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# Rapamycin Causes Poorly Reversible Inhibition of mTOR and Induces p53-independent Apoptosis in Human Rhabdomyosarcoma Cells[1]

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## ABSTRACT

**The mammalian target of rapamycin (mTOR) has been shown to link growth factor signaling and posttranscriptional control of translation of proteins that are frequently involved in cell cycle progression. However, the role of this pathway in cell survival has not been demonstrated. Here, we report that rapamycin, a specific inhibitor of mTOR kinase, induces $G_1$ cell cycle arrest and apoptosis in two rhabdomyosarcoma cell lines (Rh1 and Rh30) under conditions of autocrine cell growth. To examine the kinetics of rapamycin action, we next determined the rapamycin sensitivity of rhabdomyosarcoma cells exposed briefly (1 h) or continuously (6 days). Results demonstrate that Rh1 and Rh30 cells were equally sensitive to rapamycin-induced growth arrest and apoptosis under either condition. Apoptosis was detected between 24 and 144 h of exposure to rapamycin. Both cell lines have mutant p53; hence, rapamycin-induced apoptosis appears to be a p53-independent process. To determine whether induction of apoptosis by rapamycin was specifically due to inhibition of mTOR signaling, we engineered Rh1 and Rh30 clones to stably express a mutant form of mTOR that was resistant to rapamycin ($Ser^{2035} \rightarrow Ile$; designated mTOR-rr). Rh1 and Rh30 mTOR-rr clones were highly resistant (>3000-fold) to both growth inhibition and apoptosis induced by rapamycin. These results are the first to indicate that rapamycin-induced apoptosis is mediated by inhibition of mTOR. Exogenous insulin-like growth factor (IGF)-I protected both Rh1 and Rh30 from apoptosis, without reactivating ribosomal p70 S6 kinase ($p70^{S6K}$) downstream of mTOR. However, in rapamycin-treated cultures, the response to IGF-I differed between the cell lines: Rh1 cells proliferated normally, whereas Rh30 cells remained arrested in $G_1$ phase but viable. Rapamycin is known to inhibit synthesis of specific proteins but did not inhibit synthesis or alter the levels of mTOR. To examine the rate at which the mTOR pathway recovered, the ability of IGF-I to stimulate $p70^{S6K}$ activity was followed in cells treated for 1 h with rapamycin and then allowed to recover in medium containing ≥100-fold excess of FK506 (to prevent rapamycin from rebinding to its cytosolic receptor FKBP-12). Our results indicate that, in Rh1 cells, rapamycin dissociates relatively slowly from FKBP-12, with a $t_{1/2}$ of ~17.5 h. in the presence of FK506, whereas there was no recovery of $p70^{S6K}$ activity in the absence of this competitor. This was of interest because rapamycin was relatively unstable under conditions of cell culture having a biological $t_{1/2}$ of ~9.9 h. These results help to explain why cells are sensitive following short exposures to rapamycin and may be useful in guiding the use of rapamycin analogues that are entering clinical trials as novel antitumor agents.**

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## INTRODUCTION

The mammalian target of rapamycin, mTOR (also designated FRAP, RAFT1, and RAPT1; Refs. 1–4), has been shown to link mitogen stimulation to protein synthesis and cell cycle progression. However, although activation of PI3K[5] and, potentially, AKT/PKB, considered to lie upstream of mTOR (5, 6), can protect cells from apoptosis induced by stress (7–9), 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 macrolide antibiotic rapamycin, which potently inhibits mTOR, prevents activation of T cells (Refs. 10–12; reviewed in Refs. 13–15), and inhibits growth of nonmalignant cell types (16–19) and malignant cell lines in culture (20–22). Rapamycin competes with a structural analogue, FK-506, for binding to a $M_r$ 12,000 cytosolic protein designated FKBP-12 (23, 24). The FKBP-FK506 complex inhibits calcineurin, preventing dephosphorylation, nuclear translocation of NF-ATp, and activation of interleukin 2 transcription (25–27). The FKBP-rapamycin complex binds and inhibits the function of a serine/threonine kinase, mTOR (1–4, 28), blocking growth factor stimulation of ribosomal $p70^{S6K}$ (29–32) and phosphorylation (33–35) of the eIF 4E-binding protein PHAS-I (also designated 4E-BPI). mTOR kinase activity is necessary for activation of $p70^{S6K}$, but evidence for a direct interaction between mTOR and $p70^{S6K}$ remains controversial (36–38). Also unclear is the relationship between inhibition of $p70^{S6K}$ and cell cycle arrest (39, 40). PHAS-I is considered to be a direct substrate for mTOR kinase activity in cells (41, 42). In resting cells, association of eIF-4E with the multifunctional scaffolding protein eIF-4G is inhibited by PHAS-I and PHAS-II (33, 35). Upon growth factor or serum stimulation, phosphorylation occurs, leading to dissociation of PHAS-I, assembly of the multisubunit complex (eIF-4F), and efficient translation of mRNA having highly structured 5′-untranslated regions (43–45). In many cell lines, exposure to rapamycin results in a relatively small decrease in overall protein synthesis (~15–20%) but a specific $G_1$ cell cycle arrest. This can, in part, be explained by certain cell cycle regulators (cyclin D1, c-MYC, and ornithine decarboxylase) and growth factors (IGF-II) being controlled by either the eIF4E or $p70^{S6K}$ pathway, which are both dependent on mTOR function (44–47). In addition, rapamycin appears to stabilize the cyclin-dependent kinase inhibitor $p27^{kip1}$ in mitogen-stimulated cells (48, 49).

We have shown previously (21) that RMS cells that are dependent on IGF-IR signaling for growth are highly sensitive to inhibition by rapamycin. This is consistent with findings that IGF-I and insulin rapidly stimulate $p70^{S6K}$ activation through a rapamycin-sensitive pathway (50). We found also that one of these cell lines (Rh1) was resistant to rapamycin ($IC_{50}$ > 5000 ng/ml) under normal serum-containing growth conditions, and under serum-free conditions, it was not inhibited by neutralizing antibody against the IGF-IR. These results were consistent with IGF-IR-independent growth. However,

[5] The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; FKBP, FK-binding protein; $p70^{S6K}$, ribosomal p70 S6 kinase; eIF, eukaryotic initiation factor; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; RMS, rhabdomyosarcoma; FBS, fetal bovine serum.

under serum-free conditions, Rh1 cells became highly sensitive to inhibition by rapamycin (21). We were, therefore, interested in understanding how serum or growth factors could protect certain RMS cells from inhibitory effects of rapamycin. Although rapamycin exerts cytostatic effects against many cell lines, causing $G_1$ phase cell cycle arrest, there are examples in which this agent induces apoptosis as a single agent or promotes apoptosis induced by cellular stress or cytotoxic agents. Rapamycin induced apoptosis in BKS-2, an immature B cell lymphoma, and was synergistic with suboptimal concentrations of IgM antibodies in inducing programmed cell death (51). This effect was inhibited by the competitive analogue, FK506, indicating that rapamycin binding to FKBP-12 was necessary to induce apoptosis. Rapamycin-enhanced apoptosis induced in S49 cells treated with steroids (52), in the murine T-cell line CTLL-2 after interleukin 2 withdrawal and in *myc*-transformed RAT-1 fibroblasts (53). Furthermore, although it did not induce apoptosis itself, rapamycin was shown to promote apoptosis in HL-60 promyelocytic leukemic cells and SKOV3 ovarian carcinoma cells treated with cisplatin (53). Results presented here show that rapamycin induces apoptosis in RMS cells under conditions of autocrine growth. Apotosis is a consequence of inhibiting mTOR and can be induced by a short exposure to rapamycin.

## MATERIALS AND METHODS

**Cell Lines and Growth Conditions.** Rh30 cells, derived from an alveolar RMS, and Rh1, derived from an embryonal RMS, 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 in which 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 N2E medium, and Rh1 cells were grown in N2E with addition of fibronectin (10 $\mu$g/ml) and albumin (1 mg/ml; insulin RIA grade; Sigma Chemical Co., St. Louis, MO).

**Determination of *p53* Genotype and Function.** Rh30 cells have been characterized previously (54). This line has a single point mutation conferring an amino acid substitution, $Arg^{273} \rightarrow Cys^{273}$ that abrogates transactivator function (54). Genomic DNA was extracted from Rh1 cells with QIAamp tissue kit and exons 2–11 of the *p53* gene were amplified by PCR. The primers for sequencing *p53* exons 2–11 are as described by Mashiyama *et al.* (55). DNA sequencing was performed with an fmol sequencing kit in accordance with the manufacturer's instructions. p53 function in Rh1 cells and in NB-1643 cells that have wild-type p53[6] was determined by Western blot analysis of induction of p53 (DO-1 conjugated to horseradish peroxidase; Santa Cruz Biotechnology, Santa Cruz, CA) and $p21^{cip1}$ (C-19, polyclonal; Santa Cruz Biotechnology) 4 h after 10 Gy of ionizing radiation.

**Rapamycin Sensitivity.** Rh1 and Rh30 cells were plated in triplicate ($5 \times 10^4$ cells per 35-mm dish) in six-well culture plates (Corning, NY). The following day, the medium was removed from the adherent cells, and 2 ml of medium containing serial concentrations of rapamycin (0–10,000 ng/ml) were added to each well. After 7 days, the RMS cells were lysed under hypotonic conditions, and nuclei were counted using a Coulter counter (21). Alternatively, cells were exposed for 1 h, washed extensively, and cell number was quantitated by counting nuclei after 7 days as described.

**Ribosomal $p70^{S6K}$ Determinations.** Cells ($3 \times 10^6$) were seeded in 100-mm dishes and allowed to attach overnight. The cells were serum-starved for 24 h and then stimulated with IGF-I (10 ng/ml) in the presence or absence of rapamycin. Stimulation was terminated by removing the medium and washing cells with cold PBS, and cells were lysed by gently rocking at 4°C in 1 ml of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, and 1 mM NaF] containing 10 $\mu$g/ml each aprotinin, leupeptin, and pepstatin. Lysates were centrifuged (15,000 $\times$ *g*, 4°C, 5 min) to remove nuclei. Ten $\mu$l of anti-$p70^{S6K}$ polyclonal antibody (1 $\mu$g: Santa Cruz Biotechnology) and 50 $\mu$l of A/G Protein Plus beads (Santa Cruz Biotechnology) were added to the lysates and incubated overnight. The beads were washed three times with PBS and resuspended in 20 $\mu$l of $p70^{S6K}$ assay buffer [20 mM MOPS (pH 7.2), 25 mM $\beta$-glycerophosphate, 5 mM EGTA, 1 mM $Na_3VO_4$, and 1 mM DTT]. $p70^{S6K}$ activity was assayed using the S6 kinase assay kit (Upstate Biotechnology Inc., Lake Placid, NY), according to the manufacturer's instructions.

**Determination of Apoptosis.** Cells (Rh1, $8.5 \times 10^5$ cells per 162-$cm^2$ flask; Rh30, $1.7 \times 10^6$ cells per 162-$cm^2$ flask) were grown overnight in N2E serum-free medium. On day 1, combinations of rapamycin (100 ng/ml) and IGF-I (10 ng/ml) were added, and cells were exposed to rapamycin (10 or 100 ng/ml) for up to 6 days with or without IGF-I (10 ng/ml). Control cells in RPMI 1640 containing 10% FBS or N2E were grown for the corresponding periods without addition of rapamycin or IGF-I. Cells were trypsinized, washed with PBS, resuspended in 200 $\mu$l of binding buffer (Clontech), and incubated with 10 $\mu$l of annexin V-FITC (final concentration, 1 $\mu$g/ml; Clontech) and 500 ng of propidium iodide in a final volume of 410 $\mu$l. Cells were incubated at room temperature in the dark for 10 min before flow cytometric analysis (FACSCalibur; Becton Dickinson).

**Expression of a Rapamycin-resistant mTOR.** The AU-1-tagged mutant mTOR cDNA (41, 56) and pcDNA3 control vector were transfected into Rh1 and Rh30 cells using Lipofectamine (Life Technologies, Inc.). This mutant (designated mTOR-rr) has a single amino acid substitution ($S^{2035} \rightarrow I^{2035}$) in the FKBP-rapamycin binding domain that reduces the binding affinity of the FKBP-rapamycin complex (56). Cells were selected for G418 resistance and cloned. Individual clones were screened for expression by Western analysis using the AU-1 monoclonal antibody. A mouse monoclonal antibody against mTOR (26E3) was generated using a synthetic peptide (KPQWYRHTFEE), representing residues 230–240 in the $NH_2$ terminus of mTOR (1).

**Recovery of mTOR Function.** To examine the rate at which mTOR function recovered, Rh1 and Rh30 cells were serum-starved overnight. Following starvation, cells were either exposed for 15 min without (control) or with rapamycin (10 ng/ml). After 15 min, monolayers were washed extensively and incubated in the absence or presence of 1000 ng/ml FK506. At various times (0–24 h), cells were stimulated with IGF-I (10 ng/ml), and ribosomal $p70^{S6K}$ activity was determined as described.

**Pulse-Chase Analysis of mTOR Synthesis.** To determine whether rapamycin suppressed the rate of synthesis of mTOR, Rh30 cells were plated in complete or serum-free medium in 100-mm dishes ($3.0 \times 10^6$ cells per dish) and pulsed with rapamycin (10 ng/ml for 15 min). Rapamycin was removed by multiple washes in Hanks' buffer, and the medium was replaced. The cells were cultured in medium for a further 24 h and then placed in methionine-deficient medium for 4 h. The cultures were pulsed with [$^{35}$S]methionine (100 $\mu$Ci/ml) for an additional 4 h. mTOR was immunoprecipitated using 1 $\mu$g of the anti-mTOR mouse monoclonal antibody 26E3, and the immunoprecipitates were resolved on 10% SDS-polyacrylamide gels using standard methods.

## RESULTS

**Sensitivity of RMS Cells to Rapamycin.** To examine how serum or growth factors could protect certain RMS cell lines from the inhibitory effects of rapamycin, we established conditions for serum-free growth of Rh1 (sensitive to rapamycin only under serum-free conditions) and Rh30 cells, which are sensitive in the presence or absence of serum. Both cell lines were sensitive to rapamycin inhibition with 50% inhibition at 0.1 and 0.5 ng/ml for Rh1 and Rh30 cells, respectively (Fig. 1*A*). Consistent with our previous results (21), IGF-I did not significantly stimulate growth of Rh1 cells but did stimulate growth of Rh30 cells (increased by 84%). Shown also in Fig. 1*A* is that a low concentration of IGF-I (10 ng/ml) completely abrogated the growth-inhibitory effects of rapamycin on Rh1 cells. In the presence of IGF-I, Rh1 cells maintained the same rate of growth as untreated controls, up to 1000 ng/ml rapamycin. In contrast, IGF-I did not rescue Rh30 cells from rapamycin inhibition.

**IGF-I Reverses Rapamycin Inhibition without Reactivating Ribosomal $p70^{S6K}$.** The target of rapamycin, mTOR, lies in a signaling pathway that is intermediate between IGF-IR and ribosomal $p70^{S6K}$.

[6] L. Harris, personal communication.

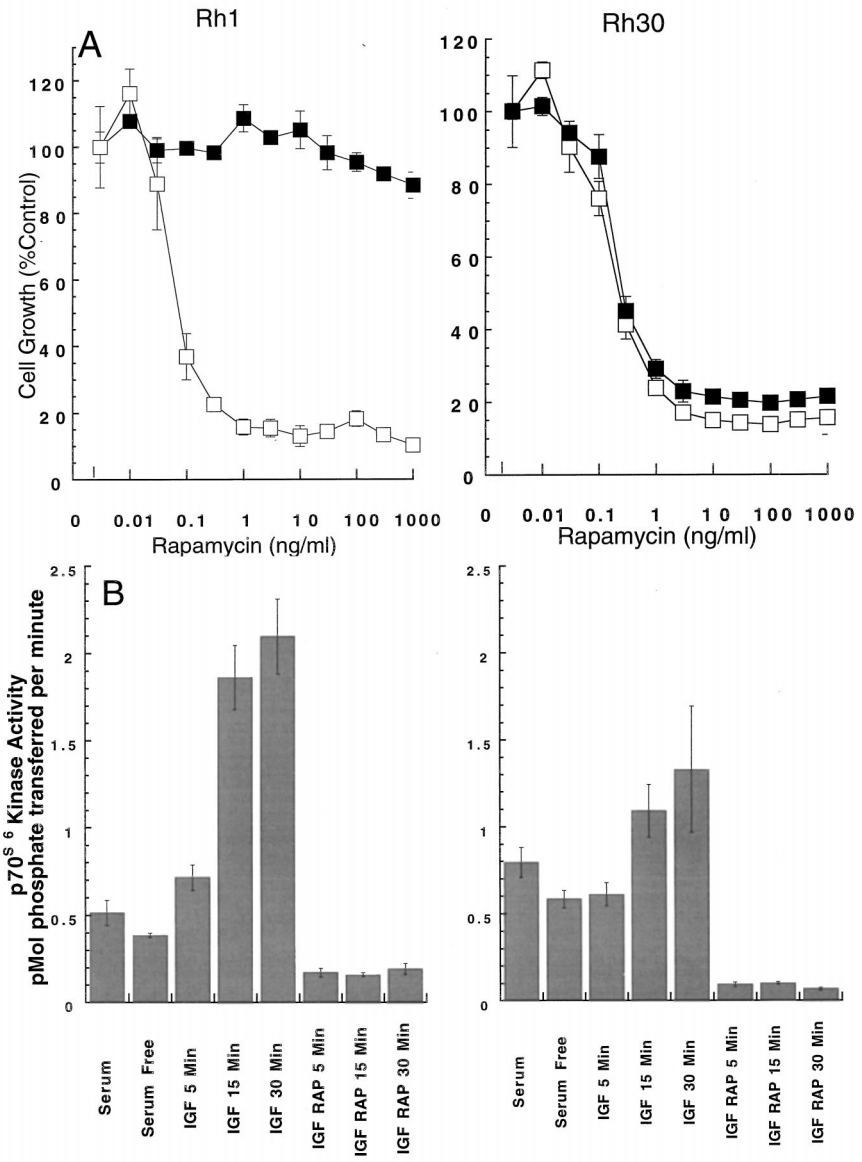


Fig. 1. Inhibitory effect of rapamycin and effect of IGF-I on Rh1 (*left*) and Rh30 (*right*) RMS cells. *A*, rapamycin sensitivity. Cells were seeded and allowed to attach overnight, washed, and cultured in serum-free N2E in medium containing serial concentrations of rapamycin with (■) or without (□) addition of IGF-I (10 ng/ml) for 7 days. Growth was assessed by lysing cells and counting nuclei. *Data points*, percentages of control. Cell number for control Rh1 cells was $1.74 \times 10^5$, and that for Rh30 was $5.7 \times 10^5$. *B*, activation of $p70^{S6K}$ by IGF-I is blocked in Rh1 (*left*) and Rh30 (*right*) by rapamycin. Cells were serum-starved overnight and then stimulated with IGF-I (10 ng/ml) without or with preincubation for 15 min with rapamycin (100 ng/ml). Ribosomal $p70^{S6K}$ assays were performed on immunoprecipitates derived from $3 \times 10^6$ cells. *Columns*, means for four determinations; *bars*, SD. A representative experiment is shown.

Consequently, we used activation of ribosomal $p70^{S6K}$ as a read-out for mTOR inhibition. To determine whether exogenous IGF-I could activate ribosomal $p70^{S6K}$ in the presence of rapamycin, we next serum-starved both cell lines and exposed them to rapamycin or drug vehicle (medium containing 0.1% DMSO) for 15 min, followed by IGF-I stimulation. IGF-I rapidly activated $p70^{S6K}$ in control cells, but this was completely inhibited by rapamycin in both cell lines (Fig. 1*B*). Further studies showed that, in both cell lines, $p70^{S6K}$ activity was inhibited for ≥24 h after 15 min of exposure to rapamycin (data not shown).

**IGF-I Protects RMS Cells from Rapamycin-induced p53-independent Apoptosis.** Our previous data (21) had suggested that IGF-I did not stimulate the growth rate of Rh1 cells growing under serum-free conditions. However, IGF-I completely reversed the inhibitory effect of rapamycin. One explanation for the results obtained for Rh1 cells was that IGF-I was promoting survival in rapamycin-treated cells. Recently, it has been shown that expression of a constitutively active PI3K, exogenous IGF-I (which can signal through PI3K), or AKT/PKB can protect cells from apoptosis under conditions of serum starvation (7–9). Consequently, we considered that inhibition of mTOR by rapamycin may prevent autocrine signaling required for growth and potentially survival of RMS cells. For example, autocrine growth of some RMS cells is regulated by secretion of IGF-II (57), and the translation of its mRNA is under mTOR regulation (46). To test the hypothesis that inhibition of mTOR function may induce apoptosis, Rh1 and Rh30 cells were grown under serum-free conditions and exposed to rapamycin (10 or 100 ng/ml) with or without exogenous IGF-I. Cells were harvested, and apoptosis determined by morphological criteria and quantitated by a flow cytometric assay. Quantitative assessment of apoptosis showed that apoptotic cells could be detected at 24 and 48 h. Representative results are shown in Table 1. The average frequencies of annexin V-positive, propidium iodide-negative cells in controls were 13.64 and 2.92% for Rh1 and Rh30 cells, respectively ($n = 4$ experiments). This rate was similar in serum-free and FBS-containing medium and probably represents an intrinsic cell death property of these cell lines. Within 24 h, the mean frequency of cells positive for annexin V had increased to 18.7% (representing an increase of 54.5% over control) in cultures of Rh1 cells. Relative to control Rh30 cells, there were increases in annexin V-positive cells to 22.7 and 36.5% at 24 and 48 h, respectively, in rapamycin-treated populations, representing increases of 34 and 66% over the respective controls. At 6 and 4 days of exposure, respectively,

~86 and 63% of Rh1 and Rh30 cells were positive for both annexin V and propidium iodide. Thus, RMS cells exposed to rapamycin appear to lose membrane integrity late in apoptosis yet remain attached to the culture dish (Table 2). As shown in Fig. 2, addition of IGF-I (10 ng/ml) completely protected Rh1 cells, and in Rh30 cells suppressed by 50% the increase in annexin V-positive, propidium iodide-positive cells. Data are quantitated in Table 2. In Rh1 cells, addition of IGF-I prevented cell death and allowed cell proliferation to continue (as shown in Fig. 1), whereas in Rh30 cells, IGF-I significantly prevented rapamycin-induced cell death but did not permit cellular proliferation. Previously it has been shown that Rh30 has mutant p53 ($Arg^{273}\rightarrow Cys^{273}$) and does not support expression of a p53-reporter plasmid (54). To determine whether, in Rh1 cells, apoptosis was p53 dependent, genomic DNA was sequenced. Rh1 cells have mutant p53 ($A^{1238}\rightarrow G$, corresponding to $Tyr^{220}\rightarrow Cys^{220}$). Functionally, p53 appeared attenuated because there was no increase in p53 or $p21^{cip1}$ expression after 10 Gy of ionizing radiation, whereas p53 and $p21^{cip1}$ were increased in NB-1643 cells that have wild-type p53 (Fig. 3). Thus, rapamycin-induced apoptosis in both Rh1 and Rh30 appears to be independent of p53 function.

**Expression of a Rapamycin-resistant mTOR Prevents $G_1$ Arrest and Apoptosis Induced by Rapamycin.** Rapamycin binds initially to a cytosolic protein, designated FKBP-12, and this complex inhibits the kinase function of mTOR. However, as with many small molecule inhibitors of protein kinases, it was important to determine that apoptosis was a consequence of selective inhibition of the putative target, mTOR. To determine whether apoptosis was mediated by the action of rapamycin on mTOR, we established clones of Rh1 and Rh30 cells that stably expressed an $NH_2$-terminal epitope-tagged (AU-1) form of mTOR that has reduced binding of FKBP-rapamycin. This mutant (designated mTOR-rr) has a single amino acid substitution ($Ser^{2035}\rightarrow Ile^{2035}$) in the FKBP-rapamycin binding domain. As shown in Fig. 4*A*, a protein band at $M_r$ ~240,000 was detected using the anti-AU-1 antibody only in Rh1 and Rh30 clones transfected with the epitope-tagged mutant mTOR. This had identical mobility on SDS-PAGE to wild-type mTOR (detected by 26E3 monoclonal antibody). In some experiments, a band of slower mobility was detected using the 26E3 antibody in lysates from Rh30 cells but not from vector control or Rh30/mTOR-rr cells. Vector-transfected clones (Rh1pcDNA and Rh30pcDNA) showed no reactivity with the antibody directed against the AU-1 epitope (Fig. 4*A*). Both Rh1 and Rh30 clones expressing mTOR-rr were highly resistant to growth inhibitory effects of rapamycin. On the basis of the $IC_{50}$ concentrations, Rh1 and Rh30 mTOR-rr expressing cells were >3000- and >20,000-fold

Table 1 *Accumulation of annexin V- and propidium iodide-positive cells after rapamycin treatment*

| Cell line[a] | Serum-free control (24 h) | + FBS control (24 h) | Serum-free Rap 100[b] (24 h) | Serum-free Rap 100 (48 h) | Serum-free Rap 100[c] |
|---|---|---|---|---|---|
| Rh1 | | | | | |
| $A^-/P^+$ | 0.15 | 0.41 | 0.48 | | 5.10 |
| $A^+/P^+$ | 3.98 | 5.05 | 5.76 | | 85.02 |
| $A^-/P^-$ | 82.23 | 83.24 | 75.20 | | 8.91 |
| $A^+/P^-$ | 13.64 | 11.3 | 18.68 | | 1.0 |
| % apoptotic | 17.62 | 16.35 | 24.44 | | 86.02 |
| Rh30 | | | | | |
| $A^-/P^+$ | 0.09 | 1.52 | 0.21 | 0.19 | 2.77 |
| $A^+/P^+$ | 14.02 | 20.02 | 18.99 | 28.76 | 62.77 |
| $A^-/P^-$ | 82.97 | 73.42 | 77.07 | 63.36 | 28.12 |
| $A^+/P^-$ | 2.92 | 5.04 | 3.72 | 7.69 | 6.35 |
| % apoptotic | 16.94 | 25.06 | 22.71 | 36.45 | 69.12 |

[a] A, annexin V; P, propidium iodide.
[b] Rap 100, 100 ng/ml rapamycin.
[c] Rh1, 144 h; Rh30, 96 h.

Table 2 *Quantitation of annexin-V and propidium iodide-positive cells in populations of RMS cells treated with rapamycin with or without IGF-I*

| Cell line[a] | + serum-free control | Serum-free + IGF-I | Serum-free + rapamycin | Serum-free + rapamycin + IGF-I |
|---|---|---|---|---|
| Rh1 | | | | |
| $A^-/P^+$ | 5.29 | 2.72 | 6.58 | 8.74 |
| $A^+/P^+$ | 15.37 | 4.67 | 85.58 | 7.98 |
| $A^-/P^-$ | 76.99 | 90.62 | 7.65 | 81.02 |
| $A^+/P^-$ | 2.35 | 1.99 | 0.19 | 2.26 |
| % apoptotic | 17.72 | 6.66 | 85.77 | 10.24 |
| Rh30 | | | | |
| $A^-/P^+$ | 0.91 | 2.70 | 2.77 | 2.91 |
| $A^+/P^+$ | 16.87 | 23.41 | 62.77 | 29.61 |
| $A^-/P^-$ | 77.94 | 71.42 | 28.12 | 63.62 |
| $A^+/P^-$ | 4.27 | 2.47 | 6.35 | 3.86 |
| % apoptotic | 21.14 | 25.88 | 69.12 | 33.47 |

[a] A, annexin V; P, propidium iodide.

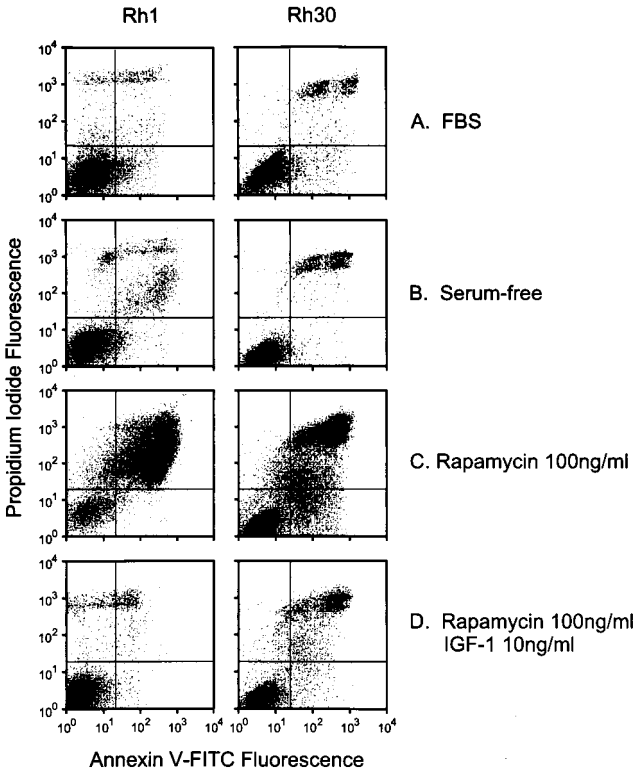


Fig. 2. Rapamycin induces apoptosis that is blocked by IGF-I. Rh1 (*left*) and Rh30 (*right*) cells were grown under serum-containing (*FBS*) or serum-free (N2E) culture conditions supplemented with rapamycin (100 ng/ml) or rapamycin plus IGF-I (10 ng/ml). Cells were harvested after 6 (Rh1) or 4 days (Rh30), and apoptosis was measured by the ApoAlert method. Cells were analyzed by fluorescence-activated cell sorting for annexin-V-FITC and propidium iodide fluorescence.

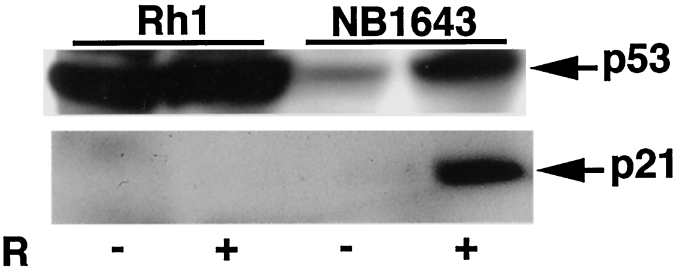


Fig. 3. Rh1 cells have abrogated p53 function. The function of p53 was determined by induction of p53 and $p21^{cip1}$ 4 h after 10 Gy of ionizing radiation (designated *R*, *Lanes* +). Cell lysates were prepared and analyzed by Western blot as described in "Materials and Methods." The human neuroblastoma cell line, NB-1643, was used as a wild-type control for p53 function.

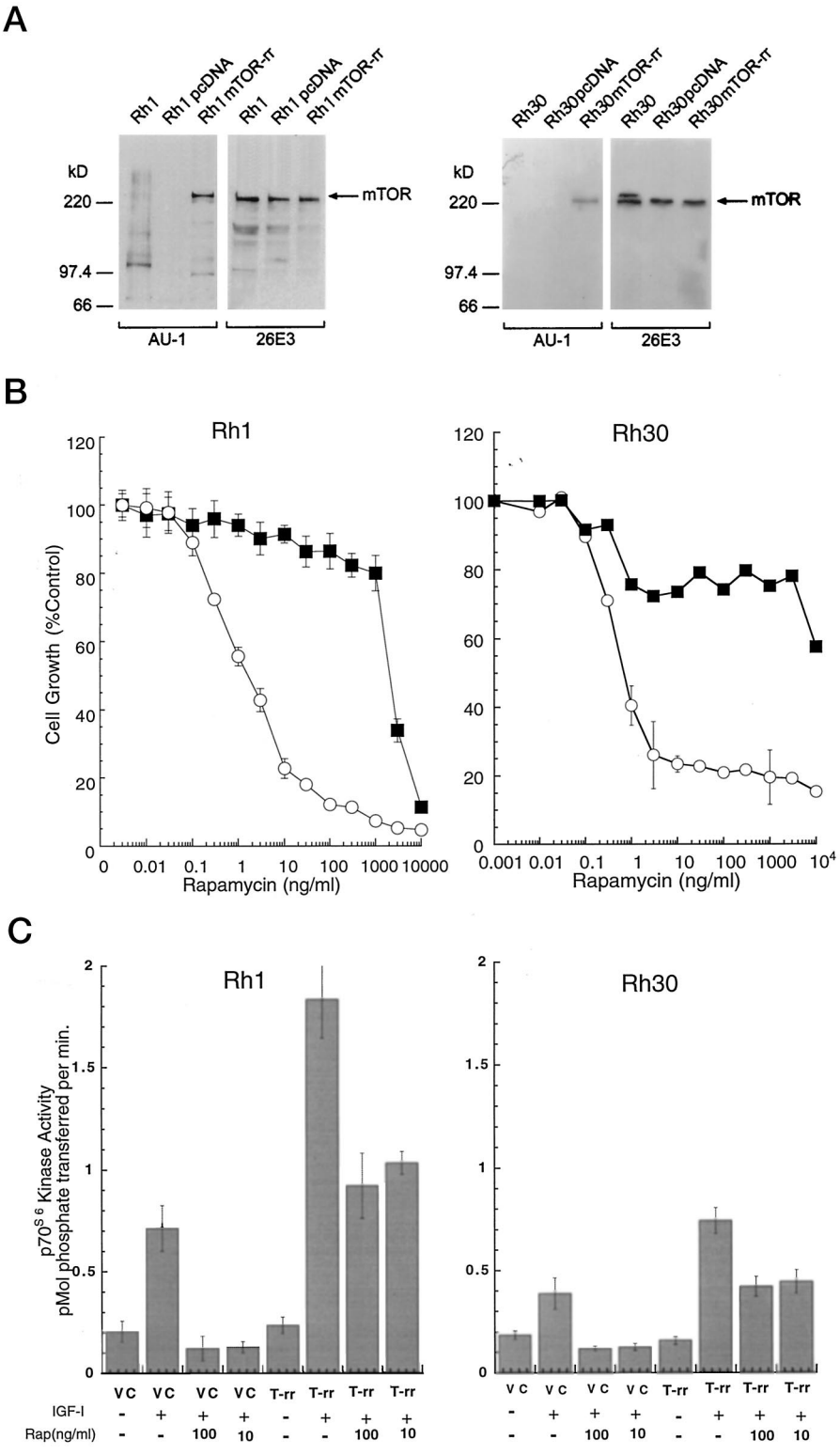


Fig. 4. RMS clones expressing mutant mTOR-rr ($S^{2035} \rightarrow I^{2035}$) are resistant to rapamycin-induced growth inhibition and apoptosis. Rh1 (*left*) and Rh30 (*right*) were transfected with pcDNA3 plasmids encoding mTOR-rr or empty vector (Rh1 pcDNA, Rh30pcDNA) and selected for resistance to G418. *A*, stable clones were characterized by Western blot analysis using anti-AU1 monoclonal antibody directed to the $NH_2$-terminal epitope tag or using 26E3 monoclonal antibody specific for mTOR. *B*, rapamycin sensitivity of mTOR-rr (■) and vector (○) transfected Rh1 and Rh30. Clones were exposed to rapamycin under serum-free conditions as described in Fig. 1. *Data points*, cell numbers, determined after 7 days ($n = 3$); *bars*, SD. *C*, IGF-I (10 ng/ml) stimulation of $p70^{S6K}$ activity in vector controls (*VC*) and mTOR-rr (*T-rr*)-expressing clones without rapamycin or with pretreatment for 15 min with 10 or 100 ng/ml rapamycin. *Columns*, mean values ($n = 3$); *bars*, SD.

resistant to rapamycin, respectively, relative to the appropriate vector control clones (Fig. 4*B*). To determine whether mTOR-rr was functional in rapamycin-treated cells, we next examined the stimulation of $p70^{S6K}$ in vector control and clones expressing mTOR-rr. As shown in Fig. 4*C*, IGF-I caused enhanced stimulation of $p70^{S6K}$ activity in both Rh1 and Rh30 clones expressing the mutant mTOR-rr. This is consistent with increased levels of mTOR (*i.e.*, wild-type plus mutant). Rapamycin only inhibited $p70^{S6K}$ activation by ~50% in these cells, consistent with inhibition of only the endogenous mTOR but not mTOR-rr activity. Thus, in the presence of rapamycin (100 ng/ml), activation of $p70^{S6K}$ in mTOR-rr-expressing clones was equivalent to that in vector control clones in the absence of rapamycin. In contrast, rapamycin completely inhibited activation in Rh1 and Rh30 clones selected in G418 after transfection of vector plasmid (Fig. 4*C*). Expression of the mutant mTOR also protected against rapamycin-induced apoptosis. As shown in Table 3, after 4 days of exposure to

rapamycin (100 ng/ml), there was no increase in the proportion of cells scored annexin V-positive in the ApoAlert assay compared to controls. IGF-I had no significant effect on these rapamycin-resistant cells. Furthermore, when Rh1 or Rh30 cells expressing the mutant mTOR were synchronized in mitosis using nocodozole and released into $G_1$ with or without rapamycin, there was no significant inhibition of $G_1$-S phase progression (Table 4). Rapamycin treatment increased the percentage of Rh1pcDNA cells in $G_1$ phase from 59.1 to 86.9% but had a lesser effect on Rh1/mTOR-rr-expressing cells. In Rh30pcDNA cells, 64% of cells had entered S phase within 24 h of release from mitotic block, whereas in the presence of rapamycin, only 37% had DNA content, indicative of S-phase progression. The percentage in $G_1$ phase increased from 21.2 to 52.0% in the presence of rapamycin. In contrast, rapamycin did not significantly retard cell cycle progression in Rh30/mTOR-rr cells, in which the drug-treated $G_1$ fraction increased from 20.4 to 28.0%. These results strongly support rapamycin-induced $G_1$ arrest and apoptosis being mediated through its inhibitory effect on mTOR kinase activity.

**Rapamycin-induced Apoptosis Is Independent of Drug Exposure Time.** Rapamycin is relatively unstable under conditions of tissue culture. Fresh solutions of rapamycin or solutions incubated in tissue culture medium (no cells; 37°C, 5% $CO_2$) containing 10% FBS for 7 days, were bioassayed using Rh30 cells. Incubation for 7 days under culture conditions resulted in a decrease of 1426-fold in potency compared to the fresh sample. This would correspond to a $t_{1/2}$ of ~9.9 h. for degradation. In contrast, our preliminary data suggested prolonged inhibition of mTOR function. To investigate the kinetics of rapamycin-induced inhibition of cell growth and induction of apoptosis, the effect of exposure for 1 h or 7 days was examined. Growth inhibition was essentially identical whether Rh1/pcDNA and Rh30 cells were exposed for 1 h and washed extensively or exposed continuously to rapamycin for 7 days (Fig. 5). Similarly, exposure to rapamycin for 1 h induced apoptosis at the same rate and to the same extent as continuous exposure. Results in Fig. 5 show the fold increase over the appropriate control (no treatment at the same time point) for Rh1/pcDNA cells positive only for annexin V or demonstrating dual positivity (annexin V and propidium iodide positive) and for the total annexin V-positive cells relative to the control. For Rh1pcDNA3 cells, increases in annexin V-positive and dual-positive cells were linear with time and significantly elevated within 24 h of exposure to rapamycin for 1 h or continuously. For Rh30 cells the increase over control was less pronounced, in part because there was a higher level of apoptotic cells in controls. The percentage increases in total annexin V-positive cells over controls were 21% at 24 h and 34% at 48 h following 1-h treatment with rapamycin (data not shown).

Table 3 *Quantitation of annexin V- and propidium iodide-positive cells in control and rapamycin-treated populations of RMS cells expressing a rapamycin-resistant mTOR mutant*

| Cell line[a] | Control | Rap 100[b] | Rap 100 + IGF-I |
|---|---|---|---|
| Rh1/mTOR-rr | | | |
| $A^-/P^-$ | 0.28 | 0.48 | 0.48 |
| $A^+/P^+$ | 6.57 | 5.22 | 4.18 |
| $A^-/P^-$ | 88.85 | 90.11 | 93.57 |
| $A^+/P^-$ | 4.30 | 4.19 | 1.76 |
| % apoptotic | 10.87 | 9.41 | 5.94 |
| Rh30/mTOR-rr | | | |
| $A^-/P^-$ | 0.82 | 0.56 | 0.76 |
| $A^+/P^+$ | 10.09 | 8.78 | 8.38 |
| $A^-/P^-$ | 83.62 | 85.51 | 84.76 |
| $A^+/P^-$ | 5.47 | 5.15 | 6.1 |
| % apoptotic | 15.56 | 13.93 | 14.48 |

[a] A, annexin V; P, propidium iodide.
[b] Rap 100, 100 ng/ml rapamycin.

Table 4 *Cell cycle progression in control and rapamycin-treated cells 24 h after release from mitotic synchronization*

| Cell clone | Rapamycin (ng/ml) | % $G_1$ | % S | % $G_2$ + M |
|---|---|---|---|---|
| Rh30pcDNA | 0 | 21.2 | 64.6 | 14.2 |
| Rh30pcDNA | 100 | 52.0 | 37.2 | 10.8 |
| Rh30/mTOR-rr | 0 | 20.4 | 66.7 | 12.9 |
| Rh30/mTOR-rr | 100 | 28.0 | 60.4 | 11.6 |
| Rh1pcDNA | 0 | 59.1 | 30.4 | 10.5 |
| Rh1pcDNA | 100 | 86.9 | 11.0 | 2.1 |
| Rh1/mTOR-rr | 0 | 59.6 | 32.5 | 7.8 |
| Rh1/mTOR-rr | 100 | 68.7 | 25.6 | 5.7 |

**Rapamycin Dissociates Slowly from Intracellular Binding Sites.** Several possibilities could explain the prolonged action of rapamycin. Rapamycin inhibits translation of specific mRNA species, although it reduces overall protein synthesis by only ~15%. We first tested whether prolonged activity could be explained by rapamycin inhibiting new synthesis of its target protein, mTOR. By Western blot analysis, mTOR levels did not decrease over 48 h in rapamycin-treated cells, and rapamycin did not alter the rate of [$^{35}$S]methionine incorporation into immunoprecipitated mTOR (data not shown). Thus, it is unlikely that prolonged inhibition of the mTOR pathway is due to rapamycin inhibiting translation of mTOR mRNA. Recently, using [$^{14}$C]rapamycin [at very high concentrations (20 $\mu$M) due to the low specific activity], we showed that there was a tight-binding, poorly exchangeable component within cells exposed to this drug (58). We considered, therefore, that binding to FKBP-12 could stabilize rapamycin, allowing for a longer biological half-life within cells. To test this, we have used IGF-I stimulation of $p70^{S6K}$ activity as a surrogate for determining the level of mTOR inhibition. Rh1 cells were serum-starved overnight and incubated with or without rapamycin (10 ng/ml) for 15 min. IGF-I stimulation was determined in control and rapamycin-treated cells at the end of the incubation (0 time; Fig. 6*A*). Alternatively, monolayers were washed extensively to remove unbound rapamycin, and monolayers were incubated in medium containing 1000 ng/ml FK506 or without FK506. This structural analogue of rapamycin competes with similar affinity (~2-fold lower) for binding to FKBP-12, hence allowing determination of the off-rate of rapamycin from this binding protein. At various times for up to 24 h, cells were stimulated with IGF-I and $p70^{S6K}$ activity determined after 1 h. As shown in Fig. 6*A*, there was a progressive recovery of $p70^{S6K}$ stimulation by IGF-I in the presence of FK506. Percentage inhibition of IGF-I stimulation of $p70^{S6K}$ activity with time is presented in Fig. 6*B*. The times for recovery to 50% maximal stimulation of $p70^{S6K}$ activity by IGF-I were calculated to be 16.9 and 17.5 h in two separate experiments in the presence of FK506. There was no recovery of the mTOR pathway in cells exposed briefly (15 min) to rapamycin but in which FK506 was not added after removal of rapamycin.

## DISCUSSION

Under serum-free conditions, malignant RMS cells maintain proliferation through autocrine mechanisms (21, 57, 59) and exhibit a relatively low level of spontaneous apoptosis, similar to that observed when cells are cultured under serum-containing conditions. These malignant cells differ, therefore, from several nonmalignant cell types, which undergo apoptosis under conditions of serum withdrawal. We showed previously that rapamycin potently induces growth arrest in Rh1 and Rh30 RMS cells under these conditions of culture. However, in the presence of serum, Rh1 cells were highly resistant to growth inhibition by rapamycin. Consequently, it was of interest to determine which components in serum protected these cells. We found that,

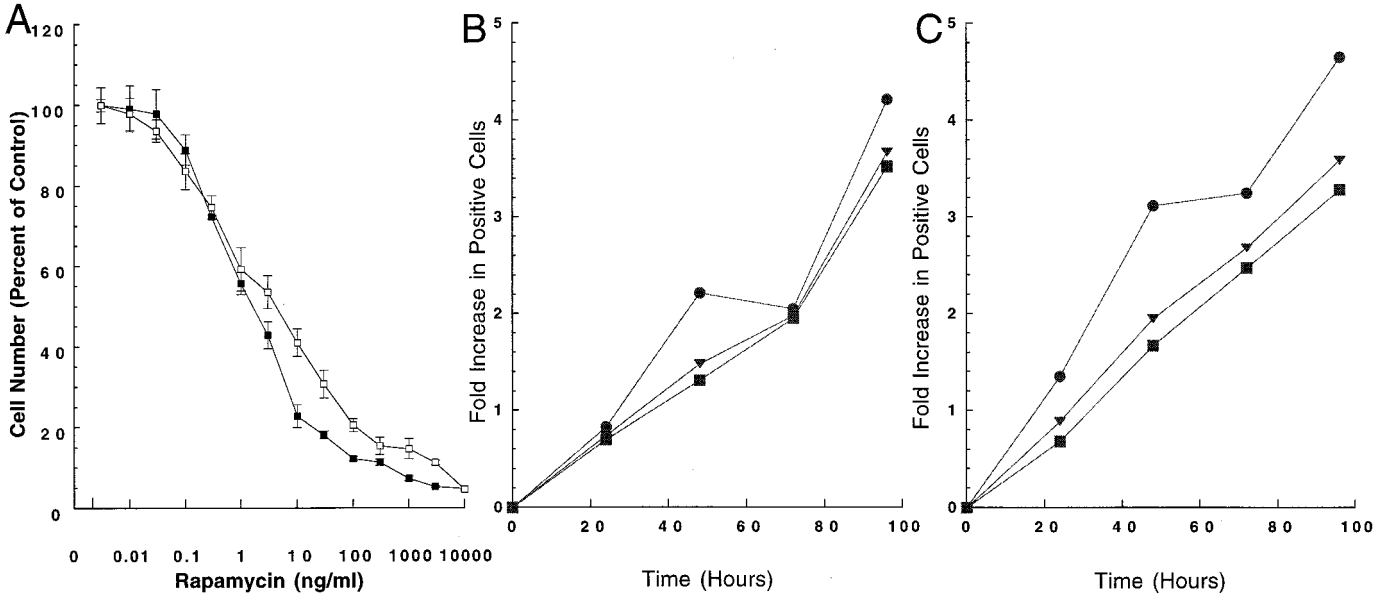


Fig. 5. Growth inhibition and induction of apoptosis in Rh1pcDNA3 following rapamycin treatment for 1 h or continuous exposure. *A*, growth-inhibitory effect of rapamycin on Rh1/pcDNA (vector control) cells exposed for 1 h (□) or continuously for 7 days (■). Cells were seeded and allowed to attach overnight, washed, and cultured in serum-free N2E in medium. Growth was assessed by lysing cells and counting nuclei, as described in Fig. 1. *Data points*, means ($n = 3$) for a representative experiment; *bars*, SD. *B* and *C*, time course for induction of apoptosis in Rh1/pcDNA cells treated with rapamycin: *B*, continuous exposure; *C*, 1-h exposure. Cells were seeded and allowed to attach overnight, washed, and cultured in serum-free N2E in medium. Cells were exposed to rapamycin continuously or for 1 h and then washed extensively. Apoptosis was determined at various times by ApoAlert assay as described in Fig. 2. ●, annexin V-positive, propidium iodide-negative; ■, annexin V-positive, propidium iodide-positive; ▼, sum of all annexin V-positive cells. *Data points*, fold increases in each population over the appropriate untreated control (no drug) at the same time point.

under serum-free conditions of growth, IGF-I completely substituted for serum in causing rapamycin resistance in Rh1 cells. Exogenous IGF-I abrogated growth arrest in Rh1 cells, whereas Rh30 cells remained arrested even in the presence of IGF-I. Rescue was not due to reactivation of mTOR because IGF-I did not reactivate $p70^{S6K}$ in the presence of rapamycin. These results further substantiate that cells (*e.g.*, Rh1) may proliferate normally even when $p70^{S6K}$ activity is inhibited (39, 40).

Proliferation of Rh1 cells is not dependent on IGF-IR function (21). Thus, one possibility was that rapamycin was inducing apoptosis and IGF-I was acting as a survival factor rather than a mitogen (60–63). To test this, we examined whether rapamycin-treated cells were undergoing apoptosis using binding of annexin V and uptake of propidium iodide as markers for this process. In both cell lines, there was a significant increase in annexin V-positive cells within 24 h of exposure to rapamycin. With increasing time, the predominant population became positive for both annexin V and propidium iodide, suggesting a loss of membrane integrity late in apoptosis. Interestingly, these propidium iodide positive "ghosts" remained stable and attached to the culture dish for several days. Because both Rh1 and Rh30 (54) cells have mutant nonfunctional p53 (as determined by failure to support a p53-promoter reporter plasmid or induce either p53 or $p21^{cip1}$ after ionizing radiation), rapamycin-induced apoptosis appears to be p53 independent. Addition of IGF-I at the same time as rapamycin completely protected Rh1 cells from apoptosis and reduced the annexin V-positive population of Rh30 cells by ~50%. The

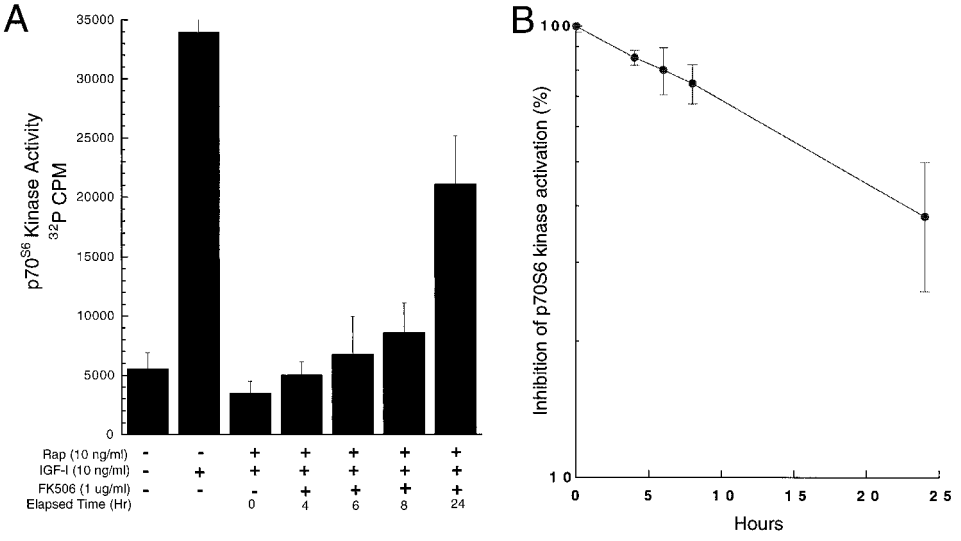


Fig. 6. *A*, recovery of IGF-I stimulation of $p70^{S6K}$ activity in the presence of FK506. Cells were serum-starved overnight and either stimulated with IGF-I in the absence of rapamycin or after 1 h exposure to rapamycin (time 0). Alternatively, after extensive washing cells were incubated in serum-free medium supplemented with 1000 ng/ml FK506. At various times (4–24 h), cells were stimulated with IGF-I and $p70^{S6K}$ activity determined after 1 h. *Columns*, means ($n = 3$) of a representative experiment; *bars*, SD. *B*, calculation of recovery of the mTOR pathway after rapamycin treatment. Data from *A* were replotted as percentage inhibition of IGF-I stimulation of $p70^{S6K}$ activity with time after adding excess FK506 to cell cultures. Maximal stimulation (0% inhibition) was taken as that induced by IGF-I in the absence of rapamycin (*A*, *second column*). Maximal inhibition (100%) was taken as the stimulation by IGF-I immediately after a 1-h exposure to rapamycin.

mechanism by which IGF-I protects cells is unknown and is currently under investigation. However, results presented here show that rescue is effected through an mTOR-independent pathway because IGF-I did not stimulate $p70^{S6K}$ activity in cells exposed to rapamycin.

Rapamycin complexed with FKBP-12 binds and inhibits the serine/threonine kinase activity of mTOR. However, definitive proof that apoptosis is a consequence of inhibition of mTOR is lacking. To establish mTOR as the rapamycin target critical for inducing apoptosis in RMS cells, clones that stably expressed an epitope-tagged mutant mTOR (mTOR-rr) resistant to inhibition by rapamycin-FKBP were selected and characterized. In clones expressing mTOR-rr, IGF-I stimulated $p70^{S6K}$ activity to a level that was ~2-fold greater than that in parental or vector control cells, indicating a similar contribution of wild-type and mutant mTOR to this pathway. Rapamycin could not inhibit activation of $p70^{S6K}$ to a level below that determined in IGF-I stimulated vector-control cells. Importantly, expression of mTOR-rr conferred high-level resistance (>3000-fold) to growth-inhibitory and apoptosis-inducing effects of rapamycin. These data strongly suggest that rapamycin effects on growth and survival are a consequence of specific inhibition of mTOR function. These data also indicate that mTOR must transduce a survival signal in RMS cells under autocrine growth conditions. This result is consistent with the report that overexpression of eIF4E blocks apoptosis in growth factor-restricted fibroblasts (64). At concentrations of >2000 ng/ml, rapamycin inhibited growth even in cells expressing mTOR-rr. This could suggest a second site for rapamycin activity at very high concentrations that is independent of mTOR.

Our initial data indicated that IGF-I could prevent rapamycin-induced apoptosis without reactivating $p70^{S6K}$ downstream of mTOR. Furthermore, even in the presence of IGF-I, recovery of $p70^{S6K}$ activity in the presence of rapamycin was slow. This was of interest because rapamycin was relatively unstable under conditions of cell culture. Incubation of a solution of rapamycin under culture conditions for 7 days caused a >1400-fold decrease in potency when it was bioassayed using Rh30 cells. This decreased activity suggests that the $t_{1/2}$ is ~9.9 h. for degradation under these conditions. It seemed likely, therefore, that rapamycin is stabilized within the cell and that relatively short exposures to drug could have biological effects equivalent to continuous exposure. To test this, we compared the sensitivity of RMS cells to rapamycin when exposure was for 1 h or for 7 days. Results demonstrated essentially equivalent potency for either exposure. Furthermore, the rate and level of cells entering apoptosis, as determined by FACs analysis, was similar in cells exposed continuously or for 1 h only. To determine a biochemical basis for this observation, we examined two possibilities. First, we considered whether synthesis of mTOR was under control of the mTOR pathway (*i.e.*, inhibition of mTOR kinase activity by rapamycin inhibited translation of mTOR mRNA leading to prolonged inhibition of this pathway). However, rapamycin did not cause a decrease in levels of mTOR or prevent incorporation of [$^{35}$S]methionine into immunoprecipitated mTOR (data not shown). Second, we considered that the prolonged action of rapamycin could be a consequence of intracellular binding to FKBP-12 and stabilization of rapamycin. The analogue FK506 prevents rapamycin-induced growth inhibition in both Rh1 and Rh30 cells, confirming that rapamycin has to bind to FKBP-12 for activity (21). To determine the rate at which rapamycin dissociated from FKBP-12 within cells, recovery of IGF-I stimulated $p70^{S6K}$ activity was determined directly after rapamycin treatment or after removal of rapamycin and incubation with an excess of FK506. FK506 binds to FKBP-12 with approximately one-half the affinity as rapamycin (13, 24, 65). The results show that, in the presence of a great excess of FK506, which prevents reassociation of rapamycin to FKBP-12, the mTOR pathway recovers 50% in ~17 h. Without FK506 as a competitor, there was no recovery of the mTOR pathway for ≥20 h. This result is consistent with reassociation of rapamycin with FKBP-12 under noncompetitive conditions. Failure of $p70^{S6K}$ activity to recover could not be explained by the relatively low level of cell death during this time period. Thus, intracellular stabilization of rapamycin, and prolonged inhibition of mTOR signaling probably explains why short exposures to rapamycin have effects equivalent to continuous exposure under the conditions reported here.

In summary, the potent immunosuppressive agent rapamycin rapidly induces p53-independent apoptosis in human RMS cells under conditions of autocrine growth. Protection from apoptosis was conferred by expression of a mutant mTOR resistant to rapamycin, demonstrating mTOR as the critical target for inducing cell death and indirectly indicating that mTOR transduces a survival signal in RMS cells. Because rapamycin and certain analogues potently inhibit growth or induce apoptosis of certain tumor cells, these agents have potential as antitumor agents. Our data demonstrating high potency and relatively slow recovery of mTOR function following a short exposure to rapamycin may be of value in developing schedules of drug administration that may optimize the differential between antitumor and immunosuppressive properties of this class of agent.

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