Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade

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Abstract

Mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin and CCI-779, have shown preclinical potential as therapy for multiple myeloma. By inhibiting expression of cell cycle proteins, these agents induce G_1 arrest. However, by also inhibiting an mTOR-dependent serine phosphorylation of insulin receptor substrate-1 (IRS-1), they may enhance insulin-like growth factor-I (IGF-I) signaling and downstream phosphatidylinositol 3-kinase (PI3K)/AKT activation. This may be a particular problem in multiple myeloma where IGF-I-induced activation of AKT is an important antiapoptotic cascade. We, therefore, studied AKT activation in multiple myeloma cells treated with mTOR inhibitors. Rapamycin enhanced basal AKT activity, AKT phosphorylation, and PI3K activity in multiple myeloma cells and prolonged activation of AKT induced by exogenous IGF-I. CCI-779, used in a xenograft model, also resulted in multiple myeloma cell AKT activation *in vivo*. Blockade of IGF-I receptor function prevented rapamycin's activation of AKT. Furthermore, rapamycin prevented serine phosphorylation of IRS-1, enhanced IRS-1 association with IGF-I receptors, and prevented IRS-1 degradation. Although similarly blocking IRS-1 degradation, proteasome inhibitors did not activate AKT. Thus, mTOR inhibitors activate PI3-K/AKT in multiple myeloma cells; activation depends on basal IGF-R signaling; and enhanced IRS-1/IGF-I receptor interactions secondary to inhibited IRS-1 serine phosphorylation may play a role in activation of the cascade. In cotreatment experiments, rapamycin inhibited myeloma cell apoptosis induced by PS-341. These results provide a caveat for future use of mTOR inhibitors in myeloma patients if they are to be combined with apoptosis-inducing agents. [Mol Cancer Ther 2005;4(10):1533–40]

Introduction

Preclinical studies suggest that the mammalian target of rapamycin (mTOR) inhibitors rapamycin and CCI-779 have significant potential in multiple myeloma (1–3). These drugs prevent mTOR-dependent phosphorylation of the p70S6-kinase (p70) and 4E-BP1 (4), resulting in a decreased expression of cyclins and c-*myc* (5, 6), increased expression of the p27 cyclin-dependent kinase inhibitor (7), and subsequent G_1 arrest (8). Myeloma cells with PTEN (2) or RAS (3) mutations are particularly sensitive to mTOR inhibitors.

One additional downstream target of mTOR is the insulin receptor substrate-1 (IRS-1), a key adapter transmitting signals from activated insulin/insulin-like growth factor-I (IGF-I) receptors. An mTOR-dependent serine phosphorylation of IRS-1 results in its dissociation from IGF-I receptors, redistribution from low-density microsomes to cytosol, and proteasomal degradation (9–11) with subsequent down-regulation of insulin or IGF-I signaling. This presents a potential disadvantage with the use of mTOR inhibitors, because prevention of IRS-1 serine phosphorylation might result in enhanced signaling downstream. This could be particularly problematic in myeloma, where IGF-I-induced activation of AKT is such a key pathway for maintaining myeloma cell viability (12, 13). In fact, a recent study by Mitsiades et al. (14) suggests that IGF-I is a key mediator of serum for stimulation of proliferation and survival of multiple myeloma cells *in vitro* or *in vivo*. Thus, although clinical use of mTOR inhibitors might induce G_1 arrest, by further enhancing IGF-I-induced activation, an antiapoptotic effect might ensue. To address this issue, we initiated the current investigation, testing whether mTOR inhibitors affect AKT activity in multiple myeloma cells.

Materials and Methods

Cell Lines and Reagents

OPM-2, 8226, MM1.S, and HS-Sultan cell lines were purchased from the American Type Culture Collection

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(Rockville, MD) and maintained in culture as described previously (1–3). The IRS-1-expressing plasmid was a gift from Dr. R.A. Roth (Stanford University) and described previously (15). The anti-IGF-R blocking antibody was purchased from Oncogene Science (Uniondale, NY). The antibody specific for IRS-1 phosphorylated on Ser^{312} (human)/Ser^{307} (murine) was purchased from Upstate Biotechnology (Lake Placid, NY). All other antibodies used in immunoprecipitation or immunoblot assays were purchased from Cell Signaling, Inc. (Beverly, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). Rapamycin was purchased from Calbiochem (La Jolla, CA), dissolved in ethanol at 1 mmol/L, and stored at −20°C. CCI-779, a gift of Wyeth-Ayerst (Pearl River, NY), was also dissolved in ethanol at 1 mmol/L and stored similarly. PS-341 was provided by Millennium Pharmaceuticals (Cambridge, MA), diluted in DMSO at 10 mmol/L, and stored at −20°C as previously described (16). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Use of CCI-779 *In vivo*

Six nonobese diabetic/severe combined immunodeficient mice were each challenged with 3×10^7 OPM-2 cells admixed with matrigel by s.c. injection. When tumor size reached 200 mm^3, three of the mice were randomly selected to receive five daily i.p. injections of CCI-779 used at 20 mg/kg, whereas the other three received vehicle alone. Eighteen hours after the last injection, mice were sacrificed, tumors were removed, and protein was extracted and pooled for immunoblot assay.

AKT Kinase Assay

The assay used a nonradioactive kit purchased from New England Biolabs (Beverly, MA). AKT was first immunoprecipitated from cell extracts and then incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. AKT-dependent GSK-3 phosphorylation was then measured by immunoblotting using a phospho-GSK-3 antibody that recognizes GSK-3 when phosphorylated.

Phosphatidylinositol 3-Kinase Assay

As previously described (17), protein was extracted in lysis buffer and phosphatidylinositol 3-kinase (PI3K) activity was immunoprecipitated by anti-p85/protein A-agarose. After exhaustively washing the immunoprecipitates, the PI3K reaction was run in a mixture containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 20 mmol/L $MgCl_2$, 0.2 mmol/L EGTA, 20 μg of phosphatidyl-4-monophosphate as substrate, 10 μmol/L ATP, and 10 μCi of (γ-^{32}P) ATP. After proceeding for 15 to 30 minutes, the reaction was terminated and lipids extracted in chloroform/methanol/HCl (100:200:2). The organic phase was collected, dried and redissolved in chloroform/methanol (1:1), and spotted on TLC plates. The plates were developed with chloroform/methanol/H_2O/NH_4OH (43:38:7:5), dried, and exposed to film. The location of PI(3,4)P was determined by comparison with standards in iodine-stained TLC plates.

IGF-R Blocking Experiments

Myeloma cells at 5×10^5/mL were incubated with blocking anti-IGF-R antibody (Oncogene Sciences) or control antibody (identical isotype) at 1 μg/mL for 1 hour. Cells were then either treated with or without rapamycin at 10 nmol/L for an additional 3 hours, after which AKT was immunoprecipitated for an AKT kinase assay. To confirm IGF-R blockade, we assayed tyrosine phosphorylation of the IGF-R. After 1 hour of incubation of cells with the blocking anti-IGF-R antibody or control antibody, the IGF-R was immunoprecipitated with a different antibody (Santa Cruz Biotechnology) and the immunoprecipitate was immunoblotted with an anti-phosphotyrosine antibody.

Isolation of Primary Myeloma Cells

As described previously (17), bone marrow cells were first separated by Ficoll-Hypaque density centrifugation and plasma cells were then isolated on an immunoabsorption column using biotinylated anti-CD38 antibody. Separated cells consisted of >98% plasma cells.

Assessment of Endogenous IRS-1 Function

Endogenous IRS-1 was assayed as previously described (12). Briefly, after cell lysis, IRS-1 was immunoprecipitated with an antibody obtained from Upstate Biotechnology and subsequently bound to protein A–coupled beads. Eluted proteins were electrophoresed in 10% SDS-PAGE and immunoblotted with antibodies to detect IRS-1 Ser^{312} phosphorylation, IRS-1 tyrosine phosphorylation, and IRS-1 interaction with IGF-R.

Apoptosis Assay

Apoptosis was identified by neo expression of membrane Annexin V as previously described (3).

Use of Mutant IRS-1

Wild-type human IRS-1, subcloned in pcDNA3, was a gift of Dr. R.A. Roth. Ser^{312} was mutated to alanine with the QuikChange XL mutagenesis kit using the sense primer 5′-GCATCACCGCCACCGCCCCGGCCAGCA-3′ and the antisense primer 5′-TGCTGGCCGGGGCGGTGGCGGTGATGC-3′. The plasmids and mutant sites were verified by sequencing. Both wild-type and mutant IRS-1 were transfected into myeloma cells by electroporation as previously described (18), using 250 V and a 25-millisecond pulse. Viabilities of multiple myeloma cells after electroporation averaged 605 to 75% with transduction efficiencies of 15% to 25%.

Results

mTOR Inhibitors Enhance Activation of AKT in Myeloma Cells

To assess effects of mTOR inhibitors on AKT activity, four separate myeloma cell lines were treated with increasing concentrations of rapamycin for 4 hours, AKT was immunoprecipitated, and its activity tested by its ability to induce phosphorylation of the GSK-3 AKT substrate. Two of these lines contain PTEN mutations (OPM-2 and HS-Sultan) and two express wild-type PTEN (8226 and MM1.S). As shown in Fig. 1A, rapamycin induced an increase in AKT activity in all four multiple myeloma lines above its basal level even when concentrations as low as 0.1 nmol/L were used. This experiment was repeated two additional times. By densitometric

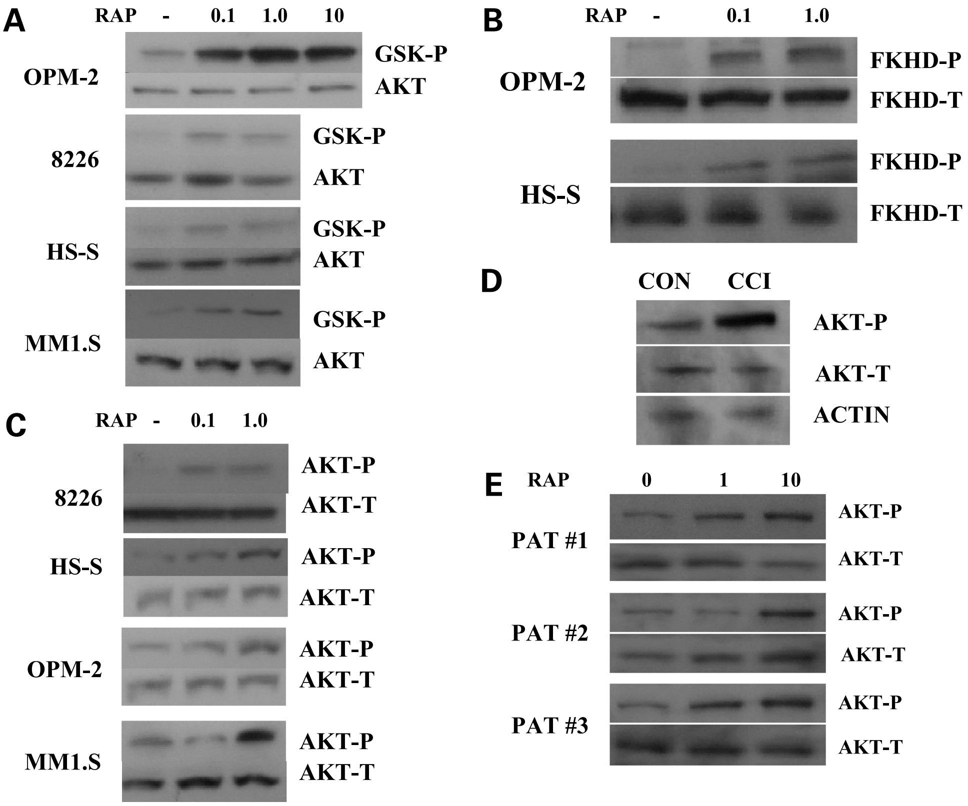

Figure 1. mTOR inhibitors increase AKT activity in myeloma cells. **A,** OPM-2, 8226, MM1.S, and HS-Sultan (*HS-S*) cell lines were incubated with increasing concentrations of rapamycin (shown above blot in nmol/L) for 4 h, after which, AKT activity was assessed by its ability to phosphorylate the GSK substrate as shown by immunoblot (labeled GSK-P). To ensure equal amounts of AKT were immunoprecipitated, we also immunoblotted the precipitate for total AKT. This experiment was repeated two additional times with identical results. **B,** OPM-2 or HS-Sultan cells were treated with rapamycin (concentrations above blot in nmol/L) for 4 h and extracts immunoblotted for total forkhead transcription factor (*FKHR-T*) and phosphorylated forkhead (*FKHR-P*). **C,** 8226, HS-Sultan, MM1.S, or OPM-2 cell lines treated with or without rapamycin (0.1 or 1.0 nmol/L) for 4 h and extracts immunoblotted for total AKT (*AKT-T*) and AKT phosphorylated on Thr^{308} (*AKT-P*). This experiment was repeated two additional times with identical results. **D,** mice with progressively growing s.c. OPM-2 tumors were injected i.p. with vehicle or CCI-779 (20 mg/kg each day × 5 d). Tumors were then harvested and extracted protein pooled and immunoblotted for total AKT, phosphorylated AKT (on threonine), and actin. **E,** three bone marrow aspirates from patients with untreated myeloma were obtained; plasma cells isolated and treated with or without rapamycin (1 or 10 nmol/L) for 4 h, after which, total and phosphorylated AKT were assayed by immunoblot.

analysis, the rapamycin-induced enhancement of AKT activity (at 1 nmol/L of rapamycin) was 8 ± 2-fold in OPM-2, 2.8 ± 0.5-fold in 8226, 2.7 ± 0.3-fold in HS-Sultan, and 4.5 ± 0.8-fold in MM1.S cells (mean ± SD, $n = 3$). In experiments not shown, at an extremely low concentration of 0.01 nmol/L, rapamycin had no effect on AKT kinase activity. Two of the four lines were further studied for rapamycin-induced phosphorylation of the forkhead transcription factor, a well-known substrate of AKT. As shown (Fig. 1B), rapamycin markedly enhanced phosphorylation of forkhead transcription factor in OPM-2 and HS-Sultan cells (6 ± 1-fold increase in OPM-2, 3.4 ± 0.7-fold in HS-Sultan; mean ± SD, $n = 3$). In Western blot analysis, rapamycin also significantly increased phosphorylation of AKT on threonine residues (at Thr^{308}) in all four lines (Fig. 1C). In three of the lines (OPM-2, MM1.S, and HS-Sultan), 1 nmol/L seemed slightly more effective than 0.1 nmol/L. Nevertheless, enhanced phosphorylation of AKT indicated that the effect of rapamycin occurred upstream with probable activation of PI3K and/or PDK-1. This latter experiment was also repeated two additional times with identical results. Again, a concentration of 0.01 nmol/L was ineffective in inducing AKT threonine phosphorylation (data not shown).

We also were able to test the effects of the mTOR inhibitor CCI-779 *in vivo* on myeloma cell AKT. Nonobese diabetic/severe combined immunodeficient mice were challenged with OPM-2 cells implanted s.c. admixed with matrigel. When tumor growth reached 200 mm^3, mice were treated with five daily i.p. injections of CCI-779 at 20 mg/kg or vehicle alone. This treatment significantly slowed tumor growth, whereby the tumor volume in control mice after 5 days of treatment was 750 ± 50 mm^3 (mean ± SD, $n = 3$) and tumor volume in CCI-779-treated mice was 300 ± 25 mm^3. A larger experiment, using 10 to 16 mice per group, confirmed the antitumor response and will be presented elsewhere. On day 6, tumors were harvested from three mice per group and protein was extracted and pooled and immunoblotted for phosphorylated and total AKT. Although inducing an inhibition of tumor growth, as shown in Fig. 1D, administration of CCI-779 *in vivo* resulted in enhanced myeloma cell AKT phosphorylation.

To test if similar rapamycin-induced AKT activation occurred in primary explanted tumor cells, multiple myeloma cells were purified from the bone marrow of three patients by selection for the CD38 membrane protein and treated with or without rapamycin for 4 hours. As shown in Fig. 1E, a significant induction of AKT phosphorylation was seen in all three preparations.

In time course experiments (Fig. 2A), rapamycin was shown to enhance AKT phosphorylation within 1 to 2 hours of exposure in both 8226 and OPM-2 cell lines. As shown, this was specific for phosphorylation of the Thr^{308} residue of AKT. Phosphorylation of Ser^{473} was unaffected. Recent work (19) identifies the mTOR/Rictor complex as being the kinase responsible for phosphorylating Ser^{473} on AKT. However, the mTOR/Rictor complex is resistant to inhibition with rapamycin (19). This would be consistent with the inability of rapamycin to inhibit Ser^{473} phosphorylation on AKT at least as shown for OPM-2 cells in Fig. 2A.

Rapamycin was also capable of prolonging AKT activation induced by exposure to exogenous IGF-I, a myeloma growth factor. As shown in Fig. 2B, exposure of untreated 8226 cells to exogenous IGF-I induces a transient activation of AKT at 10 and 90 minutes as shown by an *in vitro* kinase assay. However, following treatment with rapamycin, 8226 cells show AKT activation continuing at least up to 180 minutes following exposure to IGF-I. Similar results were seen when immunoblot assay for AKT threonine phosphorylation was done (Fig. 2B). As shown, IGF-I induced a transient phosphorylation of AKT in control cells at 10 minutes with a decrease back to undetectable levels by 60 minutes. However, following rapamycin treatment, AKT phosphorylation was prolonged with maximal levels seen up to 60 minutes and some detectable phosphorylation still present at 90 minutes. The experiments shown in Fig. 2 were repeated once with identical results.

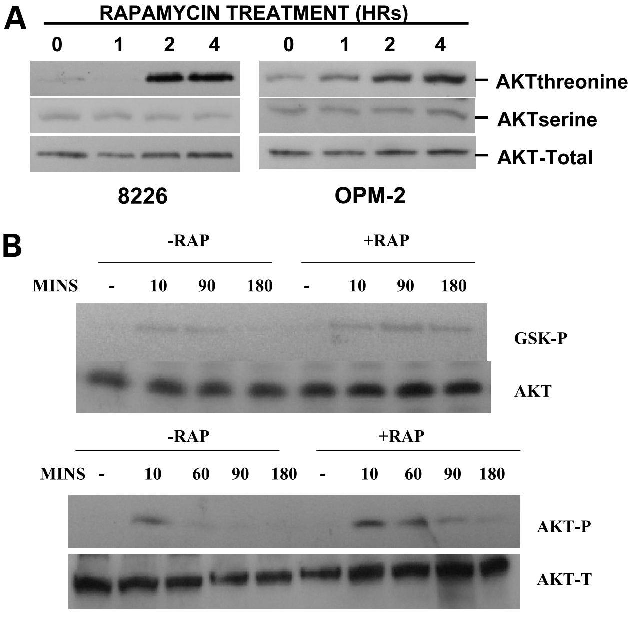

Figure 2. Kinetics of rapamycin effect and prolongation of IGF-I-induced AKT activation. **A,** 8226 or OPM-2 cells were treated with rapamycin (10 nmol/L) for 0, 1, 2, or 4 h and AKT serine or threonine phosphorylation was assayed by immunoblotting. **B,** 8226 cells were treated with or without rapamycin (10 nmol/L for 4 h) and stimulated with IGF-I (400 ng/mL). At varying times after IGF-I stimulation (10 – 180 min, shown above gels), extracts were assayed for AKT activity by ability of immunoprecipitated AKT to phosphorylate GSK substrate (*GSK-P*) and AKT phosphorylation (by immunoblot assay).

mTOR Inhibitors Enhance Activation of PI3K in Myeloma Cells

Because AKT phosphorylation and activation is a direct result of PI3K activity through the latter's phosphorylation of phosphoinositols, we next studied PI3K. OPM-2 cells were treated with or without rapamycin for 4 hours and PI3K was immunoprecipitated and its kinase activity assayed. IGF-I treatment served as a positive control for PI3K activation. As shown in Fig. 3A, rapamycin activated PI3K kinase activity (*lane 2*) and the PI3K inhibitor wortmannin prevented activation. Similar activation of PI3K by rapamycin was seen in 8226 and HS-Sultan cells (data not shown).

PI3K activation is regulated by the p85 subunit of the kinase, which becomes tyrosine phosphorylated. To assess effects of rapamycin on tyrosine phosphorylation of p85, OPM-2 cells were treated with rapamycin for 4 hours, p85 was immunoprecipitated, and the precipitate was immunoblotted with an anti-phosphotyrosine antibody. As shown in Fig. 3B, rapamycin treatment also induced tyrosine phosphorylation of p85 in multiple myeloma cells.

Rapamycin-Induced Activation of the PI3K/AKT Cascade Is Dependent on IGF Signaling

The ability of rapamycin to prolong AKT activation during exposure to exogenous IGF-I (Fig. 2) suggested to us that rapamycin-induced AKT activation in unstimulated multiple myeloma cells could be due to effects on basal signaling from the IGF-I receptor. IGF-I is a known major myeloma growth factor (12, 13), and a recent study documents that sufficient amounts of IGF-I are present in serum *in vitro* and *in vivo* to provide a potent growth signal to multiple myeloma cells (14). To directly test if low level basal IGF-I signaling due to IGF-I in serum was crucial to the resulting rapamycin-induced activation of PI3K/AKT, we prevented basal signaling in OPM-2 cells with a blocking anti-IGF-R antibody. As shown in Fig. 4, the anti-IGF-R antibody completely prevented the ability of rapamycin to activate AKT. Confirmation of IGF-R blockade was shown by the antibody's inhibition of basal IGF-R tyrosine phosphorylation (Fig. 4B). These data support the notion that rapamycin-induced activation of AKT is dependent on basal IGF-I/IGF-R signaling.

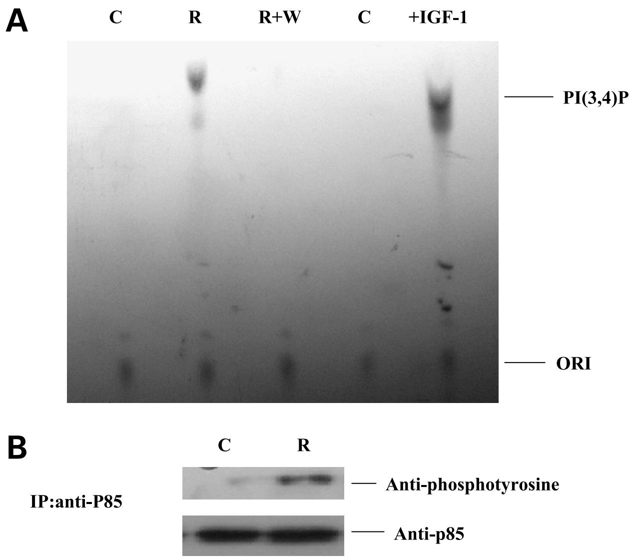

Figure 3. Rapamycin activates PI3K and induces p85 phosphorylation. **A,** OPM-2 multiple myeloma cells treated with (*R*) or without (*C*) rapamycin (10 nmol/L) and with or without wortmannin (*W*, at 0.1 μmol/L) for 4 h. Separate OPM-2 cells treated with (*+IGF-I*) or without (*C*) recombinant IGF-I for 10 min (at 400 ng/mL). Extracts assayed for PI3K activity as described in Materials and Methods. *Right,* positions of the reaction product PI(3,4)P and the origin (*ORI*). **B,** OPM-2 cells were treated with (*R*) or without (*C*) rapamycin for 4 h (10 nmol/L); p85 was immunoprecipitated and the precipitate was immunoblotted with anti-phosphotyrosine and anti-p85 antibodies.

Effects of Rapamycin on IRS-1

Following stimulation with exogenous insulin or IGF-I, IRS-1 becomes phosphorylated on Ser^{312} in humans and Ser^{307} in mice/rats in an mTOR-dependent fashion that inhibits signaling thus functioning as a negative feedback circuit. We, thus, tested if rapamycin-induced activation of AKT in multiple myeloma cells was mediated by such effects on IRS-1 in the presence of serum but absence of exogenously added IGF-I. After treatment with or without rapamycin, IRS-1 was immunoprecipitated and then immunoblotted with antibodies to detect Ser^{312} phosphorylation. As shown in Fig. 5A, rapamycin significantly inhibited serine phosphorylation of IRS-1 in both 8226 and OPM-2 cell lines.

Serine phosphorylation of IRS-1 results in its dissociation from IGF-I receptors (11). Thus, prevention of IRS-1 serine phosphorylation could theoretically increase downstream signaling by increasing its interaction with IGF-I receptors with subsequent enhanced stimulation of IRS-1 tyrosine phosphorylation. To test this, we reprobed the IRS-1 immunoprecipitates with antibody to identify tyrosine phosphorylation or associated IGF-R. As shown in Fig. 5A, concurrent with the inhibition of serine phosphorylation, rapamycin enhanced an interaction with IGF-R and tyrosine phosphorylation of IRS-1.

Similar results could be shown by transient transfection with a FLAG-tagged IRS-1 construct (Fig. 5B). Transfected cells were treated with or without rapamycin for 4 hours; the IRS-1 was immunoprecipitated with a FLAG antibody and immunoblotted with antibody to detect serine phosphorylation and association of IRS-1 with IGF-R. As shown in Fig. 5B (*left*), rapamycin was capable of inhibiting serine phosphorylation of FLAG-tagged IRS-1 and enhanced its interaction with IGF-R. To specifically test the role of Ser^{312} phosphorylation, we also transiently transfected a FLAG-tagged IRS-1 with a serine-to-alanine mutation at residue 312. Transfected cells were similarly treated with or without rapamycin and the mutant IRS-1 was immunoprecipitated. As shown in Fig. 5B (*right*), the mutant protein could not be phosphorylated at Ser^{312} as expected. In addition, although the mutant protein constitutively associated with a higher amount of IGF-R compared with the wild-type IRS-1, rapamycin was unable to increase this association as it did for the wild-type IRS-1. These data prove that the rapamycin-enhanced interaction between IRS-1 and IGF-R is due to its ability to prevent Ser^{312} phosphorylation.

Effects of Rapamycin on IRS-1 Degradation

Serine phosphorylation of IRS-1 also results in its targeting for exit from low-density microsomes into cytosol where it is ultimately degraded by the 26S proteosome (9–11, 20). Thus, prevention of IRS-1 serine phosphorylation could also theoretically enhance downstream signaling by preventing its proteosomal degradation. To address this possible mechanism, we treated multiple myeloma cells with the proteasome inhibitors lactocystin or PS-341. As shown in Fig. 6A, neither drug was able to activate multiple myeloma cell AKT even when used in up to 6 hours of incubations. To test an effect on IRS-1 degradation, multiple myeloma cells were transfected with the tagged wild-type IRS-1 and kept overnight in low serum. The next day, cells were moved to 10% FCS and incubated with or without rapamycin (10 nmol/L) or PS-341 (1 μmol/L). At 1, 3, and

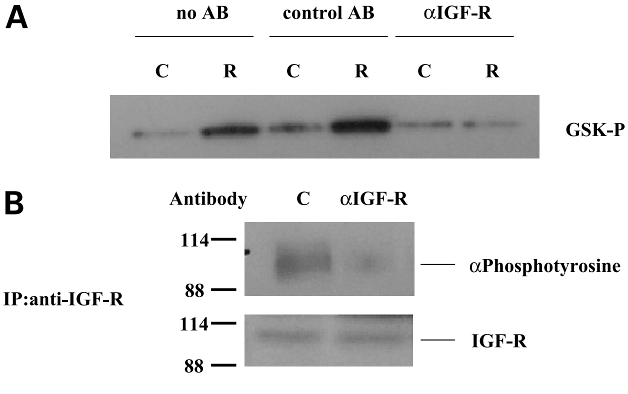

Figure 4. Rapamycin-induced activation of AKT is dependent on IGF-I signaling. **A,** multiple myeloma cells were first incubated with media alone (*no AB*), control antibody (*control AB*), or blocking anti-IGF-R antibody at 1 μg/mL for 1 h. Cells were then treated with (*R*) or without (*C*) rapamycin (10 nmol/L for an additional 3 h), after which, AKT activity was assessed by *in vitro* kinase assay [phosphorylation of GSK substrate (*GSK-P*)]. **B,** the same cells were treated with the control (*C*) or anti-IGF-R antibody (1 μg/mL for 1 h), after which, the IGF-R was immunoprecipitated and immunoblotted with anti-phosphotyrosine or anti-IGF-R antibodies.

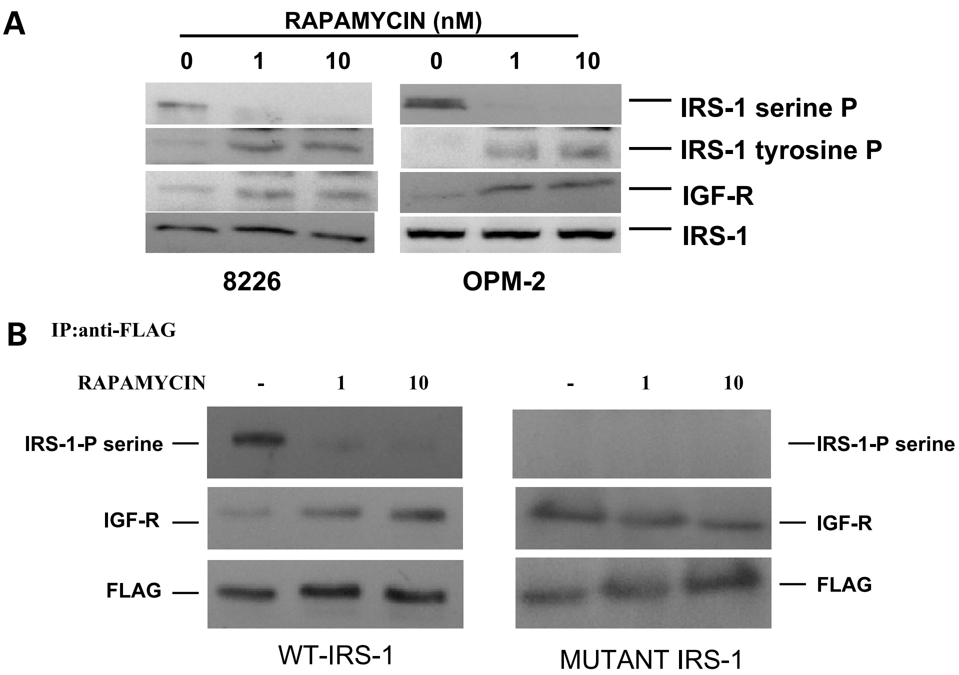

Figure 5. Effect of rapamycin on endogenous IRS-1 and tagged IRS-1 constructs. **A,** 8226 or OPM-2 cells were treated with 0, 1, or 10 nmol/L rapamycin for 4 h, after which, IRS-1 was immunoprecipitated and immunoblotted for serine-phosphorylated IRS-1, total IRS-1, tyrosine-phosphorylated IRS-1 (with a phosphotyrosine antibody), and IGF-R association. **B,** OPM-2 multiple myeloma cells were transiently transfected with FLAG-tagged IRS-1 or a serine-to-alanine mutated IRS-1 at residue 312. After treatment with 0, 1, or 10 nmol/L rapamycin for 4 h, cell extracts were immunoprecipitated with anti-FLAG antibody and immunoblotted with antibodies specific for IRS-1 phosphorylated at Ser^{312}, associated IGF-R and FLAG.

5 hours, IRS-1 was immunoprecipitated and immunoblotted for either total IRS-1 or serine-phosphorylated IRS-1. As shown in Fig. 6B, following exposure to 10% FCS, multiple myeloma cells show a slow degradation of IRS-1 between 1 and 5 hours (*left*). However, when exposure to 10% FCS occurs in the presence of either rapamycin or PS-341, IRS-1 degradation is prevented. Although PS-341 prevented degradation of IRS-1, it could not inhibit IRS-1 serine phosphorylation, whereas, once again, rapamycin was effective in this regard (Fig. 6C).

Effect of Rapamycin on Multiple Myeloma Cell Apoptosis Induced by PS-341

Because the PI3K/AKT pathway may critically support viability in multiple myeloma cells, we next asked whether its up-regulation by rapamycin was associated with an inhibition of apoptosis induced by PS-341. We tested PS-341 because a prior study (21) showed that AKT protects against this agent in multiple myeloma cells. Thus, both OPM-2 and 8226 cells were treated with or without increasing concentrations of rapamycin combined without or with increasing concentrations of PS-341 and apoptosis was examined (Fig. 7). Both OPM-2 and 8226 cell lines were sensitive to PS-341-induced apoptosis. In OPM-2 cells, 18 ± 3%, 29 ± 5%, and 68 ± 2% apoptosis was seen with 10^{-7}, 10^{-6}, and 10^{-5} mol/L PS-341, respectively. The 8226 cell line was considerably more sensitive to PS-341 with an ED_{50} of 3×10^{-8} mol/L. Rapamycin, by itself, did not result in any apoptosis. When combined with PS-341,

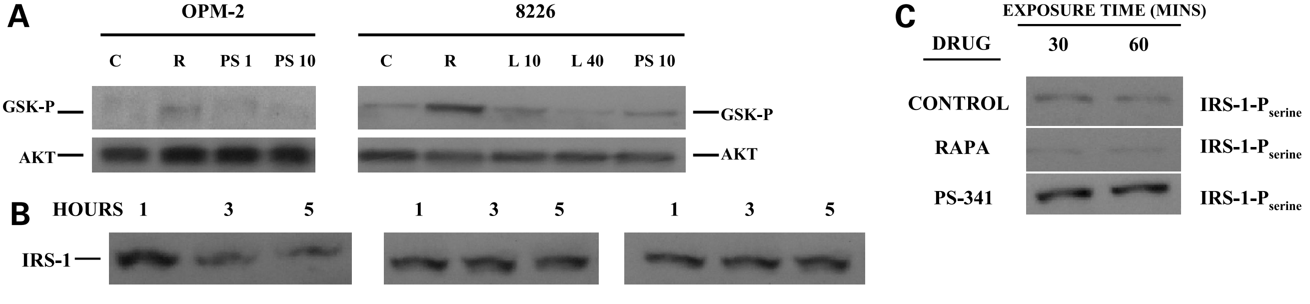

Figure 6. Effect of proteasome inhibitors on AKT and IRS-1. **A,** OPM-2 or 8226 cells were treated for 6 h without drugs (*C*, control) or with rapamycin (*R* at 1 nmol/L), lactocystin at 10 or 40 μmol/L (*L10* or *L40*), or PS-341 at 1 or 10 μmol/L (*PS1* or *PS10*). AKT was immunoprecipitated and *in vitro* kinase assay was done. This experiment was also repeated with a 4-h incubation with identical results. **B,** OPM-2 cells were transiently transfected with a tagged IRS-1 construct and kept in low serum overnight. The next day, cells were moved to 10% serum alone (*control*) or to 10% serum in the presence of rapamycin (10 nmol/L) or PS-341 (1 μmol/L). At 1, 3, and 5 h, the tagged IRS-1 was immunoprecipitated and immunoblotted for total IRS-1. **C,** OPM-2 cells were similarly transfected as in (*B*). The next day, they were moved to 10% serum alone (*control*) or in the presence of rapamycin (10 nmol/L) or PS-341 (1 μmol/L). At 30 and 60 min, the tagged IRS-1 was immunoprecipitated and immunoblotted for IRS-1 phosphorylated on Ser^{307}.

however, a modest but significant protection against apoptosis occurred in both cell lines. In OPM-2 cells, apoptosis induced by 10^{-5} mol/L PS-341 decreased from 68 ± 2% to 44 ± 4% (mean ± SD of three separate experiments) when rapamycin was present at 1 nmol/L. A significant ($P < 0.05$) decrease was also seen when rapamycin was present at 0.1 nmol/L, but 0.01 nmol/L had no effect (Fig. 7). In 8226 cells, 1 and 0.1 nmol/L rapamycin significantly decreased ($P < 0.05$) apoptosis induced by PS-341 when the latter was used at both 10^{-7} and 10^{-6} mol/L concentrations, but 0.01 nmol/L rapamycin had no significant effect.

Discussion

The results of this study show that mTOR inhibitors can enhance PI3K/AKT activity in multiple myeloma cells. This effect was independent of target cell PTEN status and achieved at low concentrations of rapamycin (i.e., as low as 0.1 nmol/L), which are sufficient to inhibit mTOR activity. Stimulation of the PI3K/AKT cascade was dependent upon low-level, basal IGF-I/IGF-R signaling. As IGF-I-induced activation of AKT is a protumoral stimulant of multiple myeloma cells (12, 13), these results provide a caveat for future clinical trials of mTOR inhibitors in myeloma patients. Although the mTOR inhibitor CCI-779 inhibited multiple myeloma tumor growth *in vivo*, treatment with CCI-779 also induced tumor AKT phosphorylation. Further *in vivo* xenograft studies will be required to determine if the enhanced AKT activity prevents a potentially greater antitumor effect of CCI-779 or if it antagonizes the *in vivo* effect of other agents.

The rapamycin-induced stimulation of PI3K/AKT activity was associated with prevention of IRS-1 phosphorylation at Ser^{312}. In other cell types, Ser^{312} phosphorylation of IRS-1 is an integral piece of a feedback inhibition pathway that down-regulates signaling. Following IGF-I/insulin-induced stimulation of the PI3K/AKT/mTOR pathway, an mTOR-dependent serine phosphorylation of IRS-1 uncouples it from its IGF-I/insulin receptors thus inhibiting its tyrosine phosphorylation and further capacity to signal downstream (22). The ability of rapamycin to prevent IRS-1 serine phosphorylation in multiple myeloma cells in addition to the enhanced interaction between IRS-1 and IGF-I receptors suggests the following scenario: low-level, serum-containing IGF-I stimulation of multiple myeloma cells results in IRS-1 binding to stimulated IGF receptors (IGF-R) and downstream signaling through PI3K/AKT to mTOR and a secondary mTOR-dependent serine phosphorylation of IRS-1. The resulting balance between low-level positive signaling and feedback inhibition-negative signaling provides the continuous basal degree of signaling of multiple myeloma cells in serum. However, when negative feedback inhibition is prevented by a rapamycin-induced block of mTOR-dependent IRS-1 serine phosphorylation, a significant enhancement of IRS-1 binding to IGF-I receptors ensues with resulting increases in IRS-1 tyrosine phosphorylation, binding to PI3K p85, p85 tyrosine phosphorylation, PI3K activity, and AKT activity. Results presented in Fig. 5, with a mutated IRS-1 construct, confirm the role of effects on IRS-1 Ser^{312} phosphorylation in rapamycin's ability to enhance binding of IRS-1 to IGF-R. A similar prevention of the negative feedback effect of IRS-1 serine phosphorylation presumably explains the prolongation of AKT activation in IGF-I-stimulated 8226 multiple myeloma cells.

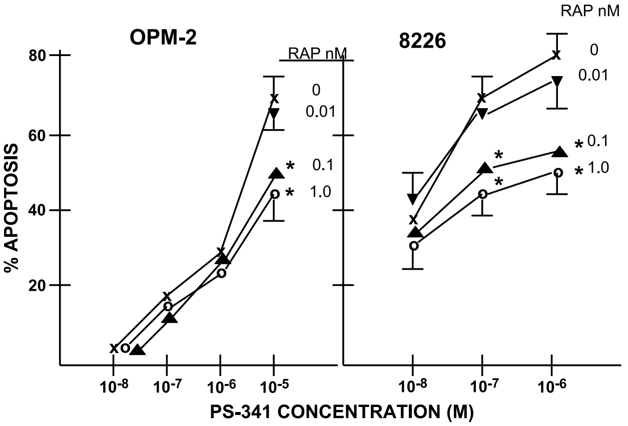

Figure 7. Effect of rapamycin on PS-341-induced myeloma cell apoptosis. OPM-2 or 8226 cells were treated for 48 h with increasing concentrations of PS-341 along with increasing concentrations of rapamycin (0 – 1 nmol/L) and percentage of cells undergoing apoptosis assessed by Annexin V staining. Rapamycin concentrations: ×, 0 nmol/L; ○, 1 nmol/L; ▲, 0.1 nmol/L; ▼, 0.01 nmol/L. *Points,* means of three separate experiments; *bars,* ±SD. *, $P < 0.05$, significant differences from control (no rapamycin).

In addition to uncoupling IRS-1 from IGF-Rs, serine phosphorylation targets it for exit from low-density microsomes into the cytosol, where it is degraded by the 26S proteasome (9–11). Thus, the ability of rapamycin to inhibit IRS-1 phosphorylation and subsequent proteasome degradation could theoretically participate in up-regulating signaling downstream through AKT. However, the results shown in Fig. 6, in which proteasome inhibitors prolong IRS-1 protein survival but do not activate multiple myeloma cell AKT, indicate that a simple prevention of IRS-1 degradation is not sufficient to activate AKT. A rapamycin-dependent prevention of redistribution of IRS-1 from microsomes to cytosol, however, may prolong its colocalization with p85 PI3K thus also contributing to activation of PI3K/AKT.

Recent studies suggest additional layers of complexity by which mTOR and mTOR inhibitors affect AKT function. Harrington et al. (23) have shown that the mTOR substrate p70S6kinase (p70) can also affect IRS-1 function by direct phosphorylation on Ser^{302}, which prevents IRS-1 binding to insulin receptors. Thus, in a similar fashion to the current study, an mTOR inhibitor could activate AKT function by preventing mTOR-dependent, p70-mediated phosphorylation of IRS-1. Quite possibly, phosphorylation at Ser^{302} (by p70) and Ser^{312} (by mTOR) is additive in causing inhibition of IRS-1 function. In contrast, when mTOR is complexed with rictor, it can directly phosphorylate and activate AKT

(19). However, this mTOR-rictor activity is not inhibited by mTOR inhibitors like rapamycin (19). Thus, a fragile balance may exist where, by inducing phosphorylation of IRS-1 in cells stimulated by IGF-I, mTOR inhibits AKT activation, but via direct interaction, it activates AKT. However, when mTOR inhibitors like rapamycin are introduced, the balance is disrupted as only the rapamycin-sensitive phosphorylation of IRS-1 is interrupted, whereas direct AKT activation by mTOR-rictor is maintained. The result would be significant rapamycin-induced AKT activation.

In a prior study (2), we found that heightened AKT activity, due to PTEN mutations, sensitized myeloma cells to G_1 arrest induced by mTOR inhibitors. It is, thus, interesting that, by inducing AKT activation, mTOR inhibitors may be able to sensitize cells to their own cytostatic effect, although one would expect that a PTEN-null cell line would still be more sensitive than a PTEN wild type–containing cell line. However, by activating AKT, mTOR inhibitors could theoretically enhance an antiapoptotic mechanism in multiple myeloma cells. Indeed, we found that rapamycin modestly but significantly inhibited apoptosis induced by the anti–multiple myeloma agent PS-341. The rapamycin concentrations that inhibited apoptosis correlated with those capable of activating AKT. In contrast, combination or rapamycin with dexamethasone (24) or with Revlimid (25) resulted in enhanced multiple myeloma cell death. Thus, some interactions between mTOR inhibitors and anti-myeloma agents may be antagonistic possibly due to activation of the antiapoptotic AKT, and other interactions may be synergistic. Additional preclinical studies will be valuable to learn how best to combine this potentially efficacious drug with other agents.

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