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p53/p21^{CIP1} Cooperate in Enforcing Rapamycin-induced G_1 Arrest and Determine the Cellular Response to Rapamycin[1]

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ABSTRACT

The relationship between G_1 checkpoint function and rapamycin-induced apoptosis was examined using two human rhabdomyosarcoma cell lines, Rh1 and Rh30, that express mutated *p53* alleles. Serum-starved tumor cells became apoptotic when exposed to rapamycin, but were completely protected by expression of a rapamycin-resistant mutant mTOR. Exposure to rapamycin (100 ng/ml) for 24 h significantly increased the proportion of Rh1 and Rh30 cells in G_1 phase, although there were no significant changes in expression of cyclins D1, E, or A in drug-treated cells. To determine whether apoptosis was associated with continued slow progression through G_1 to S phase, cells were exposed to rapamycin for 24 h, then labeled with bromodeoxyuridine (BrdUrd). Histochemical analysis showed that >90% of cells with morphological signs of apoptosis had incorporated BrdUrd. To determine whether restoration of G_1 arrest could protect cells from rapamycin-induced apoptosis, cells were infected with replication-defective adenovirus expressing either p53 or p21^{CIP1}. Infection of Rh30 cells with either Ad-p53 or Ad-p21, but not control virus (Ad-β-gal), induced G_1 accumulation, up-regulation of p21^{CIP1}, and complete protection of cells from rapamycin-induced apoptosis. Within 24 h of infection of Rh1 cells with Ad-p21, expression of cyclin A was reduced by >90%. Similar results were obtained after Ad-p53 infection of Rh30 cells. Consistent with these data, incorporation of [^{3}H]thymidine or BrdUrd into DNA was significantly inhibited, as was cyclin-dependent kinase 2 activity. These data indicate that rapamycin-induced apoptosis in tumor cells is a consequence of continued G_1 progression during mTOR inhibition and that arresting cells in G_1 phase, by overexpression of p53 or p21^{CIP1}, protects against apoptosis. The response to rapamycin was next examined in wild-type or murine embryo fibroblasts nullizygous for *p53* or *p21*^{CIP1}. Under serum-free conditions, rapamycin-treated wild-type MEFs showed no increase in apoptosis compared to controls. In contrast, rapamycin significantly induced apoptosis in cells deficient in p53 (~2.4-fold) or p21^{CIP1} (~5.5-fold). Infection of p53$^{-/-}$ MEFs with Ad-p53 or Ad-p21 completely protected against rapamycin-induced apoptosis. Under serum-containing conditions, rapamycin inhibited incorporation of BrdUrd significantly more in wild-type murine embryo fibroblasts (MEFs) than in those lacking p53 or p21^{CIP1}. When BrdUrd was added 24 h after rapamycin, almost 90% and 70% of cells lacking p53 or p21^{CIP1}, respectively, incorporated nucleoside. In contrast, only 19% of wild-type cells incorporated BrdUrd in the presence of rapamycin. Western blot analysis of cyclin levels showed that rapamycin had little effect on levels of cyclins D1 or E in any MEF strain. However, cyclin A was reduced to very low levels by rapamycin in wild-type cells, but remained high in cells lacking p53 or p21^{CIP1}. Taken together, the data suggest that p53 cooperates in enforcing G_1 cell cycle arrest, leading to a cytostatic response to rapamycin. In contrast, in tumor cells, or MEFs, having deficient p53 function the response to this agent may be cell cycle progression and apoptosis.

INTRODUCTION

The mammalian target of rapamycin, mTOR [also designated FRAP, RAFT1, or RAPT1 (1–3)], has been shown to link mitogen stimulation to protein synthesis and cell cycle progression. Both phosphatidylinositol 3′-kinase and, potentially, AKT/PKB considered to lie upstream of mTOR, can protect cells from apoptosis induced by stress. However, a role for mTOR in cell survival has not been established. To examine the potential role of mTOR in tumor cell survival, we used the macrocyclic lactone antibiotic rapamycin that potently inhibits mTOR. Rapamycin competes with a structural analogue, FK-506, for binding to a M_r 12,000 cytosolic protein designated FKBP-12. The FKBP-rapamycin complex inhibits the function of a serine/threonine kinase, mTOR, blocking growth factor stimulation of ribosomal p70S6 kinase, and phosphorylation of the eIF4E[4] binding protein (also designated PHAS-I). 4E-BP1 is a direct substrate for mTOR kinase activity in cells (4, 5), whereas certain evidence indicates the potential for intermediate steps in the mTOR-mediated activation of p70S6 kinase (6–8). In growth factor-deprived cells, association of eIF4E with the multifunctional scaffolding protein eIF-4G is inhibited by 4E-BP1 and 4E-BPII (9, 10). Upon growth factor or serum stimulation, phosphorylation occurs leading to dissociation of 4E-BP proteins, assembly of the multisubunit complex (eIF-4F), and efficient translation of mRNA having highly structured 5′-untranslated regions (11). In many cell lines, exposure to rapamycin results in a relatively small decrease in overall protein synthesis (~15–20%), but results in a specific G_1 cell cycle arrest. We have shown previously that rhabdomyosarcoma cells are highly sensitive to growth inhibition by rapamycin (12), and under serum-free conditions rapamycin induced p53-independent apoptosis (13).

The tumor suppressor p53 is a transcription factor that has been found to be mutated in >50% of human cancers (14, 15). In response to genotoxic damage, such as exposure to chemotherapeutic agents, γ-irradiation, or UV irradiation, p53 is up-regulated (16–18). Up-regulation of p53 either results in arrest of cells in G_1 phase and participates in DNA repair (18–21) or drives cells toward apoptosis (19, 22, 23). The functions of p53 are dependent on cell type and developmental stage (24), but the mechanisms by which p53 exerts its functions is still not fully understood (25). Many reports show that p53 functions as a tumor suppressor by promoting apoptosis (15, 25). On the other hand, recent studies also demonstrate that p53 may retard tumor progression by another mechanism based on irreversible growth arrest which may lead to senescence (26, 27).

p53-dependent cell cycle arrest is in part a consequence of up-regulation of p21^{CIP1}, a p53-inducible gene (28–30). p21^{CIP1} inhibits G_1 cdk which phosphorylate pRb and related family members, leading to a G_0-G_1 arrest of the cell cycle (31–34). Interestingly, recent findings further reveal that p21^{CIP1}-induced cycle arrest in G_1 phase protects cells from apoptosis induced by ionizing radiation or chemical exposure (35–42). Down-regulation of p21^{CIP1} using antisense

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[4] The abbreviations used are: eIF-4E, eukaryotic initiation factor 4E; 4E-BP1, 4E-binding protein; cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; MN2E, modified N2E; FBS, fetal bovine serum; MOI, multiplicity of infection; BrdUrd, bromodeoxyuridine; Ad-β-gal, adenovirus β-galactosidase; DAPI, 4,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting.

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oligonucleotides has also been shown to radiosensitize cells by converting growth arrest to apoptosis (43). In contrast to the role of p53 in response to DNA damage, p53 appears also to act as a sensor, causing G_1 arrest of cells prior to damage. In cells treated with the antimetabolite *N*-(phosphonacetyl)-L-aspartic acid, an inhibitor of carbamoylphosphate synthetase that depletes cellular pools of both purines and pyrimidines p53 initiates a G_1 block prior to initiation of DNA replication (44, 45).

The observation that tumor cells with mutated p53 undergo apoptosis when exposed to rapamycin, in contrast to G_1 arrest, prompted us to investigate whether p53 acted as a sensor to prevent G_1 progression in drug-treated cells in which synthesis of a subset of proteins had been inhibited. Our results reveal that rapamycin-induced death is a consequence of continued cell cycle progression despite inhibition of mTOR function. The results suggest that p53 cooperates in blocking G_1 progression in rapamycin-treated cells and that protection from drug-induced apoptosis requires $p21^{CIP1}$.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. Rh1 and Rh30 human rhabdomyosarcoma cell lines have been described previously (13, 46). Both express mutant *p53* alleles (Rh1: $Tyr^{220} \rightarrow Cys^{220}$; Rh30 $Arg^{273} \rightarrow Cys^{273}$) and were grown in antibiotic-free RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine at 37°C and 5% CO_2. For experiments where cells were deprived of serum overnight, cell monolayers were washed with RPMI 1640 containing 2 mM L-glutamine and incubated in the same medium. For prolonged serum-free conditions, Rh30 cells were cultured in MN2E (DMEM/F12 supplemented with 1 μg/ml human transferrin, 30 nM sodium selenate, 20 nM progesterone, 100 μM putrescine, 30 nM vitamin E phosphate, 50 μM ethanolamine, and 1 mg/ml BSA). Rh1 cells were grown in MN2E with addition of fibronectin (10 μg/ml). Rh1 and Rh30 clones that stably express an AU-1-tagged mutant mTOR cDNA have been described previously (13). This mutant (designated mTOR-rr) has a single amino acid substitution ($Ser^{2035} \rightarrow Ile^{2035}$) in the FKBP-rapamycin binding domain that reduces the binding affinity of the FKBP-rapamycin complex (47). Wild-type and $p53^{-/-}$ cells were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA) and used within five passages. Wild-type and $p21^{-/-}$ MEFs were kindly provided by Charles Sherr (Howard Hughes Medical Institute, St. Jude Children's Research Hospital). For prolonged serum-free conditions, cells were cultured in MN2E as described above.

Adenoviral Infections. Replication-deficient (ΔE1A and E3) adenoviral recombinants (Genetic Therapy, Inc., Gaithersburg, MD) expressing β-galactosidase (Ad-β-gal) (48), wild-type p53 (Ad-p53; from Linda Harris, St. Jude Children's Research Hospital), or wild-type human $p21^{CIP1}$ (Ad-p21; from Wafik El-Deiry, University of Pennsylvania, Philadelphia, PA) were prepared using 293 cells by conventional procedures. The adenoviral recombinants were titered by a plaque-forming assay after infection of 293 cells as described elsewhere (48). All infections were performed firstly in 2% FBS/DMEM for 2 h, then continued in 10% FBS/DMEM for 22 h. The infectivity of the cell lines was compared by infecting cells with Ad-β-gal and then staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as reported previously (48). The MOI was defined as the ratio of the total number of plaque-forming units used in a particular infection per total number of cells to be infected. Controls included Ad-β-gal infection and mock infection in which the cells were incubated with corresponding medium only.

Determination of Apoptosis. Cells (Rh1, 8.5×10^5; Rh30, 1.7×10^6/162-cm^2 flask) were grown overnight in serum-free N2E medium. On day 1 rapamycin (100 ng/ml) was added, and cells were exposed for up to 6 days. Control cells in RPMI 1640 containing 10% FBS or N2E were grown for the corresponding periods without addition of rapamycin (100 ng/ml). Cells were trypsinized, washed with PBS, resuspended in 200 μl of binding buffer (Clontech Laboratories, Inc., Palo Alto, CA), and incubated with 10 μl of Annexin V^{FITC} (final concentration 1 μg/ml; Clontech Laboratories, Inc.) and 500 ng of propidium iodide in a final volume of 410 μl. Cells were incubated at room temperature in the dark for 10 min before flow cytometric analysis (FACSCalibur, Becton Dickinson, Mountain View, CA) as described previously (13).

Flow Cytometry for Cell Cycle Analysis. Cultured cells were briefly washed with PBS and trypsinized. Cell suspensions were centrifuged at 1000 rpm for 5 min and pellets were resuspended in propidium iodide staining solution (50 ng/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100) at 1×10^6 cells/ml. The cells were then pretreated with RNase (DNase-free) solution (0.2 mg/ml RNase in 15 mM NaCl, 10 mM Tris-HCl, pH 7.5) and filtered through 40-μm diameter mesh to remove clumps of nuclei. Percentages of cells within each of the cell cycle compartments (G_0-G_1, S, or G_2-M) were determined by flow cytometry (FACSCalibur; Becton Dickinson).

Western Blot Analysis. Cultured cells were briefly washed with cold PBS and on ice lysed in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mM Tris, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Protein concentration was determined by the bicinchoninic acid assay using BSA as the standard (Pierce, Rockford, IL). Equivalent amounts of protein were separated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon polyvinylidene difluoride; Millipore, Bedford, MA). Membranes were blocked with PBS containing 0.05% Tween 20 and 5% nonfat dry milk and probed with rabbit polyclonal anti-$p21^{CIP1}$ (1:800; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti cyclin A or E (1:500; Santa Cruz Biotechnology, Inc.) followed by incubation with goat anti-rabbit IgG-conjugated horseradish peroxidase (1:16,000; Sigma, St. Louis, MO). For detection of cyclin D1 a monoclonal antibody (1:500; PharMingen, San Diego, CA) was used, followed by goat antimouse IgG-conjugated horseradish peroxidase (1:20,000; Pierce). Immunoreactive bands were visualized using Renaissance chemiluminescence reagent (NEN; Life Science Products, Inc., Boston, MA). To check the protein loading, the immunoblots were treated with stripping solution (62.5 mM Tris buffer, pH 6.7, containing 2% SDS and 100 mM β-mercaptoethanol) for 30 min at 50°C and reprobed with mouse monoclonal anti-β-tubulin antibody (1:2,000; Sigma), followed by incubation with horseradish peroxidase-coupled rabbit antimouse IgG (1:8,000; Sigma).

BrdUrd Immunohistochemistry and DAPI Staining. Rh30 cells (4×10^4/well) were seeded on 2-well glass chamber slides (Nunc, Naperville, IL) in 10% FCS-RPMI 1640 and infected with Ad-β-gal or Ad-p53, followed at 24 h by treatment with or without rapamycin (100 ng/ml) in MN2E medium for 24 h. BrdUrd (20 μM; Sigma) was then added and cells were incubated for an additional 5 days. To control for potential artifactual staining of apoptotic nuclei by anti-BrdUrd antibody, aphidicolin was added to control or rapamycin-treated cells prior to addition of BrdUrd. Cells were briefly washed with PBS and fixed with 70% ethanol (in 50 mM glycine buffer, pH 2.0) for 30 min at −20°C. Incorporation of BrdUrd into nuclear DNA was detected using an immunofluorescence assay kit (Boerhinger Mannheim GmbH, Mannheim, Germany). After washing with PBS, slides were incubated with mouse monoclonal anti-BrdUrd antibody containing nucleases (1:100, 66 mM Tris buffer, 0.66 mM $MgCl_2$, and 1 mM 2-mercaptoethanol) for 30 min at 37°C, followed by washing with PBS and immunostaining with sheep antimouse IgG-FITC conjugates (1:100, diluted in PBS) for 30 min at 37°C. To visualize nuclear morphology, slides were further counterstained with DAPI (4 μg/ml in deionized water; Sigma) for 5 min at room temperature. Following a brief washing with PBS, slides were mounted in glycerol/PBS (1:1, v/v) containing 2.5% 1,4-diazabiclo-(2,2,2)octane (Sigma) and photographed by fluorescence microscopy using Insight software. Data were statistically analyzed with a two-tailed paired Student's *t* test. The labeling index (cells stained for BrdUrd/total cells) was calculated from counting approximately 1500 cells.

***In Vitro* cdk Assay.** Cells were infected with adenovirus vectors expressing β-gal, p53, or $p21^{CIP1}$ for 24 h as described above, then incubated another 24 h in N2E medium with or without rapamycin (100 ng/ml). After two brief washes with cold PBS, cells were lysed in RIPA buffer. The lysates were cleared of insoluble material by centrifugation (14,000 rpm, 10 min, 4°C). Protein concentration in the supernatants was determined by the bicinchoninic acid assay using BSA as the standard (Pierce). Equivalent amounts of protein (300 μg/sample) were immunoprecipitated at 4°C with rabbit anti-cdk2, cdk4, or cdk6 polyclonal antibodies (all from Santa Cruz Biotechnology, Inc.) and protein A-coupled agarose beads (Santa Cruz Biotechnology, Inc.) for 4 h. The immunoprecipitates were washed twice with RIPA buffer, followed by two washes with fresh kinase buffer (20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM

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DTT, and 10 mM β-glycerophosphate). Phosphorylation reactions were initiated by addition of 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham, Arlington Heights, IL), 20 μM ATP, and 0.5 mg of histone H1 (Sigma, for cdk2 assay) or 0.5 μg of recombinant pRb fusion protein (Santa Cruz Biotechnology, Inc.) for cdk4 or cdk6 assay. After 30 min at 30°C, the reaction was terminated with 1 volume of ice-cold EDTA (20 mM, pH 8.0). Duplicate aliquots of the sample supernatant were spotted onto phosphocellulose paper squares (Upstate Biotechnology, Inc., Lake Placid, NY). The papers were immersed in 0.75% H_3PO_4 solution and washed for 6×5 min. Radioactivity incorporated into paper-bound histone or pRb was determined by liquid scintillation counting.

RESULTS

Expression of a Rapamycin-resistant mTOR Prevents Apoptosis Induced by Rapamycin. Previously we reported that rapamycin induced growth arrest and p53-independent apoptosis when Rh1 and Rh30 cells were exposed to drug under serum-free conditions (13). Expression of a rapamycin-resistant mTOR (Ser$^{2035} \rightarrow$ Ile^{2035}) that has reduced binding of FKBP-rapamycin prevented growth inhibition (13). Consistent with these results, rapamycin (100 ng/ml) did not induce apoptosis in Rh30 and Rh1 cells expressing mutant mTOR (data not shown). These results strongly support rapamycin-induced apoptosis being mediated through its interaction with mTOR and not through inhibition of a secondary target.

Rapamycin-induced Apoptosis Is a Consequence of Continued Cell Cycle Progression. The cytotoxic effect of rapamycin in these malignant cell lines is in contrast to most reports in which rapamycin exerts a cytostatic effect. We considered whether the cellular response to rapamycin (cytostasis or apoptosis) was determined by loss of a G_1 checkpoint function. Both cell lines express mutant p53 alleles (Rh1: Tyr$^{220} \rightarrow$ Cys^{220}; Rh30: Arg$^{273} \rightarrow$ Cys^{273}) and fail to induce p21^{CIP1} in response to ionizing radiation (13), prompting us to focus on the loss of this tumor suppressor as being a common characteristic of these malignant cells. Rapamycin-mediated G_1 cell cycle arrest appears to be independent of *p53* (49), and consistent with this is the finding that both Rh1 and Rh30 cells accumulate in G_1 phase in the presence of rapamycin (13). Conversely, we wondered whether rapamycin-induced apoptosis is a consequence of slowed, but continued progression through G_1 phase in the absence of a p53-mediated checkpoint. Under serum-free conditions of culture, rapamycin (100 ng/ml, 24 h) slowed growth (~70–80%) and caused a significant increase in the proportion of Rh1 and Rh30 cells in the G_1 phase (Table 1). For Rh1, drug treatment increased the fraction of cells in G_1 phase from 67 to 83%. For Rh30, the proportion of cells in G_1 phase increased from 51.5 to 70.3%. However, there was no obvious change in the expression of G_1 cyclins, D1, E, and A in the presence of rapamycin (Fig. 1). Importantly, expression of cyclin A, a marker of late G_1-S phase interface was expressed at very high levels relative to cyclin D1 or cyclin E in control and rapamycin-treated cells.

Together, these data suggested that cells were progressing through G_1 phase, and potentially entering S phase, and committing to apoptosis. To test this hypothesis, Rh30 cells were grown under serum-free conditions with or without rapamycin (100 ng/ml) for 24 h. BrdUrd was then added with or without aphidicolin. Cells were grown for an additional 5 days, fixed, and processed for BrdUrd immunohistochemistry and stained with DAPI to determine nuclear morphology. Apoptotic cells were characterized as exhibiting either membrane blebbing, shrinkage, chromatin condensation, or micronucleation. As shown in Table 2, the BrdUrd labeling index for control cultures was 100% (all cells had undergone at least one round of replication within the 5-day labeling period). Rapamycin had only a slight effect on the labeling index, indicating continued DNA synthesis during the period of rapamycin exposure. Rapamycin increased the incidence of apoptosis from 8 to 25%, with >93% of apoptotic cells having incorporated BrdUrd in the presence of rapamycin. In contrast, ~2% of apoptotic cells incorporated BrdUrd into DNA when treated with aphidicolin with or without rapamycin treatment. Thus, nuclear immunostaining for BrdUrd was not an artifact of nuclear condensation, as apoptotic cells did not score positive for nucleotide incorporation in the presence of aphidicolin, a DNA polymerase inhibitor. These data strongly support the contention that apoptosis is a consequence of progression through G_1 to S phase in rapamycin-treated tumor cells.

Table 1 *Cell cycle analysis*

Treatment	G_1-G_0	S phase	G_2-M
Rh1 control	67.2	26.2	6.6
Rh1 + Rap[a] (100 ng/ml)	83.0	12.9	4.1
Rh30 control	51.5	39.3	9.4
Rh30 + Rap (100 ng/ml)	70.3	21.9	7.8

[a] Rap, rapamycin.

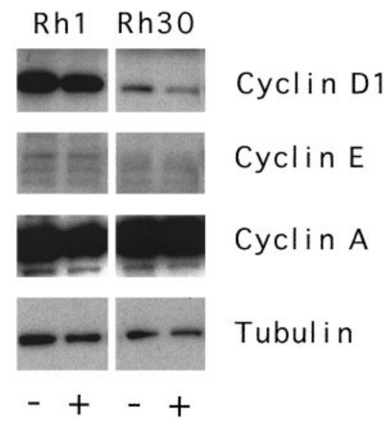

Fig. 1. Expression of G_1 cyclins in control or rapamycin-treated Rh1 and Rh30 cells growing under serum-free conditions. Cells were grown under serum-free conditions described. Control and rapamycin (100 ng/ml, 24 h)-treated cells were harvested and expression of cyclins D1, E, and A, relative to tubulin (loading control), was determined by Western blot analysis. Similar results were obtained in three separate experiments.

Wild-Type p53 Induces G_1 Arrest and Protects Cells from Rapamycin-induced Apoptosis. To determine whether expression of *p53* altered the cell cycle response to rapamycin, cells were infected with Ad-p53 or a control adenovirus Ad-β-gal. Infection of Rh1 cells with Ad-p53 resulted in dramatic apoptosis, preventing further analysis. In contrast, Rh30 cells tolerated infection. Ad-p53 infected Rh30 cells, but not those infected with Ad-β-gal accumulated in G_1 phase (Table 3). One day postinfection with Ad-p53, 80% of Rh30 cells were in G_1 phase in contrast to 47% for Ad-β-gal-infected cells. Rapamycin (100 ng/ml, 24 h) increased the G_1 fraction to 86% in Ad-p53-infected cells and to 70% in control cultures (Ad-β-gal infected). The mechanism of p53-induced G_1 arrest involves up-regulation of cdk inhibitor p21^{CIP1} (28–30). As shown in Fig. 2*A*, when infected with Ad-p53, Rh30 cells expressed p21^{CIP1} in a dose-dependent manner. A high level of p21^{CIP1} protein could be detected 24 h postinfection, which could persist for at least 4 days (Fig. 2*B*). In contrast, when Rh30 cells were mock infected, or infected with Ad-β-gal, the p21^{CIP1} band was very faint or undetectable (data not shown).

Apoptosis was determined by the ApoAlert FACS analysis after 6 days of exposure to rapamycin (100 ng/ml). As shown in Fig. 3, control cells (uninfected) or those infected with Ad-β-gal demonstrated very low levels of annexin V-positive cells (~4–6%). In contrast, rapamycin induced a significant increase in the proportion of cells positive for annexin V and propidium iodide in uninfected

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Table 2 *BrdUrd labeling of rapamycin-treated Rh30 cells*

Treatment	Total cells	BrdUrd-labeled cells	Labeling index (%)	Labeled apoptotic cells	Unlabeled apoptotic cells	% Apoptotic cells labeled	% apoptosis
Control	1398	1398	100				7.8
Control + Rap[a] (100 ng/ml)	1367	1192	97.2	323	22	93.6	25.2
Aphidicolin	1487	16	1.1			1.8	56.1
Aphidicolin + Rap (100 ng/ml)	1496	17	1.1	16	779	2.1	53.2

[a] Rap, rapamycin.

cultures or after infection with Ad-β-gal virus (26–28% viable as judged by annexin V-negative staining and exclusion of propidium iodide). Infection of Rh30 cells with Ad-p53 did not result in any increase in cells positive for annexin V or propidium iodide, but expression of p53 completely abrogated rapamycin-induced apoptosis in these cells (93% viable).

Ad-p21 Mimics Ad-p53, Protecting Cells from Rapamycin-induced Apoptosis and Inducing G_1 Block. As described above, the mechanism by which *p53* protected cells could be mediated by $p21^{CIP1}$-induced G_1 arrest. We therefore investigated whether direct expression of $p21^{CIP1}$ could protect cells from rapamycin-induced apoptosis and whether such protection results in $p21^{CIP1}$-induced G_1 block. Rh30 cells infected with Ad-p21 (MOI = 10) for 24–48 h expressed almost the same level of $p21^{CIP1}$ as that in cells infected with Ad-p53 (MOI = 1). Expression of $p21^{CIP1}$ was related to MOI, and a high level of $p21^{CIP1}$ protein could be detected 24 h postinfection and maintained for at least 5 days (Fig. 4, *A* and *B*). Expression of $p21^{CIP1}$after infection with Ad-p53 or Ad-p21 was not altered by rapamycin treatment (Fig. 4*C*). In contrast to the effect of Ad-p53, Rh1 cells tolerated infection with Ad-p21 virus. Infection of Rh1 and Rh30 cells with Ad-p21 caused G_1 accumulation (data not presented) and significantly suppressed DNA replication (determined by [^{3}H]thymidine incorporation). Inhibition of DNA synthesis by p21 (Rh1 and Rh30) or p53 (Rh30 cells) was significantly more effective than that induced by high concentrations of rapamycin, as shown in Fig. 5. This observation is consistent with the relative effects on cdk2 activity. Cells were treated as above or infected with Ad-βgal (control), then grown for 24 h with or without rapamycin (100 ng/ml). Activity of kinase activity of immunoprecipitated cdk2 is shown in Fig. 6.

Infection of Rh1 and Rh30 cells with Ad-p21 virus protected from rapamycin induced apoptosis (Fig. 7). These results suggest that the

Table 3 *Cell cycle analysis for Rh30 cells*

Treatment	G_1-G_0	S phase	G_2-M
Ad-β-gal	46.8	42.1	11.1
Ad-β-gal + Rap[a] (100 ng/ml)	70.2	21.0	8.8
Ad-p53	81.6	9.4	9.1
Ad-p53 + Rap (100 ng/ml)	86.3	5.4	8.3

[a] Rap, rapamycin.

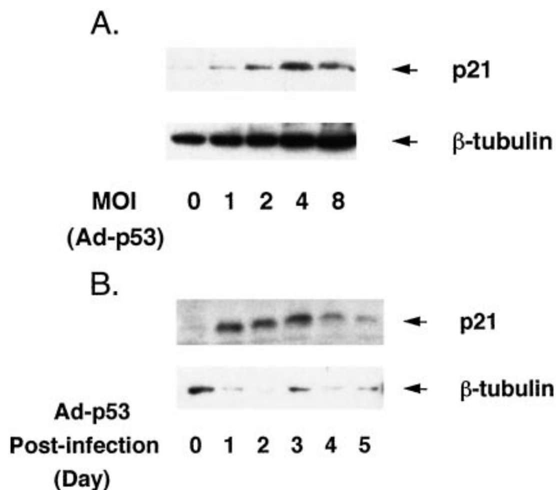

Fig. 2. Infection of Rh30 cells with Ad-p53 induces prolonged expression of $p21^{CIP1}$. *A*, Rh30 cells were infected with increasing MOI of Ad-p53 and $p21^{CIP1}$ expression determined 24 h after infection. *B*, Rh30 cells were infected with Ad-p53 (MOI = 1) and grown for up to 5 days. $p21^{CIP1}$ and β-tubulin were determined by Western blot analysis as described in "Materials and Methods." Similar results were obtained in three independent experiments.

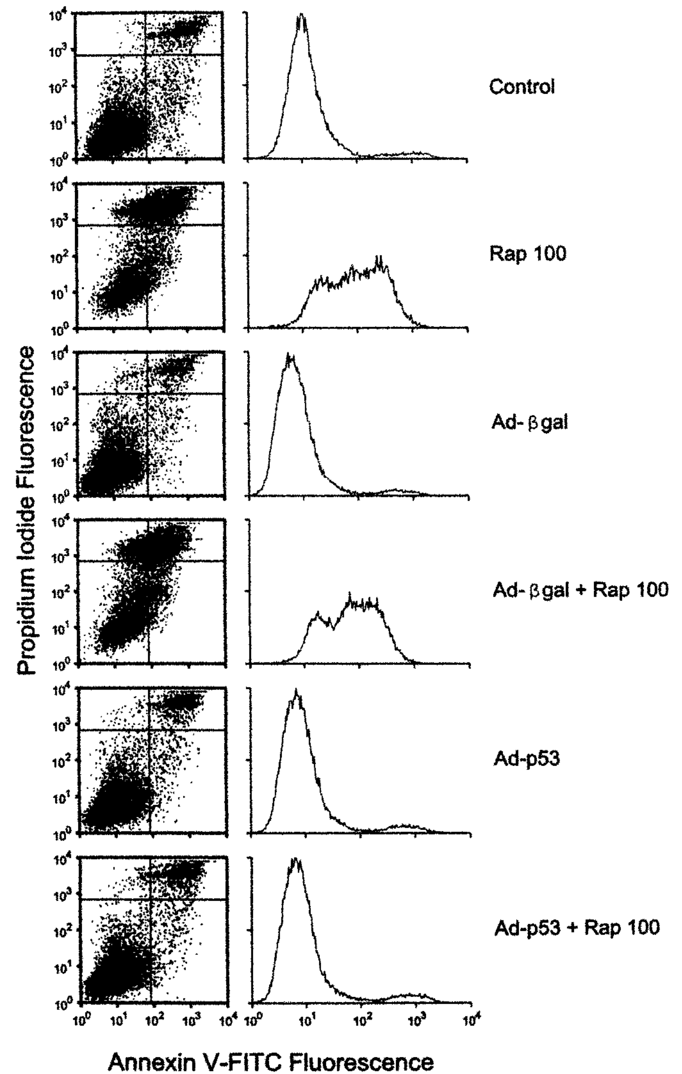

Fig. 3. Expression of *p53* protects Rh30 cells from rapamycin-induced apoptosis. Rh30 cells were infected with either Ad-p53 or Ad-β-gal adenovirus (MOI = 1). After 24 h, cell medium was replaced with serum-free N2E, and cells were grown for another 6 days without or with rapamycin (100 ng/ml). Cells were harvested and apoptosis was determined by quantitative FACS analysis (ApoAlert) as described in "Materials and Methods." *Left panels*, dual staining for propidium iodide uptake and Annexin V^{FITC}. *Right panels*, corresponding distribution of Annexin V^{FITC} staining in populations of cells. Similar results were obtained in at least four independent experiments.

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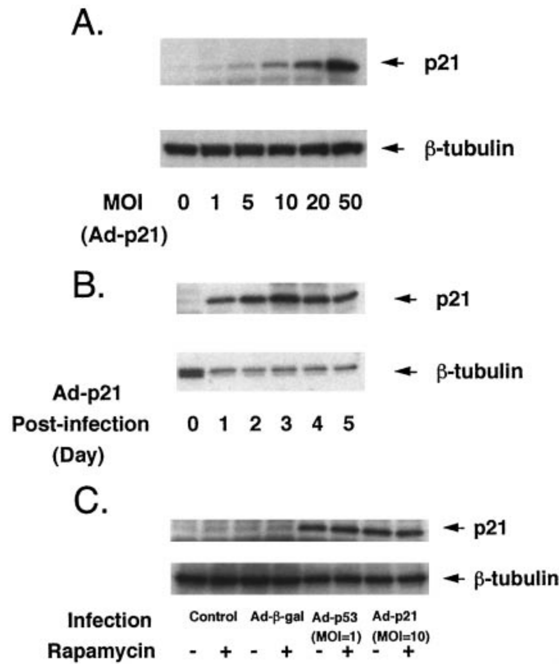

Fig. 4. Expression of p21^CIP1 after infection with Ad-p21 adenovirus in Rh30 cells. Cells were infected with Ad-p21 as described in the legend to Fig. 2. *A*, p21^CIP1 expression with increasing MOI. *B*, time course for p21^CIP1 expression after infection (MOI = 10). *C*, comparison of p21^CIP1 expression with or without rapamycin (100 ng/ml) after infection with Ad-p53 (MOI = 1) or Ad-p21 (MOI = 10). β-Tubulin was used as a loading control. Similar results were obtained in three separate experiments.

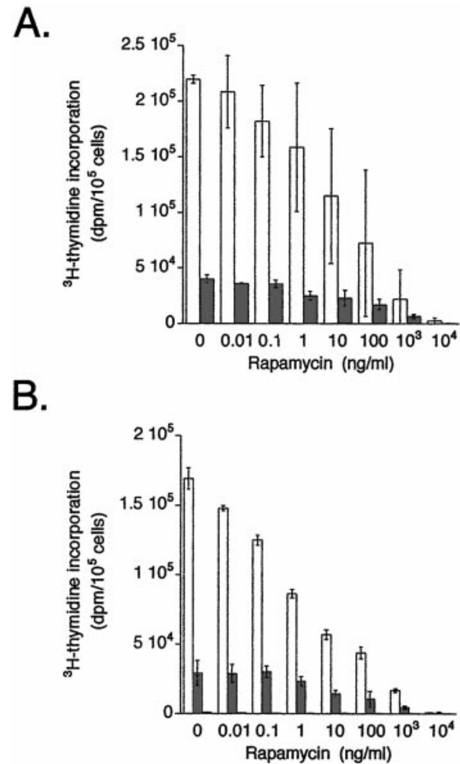

Fig. 5. Inhibition of DNA synthesis by rapamycin, Ad-p21, or Ad-p53. Cells were either unmanipulated or infected with either Ad-p53 (Rh30 only) or Ad-p21 (Rh1 and Rh30). *A*, Rh1 control cells or cells infected with Ad-p21 with or without rapamycin treatment. *B*, Rh30 cells were infected with either Ad-p21 or Ad-p53 adenovirus with or without treatment with rapamycin (0–10,000 ng/ml). After 24 h, medium was changed with fresh N2E medium, and cells were incubated for an additional 2 h with [^{3}H]thymidine. Incorporation of [^{3}H]thymidine into cells was determined as described in "Materials and Methods." *Bars*, mean ± SD values of three separate experiments.

mechanism of Ad-p53 protection is at least partially through p21^CIP1-induced G_1 cell cycle arrest. To test this, BrdUrd-labeling experiments were repeated in the absence or presence of rapamycin, as described above, in Rh30 cells infected with either Ad-p53 or Ad-p21 or control virus (Ad-β-gal). Results are presented in Table 4. All cells infected with Ad-β-gal incorporated BrdUrd during the 5-day labeling period. The effect of rapamycin was similar to that in uninfected cells (see Table 2). Ninety-seven percent of cells incorporated BrdUrd and ~25% of cells demonstrated morphological signs of apoptosis. Ad-p21 suppressed BrdUrd labeling (16% labeling) and protected against rapamycin-induced apoptosis. The effect of Ad-p53 was slightly greater with only 7% of cells labeling with BrdUrd over 5 days. Rapamycin did not increase levels of apoptosis over those in cultures infected with Ad-p21 or Ad-p53 alone. Taken together, these data suggest that rapamycin-induced apoptosis is a consequence of continued cell cycle progression, and that inhibiting progression by inducing a G_1 block protects cells.

p53 and p21^CIP1 Determine the Cellular Response to Rapamycin in Murine Embryo Fibroblasts. Results presented above demonstrate that forced expression of p53 or p21^CIP1 can protect against rapamycin-induced toxicity. However, to define the role of these gene products under normal cellular control, we examined the response to rapamycin in wild-type or mutant MEFs that were nullizygous for *p53* or *p21*^CIP1. Under serum-free conditions, rapamycin did not increase the proportion of apoptotic cells in cultures of wild-type MEFs (control 20% *versus* 17% in treated cultures). In contrast, rapamycin markedly induced apoptosis in cells lacking *p53* or *p21*^CIP1 (Fig. 8*A*). As with Rh30 cells, infection of $p53^{-/-}$ MEFs with Ad-p53 protected against rapamycin toxicity (Fig. 8*B*). Similar protection was obtained when $p53^{-/-}$ MEFs were infected with Ad-p21 (data not shown).

We next determined whether rapmycin induced a more persistent G_1 arrest in wild-type MEFs than in those lacking either *p53* or *p21*^CIP1. Cells were grown under serum-containing conditions and treated with rapamycin for 24 h, at which time BrdUrd was added.

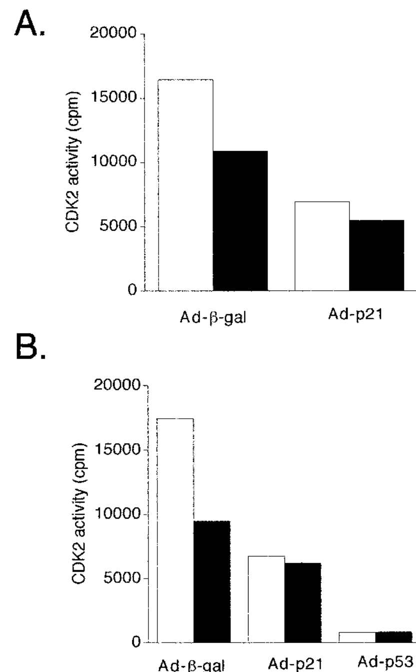

Fig. 6. Inhibition of cdk2 activity by rapamycin, Ad-p21, or Ad-p53. *A*, Rh1 cells were infected with Ad-β-gal (control) or Ad-p21 adenovirus with (■) or without (□) rapamycin treatment (100 ng/ml, 24 h). cdk2 activity was determined as described in "Materials and Methods." *B*, the experiment above was repeated with Rh30 cells, but included groups where cells were infected with Ad-p53. Similar results were obtained in three independent experiments.

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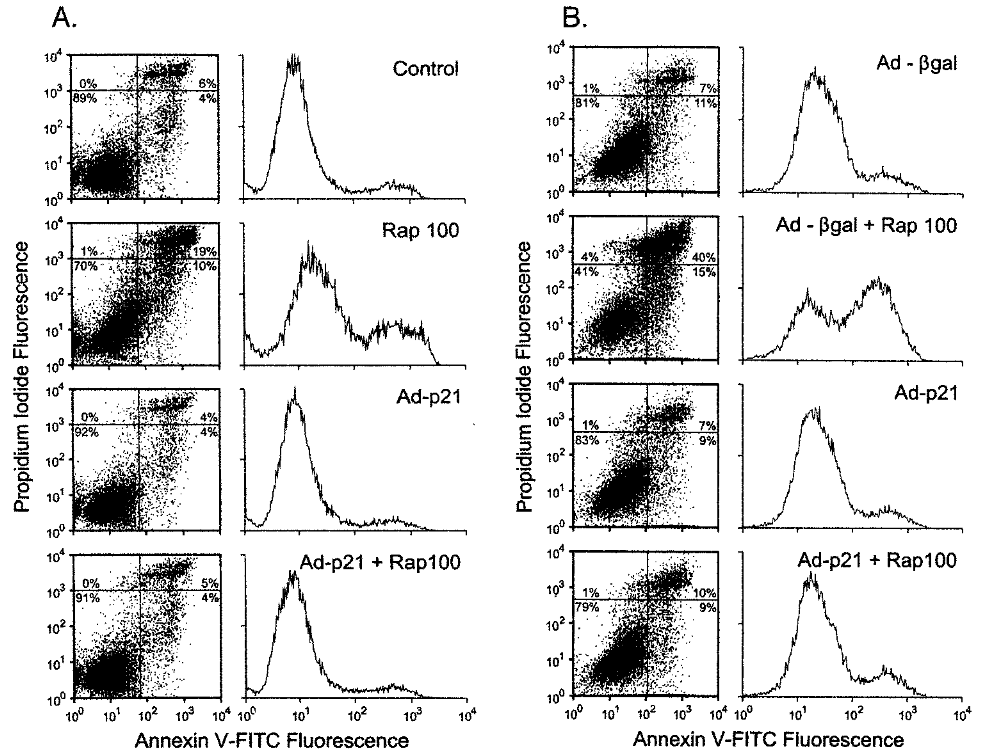

Fig. 7. Rh1 (*A*) and Rh30 (*B*) cells were mock infected or infected with Ad-p21 (MOI = 10) and grown without or with rapamycin (100 ng/ml) for 4 days (for Rh1) or 6 days (for Rh30). Cells were harvested and apoptosis was determined by quantitative FACS analysis (ApoAlert) as described in the legend to Fig. 3. Percent distribution of cells in each quadrant is presented. Similar results were obtained in three independent experiments.

After an additional 5 days, labeling of cells with BrdUrd was determined as described above. In control cultures (no rapamycin), the BrdUrd labeling index was 49.6, 100, and 100% for wild-type, $p53^{-/-}$, and $p21^{-/-}$ cells, respectively. Rapamycin significantly decreased BrdUrd labeling in wild-type MEFs with only 19% of cells labeling after 5 days. In contrast, 89% of $p53^{-/-}$ cells and 70% of $p21^{-/-}$ cells labeled with BrdUrd in the presence of rapamycin (Fig. 9*A*). Analysis of cyclin expression after 24-h treatment with rapamycin showed little effect of this agent on levels of cyclin D1 or cyclin E in any MEF strain. However, cyclin A was reduced to far lower levels in wild-type MEFs than in either $p53^{-/-}$ or $p21^{-/-}$ strains, consistent with a reduced proportion of cells in S phase (Fig. 9*B*).

DISCUSSION

Under serum-free conditions, malignant rhabdomyosarcoma cells maintain viability and proliferate through autocrine mechanisms (2, 50, 51). However, under these conditions the cytostatic agent, rapamycin, induces p53-independent apoptosis (13). Rapamycin is generally regarded as a cytostatic agent as in most reports the cellular response to rapamycin is G_1 arrest without apoptosis. Thus, we were interested in determining whether rapamycin-induced apoptosis in these malignant cells was a consequence of continued progression through G_1 phase and entry into S phase in the presence of drug.

Extending our previous findings (13), expression of the rapamycin-resistant $Ser^{2035} \rightarrow Ile^{2035}$ mutant mTOR prevented rapamycin-induced apoptosis, strongly suggesting that apoptosis is a consequence of attenuated mTOR signaling in rapamycin-treated cells. As shown previously, within 24 h rapamycin caused an increased fraction of Rh1 and Rh30 cells to accumulate in G_1 phase of the cell cycle. However, this was not associated with any detectable change in expression of cyclin D1, E, or A, which is in contrast to other reports (52–54). For example, rapamycin inhibits the expression of interleukin 2-dependent cyclin A and E and the activity of cyclin-A or E-associated kinases $p33^{cdk2}$ and $p34^{cdc2}$, causing a mid to late G_1 block (52). In NIH3T3 cells, although rapamycin does not affect cyclin D- or E-dependent kinases, it delays the expression of cyclin A and its associated kinase activities (53). Furthermore, rapamycin decreases the cyclin D1 mRNA level and protein stability, resulting in inhibition of the G_1 to S transition in NIH3T3 cells (54). It has also been shown that rapamycin induces G_1 arrest, either by inhibiting cyclin E-associated kinase by preventing interleukin 2-mediated elimination of the cdk inhibitor $p27^{Kip1}$ in T lymphocytes (55) or by inhibiting pRb phosphorylation in vascular smooth muscle cells (56). However, we did not find any effect of rapamycin either on the levels of $p21^{CIP1}$, $p27^{Kip1}$, or $p57^{Kip2}$ or the phosphorylation levels of pRb, p130, and p107 in the tumor cells (data not shown). Since all above-mentioned experiments were done using p53 wild-type cells, we speculated that loss of p53 function may allow rapamycin-treated cells to progress

Table 4 *BrdUrd labeling of rapamycin-treated Rh30 cells*

Treatment	Total cells	BrdUrd-labeled cells	Labeling index (%)	Labeled apoptotic cells	Unlabeled apoptotic cells	% Apoptotic cells labeled	% apoptosis
Ad-β-gal	1502	1502	100				7.6
Ad-β-gal + Rap[a] (100 ng/ml)	1476	1293	97.6	342	24	93.4	24.8
Ad-p21	1503	237	15.8	89	30	74.8	7.9
Ad-p21 + Rap (100 ng/ml)	1521	156	10.3	93	29	76.2	8.0
Ad-p53	1588	106	6.7	101	29	77.7	8.2
Ad-p53 + Rap (100 ng/ml)	1517	93	6.1	90	33	73.8	8.0

[a] Rap, rapamycin.

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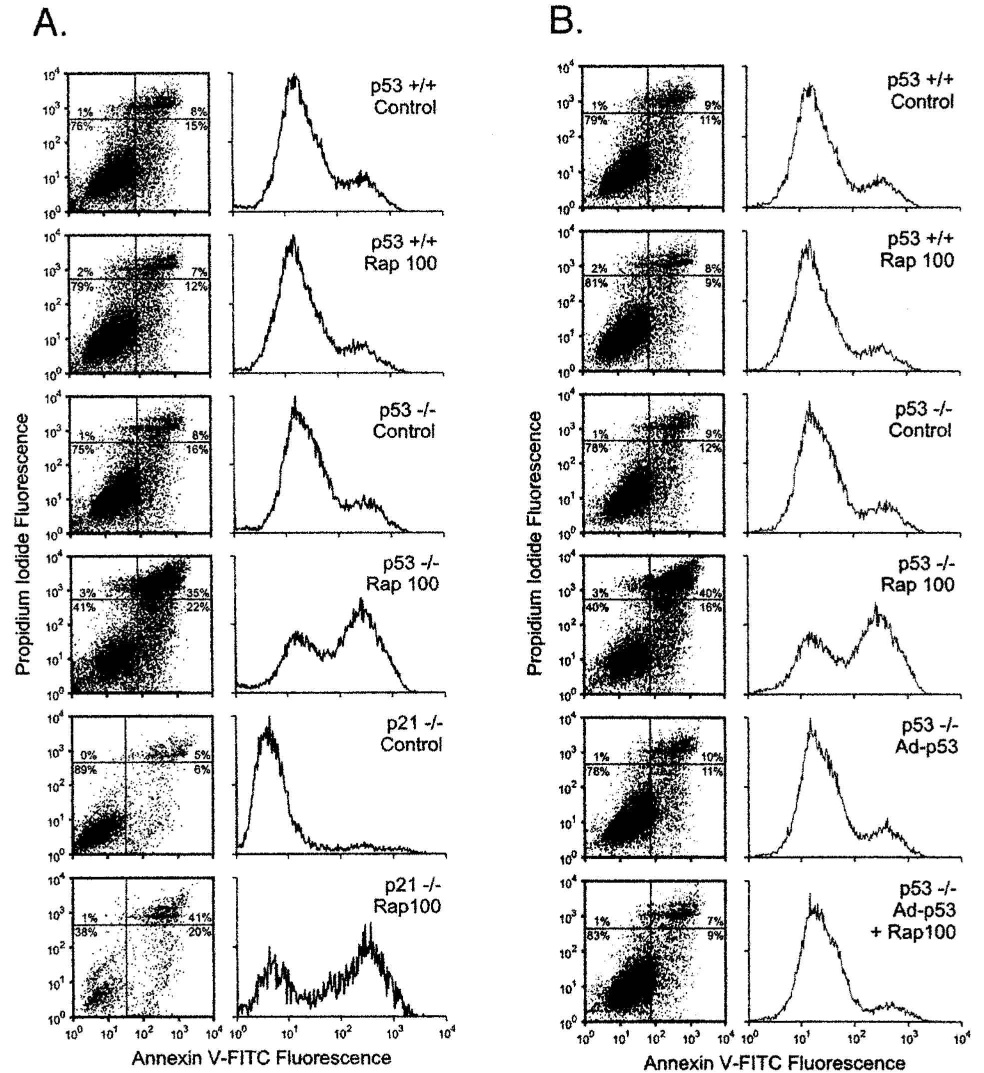

Fig. 8. The cellular response to rapamycin is dependent on functional p53 or *p21*^CIP1^ in murine embryo fibroblasts. *A*, wild-type, $p53^{-/-}$, and $p21^{-/-}$ MEFs were grown under serum-free conditions with or without rapamycin (100 ng/ml). After 5 days, cells were harvested and apoptosis was determined by ApoAlert assay as described in the legend to Fig. 3. Results show a representative experiment. Percent distribution of cells in each quadrant is presented. *B*, wild-type and $p53^{-/-}$ MEFs and $p53^{-/-}$ MEFs infected with Ad-p53 (MOI = 100) were grown without or with rapamycin (100 ng/ml). Cells were harvested after 5 days and apoptosis was determined by quantitative FACS analysis (ApoAlert) as described in the legend to Fig. 3. The percent distribution of cells in each quadrant is presented. Results show a representative experiment.

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through G_1 and enter S phase before initiating apoptosis. To test this, Rh30 cells were used as an experimental model, since Rh1 did not tolerate Ad-p53 infection for unknown reasons. Rh30 cells were exposed to rapamycin for 24 h to accumulate cells in G_1, then exposed further to rapamycin in the presence of BrdUrd. Cells were scored for morphological signs of apoptosis and immunostained to detect BrdUrd incorporation into DNA. Virtually all (>93%) apoptotic cells were positive for BrdUrd, indicating that these cells had initiated DNA synthesis in the presence of rapamycin. In addition, when rapamycin-treated Rh30 cells were exposed continuously to BrdUrd for 5 days, almost 100% of the cells were BrdUrd labeled, suggesting that rapamycin only slowed, but could not stop cell cycle progression of the *p53* mutant tumor cells.

We next determined whether enforced expression of *p53* could protect Rh30 cells from rapamycin-induced apoptosis. Unmanipulated cells or those infected with Ad-p53 or Ad-β-gal were grown with or without rapamycin. After 5 days, apoptosis was determined using the ApoAlert FACS assay. Viability of control, Ad-p53-, and Ad-β-gal-treated cultures was similar (>93%). Rapamycin induced massive apoptosis in unmanipulated cells and in those infected with Ad-β-gal. Ad-p53 dramatically protected Rh30 cells from rapamycin-induced apoptosis. Since up-regulation of p53 may drive cells to apoptosis, or protect cells from death by inducing cell cycle arrest (18–23), we further investigated the mechanism by which p53 protected Rh30 cells from rapamycin-induced apoptosis. Western blot analysis showed that infection of Rh30 cells with Ad-p53 induced p21^CIP1^ within 24 h and persisted for at least 4 days. In addition, although expression of *p53* did not significantly affect levels of cyclin D1 and E and the activity of cyclin D-associated kinases cdk4 or cdk6 (data not shown), expression of p53 dramatically reduced the levels of cyclin A and cdk2 activity. This was accompanied by a sharp decrease of phosphorylation levels of pRb (data not shown). Consistently, labeling experiments revealed that only 7% of Ad-p53-infected Rh30 cells were BrdUrd positive following incubation with BrdUrd for 5 days, indicating that expression of *p53* prevented the cell cycle progression. Moreover, expression of *p53* resulted in G_1 accumulation, and rapamycin treatment further increased the proportion of cells in G_1 to 86%. Infection of Rh30 cells with control adenovirus (Ad-β-gal) did not affect either levels of p21^CIP1^ and cyclin A, or cdk2 activity, or pRb phosphorylation, and did not induce G_1 accumulation. Taken together, these data suggest that rapamycin-induced apoptosis is a consequence of entering S phase while mTOR activity is inhibited by rapamycin. In contrast, forced expression of *p53* blocked the cell cycle progression and arrested cells in G_1 phase rescuing from rapamycin-induced apoptosis.

To test whether p21^CIP1^ could mimic p53, Rh1 and Rh30 cells were infected with Ad-p21 adenovirus. In contrast to Ad-p53, both cell lines tolerated infection with Ad-p21 and expressed p21^CIP1^ at high

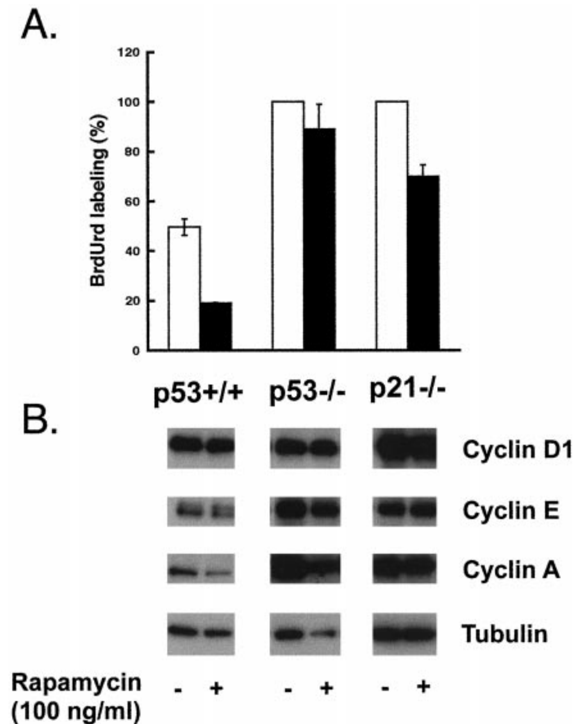

Fig. 9. Rapamycin treatment results in greater suppression of DNA synthesis and cyclin A in wild-type MEFs. *A*, BrdUrd labeling index of control and rapamycin-treated MEFs. Wild-type or mutant MEFs were exposed to rapamycin (100 ng/ml) for 24 h, at which time BrdUrd was added. After incubation for an additional 5 days, cells were fixed and stained for BrdUrd incorporated into DNA as described in "Materials and Methods." Results show the percentage of cells labeled (mean ± SD) for three separate experiments. *B*, rapamycin-induced changes in cyclin expression. Wild-type or mutant MEFs were grown in serum-replete conditions with or without rapamycin (100 ng/ml). After 24 h, cells were harvested and cyclin expression was determined as described in "Materials and Methods." Results show a representative experiment.

levels. Ad-p21 infection resulted in accumulation of cells in G_1 phase, significant suppression of both DNA synthesis, as determined by [^{3}H]thymidine and BrdUrd incorporation, and cdk2 activity, causing G_1 arrest. Ad-p21 completely protected both Rh1 and Rh30 cells from rapamycin-induced apoptosis. These results further strengthen the conjecture that arresting cell cycle progression prevented killing by rapamycin. Conversely, continued cell cycle progression in tumor cells results in rapamycin inducing a cytotoxic response.

These results indicate that forced expression of *p53* or *p21*^{CIP1} can protect tumor cells from rapamycin-induced apoptosis, leading to the speculation that in cells with proficient p53 the response to rapamycin may be cytostasis in G_1 phase. Since tumor cells with wild-type *p53* appear to have other defects in the ARF-p53-Rb pathway, we chose to examine the role of *p53* and *p21*^{CIP1} in MEFs having defined gene disruptions. The response of wild-type MEFs to rapamycin was dramatically different from those with disruptions of *p53* or *p21*^{CIP1}. Rapamycin induced significant apoptosis in *p53*$^{-/-}$ and *p21*$^{-/-}$ cells, but not in wild-type cells. These results suggest that at least part of the p53 protection is mediated by p21^{CIP1} in MEFs treated with rapamycin. Consistent with data derived from Rh30 cells, forced expression of *p53* or *p21*^{CIP1} in *p53*$^{-/-}$ MEFs also protected against rapamycin cytotoxicity. The response to rapamycin under normal culture conditions (serum containing) was also different between wild-type and mutant MEFs. Rapamycin induced growth arrest in wild-type cells and reduced the BrdUrd labeling index more effectively than in *p53*$^{-/-}$ or *p21*$^{-/-}$ cells. Ninety percent of *p53*$^{-/-}$ cells incorporated BrdUrd over 5 days in the presence of rapamycin, compared to 19% in wild-type cells. Results for *p21*$^{-/-}$ were intermediate, with 70% of rapamycin-treated cells incorporating BrdUrd. However, this latter result may represent an underestimate, as many *p21*$^{-/-}$ cells in rapamycin-treated experiments became apoptotic and detached from the microscope slide. Thus, loss of p53 or p21^{CIP1} function decreases the ability of cells to arrest in G_1 phase of the cell cycle in response to rapamycin treatment.

The results presented here support the notion that a p53-dependent event cooperates by enforcing G_1 arrest in response to inhibition of cap-dependent translation caused by rapamycin. The exact mechanism by which this is accomplished is under investigation. However, potentially, loss of p53 function could provide a basis for tumor-selective cytotoxicity of agents that target mTOR. We have shown previously (13) that rapamycin-induced apoptosis can be prevented by insulin-like growth factor I, hence it remains to be determined whether the tumor millieu *in situ* also protects cells from rapamycin-induced apoptosis in patients treated with the rapamycin ester CCI-779 that is currently in early clinical development. As CCI-779 induced objective tumor regressions in patients enrolled in the Phase I trial (57), it suggests that a cytotoxic response to mTOR inhibition may be achieved in some tumors. However, whether these "responsive" tumors had mutant p53 was not determined.

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