Role for \(p27^{\text{Kip1}}\) in Vascular Smooth Muscle Cell Migration

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Background—Rapamycin is a potent inhibitor of smooth muscle cell (SMC) proliferation and migration. Rapamycin-mediated inhibition of SMC proliferation is associated with upregulation of the cyclin-dependent kinase inhibitor \(p27^{\text{Kip1}}\). Previously, we showed that mixed embryonic fibroblasts obtained from \(p27^{\text{Kip1}}(-/-)\) mice were relatively rapamycin-resistant, suggesting that \(p27^{\text{Kip1}}\) plays an integral role in modulating the antiproliferative effects of rapamycin. We hypothesized that the antimigratory effect of rapamycin may also be mediated by \(p27^{\text{Kip1}}\).

Methods and Results—Rapamycin (1 to 10 nmol/L) inhibited basic fibroblast growth factor–induced migration of wild-type (WT) but not \(p27^{\text{Kip1}}(-/-)\) SMCs in a dose-dependent manner \((P<0.05)\) in a modified Boyden chamber. The effects of rapamycin on aortic SMC explant migration were also studied with WT, \(p27(+/+)/\), and \(p27(-/-)\) mice. Rapamycin 4 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\) IP for 5 days inhibited SMC migration by 90% in the WT and \(p27^{\text{Kip1}}(+/+)\) \((P<0.05)\) but not \(p27^{\text{Kip1}}(-/-)\) animals.

Conclusions—Lack of \(p27^{\text{Kip1}}\) reduces rapamycin-mediated inhibition of SMC migration. These novel findings suggest a role for \(p27^{\text{Kip1}}\) in the signaling pathway(s) that regulates SMC migration. (Circulation. 2001;103:2967-2972.)

Key Words: muscle, smooth ▶ cells ▶ inhibitors ▶ rapamycin

Vascular smooth muscle cell (SMC) migration is believed to play a major role in the pathogenesis of many vascular diseases, including atherosclerosis and restenosis after PTCA and coronary stenting.\(^1\) Previously, we and others demonstrated that rapamycin, a macrolide antibiotic, inhibited SMC proliferation in vitro and in vivo by blocking cell-cycle progression at the G1/S transition.\(^2\) \(^4\) The inhibition of cellular proliferation was associated with a marked reduction in cyclin-dependent kinase (CDK) activity and in retinoblastoma protein phosphorylation.\(^2\) \(^4\) Pretreatment of rat and human SMCs with rapamycin (2 nmol/L) for 48 hours inhibited platelet-derived growth factor (PDGF)-induced migration in a modified Boyden chamber. Acute rapamycin treatment (6 hours) of rat and human SMCs, however, had no effect on migration, suggesting that longer exposure to rapamycin is essential for its antimigratory actions.\(^5\) The finding that rapamycin possessed both antiproliferative and antimigratory properties led us to propose that rapamycin may have important applications in the treatment of such disorders as accelerated arteriopathy that occurs in transplanted hearts and restenosis after PTCA and placement of coronary stents.\(^5\) \(^6\) Recently, implantation of rapamycin (sirolimus)-coated stents in de novo lesions was shown to be safe and effective in inhibiting neointimal formation at 4 months of follow-up.\(^7\)


downregulation of the CDK inhibitor (CDKI) \(p27^{\text{Kip1}}\) by mitogens is blocked by rapamycin.\(^6\) In \(p27^{\text{Kip1}}(+/+)\) mice, relative rapamycin resistance was demonstrated in mixed embryonic fibroblasts and splenic T lymphocytes.\(^9\) In rapamycin-resistant myogenic cells, constitutively low levels of \(p27^{\text{Kip1}}\) were observed, which did not increase with serum withdrawal and rapamycin.\(^9\) Rapamycin (1 nmol/L) inhibited p70S6k phosphorylation and activity in both \(p27^{\text{Kip1}}(-/-)\) mixed embryonic fibroblasts and rapamycin-resistant myogenic cells.\(^9\) Although rapamycin blocks p70S6k in both \(p27^{\text{Kip1}}(-/-)\) cells and rapamycin-resistant myogenic cells, constitutively low levels or absence of \(p27^{\text{Kip1}}\) inhibits the antiproliferative properties of rapamycin. Decreased levels of \(p27^{\text{Kip1}}\) in the vessel wall have been associated with increased neointimal response after PTCA.\(^10\) Overexpression of \(p27^{\text{Kip1}}\) in SMCs inactivated cdk2 and cdk4 activity; adenoviral gene transfer of \(p27^{\text{Kip1}}\) after femoral artery balloon angioplasty significantly inhibited intimal cell proliferation.\(^11\) Similar inhibition of neointima formation has also been reported for adenovirus-mediated overexpression of \(p21^{\text{Cip1}}\) in the rat carotid artery after angioplasty.\(^12\) In this study, we demonstrate that \(p27^{\text{Kip1}}\) plays a critical role in the mediation of the antimigratory properties of rapamycin.

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Methods

Reagents
DMEM and trypsin were from Gibco, recombinant basic fibroblast growth factor (bFGF) was from Biosource International, pacitaxel was from Sigma, and the mouse monoclonal p27<sup>Kip1</sup> antibody was from Santa Cruz. Rapamycin was a gift from Dr Suren Sehgal (Wyeth-Ayerst Laboratories).

Expression of C3 Exoenzyme
The expression of GST-C3 exoenzyme (cDNA was a gift from Dr Judy Meinkoth, University of Pennsylvania) was prepared as previously described.13,14

Cell Culture
The aortic SMCs (≥5 passages) were obtained from the explant migration experiments described below and were subcultured in DMEM containing 20% FBS at 37°C in a humidified 95% air–5% CO<sub>2</sub> atmosphere. Identification of SMCs was performed with α-actin immunofluorescence.

Fluorescence-Activated Cell Sorter Analysis
Wild-type (WT) [p27<sup>Kip1</sup>(+/-)], heterozygous [p27<sup>Kip1</sup>(+/-)], and homozygous [p27<sup>Kip1</sup>(--/-)] SMCs were placed in DMEM+0.5% FBS and various concentrations of rapamycin for 48 hours to achieve quiescence. The cells were washed, harvested, and labeled with propidium iodide solution–RNase for 1 hour. The cells were analyzed on a fluorescence-activated cell sorter (FACS), with a minimum of 15 000 cells counted as previously described.6

Assessment of Cell Viability
Trypan blue exclusion assays were performed at the conclusion of all experiments and demonstrated >95% viability. The effects of rapamycin (0.1 to 100 nmol/L, 48-hour incubation) on SMC viability were also determined by a cytotoxicity assay (Promega CytoTox 96).

SMC Adhesion Assay
SMCs were treated with rapamycin or vehicle for 48 hours. SMCs (5×10<sup>3</sup>/mL in DMEM with 0.2% BSA) were loaded onto plates precoated with laminin or fibronectin. After 3 hours, the media containing nonadherent cells were removed, and cell numbers were determined in triplicate with a Coulter Counter.

SMC Migration Assay
Migration was measured by use of a 48-well modified Boyden chamber housing a polycarbonate filter with 8-μm pores as described.8 Rapamycin or FK506 was added to the growth medium for 48 hours, or C3 exoenzyme was added for 16 hours. The lower chambers were filled with either 50 ng/mL of bFGF or 0.2% BSA (negative control) in DMEM. An equal number of cells (2×10<sup>3</sup>/mL) in 50 μL were loaded into the top chamber of each well. After 6 hours, nonmigrating cells were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with Giemsa stain. The number of SMCs on the lower surface of the filter was determined by counting 4 high-power (×200) fields of constant area per well. Values are expressed as the percentage of cells migrating in response to bFGF after subtraction of the negative control (DMEM+BSA). Experiments were performed at least twice in quadruplicate wells.

Aortic SMC Explant Migration
The p27<sup>Kip1</sup>(+/-) and p27<sup>Kip1</sup>(--/-) knockout mice were kindly provided by Dr Andrew Koff.13 WT controls were obtained from 2 sources: C57BL/6 mice and p27<sup>Kip1</sup>(+/-) littermates were purchased from Jackson Laboratory (Bar Harbor, Me). No differences in sensitivity to rapamycin were detected between the 2 groups. All mice used in these experiments were genotyped by Southern hybridization as previously described15 and immunoblot analysis of p27<sup>Kip1</sup>. The mice received 1 of 3 treatment protocols of rapamycin: 9 mg·kg<sup>-1</sup>·d<sup>-1</sup> IP for 7 days, 4 mg·kg<sup>-1</sup>·d<sup>-1</sup> IP for 5 days, or 2 mg·kg<sup>-1</sup>·d<sup>-1</sup> IP for 2 days. The control group was treated with vehicle alone (0.2% sodium carboxymethyl cellulose, polysorbate 0.25%; Sigma).

At the conclusion of the protocol, the mice were euthanized with 100 mg/kg of pentobarbital, the aortas were excised, and the adventitia and surrounding connective tissue were removed, and the aortas were opened by a longitudinal cut, and the intima and a thin portion of the subjacent media were removed. The descending aorta was weighed and trimmed to normalize the weight to 10 mg. The aortas were cut into 4 pieces (~2×2 mm), and each piece was placed into a separate well of a 6-well plate containing DMEM+20% FBS. The number of cells that explanted from each piece was counted daily and the total number of cells/animal added. The results in Figure 4 are presented as the mean percentage (±SD) of inhibition of migration compared with control for ≥4 animals from each group.

Immunoblots
Lysates from SMCs growing in log phase or treated with rapamycin (100 nmol/L for 48 hours) were prepared as previously described.9

Statistics
Data are presented as the mean±SD of the independent experiments. Statistical significance was determined by 1-way ANOVA and Fisher’s protected least significant difference test. A paired t test (StatView 4.01) was used to analyze all data. A value of P<0.05 was considered statistically significant.

Results
The inhibitory effects of rapamycin on the migration of SMCs isolated from WT and p27<sup>Kip1</sup>(--/-) mice were determined. In WT SMCs, rapamycin treatment for 48 hours demonstrated a significant inhibitory effect on bFGF-induced migration (Figure 1A, open bars). The inhibition was concentration dependent between 1 and 100 nmol/L, with an IC<sub>50</sub> of ~2 nmol/L. In contrast, no significant inhibition of migration by rapamycin (1 to 10 nmol/L) was observed in p27<sup>Kip1</sup>(--/-) SMCs (Figure 1B, open bars). At higher concentrations (100 nmol/L), an ~35% inhibition was observed; the IC<sub>50</sub> in p27<sup>Kip1</sup>(--/-) cells was ~200 nmol/L, representing an ~100-fold increased IC<sub>50</sub> compared with WT SMCs. Addition of rapamycin to either the upper or lower chamber immediately before incubation had no effect on SMC migration (data not shown). FK506, an agent that binds to the same cytosolic receptor (FKBP12) as rapamycin, had no effect on SMC migration (Figure 1A and 1B, solid bars). The antimitogenic effect of rapamycin was competitively inhibited by a molar excess of FK506 (Figure 1C and 1D). These data indicate that rapamycin inhibits SMC migration by binding to FKBP12.

Treatment of WT SMCs with rapamycin (100 nmol/L for 48 hours) caused an increase in p27<sup>Kip1</sup> protein levels (Figure 1A, inset); in contrast, no p27<sup>Kip1</sup> was detected in p27<sup>Kip1</sup>(--/-) SMCs (Figure 1B, inset). Although rapamycin inhibits SMC proliferation,2 these findings cannot be explained by differences in proliferation, because equal numbers of cells were loaded into the Boyden chamber, and the total number of cells in the upper and lower chambers after the 6-hour incubation was equal in the untreated and treated WT and p27<sup>Kip1</sup>(--/-) SMCs. This is to be expected, because the cell cycle requires ~24 hours in the murine SMCs and rapamycin inhibits cells in late G<sub>1</sub>. In addition, we counted the number of cells in the upper chamber after 6 hours of incubation without bFGF in the lower chamber. Rapamycin...
had no significant effect on the total number of cells in the upper chamber [WT and p27\(^{kip1}\)(−/−)] (P=NS) (Figure 1E).

No differences in cell viability were noted between untreated and rapamycin-treated SMCs obtained from WT and p27\(^{kip1}\)(−/−) animals as measured by trypan blue exclusion. To further assess cellular viability, we determined that rapamycin treatment (100 nmol/L) did not increase LDH (a marker for cell viability) release in either WT, p27\(^{kip1}\)(+/+)\(\times\)animals as measured by trypan blue exclusion. Similar results were obtained with 0.1 and 10 nmol/L rapamycin. Because migration is dependent on the adhesion of the SMCs to the Boyden chamber membrane, we performed adhesion assays with fibronectin- and laminin-coated plates. SMCs obtained from p27\(^{kip1}\)(−/−) animals demonstrated no differences in adhesion compared with SMCs obtained from WT animals on both fibronectin- and laminin-coated plates. Furthermore, rapamycin treatment (100 nmol/L for 48 hours) did not alter the antimigratory properties of rapamycin (Figure 2B).

Figure 1. Low concentrations of rapamycin (Rapa) inhibit migration in WT but not in p27\(^{kip1}\)(−/−) SMCs. A, Migration of SMCs isolated from WT mice was determined in modified Boyden chamber after Rapa and FK506 treatment. Rapa (open bars; 1, 10, and 100 nmol/L) significantly inhibited SMC migration, whereas FK506 demonstrated no effect (solid bars). *P<0.05 vs control. Inset, Immunoblot demonstrating increased p27\(^{kip1}\) levels after Rapa (100 nmol/mL for 48 hours) treatment (lane 2) vs untreated proliferating SMCs (lane 1). B, Migration of SMCs isolated from p27\(^{kip1}\)(−/−) mice was determined in modified Boyden chamber after Rapa and FK506 treatment. Rapa (open bars; 1, 10, and 100 nmol/L) significantly inhibited SMC migration, whereas FK506 demonstrated no effect (solid bars). *P<0.05 vs control. Inset, Immunoblot demonstrating absence of p27\(^{kip1}\). C and D, FK506 competes with Rapa for binding to FKBP12 and inhibits effects of Rapa on WT (C) and p27\(^{kip1}\)(−/−) (D) SMC migration. E, Proliferation of p27\(^{kip1}\)(+/+) and p27\(^{kip1}\)(−/−) was determined in upper chamber of modified Boyden chamber (without bFGF in lower chamber) after 6 hours. Rapa had no significant effect on total number of cells in upper chamber (P=NS).

Because migration is dependent on the adhesion of the SMCs to the Boyden chamber membrane, we performed adhesion assays with fibronectin- and laminin-coated plates. SMCs obtained from p27\(^{kip1}\)(−/−) animals demonstrated no differences in adhesion compared with SMCs obtained from WT animals on both fibronectin- and laminin-coated plates. Furthermore, rapamycin treatment (100 nmol/L for 48 hours) did not alter the antimigratory properties of rapamycin (Figure 2B).

Figure 2. Low concentrations of rapamycin (Rapa) inhibit migration in WT but not in p27\(^{kip1}\)(−/−) quiescent SMCs. A, FACS analysis of untreated and Rapa-treated p27\(^{kip1}\)(+/+)\(\times\)quiescent SMCs. Analysis was based on a minimum of 15 000 cells. Percent in each phase of cell cycle is shown. B, Migration of quiescent SMCs isolated from p27\(^{kip1}\)(+/+) (left), p27\(^{kip1}\)(+/−) (middle), and p27\(^{kip1}\)(−/−) (right) mice was determined in modified Boyden chamber. Open bars (untreated) and solid bars (Rapa 1, 10, 100, and 1000 nmol/L). *P<0.05 vs control.
did not affect cell adhesion in either WT or p27\textsuperscript{kip1}(-/-) SMCs (Figure 3).

To assess the in vivo effects of rapamycin on SMC migration in the p27\textsuperscript{kip1}(-/-) animals, we examined the ability of SMCs to migrate out from aortic explants and establish cell cultures. Rapamycin was not added to the culture medium after the aortas were explanted. Explant migration of aortic SMCs was performed with p27\textsuperscript{kip1}(+/+) littersmates, p27\textsuperscript{kip1}(-/-), or p27\textsuperscript{kip1}(-/-) mice. SMCs from p27\textsuperscript{kip1}(+/+), p27\textsuperscript{kip1}(+/+), and p27\textsuperscript{kip1}(-/-) mice migrated out of the aortic explants by day 2. In animals treated with rapamycin 4 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} IP for 5 days, ~85% inhibition of migration compared with untreated animals was observed in the p27\textsuperscript{kip1}(+/+) and p27\textsuperscript{kip1}(-/-) groups (P < 0.05). In contrast, no rapamycin-mediated inhibition of migration was observed in the p27\textsuperscript{kip1}(+/+) group (P > 0.05, Figure 4A), indicating that p27\textsuperscript{kip1} plays a critical role in the rapamycin-mediated inhibition of SMC migration. At higher doses, equivalent levels of rapamycin-mediated inhibition of migration were observed in WT, p27\textsuperscript{kip1}(+/+), and p27\textsuperscript{kip1}(-/-) cells (Figure 4B). At lower doses (2 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} for 2 days), no rapamycin-mediated inhibition of migration was observed (data not shown). This assay, while primarily assessing SMC migration, may also reflect proliferation, especially after several days of culture. These results are consistent with the findings obtained in the modified Boyden chamber for p27\textsuperscript{kip1}(-/-) cells and suggest that both p27\textsuperscript{kip1}-dependent and p27\textsuperscript{kip1}-independent pathways mediate the SMC antimitragory actions of rapamycin. To demonstrate that p27\textsuperscript{kip1}(-/-) SMC migration could be inhibited by agents that act independently of the p27\textsuperscript{kip1} pathway, we treated WT and p27\textsuperscript{kip1}(-/-) mice with taxol 20 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} IP for 7 days.\textsuperscript{16} No differences in taxol-mediated inhibition were observed in the 2 groups (Figure 4C).

Recent data suggest that the Ras/RhoA mitogenic pathway regulates the degradation of p27\textsuperscript{kip1},\textsuperscript{17} C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, inhibited PDGF-induced p27\textsuperscript{kip1} degradation. These findings suggest that activation of RhoA by mitogens is necessary for degradation of p27\textsuperscript{kip1}.\textsuperscript{17} In addition, thrombin-induced vascular SMC DNA synthesis and migration were inhibited by C3 exoenzyme.\textsuperscript{14} We sought to determine whether this inhibition of migration was mediated, in part, by regulating p27\textsuperscript{kip1} levels. SMCs from WT and p27\textsuperscript{kip1}(-/-) animals were exposed to either 2 or 20 µg/mL for 16 hours, trypsinized, and loaded into the upper chamber of the Boyden chamber. Higher concentrations of C3 exoenzyme (40 µg/mL) were toxic to the cells, because we observed increased trypan blue uptake; therefore, higher concentrations of C3 exoenzyme were not used in these experiments. C3 exoenzyme significantly inhibited bFGF-mediated SMC migration in WT cells (Figure 5, open bars). SMCs from p27\textsuperscript{kip1}(-/-) animals demonstrated a 25% relative resistance to C3 exoenzyme (Figure 5, solid bars). SMCs that were acutely exposed to C3 exoenzyme demonstrated no inhibition of migration. These results implicate p27\textsuperscript{kip1} as a regulator, in part, of both rapamycin- and C3 exoenzyme–mediated inhibition of SMC migration.
Discussion

Rapamycin has been shown to inhibit rat, porcine, and human SMC migration. In addition, rapamycin reduces intimal thickening by 50% after PTCA in a porcine model. The rapamycin antiestenotic effect is characterized by an inhibition of the SMC response to coronary injury with a concomitant decrease in retinoblastoma protein phosphorylation as well as an increase in p27Kip1 levels, thereby resulting in cell-cycle arrest. The data indicate that the CDKI p27Kip1 plays a critical role in mediating the antiproliferative and antimigratory properties of rapamycin. The lack of p27Kip1 in the null mice was consistent with previous reports in rats demonstrating that the antiproliferative properties of rapamycin are mediated through FKBP12 binding. p70S6k phosphorylation and 4E-BP1 phosphorylation. FK506 competes for binding of the FK506 binding protein (FKBP12) to the cytosolic receptor of rapamycin. Rapamycin-FKBP12 also prevents mitogen-induced downregulation of p27Kip1 through an unknown mechanism (lines with bars indicate inhibitory effects; arrows indicate stimulatory effects). C3 exoenzyme, which specifically ADP-ribosylates and inactivates RhoA, inhibits SMC migration through p27Kip1-dependent and -independent (cytoskeleton effects) pathways.

Figure 5. Impaired migration-inhibitory response to C3 exoenzyme in SMCs derived from p27Kip1(-/-) mice. Migration of SMCs isolated from WT mice (open bars) and p27Kip1(-/-) (solid bars) was determined in modified Boyden chamber after C3 exoenzyme (2 and 20 μg/mL) treatment for 16 hours. SMCs derived from p27Kip1(-/-) mice demonstrated 25% relative migratory resistance to C3 exoenzyme. *P<0.05 vs control.

Figure 6. Rapamycin and C3 exoenzyme inhibit SMC migration through p27Kip1-dependent and -independent pathways. Growth factor receptor activation by mitogens/nutrients activates PI3-kinase, which indirectly stimulates mTOR, p70S6k, and RhoA. Rapamycin (RAPA)-FKBP12 inhibits mTOR-mediated activation/phosphorylation of protein translation modulators (p70S6k) and prevents mitogen-induced downregulation of p27Kip1 through an unknown mechanism (lines with bars indicate inhibitory effects; arrows indicate stimulatory effects). C3 exoenzyme, which specifically ADP-ribosylates and inactivates RhoA, inhibits SMC migration through p27Kip1-dependent and -independent (cytoskeleton effects) pathways.

We have previously shown that splenic T lymphocytes derived from p27Kip1(-/-) mice required a 15- to 30-fold higher concentration of rapamycin than do WT cells for a comparable level of growth inhibition. The inhibition of migration in both WT and p27Kip1(-/-) SMCs was inhibited by excess FK506, implying that the antimigratory properties of rapamycin are mediated by FKBP12. Similarly, we have previously demonstrated that the antiproliferative properties of rapamycin are mediated through FKBP12 binding. Approximately 20% of WT SMCs were resistant to the antimigratory effects of rapamycin; this is consistent with findings that suggested that ~10% to 20% of rat aortic SMCs were resistant to the antiproliferative effects of rapamycin. In BC3H1 myogenic cells, we were able to enrich for this population by serially passaging these cells in the presence of rapamycin (100 or 1000 nmol/L). Interestingly, these cells demonstrated constitutively low levels of p27Kip1, which did not increase on serum withdrawal and treatment with rapamycin. SMCs derived from p27Kip1(-/-) animals did not demonstrate increased proliferation or migration in Boyden chamber or explant assays. Rapamycin (100 nmol/L) inhibited WT SMC migration by ~80%, compared with 35% in p27Kip1(-/-) cells. Thus, in a subset of SMCs (~35%), rapamycin inhibits migration via a pathway that is independent of p27Kip1. Indeed, rapamycin has multiple effects on cell signaling (Figure 6). A common step is binding of the FK506 binding protein (FKBP12) to the cytosolic receptor of rapamycin. Rapamycin-FKBP12 also inhibits the mammalian target of rapamycin (mTOR), p70S6k, and 4E-BP1 phosphorylation. FK506 competes for binding to FKBP12 with rapamycin, but FK506 does not inhibit mTOR, p70S6k, or 4E-BP1. Molar excesses of FK506 compete rapamycin off from FKBP12 and block the inhibition of migration by rapamycin in p27Kip1(-/-) SMCs, indicating that both the p27Kip1-dependent and p27Kip1-independent antimigratory properties of rapamycin are mediated through FKBP12 (Figure 6). p70S6k phosphorylation and activity, however, are completely inhibited by 1 nmol/L.
rapamycin in WT and p27Kip1(-/-) cells. These findings suggest that in a subset of SMCs, rapamycin inhibits migration via a p70S6k- and p27Kip1-independent pathway that has yet to be identified (Figure 6). p27Kip1 levels are regulated in part by the Ras/RhoA mitogenic pathway (Figure 6). Overexpression of a dominant negative Ras or RhoA inhibits PDGF-induced degradation of p27Kip1. C3 exoenzyme, which ADP-ribosylates and inactivates RhoA, inhibits PDGF-induced p27Kip1 degradation13,24 and inhibits thymbin-mediated vascular SMC proliferation and migration.14 Because loss of p27Kip1 reduced C3 exoenzyme (20 μg/mL) inhibition by 25%, the Rho pathway mediates migration through both p27Kip1-dependent and independent pathways. Higher concentrations of C3 exoenzyme led to decreased cell viability. In Swiss 3T3 fibroblasts, it has been shown that Rho can be activated by extracellular ligands and that Rho activation can lead to the assembly of contractile actin-myosin filaments and focal adhesion complexes.25 These observations suggest that the Rho GTPase family is one of the key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton. Rapamycin has not been shown to interact with the Rho GTPase family, although it is interesting that inhibition of Rho17,24 and of mTOR26 are both associated with increased levels of the CDK1 p27Kip1 (Figure 6).

The function of p27Kip1 is clinically relevant because of the connections that have been made between the downregulation and enhanced degradation of p27Kip1 in colorectal, stomach, breast, and small-cell lung cancers.26 Our findings suggest that agents that increase p27Kip1 levels in vivo may have both an antiproliferative and antiangiogenic effect. Rapamycin-mediated inhibition of SMC migration appears to depend on the presence of p27Kip1. This intriguing finding implicates p27Kip1 in the signaling pathway(s) that regulates both SMC proliferation and migration. Pharmacological and/or recombinant technologies aimed at increasing p27Kip1 may have dramatic effects on the amelioration of restenosis after angioplasty/stent placement and accelerated arteriopathy after cardiac transplantation.

Acknowledgments

This work was supported by the Sol and Margaret Berger Foundation, Clifton, NJ (Dr Rabban); an American Heart Association Clinician-Scientist Award to Dr Marx; and NIH grants R01-HL-56180, R01-HL-30290, R01-A1-39794, and RO3-TW-00949 and a Doris Duke Distinguished Clinical Scientist. We gratefully acknowledge Dr Andrew Koff for generously providing the p27-null mice used in this study.

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Circulation. 2001;103:2967-2972
doi: 10.1161/01.CIR.103.24.2967
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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