Identification of S6 Kinase 1 as a Novel Mammalian Target of Rapamycin (mTOR)-phosphorylating Kinase*

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Here we demonstrate that mammalian target of rapamycin (mTOR) is phosphorylated in a rapamycin-sensitive manner. We show that S6 kinase 1 (S6K1), but not Akt, directly phosphorylates mTOR in cell-free in vitro system and in cells. Expression of a constitutively active, rapamycin- and wortmannin-resistant S6K1 leads to constitutive phosphorylation of mTOR, whereas knock-down of S6K1 using small inhibitory RNA greatly reduces mTOR phosphorylation despite elevated Akt activity. Importantly, phosphorylation of mTOR by S6K1 occurs at threonine 2446/serine 2448. This region has been shown previously to be part of a regulatory repressor domain. These sites are also constitutively phosphorylated in the breast cancer cell line MCF7 carrying an amplification of the S6K1 gene, but not in a less tumorigenic cell line, MCF10a. Many models for Akt signaling to mTOR have been presented, suggesting direct phosphorylation by Akt. These models must be reconsidered in light of the present findings.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family (reviewed in Ref. 1). The bacterial macrolide rapamycin specifically inhibits mTOR signaling to its downstream targets and is thus a useful tool in investigating mTOR function. mTOR regulates cell growth through regulation of protein synthesis by integrating signals from growth factors and hormones such as insulin (2) and nutrients such as amino acids (3, 4).

Signals integrated by mTOR are propagated by its two important downstream effectors, the eukaryotic initiation factor 4E (eIF4E) and the ribosomal S6 kinase 1 (S6K1). eIF4E and S6K1 are sensitive to amino acid levels (3, 5), mediate mTOR-potentiated translation, and contribute to efficient G1 cell cycle progression (7).

Although the mechanism of mTOR regulation by nutrients and mitogens remains to be elucidated, major advances have recently been made. The tumor suppressor protein tuberous sclerosis complex 2 (TSC2) inhibits insulin signaling (8). TSC2 does so by inhibiting the small GTPase Rheb through its GTPase-activating domain (9–13). Rheb positively modulates mTOR function by an as yet unknown mechanism. Several signaling pathways have been found to converge at the level of TSC2, including AMP-activated protein kinase (14), RSK, an effector of the Ras pathway (15), and Akt, an effector of the phosphatidylinositol 3-kinase pathway (16–20). Each of these kinases has been shown to phosphorylate and thereby regulate TSC2.

mTOR contains two conserved phosphorylation sites, threonine 2446 and serine 2448, which conform to the consensus phosphorylation motif of the AGC family of kinases, which includes Akt, RSK, and S6K1. The growth factor stimulation of mTOR function has been correlated to the increase in phosphorylation of serine 2448, and it has been argued that this site is regulated by the phosphatidylinositol 3-kinase/Akt pathway because it is wortmannin-sensitive (21–23). Threonine 2446 phosphorylation has been linked to regulation of mTOR by nutrients (24). Intriguingly, an mTOR antibody whose binding site overlaps these consensus phosphorylation sites stimulated mTOR activity in vitro (25). Moreover, deletion of the region containing these phosphorylation sites, amino acids 2430–2450, enhances mTOR activity. Based on these results, the region containing these phosphorylation sites was defined as a repressor domain (22). However, because nutrient signaling has not been linked to Akt activation and phorbol myristate acetate activates S6K1 without Akt stimulation, we sought to re-investigate the molecular mechanism of mTOR phosphorylation upon stimulation with growth factors and phorbol esters.

EXPERIMENTAL PROCEDURES

Plasmids—We have previously described the generation of the hemagglutinin-S6K1 constructs in pRK7 expression vector (26, 27). FLAG-S6K1 in pRK7 was generated by replacing the tag of hemagglutinin-S6K1 with the FLAG tag. pcDNA3/AU-mTOR constructs were kindly provided by Robert Abraham (Burnham Institute, San Diego, CA) and have been described (28).

Antibodies—Anti-hemagglutinin monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania, Philadelphia). Anti-AU1 antibodies were from Covance. Anti-FLAG monoclonal antibody M2 was purchased from Sigma. Anti-phospho-S6 antibodies were kindly provided by Morris Birnbaum (University of Pennsylvania and Howard Hughes Medical Institute, Philadelphia, PA). Anti-S6K1 antibody was raised against a C-terminal peptide of the protein and described previously (29). Anti-Akt, anti-RRX-RRX-phospho-(S/T), anti-phospho-S6K1, and anti-phospho-Akt antibodies were purchased from Cell Signaling Technology. Anti-mTOR antibodies were from Santa Cruz Biotechnology. Anti-phosphoserine 2448 antibodies were from BIOSOURCE. For immunoblotting, anti-rabbit, anti-mouse, and antigoat horseradish peroxidase-conjugated antibodies were purchased from Amersham Biosciences, Chemicon, and Santa Cruz Biotechnology, respectively.

Cell Culture and Transfections—Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. MCF10a cells were from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium/F12 supplemented with 5% horse serum, 0.02 µg/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin, and 10 µg/ml insulin. For transfection studies, HEK293E cells were transfected using calcium phosphate.
3 × 10^6 cells were seeded in a 100-mm dish 3–4 h prior to transfection. Cells were incubated with the calcium phosphate-DNA mixture overnight, washed once with calcium-buffered saline, and then the medium was replaced or the cells were starved in serum-free Dulbecco's modified Eagle's medium for 16–24 h prior to lysis. Lysates were prepared at 48 h post-transfection. For the small inhibitory RNA (siRNA) studies, 21 nucleotide complementary RNAs with symmetrical two-nucleotide overhangs were obtained from Qiagen. HEK293E cells were transfected using the calcium phosphate method described above with duplexes targeting 5'-AAGAGGATATCCTTTCATTA-3' and 5'-CAGAGAGTCAATGTCATTACA-3' sequences of S6K1 or a scrambled control duplex.

Cell Extract Preparation—Cells were stimulated as indicated in the legends to Figs. 1–6 and lysed in 300 μl of lysis buffer (10 mM KPO4, 1 mM EDTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40, 0.1% Brij 35, 1 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml pepstatin, pH 7.28) for 1 h at room temperature. Kinase activity toward mTOR in washed immunoprecipitates was assayed in a reaction containing eluted S6K1 and 20 mM HEPES, 10 mM MgCl2, 50 μM unlabeled ATP, 5 μCi of [γ-32P]ATP (PerkinElmer Life Sciences), and 3 ng/μl protein kinase A inhibitor, pH 7.2, for 20 min at 30 °C. Reactions were subjected to electrophoresis on 4–12% SDS-polyacrylamide gels, and the amount of 32P incorporated into mTOR was assessed by autoradiography using phosphorimaging analysis (Bio-Rad).

RESULTS

Given the regulation of mTOR phosphorylation by nutrients and phorbol esters, our initial goal was to re-examine the role of Akt in mTOR phosphorylation by insulin-mediated signaling in HEK293E cells. The analysis of phosphorylation employed an antibody recognizing the RRXX(Phospho-(S/T)] motif. As shown in Fig. 1A, phosphorylation of mTOR at sites recognized by this antibody was strongly stimulated by insulin and inhibited by wortmannin as reported previously (21–23). Intriguingly, we also found that rapamycin strongly inhibited this mTOR phosphorylation. Importantly, Akt remained active following treatment with rapamycin (Fig. 1A), indicating a possible Akt-independent mechanism for mTOR phosphorylation. We next confirmed our observations using an antibody against phosphoserine 2448, a site regulated by growth factor stimulation. As shown in Fig. 1B, staining with this antibody as well indicated that phosphorylation of mTOR is rapamycin-sensitive. To test whether phosphorylation of mTOR could be induced in an Akt-independent manner, we stimulated cells with phorbol 12-myristate 13-acetate (PMA), which has been previously shown to lead to phosphorylation of RRXX(Phospho-(S/T)] motifs by basophilic kinases other than Akt (15, 30). As shown in Fig. 1C, PMA treatment was as effective as insulin in stimulating mTOR phosphorylation, whereas Akt was not activated by PMA, ruling out direct involvement of Akt. In addition, rapamycin sensitivity suggested that mTOR phosphorylation was not due to direct phosphorylation by the PMA-induced basophilic kinases, RSK, or protein kinase C isoforms.

![Image](https://www.jbc.org/content/269/52/26090/F1.large.jpg)
Akt phospho-S473 (S6K1), total Akt, and phospho-S6 (mTOR). Total cell extracts were analyzed for levels of Au1-mTOR. Levels of mTOR in the immunoprecipitates were analyzed by Au1 antibody (Au1-mTOR). Total cell extracts were analyzed for levels of Akt phospho-S473 (P-Akt), total Akt, and phospho-S6 (P-S6).

The rapamycin-sensitivity of mTOR phosphorylation observed in Fig. 1, A–C indicated that either mTOR itself or a kinase downstream of mTOR mediated its phosphorylation. We then asked if the rapamycin-sensitivity of phosphorylation was due to mTOR-mediated autophosphorylation. As shown in Fig. 2, phosphorylation occurred in the kinase-inactive allele of mTOR as well as in the wild-type protein in a rapamycin-sensitive manner. This indicated that a kinase downstream of mTOR might be responsible for mTOR phosphorylation.

Based on these results, we investigated whether an mTOR target, S6K1, was responsible for mTOR phosphorylation. First, we examined whether S6K1 could phosphorylate mTOR in a cell-free system. Indeed, activated S6K1 robustly phosphorylated purified mTOR (Fig. 3A). We then determined whether S6K1 regulated mTOR phosphorylation in cells. Fig. 3B depicts the location of mutations in the alleles of S6K1 used in this experiment. As shown in Fig. 3C, overexpression of a wild-type allele of S6K1 led to an increase in basal phosphorylation of mTOR as compared with control overexpression of a kinase-inactive S6K1 (containing a K100R mutation). Once again, phosphorylation was rapamycin-sensitive. Overexpression of an activated rapamycin- and wortmannin-resistant allele of S6K1 (F5A-E389-R3A) (31) led to constitutive phosphorylation of mTOR that was rapamycin- and wortmannin-insensitive. Taken together, these results suggested that S6K1 directly phosphorylates mTOR in both cell-free and cell-based systems.

To verify the contribution of S6K1 to mTOR phosphorylation in cells, we eliminated S6K1 expression by means of siRNA technology. As shown in Fig. 4A, siRNA against S6K1 led to a significant inhibition of S6K1 expression and, concurrently, potently reduced mTOR phosphorylation as compared with the scrambled control. This inhibition of mTOR phosphorylation was seen when immunoblotting with the antibody recognizing the RXRXX-phospho-(S/T) motif as well as the antibody against phosphoserine 2448. Reduction of S6K1 levels also resulted in elevated Akt activation. These results were quantified in Fig. 4B. Together, these data solidify the notion that endogenously expressed S6K1, and not Akt, is the mTOR kinase.

Because both threonine 2446 and serine 2448 are found in the context of a motif that could be phosphorylated by S6K1 (Fig. 5A), we evaluated the relative contribution of these sites to mTOR phosphorylation by S6K1. As shown in Fig. 5B, mutation of each of these sites decreased the phosphorylation of mTOR, as detected by the antibody recognizing the RXRXX-phospho-(S/T) motif by ~50% relative to phosphorylation levels in the wild-type protein, whereas mutation of both sites completely eliminated this phosphorylation. The results of this experiment are quantified in Fig. 5C. It is important to mention that whereas phosphorylation of the wild-type and the single-site mutants was rapamycin-sensitive, phosphorylation of the rapamycin-resistant mTOR was partially refractory to rapamycin, further proving that a downstream kinase, i.e., S6K1, is responsible for mTOR phosphorylation.

MCF7 breast cancer cells carry an amplification of the chromosomal region containing the RPS6KB1 gene encoding for the S6K1 protein (32, 33). Thus, we hypothesized that in MCF7 cells mTOR would be hyperphosphorylated. We therefore compared the status of phosphorylation of proteins in the mTOR pathway between MCF7 cells and in an immortalized, non-transformed breast cell line, MCF10a. As shown in Fig. 6A, in growing MCF7 cells the levels of S6K1 expressed were much greater than those in MCF10a cells. Moreover, the difference in phosphorylation of S6K1 on threonine 389 (mTOR-regulated site) was even greater, also reflected by the level of phosphorylation of the S6K1 target protein S6. The levels of Akt, however, were similar in both cell lines, and Akt exhibited similar extent of activation. Significantly, mTOR was present in a rapamycin-sensitive, highly phosphorylated form in MCF7 cells, whereas mTOR phosphorylation in MCF10a cells was below our detection limits. These data are quantified in Fig. 6B. Thus, the status of S6K1 expression in breast cancer cell lines MCF7 and MCF10a strongly correlates with the phosphorylation of mTOR on the Thr-2446/Ser-2448 motif.

**DISCUSSION**

Previous work has provided evidence that Akt directly phosphorylates mTOR at Thr-2446/Ser-2448 in vitro and in vivo. The in vivo results were based on loss of phosphorylation at these sites in the presence of the phosphatidylinositol 3-kinase inhibitor wortmannin and increased phosphorylation with expression of a constitutively activated Akt (21, 34). Based on these experiments, the conclusion that Akt might be the mTOR kinase was sound; however, these experimental conditions are now known to also regulate the activity of mTOR effectors such as S6 kinase 1, which possesses similar regulation and biochemical properties such as sensitivity to wortmannin following activation from PI3K inputs (35) and a similar basopholic phosphorylation motif (1, 36). Furthermore, Akt also contributes to phosphatidylinositol 3-kinase-mediated S6K1 activation through Akt-dependent phosphorylation and inactivation of the Rheb-GTPase-activating protein TSC2 (16–20).

In this study we provide evidence that S6K1 and not Akt is responsible for Thr-2446/Ser-2448 phosphorylation. First, phosphorylation at Thr-2446/Ser-2448 is sensitive to rapamycin, which antagonizes S6K1 but not Akt activation. Second, tumor-promoting phorbol esters, such as phorbol myristate acetate, activate S6K1 without Akt activation and stimulate Thr-2446/Ser-2448 phosphorylation. Third, expression of a constitutively activated, rapamycin-resistant mutant of S6K1 results in constitutive and rapamycin-resistant Thr-2446/Ser-2448 phosphorylation. Fourth, RNA interference-mediated knockdown of S6K1 results in a reduction of mTOR Thr-2446/Ser-2448 phosphorylation. Finally, human breast MCF7 cancer cells with amplified S6K1 exhibit high level, rapamycin-sensitive mTOR phosphorylation.

These results clearly support the model presented in Fig. 7. In contrast to what is published elsewhere, Akt is likely not to regulate mTOR Thr-2446/Ser-2448 phosphorylation directly. Instead, phosphorylation of these sites is regulated by S6K1. These results suggest that the main route for Akt signaling to S6K1 is mediated via Akt-dependent phosphorylation and inactivation of the Rheb GTPase-activating protein TSC2 (16–20). TSC2 can also be inactivated by the Ras pathway via the RSK (15). The result of TSC2 inactivation by Akt and/or RSK is activation of Rheb and mTOR signaling. One mTOR effector is S6K1, which in turn phosphorylates mTOR as part of a poten-
tial feedback loop. At this time we cannot determine whether this is a positive, negative, or inconsequential regulatory phosphorylation. Based on many studies, we can only hypothesize that mTOR phosphorylation within this region may participate in positive feedback signaling to mTOR. This hypothesis is based on the observation that deletion of the region containing the phosphorylation motif, but not mutation of the individual residues, increases mTOR activity. It has been postulated that the region contained within amino acids 2430–2450 constitutes a repressor domain (22). Moreover, because this region contains several additional serine residues, it has been proposed that other inputs, in addition to phosphorylation of Thr-2446/Ser-2448 by S6K1, are part of the mechanism involved in inhibiting this repressor domain. Additional mapping experiments will be necessary to identify these other phosphorylation sites and consequences of cumulative phosphorylation.

Another area of investigation is to determine whether both

FIG. 3. S6K1 can phosphorylate mTOR in a cell-free system and in cells. A, S6K1 phosphorylates mTOR (P-mTOR). mTOR was immunoprecipitated from starved HEK293E cells. FLAG-S6K1 was immunoprecipitated from insulin-stimulated cells and eluted with FLAG peptide. mTOR beads were incubated with either control elution or with S6K1 eluted from FLAG beads. The reactions were resolved by SDS-PAGE and analyzed by autoradiography. B, schematic representation of the mutations in the S6K1 alleles used. K100R mutation creates a kinase-dead S6K1, and the combination of F5A-Glu-389-R3A (F5A-E389-R3A) creates an activated kinase. C, S6K1 phosphorylates mTOR in cells. HEK293E cells were transfected with kinase-dead, wild-type, or activated alleles of S6K1, serum-starved for 16–18 h, and stimulated with 100 nM insulin for 30 min or pre-treated for 30 min with either rapamycin (Rap; 20 ng/ml) or wortmannin (WM; 100 nM). mTOR was immunoprecipitated and immunoblotted for phosphorylation on the consensus motif RXRXX-phospho-(S/T) (P-mTOR). The nitrocellulose membrane was stripped and re-probed for total mTOR levels. Total cell extracts were analyzed for levels of Akt phosphoryserine 473 (P-Akt), phospho-S6 (P-S6), S6K1 phosphothreonine 389 (P-S6K1), S6K1, and Akt.

FIG. 4. Knock-down of S6K1 leads to inhibition of mTOR phosphorylation. A, HEK293E cells were transfected with either scrambled (Scr) or S6K1 siRNA. mTOR was immunoprecipitated and immunoblotted for phosphorylation of mTOR (P-mTOR) using antibodies directed against the consensus motif RXRXX-phospho-(S/T) (α-RXRXXpS/T) and phosphoserine 2448 (α-pS2448). The nitrocellulose membrane was stripped and re-probed for total mTOR levels. Total cell extracts were analyzed for levels of S6K1, Akt, phosphoserine 473 (P-Akt), and Akt. B, quantitative representation of the results in panel A. Phosphorylation levels of mTOR and Akt were assessed by densitometry, normalized to total levels of these proteins, and presented in a histogram.

FIG. 5. mTOR is phosphorylated on the important residues Thr-2446 and Ser-2448. A, sequence context of Thr-2446 and Ser-2448. B, HEK293E cells were transfected with the alleles of mTOR, namely wild-type (WT), S2448A, T2446A, S2448A/T2446A (TSAA), kinase-dead (KD), and rapamycin-resistant (RR) mTOR, serum-starved for 16–18 h, and treated with 100 nM insulin for 30 min or pre-treated for 30 min with rapamycin (Rap; 20 ng/ml). mTOR was immunoprecipitated and immunoblotted for phosphorylation on the consensus motif RXRXX-phospho-(S/T) (P-mTOR). The nitrocellulose membrane was stripped and re-probed for total mTOR levels. C, quantitative representation of the results in panel B. Phosphorylation levels of mTOR using antibodies directed against the consensus motif RXRXX-phospho-(S/T) were assessed by densitometry, normalized to total mTOR protein levels, and presented in a histogram.
PKC as well as through activation of S6K1 by protein kinase C (independently of Akt through inhibition of the TSC1/2 complex via activation of mTOR via Rheb. PMA can lead to mTOR phosphorylation through inhibition of the TSC1/2 complex and activation of mTOR phosphorylation at these sites, under various biological conditions, on mTOR function.

FIG. 6. mTOR is phosphorylated in breast cancer MCF7 cell line but not in less tumorigenic MCF10a cells. A, cells were grown in full media or treated with rapamycin (Rap; 20 ng/ml) for 24 h where indicated. mTOR was immunoprecipitated and immunoblotted for phosphorylation on the consensus motif RXXXX-phospho-(S/T) (P-mTOR). The nitrocellulose membrane was stripped and re-probed for total mTOR levels. Total cell extracts were analyzed for levels of Akt phospho-S473 (P-Akt), phospho-S6 (P-S6), S6K1 phosphothreonine 389 (P-S6K1), S6K1, and Akt. p70 and p85 denote the isoforms of S6K1 recognized by the S6K1 and S6K1 phosphothreonine 389 antibodies. B, quantitative representation of the results in panel A. Phosphorylation levels of mTOR (P-mTOR), S6K1 (P-S6K1), and Akt (P-Akt) were assessed by densitometry, normalized to total protein levels, and presented in a histogram.

FIG. 7. Model of mTOR phosphorylation. S6K1 directly phosphorylates mTOR on residues Thr-2446/Ser-2448. Akt may contribute to mTOR phosphorylation through inhibition of the TSC1/2 complex and activation of mTOR via Rheb. PMA can lead to mTOR phosphorylation independently of Akt through inhibition of the TSC1/2 complex via RSK1 as well as through activation of S6K1 by protein kinase C (PKC).

threonine 2446 and serine 2448 are simultaneously phosphorylated by S6K1 or if serine 2448 is preferred. Data from Cheng et al. (24) suggest that AMP-activated protein kinase can phosphorylate threonine 2446 and that this prevents serine 2448 phosphorylation under nutrient starvation conditions. Because S6K1 is normally not activated under these conditions, it is difficult to determine the significance of this data with regard to nutrient and growth factor signaling where AMP-activated protein kinase would presumably be inactive. It is possible that dual phosphorylation of mTOR by S6K1 under nutrient-rich and growth factor-stimulated conditions alters mTOR function in a manner very different from single phosphorylation at threonine 2446 as regulated by AMP-activated protein kinase under nutrient-deprived conditions. It is also possible that under certain disease conditions both pathways could be activated. Thus, it will be important to continue to investigate the role of mTOR phosphorylation at these sites, under various biological conditions, on mTOR function.

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