MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 6744–6751 Vol. 16, No. 12
0270-7306/96/$04.00+0

Rapamycin Resistance Tied to Defective Regulation of p27^{Kip1}

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Received 30 April 1996/Returned for modification 13 June 1996/Accepted 5 September 1996

The potent antiproliferative activity of the macrolide antibiotic rapamycin is known to involve binding of the drug to its cytosolic receptor, FKBP12, and subsequent interaction with targets of rapamycin, resulting in inhibition of p70 S6 kinase (p70^{S6K}). However, the downstream events that lead to inhibition of cell cycle progression remain to be elucidated. The antiproliferative effects of rapamycin are associated with prevention of mitogen-induced downregulation of the cyclin-dependent kinase inhibitor p27^{Kip1}, suggesting that the latter may play an important role in the growth pathway targeted by rapamycin. Murine BC3H1 cells, selected for resistance to growth inhibition by rapamycin, exhibited an intact p70^{S6K} pathway but had abnormally low p27 levels that were no longer responsive to mitogens or rapamycin. Fibroblasts and T lymphocytes from mice with a targeted disruption of the p27^{Kip1} gene had impaired growth-inhibitory responses to rapamycin. These results suggest that the ability to regulate p27^{Kip1} levels is important for rapamycin to exert its antiproliferative effects.

Rapamycin inhibits the mitogen-stimulated serine/threonine kinase p70^{S6K} (27, 41) and prevents cyclin-dependent kinase (CDK) activation, retinoblastoma protein (Rb) phosphorylation, and G_1 progression (1, 6, 10, 19, 22, 33, 35). Regulation of CDKs, which is crucial for orderly initiation and progression of the cell division cycle, involves CDK inhibitors among other mechanisms (34, 49). CDK inhibitors act stoichiometrically, and oscillations in their levels can have profound effects on cell proliferation (43). The CDK inhibitor p27^{Kip1} (39, 51) is present at high levels in quiescent cells and is downregulated by mitogenic stimulation (23, 37, 40, 43). Downregulation of p27 by mitogens can be blocked by rapamycin (37). The requirement of p27 for the antimitogenic response to rapamycin remains to be established.

Rapamycin acts by binding to the cytosolic protein FKBP12 (46), and this complex in turn binds to and inhibits FRAP (4). Activation of p70^{S6K} requires an additional input that is provided by FRAP (also known as RAFT) (3, 45), a mammalian kinase homologous to the yeast target of rapamycin (TOR) molecules (5, 19, 26). FRAP itself is not a mitogen-activated p70^{S6K} kinase but is required to render p70^{S6K} susceptible to activation by mitogenic signals (4, 7). As a result, rapamycin selectively prevents mitogen-induced p70^{S6K} activation without disrupting other known pathways (8, 25).

p27 levels are increased in response to mitogen deprivation, cell-cell contact, or addition of the antimitogen transforming growth factor β (39, 43, 50) and during myeloid cell differentiation (31) and neurogenesis (29). The high levels of p27 in quiescent macrophages, fibroblasts, or T lymphocytes are decreased in response to the mitogens colony-stimulating factor 1, serum, and interleukin-2 (IL-2), respectively (23, 37). Although p27 can be regulated at the mRNA level (31), its downregulation by mitogens occurs posttranscriptionally and involves ubiquitin-dependent degradation (38).

Inhibition of mitogen-stimulated downregulation of p27 by rapamycin raises the possibility that the regulation of p27 levels is critical for the antiproliferative activity of rapamycin. To address this question, we have taken several complementary approaches. We established rapamycin-resistant murine BC3H1 cells by culturing in the presence of the drug without any induced mutagenesis. Multiple independent rapamycin-resistant (RR) cell lines had intact p70^{S6K} regulatory responses but exhibited constitutively low p27 levels even after mitogen deprivation. Rapamycin no longer increased the p27 levels in these cells. Moreover, p27 null fibroblasts and T lymphocytes derived from *p27*$^{-/-}$ mice exhibited a significant resistance to growth inhibition by rapamycin. These results argue that the ability to block p27 downregulation contributes to the growth-inhibitory action of rapamycin.

MATERIALS AND METHODS

Establishment of RR cells. BC3H1 cells were grown in Dulbecco's modified essential medium (DMEM) with the addition of 20% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Medium was changed every 48 to 72 h; rapamycin was added directly to the culture medium (at final concentrations ranging from 0.1 to 1 μM). Cells were cultured for multiple passages over a 6-month period in the presence of high concentrations of rapamycin (0.1 to 1 μM) followed by dilutional cloning. Mv1Lu cells were from the American Type Culture Collection (CCL64). The rapamycin-resistant cell line RR-3 has been deposited in the American Type Culture Collection, where it is available on request.

DNA synthesis and cell proliferation assays. DNA synthesis in BC3H1 cells was determined by measuring the incorporation of [^{3}H]thymidine into DNA. Microcultures (~5,000 cells) were established in flat-bottom 96-well microtiter plates in the presence of various concentrations of rapamycin. After 48 h, cells were pulsed with [^{3}H]thymidine and harvested 16 to 20 h later. ^{3}H incorporation was measured in a liquid scintillation counter.

p27 immunoblot assay. Exponentially growing cells (BC3H1 or Mv1Lu) were cultured in medium containing 0.5 or 20% FBS in the presence of 20 nM rapamycin, 50 μM MG101, or 100 nM wortmannin for 24 h (Mv1Lu cells) or 16 h (BC3H1 cells) as indicated below. The cells were washed with phosphate-buffered saline (PBS) and lysed by scraping into hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], leupeptin [10 μg/ml], antipain [10 μg/ml], benzamidine hydrochloride [100 μg/ml], aprotinin [50 μg/ml], soybean trypsin inhibitor [100 μg/ml], pepstatin [10 μg/ml] with sonication. Protein concentration in the lysates were measured using the Bradford reagent (Bio-Rad), with bovine serum albumin as a standard. Cell lysates containing 50 μg of total protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12% gel) and Western blotting (immunoblotting) using a polyclonal anti-p27^{Kip1} antibody (43). The filters were washed

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and incubated with the secondary antibody conjugated to peroxidase for 20 min, and p27 was detected by chemiluminescence using the Amersham ECL system.

Rb immunoblot assay. Exponentially growing cells were plated at approximately 30% confluence. After 24 h, plates were washed twice with PBS and placed in low-serum medium (DMEM plus 0.5% FBS) for 72 h. Plates were then stimulated with 20% FBS and treated with either no drug (control) or 100 nM rapamycin. At the indicated time points, plates were washed with ice-cold PBS and lysates were prepared by scraping cells into lysis buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, 0.1 mM NaF, 0.2 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin [1 μg/ml], leupeptin [1 μg/ml], soybean trypsin inhibitor [10 μg/ml], 0.5% Nonidet P-40). Protein concentration in the lysates were measured by using the Bradford reagent (Bio-Rad), with bovine serum albumin as a standard. Cell lysates containing 50 μg of total protein were subjected to SDS-PAGE (7% gel). Filters were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% dry milk for 1 h, followed by incubation overnight with anti-pRb antibody (1/1,000 dilution) at 4°C. The filters were washed with PBS-T and then incubated with the secondary antibody conjugated to peroxidase for 1 h. Detection was with the ECL system.

FKBP12, RAFT/FRAP, and p27 reverse transcription-PCR assays. FKBP12, FRAP, and p27 cDNAs were isolated from parental and rapamycin-resistant BC3H1 cells with reverse transcription of mRNA as previously described (21). Primers were synthesized based on the sequence of the murine FKBP12 5′ and 3′ untranslated regions: sense, 5′-GCCACCCGCGTCCTTTTCC-3′; and antisense, 5′-GGCAGATCCACGTGCAGAGC-3′. Degenerate oligonucleotide primers were prepared based on the reported protein sequence for FRAP/RAFT (45): sense, 5′-AA(A/G)AA(C/T)ATGTG(C/T)GA(G/A)CA-3′; and antisense, 5′-CACCA(T/C)TC(T/C)TGiGC(T/C)TCCAT-3′. Primers were synthesized based on the sequence of the murine p27 (39); sense, 5′-CTCCATCCGTGGC GTTT-3′; and antisense, 5′-CATTAACCCACCG-3′. PCR was performed for 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 30 s. Products were ligated into pBluescript and sequenced on both strands.

p70^{S6K} phosphorylation and activity assays. Cells were incubated in medium containing 20% FBS in the presence or absence of 20 nM rapamycin for 45 min. Cells were washed with PBS and lysed at 4°C in buffer containing 10 mM $K(PO_4)$ (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_5, 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40. Cell lysates containing 50 μg of total protein were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting using anti-p70^{S6K} antibody C2 (9). Loading on Western blots was controlled by measuring the protein concentration, using the Bradford assay. For S6 kinase activity assays, cell lysates containing 1 mg of total protein were immunoprecipitated with anti-p70^{S6K} antibody N2 (9). The kinase activity of the immune complexes was determined by using an S6 peptide as a substrate as instructed by the supplier (Upstate Biotechnology, Inc.). Kinase activity was quantitated as the counts per minute of ^{32}P incorporated into the S6 peptide.

Determination of p27 half-life. Cells cultured under the indicated conditions for 24 h were placed in methionine-free medium for 30 min and then labeled with 200 μCi of [^{35}S]methionine per ml for 40 min. Cells were then placed in full medium to chase the metabolic label and harvested at the indicated time points for lysis with hypotonic buffer. The lysates were immunoprecipitated with anti-p27 antibody, and the precipitates were analyzed by SDS-PAGE (12% gel) and with a PhosphorImager. The radioactive signal associated with the p27 band was quantitated by using ImageQuant software (Molecular Dynamics).

DNA and nuclear fragmentation assays. Exponentially growing cells were incubated in medium containing 0.5 or 20% FBS for 24 h. For nuclear staining assays, cells that had spontaneously detached from the dishes and attached cells released by trypsinization were collected washed with PBS and pooled. For nuclear staining assays (2), the cell suspension was fixed with 3% paraformaldehyde for 10 min at room temperature, stained with DNA-binding fluorochrome bisbenzimide (16 μg/ml; Hoechst 33085) in 1% paraformaldehyde for 15 min, spread on a coverslip, and examined under the microscope. At least 600 cells were counted to determine the proportion showing fragmented or condensed nuclei. For DNA fragmentation assays (15), cells were lysed in a solution containing 5 mM Tris-Cl (pH 8.0), 5% glycerol, 0.05% bromophenol blue, and 5 mg of RNase A per ml. The sample was loaded onto a discontinuous agarose gel with the part above the sample wells containing 1% agarose, 2% SDS, and 64 μg of proteinase K per ml and the part below the wells being 2% agarose. Gels were run at 60 mV for 14 h at room temperature. DNA was visualized by ethidium bromide staining.

Generation and analysis of p27 null mouse cells. Mice homozygous for a targeted disruption of the *p27^{Kip1}* gene were obtained as described elsewhere (24). Embryonic primary fibroblasts were prepared from mouse embryos 13.5 days postcoitus as previously described (44). The fibroblasts were cultured in DMEM supplemented with 10% FBS. Exponentially growing fibroblasts (passage 4) were used in all experiments. p70^{S6K} assays were done as described above. [^{125}I]iododeoxyuridine incorporation into DNA was determined in sparse cultures of wild-type and *p27$^{-/-}$* mouse embryo fibroblasts after 24 h of incubation in the presence of the indicated concentrations of rapamycin. Cells were pulsed with [^{125}I]iododeoxyuridine and harvested 4 h later. ^{125}I incorporation was measured in a gamma counter.

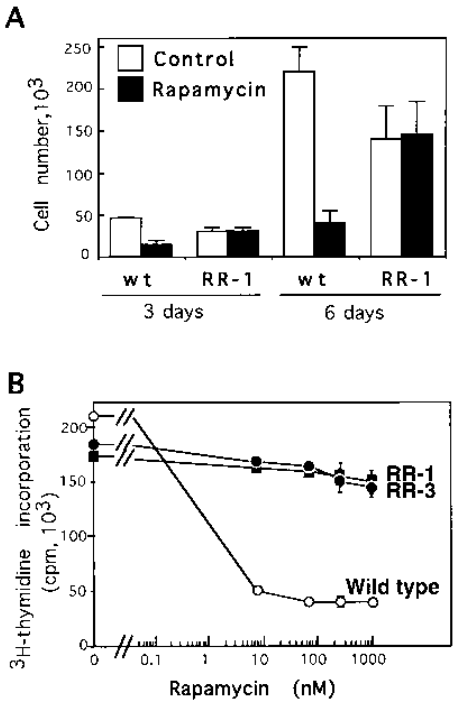

FIG. 1. Growth characteristics of RR BC3H1 cells. (A) Rapamycin inhibited cell growth in parental BC3H1 cells but not in RR cells. Cell cultures (10^4 cells each) were plated on day 0 in growth medium alone or containing 1 μM rapamycin. Cells counts were determined at the indicated times. Data are the averages of triplicate samples ± standard deviations. wt, wild type. (B) Rapamycin inhibited DNA synthesis in parental BC3H1 cells but not in two independent RR cell lines. Cells were cultured with various concentrations of rapamycin for 48 h, at which point 1 μCi of [^3H]thymidine was added. Cells were harvested 20 h later, and the [^3H]thymidine incorporated into DNA was counted. Data are the averages of four determinations ± standard deviations.

Splenic lymphocytes were obtained by gently pressing the spleen against the bottom of a culture dish with a bent syringe needle. Cells were collected and incubated for 5 min at room temperature in 0.15 M NH_4Cl–1 mM $KHCO_3$–0.1 mM EDTA (pH 7.2) to lyse erythrocytes. Cells were pelleted, washed, and resuspended in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Splenic T lymphocytes were activated in RPMI 1640 with 10% FBS, 3 μg of hamster anti-CD3 antibody (Pharmingen) per ml, 10 μg of goat anti-hamster immunoglobulin G antibody (Chappel) per ml, 100 U of IL-2 per ml, and different concentrations of rapamycin. At 48 h after activation, cells were labeled with 10 μCi of [^3H]thymidine per ml for 4 h. The labeled cells were harvested, and the incorporated radioactivity was measured by liquid scintillation counting.

RESULTS

p70^{S6K} regulation in RR cells. BC3H1 cells are a murine myogenic cell line (47) that is growth inhibited by nanomolar concentrations of rapamycin (22). Rapamycin, as low as 0.2 nM, blocks cell cycle progression in BC3H1 cells (22). Prolonged incubation of these cells with rapamycin resulted in the selection of two independent clones, RR-1 (selected in the presence of 1 μM rapamycin) and RR-3 (selected at 0.1 μM rapamycin), that are resistant to growth inhibition by rapamycin, as determined by cell counts and thymidine incorporation assays (Fig. 1). Proliferation of RR-1 and RR-3 cells was unaffected by as much as 1 μM rapamycin (Fig. 1B).

The effects of rapamycin on p70^{S6K} activity were examined in exponentially growing cells that were either serum starved, cultured in 20% FBS, or cultured in 20% FBS plus rapamycin (20 nM). Immunoblot analyses of total cell lysates indicated that rapamycin induced a switch in p70^{S6K} from the hyperphos-

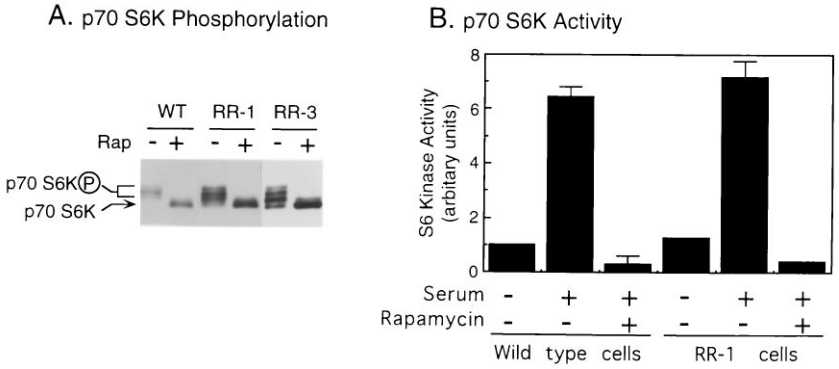

FIG. 2. Regulation of $p70^{S6K}$ is intact in cells resistant to growth inhibition by rapamycin. (A) Parental BC3H1 cells (wild type [WT]) or two RR BC3H1 cell lines (RR-1 and RR-3) were cultured with 20% FBS and received 20 nM rapamycin for 45 min or no additions. Cell lysates (50 μg of total protein) were analyzed by Western immunoblotting using an anti-$p70^{S6K}$. The phosphorylated (active) forms of $p70^{S6K}$ are the slow-migrating forms (9). (B) Parental BC3H1 cells and RR-1 cells were placed in medium containing 0.5% FBS, 20% FBS alone, or 20% FBS with 20 nM rapamycin. Cell lysates (1 mg of total protein) were immunoprecipitated with anti-$p70^{S6K}$ antibody, and the kinase activity of the immune complexes was assayed with S6 peptide as the substrate. Data are expressed as relative activity compared to the S6 kinase activity in untreated wild-type cells. Data are the averages of two determinations, and bars show the range of values.

phorylated, active state (slower-migrating bands) to the hypophosphorylated state (faster-migrating bands) in both parental and RR BC3H1 cells (Fig. 2A). Similarly, in parental and RR cells, both serum starvation and rapamycin inhibited $p70^{S6K}$ activity to levels as low as 5% of those seen with serum stimulation (Fig. 2B). Compared to wild-type cells, the levels of $p70^{S6K}$ were 3-fold higher in RR-1 cells and 1.5-fold higher in RR-3 cells, based on immunoblot analyses (Fig. 2A). Although there was more $p70^{S6K}$ protein in the RR cells, the levels of the active form (the most phosphorylated or slowest-migrating band) were similar. To ensure that the activity assays were accurate despite the increased level of $p70^{S6K}$ protein in the RR cells, we performed $p70^{S6K}$ activity assays with various amounts of cell lysate and determined that under the conditions used, the assay was in the linear range, indicating that the increased amount of $p70^{S6K}$ protein in the RR cells was not saturating (data not shown). These results indicate that the $p70^{S6K}$ inhibitory pathway is intact in the RR cells. This pathway is thought to involve rapamycin binding to FKBP12 and binding of this complex to FRAP (4). Indeed, cloning and sequencing of the endogenous FKBP12 and part of FRAP (corresponding to the region that confers rapamycin resistance when mutated in the yeast homolog [26]) showed that they were both wild type in the RR-1 cells, and the levels of FKBP12 protein as determined by immunoblot were also normal in these cells (data not shown).

Deregulated p27 levels in RR cells. p27 protein levels were determined in exponentially growing cells subjected to serum withdrawal, serum stimulation, serum plus rapamycin, or serum plus MG101 (an inhibitor of the ubiquitin-dependent degradation pathway involved in mitogen-induced p27 degradation [38]). Equal amounts of cell lysates were immunoblotted with an anti-p27 antibody. The p27 levels after serum starvation were >5-fold lower in the RR-1 and RR-3 cells than in the parental cells and were comparable to the p27 levels in serum-stimulated parental cells (Fig. 3A). Furthermore, p27 levels in the RR cells were no longer regulated: they were neither decreased in response to serum nor increased in response to rapamycin (Fig. 3A).

The low levels of p27 in the RR-1 cells were not due to constitutive activation of a ubiquitin-dependent degradation process, since MG101, which prevented the downregulation of p27 by serum in parental BC3H1 cells, failed to stimulate the accumulation of p27 in RR cells (Fig. 3A). The lack of ubiq-

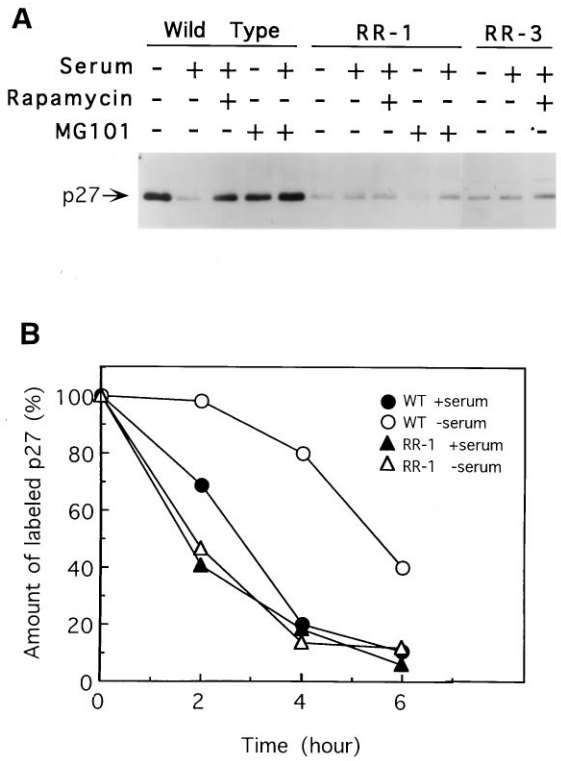

FIG. 3. p27 Levels are constitutively low in rapamycin-resistant cells. (A) Levels of p27 protein. Proliferating cell monolayers were placed in medium containing 0.5% FBS and received 20% FBS, 20 nM rapamycin, and/or 50 μM MG101 as indicated. After 24 h, cell lysates were prepared and aliquots (50 μg of total protein) were analyzed by Western immunoblotting using anti-$p27^{Kip1}$ antibody C2. (B) Half-life of p27 in BC3H1 cells. Cell monolayers were incubated with 20 or 0.5% FBS for 24 h. Cells were then metabolically labeled with [^{35}S]methionine for 40 min, and the label was chased for the indicated times in complete medium. Cell lysates were immunoprecipitated with anti-p27 antibody N2, and the immune complexes were analyzed by SDS-PAGE and exposure in a PhosphorImager. The signal associated with the p27 bands was quantitated and is plotted as a percent of the signal at the start of the chase in each condition. The experiment was repeated twice with similar results in each case. WT, wild type.

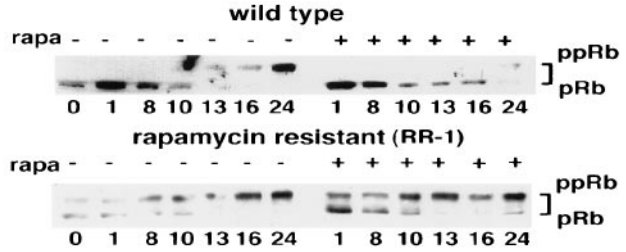

FIG. 4. Rb phosphorylation in RR cells is not affected by rapamycin and persists in mitogen-poor medium. Exponentially growing cells were placed in low-serum medium (DMEM plus 0.5% FBS) for 72 h. Cells were then stimulated with 20% FBS and treated with either no drug or 100 nM rapamycin (rapa). pRb was examined at the indicated time points (in hours) by Western immunoblotting using an anti-pRb antibody. The slow-migrating forms of pRb correspond to hyperphosphorylated forms (12).

uitin-mediated degradation of p27 in RR cells was not due to a generalized defect in the ubiquitin pathway, since MG101 did inhibit the degradation of cyclin B1 in both BC3H1 and RR-1 cells (data not shown). The loss of p27 in serum-deprived RR-1 cells incubated with MG101 (Fig. 3A) was a reproducible finding that we presume is due to a nonspecific toxic effect of MG101 on these cells when serum starved. A specific effect of MG101 would be to increase p27 levels rather than decrease them.

The p27 mRNA levels in parental and RR cells were similar, as determined by Northern (RNA) analysis (data not shown). However, the half-life of p27 in the RR cells was markedly decreased, as determined by immunoprecipitation and quantitation of labeled p27 from pulse-chase metabolically labeled cells (Fig. 3B). The half-life of p27 in RR1 cells was less than 2 h either in the presence or in the absence of serum, which was similar to the half-life of p27 in serum-stimulated parental cells and significantly less than the half-life (~5 h) in serum-starved parental cells. Therefore, the low levels of p27 in the RR cells are due to a high rate of ubiquitin-independent degradation. Sequencing of the p27 mRNA from RR-1 cells showed that it was wild type (data not shown).

Rb phosphorylation and apoptosis in serum-starved RR cells. In various cell types, the decrease in p27 levels upon addition of mitogens helps activate CDKs, leading to Rb hyperphosphorylation and cell cycle progression (23, 37). Since the p27 levels in our RR cells were low already in the absence of serum, it was of interest to determine whether this anomaly would result in mitogen-independent Rb phosphorylation. Indeed, immunoblot analysis showed the presence of hyperphosphorylated (slow-migrating) Rb in serum-starved RR-1 cells, whereas this form was absent in serum-starved parental BC3H1 cells (Fig. 4). Furthermore, the progressive accumulation of hyperphosphorylated Rb upon mitogenic stimulation of serum-starved cells was blocked by rapamycin in parental BC3H1 but not in RR-1 cells (Fig. 4), which is consistent with the fact that the latter cells were able to proliferate.

In the course of this work, we noticed that the RR cells remained in a proliferative state when placed in mitogen-poor medium, as determined by [^{3}H]thymidine or [^{125}I]iododeoxyuridine incorporation into DNA, but failed to accumulate because of continued detachment from the culture dish and death. In several instances, it has been shown that blocking or bypassing Rb function causes apoptosis when cells are placed in mitogen-poor medium (11, 42, 48, 52). These and other studies have suggested that conflicts between activation of the Rb pathway in the absence of concurrent activation of other mitogenic pathways can induce apoptosis. Thus, the presence of inactivated (hyperphosphorylated) Rb in mitogen-deprived RR-1 cells raised the possibility that cells in this population could be driven to apoptosis, rather than growth arrest, by serum deprivation. Confirming this possibility, we found that following 24 h of serum deprivation, the RR cells were detaching from the dishes and showed apoptotic features including a typical DNA fragmentation ladder on agarose gels (Fig. 5A), as well as chromatin condensation and nuclear fragmentation in 35 to 43% of the entire (attached plus floating) cell population (Fig. 5B). The attached cell population, which was the population used for the p27 and p70^{S6K} assays described above, showed little evidence of apoptosis (<3% nuclear fragmentation) when analyzed separately from the floating population of cells.

Impaired rapamycin response in *p27*$^{-/-}$ fibroblasts and T lymphocytes. The finding that selection for cell resistance to growth inhibition by rapamycin yields clones with constitutively low and deregulated p27 levels is consistent with the possibility that p27 plays a critical role in the growth-inhibitory action of rapamycin. To directly test this hypothesis, we used *p27* null cells derived from mice that have a targeted disruption of the *p27* gene (24). Primary embryo fibroblasts from *p27*$^{-/-}$ animals showed a normal p70^{S6K} inhibition response to rapamycin. p70^{S6K} phosphorylation was inhibited half-maximally at 0.1 nM rapamycin and fully at 1 nM rapamycin (Fig. 6A), which is very similar to the effect in wild-type fibroblasts T lymphocytes and other cell types (data not shown) (27, 41). However, exponentially growing *p27*$^{-/-}$ fibroblasts had an impaired antiproliferative response to rapamycin (0.1 to 1 nM), as determined by the rate of [^{125}I]iododeoxyuridine incorporation into DNA. The extent of inhibition of DNA synthesis in *p27*$^{-/-}$ fibroblasts was only half that observed in *p27*$^{+/+}$ fibroblasts (Fig. 6B). Cell density did not affect the rapamycin response, since similar effects were observed with sparsely seeded and densely seeded cells (data not shown).

The impairment of antimitogenic responsiveness to rapamycin in *p27* null cells was confirmed using splenic T lymphocytes (Fig. 6C). This cell type was of particular interest because one of the most apparent phenotypic traits observed in *p27*$^{-/-}$ animals is an enlarged thymus and spleen owing to an increase in the number of T cells and the proportion of cycling cells (16, 24, 36). The inhibitory effect of rapamycin was less extensive in *p27*$^{-/-}$ T cells than in *p27*$^{+/+}$ T cells. The sensitivity to growth inhibition by rapamycin was 15- to 30-fold lower in *p27*$^{-/-}$ cells (50% inhibitory dose = 0.6 to 1 nM rapamycin) than in *p27*$^{+/+}$ cells (50% inhibitory dose = 0.03 nM rapamycin) (Fig. 6C). These results suggest that rapamycin exerts antimitogenic effects through several mechanisms; one requires p27 and is activated by rapamycin concentrations in the 0.03 to 1 nM range, whereas the other(s) is p27 independent.

DISCUSSION

Rapamycin has been used as a molecular probe to dissect the mitogen-stimulated signaling pathways that control cell proliferation. The rapamycin-FKBP12 complex binds to FRAP/RAFT and inhibits p70^{S6K} activation. A hallmark of rapamycin-induced interference with mitogenic signals is the inhibition of CDKs and Rb phosphorylation. Moreover, the antiproliferative response to rapamycin is associated with an increase in the levels of the CDK inhibitor p27^{Kip1}. Therefore, it has been proposed that regulation of p27, possibly involving p70^{S6K}, is critical to the antiproliferative activity of rapamycin, although there is no evidence for a direct link between these molecules. In this present study, we provide evidence that

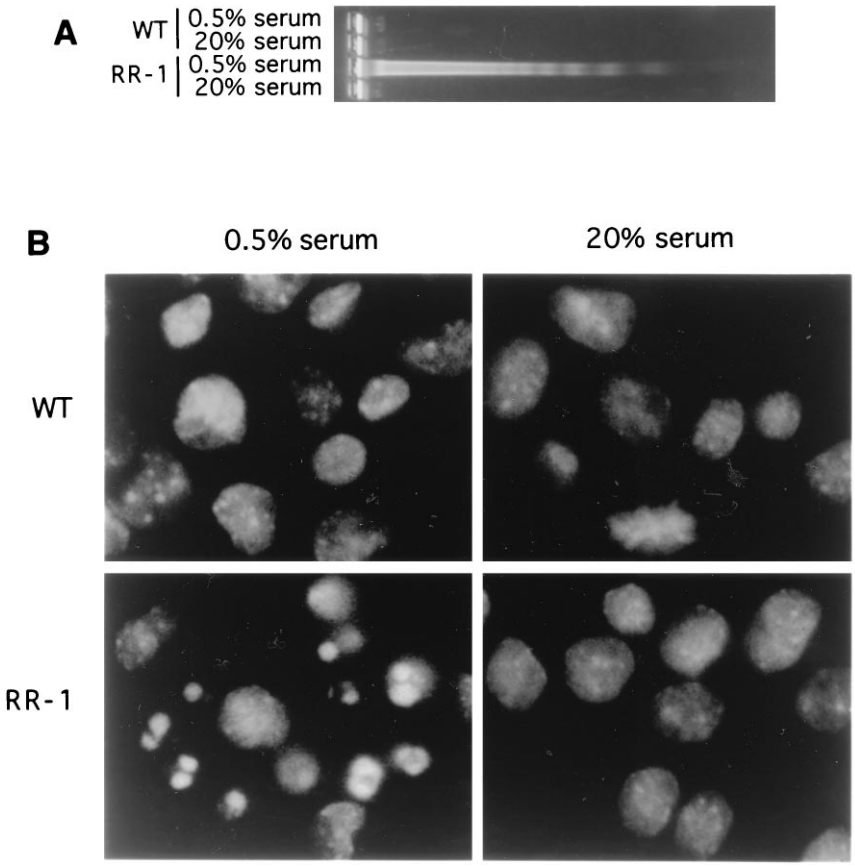

FIG. 5. Serum withdrawal induces apoptosis in the RR cells. Cells were cultured for 24 h in medium containing the indicated amounts of FBS. Cells that were attached to the culture dishes and cells that became spontaneously detached were pooled, and DNA fragmentation (A) and nuclear morphology (B) were examined as described in Materials and Methods. WT, wild type.

establishes the importance of p27 regulation in the antiproliferative activity of rapamycin.

Previous work showed that inhibition of Cdk2 by rapamycin is associated with increasing p27 levels in mitogenically stimulated T lymphocytes and human diploid fibroblasts (37). In the present study, independent BC3H1 cell lines that are resistant to inhibition of cell cycle regulators by rapamycin all exhibit the same abnormality: defective regulation of p27 levels. The constitutively low levels of p27 observed in the RR BC3H1 cells are explained by increased p27 degradation, as evidenced by a decrease in the half-life of the protein. Earlier studies demonstrated that changes in p27 levels quantitatively comparable to those observed between parental and RR BC3H1 cells can determine whether a cell will proliferate or arrest. For example, it has been shown that in an inducible p27 expression system, as little as a threefold increase in p27 levels can result in cell cycle arrest (43).

Rapamycin inhibits $p70^{S6K}$ activation (9, 27, 41), which is essential for G_1 progression in various cell types (28). However, in the RR cells, transition through G_1 to S does not depend on $p70^{S6K}$ activation, as these cells proliferate in the presence of rapamycin, even though $p70^{S6K}$ is inactivated. In the RR cells, constitutively low levels of p27 protein uncouple $p70^{S6K}$ from the antimitogenic effects of rapamycin. There are several possible explanations for this uncoupling. The best-known substrate of $p70^{S6K}$ is the 40S ribosomal protein S6 (8, 25), and S6 phosphorylation is thought to increase the translation rate of certain mRNAs required for G_1-phase progression. Moreover, in addition to ubiquitin-dependent degradation of p27, translational control also plays a role in regulating p27 levels during the cell cycle (20). It is conceivable, therefore, that under normal circumstances, $p70^{S6K}$ activates translation of an mRNA encoding a protein involved in p27 degradation, but that the abnormally short half-life of the p27 protein in the RR BC3H1 cells prevents significant changes in the levels of p27 protein. It is also possible that a protease is mutated in the RR cells such that it is constitutively activated resulting in low levels of p27 throughout the cell cycle.

In contrast to the mechanism of p27 downregulation by mitogens in parental BC3H1 cells, the short half-life of p27 in RR cells is not due to constitutive activation of a ubiquitin-dependent mechanism. This point is based on the observation that an inhibitor of ubiquitin-dependent degradation, MG101, prevents the serum-induced loss of p27 in parental cells but not the constitutive degradation of p27 in RR cells. The mechanism that leads to the short half-life of p27 in the RR BC3H1 cells remains to be determined. Characterization of RR mutants of YAC-1 murine T cells indicated that in these cells, the mutation was dominant (13, 14). This would be consistent with the constitutive activation of a protease that degraded p27.

A critical finding in the present study is that selection for cell resistance to the antimitogenic action of rapamycin yields clones with constitutively low levels of p27. The p27 levels in these cells are no longer affected by serum or rapamycin. Rapamycin resistance was established in BC3H1, a cell line that is normally growth inhibited by low concentrations of

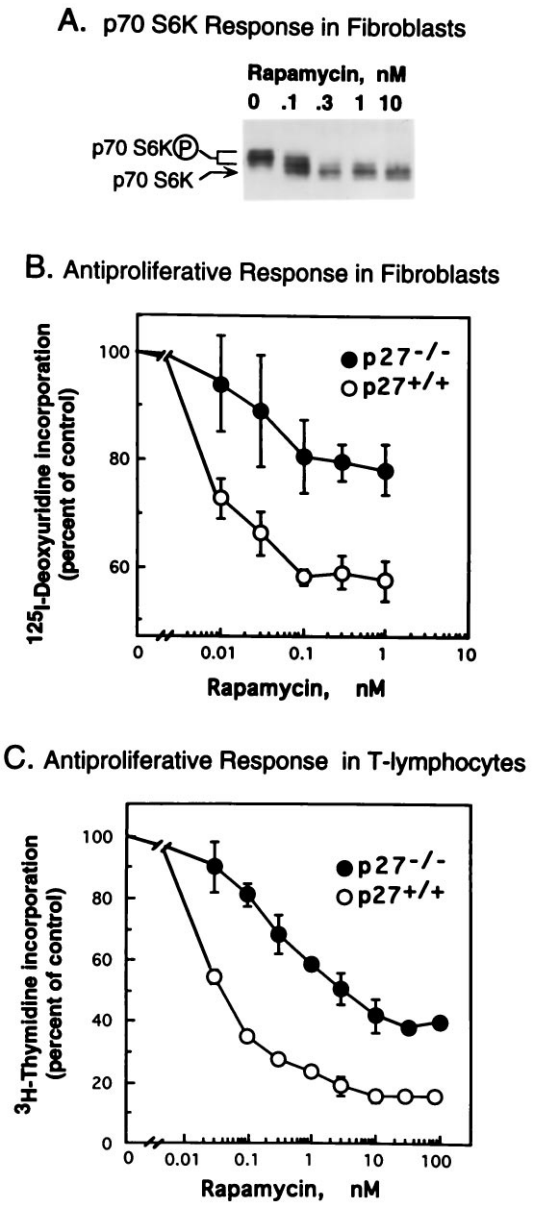

FIG. 6. Impaired growth-inhibitory response to rapamycin in p27 null cells. (A) The phosphorylation state of p70^{S6K} was assessed by Western immunoblotting in extracts from *p27*$^{-/-}$ mouse primary embryo fibroblasts treated with the indicated concentrations of rapamycin for 45 min. (B) [^{125}I]iododeoxyuridine incorporation into DNA was determined in sparse cultures of wild-type and *p27*$^{-/-}$ mouse embryo fibroblasts after 24 h of incubation in the presence of the indicated concentrations of rapamycin. (C) Wild-type and *p27*$^{-/-}$ primary T lymphocytes were activated with anti-CD3 antibody and incubated with IL-2 and various concentrations of rapamycin for 48 h. [^{3}H]thymidine incorporation into DNA was then determined. In panels B and C, data are presented as the percent incorporation relative to controls that did not receive rapamycin and as the averages of triplicate determinations ± standard deviations. The experiments were repeated four times with similar results.

rapamycin in a manner very similar to that described for other rapamycin-sensitive cell types (22). The method chosen for establishing RR BC3H1 cells, selecting for cells that could grow in high concentrations of rapamycin, did not involve any mutagenesis protocols. The findings in the present study are based on parallel experiments using two entirely independent RR cultures. Importantly, p70^{S6K} regulation by serum or rapamycin is intact in these cells. The fact that independent RR populations, selected without forced mutagenesis, both exhibited abnormal regulation of p27 supports the notion that p27 downregulation is a critical step in mitogenic stimulation of G_1 progression. To assess whether other cyclin inhibitors besides p27 were regulated abnormally in RR cells, we examined the levels of p15 and p21 in BC3H1, RR-1, and RR-3 cells. However, none of these CDK inhibitors are present at detectable levels in BC3H1 cells or in the RR cells. These data suggest that p27 is the major CDK inhibitor in BC3H1 cells, and since the levels of p15 and p21 were so low in BC3H1 and RR cells, we cannot comment on whether these other CDK inhibitors were also downregulated in RR cells.

The ability of rapamycin to inhibit p70^{S6K} activation indicates that the rapamycin-FKBP12-FRAP pathway is intact in these resistant cells. Indeed, the levels of FKBP12 protein are normal in these cells, and we found no mutations in either FKBP12 or the region of FRAP that in the yeast homolog TOR2 contains a mutation that confers rapamycin resistance (26). Recently, additional mutations in TOR1 and TOR2 that confer rapamycin resistance by preventing rapamycin-FKBP12 binding to TOR1 and TOR2 have been described (32). Similar mutations in FRAP also block rapamycin-FKBP12 binding. However, the presence of these mutations in the RR BC3H1 cells is highly unlikely since the sensitivity of p70^{S6K} activation to rapamycin is normal in these cells (4).

Two additional observations illustrate the functional significance of low p27 levels in these RR cells. First, when deprived of serum, the RR cells still contain hyperphosphorylated Rb and progress into S phase, both events are indicative of the presence of G_1 CDK activity. Second, the cells have a high propensity to undergo apoptosis under these conditions. Apoptosis in serum-free media has been found in systems in which the Rb pathway is activated without the concurrent activation of other mitogenic pathways. For example, blocking Rb action by overexpression of adenovirus E1A protein (11) or circumventing it by E2F overexpression (42, 48, 52) induces apoptosis when cells are placed in serum-free medium. This process appears to involve a p53-dependent checkpoint that triggers apoptosis in response to an imbalance of mitogenic signals (11, 42, 52). The high rate of apoptosis in our serum-deprived RR cells is therefore consistent with the presence of mitogen-independent Rb kinase activation.

The demonstration that cells from mice with a targeted disruption of the *p27* gene are partially resistant to the antiproliferative effects of rapamycin provides further support for the hypothesis that rapamycin exerts its antimitogenic action in part by preventing mitogen-induced downregulation of p27. Although rapamycin blocks p70^{S6K} activation in both primary cultures and BC3H1 cells, constitutively low levels or absence of p27 interferes with the antimitogenic effects of the drug. Moreover, the antimitogenic effect of rapamycin varies with the cell type, suggesting different degrees of dependence on rapamycin-sensitive mitogenic mechanisms. Serum-stimulated BC3H1 cells and IL-2-stimulated splenic T lymphocytes are profoundly growth inhibited by rapamycin, whereas primary mouse embryo fibroblasts are inhibited less extensively. More importantly, the results indicate that antimitogenic responses to rapamycin in both cell types are at least partially dependent on p27, since these responses are impaired in the *p27*$^{-/-}$ counterparts. p27 deficiency is associated with a decrease in both the amplitude and the sensitivity of the antiproliferative response to rapamycin. The decrease in sensitivity is most apparent in the *p27*$^{-/-}$ T lymphocytes, which require a 15- to 30-fold-higher concentration of rapamycin than do wild-type cells for a comparable level of growth inhibition. The maximal

difference in the rapamycin response of wild-type and *p27* null cells is achieved at the low rapamycin concentration range that also achieves maximal inhibition of $p70^{S6K}$.

One interpretation of the results is that rapamycin may inhibit T-lymphocyte proliferation through at least two separate mechanisms, one that correlates with $p70^{S6K}$ inhibition and requires p27 and another that is p27 independent and occurs at higher rapamycin concentrations. Indeed, the partial sensitivity of the *p27* null cells to rapamycin indicates that there is more than one pathway that determines rapamycin sensitivity. BC3H1 cells which are completely growth inhibited at the lowest concentrations of rapamycin (e.g., 10 pM) may have only the p27-dependent pathway, since rapamycin sensitivity is completely abrogated in the RR cells. However, it is also possible that p27-dependent and -independent mechanisms mediating rapamycin sensitivity are present in BC3H1 cells and are both lost in the RR cell lines.

Our observation of significant differences in rapamycin sensitivity between wild-type and *p27* null fibroblasts and T cells is in contrast to the results of Nakayama et al. (36), who did not detect a difference in the effects of rapamycin on cell growth between wild-type and *p27* null thymocytes. The discrepancy between our results and those of Nakayama et al. could be due to differences in cell culture and assay conditions. Nakayama et al. activated T cells by anti-CD3 plus anti-CD28 monoclonal antibodies and examined the effect of rapamycin on T-cell proliferation after 6 days in culture (36), whereas we activated T cells by anti-CD3 antibody plus its secondary antibody and our assays were done with T cells that had been in culture for only 2 days.

Kiyokawa et al. (24) have shown that there is no difference in apoptosis induced by either ionizing radiation or dexamethasone between wild-type and *p27* null thymocytes. This finding is in agreement with our observation that RR cells can undergo apoptosis despite having low levels of p27.

The properties of the $p27^{-/-}$ cells described here support the conclusion that loss of p27 CDK-inhibitory function enhances the proliferative ability of the cell. Disruption of *p27* in mice enhances postnatal growth owing to an increase in cell number in most organs (17, 24, 36). This phenotype is particularly apparent in the thymus and spleen, two tissues with relatively high p27 levels in normal mice. The increase in cell number is accompanied by an increase in the proportion of cycling cells, suggesting a propensity of *p27* null cells to avoid quiescence or terminal differentiation. Our observation that cells from these animals have an impaired antimitogenic response to rapamycin supports the view that increased growth may result from an intrinsic hyperproliferative potential of the *p27* null cells rather than the effect of a circulating somatotropic factor (24).

In conclusion, loss of p27 activity, whether as a result of cell selection in the presence of rapamycin or from targeted disruption of its gene, is associated with resistance to rapamycin. The level of resistance ranges from limited (e.g., in *p27* null cells) to complete (e.g., in BC3H1 RR cells). Thus, rapamycin inhibits cell proliferation in part by opposing the mitogen-induced downregulation of p27. It also implies that p27 downregulation by serum or IL-2 is an important event in the mitogenic response to these agents. Thus, rapamycin represents a class of growth inhibitors that oppose the action of mitogens by blocking their signaling pathways, in the present case a pathway leading to downregulation of a CDK inhibitor. This mode of action distinguishes rapamycin from another class of antimitogens, represented by transforming growth factor β, which oppose the action of mitogens by regulating the expression of CDKs and their inhibitors (16, 18, 43) rather than by inhibiting mitogenic signaling pathways (30).

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

We are grateful to J. Blenis for anti-$p70^{S6K}$ antibody, to J. Avruch for $p70^{S6K}$ cDNA, to R. Kolesnick for advice with the apoptosis assays, and to M. Ono for help with lymphocyte preparation.

This work was supported by grants from the National Institutes of Health to J.M. and A.R.M. and a MERCK/ACC fellowship to S.O.M. J.M. is a Howard Hughes Medical Institute Investigator. A.R.M. is a Bristol-Meyers Squibb Established Investigator of the American Heart Association.

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