2 Results

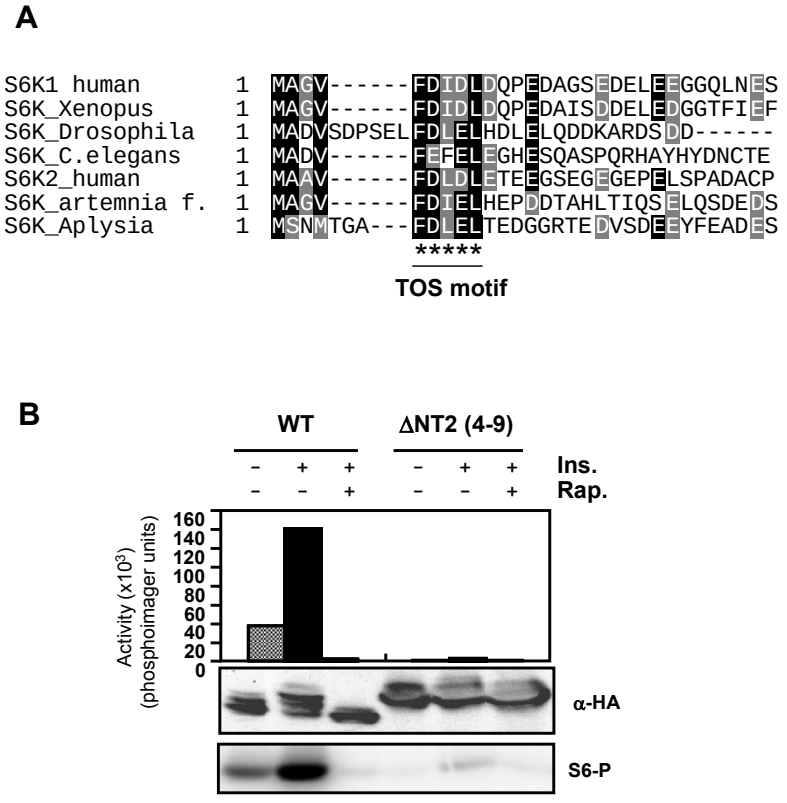
2.1 Identification of the TOS motif inS6K1

2.1.1 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 α2 isoform (or 53 amino acids in the α1 isoform) inhibits S6K1 activity and phosphorylation similar to the mTOR inhibitor rapamycin (Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995; Dennis, Pullen et al. 1996). Thus, the N-terminus of S6K1 has been suggested to participate in S6K1 regulation by the mTOR pathway. S6 kinases from all species tested to date are rapamycin-sensitive and therefore regulated by TOR, suggesting that a conserved N-terminal domain might mediate the regulation of S6 kinases by TOR (Chung, Kuo et al. 1992; Stewart, Berry et al. 1996; Shima, Pende et al. 1998; Schwab, Kim et al. 1999; Khan, Pepio et al. 2001). 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 2.1.1A). To determine the importance of this sequence for S6K1 function, the five amino acid sequence in S6K1 (S6K1-ΔNT2) were deleted and the effect of this deletion on kinase activity in starved, insulin-stimulated, or rapamycin-treated cells was tested. Similar to the effect of deleting the first 30 amino acids in S6K1 (S6K1-ΔNT) (Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995), deletion of these five amino acids almost completely abrogated the activity of S6K1 (Figure 2.1.1B). Similar results were seen for the deletion of the conserved sequence in S6K2 (data not shown).

Next, point mutations in S6K1 within this conserved sequence were introduced to determine which amino acids are critical for the regulation of S6K1 activity. The bulky phenylalanine residue number 5 (numbering for rat S6K1 αII isoform) was mutated to alanine (F5A), the two charged aspartic acid residues numbers 6 and 8 to were mutated to alanines (D6/8A), or the hydrophobic leucine

and isoleucine residue numbers 7 and 9 were changed to alanine (L7/9A). Each of the introduced point mutations had a dramatic inhibitory effect on S6K1 activity (Figure 2.1.1C). These results demonstrate that this highly conserved N-terminal motif is essential for S6K1 activation and that several of its residues are required for S6K1 activity. This sequence was referred as the TOS motif and evidence will be provided that the TOS motif 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, this construct was used for further characterization.



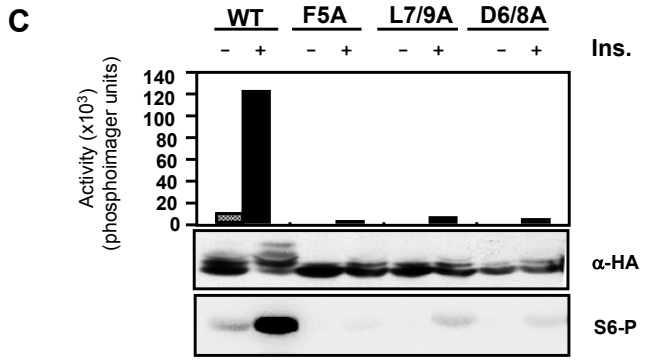


Figure 2.1.1 Identification of a conserved domain in the N-terminus of S6 kinases essential for kinase activity. (A) Sequence alignment of the first 30 amino acids of S6K1 (human), S6K (*Xenopus laevis*), S6K (*Drosophila melanogaster*), S6K (C.elegans), S6K2 (human), S6K (Artemnia), and S6K (Aplysia). Identical (black box) and conserved (shadowed box) amino acids are highlighted. **(B)** and **(C)** Deletion of - or point mutations in conserved sequence cause loss of S6K1 kinase activity. HEK293 cells were transfected with **(B)** HA-S6K1 wild-type (WT), or HA-S6K1-ΔNT2 (4-9) (ΔNT2 (4-9)) 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, rapamycin-treated (Rap.), insulin-stimulated (Ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described. Quantification of kinase assay *(upper panel)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.

2.1.2 The F5A mutation inhibits phosphorylation of S6K1 at Thr389 and Thr229 Since S6K1 activation requires multi-site phosphorylation, it was next determined which phosphorylation events of S6K1 required an intact TOS motif. Therefore, phospho-specific antibodies raised against various S6K1 phosphorylation sites were employed (Figure 2.1.2A). The F5A mutation completely inhibited insulin-stimulated Thr389 phosphorylation (Figure 2.1.2B). The effect was similar to the inhibition of Thr389 phosphorylation observed with rapamycin (Pearson, Dennis et al. 1995). The F5A mutation significantly, but not completely, inhibited Thr229 phosphorylation (Figure 2.1.2B).

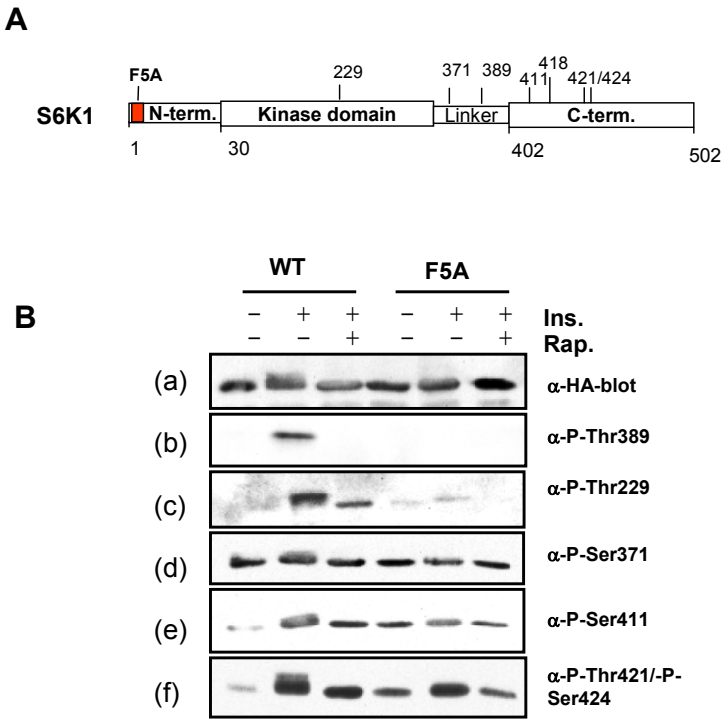


Figure 2.1.2 The F5A mutation inhibits Thr389 and Thr229 phosphorylation. (A) Schematic representation of S6K1 showing phosphorylation sites and F5A mutation. **(B)** HEK293 cells were transfected with HA-S6K1 wild-type (WT) or HA-S6K1-F5A (F5A). Transfected cells were starved, rapamycin-treated (Rap.), insulin-stimulated (Ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(panel a)*. 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 evaluated by immunoblotting with *α*-S6K1-P-Thr389 phosphopeptide-specific anibody *(panel b),* *α*-S6K1-P-Thr229 phosphopeptide-specific anibody *(panel c),* *α*-S6K1-P-Thr371 phosphopeptide-specific anibody *(panel d),* *α*-S6K1-P-Ser-411 phosphopeptide-specific anibody *(panel e),* and *α*-S6K1-P-Thr421/Ser424 phosphopeptide-specific anibody *(panel f)*.

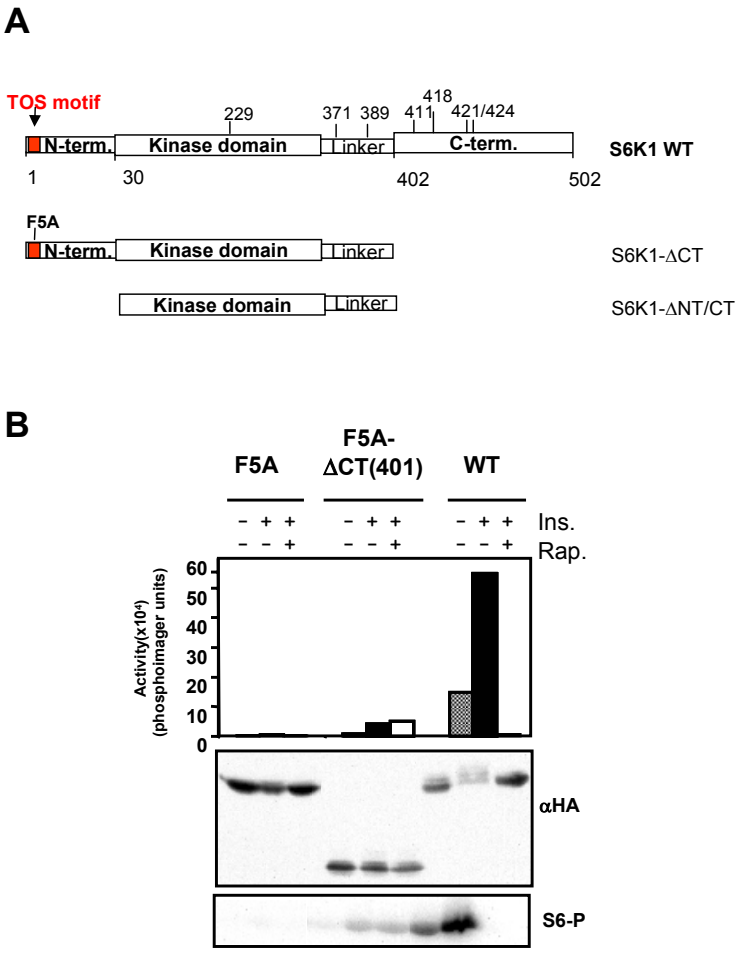
The inhibition of Thr229 phosphorylation could be a primary effect of the F5A mutation or a secondary effect caused by the inhibition of Thr389 phosphorylation and/or activation of a phosphatase. The F5A mutation did not significantly affect

the phosphorylation of the proline-directed Ser371 site, also located in the linker region (Figure 2.1.2B). 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 2.1.2B). The phosphorylation pattern of these sites in F5A is comparable to their phosphorylation in S6K1-ΔNT (Dennis, Pullen et al. 1996) and in rapamycin-treated wild type S6K1 (Figure 2.1.2B), which is once again consistent with the TOS motif being critical for mTOR signaling to S6K1.

2.1.3 The F5A point mutation mimics deletion of the N-terminus

Next, it was analyzed whether the F5A point mutation mimics 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 (Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995). 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 2.1.3B). Furthermore, the insulin-stimulated activities of S6K1-ΔNT/CT to S6K1 wild type and S6K1-ΔCT were compared (Figure 2.1.3C). 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 2.1.3C; Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995). 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 2.1.3B and 2.1.3C). 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 (Cheatham, Monfar et al. 1995; Weng, Andrabi et al. 1995)).



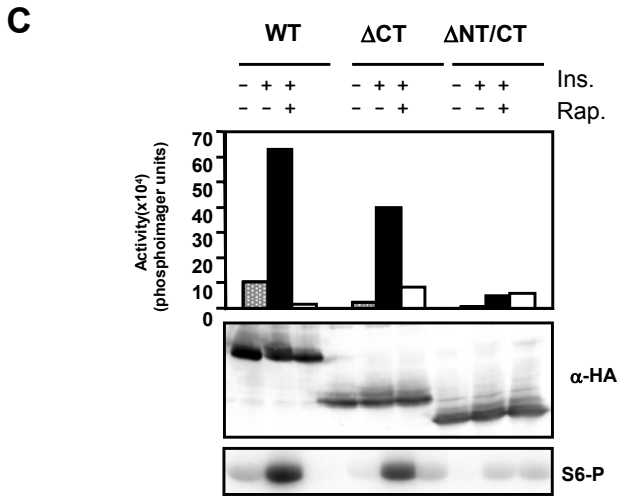


Figure 2.1.3 The F5A point mutation mimics deletion of the 30 amino acids and is partially rescued by deletion of the C-terminus. (A) Schematic representation of different S6K1 mutants. **(B)** and (C) HEK293 cells were transfected with **(A)** HA-S6K1-F5A (F5A), S6K1-F5A-ΔCT (F5A-ΔCT), or HA-S6K1 wild-type (WT) and **(B)** HA-S6K1 wild type (WT), HA-S6K1ΔCT (ΔCT), or HA-S6K1-ΔNT/CT (ΔNT/CT). Transfected cells were starved, rapamycin-treated (Rap), stimulated with insulin (Ins), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(bottom panel)*. Quantification of kinase activity *(upper panel)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel) are provided.*

2.1.4 Mutation of phosphorylation sites in S6K1 to acidic residues partially rescues the activity of S6K1-F5A mutant

Since the F5A mutation had the most severe effect on Thr389 phosphorylation (Figure 2.1.2B), it was of interest whether mutation of this site to an acidic residue in order 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 effect was largely rapamycin-resistant (Figure 2.1.4B). F5A-E389, however, had

significantly reduced activity compared to E389 in the wild type background (about 30% activity). The T389E mutation alone in the wild type background also caused reduction of insulin-stimulated kinase activity compared to wild type S6K1 (Figure 2.1.4B), and (Pearson, Dennis et al. 1995; Weng, Kozlowski et al. 1998). Therefore, additional mutations of all C-terminal sites to acidic residues ($E389D_3E$) were incorporated to determine if it was possible to overcome the inhibitory effect of the E389 mutation on kinase activity in the background of wild type S6K1. $E389D_3E$ showed elevated basal activity with increased insulin-stimulated activity compared to wild type, and was partially rapamycin-sensitive (Figure 2.1.4B; Han, Pearson et al. 1995; Dennis, Pullen et al. 1996). To determine whether additional mutations of the C-terminal sites of F5A-E389 have an effect on its activity, $E389D_3E$ was placed in the context of F5A (F5A-$E389D_3E$). F5A-$E389D_3E$ was significantly less active than $E389D_3E$ (30-40% of activity), indicating that mutation of Thr389 and the C-terminal sites to acidic residues did not completely restore activity to the F5A mutant (Figure 1.4B). However, F5A-$E389D_3E$ had a 3-5 fold higher specific activity compared to F5A-E389, likely due to phosphorylation of the C-terminal pseudosubstrate domain resulting in a more open conformation. The data are consistent with the hypothesis that the TOS motif serves a function in addition to regulation of Thr389 and C-terminal phosphorylation.

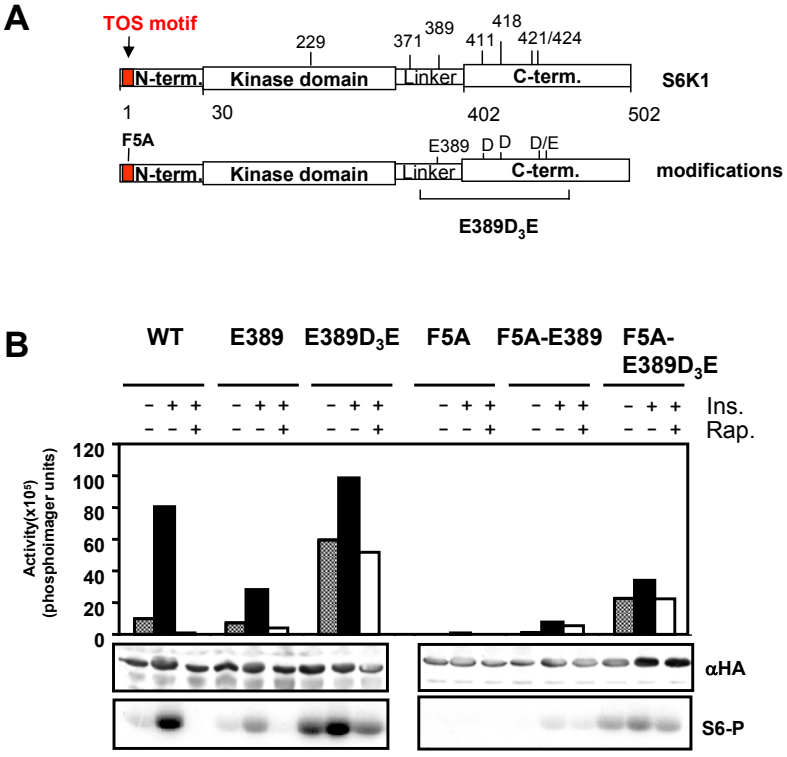


Figure 2.1.4 Partial rescue of F5A activity by substitution of C-terminal phosphorylation sites and/or Thr389 by acidic residues. (A) Schematic representation of S6K1 mutants. **(B)** HEK293 cells were transfected with HA-S6K1 wild-type (WT), HA-S6K1-E389 (E389), HA-S6K1-E389D3E (E389D3E), HA-S6K1-F5A (F5A), HA-S6K1-F5A-E389 (F5A-E389), HA-S6K1-F5A-E389D3E (F5A-E389D3E) in pRK7. Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.

2.1.5 Deletion of the C-terminus and Thr389Glu substitution rescues F5A activity completely

Next, C-terminal deletion and the E389 point mutation were combined in wild type and F5A S6K1s, creating E389-ΔCT and F5A-E389-ΔCT, respectively (Figure

2.1.5A). 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 to E389-ΔCT (Figure 2.1.5B). 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 2.1.5B). Thus, overcoming the inhibitory effect of the C-terminus appears to require more than phosphorylation of known C-terminal sites.

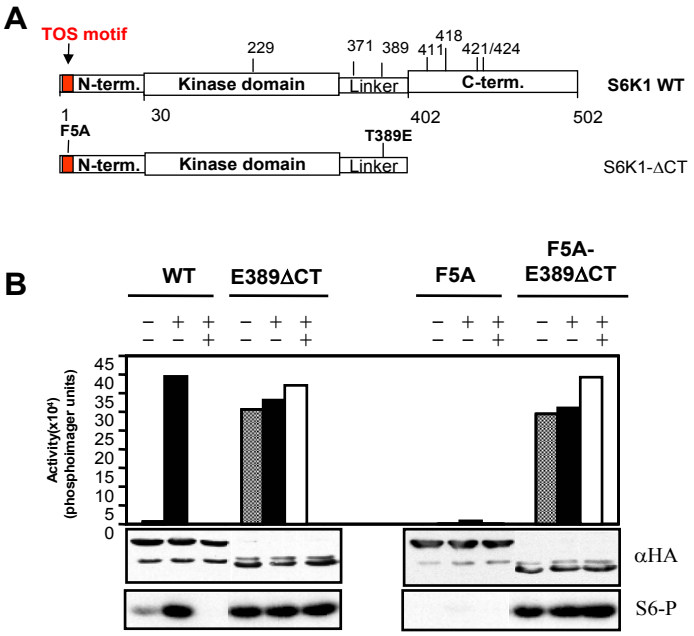


Figure 2.1.5 Deletion of the C-terminus and Thr389Glu substitution rescues F5A activity completely. (A) Schematic representation of different S6K1 mutants. **(B)** HEK293 cells were transfected with HA-S6K1 wild-type (WT), HA-S6K1- E389ΔCT (E389ΔCT), HA-S6K1- F5A (F5A), and HA-S6K1-F5A- E389ΔCT (F5A- E389ΔCT). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase

assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.

2.1.6 4E-BP1 phosphorylation is inhibited by overexpression of S6K1 with an intact TOS motif

Previous studies suggest that 4E-BP1 and S6K1 share a limiting, rapamycin-sensitive regulator that functions as a bifurcation point immediately upstream of S6K1 (von Manteuffel, Dennis et al. 1997). Insulin-stimulated phosphorylation of 4E-BP1, as assessed by its altered mobility on SDS-PAGE to the higher resolving isoform, 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 (von Manteuffel, Dennis et al. 1997).

It is possible that the TOS motif mediates 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 completely blocked by rapamycin (Figure 2.1.6). Like rapamycin treatment, overexpression of wild type, kinase dead (K100R), or E389D_3E S6K1 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 2.1.6). Inhibition of 4E-BP1 phosphorylation was independent of S6K1 activity and strictly correlated with the presence of an intact TOS motif.

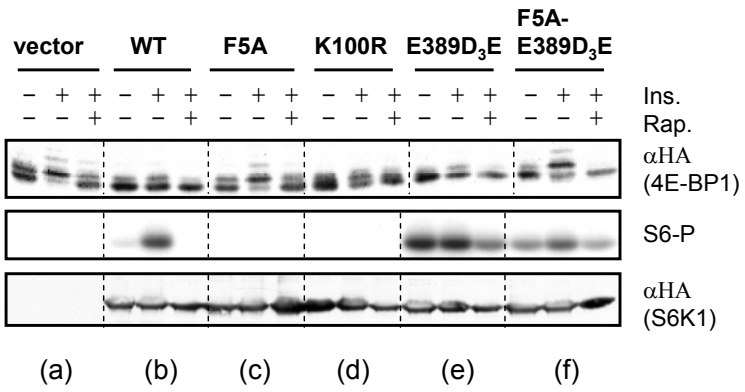


Figure 2.1.6. Expression of S6K1 with an intact TOS domain is required to inhibit 4E-BP1 phosphorylation. HEK293 cells were co-transfected with 3xHA-4E-BP1 and the empty vector pRK7 (a), HA-S6K1 wild-type (WT) (b), HA-S6K1- F5A (F5A) (c), HA-S6K1-K100R (K100R) (d), HA-S6K1-E389D3E (E389D3E) (e), or HA-S6K1-F5A-E389D3E (F5A-E389D3E) (f). Transfected cells were starved, rapamycin treated (rap.), insulin-stimulated (ins), and lysed as described in 'Materials and methods'. Protein expression levels were assayed 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 is provided *(second panel).*

2.1.7 The S6K1 F5A mutation prevents amino acid signaling via mTOR towards S6K1

To further investigate whether the TOS motif specifically mediates mTOR signaling to S6K1, it was asked whether the F5A mutation makes S6K1 insensitive to changes in amino acid levels. Amino acid withdrawal selectively inhibits mTOR signaling similar to rapamycin. In contrast, mitogen-regulated signaling pathways that are involved in S6K1 regulation, like the PI3K and MAPK pathways, are not sensitive to changes in amino acids (Hara, Yonezawa et al. 1998; Wang, Campbell et al. 1998; Iiboshi, Papst et al. 1999).

Here F5A-ΔCT instead of F5A was used, because the F5A allele has no activity. If S6K1-ΔCT is sensitive to changes in the level of amino acids but F5A-ΔCT is not, it could be concluded that the TOS motif is necessary for amino acid

signaling towards S6K1 and therefore responsible for its regulation by mTOR. S6K1ΔCT and F5A-ΔCT were modestly activated by insulin after 60 minutes of amino acid withdrawal. A more robust activation of S6K1-ΔCT was seen with a combination of amino acids and insulin (Figure 2.1.7), consistent with the model that S6K1 requires a nutrient sensing and a growth factor-mediated input for its full activation. F5A-ΔCT (Figure 2.1.7), like ΔNT/CT (data not shown and Hara, Yonezawa et al. 1998), 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 towards S6K1.

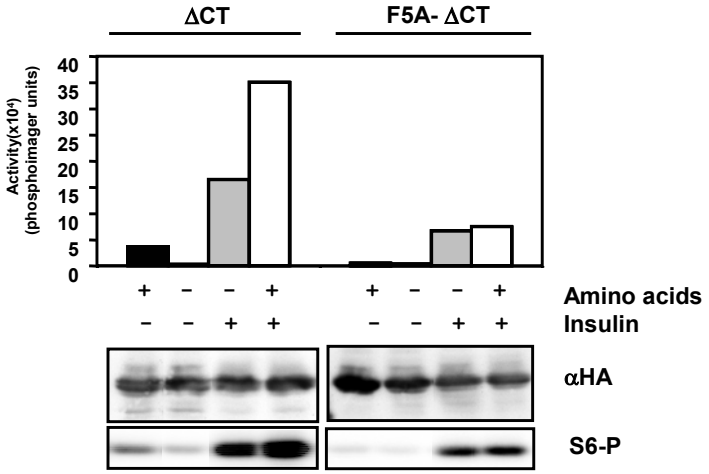


Figure 2.1.7 The F5A mutation blocks amino acid signaling via mTOR towards S6K1. HEK293 cells were transfected with HA-S6K1ΔCT (ΔCT), or HA-S6K1-F5A ΔCT (F5A-ΔCT). Transfected cells were quiesced in serum-free medium for 20 h, followed by 60 min incubation in D-PBS for withdrawal of amino acids. Cells were then stimulated for 20 min with insulin (100 nM) and/or 5x amino acids. Cells were lysed, and protein expression levels were assayed by immunoblotting with α-HA-antibody *(middle panels)* and kinase assays were performed as described. Quantification of kinase assay *(upper panels),* and autoradiogram of *in vitro* S6 phosphorylation *(lower panels)* is provided.

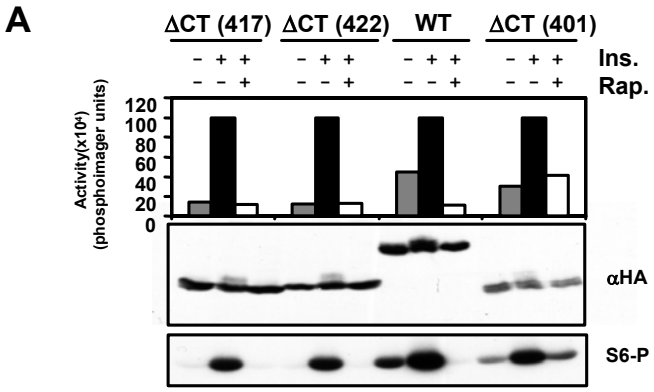
2.1.8 Characterization of C-terminal inhibitory effect of S6K1

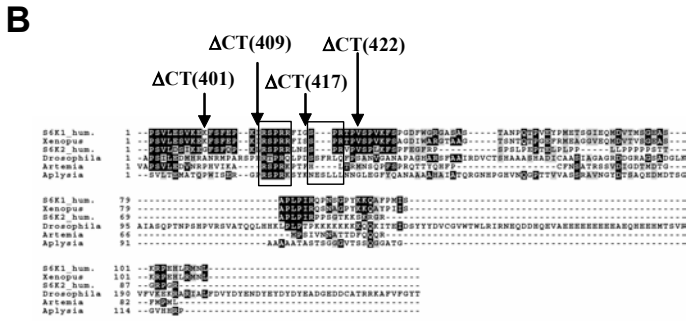
Deletion of the S6K1 C-terminus (S6K1-ΔCT(401)) or the S6K2 C-terminus renders these kinases partially rapamycin-resistant (Figure 2.1.3 C and data not shown). Thus, a rapamycin-sensitive, and therefore mTOR-dependent, inhibitory function for the C-terminus of S6 kinases has been proposed. To identify the region in the C-terminus responsible for this inhibitory effect, a series of C-terminal truncation mutants were generated and their kinase activity was assayed under starved, insulin-stimulated, or rapamycin-treated conditions. In contrast to the partial rapamycin-resistance of the S6K1-ΔCT(401) mutant, C-terminal truncation mutants at amino acid 418 (ΔCT (418)) and 421 (ΔCT(421)) were completely sensitive to rapamycin (Figure 2.1.8 A). These results suggest that the region between amino acid 401 and 421 within the C-terminus mediates the inhibitory effect of S6K1. This region contains two interesting features: a rapamycin-sensitive phosphorylation site Ser 404, and a conserved five amino acids motif (RSPRR) found in all S6Ks described at date (Figure 2.1.8 B). A second partially conserved repeat of the SPR motif (amino acids 418-420, Figure 2.1.8 B) seems to be dispensable from the inhibitory effect, as its presence (ΔCT(421)) or absence (S6K1 ΔCT(418)) did not affect the rapamycin sensitivity of S6K1 (Figure 2.1.8 A).

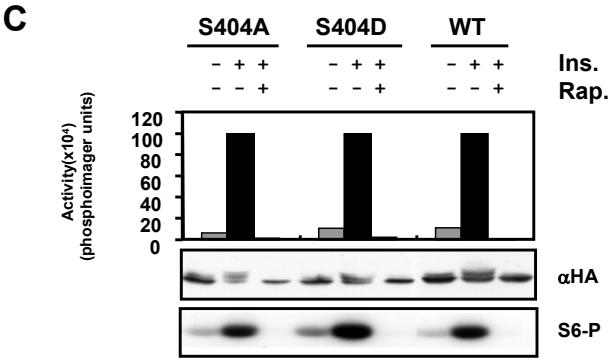
To test whether Ser404 phosphorylation contributes to the inhibitory effect of the S6K1 C-terminus, Ser404 was mutated to Asp to mimic phosphorylation or to Ala to prevent phosphorylation. As shown in Figure 2.1.8 C, mutation of Ser 404 to Asp or Ala did not affect the rapamycin sensitivity of S6K1. Based on these results we conclude that Ser404 phosphorylation does not contribute to the inhibitory function of the S6K1 C-terminus.

Next, an additional S6K1 truncation mutant within the C-terminal amino acid sequence from residue 401 to residues 421 to narrow down the location of the inhibitory sequence. Similar to S6K1-ΔCT(401), S6K1-ΔCT(409) was partially

rapamycin-resistant (Figure 2.1.8 D). Since these constructs contain an intact RSPRR motif, this motif may mediate the rapamycin sensitivity of the C-terminus.







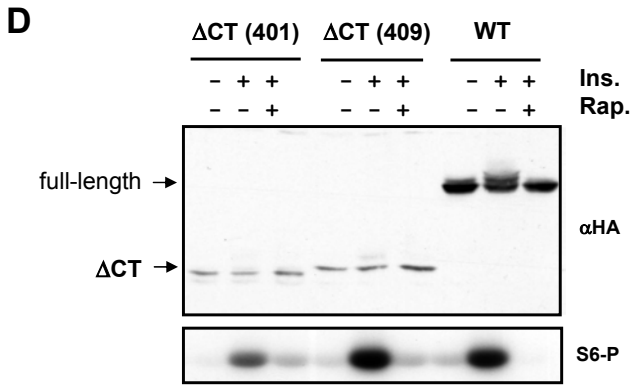
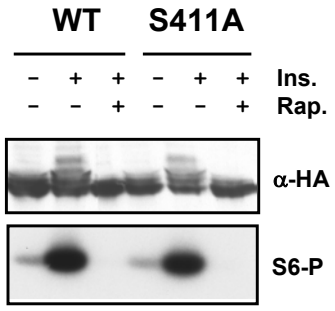


Figure 2.1.8 Characterization of C-terminal inhibitory effect of S6K1. (A) HEK293 cells were transfected with HA-S6K1-ΔCT (417) (ΔCT (417)), HA-S6K1-ΔCT (422) (ΔCT (422)), HA-S6K1 wild-type (WT), or HA-S6K1-ΔCT (401) (ΔCT (401)). Transfected cells were starved, rapamycin-treated (rap.), insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided. **(B)** C-terminal sequence alignment of S6Ks. Identical (black box) and conserved (shadowed box) amino acids are highlighted. The truncation mutants of S6K1 (human) are indicated accordingly. Conserved RSPRR and SPR sequences are highlighted by boxes. HEK293 cells were transfected with HA-S6K1 wild-type (WT), or HA-S6K1-S404A. Transfected cells were starved, rapamycin-treated (rap.), insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* is provided.

2.1.9 Ser411 phosphorylation does not affect rapamycin sensitivity

The RSPRR motif is located within the S6K1 pseudo-substrate domain, which is thought to occlude the S6K1 kinase domain and inhibit the phosphotransferase activity of S6K1. Phosphorylation of the several proline-directed sites within the pseudo-substrate domain is thought to destabilize the interaction between pseudo-substrate domain and allow further activation of S6K1. The RSPRR motif contains Ser411, which is one of the mitogen-regulated phosphorylation sites within the

pseudo-substrate domain. To determine whether phosphorylation of Ser411 is important for the C-terminal mediated rapamycin sensitivity of S6K1, Ser411 was mutated to Ala (S6K1-S411A) to prevent phosphorylation at this site (Figure 2.1.9). Similar to wild type S6K1, S6K1-S411A was completely rapamycin sensitive, indicating that Ser411 phosphorylation does not regulate rapamycin-sensitivity of S6K1 (Figure 2.1.9). In addition, mutating all the phosphorylation sites within the pseudo-substrate domain to acidic residues to mimic phosphorylation did not affect the rapamycin sensitivity of S6K1 (data not shown). The data suggest that the mTOR-regulated inhibitory function of the S6K1 C-terminus is not regulated by phosphorylation of Ser404/411 but by other residues within the RSPRR motif.



2.1.10 The inhibitory effect of the F5A mutation requires the RSPRR motif

Deletion of the last 100 C-terminal residues (ΔCT 401) partially rescued the inhibitory effect of the F5A mutant (Figure 2.1.3B). Similar to F5A-ΔCT (401), deletion of 92 C-terminal amino acids (ΔCT409) partially rescues the inhibitory effect of the F5A mutant (Figure 2.1.10B). In contrast, deletion of only 83 C-terminal acids (F5A-ΔCT (418) creating a mutant with intact RSPRR motif, did not rescue the inhibitory effect of the F5A mutation (Figure 2.1.10B), indicating that the conserved N-terminal TOS motif and the conserved C-terminal RSPRR motif cooperate in S6K1 regulation by mTOR signaling.

2.1.10 The inhibitory effect of the F5A mutation requires the RSPRR motif

Deletion of the last 100 C-terminal residues (ΔCT(401)) partially rescued the inhibitory effect of the F5A mutant (Figure 2.1.3B). Thus, it was of interest to determine whether specific deletion of the RSPRR motif might be responsible for

the observed rescue of the kinase activity. Similar to F5A-ΔCT(401), deletion of 92 C-terminal amino acids (ΔCT(409)) partially rescues the inhibitory effect of the F5A mutant (Figure 2.1.10B). In contrast, deletion of only 83 C-terminal acids (F5A-ΔCT(418)) creating a mutant with intact RSPRR motif, did not rescue the inhibitory effect of the F5A mutation (Figure 2.1.10B), indicating that the conserved N-terminal TOS motif and the conserved C-terminal RSPRR motif cooperate in S6K1 regulation by mTOR signaling.

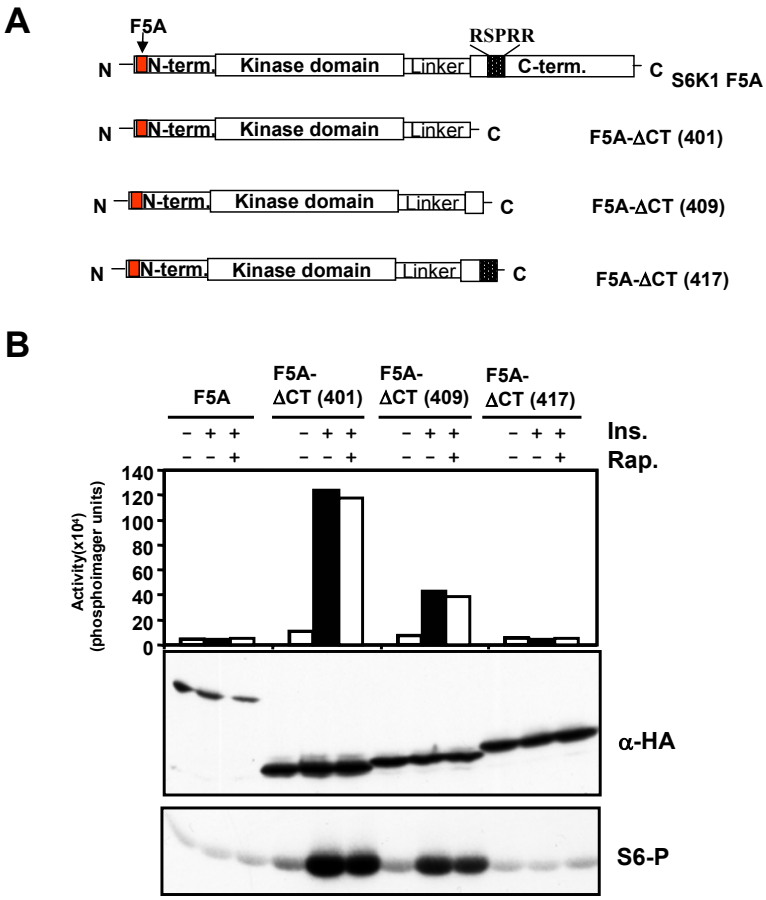


Figure 2.1.10 The inhibitory effect of the F5A mutation requires the RSPRR motif. (A) Schematic representation of the truncation mutants. **(B)** HEK293 cells were transfected with HA-S6K1-F5A (F5A), HA-S6K1-F5A-ΔCT(401) (F5A-ΔCT (401)), HA-S6K1-F5A-ΔCT(409) (F5A-

ΔCT (409)), or HA-S6K1-F5A-ΔCT(417) (F5A-ΔCT (417)). Transfected cells were starved, rapamycin-treated (rap.), insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.

2.1.11 RSPRR motif is not required for interaction with common mTOR activator

The TOS motif may mediate the binding of S6K1 and 4E-BP1 to a common upstream activator, since only overexpression of S6K1 with a functional TOS motif is required to inhibit 4E-BP1 phosphorylation (Figure 2.1.6). Thus, it was of interest to determine whether the RSPRR motif is also necessary for S6K1 binding to this common activator. Co-expression of vector control or S6K1-F5A did not affect insulin-stimulated 4E-BP1 phosphorylation. In contrast, overexpression of wild type S6K1 inhibited 4E-BP1 phosphorylation, indicating that an intact TOS motif is required to block signaling by a common 4E-BP1 and S6K1 regulator (Figure 2.1.11). No differences in the ability of different C-terminal S6K1 truncation mutants to inhibit 4E-BP1 phosphorylation depending on presence (S6K1-ΔCT (418)) or absence (S6K1-ΔCT (401)) of the RSPRR motif were detected, suggesting that the RSPRR motif is not required for the binding of S6K1 to its upstream activator (Figure 2.1.11). Interestingly, overexpression of wild-type S6K1 inhibited 4E-BP1 phosphorylation more potently than overexpression of the C-terminal truncated S6K1 mutants, indicating that the S6K1 C-terminus is required for optimal binding to an upstream activator (Figure 2.1.11). The lower binding affinity of S6K1-ΔCT401 to the common activator may also explain why this mutant is 30% less active than wild-type S6K1 under insulin-stimulated conditions.

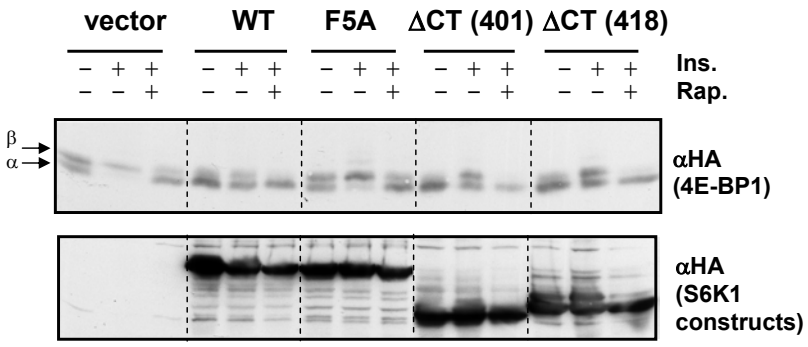


Figure 2.1.11 Expression of S6K1 with an intact TOS domain is required to inhibit 4E-BP1 phosphorylation. HEK293 cells were cotransfected with 3xHA-4E-BP1 and the empty vector pRK7, HA-S6K1 wild-type (WT), or various HA-tagged S6K1 mutants. Transfected cells were starved, rapamycin treated (rap.), insulin-stimulated (ins), and lysed as described in 'Materials and methods'. Protein expression levels were assayed 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)*.

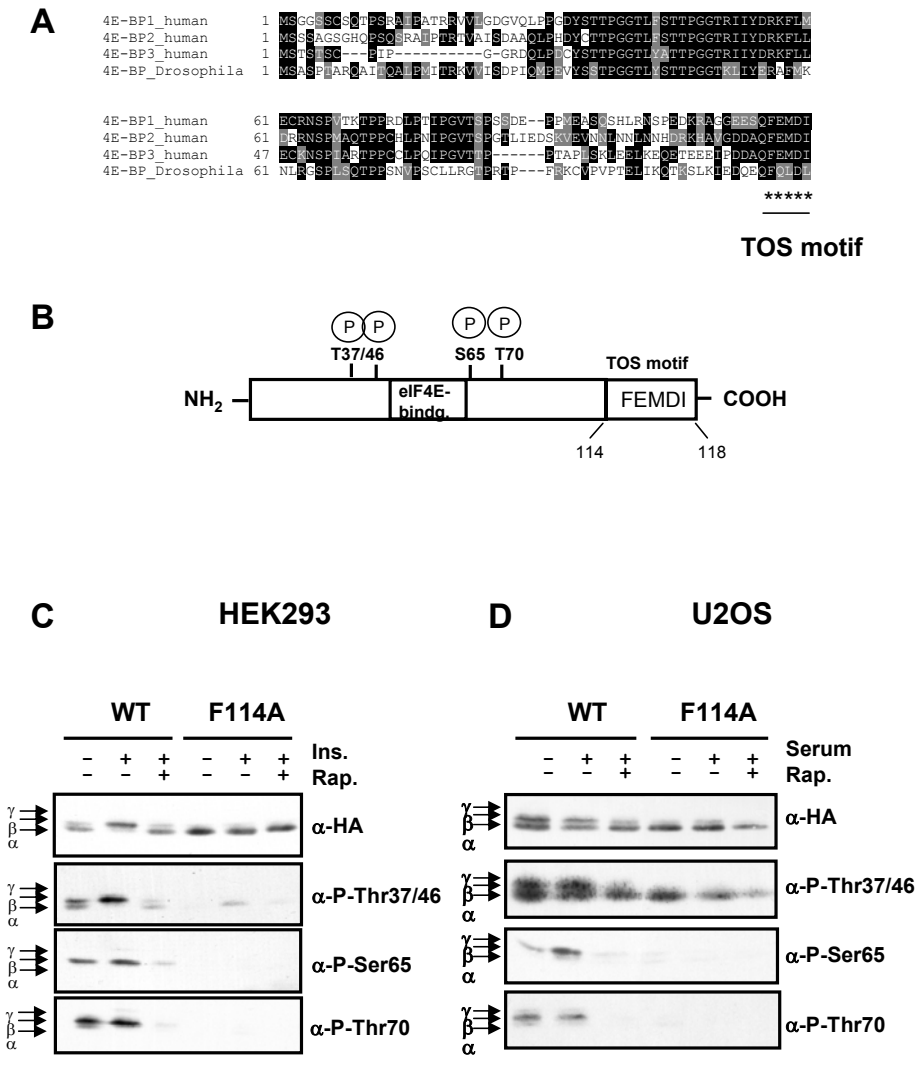
2.2 Identification of a TOS motif in 4E-BP1

2.2.1 The TOS motif in 4E-BP1 is required for its phosphorylation

The TOS motif may play a general role in mTOR signaling and could therefore be presented in other mTOR targets. Comparison of the amino acids sequences of all three human 4E-BPs and the Drosophila 4E-BP revealed a potential TOS motif, which is conserved in the C-terminus of the 4E-BPs (Figure 2.2.1A). Mutation of the Phenylalanine 114 to Alanine in the potential TOS motif (FEMDI) of 4E-BP1 inhibited its insulin-dependent phosphorylation (Figure 2.2.1C), suggesting that this sequence is indeed crucial for 4E-BP1 phosphorylation.

mTOR regulates hierarchical 4E-BP1 phosphorylation (Thr37/46, Ser65, and Thr70), which is required for 4E-BP1's release from eIF4E (Gingras, Raught et al. 2001). To investigate which phosphorylation events on 4E-BP1 are dependent on the potential TOS motif (Figure 1A), phosphorylation of the non-functional TOS motif-containing mutant, 4E-BP1-F114A, was analyzed by employing various 4E-BP1 phospho-specific antibodies. Basal and insulin-stimulated Thr37/46 phosphorylation was reduced in the 4E-BP1-F114A mutant protein when compared to wild-type 4E-BP1 in HEK293E cells (Figure 2.2.1C). The F114A mutation had a more pronounced inhibitory effect on phosphorylation of the later 4E-BP1 phosphorylation sites Thr70 and Ser65 (Figure 2.2.1C), of which residual phosphorylation was only detected on longer film exposures (data not shown). Therefore, the TOS motif is required for efficient 4E-BP1 phosphorylation at all mTOR-regulated sites. The residual phosphorylation of Thr37/46 (Figure 2.2.1C and D), Ser65, and Thr70 (data not shown) in 4E-BP1-F114A was still sensitive to the mTOR inhibitor, rapamycin, suggesting that either the single F114A point mutation of the TOS motif does not completely block the function of this TOS motif to mediate mTOR-dependent phosphorylation of 4E-BP1 or that the rapamycin-sensitive phosphorylation of Thr37/46, Ser65, and Thr70 can also occur independently of this motif. TOS motif-independent dephosphorylation of 4E-BP1 could be caused by a rapamycin-activated, and thus

mTOR-inhibited phosphatase. Regulation of 4E-BP1 phosphorylation by such a phosphatase has already been suggested (Lin and Lawrence 1997; Peterson, Desai et al. 1999). Similar regulation of these phosphorylation sites was also detected in U2OS cells (Figure 2.2.1D).



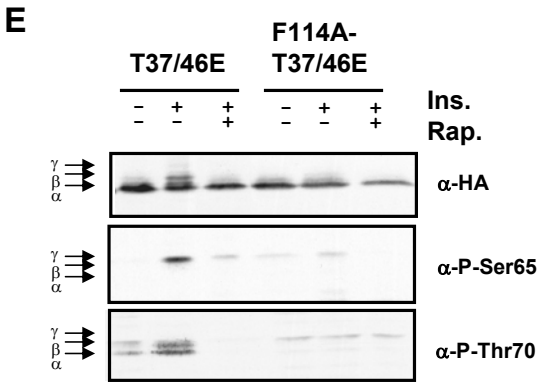


Figure 2.2.1. TOS motif (FEMDI) is required for multi-site 4E-BP1 phosphorylation. (A) Sequence alignment of human 4E-BP1, -2, and -3 and *Drosophila* 4E-BP (d4E-BP). Identical (black box) and conserved (shadowed box) amino acids are highlighted. **(B)** Schematic representation of 4E-BP1 with TOS motif, mTOR-regulated phosphorylation sites (Thr37/46, Ser65 and Thr70), and eIF4E-binding site (eIF4E-bindg.) are indicated. **(C)** HEK293E or **(D)** U2OS cells were transfected with wild-type HA-4E-BP1 (WT) or HA-4E-BP1-F114A (F114A). Transfected cells were starved, rapamycin-treated (Rap.), insulin-stimulated (Ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with anti-HA-antibody *(upper panels). In vivo* phosphorylation of 4E-BP1 at Thr37/46 (anti-P-Thr37/36), Ser65 (anti-P-Ser65), and Thr70 (anti-P-Thr70) was evaluated by immunoblotting with 4E-BP1 phosphopeptide-specific antibodies. **(E)** HEK293E cells were transfected with HA-4E-BP1-T37/46E (T37/46E) and HA-4E-BP1-F114A-T37/46E (F114A-T37/46E). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with anti-HA-antibody *(upper panel). In vivo* Ser65 (anti-P-Ser65) and Thr70 (anti-P-Thr70) phosphorylation was evaluated by immunoblotting with phosphopeptide-specific antibodies.

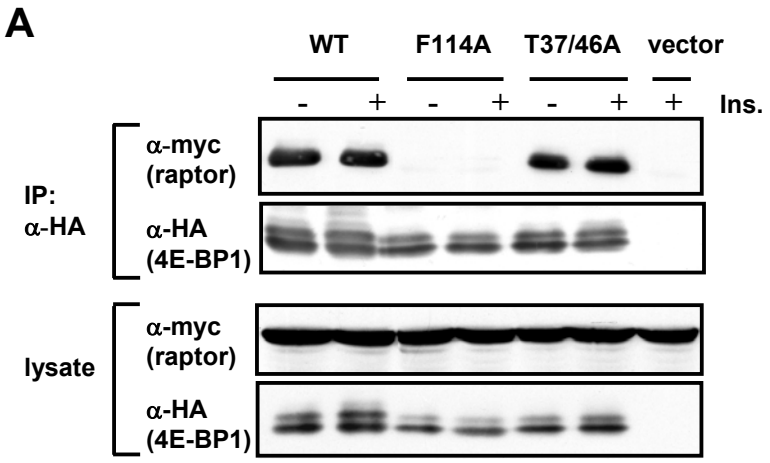
To role out the possibility that the inhibitory effect of the TOS motif mutant (4E-BP1-F114A) on Ser65 and Thr70 phosphorylation could be a secondary consequence of reduced phosphorylation of the priming sites (Thr37/46), the phosphorylation of Ser65 and Thr70 in wild type and F114A-4E-BP1 that contain acidic mutations in the priming sites Thr37/46 (4E-BP1-T37/46E) to mimic Thr37/46 phosphorylation was compared. It has been reported that mutation of Thr37/46 to glutamic residues (4E-BP1-T37/46E) partially

restores phosphorylation of Ser65 and Thr70, whereas mutation of Thr37/46 to alanines abolishes phosphorylation of Ser65 and Thr70 (Gingras, Gygi et al. 1999). Phosphorylation of Ser65 and Thr70 in the TOS motif mutant containing acidic Thr37/46 mutations (4E-BP1-F114A) was significantly less than in a 4E-BP1 mutant with the acidic substitutions for Thr37/46 but with a functional TOS motif, suggesting that the TOS motif is required for efficient phosphorylation of Ser65 and Thr70 even when 4E-BP1 is primed by phosphorylation at Thr37/46. Therefore, the TOS motif is required for mTOR-dependent phosphorylation of 4E-BP1 at all known rapamycin sensitive sites (Figure 2.2.1C-E), similar to its role in regulating the phosphorylation status of several sites in S6K1 (Schalm and Blenis 2002).

2.2.2 The TOS motif is required for 4E-BP1-raptor complex formation

How does the TOS motif mediate mTOR regulation of 4E-BP1? Recently, raptor and its yeast homolog, KOG1, were identified as TOR interacting regulatory proteins (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002; Loewith, Jacinto et al. 2002). Raptor forms a complex with 4E-BP1 and mTOR and is thought to function as a scaffolding protein that recruits mTOR in close proximity to its downstream substrates (Hara, Maruki et al. 2002). To investigate whether the TOS motif is required for 4E-BP1 regulation by the raptor-mTOR complex, we co-transfected HA-tagged 4E-BP1 and myc-tagged raptor in HEK293E cells. The TOS motif mutant 4E-BP1 (F114A) failed to co-immunoprecipitate with raptor whereas wild-type 4E-BP1 co-immunoprecipitated, as previously reported (Figure 2.2.2 A, 2B, 2D, and Hara, Maruki et al. 2002), indicating that the TOS motif in 4E-BP1 is required for its binding to raptor. The inability of 4E-BP1-F114A to bind to raptor is not a consequence of its hypophosphorylated status, as the similarly hypophosphorylated 4E-BP1-T37/46A mutant bound raptor as efficiently as wild-type 4E-BP1 (Figure 2.2.2A). Additional mutations in other residues

within the TOS motif of 4E-BP1 were generated and tested for their effect on 4E-BP1 phosphorylation (as assayed by enhanced mobility on SDS-PAGE) and 4E-BP1 co-immunoprecipitation with myc-tagged raptor. Different mutations in the TOS motif inhibited the ability of 4E-BP1 to co-immunoprecipitate with raptor, paralleling their effect on 4E-BP1 phosphorylation (Figure 2.2.2B and 2.2.2C). Importantly, the TOS motif was also required for 4E-BP1 to bind raptor under conditions (Kim, Sarbassov et al. 2002) that preserve mTOR complexes (0.2% CHAPS as detergent and crosslinker DSP); Upon co-transfection of WT-and F114A-4E-BP1 with raptor followed by lysis under these conditions, raptor and endogenous mTOR co-immunoprecipitated with wild-type but not with the TOS motif mutant of 4E-BP1 (Figure 2.2.2D). In summary, the data indicate that a functional TOS motif is required for 4E-BP1-raptor complex formation.



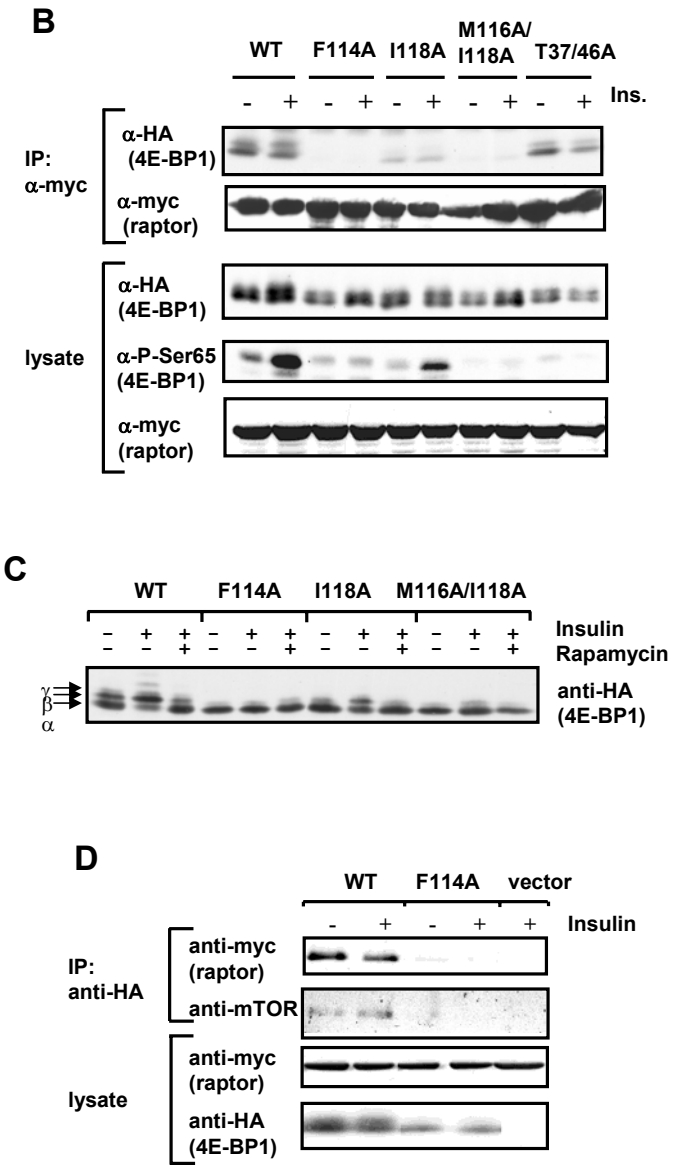


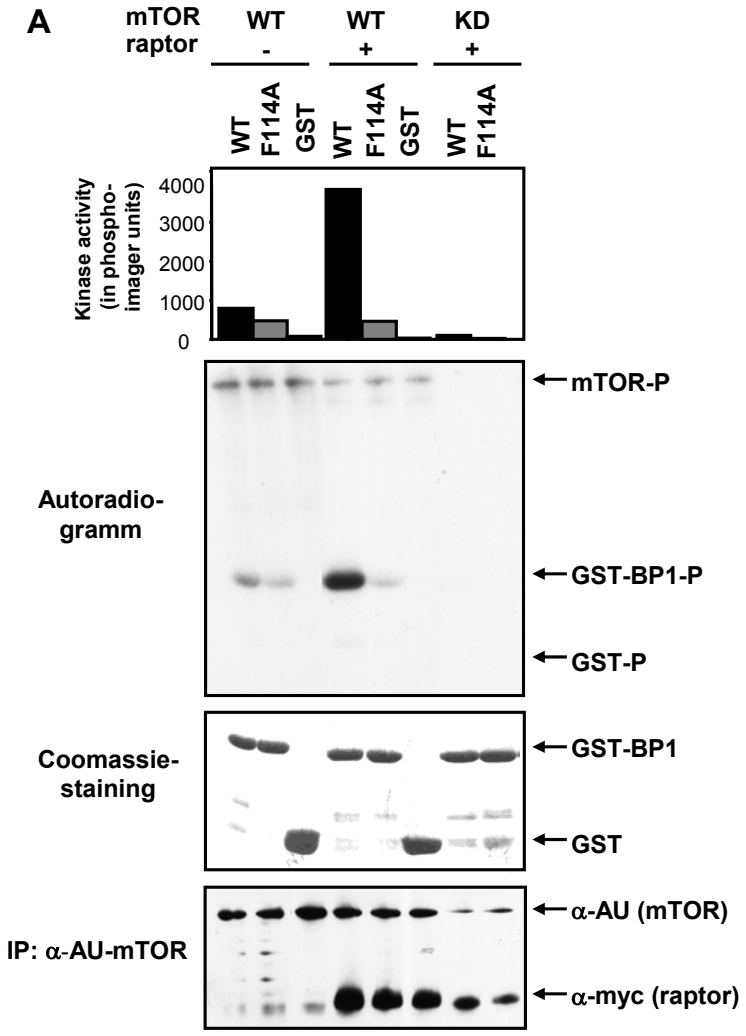
Figure 2.2.2. A functional TOS motif is required for 4E-BP1 to bind to raptor. HEK293E cells were cotransfected with **(A)** myc-raptor and either HA-4E-BP1 wild-type (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-T37/46A (T37/46A), or the empty pACTAG-2 vector (vector) and **(B)** with myc-raptor and either with wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-I118A (I118A), or HA-4E-BP1-M116A/I118A (M116A/I118A). Transfected cells were starved, insulin-stimulated (Ins.), and lysed as described in 'Materials and methods'. **(A)** anti-HA immunoprecipitations were analyzed by anti-HA (HA-4E-BP1) and anti-myc (myc-raptor) immunoblotting. Myc-raptor expression levels was assayed by immunoblotting of cell lysate with

anti-myc antibody**. (B)** α-myc immunoprecipitations were analyzed by anti-HA (HA-4E-BP1) and anti-myc (myc-raptor) immunoblotting. Protein expression levels were assayed by immunoblotting cell lysate with anti-myc antibody. Ser65 phosphorylation was evaluated by immunoblotting with anti-4E-BP1-P-Ser65 phosphopeptide-specific antibody. **(E)** HEK293E cells were transfected with wild-type HA-4E-BP1 (WT), HA-4E-BP1-F114A (F114A), HA-4E-BP1-I118A (I118A), and HA-4E-BP1-M116A/I118A (M116A/I118A). Transfected cells were starved, rapamycin treated, stimulated, and lysed as described in 'Materials and methods'. 4E-BP1 was detected by immunoblotting with anti-HA-antibody.

2.2.3 TOS motif is required for efficient *in vitro* phosphorylation of 4E-BP1 by mTOR

Since the TOS motif is required for efficient 4E-BP1 phosphorylation at all mTOR-regulated sites *in vivo* (Figure 2.2.1C, 2.2.1D and 2.2.1E), it was examined next whether the TOS motif is required for mTOR phosphorylation of 4E-BP1 *in vitro*. Therefore, the phosphorylation of recombinant wild-type GST-4E-BP1and GST-4E-BP1-F114A by AU-tagged mTOR immunoprecipitated from cells transfected with AU-mTOR in the absence or presence of myc-raptor were compared. Importantly, cell extracts under were prepared under conditions that retain the mTOR-raptor complex and as expected, myc-raptor co-immunprecipitatetd with AU-mTOR (Figure 3A and Kim, Sarbassov et al. 2002). AU-mTOR phosphorylated wild-type GST-4E-BP1 to a much higher extent than GST-4E-BP1-F114A (Figure 2.2.3A), indicating that direct phosphorylation of 4E-BP1 by mTOR requires an intact TOS motif. As previously reported, AU-mTOR/myc-raptor immunocomplexes significantly enhances *in vitro* phosphorylation of wild-type GST-4E-BP1 (Figure 2.2.3A and Hara, Maruki et al. 2002). Interestingly, AU-mTOR/myc-raptor immunocomplexes did not enhance the phosphorylation of GST-4E-BP1-F114A as potently as wild-type GST-4E-BP1 under the same conditions *in vitro* (approximately 8 times lower; Figure 2.2.3A). Importantly, the kinase-inactive mutant of mTOR (AU-mTOR-KD) did not phosphorylate GST-4E-BP1 or GST-4E-BP1-F114A (Figure 2.2.3A), indicating that mTOR, rather than an associated kinase, directly phosphorylates GST-4E-BP1

in this assay. In contrast, activated recombinant ERK2 phosphorylated both wild-type 4E-BP1 (Chen, Sarnecki et al. 1992) and the TOS motif mutant F114A to a similar extent, indicating that the proline-directed sites in both recombinant proteins are accessible for phosphorylation (Figure 2.2.3B). As expected, phosphorylation of GST alone by mTOR or ERK2 was not detected (Figure 2.2.3A and 2.2.3B). Immunoprecipitated endogenous mTOR from HEK293E cells also phosphorylated wild-type GST-4E-BP1 much more efficiently than the F114A mutant (data not shown).



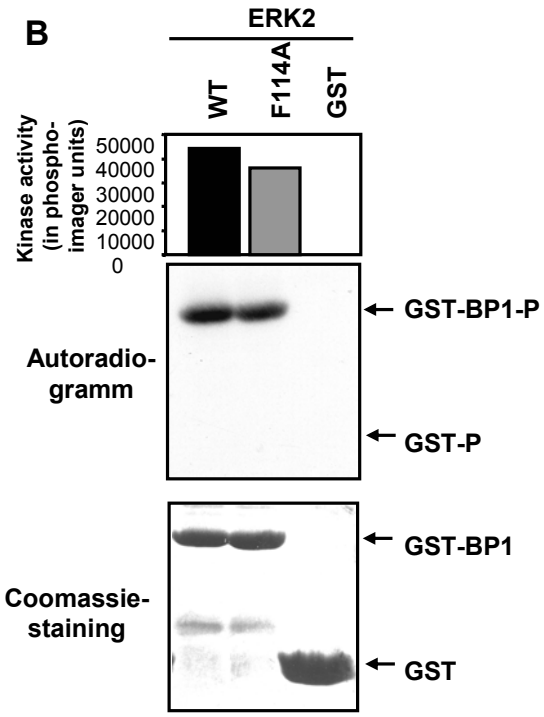


Figure **2.2.3. TOS motif is required for 4E-BP1 *in vitro* phosphorylation by mTOR but not ERK2. (A)** AU-mTOR wild-type (WT) or kinase-dead (KD) co-expressed in HEK293E cells with myc-raptor or vector control were purified by anti-AU immunoprecipitation and subjected to *in vitro* mTOR kinase assay using recombinant wild-type GST-4E-BP1, GST-4E-BP1-F114A, or GST as substrates as described in 'Materials and methods'. Quantification of the kinase assay (first panel) and autoradiogram of phosphorylated proteins (second panel) is shown. A coomassie staining of the SDS-PAGE gel reveals the relative amounts of each fusion protein in the assay (third panel). An anti-AU immunoblot of the kinase assay (forth panel) is shown. **(B)** *In vitro* phosphorylation of recombinant wild-type GST-4E-BP1, GST-4E-BP1-F114A, and GST by activated (His)6-ERK2 was performed as described in 'Materials and methods'. Quantification of the kinase assay (first panel), and autoradiogram of phosphorylated proteins (second panel) is shown. A coomassie staining of the SDS-PAGE gel reveals the relative amounts of each fusion protein in the assay (third panel).

2.2.4 Mutation of the TOS motif strengthens the binding of 4E-BP1 to eIF4E

Next, it was investigated whether the 4E-BP1-F114A mutant could inhibit eIF4E function under conditions where wild-type 4E-BP1 does not, as during insulin stimulation. Therefore, the association of wild-type 4E-BP1 and 4E-BP1-F114A to eIF4E was compared by a m^7GTP-Sepharose pull-down assay. m^7GTP mimics the cap-structure of mRNA and can be used to affinity purify eIF4E and its associated proteins. In serum-starved HEK293E cells wild-type 4E-BP1 associated with eIF4E, which was abrogated upon insulin stimulation but recovered when cells were pretreated with rapamycin, as expected (Figure 2.2.4). The 4E-BP1-F114A mutant displayed stronger association to eIF4E than wild-type 4E-BP1 in serum-starved or rapamycin-treated HEK293E cells, consistent with decreased phosphorylation on the 4E-BP1-F114A mutant (Figure 2.2.1C and 2.2.3A). The amount of 4E-BP1-F114A bound to eIF4E decreased upon insulin stimulation of serum-starved HEK293E cells, but significant binding was still detectable. Indeed, the association of 4E-BP1-F114A to eIF4E in insulin-stimulated cells was significantly higher than association of wild-type 4E-BP1 to eIF4E in serum-starved cells (Figure 2.2.4). As a negative control, the eIF4E-binding site (4E-BP1-Y54A/L59A) on 4E-BP1 was mutated (Mader, Lee et al. 1995). Binding of both wild-type and 4E-BP1-F114A to eIF4E was abrogated by Y54A/L59A mutations (Figure 2.2.4). These data suggest that 4E-BP1-F114A binds to eIF4E specifically via the defined eIF4E-binding site and therefore functions as an inhibitor of eIF4E. Similar data were observed using U2OS cells (data not shown).

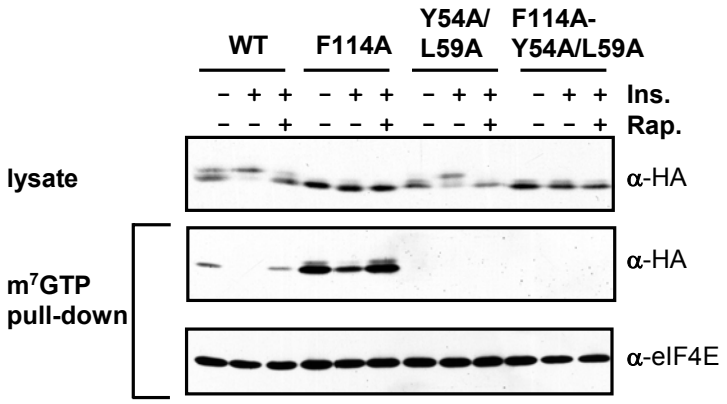


Figure 2.2.4. Mutation of the TOS-motif induces stronger binding to eIF4E. HEK293E cells overexpressing wild-type HA-tagged 4E-BP1 (WT) and HA-tagged 4E-BP1 mutants (F114A, Y54A/L59A, or Y54A/L59A-F114A) were starved, rapamycin treated (rap.), insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with anti-HA-antibody *(top panel).* m7GTP-Sepharose pull down was analyzed by immunoblotting for anti-HA (HA-4E-BP1) *(middle panel)* and anti-eIF4E *(bottom panel)*.

2.2.5 Overexpression of 4E-BP1-F114A reduces cell size

Reduction of raptor expression by siRNA (Kim, Sarbassov et al. 2002) or inhibition of mTOR-dependent signaling with rapamycin (Fingar, Salama et al. 2002) results in reduced cell size, suggesting that raptor mediates mTOR-dependent regulation of cell growth. To determine whether TOS-mediated 4E-BP1 phosphorylation by the raptor/mTOR complex is important for the control of cell growth, it was analyzed whether overexpression of 4E-BP1-F114A would reduce the size of proliferating U2OS cells. U2OS cells were used for this study as their cell size is fairly homogenous, and they have been well characterized for cell size regulation by mTOR signaling (Fingar, Salama et al. 2002). Furthermore, the regulation of 4E-BP1 phosphorylation is similar in U2OS and HEK293 cells (Figure 2.2.1C and 2.2.1D). The relative size of G_1-phase cells was measured by flow cytometry using the parameter forward scatter-height. In the first set of experiments the FSC-H histogram of cells transfected with pACTAG-2 vector

control was compared to cells treated with rapamycin or transfected with WT-, F114A-, or T37/46-4E-BP1 (Figure 2.2.5A). Overexpression of 4E-BP1-F114A induced a shift to reduced cell size compared to vector control (Figure 2.2.5A), similar to the previously reported effect that rapamycin or the dominant 4E-BP1-T37/46A mutant has on reducing cell size (Fingar, Salama et al. 2002). WT-4E-BP1 had a minimal effect, consistent with previously published data. Graphical representation of the same experiment whereby each construct was transfected in triplicates shows that the reduction in cell size induced by F114A-4E-BP1 expression is significant (Figure 2.2.5B). Importantly, mutations of the eIF4E-binding domain (Y54A/L59A) on 4E-BP1 abrogated the ability of the TOS motif mutant 4E-BP1-F114A to reduce cell size (Figure 2.2.5B), demonstrating that the growth inhibitory effect caused by 4E-BP1-F114A occurs specifically through eIF4E binding and thus inhibition of eIF4E function. These data indicate that the TOS motif is important for regulation of 4E-BP1's biological function, to control cell growth.

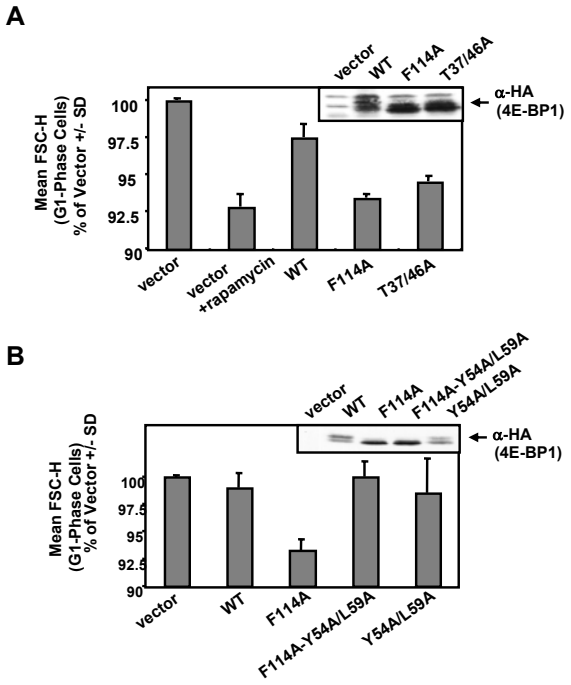


Figure 2.2.6. Overexpression of 4E-BP1-F114A causes reduction of cell size. U2OS cells were cotransfected overnight with **(A)** 1μg CD20 and 10μg of either the empty pACTAG-2 vector (vector), HA-4E-BP1 wild-type (WT), HA-4E-BP1-F114A (F114A), or HA-4E-BP1-T37/46A (T37/46A) or **(B)** with 1μg of CD20 and either 10μg of the empty pACATG-2 vector (vector), wild-type HA-4E-BP1 (WT) 4E-BP1, or the following HA-4E-BP1 mutants: F114A, Y54A/L59A-F114A, or Y54A/L59A, cultured for 72 h in DMEM/FBS, and assayed by flow cytometry to determine cell size. The mean FSC-H +/- SD of triplicate samples is shown as above.

2.3 Regulation of S6K2

S6K2 has high homology to S6K1 (Figure 2.3.1), but displays significantly lower kinase activity than S6K1 upon stimulation with a variety of known S6K1 activators (Figure 2.3.4B). However, deletion of the C-terminus increases S6K2 activity and renders the enzyme more sensitive to mitogen stimulation (Martin et al.), suggesting a more potent inhibitory effect mediated by the S6K2 C-terminus that is not found in S6K1. Identification of a unique S6K2 regulator would provide us with a better understanding of the physiological role of S6K2. Therefore, it was interesting to determine the region in the C-terminus of S6K2 that is responsible for the inhibitory effect and to identify a potential binding protein. The highest homology between these S6 kinases is found in their catalytic (84% identical) and linker (80% identical) domains (Figure 2.3.1). The N-termini and extreme C-termini of the S6 kinases represent the parts of the protein with the lowest sequence homology. S6K2 has a poly-proline stretch in its C-terminus that is not found in S6K1. Proline-rich domains often interact with SH3-domain containing proteins. The proline-rich stretch in S6K2 was therefore a good candidate to mediate the binding of a putative inhibitory protein.

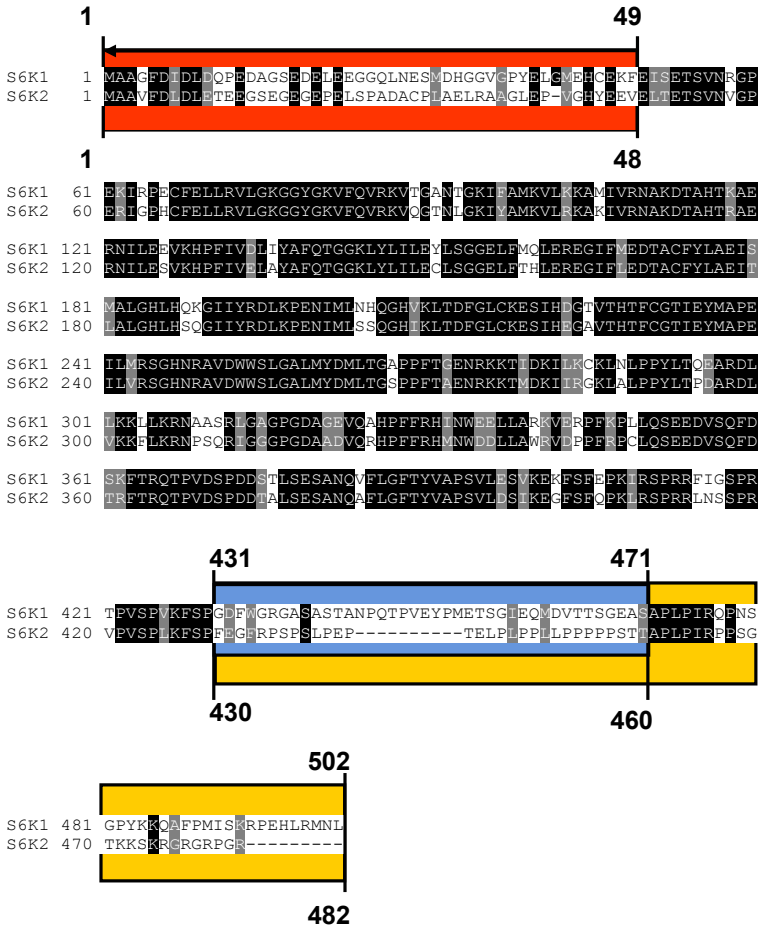


Figure 2.3.1 Sequence alignment of S6K1 (rat) and S6K2 (human) . Identical (black box) and conserved (shadowed box) amino acids are highlighted, unique N-terminal regions are highlighted in red, proline-rich domain of S6K2 is highlighted in blue, and the extreme C-terminal parts of S6K1 and S6K2 are highlighted in yellow.

2.3.1 N- and C-terminus of S6K2 inhibit its activity

First the question was addressed whether deletion of the proline-rich domain in S6K2 enhances insulin-stimulated S6K2 activity. To preserve the structural integrity of S6K2, the proline rich region of S6K2 (amino acids 430-460) was replaced by the corresponding part of S6K1 (amino acids 431-471) (S6K2/P-S6K1) and the effect on insulin-stimulated activity was measured (Figure 2.3.2B). Surprisingly, replacement of the proline rich region in S6K2 did not significantly increase the mitogen-stimulated activity of S6K2, suggesting that the proline rich

domain of S6K2 is not responsible for the additional inhibition of S6K2 kinase activity (Figure 2.3.2B). In contrast, replacing the unique region of the extreme 52 C-terminal amino acids of S6K2 with the corresponding S6K1 part (S6K2/CT-S6K1) increased the insulin-stimulated S6K2 activity significantly when compared to wild-type S6K2 (Figure 2.3.2B).

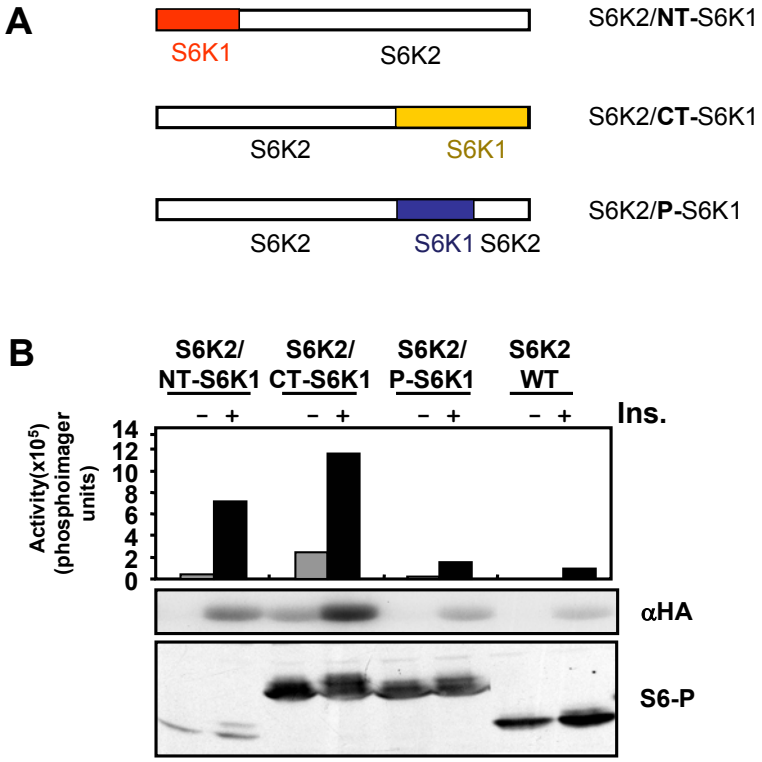


Figure 2.3.2 S6K2 proline-rich domain not required for C-terminal inhibitory effect. (A) Schematic representation of S6K2 mutants used. S6K2/**NT**-S6K1 (S6K2 fused with N-terminus of S6K1), S6K2/**CT**-S6K1 (S6K2 fused with C-terminus of S6K1), and S6K2/**P**-S6K1 (proline-rich domain of S6K2 replaced by corresponding part of S6K1). **(A)** HEK293 cells were transfected with HA-S6K2/**NT**-S6K1 (S6K2/**NT**-S6K1), HA-S6K2/**CT**-S6K1 (S6K2/**CT**-S6K1), HA-S6K2/**P**-S6K1 (S6K2/**P**-S6K1), or HA-S6K2 wild-type (S6K2-WT). Transfected cells were starved, insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.

These data, therefore, suggest that amino acid residues 460-482 of S6K2 mediate an inhibitory effect on its kinase activity. A nuclear localization sequence located in the C-terminus of S6K2 may be responsible for this effect. Indeed, mutation within the residues in the nuclear localization signal increased the kinase activity of S6K2 (Martin et al). To investigate whether the N-terminus of S6K2 also contributes to an inhibitory effect, the unique N-terminal region of S6K2 (amino acids 1-48) was replaced with the corresponding N-terminal part of S6K1 (amino acids 1-49) (referred as 'S6K2/NT-S6K1'). Interestingly, a S6K2 chimera containing the 49 N-terminal residues of S6K1 displayed a higher specific activity than S6K2 wild-type (Figure 2.3.2B).

2.3.2 N-and C-terminal regulation of S6K1

In addition, it was tested whether replacing the N-or C-terminal domain of S6K1 with the N-or C-terminal part of S6K1, respectively, effects the kinase activity of S6K1. S6K1 chimeras containing the first 48 N-terminal (S6K1/NT-S6K2) or the last 52 amino acid residues of S6K2 (S6K1/CT-S6K2) displayed a lower specific kinase activity than wild-type S6K1 (Figure 2.3.4B). The results obtained support a model of S6K2 regulation, where N- and C-terminus mediate an inhibitory effect on its kinase activity. Mutation of a potential phosphorylation site in the N-terminus of S6K2 (Ser 24) did not alter S6K2 activity (data not shown), suggesting that molecular interactions and not phosphorylation is responsible for the additional inhibitory effect mediated by the S6K2 N-terminus.

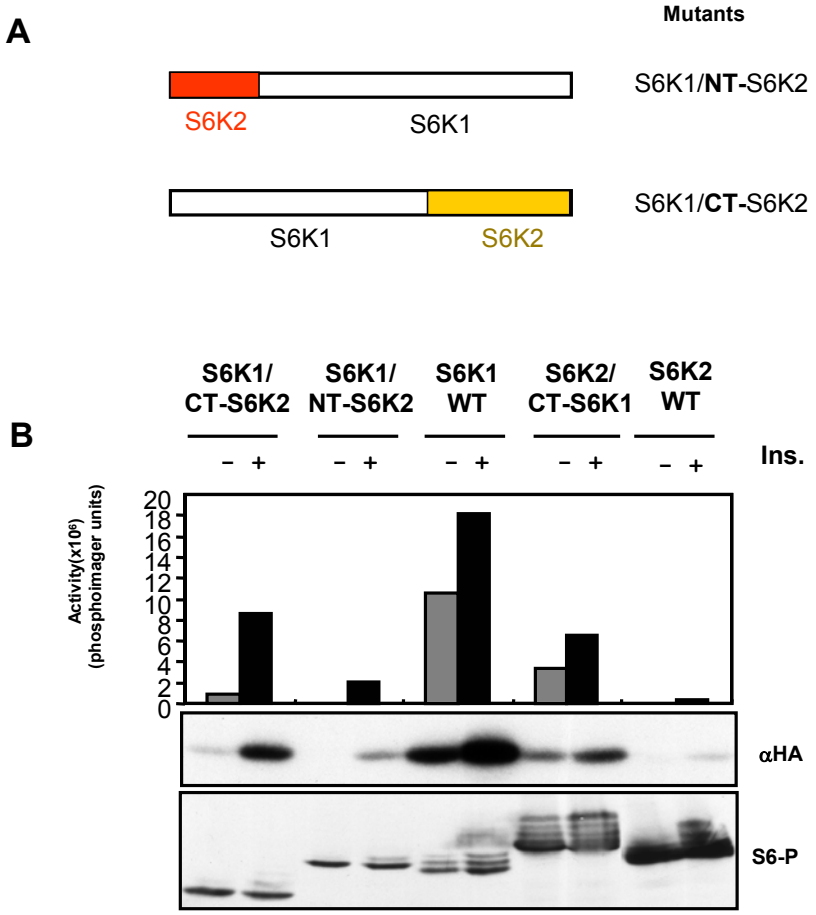


Figure 2.3.3. S6K2 proline-rich domain not required for C-terminal inhibitory effect. (A) Schematic representation of mutants used. S6K1/**NT**-S6K2 (S6K1 fused with N-terminus of S6K2), and S6K2/**CT**-S6K1 (S6K1 fused with C-terminus of S6K2). **(B)** HEK293 cells were transfected with HA-tagged S6K1 wild-type (S6K1 WT), S6K1/**NT**-S6K2, S6K2/**CT**-S6K1, S6K2 wild-type (S6K2 WT), or S6K2/**CT**-S6K1 . Transfected cells were starved, insulin-stimulated (ins.), and lysed as described in 'Materials and methods'. Protein expression levels were assayed by immunoblotting with α-HA-antibody *(second panel)* and kinase assays were performed as described *(lower panels)*. Quantification of kinase activity *(upper panels)* and autoradiogram of *in vitro* S6 phosphorylation *(lower panel)* are provided.