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Raptor, a Binding Partner of Target of Rapamycin (TOR), Mediates TOR Action

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Summary

mTOR controls cell growth, in part by regulating p70 S6 kinase α (p70α) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Raptor is a 150 kDa mTOR binding protein that also binds 4EBP1 and p70α. The binding of raptor to mTOR is necessary for the mTOR-catalyzed phosphorylation of 4EBP1 in vitro, and it strongly enhances the mTOR kinase activity toward p70α. Rapamycin or amino acid withdrawal increases, whereas insulin strongly inhibits, the recovery of 4EBP1 and raptor on 7-methyl-GTP Sepharose. Partial inhibition of raptor expression by RNA interference (RNAi) reduces mTOR-catalyzed 4EBP1 phosphorylation in vitro. RNAi of *C. elegans* raptor yields an array of phenotypes that closely resemble those produced by inactivation of Ce-TOR. Thus, raptor is an essential scaffold for the mTOR-catalyzed phosphorylation of 4EBP1 and mediates TOR action in vivo.

Introduction

The target of rapamycin proteins, TOR1 and TOR2, were first identified in *Saccharomyces cerevisiae* through mutants that confer resistance to growth inhibition induced by an immunosuppressive macrolide rapamycin (Kunz et al., 1993). Early findings in *S. cerevisiae* indicated that TOR1 and TOR2 participate in the control of translational initiation and early G1 progression in response to nutrient availability (Barbet et al., 1996; Di Como and Arndt, 1996; Schmidt et al., 1998). Recent work has identified additional functions of TOR, including the regulation of transcription, cytoskeletal organization, and protein degradation through autophagy (Schmelzle and Hall, 2000). TOR orthologs have been identified in mammals (mTOR, also known as FRAP, RAFT1, or RAPT) (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995) and many other organisms (Weisman and Choder, 2001; Long et al., 2002; Oldham et al., 2000; Zhang et al., 2000). All contain a kinase domain near their C termini, most closely related to the phosphatidylinositol kinase (PIK)-related kinase family, which includes ATM, ATR, and DNA-PK, kinases that play important parts in controlling cell cycle (Keith and Schreiber, 1995; Takahashi et al., 2000; Zakian, 1995). Other characteristic structures include N-terminal HEAT repeats, the FAT/FATC domains surrounding the kinase domain, and the FKBP-rapamycin binding (FRB) domain.

In cultured mammalian cells, rapamycin blocks phosphorylation of several translational regulators including eukaryotic initiation factor-4E binding protein 1 (4EBP1) (Beretta et al., 1996; Lawrence and Abraham, 1997) and p70 S6 kinase α (p70α) (Chung et al., 1992; Price et al., 1992). Evidence supporting the mTOR kinase as the rapamycin-sensitive element responsible for p70α and 4EBP1 is the finding that a mutant of mTOR (ST-mTOR) that no longer binds the FKBP12/rapamycin complex protects coexpressed p70α or 4EBP1 from rapamycin-induced dephosphorylation and/or inhibition, whereas the kinase-dead mutant of ST-mTOR (ST/NK-mTOR) does not (Brown et al., 1995; Brunn et al., 1997; Hara et al., 1997).

In response to insulin or mitogens, the p70α activity dramatically increases through the multisite phosphorylation in vivo (Avruch et al., 2001). p70α is proposed to play an important role in the translation of a subclass of mRNAs that contain a short oligopyrimidine sequence (Jefferies et al., 1997), although this has been disputed recently (Tang et al., 2001). 4EBP1 binds to the eIF-4E (a 7-methyl-guanosine mRNA cap binding protein) and prevents eIF-4E from binding to a scaffold protein eIF-4G, thereby inhibiting the formation of the active translational complex, eIF-4F (Sonenberg, 1996). Insulin or mitogens stimulate the phosphorylation of 4EBP1, resulting in the dissociation and disinhibition of eIF-4E. In addition to regulation by insulin or mitogens, nutrients, especially amino acids, recently have been discovered to regulate the phosphorylation of p70s and 4EBP1s and to be necessary for insulin or mitogen regulation of these targets (Fox et al., 1998; Hara et al., 1998; Minami et al., 2001; Patti et al., 1998; Shigemitsu et al., 1999; Wang et al., 1998; Xu et al., 1998). Indirect evidence implicates mTOR in this amino acid-regulated signaling (Hara et al., 1998). In addition, *Drosophila* TOR has been shown to be required for normal cell growth and proliferation during larval development (Oldham et al., 2000; Zhang et al., 2000), at least in part by regulating several translational effectors in response to the nutritional environment.

mTOR immunopurified from cultured cells or tissues phosphorylates in vitro the exogenous substrates 4EBP1 and p70α purified from *E. coli* (Brunn et al., 1997; Burnett et al., 1998). Importantly, immunopurified mTOR phosphorylates and activates mammalian recombinant p70α and p70β (also known as S6K2) (Gout et al., 1998; Koh et al., 1999; Lee-Fruman et al., 1999; Saitoh et al., 1998; Shima et al., 1998) directly in vitro in a synergistic manner with 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Alessi et al., 1997) through phosphorylation at several p70s amino acids situated in structurally unrelated motifs (Isotani et al., 1999; Minami et al., 2001). These results support the idea that mTOR, in addition

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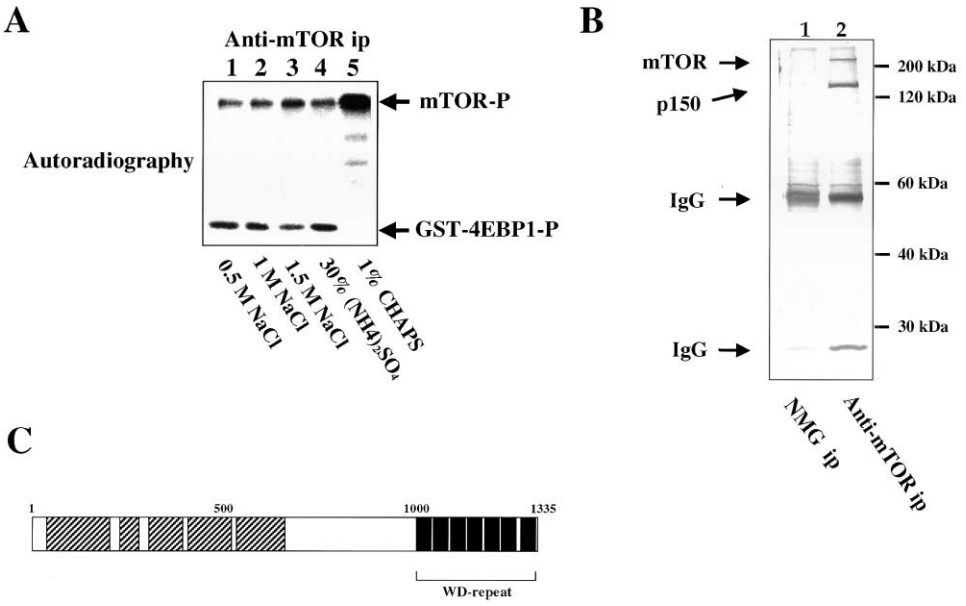

Figure 1. Purification and Cloning of mTOR Binding Protein

(A) Cell extract was prepared from HEK293 cells without detergent and subjected to immunoprecipitation with anti-mTOR Ab. The immunoprecipitates were washed with wash buffer containing either 0.5 M NaCl (lane 1), 1 M NaCl (lane 2), 1.5 M NaCl (lane 3), or 1% CHAPS and 0.5 M NaCl (lane 5). Another cell extract was subjected to 30% $(NH_4)_2SO_4$ precipitation, and the precipitate was dissolved in Buffer A, then subjected to immunoprecipitation with anti-mTOR Ab (lane 4). The immunoprecipitates were subjected to mTOR kinase assay using GST-4EBP1 as substrate and analyzed by autoradiography.

(B) After $(NH_4)_2SO_4$ precipitation and ion column chromatography, mTOR and its associated proteins were immunopurified with anti-mTOR Ab. The immunopurified proteins were eluted with buffer A with 1% NP-40 (lane 2). As a control, immunopurification was performed with normal mouse IgG and eluted with buffer A with 1% NP-40 (lane 1).

(C) A schematic structure of raptor. The highly conserved regions are shaded and the WD repeat is shown by black box.

to being an upstream regulator of these elements, is a direct kinase toward 4EBP1 and p70s in vitro and in vivo. On the other hand, several studies indicate that a phosphatase also plays a critical role in the mTOR regulation of p70α (Peterson et al., 1999); the detailed mechanism by which mTOR regulates these phosphorylations in vivo remains to be elucidated.

The mechanism by which mTOR phosphorylates 4EBP1 appears to be different from that by which mTOR phosphorylates p70s in vitro. First, mTOR-catalyzed 4EBP1 phosphorylation in vitro is severely reduced if the mTOR immunoprecipitate is washed with detergents, whereas mTOR-catalyzed p70 phosphorylation is unaffected or possibly enhanced by the same washing (Isotani et al., 1999). Second, immunopurified mTOR catalyzes phosphorylation of 4EBP1 exclusively at Ser/Thr-Pro motifs (Gingras et al., 1999; Mothe-Satney et al., 2000), whereas a Phe-Thr-Tyr motif is the major site of mTOR-catalyzed phosphorylation on p70α and p70β (Burnett et al., 1998; Isotani et al., 1999; Minami et al., 2001). Although this remarkably broad specificity suggests the operation of more than one kinase, all these mTOR-catalyzed phosphorylations are abolished by direct addition in vitro of a rapamycin/FKBP12 complex.

We explored the possibility that the mTOR-catalyzed phosphorylation of 4EBP1 in vitro requires the participation of additional, detergent-sensitive molecules that are copurified in mTOR immunoprecipitate. Herein, we describe the identification of an mTOR binding protein, raptor, that is necessary for mTOR-catalyzed 4EBP1 phosphorylation in vitro and for TOR action in vivo.

Results

Identification and Cloning of Raptor

As shown previously, the kinase activity of immunoprecipitated mTOR toward 4EBP1 is diminished by washing with a detergent such as 1% NP-40 or 1% CHAPS (Figure 1A, compare lane 1 to lane 5). In contrast, the kinase activity is fully recovered after washing with a high-salt buffer containing up to 1.5 M NaCl (Figure 1A, compare lane 1 to lanes 2 and 3). Moreover, the mTOR kinase activity toward 4EBP1 is fully recovered even after mTOR precipitation by 30% $(NH_4)_2SO_4$, (Figure 1A, lane 4). These results suggest that a molecule that is required for the mTOR kinase activity toward 4EBP1 is removed by detergent, but not by high-salt washes. We therefore sought mTOR-associated molecules exhibiting these properties.

We employed a three-step method to purify the possible mTOR-associated proteins (see Supplemental Data at http://www.cell.com/cgi/content/full/110/2/177/DC1). First, mTOR and its potential binding partners were precipitated with 30% $(NH_4)_2SO_4$. Next, the redissolved precipitate was subjected to cation exchange chromatography; finally, the peak fraction containing mTOR, identified by anti-mTOR immunoblot, was applied to protein G Sepharose beads coupled to a monoclonal anti-mTOR antibody (Ab). After washing the beads with high-salt buffers, bound proteins were eluted with 1% NP-40 (Figure 1B, lane 2). We identified an mTOR-associated polypeptide at 150 kDa in the eluate. An in-gel digest of this polypeptide was subjected to

mass spectrometric analysis for protein identification by the peptide mass fingerprinting and tandem mass spectrometry (MS/MS) ions search using Mascot. Database searches, complemented by 5′ RACE (see Supplemental Data) enabled the assembly of a full-length cDNA of human p150, which consists of an open reading frame encoding 1335 amino acids (see Supplemental Figure S1). The deduced polypeptide contains seven WD repeats encompassing the C-terminal one-third of the amino acid sequence (Figure 1C) and is homologous to the WD repeat protein Mip1p of *S. pombe*, a protein essential for cell growth (Shinozaki-Yabana et al., 2000). We named p150 as raptor. Alignment of the deduced amino acid sequence of the raptor orthologs of *S. cerevisiae* (accession number P38873), *S. pombe* (accession number P87141), *C. elegans* (Ce-raptor) (accession number T19148), *D. melanogaster* (accession number AAF46122), and *Arabidopsis thaliana* (accession number AAF07837) using the CLUSTAL W program define five highly conserved segments in the N-terminal half of the polypeptide (see Supplemental Figure S2). Each segment is separated by insertions of about 20–130 amino acids, suggesting that each segment represents an independently folding and perhaps functional domain. We also determined the full cDNA sequence of mouse raptor; human and mouse raptor exhibited 96.9% identity in the amino acid sequence (data not shown). Northern blot analysis of mouse raptor revealed that raptor was expressed ubiquitously as a single transcript in many tissues, with higher expression in brain and kidney (data not shown).

Binding of mTOR and Raptor

HA-tagged mTOR and FLAG-tagged raptor were coexpressed in HEK293 cells and their association was evaluated (Figure 2A). When cell extraction and immunoprecipitation are performed without detergent, HA-mTOR is coimmunoprecipitated with the FLAG-raptor (Figure 2A, Anti-FLAG ip, lanes 4–7; Anti-HA ip, lanes 12–15). The efficiency of coimmunoprecipitation is not affected by amino acid depletion (D-PBS, lanes 4 and 12), serum stimulation (AA+S, lanes 6 and 14), or rapamycin (AA+S+Rap, lanes 7 and 15), but is abolished if the immunoprecipitates are washed with a wash buffer containing 1% NP-40 (lanes 8 and 16).

We next examined the binding of endogenous mTOR and raptor (Figure 2B). HEK293 cells incubated under a variety of conditions were extracted without detergent, and endogenous mTOR or raptor was immunoprecipitated with either anti-mTOR Ab or anti-N-raptor Ab, respectively. The immunoprecipitate was subjected to the kinase assay using GST-4EBP1 as a substrate and to immunoblot with anti-mTOR Ab and anti-N-raptor Ab. The anti-N-raptor Ab precipitates a kinase activity toward GST-4EBP1 (Figure 2B, Anti-p150 ip, lanes 7–10), and endogenous mTOR is visualized in raptor immunoprecipitate (Anti-mTOR blot, lanes 7–10). As raptor is a noncatalytic polypeptide, it is likely that the raptor-associated kinase activity toward GST-4EBP1 is due to the coprecipitation of mTOR. Reciprocally, raptor is readily detectable in the anti-mTOR Ab immunoprecipitate, confirming that endogenous raptor coprecipitates with mTOR (Anti-p150 blot, lanes 2–5). In comparing immunoprecipitates from cells incubated in a variety of conditions, significant changes in the relative association between mTOR and raptor were not detected except for some decrease in the kinase activity and the recovery of raptor with mTOR from cells treated with rapamycin (Figure 2B, lanes 5 and 10).

Effects of Raptor on the mTOR Kinase Activity

We next examined whether raptor affects the mTOR kinase activity in vitro. HEK293 cells were transfected with cDNA encoding HA-mTOR and/or FLAG-raptor; HA-mTOR was immunoprecipitated, washed by high-salt wash buffer with or without 1% NP-40, and assayed for kinase activity toward GST-4EBP1 (Figure 3A). As shown previously (Takahashi et al., 2000), significant HA-mTOR kinase activity is detected only when HA-mTOR is immunopurified in the absence of detergent (Figure 3A, compare lanes 5 and 6). Notably, the HA-mTOR kinase activity is increased 6.3-fold when HA-mTOR is coexpressed with FLAG-raptor (Figure 3A, compare lanes 5 and 7). If, however, the HA-mTOR immunoprecipitate is washed with 1% NP-40, the coexpressed raptor is washed away, and the mTOR kinase activity is severely decreased, to the level observed when HA-mTOR is expressed alone or coexpressed with FLAG-raptor but washed in the presence of 1% NP-40 (Figure 3A, compare lanes 6–8). If the HA-mTOR immunoprecipitates are washed with different concentrations of CHAPS, the amount of coexpressed FLAG-raptor recovered with HA-mTOR decreases progressively as the concentration of CHAPS in wash buffer is increased (Figure 3B, bottom); the mTOR kinase activity, which is enhanced 5.2-fold by coexpression (and coprecipitation) with FLAG-raptor when the HA-mTOR immunoprecipitate is prepared in the absence of CHAPS (Figure 3B, compare lanes 5 and 7), declines progressively, in parallel with the decreasing recovery of FLAG-raptor as the CHAPS concentration is increased (Figure 3B, lanes 7–11).

We reported previously that the ability of immunopurified mTOR to phosphorylate and activate p70α and p70β in vitro, in contrast to 4EBP1, is not abolished (and may be enhanced) by washing the mTOR immunoprecipitate with detergent (Isotani et al., 1999; Minami et al., 2001). We therefore next examined the effect of raptor on the mTOR kinase activity toward p70α (Figure 3C). HA-tagged wild-type (wt) or kinase-dead (NK) mTOR, coexpressed with or without FLAG-raptor in HEK293 cells, was immunoprecipitated, washed with or without 1% CHAPS, and assayed for the kinase activity toward recombinant GST-p70α purified from rapamycin-treated HEK293 cells. As shown previously, wild-type mTOR, but not kinase-dead mTOR, phosphorylates GST-p70α in vitro, and the kinase activity is not abolished by washing the immunoprecipitate with 1% CHAPS (Figure 3C, lanes 2, 3, 4, and 9). Interestingly, as observed using GST-4EBP1 as substrate, the mTOR kinase activity toward GST-p70α is also enhanced (by 3.8-fold) by coexpression of HA-mTOR with FLAG-raptor (Figure 3C, compare lanes 2, 3, 5, and 6). In contrast, when the HA-mTOR immunoprecipitate is washed with detergent so that the FLAG-raptor is washed out, the mTOR kinase activity is decreased to the level observed when HA-

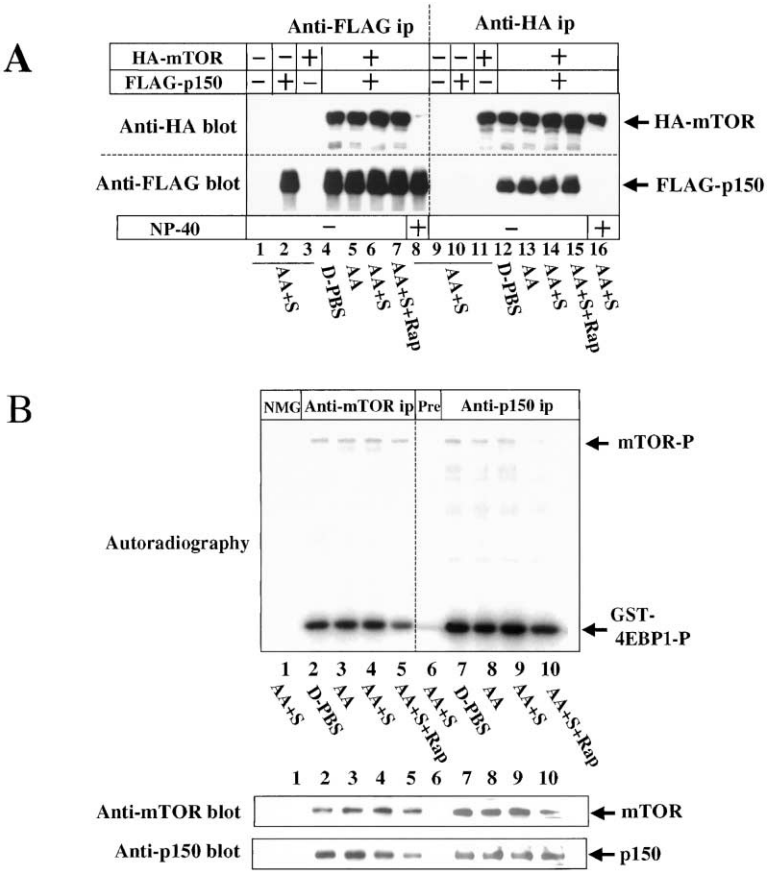

Figure 2. Coimmunoprecipitation of Raptor and mTOR

(A) HEK293 cells were transfected with cDNA encoding HA-mTOR and/or FLAG-raptor. Cells were deprived of serum for 16 hr and further incubated in D-PBS for 1.5 hr. Then, medium was replaced with DMEM alone (AA, lanes 5 and 13), DMEM with 10% FBS (AA+S, lanes 1–3, 6, 8–11, 14, and 16), or DMEM with 10% FBS and 200 nM rapamycin (AA+S+Rap, lanes 7 and 15) for 30 min. Other cells were incubated in D-PBS for additional 30 min (D-PBS, lanes 4 and 12). After extraction of the cells without detergent, the supernatant was divided into two aliquots; one was subjected to immunoprecipitation with anti-FLAG Ab (Anti-FLAG ip), and the other with anti-HA Ab (Anti-HA ip). The immunoprecipitates were washed with the high-salt wash buffer with (lanes 8 and 16) or without (lanes 1–7 and 9–15) 1% NP-40, separated by SDS-PAGE, and transferred onto PVDF membrane. The membrane was divided into two parts; the upper part was immunoblotted with anti-HA Ab, and the lower part with anti-FLAG Ab. (B) HEK293 cells were treated and cell extract was prepared as described in (A). Endogenous mTOR or raptor was immunoprecipitated with either anti-mTOR Ab or anti-N-raptor Ab, respectively, and subjected to mTOR kinase assay using GST-4EBP1 as substrate. As control, normal mouse IgG (lane 1) and preimmune serum (lane 6) were used for immunoprecipitation. The samples were separated on SDS-PAGE, transferred onto PVDF membrane, and analyzed by autoradiography (Autoradiography) and subsequent immunoblot with anti-mTOR Ab (Anti-mTOR blot) and anti-N-raptor Ab (Anti-p150 blot).

mTOR kinase is expressed alone, i.e., without coexpression of FLAG-raptor (Figure 3C, lanes 9 and 10). Thus, raptor appears to play an important, but not necessarily obligatory, role in the mTOR-catalyzed phosphorylation of p70α.

Raptor Forms Complexes with mTOR and 4EBP1 or p70α

We next examined whether raptor participates in the formation of complexes involving the proteins relevant to mTOR signaling. MYC-tagged raptor was coexpressed with GST-fusion proteins of PDK1, p70α, or 4EBP1 (Figure 4A). MYC-raptor is recovered with GST-4EBP1 and, to a much lesser extent, with GST-p70α but not with GST-PDK1. The binding of MYC-raptor to GST-4EBP1 and GST-p70α is not diminished by inclusion of 1% NP-40 during extraction of the cells and washing of the glutathione Sepharose beads. To determine whether raptor forms ternary complexes with mTOR and either 4EBP1 or p70α (Figure 4B), HEK293 cells, transfected with mixtures of these cDNAs, were extracted without detergent; FLAG-raptor, immunopurified and washed without detergent, was analyzed by immunoblot for the presence of associated proteins. GST-4EBP1 and a smaller amount of GST-p70α, but not GST alone, are specifically recovered with FLAG-raptor together with HA-mTOR (Figure 4B, lanes 5, 9, and 10). Reciprocally, the same extract was incubated with glutathione Sepharose beads, and the washed beads were analyzed by immunoblot with anti-HA Ab and anti-FLAG Ab (Figure 4C); FLAG-raptor copurifies with GST-4EBP1 and to a lesser extent with GST-p70α, but not with GST alone. In addition, HA-mTOR also copurifies with GST-4EBP1, but only when FLAG-raptor is coexpressed. Thus, the ability of HA-mTOR and GST-4EBP1 to form a complex requires raptor. Copurification of HA-mTOR with GST-p70α was not observed, even when FLAG-raptor is coexpressed; this may reflect a lower binding affinity between GST-p70 and FLAG-raptor as compared to that between GST-4EBP1 and FLAG-raptor. Nevertheless, we cannot exclude the possibility that p70α does not form a stable ternary complex with mTOR and raptor.

Copurification of Raptor and 4EBP1 on 7-Methyl-GTP-Sepharose

We wondered whether the ability of raptor to bind to 4EBP1 enabled it to recruit eIF-4E, the 5′ mRNA cap binding protein. CHO cells overexpressing human insulin receptor (CHO-IR) were transfected with cDNA encoding FLAG-raptor and treated with insulin or rapamycin or incubated in D-PBS (Figure 5A). Cell extracts, prepared without detergent, were incubated with 7-methyl-GTP Sepharose beads, and the bound proteins were analyzed by immunoblot for the presence of FLAG-raptor, 4EBP1,

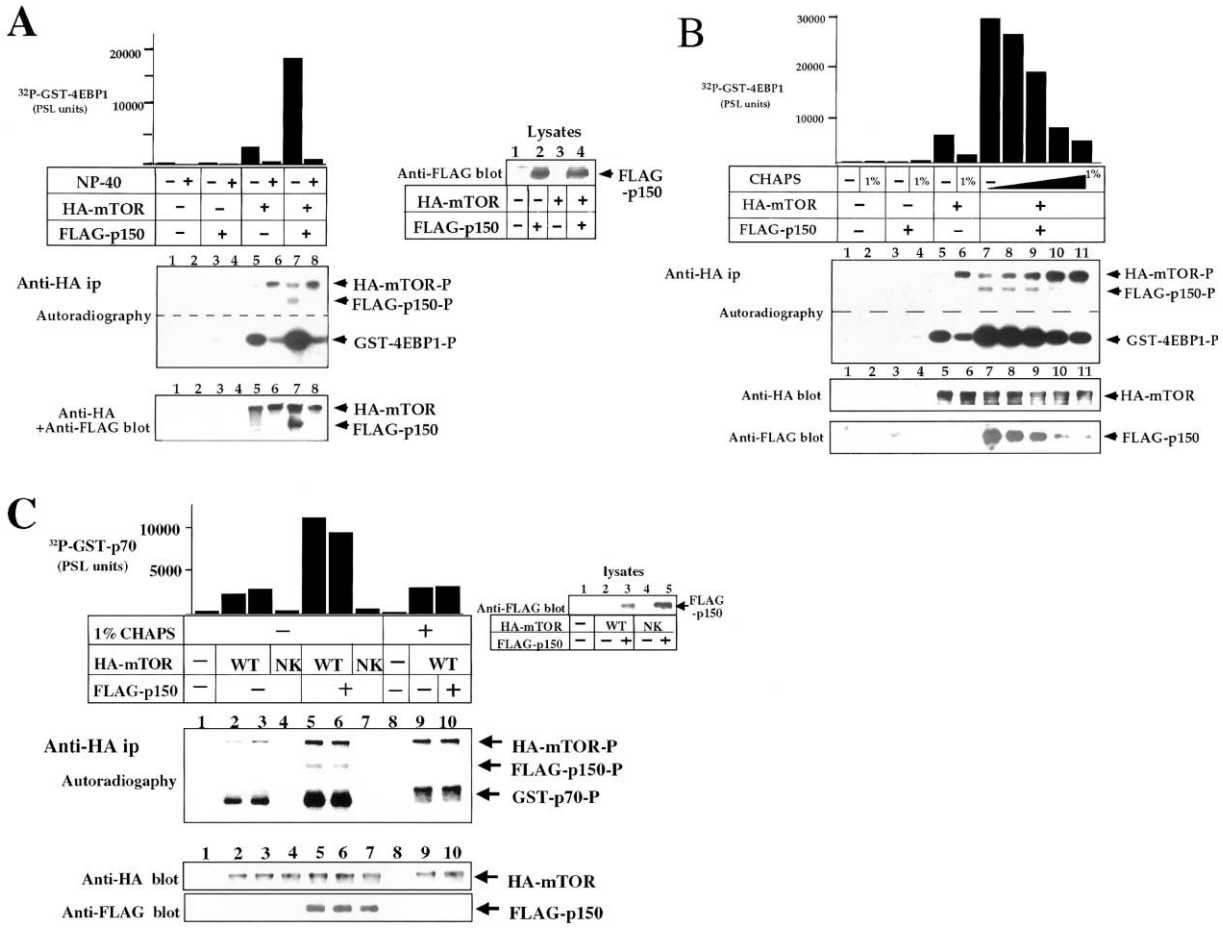

Figure 3. Effects of Raptor on the mTOR Kinase Activities toward 4EBP1 and p70α In Vitro

(A) HEK293 cells were transfected with cDNA encoding HA-mTOR and/or FLAG-raptor as indicated. After extraction of the cells without detergent, immunoprecipitation was performed with anti-HA Ab, and the immunoprecipitates were washed with the high-salt wash buffer with or without 1% NP-40 as indicated, then subjected to mTOR kinase assay using GST-4EBP1 as substrate. The samples were separated by SDS-PAGE, transferred onto PVDF membrane, and analyzed by autoradiography (Autoradiography) and subsequent immunoblot with a mixture of anti-HA Ab and anti-FLAG Ab (Anti-HA + Anti-FLAG blot). The supernatant prepared at the same time was also analyzed by immunoblot with anti-FLAG Ab to confirm the expression of FLAG-raptor (Lysates). ^{32}P incorporated into GST-4EBP1 is shown in a bar graph.

(B) The immunoprecipitates were prepared as described above and washed with the high-salt wash buffer without CHAPS (lanes 1, 3, 5, and 7) or with different concentrations of CHAPS (lanes 2, 4, 6, and 11, 1%; lane 8, 0.25%; lane 9, 0.5%; lane 10, 0.75%) as indicated, then subjected to mTOR kinase assay using GST-4EBP1 as substrate. The samples were separated by SDS-PAGE, transferred onto PVDF membrane, and analyzed by autoradiography (Autoradiography) and subsequent immunoblot with anti-HA Ab (Anti-HA blot) and anti-FLAG Ab (Anti-FLAG blot). ^{32}P incorporated into GST-4EBP1 is shown in a bar graph.

(C) HEK293 cells were transfected with cDNA encoding either wild-type HA-mTOR (wt) or kinase-negative HA-mTOR (NK) together with or without FLAG-raptor as indicated. The immunoprecipitates were prepared as described above and washed with the high-salt wash buffer with or without 1% CHAPS, then subjected to mTOR kinase assay using GST-p70α as substrate. The samples were separated by SDS-PAGE, transferred onto PVDF membrane, and analyzed by autoradiography (Autoradiography) and subsequent immunoblot with anti-HA Ab (Anti-HA blot) and anti-FLAG Ab (Anti-FLAG blot). The supernatant prepared at the same time was analyzed by immunoblot with anti-FLAG Ab to confirm the expression of FLAG-raptor (lysates). ^{32}P incorporated into GST-p70α is shown in a bar graph.

and eIF-4E and the overall phosphorylation of 4EBP1. FLAG-raptor copurifies on 7-methyl-GTP Sepharose (Figure 5A, top), and the recovery of raptor on 7-methyl-GTP Sepharose beads is positively correlated with the overall recovery of 4EBP1 and in an inverse relation to the extent of 4EBP1 phosphorylation (Figure 5A, second and fourth panels from the top). Thus, the insulin stimulation of cells prior to extraction reduces substantially the amount of raptor recovered on 7-methyl-GTP Sepharose; the decrease in recovery of raptor in response to insulin is relatively greater than the decrease in recovery of 4EBP1. Endogenous raptor also purifies on 7-methyl-GTP Sepharose beads (Figure 5B, top). Insulin stimulation reduces substantially the amount of endogenous raptor recovered on 7-methyl-GTP Sepharose, whereas rapamycin or amino acid withdrawal dramatically increases the recovery of raptor on 7-methyl-GTP Sepharose, even though the amount of 4EBP1 purified on the beads from extracts prepared from untreated cells (lane 1) and those prepared from rapamycin-treated (lane 3) or amino acid-deprived cells (lane 4) appear almost identical.

We next examined the binding of raptor with two 4EBP1 mutants: one in which five phosphorylation sites (Thr-37, Thr-46, Ser-65, Thr-70, and Ser-83) are each

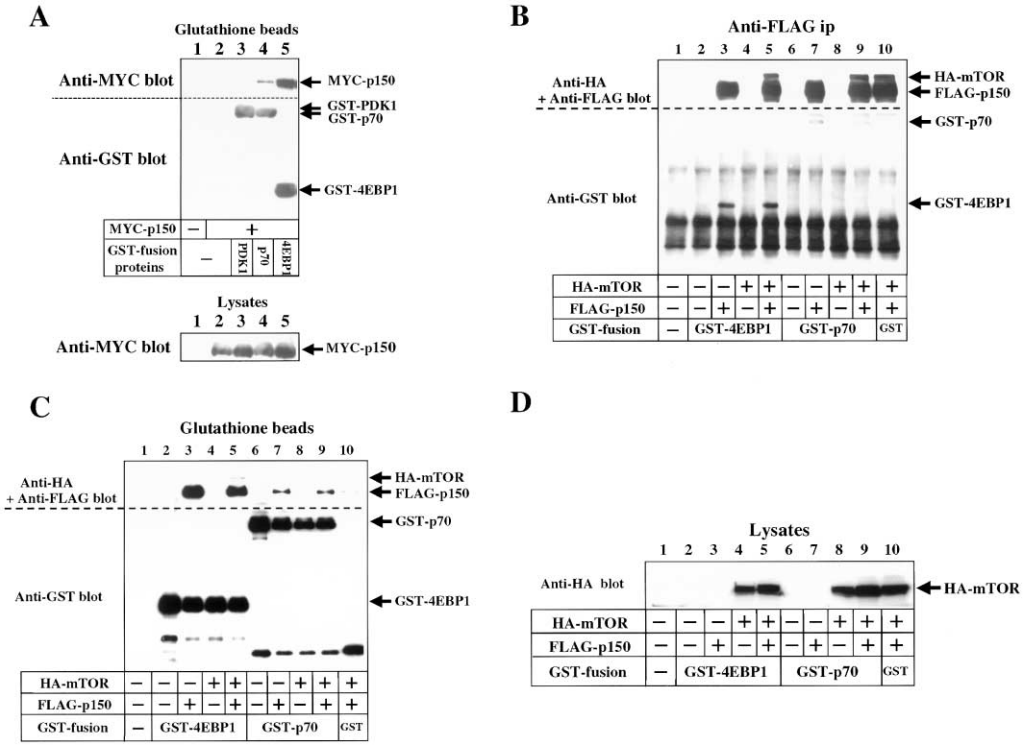

Figure 4. Raptor Binds to 4EBP1 and p70α

(A) HEK293 cells were transfected with cDNA encoding MYC-raptor alone (lane 2) or together with either GST-PDK1 (lane 3), GST-p70α (lane 4), or GST-4EBP1 (lane 5). Cells were extracted in buffer A with 1% NP-40, and the supernatants were incubated with glutathione Sepharose beads. The samples were washed with the high-salt wash buffer with 1% NP-40, separated by SDS-PAGE, and transferred onto PVDF membrane. The membrane was divided into two parts; the upper part was immunoblotted with anti-MYC Ab (Anti-MYC blot), and the lower part with anti-GST Ab (Anti-GST blot). The supernatant prepared at the same time was also analyzed by immunoblot with anti-MYC Ab to confirm the expression of MYC-raptor (Lysates).

(B–D) HEK293 cells were transfected with cDNA encoding HA-mTOR and/or FLAG-raptor and together with either GST-4EBP1, GST-p70α, or GST as indicated. After extraction of the cells without detergent, the supernatant was divided into three aliquots; the first aliquot was subjected to immunoprecipitation with anti-FLAG Ab (B), and the second aliquot was incubated with glutathione Sepharose beads (C). After washing the beads with the high-salt wash buffer without detergent, the samples were separated by SDS-PAGE and transferred onto PVDF membrane. The membrane was divided into two parts; the upper part was immunoblotted with a mixture of ant-HA-Ab and anti-FLAG Ab (Anti-HA + Anti-FLAG blot), and the lower part with anti-GST Ab (Anti-GST blot). The third aliquot was separated by SDS-PAGE and analyzed by immunoblot with anti-HA Ab to confirm the expression of HA-mTOR (D).

changed to Ala (5A-4EBP1), and one that is mutated so that these same five phosphorylation sites are changed to Glu (5E-4EBP1). MYC-raptor binds substantially more 5A-4EBP1 than wild-type 4EBP1 (Figure 5C, lanes 3 and 4), whereas no binding of 5E-4EBP1 to raptor is detectable (Figure 5C, lane 5). These results taken together indicate that raptor binds preferentially to poorly or nonphosphorylated form of 4EBP1, and this binding is critical to the ability of mTOR to catalyze their phosphorylation.

Overexpression of the Wild-Type and a Deletion Mutant of Raptor Inhibits Phosphorylation of 4EBP1 and Activation of p70α in Intact Cells

Overexpression in HEK293 cells of the wild-type and a mutant form of raptor (raptor/ΔCT) in which the C-terminal 437 residues including the WD repeat domain is deleted inhibits in a dose-dependent manner the phosphorylation of coexpressed 4EBP1, as estimated both by the mobility of 4EBP1 on SDS-PAGE (see Supplemental Figure S3A, top, at http://www.cell.com/cgi/content/full/110/2/177/DC1) and by immunoblot with the phosphospecific Ab against 4EBP1 Thr-37 and Thr-46 (see Supplemental Figure S3A, second and third from top).

A similar inhibitory effect of recombinant wild-type and mutant, truncated raptor is also observed on the activity of coexpressed p70α (see Supplemental Figures S3B and S3C). The kinase activity of p70α is severely inhibited by coexpression with wild-type and mutant raptor, whereas the activity of recombinant coexpressed ERK1 is unaffected, indicating that the overexpression of raptor does not cause nonspecific inhibition of protein kinase activity (see Supplemental Figure S3D).

Effects of RNAi of Raptor on the mTOR Kinase Activity toward 4EBP1

We next attempted to reduce the expression of endogenous raptor using RNA interference (RNAi). We employed two sets of siRNAs: one (siRNA 47–67) corresponds to cDNA encoding bp 47–67 after the start codon of raptor, and the other (siRNA 127–147) corresponds to bp 127–147. Transfection of siRNA 47–67 duplex into Hela S3

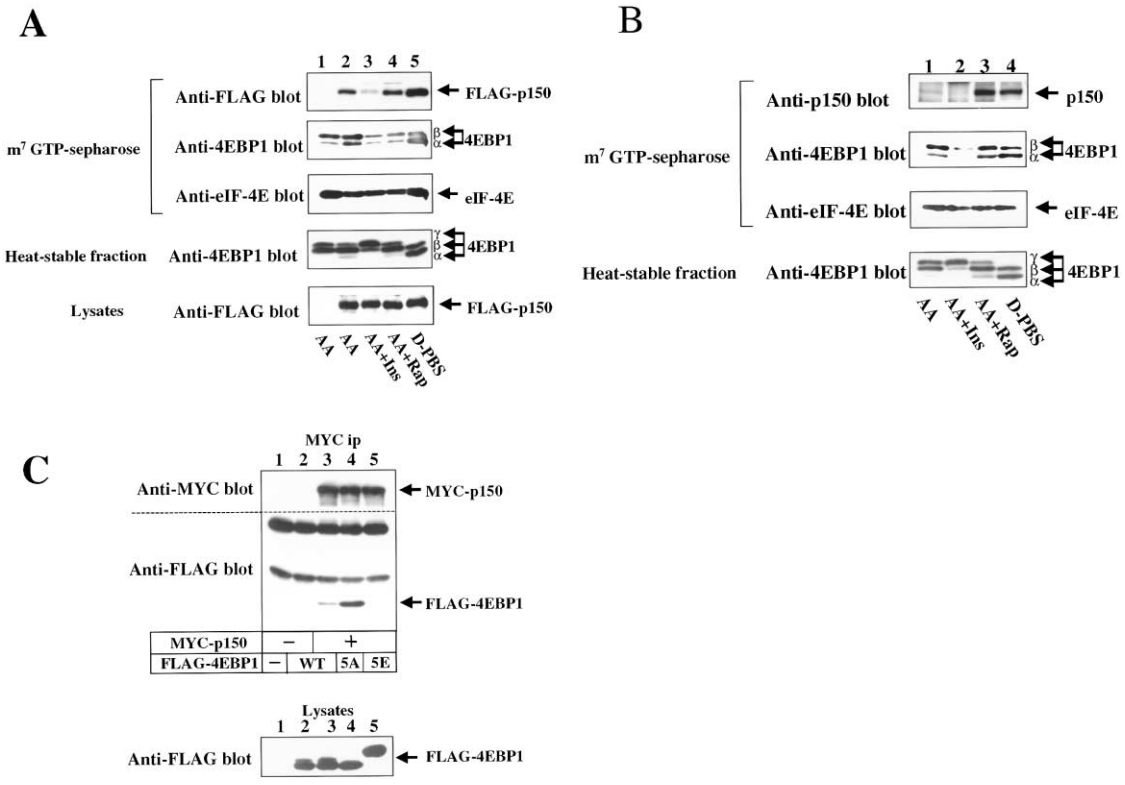

Figure 5. Copurification of Raptor and 4EBP1 on 7-Methyl-GTP-Sepharose

(A) CHO-IR cells were transfected with cDNA encoding FLAG-raptor (lanes 2–5) or mock transfected (lane 1). Cells were incubated in Ham's-F12 medium without serum for 16 hr, then treated with vehicle (lanes 1 and 2), 100 nM insulin for 10 min (lane 3), 200 nM rapamycin for 30 min (lane 4), or incubated in D-PBS for 2 hr (lane 5). Cells were extracted without detergent and divided into three aliquots. The first aliquot was incubated with 7-methyl-GTP Sepharose beads and washed, and bound proteins were analyzed by immunoblot with anti-FLAG Ab, anti-4EBP1 Ab, and anti-eIF-4E Ab. The second aliquot was heated at 100°C for 5 min, and the heat-stable fractions were analyzed by immunoblot with anti-4EBP1 Ab (Heat-stable fraction). The third aliquot was directly separated by SDS-PAGE and analyzed by immunoblot with anti-FLAG Ab to confirm the expression of FLAG-raptor (Lysates).

(B) H4IIE cells were incubated in DMEM medium without serum for 16 hr, then treated with vehicle (lane 1), 100 nM insulin for 10 min (lane 2), 200 nM rapamycin for 30 min (lane 3), or incubated in D-PBS for 2 hr (lane 4). Cells were extracted without detergent and divided into two aliquots. The first aliquot was incubated with 7-methyl-GTP Sepharose beads and washed, and bound proteins were analyzed by immunoblot with anti-GST-raptor Ab, anti-4EBP1 Ab, and anti-eIF-4E Ab. The second aliquot was heated at 100°C for 5 min, and the heat-stable fractions were analyzed by immunoblot with anti-4EBP1 Ab (Heat-stable fraction).

(C) HEK293 cells were transfected with cDNA encoding MYC-raptor and/or FLAG-tagged wild-type 4EBP1 (wt), 5A-4EBP1 (5A), or 5E-4EBP1 (5E) as indicated. Cells were extracted in Buffer A with 1% NP-40 and subjected to immunoprecipitation with anti-MYC Ab. The samples were washed with the high-salt wash buffer with 1% NP-40, separated by SDS-PAGE, and transferred onto PVDF membrane. The membrane was divided into two parts; the upper part was immunoblotted with anti-MYC Ab (Anti-MYC blot), and the lower part with anti-FLAG Ab (Anti-FLAG blot). The supernatant prepared at the same time was also analyzed by immunoblot with anti-FLAG Ab to confirm the expression of FLAG-4EBP1 mutants (Lysates).

cells resulted in severe reduction in the amount of endogenous raptor (Figure 6A, bottom, lane 3), whereas siRNA 127–147 duplex induced mild reduction in the expression (lane 4). Correspondingly, the amount of raptor coimmunoprecipitated with mTOR (Figure 6A, middle) and the mTOR kinase activity toward GST-4EBP1 (Figure 6A, top) were severely reduced in cells transfected with siRNA 47–67 duplex (lane 3) but moderately reduced with siRNA 127–147 (lane 4). These results demonstrate that raptor is required for phosphorylation of 4EBP1 by immunopurified mTOR.

RNAi of Ce-Raptor Mimics the Phenotypes Produced by Ce-TOR RNAi

To explore the role of raptor in the TOR-signaling pathway of an intact metazoan organism, we also applied the RNAi method to the nematode *C. elegans* and compared the phenotypes produced by feeding bacteria expressing RNAi of Ce-raptor with that of Ce-TOR. We have recently characterized the phenotypes of Ce-TOR deficiency in *C. elegans* (Long et al., 2002); a virtually identical array of defects occurs in Ce-TOR mutant worms as in wild-type worms fed Ce-TOR RNAi-producing bacteria (Long et al., 2002). These phenotypes are distinct from those induced by inhibiting the DAF-2 pathway. First, the progeny (F1s) of parent (P0) N2 worms fed with Ce-TOR RNAi-producing bacteria exhibit developmental arrest at the L3 stage. These F1 larvae have delayed gonadal development and by 5–8 days PEL, the gonads appear degenerate (Figure 6B, image b). Second, starting in early L3, Ce-TOR RNAi-raised F1s contain large refractile gut lysosomes that take up Neu-

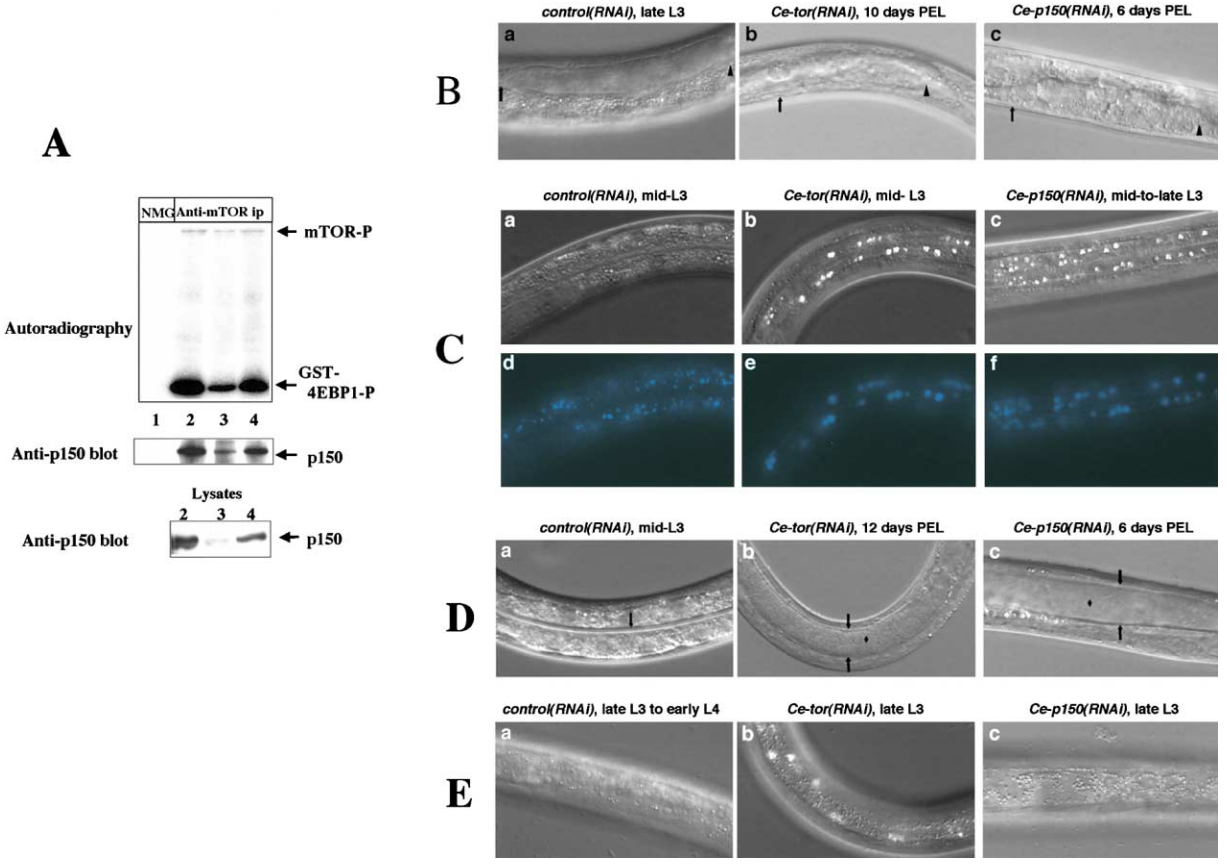

Figure 6. Disruption of Raptor by RNA Interference (RNAi) in Hela S3 Cells and *C. elegans*

(A) Hela S3 cells were transfected with siRNA duplex or mock transfected (lane 1 and 2, mock; lane 3, siRNA 47–67; lane 4, siRNA 127–147). After extraction of the cells without detergent, immunoprecipitation was performed with anti-mTOR Ab, and the immunoprecipitates were washed with the high-salt wash buffer without detergent, then subjected to mTOR kinase assay using GST-4EBP1 as substrate. The samples were separated by SDS-PAGE, transferred onto PVDF membrane, and analyzed by autoradiography (Autoradiography) and subsequent immunoblot with anti-N-raptor Ab (Anti-p150 blot). The supernatant was also analyzed by immunoblot with anti-N-raptor Ab to confirm the expression of raptor (Lysates).

(B) Ce-raptor RNAi feeding caused developmental arrest and gonadal degeneration in F1 animals. (a) The anterior gonad arm of a late L3 N2 F1 raised on control RNAi-producing bacteria. Anterior is to the right. (b) The posterior gonad arm of a late L3 N2 F1 arrested on Ce-TOR RNAi-producing bacteria (10 days PEL). Anterior is to the left. (c) The posterior gonad arm of a late L3 N2 F1 arrested on Ce-raptor RNAi-producing bacteria (6 days PEL). Anterior is to the left.

(C) F1s raised on Ce-raptor RNAi have larger refractile and autofluorescent intestinal lysosomes but are decreased in the number and size of the nonrefractile intestinal vesicles. (a–c) Nomarski optics photographs. (d–f) DAPI channel autofluorescence of (a)–(c). (a and d) The middle section of a mid-L3 stage N2 F1 raised on control RNAi. Anterior is to the right. (b and e) The middle section of a mid-L3 arrested N2 F1 raised on Ce-TOR RNAi. Anterior is to the right. (c and f) The middle section of a mid to late L3 arrested N2 F1 raised on Ce-raptor RNAi. Anterior is to the left.

(D) N2 F1s arrested on Ce-raptor RNAi-producing bacteria show decreased intestinal cytoplasmic volume and increased intestinal lumen size. (a) The intestinal lumen (arrow) in a mid-L3 stage N2 raised on control RNAi. Anterior is to the left. (b) The dramatically enlarged intestinal lumen (arrows) in a 12 day PEL N2 F1 raised on Ce-TOR RNAi. A diamond indicates undigested or half-digested bacteria filling the enlarged lumen. Anterior is to the right. (c) The dramatically enlarged intestinal lumen (arrows) in a 12 day PEL N2 F1 raised on Ce-raptor RNAi. A diamond indicates undigested or half-digested bacteria filling the enlarged lumen. Anterior is to the left.

(E) Increased hypodermal granule number and size in Ce-raptor RNAi-raised animals. (a) The middle section of a late L3 to early L4 stage N2 raised on control RNAi. Anterior is to the right. (b) The middle section of a late L3 N2 F1 raised on Ce-TOR RNAi. Anterior is to the left. (c) The middle section of a late L3 N2 F1 raised on Ce-raptor RNAi. Anterior is to the left. In (E), the focus is on the hypodermal granule level (top surface).

tral Red and autofluoresce under UV light (Figure 6C, images b and e; data not shown); however, by 5 days PEL, both the number and the autofluorescent intensity of the refractile lysosomes dramatically decrease. Third, the intestinal cell cytoplasmic volume is dramatically reduced in Ce-TOR RNAi-raised F1 (Figures 6C, image b, and 6D, image b) and the intestinal lumen is significantly enlarged (Figure 6D, image b). This is accompanied by a compromise in the intestine's ability to digest and absorb. Fourth, Ce-TOR-deficient worms exhibit larger and increased numbers of hypodermal granules containing lipid (Figure 6E, image b). Most of these abnormalities were also observed in F1s raised on RNAi corresponding to Ce-eIF-4G, Ce-eIF2α, and Ce-eIF2β but are not reproduced by interference with the expression of Ce-p70α or Ce-TAP42 (Long et al., 2002).

As with Ce-TOR RNAi, the F1s from Ce-raptor RNAi-raised P0s, fed with Ce-raptor RNAi-producing bacteria from hatching, exhibit delayed gonadal development, with developmental arrest occurring at mid to late L3 stage (data not shown); by 5–6 days PEL, gonads in Ce-raptor RNAi-raised F1s appear degenerate (compare Figure 6B, images c and a). Moreover, as observed with Ce-TOR RNAi, the L3 gonadal morphology is accompanied by L4 vulval structure in F1s arrested on Ce-raptor RNAi (data not shown; Long et al., 2002). Ce-raptor RNAi-raised F1s, starting in early L3, also exhibit large, refractile, and autofluorescent gut lysosomes (compare Figures 6C, images c and f, with 6C, images a and d), which have decreased in number by day 6 PEL. By ~50 hr PEL, the cytoplasmic volume of the intestinal cells is markedly decreased and the intestinal lumen is moderately enlarged in F1s raised on Ce-raptor RNAi (compare Figure 6C, images c and a; data not shown); the size of the intestinal lumen continues to enlarge progressively, and after a prolonged period on Ce-raptor RNAi plate, the intestinal lumen becomes filled with clumps of undigested or half-digested bacteria (Figure 6D, image c). Finally, starting at L2, significantly larger hypodermal granules are observed in Ce-raptor RNAi-raised F1s as compared with control RNAi-raised F1s (compare Figure 6E, images c and a). During no developmental stage on Ce-raptor RNAi did the F1 worms demonstrate dauer characteristics except for increased hypodermal granule size. Thus, in every aspect examined, the phenotypes caused by RNAi of Ce-raptor recapitulate those of Ce-TOR RNAi, and in turn both are very similar to those resulting from RNAi-induced deficiency of Ce-eIF2α, Ce-eIF2β, or Ce-eIF-4G, except for the absence of gonadal degeneration in the latter.

Discussion

Identification of an mTOR Binding Protein, Raptor

The raptor polypeptide, an mTOR binding protein, contains seven WD repeats near the C terminus, and a considerable portion of the N-terminal half, although lacking a known domain motif, is highly conserved among raptor orthologs in fly, worm, and yeast. The WD repeat lacks catalytic function but creates a stable platform for the assembly of reversible complexes with a variety of proteins (Smith et al., 1999). The raptor ortholog in *S. pombe*, Mip1p (Shinozaki-Yabana et al., 2000), was shown to be essential for Mei2p-driven meiosis and sexual development through the interaction with Ste11p. *S. pombe* Tor1 protein has also been reported to have a positive role in the sexual development (Weisman and Choder, 2001). These findings raise the possibility that Mip1p, the ortholog of raptor, is also involved in the TOR-dependent signaling pathway to regulate sexual development in *S. pombe*.

We find that both recombinant and endogenous mTOR and raptor can be coprecipitated from cell extracts, and as expected from our previous findings, the association of mTOR and raptor is disrupted by washing the immunoprecipitate with a detergent such as 1% NP-40 or 1% CHAPS. Nevertheless, a variety of cellular stimuli did not significantly alter the association between mTOR and raptor, as monitored by coimmunoprecipitation. This suggests a tight and relatively stable interaction between mTOR and raptor, at least as measured by coimmunoprecipitation assay. The dynamic aspects of the interaction between mTOR and raptor in living cells, if any exist, will require another approach for detection, such as fluorescence resonance energy transfer (FRET).

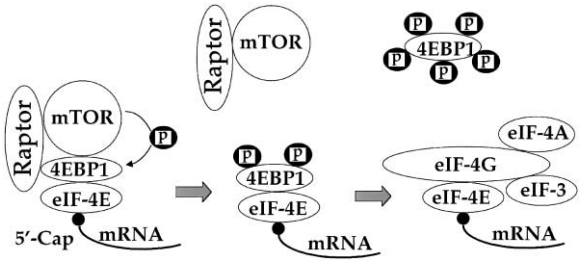

Figure 7. Model of Phosphorylation of 4EBP1 by mTOR-Raptor Complex

Raptor recruits mTOR to non- or poorly phosphorylated form of 4EBP1 bound to eIF-4E on the 5′-cap structure of mRNA and positively modulates phosphorylation of 4EBP1 by mTOR (left). Once 4EBP1 is phosphorylated, mTOR-raptor complex dissociates from 4EBP1 (middle). 4EBP1 is phosphorylated on multiple sites by other kinases, resulting in dissociation of 4EBP1 from eIF-4E, thereby allowing eIF-4G to bind to eIF-4E and make active complex with eIF-4A and eIF-3 (right).

Raptor Functions as a Scaffold for the mTOR-Signaling Pathway

Recombinant mTOR expressed in HEK293 cells exhibits 5–6 times higher kinase activity toward 4EBP1 in vitro when coexpressed with raptor. The enhanced mTOR kinase activity immunopurified from cells coexpressing raptor is decreased progressively by washing the immunoprecipitate with increasing concentrations of CHAPS, concomitant with the decrease in the amount of raptor recovered in the mTOR immunoprecipitate. This indicates that raptor must be continuously associated with mTOR to enhance the mTOR kinase activity toward 4EBP1. In addition, reduction in the expression of endogenous raptor by RNA interference results in the parallel reduction in the mTOR-catalyzed 4EBP1 phosphorylation in vitro, supporting the important role for raptor in the mTOR-signaling pathway. Similarly, the mTOR kinase activity toward p70α is also enhanced by the presence of raptor in vitro, and this enhancement is also lost by removing raptor from mTOR immunoprecipitate in vitro. Unlike 4EBP1, however, removal of raptor does not abolish the mTOR kinase activity toward p70α. Thus, raptor is required absolutely for mTOR-catalyzed 4EBP1 phosphorylation in vitro and enhances but is not absolutely required for the mTOR-mediated phosphorylation and activation of p70α.

The raptor polypeptide binds directly to 4EBP1 and p70α, independently of its association with mTOR. Although the binding affinity of raptor for p70α appears lower than for 4EBP1, both interactions are stable to washing with high salt and detergent. In addition, mTOR can enter a ternary complex with raptor and either 4EBP1 or (probably) p70α, consistent with the idea that raptor functions as a scaffold for mTOR and positively

modulates in vivo the mTOR kinase reaction toward 4EBP1 and p70α. Further support for the physiologic importance of these associations is the recovery of raptor on 7-methyl-GTP Sepharose beads; the amount of raptor recovered on 7-methyl-GTP Sepharose depends on both the amount of 4EBP1 bound to 7-methyl-GTP Sepharose and (inversely) the extent of phosphorylation of 4EBP1. Withdrawal of amino acids or rapamycin, treatments known to result in the hypophosphorylation of 4EBP1, markedly increase the amount of raptor recovered on 7-methyl-GTP Sepharose, whereas insulin stimulation results in a dramatic decrease. The recovery of raptor on 7-methyl-GTP Sepharose is likely to be affected in a complex manner by 4EBP1 phosphorylation, as site-specific phosphorylation of 4EBP1 may differentially alter its binding to eIF-4E or raptor. It has been reported that the hypophosphorylated α and β forms of 4EBP1 recovered on 7-methyl-GTP Sepharose are phosphorylated at least on Thr-37, Thr-46, and Thr-70 (Gingras et al., 2001; Mothe-Satney et al., 2000). Thus, it will be of interest to determine how each phosphorylation site in 4EBP1 contributes to the binding affinity between raptor and 4EBP1. Insulin-induced phosphorylation of eIF-4E may also affect the binding of raptor on the beads. The enhanced binding of raptor to 5A-4EBP1, in which five physiological phosphorylation sites (Thr-37, Thr-46, Ser-65, Thr-70, and Ser-83) are substituted with Ala, and the greatly decreased binding to 5E-4EBP1, in which five phosphorylation sites are substituted with Glu, indicate that raptor preferentially couples mTOR with non- or poorly phosphorylated forms of 4EBP1, enabling their phosphorylation on the 5′-Cap structure. A model summarizing these findings is illustrated in Figure 7.

Raptor Mediates mTOR Signaling Pathway in Intact Cells and in *C. elegans*

Unexpectedly, overexpression of raptor resulted in dose-dependent inhibition of 4EBP1 phosphorylation and p70α activation. Moreover, a deletion mutant of raptor (raptor/ΔCT) lacking the C-terminal 437 residues including WD repeat also induced similar inhibitory effects. The overexpression of scaffold proteins can often result in inhibition of signaling in vivo, because the excess scaffold may actually bind incomplete sets of signaling elements, resulting in an uncoupling of signal transmission. In this instance, however, we have determined that raptor/ΔCT does not bind mTOR and binds only weakly to 4EBP1 (data not shown). Thus, the inhibitory effect of overexpressed raptor/ΔCT (and possibly wild-type raptor) may not be caused by binding to mTOR or 4EBP1 and thereby disrupting the ternary signaling complex of mTOR-raptor-4EBP1; rather, wild-type and mutant raptor may sequester an as yet unknown molecule that is required for the intact mTOR-signaling pathway, probably by binding through the highly conserved regions in the N terminus.

Interestingly, coexpression of mTOR with p70α does not induce activation of p70α in intact cells, although it has been clearly shown that immunopurified mTOR activates p70α through phosphorylation in vitro in a manner synergistic with PDK1. Similarly, coexpression of raptor with p70α results only in the inhibition of p70α activity in vivo, even though raptor enhances the mTOR-catalyzed phosphorylation of p70α in vitro. It is likely that the ratio of recombinant raptor and mTOR in vivo is critical in determining the outcome as regards p70 activity. Moreover, inasmuch as phosphatases also play a critical role in the regulation of p70α in mTOR-signaling pathway, it will be of interest to examine whether raptor binds and/or regulates protein phosphatases that act on p70α, or their regulatory subunits such as α4 (Chen et al., 1998; Murata et al., 1997; Nanahoshi et al., 1999).

The comparison of the phenotype in *C. elegans* resulting from the application of RNA interference for Ce-TOR and Ce-raptor provides compelling evidence for the importance of raptor in the TOR-signaling pathway of a metazoan organism. The ability of RNAi against Ce-raptor to recapitulate essentially the entire array of very characteristic phenotypes produced by RNAi of Ce-TOR indicates that raptor mediates the pathways responsible for most of the functional outputs of Ce-TOR. The similarity of the phenotypes observed for RNAi against the general translational initiation factors (Ce-eIF-4G, Ce-eIF2α, and Ce-eIF2β) to most of those engendered by deficiency of Ce-TOR and Ce-raptor suggests that interference with overall mRNA translation is primarily responsible for these abnormalities. It would be of interest to determine the extent to which deficiency of Ce-4EBP1 could ameliorate these changes; however, there has been no report concerning the 4EBP1 orthologs in *C. elegans*, and we have been unable to identify the ortholog in the worm genomic database.

In conclusion, we have identified an mTOR binding protein, raptor. Raptor contains multiple WD repeats and plays a pivotal role as a scaffold protein in the mTOR-signaling pathway that appears to be phylogenetically conserved. Inasmuch as WD repeats are important for interacting with multiple signaling proteins, it will probably be necessary to identify all such raptor-interacting proteins to uncover the function of raptor in vivo.

Experimental Procedures

Materials

Materials are described in detail in the Supplemental Data at http://www.cell.com/cgi/content/full/110/2/177/DC1.

Antibodies

The monoclonal anti-mTOR Ab was produced as described previously (Nishiuma et al., 1998). Two kinds of polyclonal Ab against raptor were produced; one was raised against a peptide encoding the N-terminal 18 residues of raptor (anti-N-raptor), in which the first Met was acetylated, and the other one was against N-terminal 53 residues fused with GST (anti-GST-raptor), which was expressed in and purified from *E. coli*.

Purification and Identification of Raptor

The purification and identification processes are described in detail in the Supplemental Data.

cDNAs

KIAA1303 cDNA was provided by Kazusa DNA Research Institute. To make the expression vector of FLAG-tagged full-length raptor, cDNA encoding bp 1–976 was amplified from human whole brain library with the sense primer 1 (ACGGATCCATGGAGTCCGAAATGC TGCAATCGC) and the antisense primer 2. The PCR product was digested with *Bam*H I (included in the sense primer 1) and *Sph* I (836 bp). A cDNA fragment encoding bp 836–6051 (including stop

codon) was cut out from KIAA1303 cDNA and ligated together with the PCR fragment encoding bp 1–836 into pcDNA1-FLAG plasmid so as to introduce a FLAG epitope at the N terminus. To make the expression vector of MYC-tagged full-length raptor, full-length cDNA of raptor was introduced into pcDNA3-MYC so as to introduce a MYC epitope at the N terminus. To make the expression vector of FLAG-tagged raptor/ΔCT, cDNA fragment encoding bp 1–2711 (*Nae* I site) was cut out from pcDNA1-FLAG-raptor and ligated into pcDNA1 plasmid.

The expression vectors of HA-tagged p70α, HA-tagged wild-type mTOR (WT-mTOR) and kinase-negative mutant of mTOR (NK-mTOR), and FLAG-tagged 4EBP1 were described previously (Hara et al., 1997). The expression vectors of GST-tagged p70α and PDK1 and control pEBG2T vector were described previously (Alessi et al., 1998). The expression vectors of GST-tagged 4EBP1 was made by transferring 4EBP1 cDNA into pEBG2T plasmid. The expression vectors of mutants of 4EBP1 in which Thr-37, Thr-46, Ser-65, Thr-70, and Ser-83 were substituted with either Ala (4EBP1-5A) or Glu (4EBP1-5E) were created by using QuikChange™ site-directed mutagenesis kit (Stratagene).

Cell Culture and cDNA Expression

HEK293 cells and Hela S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). CHO-IR cells and H4IIE cells were cultured as described previously (Hara et al., 1998; Shigemitsu et al., 1999). Transient transfection was performed by the lipofection method using lipofectAMINE according to the manufacturer's protocol (Life Technologies).

Immunoprecipitation, Kinase Assay, and Immunoblot

These procedures were carried out by standard techniques, as described in the Supplemental Data at http://www.cell.com/cgi/content/full/110/2/177/DC1.

RNA Interference in Hela S3 Cells

Two pairs of siRNAs were chemically synthesized, annealed, and transfected into 60%–70% confluent Hela S3 cells using Oligofectamine (Life Technologies) according to Elbashir et al. (2001). The sequence of the siRNAs corresponding to the coding regions 47–67 from the start codon of raptor (siRNA 47–67) are as follows: 47-sense, GAUGAGGCUGAUCUUACAGTT; 47-antisense, CUGUAA GAUCAGCCUCAUCTT. The sequence of the siRNAs corresponding to the coding regions 127–147 (siRNA 127–147) are as follows: 127-sense, ATCCUUAGCUCAGAGCUGGTT; 127-antisense, CCAGCU CUGAGCUAAGGAUTT. Forty-eight hours after transfection, cells were lysed and the supernatant was prepared for mTOR kinase assay and immunoblot for p150.

RNA Interference in *C. elegans*, Imaging, and Image Processing

C. elegans Bristol N2 (Brenner, 1974) strain was maintained according to Brenner (1974) and grown at 20°C or 25°C. RNAi through feeding worms with bacteria that were induced to express double-stranded RNA of the gene of interest was done according to Fraser et al. (2000). Genomic segments of the gene of interest (see below) were amplified by PCR using gene-specific primer pairs, ligated into the L4440 vector (Timmons and Fire, 1998), and transformed into the HT115 (DE3) bacterial strain. Worm plates containing NGM agar + 5 mM IPTG + 25 μg/ml carbenicillin were inoculated with bacterial cultures grown 8–18 hr for either the control L4440 vector or the test RNAi construct. L4 stage worms were then placed on the dried RNAi-producing plates and allowed to produce progeny on them. Adults were removed after 2–3 days or transferred to a new RNAi-producing plate. The genomic segments used in the RNAi constructs were chosen for the gene of interest by selecting exon(s) close to the 5′ end of the gene and not interrupted or only interrupted by short introns. The genomic segment thus chosen for Ce-TOR is −36 bp (from the ATG) to +1078 bp; the genomic segments chosen for Ce-raptor are +1771 bp to +2971 bp and +2127 bp to +2927 bp. The two constructs for Ce-raptor gave similar RNA interference potency (unpublished data).

All high-magnification images were obtained using a Zeiss Axioplan (Zeiss) scope's Nomarski channel, DAPI channel (for autofluorescent signals), or Rhodamine channel (for Neutral Red signals) and the Openlab software 3.0.6. Images were processed using the Adobe Photoshop 5.0 software.

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References

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., et al. (1997). 3-phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. Curr. Biol. *7*, 776–789.

Alessi, D.R., Kozlowski, M.T., Weng, Q.P., Morrice, N., and Avruch, J. (1998). 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Curr. Biol. *8*, 69–81.

Avruch, J., Belham, C., Weng, Q.P., Hara, K., and Yonezawa, K. (2001). The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. In Progress in Molecular and Subcellular Biology, R.E. Rhoads, ed. (Berlin: Heiderberg), pp. 115–154.

Barbet, N.C., Schneider, U., Helliwell, S.B., Stansfield, I., Tuite, M.F., and Hall, M.N. (1996). TOR controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell *7*, 25–42.

Beretta, L., Gingras, A.C., Svitkin, Y.V., Hall, M.N., and Sonenberg, N. (1996). Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J. *15*, 658–664.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756–758.

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.

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.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4EBP1. Proc. Natl. Acad. Sci. USA *95*, 1432–1437.

Chen, J., Peterson, R.T., and Schreiber, S.L. (1998). Alpha 4 associates with protein phosphatases 2A, 4, and 6. Biochem. Biophys. Res. Commun. *247*, 827–832.

Chiu, M.I., Katz, H., and Berlin, V. (1994). RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proc. Natl. Acad. Sci. USA *91*, 12574–12578.

Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell *69*, 1227–1236.

Di Como, C.J., and Arndt, K.T. (1996). Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. *10*, 1904–1916.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschi, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Fox, H.L., Kimball, S.R., Jefferson, L.S., and Lynch, C.J. (1998). Amino acids stimulate phosphorylation of p70S6k and organization of rat adipocytes into multicellular clusters. Am. J. Physiol. *274*, C206–213.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature *408*, 325–330.

Gingras, A.-C., Gygi, S.P., Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R., and Sonenberg, N. (1999). Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. *13*, 1422–1437.

Gingras, A.-C., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001). Hierarchical phosphorylation of the translational initiator 4E-BP1. Genes Dev. *15*, 2852–2864.

Gout, I., Minami, T., Hara, K., Tsujishita, Y., Filonenko, V., Waterfield, M.D., and Yonezawa, K. (1998). Molecular cloning and characterization of a novel p70 S6 kinase, p70 S6 kinase beta containing a proline-rich region. J. Biol. Chem. *273*, 30061–30064.

Hara, K., Yonezawa, K., Kozlowski, M.T., Sugimoto, T., Andrabi, K., Weng, Q.P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997). Regulation of eIF-4E BP1 phosphorylation by mTOR. J. Biol. Chem. *272*, 26457–26463.

Hara, K., Yonezawa, K., Weng, Q.P., Kozlowski, M.T., Belham, C., and Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J. Biol. Chem. *273*, 14484–14494.

Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J., and Yonezawa, K. (1999). Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J. Biol. Chem. *274*, 34493–34498.

Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., and Thomas, G. (1997). Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70s6k. EMBO J. *16*, 3693–3704.

Keith, C.T., and Schreiber, S.L. (1995). PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. Science *270*, 50–51.

Koh, H., Jee, K., Lee, B., Kim, J., Kim, D., Yun, Y.H., Kim, J.W., Choi, H.S., and Chung, J. (1999). Cloning and characterization of a nuclear S6 kinase, S6 kinase-related kinase (SRK); a novel nuclear target of Akt. Oncogene *18*, 5115–5119.

Kunz, J., Henriquez, R., Schneider, U., Deuter Reinhard, M., Movva, N.R., and Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell *73*, 585–596.

Lawrence, J.C., Jr., and Abraham, R.T. (1997). PHAS/4E-BP1s as regulators of mRNA translation and cell proliferation. Trends Biochem. Sci. *22*, 345–349.

Lee-Fruman, K.K., Kuo, C.J., Lippincott, J., Terada, N., and Blenis, J. (1999). Characterization of S6K2, a novel kinase homologous to S6K1. Oncogene *18*, 5108–5114.

Long, X., Spycher, C., Han, S.Z., Rose, A.M., Müller, F., and Avruch, J. (2002). TOR deficiency in *C. elegans* causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. Curr. Biol. *12*, in press.

Minami, T., Hara, K., Oshiro, N., Ueoku, S., Yoshino, K., Tokunaga, C., Shirai, Y., Saito, N., Gout, I., and Yonezawa, K. (2001). Distinct regulatory mechanism for p70 S6 kinase β from that for p70 S6 kinase α. Genes Cells *6*, 1003–1015.

Mothe-Satney, I., Brunn, G.J., McMahon, L.P., Capaldo, C.T., Abraham, R.T., and Lawrence, J.C. (2000). Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. J. Biol. Chem. *275*, 33836–33843.

Murata, K., Wu, J., and Brautigan, D.L. (1997). B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. Proc. Natl. Acad. Sci. USA *94*, 10624–10629.

Nanahoshi, M., Tsujishita, Y., Tokunaga, C., Inui, S., Sakaguchi, N., Hara, K., and Yonezawa, K. (1999). Alpha4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases. FEBS Lett. *446*, 108–112.

Nishiuma, T., Hara, K., Tsujishita, Y., Kaneko, K., Shii, K., and Yonezawa, K. (1998). Characterization of the phosphoproteins and protein kinase activity in mTOR immunoprecipitates. Biochem. Biophys. Res. Commun. *252*, 440–444.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G., and Hafen, E. (2000). Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin. Genes Dev. *14*, 2689–2694.

Patti, M.E., Brambilla, E., Luzi, L., Landaker, E.J., and Kahn, C.R. (1998). Bidirectional modulation of insulin action by amino acids. J. Clin. Invest. *101*, 1519–1529.

Peterson, R.T., Desai, B.N., Hardwick, J.S., and Schreiber, S.L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin associated protein. Proc. Natl. Acad. Sci. USA *96*, 4438–4442.

Price, D.J., Grove, J.R., Calvo, V., Avruch, J., and Bierer, B.E. (1992). Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science *257*, 973–977.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35–43.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. *270*, 815–822.

Saitoh, M., ten Dijke, P., Miyazono, K., and Ichijo, H. (1998). Cloning and characterization of p70(S6K beta) defines a novel family of p70 S6 kinases. Biochem. Biophys. Res. Commun. *253*, 470–476.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253–262.

Schmidt, A., Beck, T., Koller, A., Kunz, J., and Hall, M.N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J. *17*, 6924–6931.

Shigemitsu, K., Tsujishita, Y., Hara, K., Nanahoshi, M., Avruch, J., and Yonezawa, K. (1999). Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. J. Biol. Chem. *274*, 1058–1065.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S.C. (1998). Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J. *17*, 6649–6659.

Shinozaki-Yabana, S., Watanabe, Y., and Yamamoto, M. (2000). Novel WD-repeat protein Mip1p facilitates function of the meiotic regulator Mei2p in fission yeast. Mol. Cell. Biol. *20*, 1234–1242.

Smith, T.F., Gaitatzes, C., Saxena, K., and Neer, E.J. (1999). The WD repeat: a common architecture for diverse functions. Trends Biochem. Sci. *24*, 181–185.

Sonenberg, N. (1996). mRNA 5′ cap-binding protein eIF4E and control of cell growth. In Translational control, W.B. Hershey, M.B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 245–270.

Takahashi, T., Hara, K., Inoue, H., Kawa, Y., Tokunaga, C., Hidayat, S., Yoshino, K., Kuroda, Y., and Yonezawa, K. (2000). Carboxyl-terminal region conserved among phosphoinositide-kinase-related kinases is indispensable for mTOR function in vivo and in vitro. Genes Cells *5*, 765–775.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M.,

Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidyl inositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol. Cell. Biol. *21*, 8671–8683.

Timmons, L., and Fire, A. (1998). Specific interference by ingested dsRNA. Nature *395*, 854.

Wang, X., Campbell, L.E., Miller, C.M., and Proud, C.G. (1998). Amino acid availability regulates p70 S6 kinase and multiple translation factors. Biochem. J. *334*, 261–267.

Weisman, R., and Choder, M. (2001). The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine. J. Biol. Chem. *276*, 7027–7032.

Xu, G., Kwon, G., Marshall, C.A., Lin, T.A., Lawrence, J.C., and McDaniel, M.L. (1998). Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic beta-cells. A possible role in protein translation and mitogenic signaling. J. Biol. Chem. *273*, 28178–28184.

Zakian, V.A. (1995). ATM-related genes: what do they tell us about functions of the human gene? Cell *82*, 685–687.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., and Neufeld, T.P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev. *14*, 2712–2724.

Accession Numbers

The GenBank accession numbers for the human and mouse raptor in this paper are AB082951 and AB082952, respectively.