

Studies on the Mechanism of Resistance to Rapamycin in Human Cancer Cells

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ABSTRACT

Rapamycin is a potent cytostatic agent that arrests cells in the G1 phase of the cell cycle. The relationships between cellular sensitivity to rapamycin, drug accumulation, expression of mammalian target of rapamycin (mTOR), and inhibition of growth factor activation of ribosomal p70S6 kinase (p70^{S6k}) and dephosphorylation of pH acid stable protein I (eukaryotic initiation factor 4E binding protein) were examined. We show that some cell lines derived from childhood tumors are highly sensitive to growth inhibition by rapamycin, whereas others have high intrinsic resistance (>1000-fold). Accumulation and retention of [¹⁴C]rapamycin were similar in sensitive and resistant cells, with all cells examined demonstrating a stable tight binding component. Western analysis showed levels of mTOR were similar in each cell line (<2-fold variation). The activity of p70^{S6k}, activated downstream of mTOR, was similar in four cell

lines (range, 11.75–41.8 pmol/2 × 10⁶ cells/30 min), but activity was equally inhibited in cells that were highly resistant to rapamycin-induced growth arrest. Rapamycin equally inhibited serum-induced phosphorylation of pH acid stable protein I in Rh1 (intrinsically resistant) and sensitive Rh30 cells. In serum-fasted Rh30 and Rh1 cells, the addition of serum rapidly induced c-MYC (protein) levels. Rapamycin blocked induction in Rh30 cells but not in Rh1 cells. Serum-fasted Rh30/rapa10K cells, selected for high level acquired resistance to rapamycin, showed ≥10-fold increased c-MYC compared with Rh30. These results suggest that the ability of rapamycin to inhibit c-MYC induction correlates with intrinsic sensitivity, whereas failure of rapamycin to inhibit induction or overexpression of c-MYC correlates with intrinsic and acquired resistance, respectively.

The macrolide antibiotic rapamycin and its analogue FK-506 have been the subject of intensive investigation because they represent agents that inhibit signal transduction processes involved in T cell activation (reviewed in Schreiber, 1991; Kunz and Hall, 1993). Both agents are potent inhibitors of T cell activation, and activity is mediated after binding to a highly conserved cytosolic protein (FKBP), of which a 12-kDa form seems to be important in drug activity (Sieki-erka *et al.*, 1989). The mechanism by which FK-506 blocks Ca²⁺-dependent signaling seems to be a consequence of the drug/protein complex inhibiting calcineurin (Liu *et al.*, 1991;

Fruman *et al.*, 1992) and failure to activate NF-AT necessary for transcriptional activation of IL-2 (Flanagan *et al.*, 1991; McCaffrey *et al.*, 1993). In contrast, rapamycin/FKBP blocks at some point distal to IL-2 induction (Schreiber, 1991; Kunz and Hall, 1993) is not inhibitory to calcineurin activity in T cells (Fruman *et al.*, 1992) and inhibits activation of T cells only when added within 6 hr of antigen stimulation (Terada *et al.*, 1993). It is now established that the rapamycin/FKBP complex inhibits the serine/threonine kinase function of a 289-kDa phosphoprotein, designated mTOR (also designated FRAP, RAFT, and RAPT; Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994). mTOR signals to two separate pathways, each of which controls translation of specific subsets of mRNA species. mTOR directly phosphorylates PHAS-I *in vitro* (Brunn *et al.*, 1997; Hara *et al.*, 1997; also designated 4E-BP1), the suppressor of the eukaryotic initiation factor eIF-4E, causing PHAS-I to dissociate from eIF-4E

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ABBREVIATIONS: FKBP, FK-binding proteins; IL, interleukin; RMS, rhabdomyosarcoma; p70^{S6k}, ribosomal p70 S6 kinase; IGF, insulin-like growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; NF-AT, nuclear factor of activated T cells; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; MOPS, 3-(N-morpholino)propanesulfonic acid; SSC, standard saline citrate; PHAS-I, pH acid stable protein I; 4E-BP1, eukaryotic initiation factor 4E binding protein.

and allowing the translation of mRNAs with highly structured 5'-untranslated regions. Thus, mTOR controls the synthesis of essential proteins involved in cell cycle progression, such as cyclin D1 (Rosenwald *et al.*, 1995) and ornithine decarboxylase (Shantz and Pegg, 1994), and survival (c-MYC) (DeBenedetti *et al.*, 1994). Whether mTOR directly or indirectly activates p70^{S6k} *in vivo* remains controversial (von Manteuffel *et al.*, 1997; Burnett *et al.*, 1998; Pullen *et al.*, 1998). The p70^{S6k} pathway controls translation of mRNA species that contain a 5' terminal oligopyrimidine tract such as those encoding ribosomal proteins, elongation factors (Jeffries *et al.*, 1994; Terada *et al.*, 1994), and IGF-II (Nielsen *et al.*, 1995). This last point may be of importance because we have shown that in many RMS cell lines, autocrine growth is mediated by secretion of IGF-II and signaling through the IGF-I receptor (El-Badry *et al.*, 1990; Dilling *et al.*, 1994; Shapiro *et al.*, 1994).

Indeed, rapamycin is a potent inhibitor of the growth of specific tumor cells, offering the possibility that this, or an analogue, may have therapeutic efficacy against some malignancies. Our data have indicated that cell lines derived from alveolar RMS that are highly dependent on signaling through the IGF-I receptor (El-Badry *et al.*, 1990; Shapiro *et al.*, 1994) are exquisitely sensitive to this agent, being several thousand-fold more sensitive than human colon cancer cell lines or cell lines evaluated in the National Cancer Institute *in vitro* screen (Dilling *et al.*, 1994). Thus, rapamycin demonstrates striking cell type selectivity in these studies. In contrast, the analogue FK-506 is 400-3000-fold less potent against these lines, indicating that inhibition of the peptidyl-prolyl isomerase activity of FKBP-12 is not critical to growth inhibition of RMS cells.

Here, we show that cell lines from other childhood carcinomas are also highly sensitive to the growth-inhibitory effects of rapamycin, suggesting that this agent may have a more general cytostatic activity in various malignant cell types. To determine whether the level of expression of the target of rapamycin, mTOR, and inhibition of p70^{S6k} activity, or PHAS-I phosphorylation, correlated with sensitivity to rapamycin-induced growth inhibition, we examined two pairs of cell lines derived from RMS and glioblastoma, respectively, that demonstrate >1000-fold difference in intrinsic sensitivity to growth inhibition by rapamycin.

Materials and Methods

Cell lines and culture. Childhood RMS lines (Rh1 and Rh30), and neuroblastomas (NB-SD, NB-1382.2, NB-1643, NB-1691) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For experiments with RMS lines under serum-free conditions, cells were cultured on fibronectin-coated dishes in N2E medium as described previously (Dilling *et al.*, 1994). Rh30/rapa10K was selected for rapamycin resistance by culturing in progressively higher drug concentrations. It was maintained continuously in medium containing rapamycin (10,000 ng/ml). SJ-G2 cells were established from a glioblastoma multiforme specimen from 5-year-old girl, and SJ-G3, also a glioblastoma multiforme specimen, was established from a 12-year-old girl. The biological and molecular characteristics of these lines will be described elsewhere. For assays of growth inhibition, cells were plated onto six-well culture plates in triplicate (10⁵ cells/well). The next day, serial drug dilutions were added to the plates, and cells were incubated for 5–7 days. Cells were lysed under hypotonic conditions, as described previously, and nuclei

were enumerated using a Coulter Electronics (Miami Lake, FL) counter (Dilling *et al.*, 1994). For clonogenic assays, cells were plated in triplicate onto six-well plates (3000 cells/well). Drugs were added the next day, and cells were incubated for 7 days. Colonies were stained with crystal violet and quantified with an Artek counter (Imaging Products International, Chantilly, VA).

Accumulation and retention of rapamycin. [¹⁴C]Rapamycin (2,6-pipecolate-¹⁴C; specific activity, 13 μ Ci/mg) was a generous gift from Dr. J. Gibbons (Wyeth-Ayerst, Pearl River, NJ). Cells were plated at 1.0×10^6 /35-mm culture plate and allowed to attach overnight. Monolayers were washed twice with RPMI 1640 and incubated with drug-containing medium (19 μ M) at 23°. At appropriate times, medium was aspirated, and radiolabel associated with the cells was determined as described previously (Houghton *et al.*, 1990). For retention studies, accumulation was stopped at 60 min by aspirating the medium, washing the monolayer extensively, and refeding cells with drug-free medium. Cultures then were returned to an incubator (37°, 5% CO₂), and samples were taken at time points up to 8 hr.

Expression of TOR. Briefly, total RNA was extracted from cells using RNeasy (Tel Test B, Friendswood, TX). RNA samples (20 μ g) were denatured (65°, for 15 min) in a solution of 50% formamide, 17.5% formaldehyde, 20 mM MOPS-EDTA, pH 7.4, and 0.25% bromophenol blue. Ethidium bromide (1 μ l of 1 mg/ml) was added to each sample before electrophoresis in an RNA-formaldehyde gel (1% agarose, 6% formaldehyde) with recirculating MOPS-EDTA buffer, pH 7.4. The RNA was transferred to Hybond-N⁺ nucleic acid transfer membrane (Amersham, Arlington Heights, IL) by capillary action, and the membrane was baked for 1 hr at 80°. The membrane was prehybridized for 15 min at 65° in rapid hybridization buffer (Amersham). Hybridization was carried out in the same buffer for 2 hr with denatured random primed ³²P-labeled cDNA probes. For detection of mTOR transcripts, a 4.8-kb carboxyl-terminal probe to mouse mTOR (RAPT; Chiu *et al.*, 1994) was used, and the signal was normalized against that for a 1.0-kbp fragment of a cDNA for G3PDH (Clontech Laboratories, Palo Alto, CA). Specific activities of the probes were 7.3×10^8 cpm/mg of DNA. After hybridization, the membranes were washed twice with 1 \times SSC and 0.1% SDS at 65° for 15 min and once with 0.1 \times SSC and 0.1% SDS at 65° for 30 min. The membranes were exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY), with an intensifying screen for autoradiography. The relative signals were quantified by densitometric analysis using a PhosphorImager and normalized to the G3PDH signal.

Development of monoclonal antibodies. Mice were immunized with 100 μ g of a synthetic peptide (KPQWYRHTFEE, designated peptide 1), representing residues 230–240 in the amino terminus of mTOR (Brown *et al.*, 1994), using procedures reported previously (Dias *et al.*, 1992). Spleen cells were fused with SpAG8 myeloma cells, and clones were screened using a solid-phase assay with the peptide. Positive clones were subcloned and characterized by Western blot analysis. One clone, designated 22C2, was used in the experiments reported.

mTOR protein levels in tumor cells. Levels of mTOR in the cell lines were examined by immunoblotting. Whole-cell lysate was extracted from cells in 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS in PBS (1 \times = 8.5 g/liter NaCl, 1.1 g/liter dibasic sodium phosphate, 0.32 g/liter monobasic sodium phosphate) (RIPA buffer) supplemented with 10 μ g/ml concentration each of leupeptin, aprotinin, and antipain; 1 mM sodium orthovanadate, and 1 mM PMSF. After centrifugation (15,000 \times g, 20 min at 4°), extracts (80 μ g of protein) were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose/polyvinylidene difluoride membrane using Tris-glycine, pH 8.3, and 20% methanol. The membrane was blocked for 1 hr in TBST with 5% (w/v) nonfat dry milk, incubated overnight with 1:10 dilution of 22C2 hybridoma supernatant in TBST with 5% milk, washed three times each for 5 min with TBST, incubated for 1 hr with horseradish peroxidase-conjugated anti-mouse secondary antibody, washed as before, and detected with the ECL kit (Amersham).

PHAS-I analysis in tumor cells. Lysates were prepared as described previously (Brunn *et al.*, 1996), analyzed by SDS-PAGE, and immunoblotted as described above. Polyclonal antibody generated by immunization of rabbits with recombinant His-tagged rat PHAS-I was used at 1:750 dilution. Detection was as described above, except that horseradish peroxidase-conjugated anti-rabbit secondary antibody was used.

Analysis of PHAS-I binding to eIF4E. Functional assay of PHAS-I was examined, essentially as described by Gingras *et al.* (1998). Rh30 and Rh1 cells were plated at a density of 3.0×10^6 cells/100-mm dish. The next day, they were shifted to serum-free conditions for 24 hr. The cells then were stimulated with IGF-I (10 ng/ml; Upstate Biotechnology, Lake Placid, NY) in the presence or absence of 10 ng/ml rapamycin. Samples were harvested 4 and 8 hr after stimulation. Extracts were prepared by scraping the cells in 1 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 50 mM β -glycerophosphate, 1 mM EGTA, 50 mM NaF, 10 mM Na-pyrophosphate, 0.1 mM Na_3VO_4 , 50 mM okadaic acid, 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 1 $\mu\text{g/ml}$ leupeptin, 2 μM benzamidin, and 10 $\mu\text{g/ml}$ soybean trypsin inhibitor). Lysis was accomplished with three freeze/thaw cycles. To bind eIF4E, 25 μl of ^7M -GTP Sepharose (Pharmacia Biotech, Alameda, CA) was added to the lysates, which were incubated overnight on a rotator at 4°. The complexes were pelleted and washed three times with lysis buffer. To dissociate bound eIF4E from the Sepharose, 50 μl of SDS-PAGE loading buffer was added to the samples, which then were heated to 95° for 3 min. Samples next were analyzed by SDS-PAGE and Western blotting using standard chemiluminescent methods. Rabbit polyclonal anti-PHAS-I antibody 11208 (generously provided by Nahum Sonenberg, McGill University, Montreal, Canada) was used to detect PHAS-I associated with eIF4E. eIF4E was detected using a commercially available monoclonal antibody (Transduction Laboratories, Lexington, KY).

Assay of ribosomal p70^{S6k}. Cells (2×10^6) were seeded in 100-mm culture dishes and allowed to attach overnight. Initial experiments determined that exposure to rapamycin for 1 hr resulted in maximal inhibition of p70^{S6k} activity. For the experiments reported, cells were exposed for 1 hr to varying concentrations of rapamycin, washed extensively, and lysed by gently rocking cells at 4° in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4 , and 1 mM NaF) containing 10 $\mu\text{g/ml}$ concentration each of aprotinin, leupeptin, and pepstatin. Lysates were transferred and centrifuged (15,000 $\times g$, 4°, 5 min) to remove nuclei. Then, 20 μl of anti-p70^{S6k} polyclonal antibody (2 μg ; Santa Cruz Biotechnology, Santa Cruz, CA) and Protein A beads were added to the supernatant, mixed, and kept overnight at 4°. After centrifugation, the beads were washed twice with PBS and resuspended in 20 μl of p70^{S6k} assay buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 , 1 mM dithiothreitol). p70^{S6k} activity was assayed using the S6 kinase assay kit (Upstate Biotechnology) according to the manufacturer's instructions.

Induction of c-MYC. Cells were plated into 2 ml of medium at a density of 5×10^5 cells/35-mm well in six-well plates (Corning Glassworks, Corning, NY). After overnight incubation at 37° and 5% CO_2 , medium was removed from adherent cells, and 2 ml of serum-free RPMI 1640 supplemented with 2 mM L-glutamine was added to each well. After an additional 24 hr, the cells were stimulated by the addition of serum to a final concentration of 10%. Cells were incubated further for the appropriate time periods, washed with ice-cold PBS, and processed as above for Western analysis using hybridoma 1-9E10 culture supernatant.

Degradation of c-MYC protein. Rh30 cells were serum-fasted for 24 hr and stimulated with 50 ng/ml IGF-I for 4 hr and then labeled with [^{35}S]methionine for 1 hr. The labeled cells were washed with serum-free medium containing 50 ng/ml IGF-I and then incubated further in the presence of IGF-I for up to 60 min in the presence or absence of rapamycin (100 ng/ml). Cells were lysed in

RIPA buffer (2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS in PBS) supplemented with 10 $\mu\text{g/ml}$ concentration each of leupeptin, aprotinin, and soybean trypsin inhibitor; 1 mM sodium orthovanadate; and 1 mM PMSF. Cells then were incubated at 4° for 1 hr with mouse monoclonal antibody against c-MYC (1-9E10) as described above. Immune complexes were absorbed to Protein G PLUS-agarose beads (Santa Cruz Biotechnology), and precipitated proteins were separated in SDS-10% polyacrylamide gels. Gels were dried, and radiolabeled species were visualized by autoradiography.

RNA extraction and Northern blot analysis. Untreated Rh30 cells and those treated with IGF-I or rapamycin were homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA was electrophoretically fractionated in 1.5% agarose/formaldehyde gels and transferred to nylon membranes (Qiagen, Chatsworth, CA). Probes for human c-myc and β -actin were radiolabeled with [α - ^{32}P]dCTP (~50 μCi ; specific activity, 3000 Ci/mmol; Amersham) by using the Random Primers DNA labeling system (Amersham). The labeled probes were purified on Sephadex G-50 nick columns (Pharmacia Biotech). Prehybridization was performed for 1 hr at 68° in 15 ml of QuickHyb solution (Stratagene, La Jolla, CA), followed by hybridization with radiolabeled probes (~ 1×10^7 cpm) for 2 hr at 68°. The blots were washed twice for 10 min each with $2 \times \text{SSC}/0.1\%$ SDS at room temperature and once for 1 hr with $0.2 \times \text{SSC}/0.1\%$ SDS at 68°. The labeled blots were exposed to BioMax film (Kodak) at -70° with an intensifying screen.

Results

Sensitivity to rapamycin. We reported previously that cell lines derived from alveolar RMS, dependent on signaling through the IGF-I receptor, were sensitive to rapamycin inhibition. These studies have been extended to neuroblastoma and glioblastoma cell lines derived from pediatric patients (Table 1). The sensitivity of each cell line to inhibition by rapamycin was examined in complete medium (containing 10% fetal calf serum). As shown, the IC_{50} concentration for Rh30 and Rh1 cells was 0.37 and 4680 ng/ml, respectively, which is in agreement with previously reported results (Dilling *et al.*, 1994). Three of five neuroblastoma lines were also sensitive, with IC_{50} concentrations of 3 ng/ml. The two brain tumor cell lines demonstrated marked differences in sensitivity; SJ-G2 was very sensitive ($\text{IC}_{50} \sim 0.5$ ng/ml), whereas SJ-G3 was completely resistant at $>10,000$ ng/ml. Complete dose-response curves for two histiotype pairs are shown in Fig. 1. For Rh1 cells, there was no significant inhibition of growth at rapamycin concentrations of <1000 ng/ml. Interestingly, SJ-G3 cells were growth stimulated by increasing concentrations of rapamycin. For Rh30 and SJ-G2 cells, rapamycin inhibition of cell growth was reversed by the

TABLE 1
Sensitivity of Cell Lines Derived from Childhood Tumors to Rapamycin

Cell type	Cell line	Rapamycin IC_{50} ng/ml
RMS	Rh1	4680
	Rh30	0.37
Neuroblastoma	Rh30/rapa 10Kc4	1370
	NB-SD	~1
	NB-1643	~1
	NB-EB	2.9
	NB-1691	18
	NB-1382.2	639
Glioblastoma	SJ-G2	0.5
	SJ-G3	$>10,000$

addition of the analog FK506 (Dilling *et al.*, 1994; and data not shown). An additional line, Rh30/rapa10K, which was selected for growth in the continuous presence of 10,000 ng/ml rapamycin, also was tested. Cells were washed extensively, plated, and allowed to attach overnight. Rh30/rapa10K cells then were exposed to rapamycin for 7 days, and the IC_{50} concentration was determined. As shown in Table 1, these cells were ~3700-fold resistant to rapamycin. To determine whether resistance could be caused by rapid catabolism of rapamycin in resistant cells, SJ-G3 glioblastoma and Rh1 RMS cells were exposed to rapamycin continuously for 7 days or under conditions in which drug-containing medium was replaced daily. Cells were equally resistant under either condition, suggesting that resistance was not due to inactivation of rapamycin (data not shown).

Accumulation and retention of rapamycin. To determine whether cellular sensitivity was determined by greater uptake or retention of drug, cells were incubated for up to 1 hr, and drug accumulation was determined during this time course. Alternatively, after accumulation, monolayer cells were washed extensively and incubated in drug-free medium in an incubator for up to 8 hr. Due to the low specific activity

of [^{14}C]rapamycin, these studies were undertaken at high drug concentrations (19 μM). Results are presented in Fig. 2. The glioblastoma cell lines accumulated higher levels of rapamycin at 1 hr (1.7 and 2.8 $\mu g/10^6$ cells for SJ-G2 and SJ-G3, respectively) compared with RMSs (1.2 and 1.4 $\mu g/10^6$ cells for Rh1 and Rh30, respectively). Thus, there was no correlation between drug accumulation over 1 hr and sensitivity to rapamycin. After washing cells, ~70–80% of radio-label was lost from each cell line. Steady-state levels were achieved at 120 min after washout of drug, after which levels of drug associated with cells remained constant for up to 8 hr in RMS cells (data not shown). The steady-state levels ranged from a low of 344 ng/ 10^6 cells (Rh1) to the highest level of 577 ng/ 10^6 cells (SJ-G3, the most resistant cell line). These levels may represent potential capacities for formation of rapamycin/FKBP complexes. However, neither initial drug accumulation nor drug retention correlated with cellular sensitivity to rapamycin.

Expression of mTOR. To determine whether the level of expression correlated with cell sensitivity, we examined expression by Northern blot analysis in histiotype pairs of cell lines that demonstrated large differences in sensitivity to rapamycin. Northern analysis of two RMS and two glioblastoma lines is shown in Fig. 3A. The transcript (~9 kb) was detected in each cell line. After normalization to the *G3PDH* signal, the ratio of transcripts was 1, 1.16, 1.57, and 2.69 for SJ-G2, SJ-G3, Rh1, and Rh30, respectively. To determine whether rapamycin altered levels of mTOR transcripts, Rh1 and Rh30 cells were treated with rapamycin (100 ng/ml) for 48 hr, and RNA extracted from control and treated cells. As shown in Fig. 3B, rapamycin treatment did not alter transcript levels in either Rh1 or Rh30 cells.

Determination of mTOR protein. To determine levels of mTOR in tumor cells, we developed monoclonal antibodies against a synthetic decapeptide (KPQWYRHTFEE), representing the unique amino acid residues from 230–240 in the amino terminus of mTOR (Fig. 4A). As shown in Fig. 4B, the 22C2 clone recognized a single band by Western blot analysis, and immunoreactivity was completely blocked by inclusion of the cognate peptide (peptide 1) but not by a peptide representing residues 920–930 of mTOR (peptide 3; SKSSQDSSDY). Immunoprecipitation/Western blot analysis detected only a single band, and immunoprecipitation was blocked only by the addition of peptide 1 (Fig. 4C). Furthermore, using an FKBP affinity column, 22C2 recognized a protein bound to the column only in the presence of rapamycin (Fig. 4D). These results indicate the specificity of 22C2 for mTOR. Western blot analysis of whole-cell extracts from each of the cell lines is shown in Fig. 5. Each cell line expressed detectable levels of mTOR, with slightly higher levels in SJ-G3, Rh1, NB-1691, and NB-EB cells, although the differential between the lowest (Rh30) and highest (SJ-G3) was <2-fold. The levels of mTOR were not increased in Rh30/rapa10K cells that were selected over time for growth in 10,000 ng/ml rapamycin.

Inhibition of ribosomal $p70^{S6k}$ activity. After immunoprecipitation, $p70^{S6k}$ activity was measured by transfer of [γ - ^{32}P]ATP to a specific substrate (AKRRRLSSLRA). Levels of $p70^{S6k}$ activity in untreated Rh1, Rh30, SJ-G2, and SJ-G3 cells were 11.75, 12.2, 41.8, and 14.06 pmol of phosphate transferred over 30 min to the substrate in extracts of 2×10^6 cells. Thus, endogenous levels of enzyme activity did not

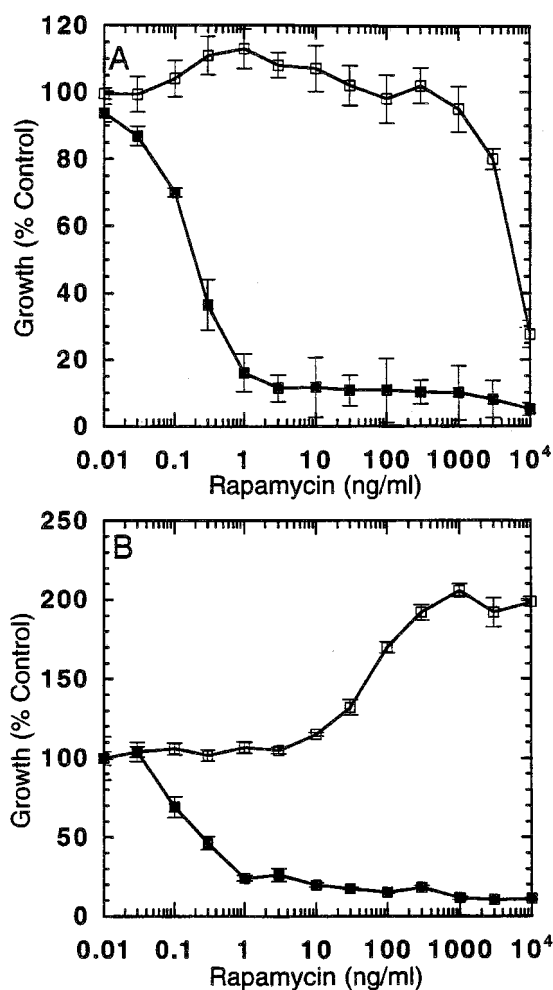


Fig. 1. Sensitivity of cell lines derived from childhood RMS and glioblastoma to rapamycin. Cells were exposed to rapamycin at increasing concentrations for 7 days and cell numbers were enumerated as described in Materials and Methods. *Top*, RMSs: □, Rh1; ■, Rh30. *Bottom*, glioblastomas: □, SJ-G3; ■, SJ-G2. Values are mean \pm standard deviation from three experiments.

seem to correlate with cellular sensitivity to rapamycin. In Rh30 cells, inhibition of p70^{S6k} activity was concentration dependent, with ~60% inhibition after exposure to 1 ng/ml rapamycin (Fig. 6A). Exposure to 100 ng/ml drug resulted in ~90% inhibition; thus, the degree of inhibition of p70^{S6k} was in good agreement with inhibition of cell growth caused by the same concentrations of rapamycin. Inhibition of activity in SJ-G2, SJ-G3, and Rh1 cells after exposure to 100 or 1000 ng/ml rapamycin is shown in Fig. 6A. In the brain tumor lines, >90% inhibition of enzyme activity occurred after exposure to 100 ng/ml rapamycin, regardless of their sensitivity to growth inhibition by this agent. In contrast, p70^{S6k} activity was reduced by ~70% at 100 ng/ml and ~85% in Rh1 cells after exposure to 1000 ng/ml. However, growth of this line was not inhibited significantly, even at the higher concentration of rapamycin. We next examined the residual activity of p70^{S6k} in Rh30/rap10K cells, growing in the presence of 10,000 ng/ml rapamycin. There was no detectable activity in extracts from these cells (data not shown). Because rapamycin was retained at high levels within cells after extensive washing, we were interested in determining whether a short exposure to this agent would result in a very prolonged inhibition of mTOR function. To examine this, RMS cells, treated as above, were exposed to rapamycin (100 ng/ml) for 15 min. Cells were washed extensively and incubated in serum-free N2E medium for up to 72 hr. The ability of IGF-I to stimulate p70^{S6k} activity was examined after 48 and 72 hr and compared with control cells grown under the same conditions. As shown in Fig. 6B, activation of p70^{S6k} by IGF-I was still completely inhibited in Rh1 cells and remained significantly inhibited in Rh30 cells at 72 hr after the removal of rapamycin. Thus, although rapamycin is quite unstable under conditions of cell culture (Houghton PJ and Germain GS, unpublished observations), once bound within cells (presumably to FKBP12), the complex seems to be highly stable, causing prolonged inhibition of mTOR signaling.

Effect of rapamycin on PHAS-I phosphorylation. Rapamycin rapidly causes dephosphorylation of PHAS-I, resulting in its association with eIF4E and suppression of

translation of specific mRNA species. We were interested, therefore, in determining whether rapamycin had a differential effect in cells with markedly different sensitivities to the cytostatic actions of this drug. PHAS-I phosphorylation was examined in serum-fasted Rh30 and Rh1 cells at varying times after the addition of serum in the absence or presence of rapamycin (100 ng/ml) (Fig. 7). In serum-fasted cells, PHAS-I was predominantly hypophosphorylated (α and β isoforms). On serum stimulation, there was a rapid conversion to the γ isoform, which was equally inhibited by rapamycin in both cell lines. As a functional assay for PHAS-I, we examined its association with eIF4E. Cells were serum-fasted for 24 hr and then stimulated with IGF-I in the absence or presence of rapamycin. After 4 or 8 hr, cells were harvested, and lysates were prepared. Samples were incubated with Sepharose-bound ⁷M-GTP to trap eIF4E and any associated PHAS-I. Western blot analysis of both proteins in lysates from control and rapamycin-treated cells is shown in Fig. 7C; eIF4E levels remained fairly constant for all samples. In serum-starved cells, PHAS-I was associated with eIF4E. Stimulation with IGF-I significantly decreased association at both 4 and 8 hr. In contrast, rapamycin treatment prevented the IGF-I-induced dissociation of PHAS-I from eIF4E. These results are consistent with changes in phosphorylation of PHAS-I.

Induction of c-MYC by serum or IGF-I. The mTOR/PHAS-I pathway is considered to control the translation of specific mRNA species, some of which are involved in cell cycle control (e.g., ornithine decarboxylase, cyclin D1, c-MYC). Because rapamycin equally inhibited p70^{S6k} activity and PHAS-I phosphorylation in Rh1 and Rh30 cells, it was of interest to determine whether cells that demonstrated different intrinsic levels of sensitivity to rapamycin could be distinguished at the level of induction of c-MYC. Rh1 and Rh30 cells were serum-fasted overnight and then serum-stimulated. Cell lysates were prepared, and c-MYC protein was detected by immunoblotting. As shown in Fig. 8, rapamycin completely inhibited c-MYC induction by serum in Rh30 cells (Fig. 8A). Similar results were obtained when IGF-I was used instead of serum (data not shown). In contrast, rapamycin

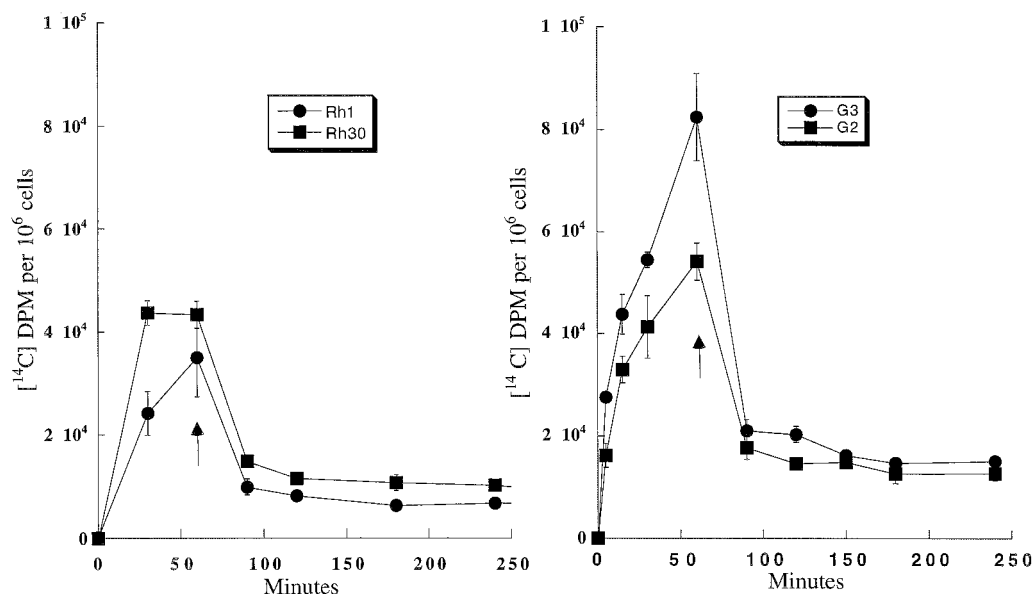


Fig. 2. Accumulation and retention of [¹⁴C]rapamycin in RMS (left) and glioblastoma (right) cell lines. Cells were incubated with rapamycin (19 μ M, 23°) for up to 60 min. Monolayers were washed extensively and then incubated at 37° (5% CO₂) for up to 3 hr. At appropriate times, medium was aspirated and radioactivity was determined as described in Materials and Methods. Values are mean \pm standard deviation from three experiments.

had essentially no effect on induction of this protein in rapamycin-resistant Rh1 cells (Fig. 8B). We next determined whether the induction of c-MYC by IGF-I was due to increased transcription, translation, or stabilization of protein. IGF-I (or serum) in the absence or presence of rapamycin (100 ng/ml) for up to 2 hr had no significant effect of levels of *c-myc* mRNA normalized to β -actin (Fig. 8C). In addition, rapamycin did not affect the relative amount of [35 S]methionine present in the immunoprecipitated c-MYC during a 60-min pulse chase (data not shown), suggesting that rapamycin did not affect the degradation of c-MYC. These data support induction of c-MYC at a translational level through an mTOR-dependent pathway. To further examine the relationship between rapamycin resistance and c-MYC expression, we examined the ability of rapamycin to inhibit serum-induced induction of c-MYC in Rh30/rapa10K cells with acquired resistance to rapamycin. Rh30/rapa10K cells, which

are grown in medium containing rapamycin (10,000 ng/ml), were serum-fasted (no rapamycin) overnight and exposed to rapamycin (100 ng/ml) 15 min before serum stimulation, as described above. c-MYC induction with or without rapamycin

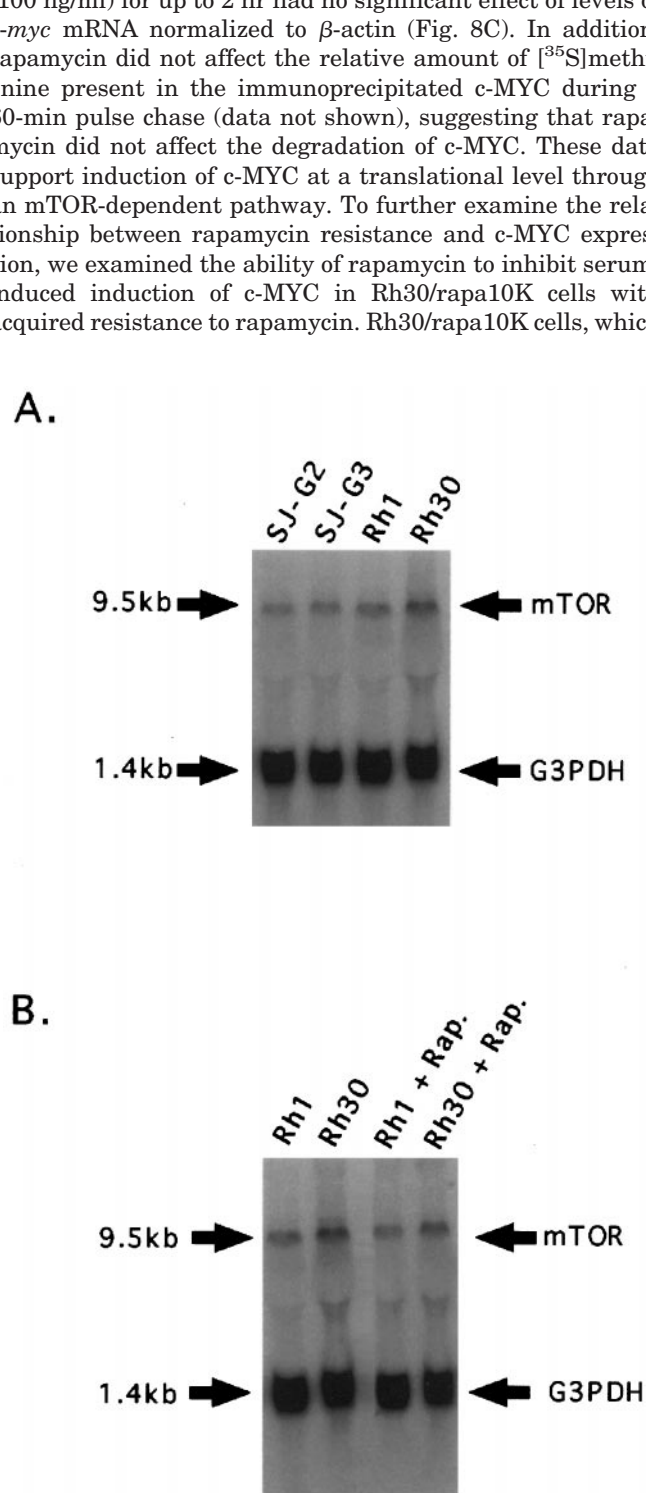


Fig. 3. Expression of *mTOR* in glioblastoma and RMS cell lines. A, Northern blot analysis of total RNA (20 μ g) extracted from rapamycin-sensitive (SJ-G2, Rh30) and -resistant (SJ-G3, Rh1) cells. B, Northern blot analysis of total RNA (20 μ g) from control Rh1 and Rh30 cells or cells treated with rapamycin (*Rap.*) (100 ng/ml, 48 hr). Expression of the housekeeping gene *G3PDH* was used as a control.

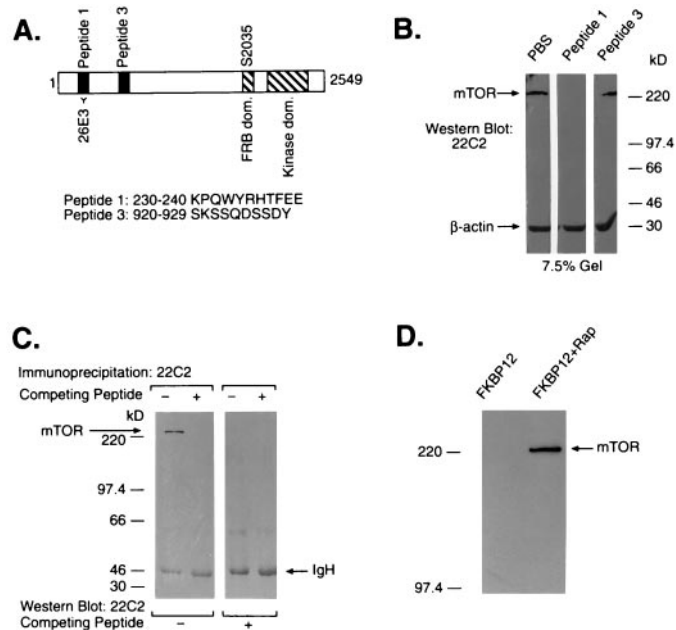


Fig. 4. Characterization of monoclonal antibody 22C2. A, Schematic of mTOR showing FKBP/rapamycin binding and kinase domains and sequences against which antibodies were raised. B, Western analysis of mTOR using monoclonal antibody 22C2 and selective competition with peptide 1. C, Immunoprecipitation/Western blot analysis of Rh30 cell extracts with and without peptide 1. D, Western blot showing reactivity of 22C2 with protein eluted from an FKBP-affinity column after binding in the absence (*FKBP12*) or presence (*FKBP12+Rap*) of rapamycin.

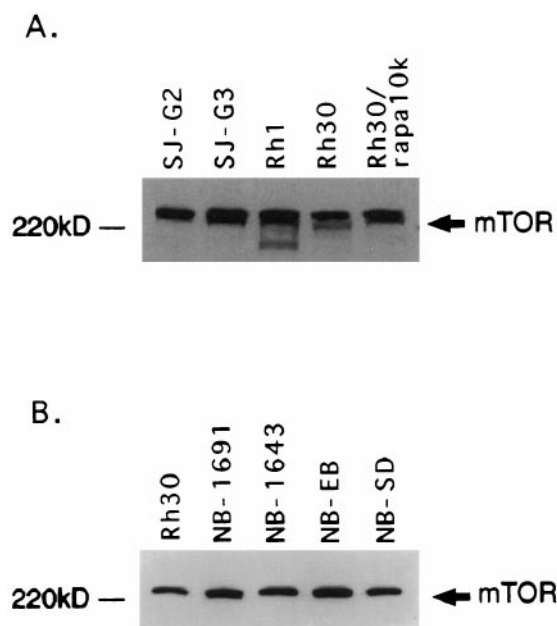


Fig. 5. Levels of mTOR in cells with different intrinsic sensitivities to rapamycin. A, Glioblastoma and RMS cell lines. B, Neuroblastomas compared with Rh30. Whole-cell lysates (80 mg of protein) were prepared from rapamycin-sensitive (Rh30, SJ-G2, NB-1643, NB-1691, NB-EB, NB-SD), intrinsically resistant (Rh1, SJ-G3), and Rh30/rapa10K cells (selected for acquired resistance). Lysates were analyzed by immunoblotting using monoclonal antibody 22C2.

was examined by Western blot analysis after 5 hr. Shown in Fig. 9A is a Western blot of lysates from serum-fasted Rh30 and Rh30rapa10K cells probed for c-MYC and a housekeeping gene product, β -tubulin. The data indicate that the basal level of c-MYC in Rh30rapa10K cells was increased ≥ 10 -fold over parental Rh30 cells. Furthermore, after the addition of serum, there was no further increase in c-MYC in Rh30/rapa10K cells, and rapamycin did not decrease c-MYC levels during this time frame (Fig. 9B).

Discussion

The macrolide antibiotic rapamycin represents a novel class of agent that inhibits signal transduction (reviewed in Schreiber, 1991; Kunz and Hall, 1993). Compounds of this class hence have the potential to inhibit growth of tumor cells in which the mitogenic stimulus is transduced through the rapamycin target. We previously reported that RMS cells dependent for growth on signals mediated by IGF-I receptor were growth inhibited at low concentrations of rapamycin.

Because the growth of neuroblastomas (El-Badry *et al.*, 1989) and some glioblastomas also is dependent on IGF signaling, we examined the sensitivity to rapamycin of several additional cell lines derived from childhood tumors. The results suggest that some neuroblastoma and glioblastoma lines also are sensitive to rapamycin; hence, this agent may have more general cytostatic activity against pediatric tumors. Within each cell type (RMS, neuroblastoma, and glioblastoma), there were both sensitive and intrinsically resistant cell lines. For the RMS and glioma lines, pairs that differed by >1000 -fold in sensitivity to rapamycin were studied further to determine whether there were differences in drug accumulation and retention, the levels of mTOR, or the effect of rapamycin on downstream targets (p70^{S6k} activity or PHAS-I) between cells with markedly different intrinsic sensitivities to rapamycin.

Accumulation of rapamycin was similar in sensitive and resistant cells. Although these studies were undertaken at very high concentrations of rapamycin, due to the low specific activity of the available radiolabeled drug, the studies are informative. Rapamycin accumulated to a very high level in glioblastoma cell lines, and with one exception (Rh30), it had not achieved steady state after 1-hr exposure to drug. The highest accumulation was in SJ-G3 cells, which demonstrate the highest level of intrinsic resistance to rapamycin. Of note was that after washing cells and incubating them for up to 8 hr, there was a significant retention of drug within both sensitive and resistant cells. This may represent tight binding to FKBP-12 (or other immunophilins) with which rapamycin complexes. However, initial uptake and the level of retention did not correlate with responsiveness to rapamycin.

Expression of mTOR was quantified by Northern blot analysis of total RNA. Results demonstrate a 3-fold range of expression. Highest and lowest expressions were detected in the two cell lines (Rh30 and SJ-G2) most sensitive to rapamycin. Thus, levels of expression, at the RNA level, did not correlate with drug sensitivity. Furthermore, in Rh1 and Rh30 cells, treatment with rapamycin (100 ng/ml, 48 hr) did not alter the level of transcripts from that determined in control cells. To detect and quantify the levels of mTOR in these cells, we developed monoclonal antibodies against a synthetic peptide representing residues 230–240. Characterization showed this reagent (22C2) to be specific for mTOR. Using this antibody, levels of mTOR were quantified by immunoblotting in each of the cell lines and were found to be quite similar (<2 -fold variation). Thus, levels of mTOR did not correlate with sensitivity of cells to rapamycin.

We next examined the relationship between cell sensitivity and inhibition of p70^{S6k} activity in RMS and glioblastoma cell line pairs. Activation of ribosomal p70^{S6k} by growth factors is rapamycin sensitive, although mTOR may not directly activate p70^{S6k}. The regulation of p70^{S6k} activation is complex (Pearson *et al.*, 1995; Dennis *et al.*, 1996; von Manthey *et al.*, 1996, 1997; Pullen *et al.*, 1998). Activation involves phosphorylation of T229, T389, S404, and S411. The principal site of rapamycin-induced dephosphorylation is T389. Consequently, we were interested to determine whether inhibition of p70^{S6k} activity correlated with growth inhibition of tumor cells by rapamycin. Furthermore, a failure to inhibit p70^{S6k} activity would be a good indication that resistance to rapamycin may be a consequence of mutation of mTOR that decreased binding of the rapamycin/FKBP com-

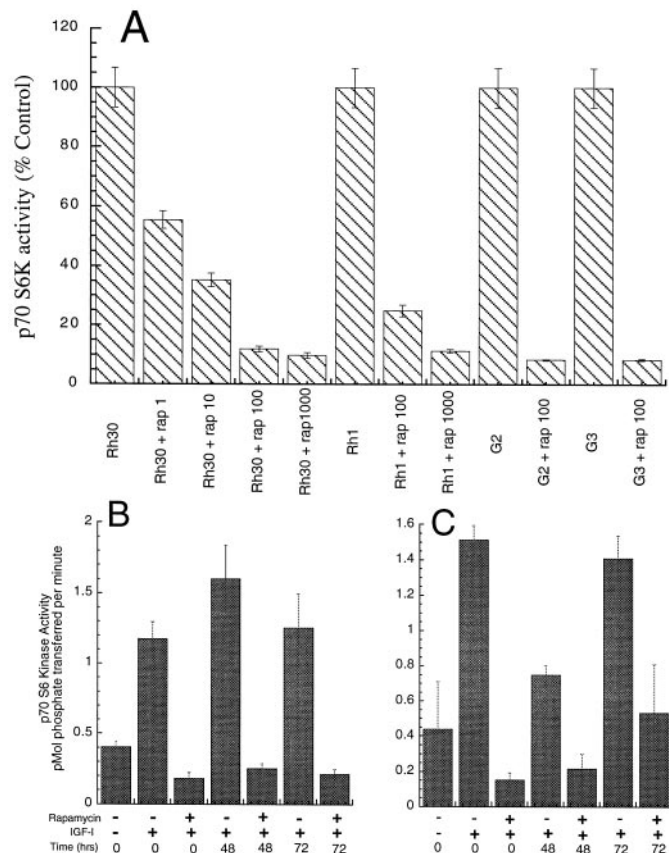


Fig. 6. Inhibition and recovery of ribosomal p70^{S6k} activation by IGF-I in rapamycin-treated cell lines. A, Concentration-dependent inhibition of enzyme activity in Rh30 cells incubated 1 hr with increasing concentrations of rapamycin (*rap*). For Rh1, SJ-G2, and SJ-G3 cells, inhibition of p70^{S6k} activity was determined after 1-hr exposure to 100 or 1000 ng/ml rapamycin. Bars, range for duplicate determinations and are representative experiments. Values are normalized to activity in equivalent protein in lysates from untreated controls (values are mean \pm standard deviation from three experiments). B, Rh1 and Rh30 cells were exposed to rapamycin (100 ng/ml) for 15 min. Cells were washed extensively and then cultured for 48 or 72 hr with or without rapamycin in serum-free N2E medium. Cells were washed and stimulated with IGF-I, and p70^{S6k} activity determined as above (values are mean \pm standard deviation from three experiments).

plex or inability to bind to a mutant FKBP-12. The activity of $p70^{S6k}$ demonstrated a 3-fold variation (11.75–41.8 pmol of phosphate transferred/30 min/ 2×10^6 cells) in extracts from the cell lines examined. The highest activity was determined in SJ-G2 cells that demonstrated high sensitivity to rapamycin inhibition. In rapamycin-treated cells, inhibition of $p70^{S6k}$ activity was rapid, being maximal within 1 hr. In Rh30 cells, there was a good correlation; IC_{50} concentrations for both inhibition of $p70^{S6k}$ activity and growth was <1 ng/ml. However, residual activity ($\sim 12\%$ control) was deter-

mined at concentrations up to 1000 ng/ml. Of particular interest was that inhibition of $p70^{S6k}$ activity was similar in both brain tumor lines, only one of which is sensitive to rapamycin. Rapamycin also inhibited $p70^{S6k}$ activity by $>85\%$ in Rh1 cells at concentrations that were not growth inhibitory. Collectively, these data show that inhibition of $p70^{S6k}$ activity does not correlate with inhibition of growth by rapamycin. Our data indicate that after a relatively brief exposure (15 min), cells retain rapamycin for prolonged periods. We therefore examined the rate at which the mTOR

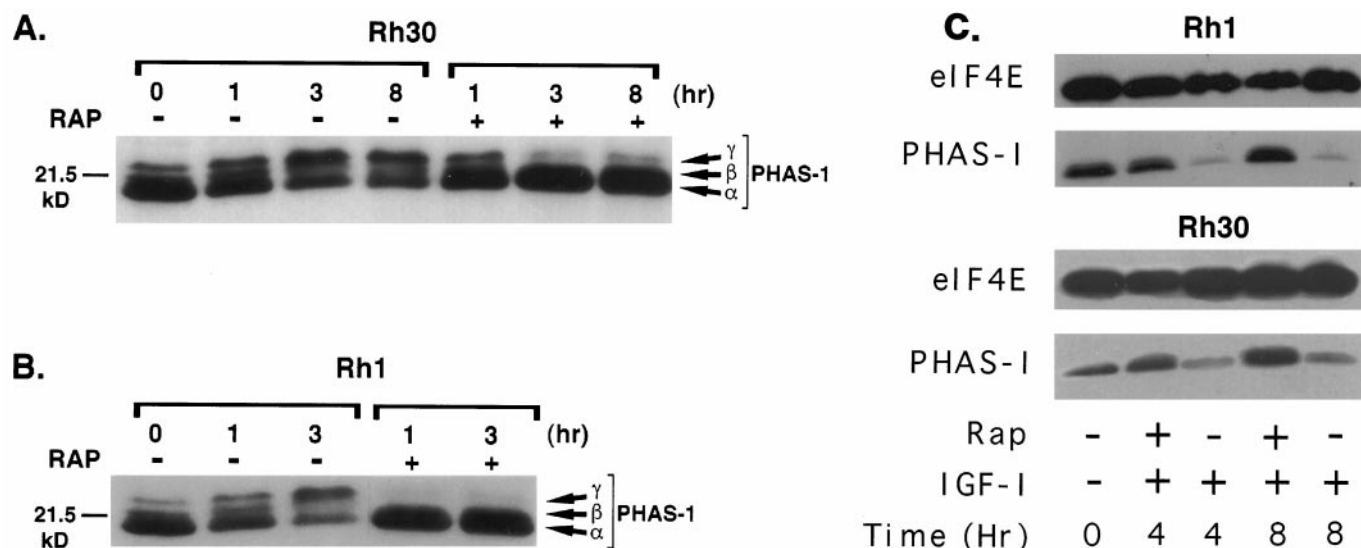


Fig. 7. Rapamycin inhibits serum-induced phosphorylation of PHAS-I in cells regardless of their intrinsic sensitivity to growth inhibition by rapamycin. Rh1 and Rh30 cells were serum-fasted (24 hr) and then stimulated by the addition of serum. Whole-cell lysates (40 μ g) were prepared just before serum addition (time 0) and for up to 8 hr after the addition of serum in the absence or presence of rapamycin (RAP) (100 ng/ml). A, Rh30. B, Rh1. C, Binding of PHAS-I to eIF4E. RMS cells were serum-fasted for 24 hr and then stimulated with IGF-I in the absence or presence of rapamycin (Rap) (100 ng/ml). eIF4E from lysates was bound to 32 P-GTP as described in Materials and Methods. Samples were analyzed by SDS-PAGE and transferred to membranes, and proteins were detected using antibodies against eIF4E and PHAS-I.

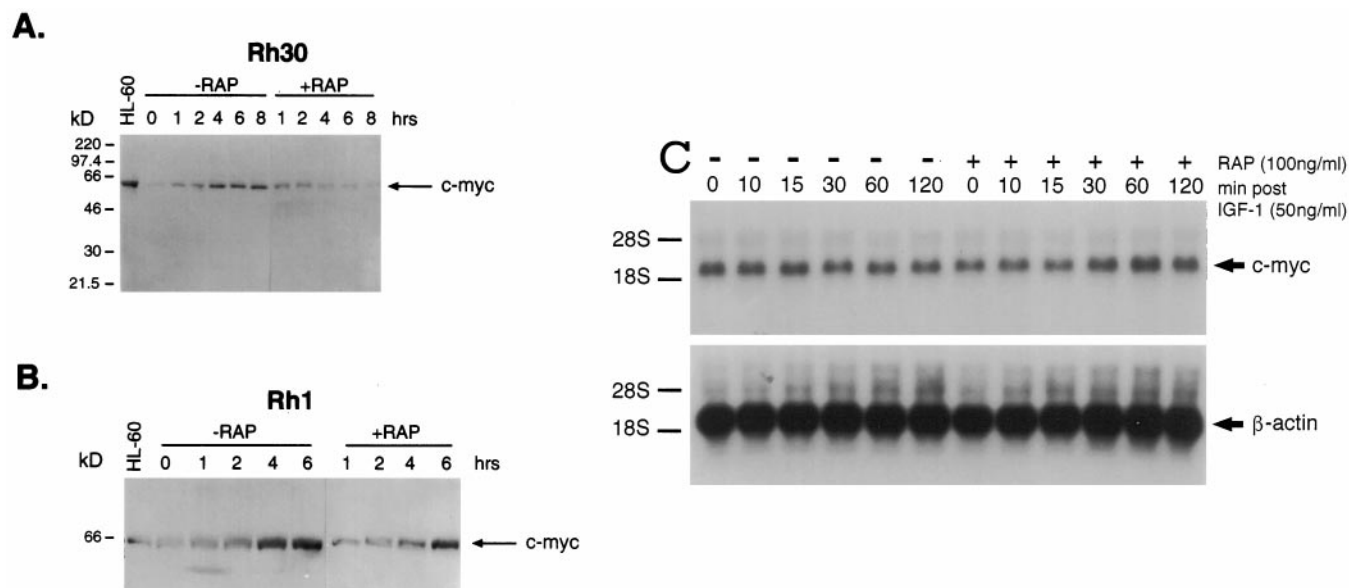


Fig. 8. Rapamycin inhibits induction of c-MYC only in Rh30 cells intrinsically sensitive to rapamycin (RAP). Cells were serum-fasted and stimulated by the addition of serum, as described in Fig. 7. Whole-cell lysates from equal numbers of cells were prepared as described in Materials and Methods and analyzed by SDS-PAGE. c-MYC was detected by immunoblotting using 1-9E10 monoclonal antibody and ECL as the detection system. Time 0, just before the addition of serum. A, Rh30 cells. B, Rh1 cells. C, Northern blot analysis of c-myc. Serum-fasted Rh30 cells were stimulated by IGF-I in the presence or absence of rapamycin (100 ng/ml) for the indicated times after serum starvation. Total RNA was isolated and transferred to a nylon membrane. The membrane was probed with radiolabeled c-myc cDNA, and c-myc mRNA was detected by autoradiography. The blots were stripped and rehybridized with a human β -actin probe for normalization.

pathway recovered in Rh1 and Rh30 cells after this brief period of exposure to rapamycin. The results show that the ability of IGF-I to activate p70^{S6k} was inhibited for very prolonged periods. There was no recovery over 72 hr in Rh1 cells and only slight recovery in Rh30 cells during the same time period. Rapamycin is relatively unstable under tissue culture conditions ($t_{1/2} \sim 12$ hr using bioassay to determine potency; Houghton PJ and Germain GS, unpublished observations). Thus, prolonged inhibition of p70^{S6k} is probably due to the intracellular stability of rapamycin when complexed to FKBP-12.

We examined next whether rapamycin treatment inhibited serum-induced phosphorylation of PHAS-I in the pair of RMS cell lines. PHAS-I was rapidly phosphorylated in both Rh1 and Rh30 cells in response to serum but was equally inhibited by rapamycin in both cell lines. Thus, the intrinsic sensitivity of these cells to rapamycin could not be distinguished by a differential effect on inhibiting PHAS-I phosphorylation. In Rh1 cells that are intrinsically resistant to the cytostatic action of rapamycin, this agent inhibited p70^{S6k} activity and inhibited phosphorylation of PHAS-I, in a manner that was indistinguishable from that in the rapamycin-sensitive cell line Rh30. We next determined whether alterations in PHAS-I phosphorylation correlated with binding to eIF4E. IGF-I stimulation led to a significant decrease in PHAS-I association with eIF4E, consistent with increased phosphorylation. In the presence of rapamycin, PHAS-I remained bound to eIF4E after stimulation of Rh1 or Rh30 cells with IGF-I. Thus, rapamycin had a similar effect on either cell line regardless of the sensitivity to this agent.

We therefore were interested in determining whether there was a differential effect of rapamycin on induction of translation of one of the mRNA species considered to be dependent on the mTOR/PHAS-I pathway. In serum-fasted

Rh1 and Rh30 cells, the addition of serum causes rapid increase in c-MYC protein, as determined by immunoblotting. The addition of serum did not induce transcription or seem to stabilize c-MYC in these cells (data not shown). Significantly, rapamycin completely inhibited induction of c-MYC in Rh30 cells but had no detectable effect on serum induction of c-MYC in Rh1 cells. Thus, despite its actions on p70^{S6k}, PHAS-I phosphorylation, or association of PHAS-I with eIF4E, rapamycin was unable to inhibit induction of c-MYC protein in Rh1 cells. These results suggest that in Rh1 cells, translation of *c-myc* mRNA may be under control of a pathway distinct from PHAS-I or p70^{S6k}. Of particular interest are the results from Rh30/rapa10K cells that were selected for acquired resistance to rapamycin. In these cells, the basal level of c-MYC, after serum starvation, was ≥ 10 -fold greater than that in Rh30 cells. Furthermore, in Rh30/rapa10K cells, there was no stimulation of c-MYC levels by serum; consequently, we were unable to demonstrate any activity of rapamycin. Our results thus implicate c-MYC overexpression in conferring acquired resistance to rapamycin. However, further studies on additional clones selected for rapamycin resistance will be required to confirm or refute this conjecture.

Taken together, these data indicate that intrinsic resistance to rapamycin is not a consequence of mutations of FKBP that prevent rapamycin binding or of mTOR that prevent binding and inactivation by the rapamycin/FKBP complex. Ribosomal p70^{S6k} activity and PHAS-I phosphorylation were inhibited, regardless of cell sensitivity to the growth-inhibitory effect of rapamycin. These results could indicate that p70^{S6k} is redundant in cells intrinsically resistant to rapamycin (Rh1 and SJ-G3) but not in sensitive cells (Rh30 and SJ-G2). Results demonstrating that c-MYC induction is refractory to the action of rapamycin suggest that in Rh1 cells, control of translation may be, at least partially, independent of the mTOR/PHAS-I pathway favoring the hypothesis of redundancy in cells that demonstrate a high level of intrinsic resistance to rapamycin. Our finding that c-MYC is overexpressed in a cell line with acquired resistance to rapamycin further suggests that the role of c-MYC is important in determining the cellular response to this agent. Data presented indicate that although inhibition of p70^{S6k} activity or PHAS-I dephosphorylation may be surrogates for rapamycin activity, such measurements are not informative about resulting growth inhibition. However, decreases in c-MYC levels in treated tumors may predict cellular response. This may be important as rapamycin, and analogues are being developed as anticancer agents, having demonstrated significant cytostatic activity in human tumor xenograft models (Senderowicz *et al.*, 1995; and Houghton PJ, unpublished observations). While this article was being prepared for publication, similar results demonstrating mTOR control of *c-myc* induction were reported by West *et al.* (1998).

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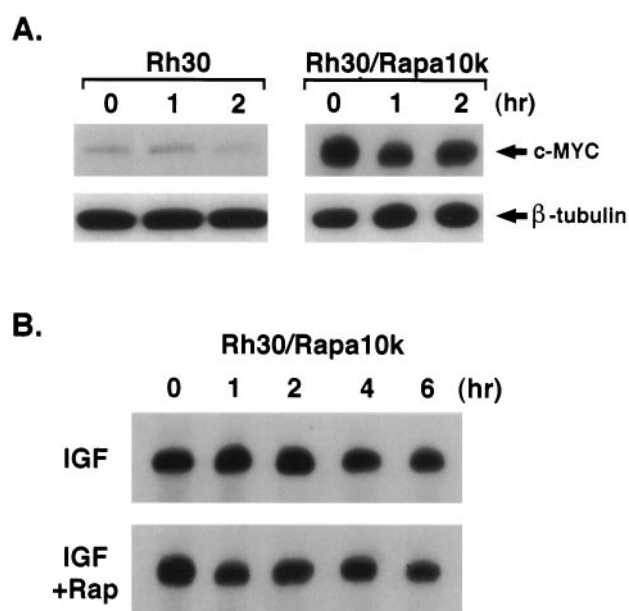


Fig. 9. A, Quantification of c-MYC levels in Rh30 and Rh30/Rapa10K cells. Western analysis of c-MYC normalized to β -tubulin in parental and resistant cell lines. B, Failure of IGF-I to induce c-MYC in Rh30/rapa10K cells after serum starvation. Rh30/rapa10K cells were serum-fasted overnight and stimulated as described in Fig. 7. c-MYC was determined by Western blot analysis at various times after serum stimulation in the absence or presence of rapamycin (Rap) (100 ng/ml).

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