Mechanistic Target of Rapamycin Complex 1 (mTORC1)-mediated Phosphorylation Is Governed by Competition between Substrates for Interaction with Raptor*[^1][^2]

Received for publication, July 18, 2012, and in revised form, November 2, 2012. Published, JBC Papers in Press, November 26, 2012, DOI 10.1074/jbc.M112.402461

Michael D. Dennis, Scot R. Kimball[^1], and Leonard S. Jefferson

From the Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Background: mTORC1 targets p70S6K1 and 4E-BP1 are phosphorylated in response to nutrient and hormonal signals.

Results: Loss of 4E-BP1/2 enhances the rate of p70S6K1 phosphorylation.

Conclusion: By competitively interacting with raptor, 4E-BP1 impairs p70S6K1 phosphorylation.

Significance: Altered 4E-BP1 expression potentially governs phosphorylation of p70S6K1 under pathophysiological states.

In this study, the interaction of mTORC1 with its downstream targets p70S6K1 and 4E-BP1 was evaluated in both mouse liver and mouse embryonic fibroblasts following combined disruption of the genes encoding 4E-BP1 and 4E-BP2. Phosphorylation of p70S6K1 was dramatically elevated in the livers of mice lacking 4E-BP1 and 4E-BP2 following feeding-induced activation of mTORC1. Immunoprecipitation of mTORC1 suggested that elevated phosphorylation was the result of enhanced interaction of p70S6K1 with raptor. These findings were extended to a cell culture system wherein loss of 4E-BP1 and 4E-BP2 resulted in elevated interaction of p70S6K1 with IGF1-induced activation of mTORC1 in conjunction with an enhanced rate of p70S6K1 phosphorylation at Thr-389. Furthermore, cotransfecting HA-p70S6K1 with 4E-BP1, but not 4E-BP1(F114A), reduced recovery of mTORC1 in HA-p70S6K1 immunoprecipitates. Together, these findings support the conclusion that, in the absence of 4E-BP proteins, mTORC1-mediated phosphorylation of p70S6K1 is elevated by a reduction in competition between the two substrates for interaction with raptor.

The mechanistic target of rapamycin (mTOR[^2]); previously known as the mammalian target of rapamycin) is an evolutionarily conserved serine/threonine protein kinase that regulates the cell signaling response to nutrient sufficiency and growth factors (1). The polypeptide mTOR functions in two distinct multiprotein complexes: mTORC1 (mTOR complex 1) and mTORC2 (2). Both complexes contain mTOR and mLST8; however, only mTORC1 contains raptor (regulatory-associated protein of mTOR), which serves as a scaffolding protein for recruitment of substrates for phosphorylation by the kinase domain of mTOR. Overexpression of raptor stimulates mTORC1-mediated phosphorylation of its substrates, whereas the inhibitor rapamycin is believed to act by disruption of the mTOR-raptor interaction (3, 4). Activation of the mTORC1 signaling pathway in response to insulin or growth factors occurs primarily through the PI3K/Akt signaling pathway (5–7). The activation of Akt is responsible for phosphorylation of at least two proteins involved in the regulation of mTORC1, TSC2 (tuberous sclerosis complex 2) and PRAS40 (proline-rich Akt substrate of 40 kDa). TSC2 functions in a heterodimeric complex with TSC1 as a GTPase-activating protein toward the small GTPase Rheb (Ras homolog enriched in brain). Association of Rheb in its GTP-bound, but not GDP-bound, state with mTORC1 leads to its activation through an incompletely defined mechanism. Phosphorylation of PRAS40 by Akt results in its dissociation from mTORC1, allowing raptor to recruit the two best characterized downstream targets of mTORC1, p70S6K1 (p70 ribosomal protein S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) (8–10).

Both p70S6K1 and 4E-BP1 contain a common mTORC1 signaling (TOS) motif that plays a critical role in substrate recognition by mediating direct interaction with raptor and consequent phosphorylation by mTORC1 (11, 12). The TOS motif contains an essential Phe residue, followed by four alternating acidic and small hydrophobic residues, and is found in the N terminus of p70S6K1 (FDIDL) and C terminus of 4E-BP1 (FEMDI). Mutation of the essential Phe residue impairs the interaction of both 4E-BP1 and p70S6K1 with raptor and thus eliminates the rapamycin-sensitive phosphorylation of the two substrates (11). Intriguingly, the interaction of PRAS40 with raptor (and thus, its inhibitory effect on 4E-BP1 and p70S6K1 phosphorylation) is also dependent on a TOS motif variant (10, 13). Overexpression of PRAS40 suppresses phosphorylation of 4E-BP1 and p70S6K1, whereas mutant PRAS40 that is deficient in binding raptor does not affect their phosphorylation (13). Furthermore, overexpression of p70S6K1 impairs mTORC1-catalyzed phosphorylation of 4E-BP1 (14); however, this effect is absent when the TOS motif is ablated (11). Thus, direct inter-
action of 4E-BP1 and p70S6K1 with raptor functions as a critical step in mediating substrate phosphorylation by mTORC1. In this study, we demonstrate accelerated phosphorylation of p70S6K1 occurring concomitant with increased interaction of p70S6K1 with mTORC1 in the livers of mice lacking 4E-BP1 and 4E-BP2 compared with control mice. Moreover, in mouse embryonic fibroblasts (MEFs) lacking 4E-BP1/2, the interaction of exogenously expressed HA-p70S6K1 with mTORC1 was elevated, and this effect was repressed by cotransfection with exogenous 4E-BP1, but not with 4E-BP1 in which the essential Phe residue in the TOS motif was altered. Together, these findings demonstrate that competitive substrate binding to raptor governs mTORC1-mediated phosphorylation of p70S6K1 and 4E-BP1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protease inhibitor mixture was purchased from Sigma, and ECL Western blotting detection reagent was from Pierce. Preparation of the anti-4E-BP1 and anti-eIF4E antibodies has been described previously (15, 16). Anti-mTOR, anti-p70S6K1, anti-eIF3c, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies were purchased from Bethyl Laboratories. Anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, and all other antibodies were purchased from Cell Signaling Technology. Protein content was measured using the DC protein assay (Bio-Rad).

**Animals**—Eif4ebp1; Eif4ebp2 mutant mice were a kind gift from Dr. Nahum Sonenberg (McGill University). Male mice weighing ~30 g were maintained on a 12:12-h light/dark cycle with food (Harlan Teklad) and water provided ad libitum. All procedures involving these mice were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Mice were fasted for 10 h, followed by a refeeding period of 45 min where indicated.

**Processing of Liver Samples**—For analysis of the protein phosphorylation state, a portion (~0.3 g) of liver was homogenized in 7 volumes of CHAPS lysis buffer (40 mM HEPES, 0.3% CHAPS, 1 mM EDTA, 50 mM NaF, 40 mM NaCl, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM benzamidine, 200 mM sodium vanadate, and 10 μl/ml protease inhibitor mixture) using a Polytron homogenizer. The homogenate was centrifuged at 1000 × g for 3 min at 4 °C, and the resulting supernatant fraction was subjected to SDS-PAGE and Western blot analysis as described previously (17). Phosphorylation of p70S6K1, Akt, 4E-BP1, and IRS1 was measured in the supernatant fraction using phosphospecific antibodies as described previously (18).

**Cell Culture and Transfections**—Cultures of wild-type and Eif4ebp1; Eif4ebp2 double knock-out (DKO) MEFs, a kind gift from Dr. Nahum Sonenberg, and Rps6k1; Rps6k2 DKO MEFs, a kind gift from Dr. Sara Kozma (University of Cincinnati), were maintained in Dulbecco’s modified Eagle’s medium lacking sodium pyruvate and containing high glucose (Invitrogen) supplemented with 10% fetal bovine serum (Atlas Biologicals) and 1% penicillin/streptomycin (Invitrogen). Transfections were performed using X-tremeGENE HP (Roche Applied Science) with a 3:1 ratio of reagent to DNA (μg/μg) according to the manufacturer’s instructions. Cells were deprived of serum for 3 h and treated with IGF1 (10 ng/ml) as indicated. Cells were harvested in 1× SDS sample buffer for analysis of cell lysate or in CHAPS lysis buffer for immunoprecipitation. Overexpression of HA-S6K1 was achieved using pRK7-HA-S6K1, which was generously provided by Dr. John Blenis (Harvard University). 4E-BP1 was exogenously expressed in cells using the pCMV6–4E-BP1 plasmid (purchased from OriGene Technologies). Generation of pCMV6–4E-BP1(F114A) was performed using the QuickChange Lightning site-directed mutagenesis kit (Stratagene) with the following primers: 5’-CAGGGCGTGAAGGCTCACAAGCTGAGATGCACTTAAGG-3’ and 5’-CCCTAAATGTCCATCTCAGCTTGATGACTTTCCACC-GCCTG-3’. Confirmation of mutagenesis was obtained by sequencing.

**Immunoprecipitations**—Immunoprecipitations were performed by incubating the 1000 × g supernatant fractions of liver homogenates or cell lysates with polyclonal anti-mTOR or monoclonal anti-raptor antibody (Cell Signaling Technology). Supernatant fractions or cell lysates containing ~1 mg of protein were incubated with 2 μg of antibody for 1 h at 4 °C. A 200-μg portion of BioMag goat anti-rabbit or anti-mouse IgG beads (Qiagen), previously blocked with CHAPS lysis buffer containing 1% BSA, was added to each sample, and the suspension was rocked at 4 °C for 2 h. For anti-α-HA immunoprecipitations, 10 μl of anti-α-HA-agarose affinity resin (Sigma) was washed twice with CHAPS lysis buffer and blocked for 1 h in CHAPS lysis buffer containing 1% BSA. Cell lysate was then added and incubated for 2 h. For all immunoprecipitations, beads were washed twice with 1 ml of ice-cold CHAPS lysis buffer, suspended in 1× SDS sample buffer, and boiled for 5 min. Supernatants were subjected to Western blot analysis using antibody to mTOR, raptor, p70S6K1, eIF3c, or 4E-BP1, and the results were normalized for the amount of the target protein in the immunoprecipitate.

**Statistical Analysis**—Data are expressed as means ± S.E. One-way analysis of variance and Student’s t test were used to compare differences among groups. p < 0.05 was considered statistically significant.

**RESULTS**

Ablation of 4E-BP1 and 4E-BP2 Enhances Phosphorylation of p70S6K1 in Response to Activation of mTORC1—Feeding-induced activation of mTORC1 led to enhanced phosphorylation of p70S6K1 (Fig. 1A) and 4E-BP1 (Fig. 1B) in the livers of wild-type mice. However, in the livers of mice lacking 4E-BP1/2, phosphorylation of p70S6K1 at Thr-389 was elevated by 22-fold compared with a 7-fold increase in wild-type mice when evaluated 45 min after feeding-induced activation of mTORC1 (Fig. 1A). One possible explanation for the enhanced phosphorylation of p70S6K1 at Thr-389 would be up-regulated stimulatory signals to mTORC1 in mice lacking 4E-BP1/2; however, it has been previously reported that signaling to mTORC1 is actually impaired in the muscle, liver, and adipose tissue of 4E-BP1/2 DKO mice compared with wild-type mice due to a p70S6K1-mediated negative feedback loop through the phosphorylation of IRS1 at Ser-636/639 and impaired phosphorylation of Akt at Ser-473 (19). In this study, there was no significant difference in either the basal or feeding-induced phosphoryla-
tion of Akt at Ser-473 or of PRAS40 at Ser-246, an Akt target site, in the livers of DKO mice compared with wild-type mice (Fig. 1, C and D). Taken together, these findings suggest that greater activation of mTORC1 by signaling inputs upstream of Akt is not likely responsible for the feeding-induced elevation in the phosphorylation of p70S6K1 in the livers of DKO mice compared with wild-type mice.

To further evaluate mTORC1 signaling to p70S6K1 in the absence of 4E-BP1 and 4E-BP2, we employed wild-type and 4E-BP1/2 DKO mouse MEFs. IGF1-induced activation of mTORC1 enhanced p70S6K1 phosphorylation at Thr-389 in 4E-BP1/2 DKO MEFs to an extent that was nearly 2-fold greater than the values observed in wild-type MEFs (Fig. 2A). Similar to the findings in DKO mouse liver, phosphorylation of p70S6K1 did not appear to be the result of a greater signaling input from Akt (Fig. 2A, compare the third and fourth lanes with the seventh and eighth lanes). Notably, unlike in the liver, where fasting levels of p70S6K1 phosphorylation at Thr-389 were almost undetectable in wild-type and DKO mice (Fig. 1A), p70S6K1 phosphorylation at Thr-389 was elevated in serum-starved DKO MEFs compared with wild-type MEFs (Fig. 2A). Whereas fasting eliminates signaling inputs to mTORC1 from both
growth factors and amino acids, serum deprivation of cell culture medium eliminates only those from growth factors. Thus, the input from amino acids maintained mTORC1 signaling in serum-deprived MEFs at levels sufficient to detect differential signaling to p70S6K1 in wild-type MEFs compared with DKO MEFs. To further evaluate phosphorylation of p70S6K1 in response to IGF1-induced activation of mTORC1, we performed a time course experiment in wild-type and DKO MEFs. At early time points (0–20 min), phosphorylation of p70S6K1 was elevated in DKO MEFs compared with wild-type MEFs. However, after 30 min, phosphorylation of p70S6K1 did not appear to differ in wild-type and DKO MEFs (Fig. 2, B and C, compare the seventh and eighth lanes; and Fig. 2D). Thus, IGF1-induced p70S6K1 phosphorylation at Thr-389 occurred more rapidly in DKO MEFs compared with wild-type MEFs. In both cell types, a similar maximal level of p70S6K1 phosphorylation was achieved, but this occurred more rapidly in the absence of 4E-BP1/2 (i.e. ~40 min for wild-type MEFs and ~20 min for DKO MEFs) (Fig. 2D).

Because the enhanced feeding-induced phosphorylation of p70S6K1 in the absence of 4E-BP1 did not appear to be due to upstream signal inputs from Akt, we sought to evaluate activation of mTORC1. The interaction of raptor with mTOR is required for the recruitment of substrates under nutrient-rich conditions; however, in the absence of nutrient-induced signaling to mTORC1, a second high-affinity interaction with raptor represses mTORC1 activity (3). In this study, the interaction of raptor with mTOR was evaluated by immunoprecipitating both mTOR and raptor from the supernatant fraction of liver homogenates and assessing the amount of each protein in the immunoprecipitate. As shown in Fig. 3A and supplemental Fig. 1, in the fasted state, the mTOR-raptor interaction was similar in the livers of both wild-type and 4E-BP1/2 DKO mice. Moreover, feeding reduced the mTOR-raptor interaction by ~25% in the livers of both wild-type and DKO mice. As a second measure of mTORC1 activation, we also assessed autophosphorylation of mTOR at Ser-2481 in the raptor immunoprecipitate. Autophosphorylation of mTOR at Ser-2481 has been previously shown to function as a biomarker for monitoring mTORC-specific catalytic activity (20). In the livers of both wild-type and DKO mice, feeding increased the autophosphorylation of the mTOR present in the mTORC1 complex by ~80% (Fig. 3B). Taken together, these findings imply that the elevation in the feeding-induced phosphorylation of p70S6K1 in the livers of 4E-BP1/2 DKO mice was not the result of enhanced mTORC1 activation but was likely due to the enhanced ability of active mTORC1 to phosphorylate p70S6K1 in the absence of 4E-BP1/2.

4E-BP1 and p70S6K1 Compete for Interaction with mTORC1

FIGURE 2. IGF1-induced phosphorylation of p70S6K1 is enhanced in MEFs lacking 4E-BP1/2. Wild-type and 4E-BP1/2 DKO MEFs were incubated in serum-free medium (SFM) for 3 h to repress phosphorylation of p70S6K1 at Thr-389. Activation of mTORC1 signaling was achieved by treating cells with IGF1 as indicated. A, phosphorylation of p70S6K1 at Thr-389 and Akt at Ser-473 15 min after the administration of IGF1 was assessed by Western blot analysis with phosphospecific antibodies in wild-type and 4E-BP1/2 DKO MEFs. Representative blots are shown. Values are means ± S.E. (n = 4). Statistical significance is denoted by the presence of different letters above the bars on the graphs. Bars with different letters are statistically different (p < 0.05). To further evaluate the rate of p70S6K1 phosphorylation, wild-type (B) and 4E-BP1/2 DKO (C) MEFs were harvested after IGF1 treatment as indicated. Phosphorylation of p70S6K1 at Thr-389 was evaluated by Western blot analysis. D, values are means ± S.E. (n = 4). *, p < 0.05.
absence of 4E-BP1/2 is a reduction in competition between the two substrates for binding to raptor, as phosphorylation of 4E-BP1 and p70S6K1 by mTORC1 depends on the interaction of raptor with their respective TOS motifs (12). To evaluate the interaction of p70S6K1 with mTORC1, we immunoprecipitated raptor from the supernatant fraction of liver homogenates and accessed the presence of p70S6K1 in the immunoprecipitate. The interaction of p70S6K1 with raptor was enhanced in the livers of both wild-type and DKO mice upon feeding-induced activation of mTORC1 relative to fasted mice (Fig. 3C). However, in the livers of 4E-BP1/2 DKO mice, the magnitude of the increase was 3-fold greater than that observed in wild-type mice (Fig. 3C, compare the first and second lanes with the third and fourth lanes). We also observed phosphorylation of p70S6K1 at Thr-389 when bound to raptor (Fig. 3D).

To further evaluate the enhanced interaction of p70S6K1 with mTORC1, we performed immunoprecipitations on the supernatant fractions of cell lysates from wild-type and 4E-BP1/2 DKO MEFs. Initially, we sought to evaluate the interaction of endogenous p70S6K1 and raptor. Unfortunately, unlike with the liver samples, the recovery of endogenous p70S6K1 from the raptor immunoprecipitate of cell lysates was poor, and the presence of background bands made analysis difficult. This result is consistent with previous reports of a weak interaction between endogenous p70S6K1 and mTORC1 in cell lysates (21, 22). To perform a more thorough analysis, we made
use of exogenously overexpressed HA-tagged p70S6K1 and evaluated its interaction with mTORC1 upon serum deprivation and following treatment with IGF1. The immunoprecipitate of HA-p70S6K1 from serum-starved MEFs contained both mTORC1 and eIF3 (Fig. 4A). When inactive, p70S6K1 interacts with eIF3 (23). In a previously reported model, activation of mTORC1 promoted its recruitment to eIF3, where mTORC1 interacted with p70S6K1 to mediate phosphorylation of multiple residues (23). Thus, the presence of mTOR and raptor in the immunoprecipitate of exogenously expressed HA-p70S6K1 potentially reflects both mTORC1 bound directly to HA-p70S6K1 and mTORC1 bound indirectly through eIF3. It seems likely that exposure to serum-free medium for 3 h was not sufficient to produce full dissociation of mTORC1 from eIF3, as there was no change in the interaction of mTORC1 with either HA-eIF3f (supplemental Fig. 2A) or HA-eIF3h (supplemental Fig. 2B) when serum-deprived wild-type or DKO MEFs were treated with IGF1. This interpretation is supported by the finding that hypophosphorylated 4E-BP1 was also present in the HA-p70S6K1 immunoprecipitate when serum-deprived wild-type or DKO MEFs were treated with IGF1. This interpretation is less appealing for two reasons. 1) Co-immunoprecipitation of 4E-BP1 with p70S6K1 was mainly observed in serum-deprived cells. 2) Co-immunoprecipitation of 4E-BP1 with p70S6K1 was principally observed in serum-deprived cells.

**FIGURE 4. IGF1-induced interaction of p70S6K1 with mTORC1 in MEFs lacking 4E-BP1 and 4E-BP2.** Wild-type and 4E-BP1/2 DKO MEFs were exposed to serum-free medium (SFM) for 3 h to repress phosphorylation of p70S6K1 at Thr-389. A and B, activation of mTORC1 signaling was achieved by stimulating cells with IGF1 as indicated. The interaction of endogenous p70S6K1 with mTORC1 was evaluated by immunoprecipitating IP: raptor and measuring the amount of p70S6K1 by Western blot analysis. Autoradiography of mTOR at Ser-2481 was also measured in the HA-p70S6K1 immunoprecipitate to assess mTORC1 activation. Expression of mTOR, raptor, eIF3c, HA-p70S6K1, and 4E-BP1 was evaluated in whole cell lysate (WCL) by Western blot analysis. Phosphorylation of p70S6K1 at Thr-389 was assessed by Western blot analysis with a phosphospecific antibody. Quantitation of mTOR (C) and raptor (D) interaction with HA-p70S6K1 was evaluated 5–25 min after IGF1 administration. Values are means ± S.E. (n = 2–4). *, p < 0.05. Representative blots are shown.
4E-BP1 and p70S6K1 Compete for Interaction with mTORC1

When serum-deprived wild-type or DKO MEFs were treated with IGF1, the amount of elf3, mTOR, and raptor recovered in the HA-p70S6K1 immunoprecipitate was reduced (Fig. 4A). As a measure of mTORC1 activation status, we evaluated autophosphorylation of mTOR at Ser-2481 in the HA-p70S6K1 immunoprecipitate (Fig. 4A). Whereas mTOR phosphorylation at Ser-2481 was low in the HA immunoprecipitate relative to the amount of total mTOR under the serum-deprived condition, IGF1 treatment produced an increase in the ratio of phosphorylated to total mTOR in the HA-p70S6K1 immunoprecipitate (Fig. 4A, compare mTOR Ser-2481-P with mTOR (total)). On the basis of these findings and the previously described model (23), we propose that, in the serum-deprived condition, mTORC1 and HA-S6K1 bind independently to elf3, whereas upon IGF1 treatment, HA-S6K1 interacts directly with raptor to allow for phosphorylation by mTOR (supplemental Fig. 2E).

To evaluate the influence of 4E-BP1/2 on the interaction of HA-p70S6K1 with mTORC1, we treated serum-deprived wild-type and DKO MEFs with IGF1 for 10, 15, or 20 min and evaluated the presence of mTOR and raptor in the HA-p70S6K1 immunoprecipitate (Fig. 4, A and B). Under the serum-deprived condition, the absence of 4E-BP1/2 did not significantly alter the interaction of either protein with HA-p70S6K1 (Fig. 4, C and D, respectively). This finding suggests that p70S6K1 and 4E-BP1 possess unique binding sites under this condition; HA-p70S6K1 was likely associated directly with elf3, with 4E-BP1 being bound indirectly to elf3 via elf4E. When wild-type cells were treated with IGF1, the amount of mTORC1 in the HA-p70S6K1 immunoprecipitate was reduced; however, in cells lacking 4E-BP1/2, the presence of both mTOR and raptor in the HA-p70S6K1 immunoprecipitate following 15 or 20 min of IGF1 treatment was significantly elevated compared with that observed in wild-type cells (Fig. 4, C and D). This result demonstrates enhanced interaction of HA-p70S6K1 with active mTORC1 in cells lacking 4E-BP1/2 compared with the wild-type condition. We also evaluated the interaction of mTORC1 with exogenously overexpressed HA-tagged 4E-BP1 in S6K1−/−/S6K2−/− MEFs (supplemental Fig. 2C). Whereas the amount of mTORC1 detected in the HA-4E-BP1 immunoprecipitate was not significantly different in the absence of S6K1/2 expression under serum-deprived conditions, the interaction of both mTOR and raptor with HA-4E-BP1 was elevated following IGF1 treatment (supplemental Fig. 2D). Together, these findings suggest that 4E-BP1 impairs the interaction of active mTORC1 with p70S6K1 in a manner that is mediated by the TOS motif of 4E-BP1.

**DISCUSSION**

In this study, we used 4E-BP1/2 DKO mice and MEFs generated from these animals to evaluate the interaction of mTORC1 with its downstream targets p70S6K1 and 4E-BP1. Phosphorylation of p70S6K1 and 4E-BP1 by mTORC1 is facilitated by a common TOS motif (11). Mutation of the TOS domain impairs the ability of both 4E-BP1 and p70S6K1 to interact with raptor (12) and thus eliminates the rapamycin-sensitive phosphorylation of mutant isoforms (11). Furthermore, the integrity of the TOS domain appears to be necessary for p70S6K1 and 4E-BP1 to compete as substrates for mTORC1-mediated phosphorylation, as overexpression of p70S6K1 that contains an altered TOS motif fails to inhibit 4E-BP1 phosphorylation (11). Although 4E-BP1 and p70S6K1 have been shown to compete as substrates for mTORC1-mediated phosphorylation (11, 14), it has not been previously demonstrated that this phenomenon is the result of mutually exclusive interaction with the substrate-binding domain of raptor upon activation of mTORC1. In the livers of mice lacking 4E-BP1 and 4E-BP2, enhanced phosphorylation of p70S6K1 was observed following feeding in conjunction with increased interaction of p70S6K1 with mTORC1. Moreover, in cells lacking 4E-BP1/2, the interaction of HA-p70S6K1 with mTORC1 was elevated in a manner that could be repressed by cotransfection with exogenous 4E-BP1, but not with 4E-BP1 containing a disruption of the TOS domain. Together, these findings suggest that mTORC1-mediated phosphorylation of p70S6K1 and 4E-BP1 is co-regulated by competition between the two substrates for interaction with mTORC1.

In response to nutrients and environmental signals, mTORC1 and p70S6K1 associate with or dissociate from elf3 in a coordinated manner (23). When inactive, p70S6K1 is bound to elf3, whereas mTORC1 only weakly interacts with elf3 in HEK293E cells after 20 h of serum starvation or following treatment with rapamycin (23). Upon stimulation with insulin, but not with insulin plus rapamycin, mTORC1 is recruited to elf3, where mTORC1 interacts with p70S6K1 to mediate phosphorylation of multiple residues (23). In support.....
of this model, we observed a feeding-induced enhancement in the interaction of p70S6K1 with mTORC1 in the livers of both wild-type and DKO mice. However, unlike in this previously reported model (23), we were initially surprised to observe a reduction in the interaction of exogenously expressed HA-S6K1 with both mTORC1 and eIF3 in serum-deprived wild-type and DKO MEFs upon stimulation with IGF1. We observed that mTORC1 remained associated with subunits of eIF3 following 3 h of serum deprivation despite a marked reduction in phosphorylation of p70S6K1. Furthermore, following serum deprivation, IGF1 treatment enhanced autophosphorylation of mTOR at Ser-2481 but was unable to increase the interaction of p70S6K1 with mTORC1 (Fig. 3D), an observation that suggests that phosphorylation of p70S6K1 proceeds more rapidly than release of p70S6K1 from mTORC1. Upon mTORC1-mediated phosphorylation, p70S6K1 is released from the elf3-mTORC1 complex such that p70S6K1 can be phosphorylated at Thr-229 in its activation loop by PDK1 and become fully activated (23). Following dissociation of phosphorylated p70S6K1 from mTORC1, the TOS motif of raptor is free to interact with either a new unphosphorylated p70S6K1 molecule or hypophosphorylated 4E-BP.

In this study, we observed co-immunoprecipitation of p70S6K1 and mTORC1 in subpopulations containing both phosphorylated and unphosphorylated p70S6K1 and mTORC1. In the livers of fasted mice, p70S6K1 interacted with mTORC1; however, upon feeding, autophosphorylation of mTOR at Ser-2481 was enhanced, and the interaction of p70S6K1 with mTORC1 was elevated. More interestingly, feeding induced phosphorylation of p70S6K1 in the raptor immunoprecipitate (Fig. 3D), an observation that suggests that phosphorylation of p70S6K1 proceeds more rapidly than release of p70S6K1 from mTORC1. Upon mTORC1-mediated phosphorylation, p70S6K1 is released from the elf3-mTORC1 complex such that p70S6K1 can be phosphorylated at Thr-229 in its activation loop by PDK1 and become fully activated (23). Following dissociation of phosphorylated p70S6K1 from mTORC1, the TOS motif of raptor is free to interact with either a new unphosphorylated p70S6K1 molecule or hypophosphorylated 4E-BP.
Thus, in the absence of 4E-BP1/2, there is a lack of competition for binding to mTORC1 in its activated state, as evidenced by increased recovery of mTOR phosphorylated at Ser-2481 in the HA-p70S6K1 immunoprecipitate from DKO MEFs compared with wild-type MEFs following stimulation with IGF1 (Fig. 4B). A similar result was obtained in vivo, where we observed enhanced interaction of p70S6K1 with mTORC1 in the livers of 4E-BP1/2 DKO mice compared with wild-type mice upon feeding (Fig. 3C). However, unlike the activated state, we observed no difference in the association of p70S6K1 with mTORC1 in the absence of 4E-BP1/2 in the livers of fasted mice or in serum-deprived MEFs. One explanation for these results is that mTORC1, p70S6K1, and the 4E-BP proteins bind independently to eIF3 when mTORC1 is inactive, but upon activation of mTORC1, the conformation of the complex is altered such that mTORC1 can interact directly with either p70S6K1 or the 4E-BP proteins, but not both at the same time (supplemental Fig. 2). Moreover, in such a model, the interaction between mTORC1 and its substrates is mediated primarily through the binding of the substrates to raptor. On the basis of this model, we conclude that enhanced phosphorylation of p70S6K1 in the absence of 4E-BP1 occurs due to a reduction in competition for binding to activated mTORC1.

The findings of this study demonstrate that competition between substrates for binding to raptor plays an important role in mTORC1-mediated phosphorylation of p70S6K1 and 4E-BP1. It is important to point out that increased substrate binding alone has been recently shown to produce limited increases in mTORC1-mediated phosphorylation (25). However, when mTORC1 is activated by upstream signaling inputs, enhanced binding of p70S6K1 is achieved by the elimination of 4E-BP1/2 and substantially increases p70S6K1 phosphorylation. Thus, conditions that alter expression of p70S6K1 or 4E-BP1 potentially modulate signaling through both pathways. We have recently shown that the expression of 4E-BP1 is elevated in the livers of mice with streptozotocin-induced type 1 diabetes (26). Furthermore, a majority of large advanced breast cancers overexpress 4E-BP1 in a manner that facilitates tumor angiogenesis and hypoxia responses (27). In these pathophysiological states, elevated 4E-BP1 expression potentially impairs phosphorylation of p70S6K1 by acting as a competitive substrate for interaction with raptor and thus impairs the insulin signaling cascade downstream of mTORC1.

Acknowledgments—We thank Dr. Nahum Sonenberg for generously providing 4E-BP1/2 DKO mice and 4E-BP1/2 DKO MEFs, Dr. John Blenis for providing the pR7-HA-S6K1 plasmid, and Dr. Sara Kozma for providing S6K1/2 DKO MEFs. We also thank Lydia Kutzler for assistance with animals and Holly Lacko for assistance with Western blotting.

REFERENCES
1. Laplante, M., and Sabatini, D. M. (2012) mTOR signaling in growth control and disease. Cell 149, 274–293
2. Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M. A., Hall, A., and Hall, M. N. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin-insensitive. Nat. Cell Biol. 6, 1122–1128
3. Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163–175
4. Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004) Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. Genes Cells 9, 359–366
5. Gingras, A. C., Kennedy, S. G., O’Leary, M. A., Sonenberg, N., and Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev. 12, 502–513
6. Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002) TSC2 is phosphorylated and inactivated by Akt and suppresses mTOR signalling. Nat. Cell Biol. 4, 648–657
7. Winter, J. N., Jefferson, L. S., and Kimball, S. R. (2011) ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. Am. J. Physiol. Cell Physiol. 300, C1172–C1180
8. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) Control of p70 S6 kinase by kinase activity of FRAP in vivo. Nature 377, 441–446
9. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr., and Abraham, R. T. (1997) Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 277, 99–101
10. Wang, L., Harris, T. E., Roth, R. A., and Lawrence, J. C., Jr. (2007) PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J. Biol. Chem. 282, 20036–20044
11. Schalm, S. S., and Blenis, J. (2002) Identification of a conserved motif required for mTOR signaling. Curr. Biol. 12, 632–639
12. Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J., and Yonezawa, K. (2003) The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. 278, 15461–15464
13. Oshiro, N., Takahashi, R., Yoshino, K., Tanimura, K., Nakashima, A., Eguchi, S., Miyamoto, T., Hara, K., Takehana, K., Avruch, J., Kikkawa, U., and Yonezawa, K. (2007) The proline-rich Akt substrate of 40 kDa (PRS40) is a physiological substrate of mammalian target of rapamycin complex 1. J. Biol. Chem. 282, 20329–20339
14. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas, G. (1997) The insulin-induced signaling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70S6K. Mol. Cell. Biol. 17, 5426–5436
15. Kimball, S. R., Horetsky, R. L., and Jefferson, L. S. (1998) Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. J. Biol. Chem. 273, 30945–30953
16. Kimball, S. R., Jurasinski, C. V., Lawrence, J. C., Jr., and Jefferson, L. S. (1997) Insulin stimulates protein synthesis in skeletal muscle by enhancing the association of eIF-4E and eIF-4G. Am. J. Physiol. 272, C754–C759
17. Dennis, M. D., Baum, J. I., Kimball, S. R., and Jefferson, L. S. (2011) Mechanisms involved in the coordinate regulation of mTORC1 by insulin and amino acids. J. Biol. Chem. 286, 8287–8296
18. Kimball, S. R., Siegfried, B. A., and Jefferson, L. S. (2009) Glucagon represses signaling through the mammalian target of rapamycin in rat liver by activating AMP-activated protein kinase. J. Biol. Chem. 279, 54103–54109
19. Le Bacquer, O., Petroulakis, E., Pagliaiuong, S., Poulin, F., Richard, D., Cianflone, K., and Sonenberg, N. (2007) Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. J. Clin. Invest. 117, 387–396
20. Soliman, G. A., Acosta-Jaquez, H. A., Dunlop, E. A., Ekim, B., Maji, N. E., Tee, A. R., and Finger, D. C. (2010) mTOR Ser-2481 autophosphorylation monitors mTORC-specific catalytic activity and clarifies rapamycin mechanism of action. J. Biol. Chem. 285, 7866–7879
21. Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110, 177–189
22. Schalm, S. S., Fingar, D. C., Sabatini, D. M., and Blenis, J. (2003) TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr. Biol.* **13**, 797–806

23. Holz, M. K., Ballif, B. A., Gygi, S. P., and Blenis, J. (2005) mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123**, 569–580

24. Yip, C. K., Murata, K., Walz, T., Sabatini, D. M., and Kang, S. A. (2010) Structure of the human mTOR complex I and its implications for rapamycin inhibition. *Mol. Cell* **38**, 768–774

25. Rapley, J., Oshiro, N., Ortiz-Vega, S., and Avruch, J. (2011) The mechanism of insulin-stimulated 4E-BP protein binding to mammalian target of rapamycin (mTOR) complex 1 and its contribution to mTOR complex 1 signaling. *J. Biol. Chem.* **286**, 38043–38053

26. Dennis, M. D., Schrufer, T. L., Bronson, S. K., Kimball, S. R., and Jefferson, L. S. (2011) Hyperglycemia-induced O-GlcNAcylation and truncation of 4E-BP1 protein in liver of a mouse model of type 1 diabetes. *J. Biol. Chem.* **286**, 34286–34297

27. Braunstein, S., Karpisheva, K., Pola, C., Goldberg, J., Hochman, T., Yee, H., Cangiarella, J., Arju, R., Formenti, S. C., and Schneider, R. J. (2007) A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer. *Mol. Cell* **28**, 501–512