Inhibition of Mammalian Target of Rapamycin Activates Apoptosis Signal-regulating Kinase 1 Signaling by Suppressing Protein Phosphatase 5 Activity*

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Under serum-free conditions, rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), induces a cellular stress response characterized by rapid and sustained activation of the apoptosis signal-regulating kinase 1 (ASK1) signaling pathway and selective apoptosis of cells lacking functional p53. Here we have investigated how mTOR regulates ASK1 signaling using p53-mutant rhabdomyosarcoma cells. In Rh30 cells, ASK1 was found to physically interact with protein phosphatase 5 (PP5), previously identified as a negative regulator of ASK1. Rapamycin did not affect either protein level of PP5 or association of PP5 with ASK1. Instead, rapamycin caused rapid dissociation of the PP2A-B′' regulatory subunit (PR72) from the PP5-ASK1 complex, which was associated with reduced phosphatase activity of PP5. This effect was dependent on expression of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Down-regulation of PP5 activity by rapamycin coordinately activated ASK1, leading to elevated phosphorylation of c-Jun. Amino acid deprivation, which like rapamycin inhibits mTOR signaling, also inhibited PP5 activity, caused rapid dissociation of PR72, and activated ASK1 signaling. Overexpression of PP5, but not the PP2A catalytic subunit, blocked rapamycin-induced phosphorylation of c-Jun, and protected cells from rapamycin-induced apoptosis. The results suggest that PP5 is downstream of mTOR and positively regulated by the mTOR pathway. The findings suggest that in the absence of serum factors, mTOR signaling suppresses apoptosis through positive regulation of PP5 activity and suppression of cellular stress.

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Rapamycin, and its analogues CCI-779, RAD001, and AP23573 now in clinical trials as anticancer agents potently inhibit tumor cell proliferation (1). These agents inhibit the function of the mammalian target of rapamycin, mTOR1 (also designated FRAP, RAFT1, or RAFT1), a 289 kDa Ser/Thr kinase, which lies downstream of phosphatidylinositol 3′-kinase, and senses mitogenic stimuli, nutrient conditions (2–4), and ATP (5), and regulates multiple cellular events required for cellular growth (size) and proliferation (6), differentiation (7–9), migration (10, 11), and survival (12–14). Rapamycin first forms a complex with a 12 kDa cytosolic protein designated FK-506-binding protein 12 (FKBP-12) and this complex binds mTOR and inhibits its function. Subsequently, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) down-regulates protein synthesis and associates with eIF4E (15, 16). This association prevents binding of eIF4E to eIF4G and formation of eIF4F initiation complex, thereby inhibiting cap-dependent translation of mRNA. Inhibition of mTOR by rapamycin also directly or indirectly inactivates ribosomal p70S6 kinase (S6K1), thereby blocking translation of mRNA species containing 5′-terminal oligopyrimidine tracts (TOP) (17, 18). However, the requirement for S6K1 activity in translation of TOP containing mRNAs has been recently challenged. Importantly, complete inhibition of mTOR by rapamycin had only a slight repressive effect on translation of TOP mRNAs leading to the conclusion that regulation by growth factors and mitogens is primarily through the PI3K pathway with a minor role for mTOR in regulation of TOP mRNA translation (19, 20). In many cell lines, exposure to rapamycin reduces overall protein synthesis only ~15–20% but results in a specific G1 accumulation.

We previously found that proliferation of human rhabdomyosarcoma cells was inhibited by low concentrations of rapamycin (21, 22). Under serum-free culture conditions rapamycin treatment induces apoptosis in these and other cells lacking functional p53 (12, 13). Ectopic expression of p53 or p21Cip1 protects cells from apoptosis (13). Rapamycin inhibition of mTOR induces a cellular stress response characterized by rapid and sustained activation of apoptosis signal-regulating kinase 1 (ASK1) signaling in p53-mutant cells. In contrast only transient activation of ASK1 signaling occurs in cells express-

1 The abbreviations used are: mTOR, mammalian target of rapamycin; ASK1, apoptosis signal-regulating kinase 1; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; PR72, PP2A-B′ regulatory subunit; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K1, p70S6 kinase; TPR, tetratricopeptide repeat; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; eIF4E, eukaryotic initiation factor 4E; eIF4F, eIF4E-eIF4G-eIF4A initiation complex; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; RAPT1, rapamycin-induced protein kinase; ASK1, apoptosis signal-regulating kinase 1; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; PR72, PP2A-B′ regulatory subunit; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K1, p70S6 kinase; TPR, tetratricopeptide repeat; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; eIF4E, eukaryotic initiation factor 4E; eIF4F, eIF4E-eIF4G-eIF4A initiation complex; FITC, fluorescein isothiocyanate.
ing functional p53/p21<sup>Cip1</sup> (14). However, little is known about the mechanism by which inhibition of mTOR causes activation of ASK1 signaling.

Protein phosphatase 5 (PP5, also named PPT) is a novel member of Ser/Thr phosphatase family, which also includes PP1, PP2A, PP2B (also termed PP3 or calcineurin A), PP2C, PP4 (also termed PPX), PP6 (also named PPV in Drosophila, or Sit4 in yeast), and PP7 (reviewed in Ref. 29). PP5 differs from the other Ser/Thr phosphatases in that it uniquely contains three tetratricopeptide repeat (TPR) motifs at its N terminus (24, 25) that serve as protein-protein interaction domains (26). Removal of the TPR domains by limited proteolysis results in a substantial increase in phosphatase activity suggesting that the TPR domains also function as autoinhibitory domains (27, 28). PP5 has been shown to interact with a number of proteins through TPR motifs (29–38). Important, PP5 associates with ASK1 (39), and other protein phosphatase subunits such as PP2A-A and -B subunits (40).

As in yeast, mTOR appears to regulate several protein phosphatases through binding to the Ig receptor-binding protein α4, the mammalian homologue of yeast Tap42. Association of α4 with PP2A, PP4, and PP6 has been linked to rapamycin sensitivity (41, 42), although it is considered that PP2A is negatively regulated by mTOR (18, 41, 43, 44), this remains controversial. In yeast Tap42 may redirect PP2A activity (61). Very recent results in yeast indicate that Tap42 can both inhibit and activate protein phosphatases (47), suggesting even greater complexity of Tor signaling. At this time it is unknown whether the mTOR pathway regulates PP5. As in yeast, contradictory information on the consequences of α4 dissociation from PP2A or PP6 has been reported. For instance, rapamycin treatment inhibits cell proliferation by decreasing PP2A activity through dissociating α4 from PP2A catalytic subunit (42). Other studies (45, 46) do not demonstrate rapamycin-induced dissociation of α4 from PP2A or PP6. ASK1, a recently identified mitogen-activated protein kinase (MAPK) kinase activator, activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways in response to a variety of cytotoxic stimuli, including oxidative stress, Fas ligand, tumor necrosis factor, nutrient depletion and antitumor agents (14, 39, 48–56). Numerous protein phosphatases can dephosphorylate and inactivate JNK and p38 MAPK (57). However, PP5 is the only protein phosphatase that has so far been identified to physically interact with, and negatively regulate ASK1 (39). Here we show that inhibition of mTOR by rapamycin or amino acid deprivation causes rapid dissociation of PP2A-α<sup>B</sup> subunit (designated PR72) from PP5, that is associated with decreased phosphatase activity of PP5, and concurrently activating ASK1. Furthermore, down-regulation of PP5 activity by inhibition of mTOR is dependent on expression of 4E-BP1. Our findings suggest that PP5 is downstream in the mTOR pathway, is positively regulated by mTOR and that mTOR regulates ASK1 activity via PP5.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Growth Conditions**—Human rhabdomyosarcoma cell line Rh30 that expresses a mutant p53 (Arg<sup>727</sup> → Cys) (14), and Rh30/mTOR-rr expressing a rapamycin-resistant mutant mTOR (Ser<sup>2481</sup> → Ile) (12) were grown in antibiotic-free RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37 °C and 5% CO<sub>2</sub>. For experiments where cells were deprived of serum overnight, 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 modified N2E medium (MN2E; DMEM/F12 supplemented with 1 µg/ml human transferrin, 30 nM sodium selenate, 20 nM progesterone, and 100 µM β-glutamylcysteine, and 5% vitamin E phosphate, 50 µM ethanolamine, and 1 mg/ml bovine serum albumin). For amino acid deprivation, cells were cultured in a specific RPMI 1640 medium (R-7130, without l-glutamine, l-leucine, l-lysine, and l-methionine) (Sigma).
protein A/G-agarose. Subsequently, the beads were washed three times with the above lysis buffer, and twice with the phosphatase assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂). The phosphatase activity of immunoprecipitated PP2A or PP5 was assayed with a Ser/Thr Phosphatase Assay kit 1 using KRpTIRR as the substrate peptide (Upstate Biotechnology) following the manufacturer’s instructions.

Growth Inhibition Assay—Cells were seeded on 6-well plates (5 × 10⁴ cells/well) in triplicate. Rapamycin (0–10,000 ng/ml) was added the next day. After incubation for 7 days, cells were enumerated by counting the nuclei as described previously (21).

FACS Analysis of Apoptosis—Cells (1.7 × 10⁶ per 162-cm² flask) were grown overnight in MN2E. Rapamycin (100 ng/ml) was added the next day, and cells were exposed for up to 6 days. Parallel control cells were not treated with rapamycin. Cells were treated with trypsin, washed with PBS, resuspended in 200 µl of binding buffer (Clontech Laboratories), and incubated with 10 µl annexin V-FITC (final concentration, 1 µg/ml; Clontech Laboratories) and 500 ng propidium iodide (final volume, 410 µl). Cells were incubated at room temperature without light for 10 min before flow cytometric analysis (FACSCalibur) (12).

RESULTS

Rapamycin Inhibits PP5 Activity in an mTOR-dependent Manner—We previously found that under conditions of serum-free growth rapamycin induced apoptosis of p53-mutant/null cells. Although detection of apoptosis was relatively delayed, early events were characterized by rapid and sustained activation of the ASK1 signaling pathway (12–14). Rapamycin did not affect the level of ASK1, but increased the kinase activity of ASK1 (14). Recently, PP5 has been shown to interact with ASK1 and identified as a negative regulator of ASK1 in HeLa cells (39). Activation of ASK1 signaling could be attributed to either down-regulation of PP5 protein level, decreased association of PP5 with ASK1, or decreased phosphatase activity of PP5 (14). To answer these questions, serum-starved Rh30 cells with mutant p53 (Arg²⁷³ → Cys) were exposed to rapamycin (100 ng/ml) for up to 24 h, followed by either direct Western blot with antibodies to ASK1, PP5, and β-tubulin (loading control), or immunoprecipitation with antibodies to PP5, and then immunoblotting with antibodies to ASK1 and PP5, respectively, or in vitro phosphatase assay for PP5. As seen previously (14), rapamycin did increase the kinase activity of ASK1 and induce high level of phospho-c-Jun over 24 h (data not shown). Rapamycin did not affect either the levels of PP5 or ASK1 proteins (Fig. 1A), or the association of PP5 with ASK1 (Fig. 1B). In contrast treatment of cells with H₂O₂ (1 mM) for 30 min enhanced association of PP5 with immunoprecipitated ASK1 (Fig. 1C) in agreement with Morita et al. (39). Of interest was the observation that rapamycin caused a rapid and significant decrease of PP5 phosphatase activity in in vitro assays (Fig. 1D). Within 30 min, the phosphatase activity was reduced by 50%, and the inhibition of PP5 was sustained for up to 24 h, which is consistent with the time course for c-Jun phosphorylation (14), an indicator of activated ASK1 signaling.

To test whether the phosphatase activity of PP5 was suppressed as the result of inhibition of mTOR function, we examined the effect of rapamycin in Rh30 cells and in engineered Rh30 cells expressing a rapamycin-resistant mTOR (Rh30/mTOR-rr; Ser²⁵⁹⁵ → Ile) with reduced binding affinity for the FKBP12-rapamycin complex (12, 59). Cells were serum-starved and exposed to rapamycin for 2 h, followed by in vitro phosphatase assay for PP5. As shown in Fig. 1E, rapamycin significantly decreased the phosphatase activity of PP5 in Rh30 cells, but not in Rh30/mTOR-rr cells. The results suggest that rapamycin suppresses the phosphatase activity of PP5 in an mTOR-dependent manner.

Rapamycin Causes Dissociation of the PR72 Regulatory Subunit from PP5—PP5 interacts with PP2A-A and -B subunits (40), but how rapamycin impacts the phosphatase activity of PP5 is unknown. PP2A is a heterotrimeric holoenzyme composed of a catalytic subunit (PP2Ac), an A subunit (also termed PR65), and members of the B subunit families, such as B (PR55), B’ (PR61), B” (PR72), and B”’ (PR93/PR110) (60). The phosphatase activity of PP2Ac is modulated by its association with PP2A-A, -B regulatory subunits or 4 protein. To further investigate the mechanism by which inhibition of mTOR by rapamycin down-regulates the phosphatase activity of PP5, serum-starved Rh30 cells were exposed to rapamycin (100 ng/ml) for up to 1 h. Levels of PP5, PP2A-A, PR72, PP2Ac, and β-tubulin (loading control) were determined in cell lysates (Fig. 2A) or following immunoprecipitation with antibodies against PP5, association of PP2A-A, -B subunits (PR72), and 4 protein
was determined by Western blot using respective antibodies. As shown in Fig. 2A, rapamycin treatment did not alter cellular levels of PP5, PP2A-A, PR72, or PP2Ac. As shown in Fig. 2B, in untreated Rh30 cells both PP2A-A and PR72 subunits were associated with PP5 immunoprecipitates. In contrast, e protein was not detected (data not shown). Rapamycin did not affect the interaction of PP5 with the PP2A-A subunit, but caused rapid dissociation of PR72 from the PP5 complex, Fig. 2B. Consistently, rapamycin did not influence the association of PP5 with either PP2A-A or PR72 subunit in rapamycin-resistant Rh30/mTOR-rr cells (Fig. 2B). These findings support the idea that inhibition of mTOR by rapamycin suppresses the phosphatase activity of PP5 by decreasing association of the PR72 regulatory subunit with PP5.

Overexpression of PP5 Inhibits Rapamycin-induced Phosphorylation of c-Jun, Protecting Cells from Death—We next investigated whether overexpression of PP5 or PP2Ac has any impact on rapamycin-activated ASK1 signaling, as well as rapamycin-induced apoptosis. To this end, stable cell lines Rh30/PP2Ac and Rh30/PP5 expressing FLAG-tagged PP2Ac catalytic subunit and FLAG-tagged PP5 were established, respectively (Fig. 3A). Overexpression of PP2Ac or PP5 did not significantly alter either Rh30 growth rate or the inhibitory effect of rapamycin on Rh30 cell proliferation (data not shown). To determine protein phosphatase activities, Rh30/PP2Ac, Rh30/PP5 and parental Rh30 cells (as controls) were serum-starved for 24 h and exposed to rapamycin (100 ng/ml) for 2 h, at which time PP2Ac or PP5 were immunoprecipitated, and phosphatase activities in immunocomplexes determined. As shown in Fig. 3A, overexpression of PP2Ac increased the phosphatase activity of PP2A by ~20%, whereas overexpression of PP5 elevated the phosphatase activity of PP5 by about 75%, in comparison with the basal levels of phosphatase activities in parental Rh30 cells (or pcDNA vector controls). In agreement with previous findings (18), rapamycin increased the PP2A phosphatase activities in both parental Rh30 and Rh30/PP2Ac cells by 60–70%. In contrast, under the same conditions, rapamycin decreased the phosphatase activity of PP5 in parental Rh30 cells by 40–45%. After treatment with rapamycin, the phosphatase activity of PP5 in Rh30/PP5 cells was lowered to a level similar to that in non-treated parental Rh30 cells. The results further support the notion that PP5 differs from PP2A in its regulation by mTOR, being positively regulated.
To further define the role of PP2A and PP5 in the regulation of ASK1 signaling, serum-starved cells were treated with rapamycin (100 ng/ml) for up to 2 h, followed by Western blot analysis with antibodies to phospho-c-Jun and c-Jun, respectively. Consistent with our previous findings (14), rapamycin increased phospho-c-Jun, a consequence of ASK1 activation, in control cells (Rh30/pcDNA) (Fig. 3C). Overexpression of PP5, but not PP2A, blocked the rapamycin-induced phosphorylation of c-Jun (Fig. 3C). As shown in Fig. 4, rapamycin induced apoptosis of control cells (parental Rh30 and Rh30/pcDNA), as seen previously (14). Overexpression of PP2A failed to protect cells from rapamycin-induced death, but overexpression of PP5 completely prevented rapamycin-induced apoptosis (Fig. 4). These results further strengthen the conjecture that PP5 negatively regulates ASK1 signaling, and rapamycin activates ASK1 signaling by suppression of the phosphatase activity of PP5.

Down-regulation of PP5 Activity Requires Expression of 4E-BP1—Previously we demonstrated that cellular stress, characterized by ASK1 activation and increased levels of P-c-Jun, induced by mTOR inhibition (by rapamycin treatment or amino acid starvation) was dependent on the expression of 4E-BP1 (14). To determine if suppression of PP5 activity was similarly dependent on 4E-BP1 expression we used siRNA to down-regulate 4E-BP1 protein expression. Rh30 cells were transfected with piNEO-4E-BP1 plasmid, or control plasmid (piNEO) as described (14). Pools of transfected cells (designated Rh30/piNEO-4E-BP1) expressed very low levels of 4E-BP1, whereas 4E-BP1 levels in the vector-transfected controls (designated Rh30/piNEO) were similar to that of parental Rh30 cells (14, and data not shown). Following exposure to rapamycin (100 ng/ml) or amino acid deprivation for 2 h, there was a significant decrease in the PP5 activity in vector controls, but not in cells where 4E-BP1 was down-regulated (Fig. 5A). The results suggest that mTOR regulates PP5 through 4E-BP1 pathway, which is consistent with our previous findings that rapamycin-activated ASK1 signaling is dependent on 4E-BP1 protein expression (14).

To further define the mechanism by which inhibition of mTOR by rapamycin or amino acid deprivation results in suppression of phosphatase activity of PP5, serum-starved Rh30/piNEO and Rh30/piNEO-4E-BP1 cells were exposed to rapamycin (Fig. 5B, bottom panel) and amino acid deprivation (Fig. 5C) caused...
mTOR-mediated ASK1 Signaling Through PP5

Results presented here indicate that phosphatase activity of PP5 is modulated by mTOR and dependent on 4E-BP1 expression. Inhibition of the phosphatase activity of PP5 by rapamycin or amino acid deprivation was determined only in cells expressing control 4E-BP1 protein levels. Down-regulation of 4E-BP1 abrogated dissociation of the PR72 subunit from PP5 induced by mTOR inhibition. However, how 4E-BP1 affects this process is unclear. Potentially PR72 binding to the PP5 complex may be dependent on ongoing cap-dependent translation, although the kinetics of dissociation are rapid and would seem to preclude this explanation. Alternatively hypophosphorylated 4E-BP1 could directly or indirectly displace PR72 from this complex. However, we have not consistently observed 4E-BP1 in the immunoprecipitated PP5 complex. Clearly, more studies are required to address the question.

In summary, our results demonstrate that PP5 activity is regulated by mTOR and hence appears to be a downstream component of the mTOR signaling pathway. Unlike PP2A, which is negatively regulated by mTOR, PP5 is positively regulated. Inhibition of mTOR by rapamycin or amino acid deprivation does not affect either PP5 protein level or binding of PP5 with ASK1. Consistent with previous findings (35, 40), we found that PP5 is associated with PP2A-A and PR72 subunits. However, in contrast to the catalytic subunit of PP2A, PP5 was not found to associate with α4 protein, suggesting that PP5 may be regulated in a different way. PP2A catalytic subunit was associated with S6K1 (data not shown), but not ASK1, whereas PP5 co-immunoprecipitated with ASK1, but not S6K1 (not shown), also indicating that PP2A and PP5 target different proteins as substrates, and may play specific roles in the regulation of different cellular functions. Our finding that rapamycin suppresses the phosphatase activity of PP5 in an mTOR-dependent manner, and specifically reduces association of PR72 with PP5 support the notion that PP5 is downstream of the mTOR signaling pathway, and unlike PP2A, is positively regulated by mTOR. Rapamycin treatment did not cause dissociation of PR72 in cells expressing a rapamycin resistant mTOR; hence the association of PR72 in the PP5 complex is mTOR-dependent. Recent evidence in yeast suggests that the Tor pathway may also positively regulate the activity of protein phosphatases through Tap42 (47). Our results are the first to suggest a similar function in mammalian cells.

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