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Identification of a Conserved Motif Required for mTOR Signaling

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Summary

Background: The mammalian target of rapamycin (mTOR) controls the translation machinery via activation of S6 kinases 1 and 2 (S6K1/2) and inhibition of the eukaryotic initiation factor 4E (eIF4E) binding proteins 1, 2, and 3 (4E-BP1/2/3). S6K1 and 4E-BP1 are regulated by nutrient-sensing and mitogen-activated pathways. The molecular basis of mTOR regulation of S6K1 and 4E-BP1 remains controversial.

Results: We have identified a conserved TOR signaling (TOS) motif in the N terminus of all known S6 kinases and in the C terminus of the 4E-BPs that is crucial for phosphorylation and regulation S6K1 and 4E-BP1 activities. Deletion or mutations within the TOS motif significantly inhibit S6K1 activation and the phosphorylation of its hydrophobic motif, Thr389. In addition, this sequence is required to suppress an inhibitory activity mediated by the S6K1 C terminus. The TOS motif is essential for S6K1 activation by mTOR, as mutations in this motif mimic the effect of rapamycin on S6K1 phosphorylation, and render S6K1 insensitive to changes in amino acids. Furthermore, only overexpression of S6K1 with an intact TOS motif prevents 4E-BP1 phosphorylation by a common mTOR-regulated modulator of S6K1 and 4E-BP1.

Conclusions: S6K1 and 4E-BP1 contain a conserved five amino acid sequence (TOS motif) that is crucial for their regulation by the mTOR pathway. mTOR seems to regulate S6K1 by two distinct mechanisms. The TOS motif appears to function as a docking site for either mTOR itself or a common upstream activator of S6K1 and 4E-BP1.

Introduction

mTOR, the mammalian target of rapamycin (also referred to as FRAP, RAFT, and RAPT) [1] is a member of a superfamily of protein serine/threonine kinases termed PIK-related kinases [2]. TOR from budding yeast and *Drosophila* functions to monitor nutrient availability, as TOR deletions in these organisms result in physiological changes characteristic of nutrient deprivation [3]. A role for mTOR in a nutritional checkpoint is most likely conserved in mammals, as downstream targets of mTOR, such as the ribosomal S6 kinase1 (S6K1), and the eukaryotic initiation factor 4E (eIF4E) binding protein1 (4E-BP1) are sensitive to amino acid levels [4].

In mammalian cells, it is believed that mTOR participates in the regulation of cell growth and proliferation through regulation of translation via the S6Ks 1 and 2 and the 4E-BPs 1, 2, and 3 [5]. In quiescent cells, hypophosphorylated 4E-BP1 binds to eIF4E and inhibits eIF4E-dependent translation of CAP containing mRNAs with highly structured 5′ untranslated regions (UTR) [5]. This subset of mRNAs tends to encode proteins important for cell proliferation, such as c-Myc and cyclin D1 [6]. S6K1 and S6K2 phosphorylate the 40S ribosomal protein S6, which may enable the efficient translation of a subset of mRNAs containing a terminal oligopolypyrimidine (TOP) track at the 5′ end. Many of these mRNAs encode proteins of the translational machinery, such as ribosomal proteins and elongation factors [7].

The work from mammalian and *Drosophila* systems suggests that S6K1 and 4E-BP1 are capable of integrating signaling from mitogen- and nutrient-regulated pathways [5]. Nutrient-dependent signaling is mediated by mTOR, while mitogen-dependent signaling is mediated through (PI3K)-dependent and -independent inputs. The mTOR inhibitor rapamycin blocks S6 kinase activation and full 4E-BP1 phosphorylation by all agonists, demonstrating the requirement for an intact mTOR signaling pathway [5, 8, 9].

The mechanisms by which mTOR regulates S6K1 and 4E-BP1 phosphorylations are controversial. It is not clear whether mTOR directly phosphorylates S6K1and 4E-BP1, activates intermediate kinases, or inhibits a phosphatase [10]. Studies using rapamycin have shown that mTOR regulates the phosphorylation of several sites in S6K1, namely, Thr389, Thr229, and Ser404 [11, 12]. Thr389 appears to be the main target of mTOR regulation in S6K1, because it is rapidly and completely dephosphorylated after rapamycin treatment [13].

Here we have focused on how mTOR signals to its downstream target, S6K1. We have identified a highly conserved sequence in S6K1 that is essential for mTOR-dependent signaling. We refer to the sequence as the TOR signaling (TOS) motif. Through the use of a variety of S6K1 deletion and phosphorylation site mutants, we show that mTOR regulation of S6K1 involves two distinct regions of the protein. One input through the N-terminal TOS motif leads to the phosphorylation of Thr389, and the other one requires the TOS motif to release a negative regulatory activity mediated by the C terminus of S6K1. The importance of the TOS motif in mTOR signaling is underscored by its identification in other mTOR-regulated proteins. The TOS motif is also conserved throughout evolution in 4E-BPs. Mutation of the domain in 4E-BP1 similarly blocks its mitogen-dependent phosphorylation.

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Results

Identification of a Conserved Region in the N Terminus of S6K1 that Is Essential for Its Activation

Deletion of the N-terminal 30 amino acids in the S6K1 αII isoform (or 53 amino acids in the αI isoform) inhibits S6K1 activity and phosphorylation as does the mTOR inhibitor rapamycin [11, 14, 15]. Thus, the N terminus of S6K1 has been suggested to participate in its regulation by the mTOR pathway. S6 kinases from all species tested to date are rapamycin sensitive and therefore regulated by TOR, indicating the possibility that a conserved N-terminal domain might mediate the regulation of S6 kinases by TOR [16–20]. While the amino acid sequence of the N terminus is highly divergent between S6K1 and S6K2 and across species, there exists a five amino acid sequence in S6K1, FDIDL (amino acids 5–9), that is evolutionary conserved (Figure 1A). To determine the importance of this sequence for S6K1 function, we deleted it in S6K1 (S6K1-ΔNT2) and tested kinase activity in starved, insulin-stimulated, or rapamycin-treated cells. Similar to the effect of deleting the first 30 amino acids in S6K1 (S6K1-ΔNT) [14, 15], deletion of the conserved N-terminal sequence almost completely abrogated the activity of S6K1 (Figure 1B). Similar results were seen for the deletion of the conserved sequence in S6K2 (data not shown).

Next we introduced point mutations in S6K1 within this conserved sequence to determine which amino acids are critical for the regulation of S6K1 activity. We mutated the bulky phenylalanine residue number 5 (numbering for rat S6K1 αII isoform) to alanine (F5A), the two charged aspartic acid residues numbers 6 and 8 to alanines (D6/8A), or the hydrophobic leucine and isoleucine residue numbers 7 and 9 to alanine (L7/9A). Each of the introduced point mutations had a dramatic inhibitory effect on S6K1 activity (Figure 1C). These results demonstrate that this highly conserved N-terminal domain is essential for S6K1 activation and that several of its residues are required for S6K1 activity. We refer to this sequence as the TOS motif and provide evidence that it is indeed required for mTOR signaling. Since the F5A mutation generated a mutant allele essentially devoid of any activity and therefore mimics the loss of the TOS motif, we used this construct for further characterization.

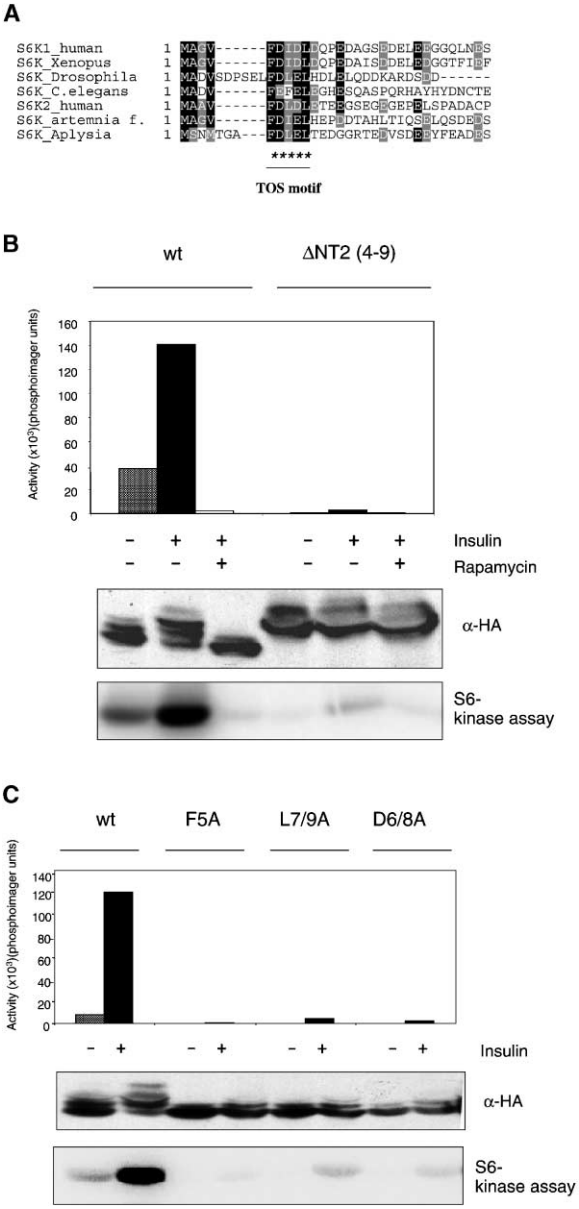

Figure 1. Identification of a Conserved Domain in the N Terminus of S6 Kinases Essential for Kinase Activity

(A) Sequence alignment of the first 30 amino acids of S6K1 (human), S6K (*Xenopus laevis*), S6K (*Drosophila melanogaster*), S6K (*C. elegans*), S6K2 (human), S6K (Artemnia fransiscana), and S6K (*Aplysia*). Identical (black box) and conserved (shadowed box) amino acids are highlighted. HEK293 cells were transfected with (B) HA-S6K1 wild-type (wt) or HA-S6K1-ΔNT2 (ΔNT2) and (C) HA-S6K1 wild-type (wt), HA-S6K1-F5A (F5A), HA-S6K1-L7/9A (L7/9A), or HA-S6K1-D6/8A (D6/8A). Transfected cells were starved, stimulated, and lysed as described in Experimental Procedures. Protein expression levels were assayed by immunoblotting with α-HA-antibody (second panel), and kinase assays were performed as described in Experimental Procedures. Quantification of kinase assay (upper panel) and autoradiogram of in vitro S6 phosphorylation (lower panel).

The F5A Mutation Inhibits Phosphorylation of S6K1 at Thr389 and Thr229

Since S6K1 activation correlates with multisite phosphorylation, we next determined which phosphorylation events of S6K1 required an intact TOS motif. To do this, we employed phosphospecific antibodies raised against various S6K1 phosphorylation sites (Figure 2). The F5A mutation completely inhibited insulin-stimulated Thr389 phosphorylation (Figure 2). The effect was similar to the inhibition of Thr389 phosphorylation observed with rapamycin [13]. The F5A mutation significantly but not completely inhibited Thr229 phosphorylation (Figure 2). The inhibition of Thr229 phosphorylation could be a primary effect of the F5A mutation or a secondary effect caused by the inhibition of Thr389 phosphorylation and/or activation of a phosphatase. The F5A mutation did not significantly affect the phosphorylation of the proline-directed Ser371 site, also located in the linker region

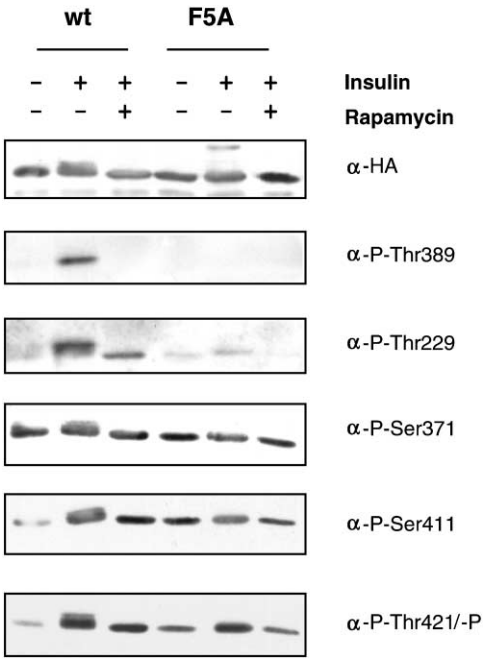

Figure 2. The F5A Mutation Inhibits Thr389 and Thr229 Phosphorylation

HEK293 cells were transfected with HA-S6K1 wild-type (wt) or HA-S6K1-F5A (F5A). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in Experimental Procedures. Protein expression levels were assayed by immunoblotting with α-HA-antibody (upper panel). For evaluation of the in vivo phosphorylation of Thr389, Thr229, Thr371, Ser411, and Thr421/Ser424, HA-S6K1 and HA-S6K1-F5A were immunoprecipitated with an anti-HA-antibody, and phosphorylation was evaluated by immunoblotting with α-S6K1-P-Thr389 phosphopeptide-specific antibody, α-S6K1-P-Thr229 phosphopeptide-specific antibody, α-S6K1-P-Thr371 phosphopeptide-specific antibody, α-S6K1-P-Ser-411 phosphopeptide-specific, and α-S6K1-P-Thr421/Ser424 phosphopeptide-specific antibody.

(Figure 2). Phosphorylation of the proline-directed C-terminal phosphorylation sites Ser411, Thr421/Ser424 were also not significantly affected by the F5A mutation under these conditions (Figure 2). The phosphorylation pattern of these sites in F5A is comparable to their phosphorylation in S6K1-ΔNT [11] and in rapamycin-treated wild-type S6K1 [13, 21], which is once again consistent with the TOS motif being critical for mTOR signaling to S6K1.

The F5A Point Mutation Mimics Deletion of the N Terminus

We wanted to determine if the F5A point mutation mimicked the effect of deleting the N terminus of S6K1. It has been reported that the additional deletion of the C terminus rescues the inhibitory effect of the N-terminal deletion and renders S6K1 (S6K1ΔNT/CT) completely rapamycin resistant [14, 15]. Deletion of the C terminus in the F5A background (F5A-ΔCT) only partially restored S6K1 activity, which, like S6K1-ΔNT/CT, was completely rapamycin resistant (Figure 3A). We compared the insulin-stimulated activities of S6K1-ΔNT/CT to S6K1 wild-type and S6K1-ΔCT. Like F5A-ΔCT, S6K1-ΔNT/CT has only about 5%–15% of the insulin-stimulated S6K1 wild-type kinase activity, whereas S6K1-ΔCT has about 50%–75% of the wild-type activity. Importantly, S6K1-ΔCT, unlike the wild-type protein, is partially rapamycin resistant (Figure 3B and [14, 15]). The activity of S6K1-ΔCT in the presence of rapamycin is comparable to the insulin-stimulated, rapamycin-resistant activity of S6K1-ΔNT/CT and F5A-ΔCT, suggesting that the deletion of the C terminus is contributing to the observed rapamycin resistance of S6K1-ΔCT and S6K1-ΔNT/CT (Figure 3B). These data suggest that deletion of the C terminus might release a rapamycin-sensitive inhibitory function, resulting in a mitogen-dependent but mTOR-independent activation of S6K1. The N terminus appears to mediate a positive mTOR-regulated input that is absent in S6K1-ΔNT and S6K1-ΔNT/CT but still inhibited by rapamycin in S6K1-ΔCT. Similar results were seen for N- and/or C-terminal truncation mutants of S6K2 (data not shown). Thus, the single F5A point mutation mimicked the effect of deleting the entire N-terminal domain (Figure 4 and [14, 15]).

Since the F5A mutation had the most severe effect on Thr389 phosphorylation (Figure 2), we wanted to determine if mutation of this site to an acidic residue to mimic phosphorylation was sufficient to overcome the inhibitory effect of the F5A mutation on S6K1 kinase activity. Replacing Thr389 with glutamic acid (E389) partially restored the activity of the F5A mutant (F5A-E389), and this was largely rapamycin resistant (Figure S2 in the Supplementary Material available with this article online).

We next combined the C-terminal deletion with the E389 point mutation in wild-type and F5A S6K1s, creating E389-ΔCT and F5A-E389-ΔCT, respectively. Deleting the C terminus and introducing the Thr389Glu mutation into F5A completely restored its activity to the level of the stimulated wild-type enzyme and conferred complete rapamycin resistance, making it indistinguishable from E389-ΔCT (Figure 3C). The data suggest that signaling through the TOS motif is required for Thr389 phosphorylation and for the inhibition of a negative regulatory effect mediated by the C terminus. In contrast to F5A-E389ΔCT, F5A-E389D_3E, only partially rescued the effect of the F5A mutation, and this mutant was still partially rapamycin sensitive (Figure S2). Thus, overcoming the inhibitory effect of the C terminus appears to require more than phosphorylation of known C-terminal sites.

The S6K1 F5A Mutation Prevents Amino Acid Signaling via mTOR toward S6K1

To further investigate the role of the TOS motif in specifically mediating mTOR signaling to S6K1, we wanted to determine if the F5A mutation makes S6K1 insensitive to changes in amino acid levels. Amino acid withdrawal selectively inhibits mTOR signaling similar to rapamycin. Mitogen-regulated signaling pathways that are involved in S6K1 regulation, like the PI3K and MAPK pathways, are not sensitive to changes in amino acids [22–24].

Here we used F5A-ΔCT instead of F5A, because the F5A allele has no activity. If S6K1ΔCT is sensitive to changes in the level of amino acids but F5A-ΔCT is not, we could conclude that the TOS motif is necessary for

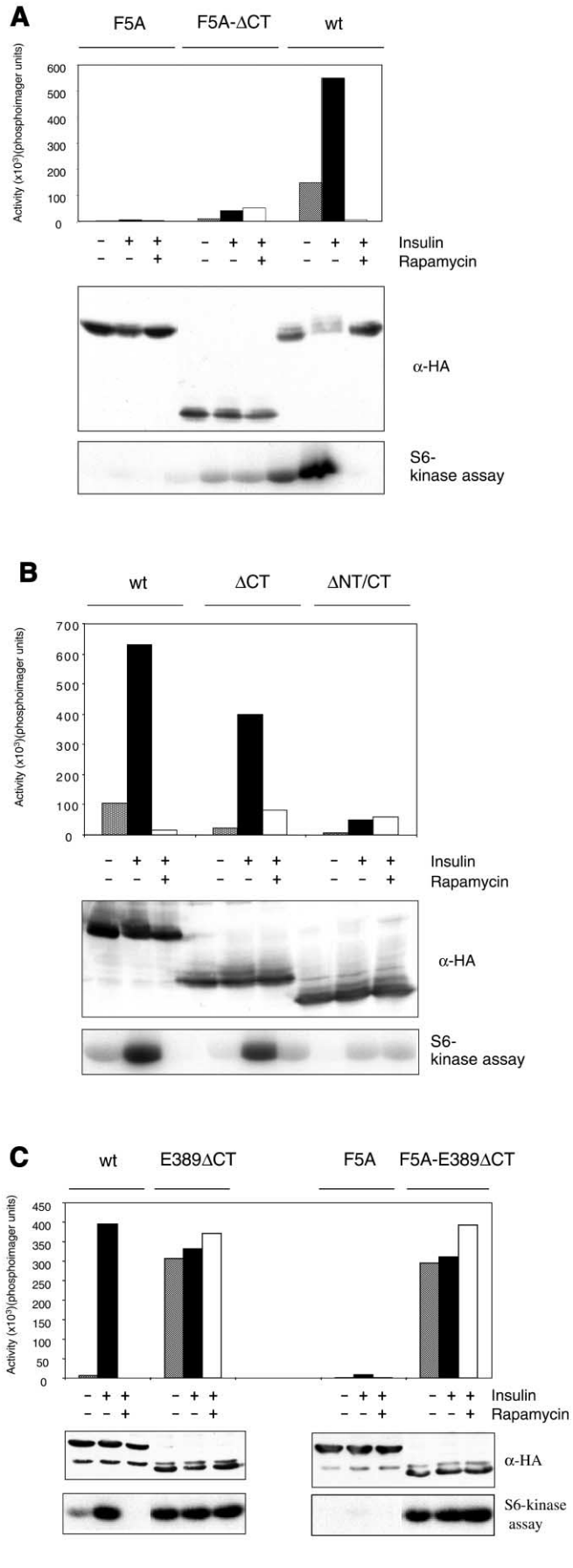

Figure 3. The F5A Point Mutation Mimics Deletion of the 30 Amino Acids, and Its Activity Is Partially Rescued by Deletion of the C Terminus

HEK293 cells were transfected with (A) HA-S6K1-F5A (F5A), S6K1-F5A-ΔCT (F5A-ΔCT), or HA-S6K1 wild-type (wt); (B) HA-S6K1 wild-type (wt), HA-S6K1ΔCT (ΔCT), or S6K1-ΔNT/CT (ΔNT/CT); and (C) HA-S6K1 wild-type (wt), HA-S6K1- E389ΔCT (E389ΔCT), HA-S6K1-F5A (F5A), and HA-S6K1-F5A- E389ΔCT (F5A- E389ΔCT). Transfected cells were starved, stimulated, and lysed as described in Experimental Procedures. Protein expression levels were assayed by immunoblotting with α-HA-antibody (second panels), and kinase assays were performed as described in Experimental Procedures. Quantification of kinase assay (upper panels) and autoradiogram of in vitro S6 phosphorylation (lower panels).

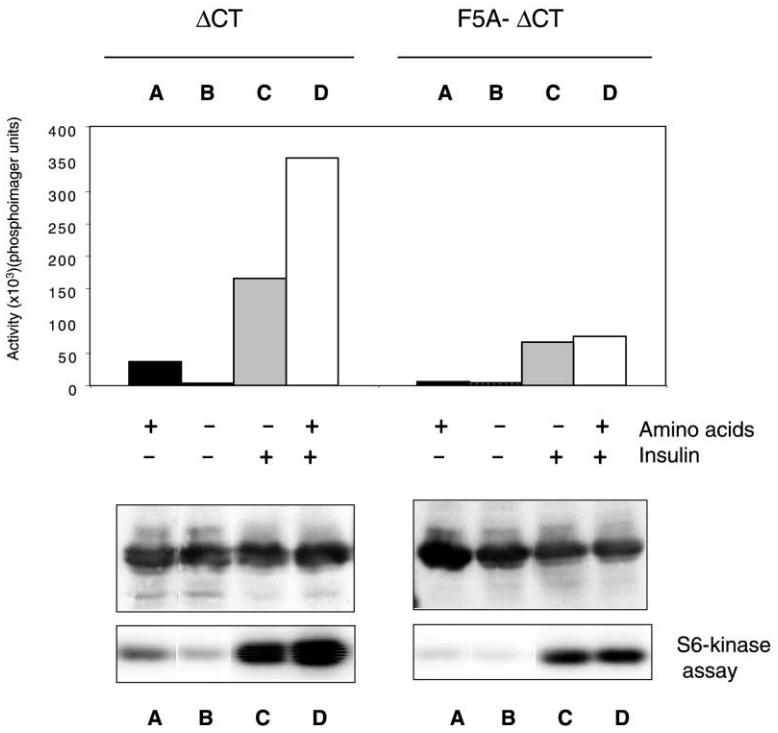

Figure 4. The F5A Mutation Blocks Amino Acid Signaling via mTOR toward S6K1

HEK293 cells were transfected with HA-S6K1ΔCT (ΔCT) or HA-S6K1-F5A-ΔCT (F5A-ΔCT). Transfected cells were stimulated and lysed as described in Experimental Procedures. Protein expression levels were assayed by immunoblotting with α-HA-antibody (middle panels), and kinase assays were performed as described in Experimental Procedures. Quantification of kinase assay (upper panels) and autoradiogram of in vitro S6 phosphorylation (lower panels).

amino acid signaling toward S6K1 and therefore responsible for its regulation by mTOR. S6K1ΔCT and F5A-ΔCT were modestly activated by insulin after 60 min of amino acid withdrawal. A more robust activation of S6K1ΔCT was seen with a combination of amino acids and insulin (Figure 4), consistent with the model that S6K1 requires a nutrient-sensing and a growth factor-mediated input for its full activation. F5A-ΔCT (Figure 4), like ΔNT/CT (data not shown and [24]), did not show a significant difference between activation of these alleles by insulin only or a combination of insulin and amino acids. These data show mutations in the TOS motif make S6K1 insensitive to changes in amino acids, demonstrating that this sequence is essential for mTOR signaling toward S6K1.

4E-BP1 Phosphorylation Is Inhibited by Overexpression of S6K1 with an Intact TOS Motif

We wanted to further establish the role for the TOS motif in mTOR signaling to S6K1. Previous studies suggest that 4E-BP1 and S6K1 share a limiting, rapamycin-sensitive regulator that functions as a bifurcation point immediately upstream of S6K1 [25]. Insulin-stimulated phosphorylation of 4E-BP1, as assessed by retarded band migration on SDS-PAGE immunoblots, is inhibited by overexpression of S6K1. Thus, overexpression of S6K1 appears to sequester a common S6K1 and 4E-BP1 regulator. Inhibition of the 4E-BP1 and S6K1 phosphorylation by ectopic S6K1 overexpression seems to be specific for the mTOR pathway, because a similar inhibition of 4E-BP1 and S6K1 phosphorylation is seen with rapamycin [25].

We hypothesized that the TOS motif might mediate signaling by this common regulator and that the F5A mutation would prevent S6K1 from sequestering an upstream mTOR regulator. If this hypothesis is correct, then overexpression of the F5A mutant should not inhibit 4E-BP1 phosphorylation. The 4E-BP1 mobility shift upon insulin stimulation was inhibited by rapamycin (Figure 5). Like rapamycin treatment, overexpression of S6K1 wild-type, kinase dead (K100R), or E389D_3E constructs inhibited insulin-induced 4E-BP1 phosphorylation. In contrast, overexpression of F5A or F5A-E389D_3E mutants did not inhibit 4E-BP1 phosphorylation, indicating that an intact TOS motif is required to block signaling by a common 4E-BP1 and S6K1 regulator (Figure 5). Inhibition of 4E-BP1 phosphorylation was independent of S6K1 activity and strictly correlated with the presence of an intact TOS motif.

Identification of a TOS Motif in 4E-BP1

If the TOS motif plays a general role in mTOR signaling, we predicted that it would be present in other mTOR targets. 4E-BP1 is a well-characterized target of mTOR signaling. Like S6K1, 4E-BP1 is in vitro phosphorylated by mTOR and several of its phosphorylation sites are sensitive to rapamycin [5]. We identified a potential TOS motif in the C terminus of 4E-BP1 (FEMDI) that is conserved between all three human 4E-BPs and *Drosophila* 4E-BP (Figure S3). Mutation of the phenylalanine 114 to alanine in this motif inhibited insulin-dependent phosphorylation of 4E-BP1 (Figure 6), suggesting that this sequence is crucial for 4E-BP1 phosphorylation and therefore also required for mTOR signaling toward 4E-BP1.

Discussion

Rapamycin inhibits S6K1 activation and 4E-BP1 phosphorylation by all known stimuli, indicating that mTOR signaling is essential for these phosphorylation-regu-

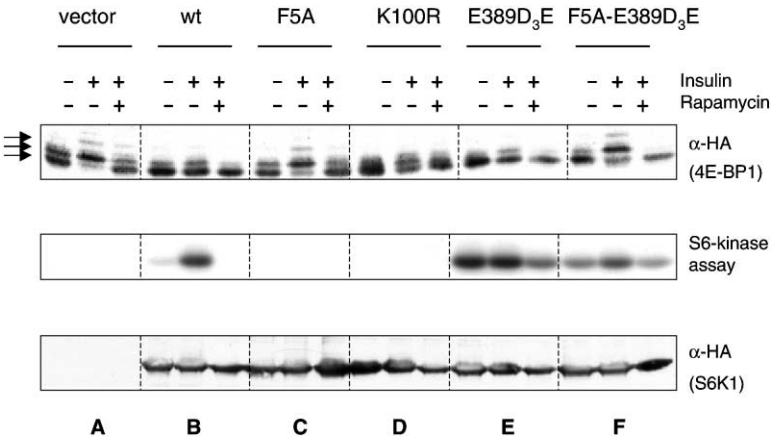

Figure 5. Expression of S6K1 with an Intact TOS Domain Is Required to Inhibit 4E-BP1 Phosphorylation

HEK293 cells were cotransfected with HA-4E-BP1 and the empty vector pRK7 (A), HA-S6K1 wild-type (wt) (B), HA-S6K1- F5A (F5A) (C), HA-S6K1-K100R (K100R) (D), HA-S6K1-E389D_3E (E389D_3E) (E), or HA-S6K1-F5A-E389D_3E (F5A-E389D_3E) (F). Transfected cells were starved and lysed as described in Experimental Procedures. Detection of HA-4E-BP1 by immunoblotting with α-HA-antibody (upper panel) and protein expression levels of S6K1 constructs were assayed by immunoblotting with α-HA-antibody (bottom panel). Autoradiogram of S6 in vitro phosphorylation (second panel).

lated processes. The molecular mechanisms whereby mTOR regulates its downstream targets are controversial. It is not clear if mTOR regulates S6 kinases and 4E-BPs via direct phosphorylation, by activating an intermediary kinase, and/or by inhibiting a phosphatase [5, 10].

We have identified a novel conserved motif (TOS motif) in the N terminus of S6K1 and in the C terminus of 4E-BP1 that is crucial for their phosphorylation by the mTOR pathway. Our results indicate that mTOR regulates phosphorylation of S6K1 by at least two mechanisms, both requiring the presence of the newly identified TOS motif. The first mechanism appears to provide a positive input regulating phosphorylation of the critical hydrophobic motif site Thr389 directly or through a mTOR-regulated kinase. Although mutations in the TOS domain (F5A) cause complete loss of kinase activity and inhibition of Thr389 phosphorylation (Figures 1C and 2), mutation of the Thr389 site to an acidic residue to mimic phosphorylation in F5A only partially rescued S6K1 activity (Figure S2). An additional deletion of the C-terminal domain (F5A-E389ΔCT) completely overcame the inhibitory effect of the F5A mutation, making this S6K1 mutant fully active and rapamycin resistant (Figure 3C), suggesting a second C-terminal-mediated regulatory mechanism that requires the TOS motif to be suppressed. Thus, mTOR might suppress the autoinhibitory function of the C terminus or inhibit the activity of a C-terminally-associated phosphatase. One possible mechanism for mTOR regulation of the C-terminal inhibitory pseudosubstrate domain is through phosphorylation. Mutation of the rapamycin-sensitive site Ser404 to Asp or all of the other known phosphorylation sites in the C terminus to acidic residues (S6K1-D_3E) did not change rapamycin sensitivity of S6K1 activity (data not shown and [13, 15]), suggesting that the mTOR input mediated by the C terminus of S6K1 is not regulated by phosphorylation of known sites but more likely by another mechanism possibly involving a phosphatase.

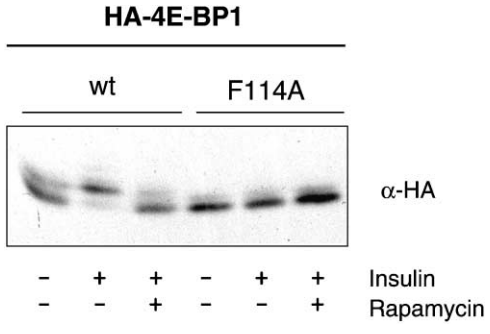

Figure 6. Identification of Conserved Motif in C Terminus of 4E-BPs

Point mutation F114A in potential TOS motif in 4E-BP1 causes loss of phosphorylation. HEK293 cells were transfected with HA-4E-BP1 or HA-4E-BP1 F114A. 4E-BP1 was detected by immunoblotting with α-HA-antibody.

Data from budding yeast provides a compelling argument that TOR may function to regulate a phosphatase [26, 27], and evidence already exists for regulation of S6K1 phosphorylation by a rapamycin-activated phosphatase [28, 29]. The rapamycin-sensitive dephosphorylation of different sites in S6K1, such as Thr229, Thr389, and Ser404 (Figure 2 and [13, 14]), may be consistent with the activation of a PP2A-like phosphatase. Therefore, in addition to providing a direct input, mTOR might regulate its targets by inhibiting a phosphatase that binds to the C terminus and is able to dephosphorylate several phosphorylation sites in S6K1. This would allow for a very dynamic regulation of S6K1 activity in response to nutrients and mitogenic agonists.

A previous model, based on the observation that S6K1-ΔCT is mitogen regulated and still rapamycin sensitive, whereas S6K1-ΔNT/CT is not rapamycin sensitive, suggested that a PP2A-like phosphatase that is inhibited by mTOR would bind to the N terminus and dephosphorylate S6K1 [11, 14]. This model does not take into account, however, the fact that S6K1-ΔCT is partially rapamycin resistant and that its activity in the presence of rapamycin is comparable to the mitogen-stimulated activity of S6K1-ΔNT/CT (Figure 3B). These previous experiments also did not address the possibility of phosphatase interaction with the C terminus. Our data suggest rather that the deletion of the C terminus contributes to the observed rapamycin resistance (Figure 3B).

Our data indicate that the TOS motif functions to mediate an interaction between S6K1 and an upstream mTOR regulator important for regulation of 4E-BP1 and S6K1. Overexpression of S6K1 with an intact TOS motif inhibited 4E-BP1 and S6K1 phosphorylation at sites known to be regulated by mTOR, suggesting the existence of a limiting activator functioning in the mTOR pathway immediately upstream of S6K1 (Figure 5). Since the inhi-

bition of kinase activity caused by deletion or mutations of the TOS motif can be rescued (Figure 3C), it is unlikely that these mutations are causing structural changes in S6K1 that render the enzyme kinase inactive. mTOR is thought to function as a nutritional checkpoint, as its downstream targets, such as S6K1, are sensitive to changes in amino acid levels [22–24]. Mutations in the TOS motif renders S6K1 insensitive to changes in amino acid levels (Figure 4), providing additional evidence that mTOR signaling is mediated by the TOS motif in S6K1.

Conclusions

S6K1 contains in its N terminus a conserved sequence (TOS motif) that is crucial for its regulation by the mTOR pathway. mTOR seems to regulate S6K1 by two distinct mechanisms. One may involve direct phosphorylation of Thr389 by mTOR or indirect regulation by a mTOR-regulated kinase, the other mechanism is required to suppress an inhibitory activity mediated by the S6K1 C terminus. This inhibitory function might be affected by a mTOR-regulated phosphatase. Similarly, we have identified a functional TOS motif in the C terminus of 4E-BP1. The identification of the TOS motif in other signaling molecules may reveal additional targets and processes regulated by mTOR.

Experimental Procedures

Plasmids and Mutagenesis

We have previously described the generation of the HA-S6K1 wild-type, HA-S6K1-ΔCT, and HA-S6K1-ΔNT/CT alleles [15]. These constructs were subcloned into a pRK7 expression vector. Deletion of amino acids 4–9 (VFDIDL) of S6K1 was performed by PCR, using HA-S6K1/pRK7 as a template, and subcloned into pKH3, resulting in 3xHA-S6K1-ΔNT2. HA-S6K1/pRK7 and 3xHA-pACTAG-2-4E-BP1 plasmids were mutagenized using the Quick-Change PCR-based method (Stratagene). HA-S6K1-F5A, HA-S6K1-D6/8A, and HA-S6K1-L7/9A were generated by mutating Phe5 to Ala, Asp6 and Asp8 to Ala, or Ile7 and Leu9 to Ala, respectively, using HA-S6K1/pRK7 as a template. The HA-S6K1-E389 and HA-S6K1-E389D_3E alleles were generated by mutating Thr389 to Glu using HA-S6K1 and HA-S6K1-D_3E [15], respectively, as templates. HA-S6K1-E389ΔCT and HA-S6K1-F5A-E389ΔCT alleles were generated by introducing a stop codon at amino acid 402 into HA-S6K1-E389 and HA-S6K1-F5A-E389, respectively. HA-S6K1-F5A-E389, HA-S6K1-F5A-E389D_3E, and HA-S6K1-F5A-ΔCT constructs were generated by swapping fragments of HA-S6K-F5A and HA-S6K1-E389, HA-S6K1-E389D_3E, or HA-S6K1-ΔCT, respectively. 3xHA-pACTAG-2-4E-BP1 was generously provided by N. Sonenberg. 3xHA-4E-BP1-F114A was generated by mutating Phe114 to Ala using 3xHA-pACTAG-2-4E-BP1 as template.

Antibodies

The anti S6K1-phospho-Thr389 antibody was obtained from Cell Signaling Inc. (Beverley, MA); the anti S6K1-phospho-Ser411 and anti S6K1-phospho-Thr421/Ser424 antibodies were obtained from New England Biolabs (Beverly, MA). The anti S6K1-phospho-Thr229 was generously provided by A. Newton and was initially characterized as an anti-phospho-Thr500 antibody for PKCβ. Additional anti-S6K1-phospho-Thr229 antibody was purchased from R&D Systems (Minneapolis, MN). The anti S6K1-phospho-Ser371 antibody was generated for us by Research Genetics Inc., using the phosphopeptide TRQTPVDS*PDDSTLS coupled to MAP and affinity purified.

Cell Culture, Transfection, and Lysis

HEK 293E cells were cultured, transfected, and lysed as described previously [30]. After 20 hr of starvation, serum-free DMEM cells were pretreated for 30 min with rapamycin (20 ng/ml) or ethanol vehicle and then stimulated with insulin (100 nM) for 30 min. For the amino acid withdrawal, cells were first incubated in serum-free DMEM for 20 hr, washed once with Dulbecco's phosphate-buffered saline (D-PBS, containing 0.1 g/liter $CaCl_2$), and incubated in the same buffer for 60 min. Readdition of amino acids involved changing the medium to D-PBS containing a "5× amino acid mixture" (MEM Amino Acids Solution, GibcoBRL).

Immunoblotting

Immunoblotting with anti HA- or phosphospecific antibodies as described previously [30].

Immune-Complex Kinase Assay

Immune-complex kinase assay was carried out as described previously [30].

Supplementary Material

Supplementary Material including figures showing a schematic representation of S6K1, the effect of mutations of phosphorylation sites in S6K1 wild-type (E389 and E389D_3E) and F5A (F5A-E389 and F5A-E389D_3E) on kinase activity, and a sequence alignment of human 4E-BP1, 2, and 3 and *Drosophila* 4E-BP is available at http://images.cellpress.com/supmat/supmatin.htm.

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