenzyme) induce synthesis from a p27 reporter without altering the available steady state amount of message from the reporter. Consistent with the activity of Rho being the target for lovastatin-induced synthesis, we find that we can overcome the effect by introducing a constitutively active mutant of Rho, RhoA^{Q63L}. This mutant cannot efficiently hydrolyze GTP but can still bind GTP and interact with effectors without the requirement for membrane localization. Furthermore, other signaling pathways inactivated by lovastatin, including the Mek1, PI3-kinase, and S6 kinase pathways, individually cannot induce synthesis. We did not examine combinatorial effects and thus cannot state that the lovastatin-induced increase in synthesis is entirely due to Rho and is not assisted by some other signaling pathway being modulated (see below).

How might Rho repress translation of the p27 mRNA? There are many effectors of Rho, ROCK and LIM kinases being the best defined. Which, if any, of these signaling pathways is utilized by Rho to repress p27 synthesis needs to be investigated further. We have found that neither cytochalasin D

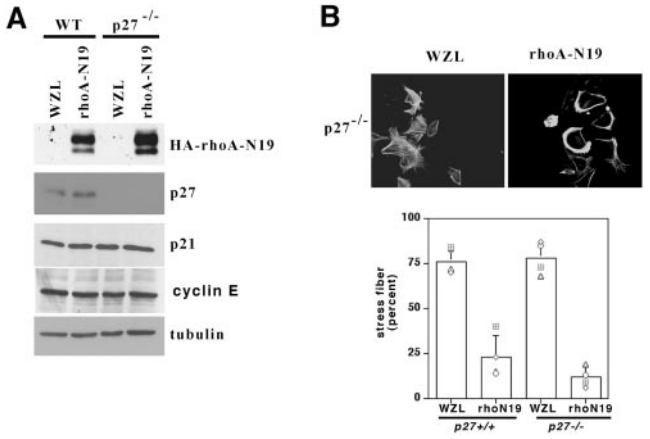


FIG. 5. **Rho^{N19} alters stress fiber formation in *p27−/−* mouse embryo fibroblasts.** Wild-type (*WT*) and *p27−/−* mouse embryo fibroblasts were transfected with retroviruses encoding a hemagglutinin-tagged RhoA^{N19}, selected in hygromycin for 4 days, and grown an additional week prior to making extracts and examining the actin cytoskeleton by phalloidin staining. *A,* immunoblots. 50 μg of extract were resolved by 12% SDS-PAGE and transferred to PVDF membranes and probed with the antibodies indicated to the *right* of each panel. The source of extract is indicated *above* each lane. *WZL* represents cells infected by the retrovirus lacking an insert. RhoA^{N19} represents cells infected by the WZL retrovirus expressing RhoA^{N19}. This is a representative experiment of three independent transductions on three individual primary cultures. *B,* RhoA^{N19} affects stress fiber formation in a p27-independent manner. Primary mouse fibroblasts were stained with FITC-phalloidin, and representative fields are shown. The transduced virus is indicated *above* each field. Four to five fields from three independent transductions of wild-type and *p27−/−* cells (indicated by a *hatch mark*) were used to determine the percentage of cells with stress fibers, and these results are plotted in the accompanying graph.

which disrupts the cytoskeleton nor plating cycling cells in the absence of integrin signaling was sufficient to induce p27 reporter activity in our assay.[2] Because both of these treatments would affect the activity of the ROCK kinase, this suggests that the Rho linkage to p27 synthesis may not be associated with signals that measure cytoskeletal events. Once we narrow down the sequences required for the Rho-dependent effect on translation and identify proteins that interact there, in a manner dependent on Rho signaling, we should be able to make some progress in elucidating this novel translational regulation pathway of Rho.

Rb and p27 Are on Different Pathways with Respect to Transformation of 3T3 Cells by Ras^{V12}—Others have shown that Rho can increase transcription of cyclin D1 (45) and decrease the transcription of p21 (44) in various model cell systems. Both effects are in some fashion dependent on cells with a high persistent level of Erk signaling. Rho is essential for the repression of p21 transcription in cells that are subject to a hyperactive Erk signal. Cyclin D1 is induced by such signals but only when maintained in the presence of Rho activity. Interestingly, the relationship of cyclin D to cell cycle control is dependent on the presence of Rb. Established evidence indicates that in the absence of this tumor suppressor, fibroblasts are no longer dependent on cyclin D-cdk4 activity for cell proliferation (47–49). Consequently, one might have expected that Rb-negative cells would be refractory to the proliferative effects of inhibiting Rho. However, we report that the transformation of *Rb−/−* cells, as well as p16-deficient cells (cells in which a reduction of the Ink4 inhibitor could increase cyclin D-cdk4 complex formation), was still modulated by the expression of dominant negative Rho. On the other hand, the transformation of *p27−/−* cells was not attenuated by the dominant negative Rho. This could not be attributed to an increase in Rho activity as measured by the formation of stress fibers in those cells and their disruption in the cells expressing dominant negative Rho. There is other supporting evidence for independent functions for p27 and Rb in transformation. We reported previously (50) that Rb and p27 could cooperate to enhance tumor development in a pituitary model independently of enhancement in the number of proliferating cells. Consequently, Rho is acting through a mechanism involving p27. This is discrete, although possibly overlapping, from the p16/Rb axis when participating in transformation by Ras^{V12}.

p27 and p21 in G_1 Progression—Singularly, neither p27 nor p21 has been implicated as an important mediator of G_1 progression in mouse fibroblasts except in the case of NIH3T3 cells that when treated with antisense oligonucleotides gain a degree of serum independence (51, 52). Primary cultures of p27- or p21-deficient mouse embryo fibroblasts were not even partially serum-independent (53–55).[2] These cells, when immortalized, retain serum dependence and are no more prone to spontaneous transformation as wild-type cells.[2] The rate at which these cells enter S-phase from a serum-starved state is indistinguishable from wild-type cells. This is similar to observations in p16 null fibroblasts that have normal growth characteristics and remain susceptible to Ras-induced senescence (56, 57).

Nevertheless, the notion that p27 is an important protein for the regulation of the cell cycle during serum-induced entry into S-phase is supported by the observation that cdk4-deficient mouse embryo fibroblasts induced to re-enter the cell cycle from serum starvation have a prolonged G_1-phase, a prolongation that was almost completely eliminated by p27 deficiency. Similar analysis has not been carried out on *p21−/−* cells. We have noted that p27 deficiency eliminates a modest delay in S-phase entry in primary passage 2 mouse fibroblasts expressing dominant negative Rho,[2] consistent with the possibility that the G_1 function of Rho is affecting accumulation of p27, but clonal variations in expression level and the resultant delay make the analysis of this problematic without a good single cell assay. Rho activity can affect progression of cells from G_0 into S-phase in at least four of the following ways: it can reduce synthesis of p27 (data here); it can reduce the transcription of p21 (39); it can increase the transcription of cyclin D1; and it can affect cyclin E-cdk2 activity. Ultimately, the only thing the cell needs is to suppress the available p27 (or p21) that can interact with cyclin E-cdk2. During the G_0 to G_1 transition, the amount of p21 in a cell coupled with both cdk2-independent proteolysis of p27 (16) and a decrease in synthesis of p27 would establish the threshold to which cyclin D-cdk4 would need to accumulate. Once cyclin D-cdk4 has passed this threshold, active cyclin E-cdk2 can accumulate, allowing the cells to progress from G_1 into S-phase and the cdk2-dependent turnover of p27. In support of the role of Rho suppressing cdk-inhibitor levels during this transition, we find that *p21−/−* cells are also resistant to the effect of RhoA^{N19} in the Ras^{V12} foci formation assay (data not shown).

Conclusions—In conclusion, our data indicate a genetic relationship between Rho and p27 as related to the ability of Rho to participate in transformation by Ras^{V12} in immortalized 3T3 cells. It suggests that this is not identical to the function of inactivating Rb, as Rb-deficient cells retain sensitivity to Rho inhibition in this assay. We speculate that at least part of the Rho-p27 connection is associated with modulating the synthesis of p27 protein, because both lovastatin and C3 exoenzyme increase translational efficiency of p27 mRNA. Nevertheless, the amount of p27 synthesis is not appreciably increased in NIH3T3 cells transfected with RhoA^{N19} at levels sufficient to disrupt stress fiber formation, indicating that inactivating Rho while necessary is not sufficient for the translational effect

(data not shown). One possibility is that other pathways must be simultaneously impaired to allow a translational effect of Rho inactivation. We have tried to co-transfect reporters with $RhoA^{N19}$ and either a p27 expression vector or a Ras^{V12} expression vector, or transfect reporters with $RhoA^{N19}$ and treat cells with either LY294002 or rapamycin (data not shown). However, in no case could we recapitulate the translational enhancement we saw with lovastatin or C3 exoenzyme.[2] Alternatively, the inability of $RhoA^{N19}$ to function in translation enhancement is somehow associated with the fact that it is a competitive inhibitor of Rho signaling, and both lovastatin and C3 exoenzyme act in a non-competitive manner. Lovastatin is a strong inhibitor of prenylation thus affecting both the overall activity of Rho family members and the activities of many other molecules involved in the activation of Rho family members. C3 exoenzyme acts catalytically to inactivate the RhoA, RhoB, and RhoC family members.

However, p27 deficiency can attenuate the ability of $RhoA^{N19}$ to suppress foci formation. It is important to note that taking cells co-transfected with Ras^{V12} and $RhoA^{N19}$, which do not have enhanced reporter activity (data not shown), is not identical to the additional selective pressures evident in a foci formation assay. Only a small proportion (~1%) of cells transduced with Ras^{V12} are able to overcome the contact inhibition growth arrest signal. We do not know why this is, but it could be related to both the amount of signal a cell needs to become transformed and the amount that it can tolerate, as well as additional cryptic mutations that might arise in the population. It is under this condition where we find the connection between Rho and p27. How much of that connection is associated with p27 translation is not yet clear; nevertheless, p27 synthesis is in some way affected by Rho status, and Rho status is less important in *p27−/−* cells transformed by Ras^{V12}.

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Rho Activity Can Alter the Translation of p27 mRNA and Is Important for Ras^{V12} -induced Transformation in a Manner Dependent on p27 Status

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