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mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery

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Summary

mTOR/RAFT1/FRAP is the target of the immunosuppressive drug rapamycin and the central component of a nutrient- and hormone-sensitive signaling pathway that regulates cell growth. We report that mTOR forms a stoichiometric complex with raptor, an evolutionarily conserved protein with at least two roles in the mTOR pathway. Raptor has a positive role in nutrient-stimulated signaling to the downstream effector S6K1, maintenance of cell size, and mTOR protein expression. The association of raptor with mTOR also negatively regulates the mTOR kinase activity. Conditions that repress the pathway, such as nutrient deprivation and mitochondrial uncoupling, stabilize the mTOR-raptor association and inhibit mTOR kinase activity. We propose that raptor is a missing component of the mTOR pathway that through its association with mTOR regulates cell size in response to nutrient levels.

Introduction

Increasing evidence indicates that in eukaryotes, cell growth (mass accumulation) is finely regulated in response to environmental and developmental conditions and can be deranged in human diseases such as cancer and diabetes (reviewed by Dixon and Fordham-Skelton, 1998; Johnston and Gallant, 2002; Katso et al., 2001; Kozma and Thomas, 2002; Schmelzle and Hall, 2000). The rate of mass accumulation is controlled not simply by the availability of nutrients, but by signaling pathways that coordinate the activity of the cell growth machinery with nutritional, hormonal, and mitogenic signals. Studies into the mechanism of action of rapamycin, an immunosuppressive and anticancer drug, led to the discovery of an evolutionarily conserved regulator of cell growth, the TOR (*t*arget *o*f *r*apamycin) pathway (Brown et al., 1994; Chiu et al., 1994; Kunz et al., 1993; Oldham et al., 2000; Sabatini et al., 1994; Sabers et al., 1995; Zhang et al., 2000). The complex of rapamycin with its receptor, FKBP12, binds directly to TOR and perturbs its function in a poorly understood fashion (Brown et al., 1995; Burnett et al., 1998; Peterson et al., 2000; Zheng et al., 1995). Studies in several organisms have shown that the TOR pathway regulates a variety of processes contributing to cell growth, including initiation of mRNA translation, ribosome synthesis, expression of metabolism-related genes, autophagy, and cytoskeletal reorganization (recently reviewed by Schmelzle and Hall, 2000, and by Gingras et al., 2001). By interfering with the function of mammalian TOR, rapamycin inhibits progression through the G1 phase of the cell cycle in various cell types. Because of these antiproliferative effects, rapamycin is a clinically valuable drug that is currently used to block immune rejection of transplanted organs (Saunders et al., 2001) and in trials for the treatment of cancer (Dudkin et al., 2001; Hidalgo and Rowinsky, 2000) and for the prevention of restenosis after angioplasty (Sousa et al., 2001).

Mammalian TOR, mTOR (also known as RAFT1 or FRAP), phosphorylates at least two regulators of protein synthesis: S6K1 (formerly called p70 ribosomal S6 kinase) and an inhibitor of translation initiation, the eIF-4E binding protein 1 (4E-BP1) (Brunn et al., 1997; Burnett et al., 1998; Hara et al., 1997; Isotani et al., 1999). In mammalian cells, amino acid deprivation leads to the dephosphorylation of both S6K1 and 4E-BP1 and to decreased rates of protein synthesis, effects that are rapidly reversed by the readdition of amino acids (Fox et al., 1998; Hara et al., 1998). Among amino acids, changes in leucine levels alone are sufficient to regulate the phosphorylation state and activity of both downstream components of the mTOR pathway (Hara et al., 1998; Lynch et al., 2000). In addition to amino acid level in the media, mitochondrial function (Xu et al., 2001), glycolysis (Dennis et al., 2001), and cell stress (Parrott and Templeton, 1999) regulate S6K1, as do growth factors such as insulin (Lawrence and Brunn, 2001).

Despite extensive efforts, how nutrients regulate the mTOR signaling pathway remains poorly understood. In particular, stimuli that activate (e.g., amino acids) or inhibit (e.g., mitochondrial uncouplers) downstream effectors of mTOR, such as S6K1 and 4E-BP1, fail to change the in vitro kinase activity of mTOR (Dennis et al., 2001; Hara et al., 1997). This discrepancy has led us to hypothesize that in vivo mTOR exists as a complex with one or more proteins that are lost during isolation of mTOR for in vitro assays. Several lines of evidence support the notion that mTOR associates with other proteins: mTOR contains HEAT repeats, which are known to be protein-protein interaction motifs; it migrates at a disproportionately large apparent molecular weight (1.5–2.0 mDa) in gel filtration chromatography (D.M.S., unpublished data); and the expression in transfected cells of mTOR fragments lacking the catalytic site has dominant-negative effects on the pathway (data not shown). However, conventional biochemical attempts to purify mTOR-interacting proteins have been fruitless, as might be expected if mTOR-containing complexes were unstable under standard purification conditions. Therefore, we devised a purification scheme that uses a reversible chemical crosslinker to stabilize putative mTOR-containing complexes. Using this strategy, we have discovered raptor (*r*egulatory *a*ssociated *p*rotein of m*TOR*), a protein that we find has a positive role

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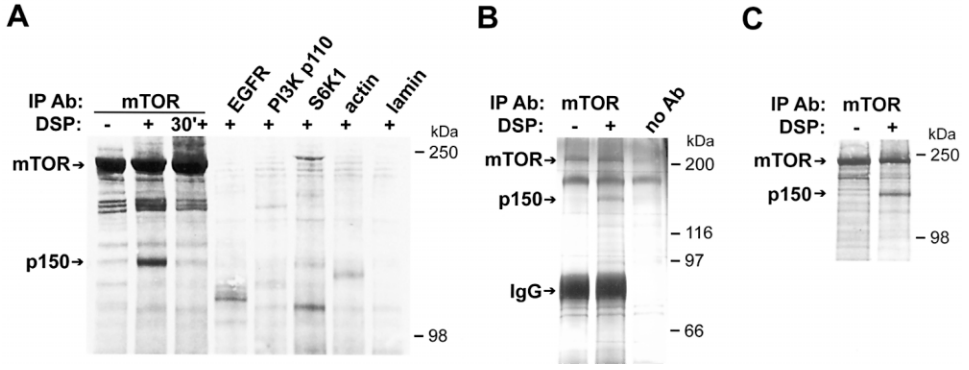

Figure 1. Identification of a 150 kDa mTOR-Associated Protein

(A) Autoradiograph of SDS-PAGE analysis of mTOR immunoprecipitates prepared from metabolically labeled HEK293T cells lysed in the absence (−) or presence (+) of the chemical crosslinker DSP, or from lysates treated with DSP 30 min after lysis (30′+). The 150 kDa protein is not coimmunoprecipitated by antibodies recognizing the EGF receptor (EGFR), the 110 kDa subunit of PI-3-kinase (PI3K p110), the ribosomal S6 kinase (S6K1), actin, or lamin.

(B) Silver stain of SDS-PAGE analysis of mTOR immunoprecipitates isolated as in (A) from unlabeled cells. Quantitation reveals a ratio of 1.0 mTOR to 0.84 p150.

(C) Autoradiograph of SDS-PAGE analysis of mTOR immunoprecipitates prepared from cells metabolically labeled to equilibrium. Quantitation reveals a ratio of 0.92 mTOR to 1.0 p150 after normalization to the methionine content of each protein.

in nutrient signaling to S6K1 and in the control of cell size. The mTOR-raptor interaction also regulates the kinase activity of mTOR and is sensitive to conditions, such as nutrient availability, that signal through the pathway. Thus, we provide a mechanism for how nutrients regulate mTOR activity in vivo. Although rapamycin has been regarded as mimicking the effects of nutrient deprivation, we find that the two conditions have opposite effects on the mTOR-raptor interaction.

Results

Identification of Raptor as an mTOR-Associated Protein

HEK293T cells were metabolically labeled with [^{35}S]methionine and lysed in the presence or absence of the reversible crosslinker dithiobis(succinimidylpropionate) (DSP). mTOR immunoprecipitates prepared from these lysates were then treated with dithiothreitol to reduce the DSP crosslinks and analyzed by SDS-PAGE and autoradiography. A protein with an apparent molecular weight of 150 kDa coprecipitated with mTOR only in immunoprecipitates recovered from lysates prepared in the presence of DSP (Figure 1A). The intensity of this band decreased significantly when DSP was added 30 min after cell lysis, suggesting that the protein interacts weakly or transiently with mTOR or that the lysis procedure disrupts the association (Figure 1A). For reasons that will become apparent, we have named the 150 kDa protein raptor (*r*egulatory *a*ssociated *p*rotein of m*TOR*). Raptor was also detected in silver- (Figure 1B) or Coomassie blue-stained gels (data not shown) of mTOR immunoprecipitates prepared from DSP-treated lysates of unlabeled cells. Quantitation of the amount of raptor isolated with mTOR indicates that it is present in near stoichiometric amounts with mTOR (Figures 1B and 1C).

Raptor Is an Evolutionarily Conserved Protein with a Novel N-Terminal Domain and Seven WD40 Repeats

To characterize raptor, we obtained sequence information by mass spectrometry and identified three peptides (SVSSYGNIR, LDDQIFLNR, and IPEEHDLESQIR), all of which are contained within KIAA1303 (NCBI # 7242961), a partial ORF for a human protein of unknown function. Using the BLAST homology search algorithm, we found that KIAA1303 likely represents an N-terminally truncated fragment of the human homolog of fission yeast Mip1p. The function of Mip1 is not clear, but, interestingly, it has been implicated in cell growth and nitrogen sensing (Shinozaki-Yabana et al., 2000), processes regulated by TOR proteins in a variety of organisms. By searching EST databases, we identified overlapping cDNAs that extended the 5′ end of the KIAA1303 cDNA and provided sequence information that allowed us to design PCR primers and assemble a full-length cDNA for human raptor. The 7051 nucleotide mRNA contains an open reading frame encoding a protein of 1335 amino acids (predicted molecular weight of 149 kDa) that is flanked by 5′ and 3′ untranslated regions of 0.9 and 2.1 kb, respectively. Human genome sequence information for the raptor locus is still incomplete but permitted us to localize the gene to human chromosome 17q25.3.

Raptor shows a high degree of conservation amongst all eukaryotes with completed genome projects, including *D. melanogaster*, *S. pombe*, *S. cerevisiae*, *C. elegans*, and *A. thaliana* (Figure 2A and see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/110/2/163/DC1). The raptor homologs in budding (Winzeler et al., 1999) and fission (Shinozaki-Yabana et al., 2000) yeasts are encoded by essential genes. All raptor homologs have a novel N-terminal domain that we have named RNC (for *r*aptor *N*-terminal *c*onserved) that consists of three blocks with at least 67%–79% sequence similarity (Figure 2B). The RNC domain is unrelated to other sequences in the public databases and is predicted to have a high propensity to form α helixes. Following the RNC domain, all raptor homologs have three HEAT repeats (Andrade and Bork, 1995), which are then followed by seven WD40 repeats in the C-terminal third of the protein (Figure 2A). Northern blot analysis shows that raptor is expressed in all human tissues in a pattern similar to that of mTOR, with the greatest levels of both

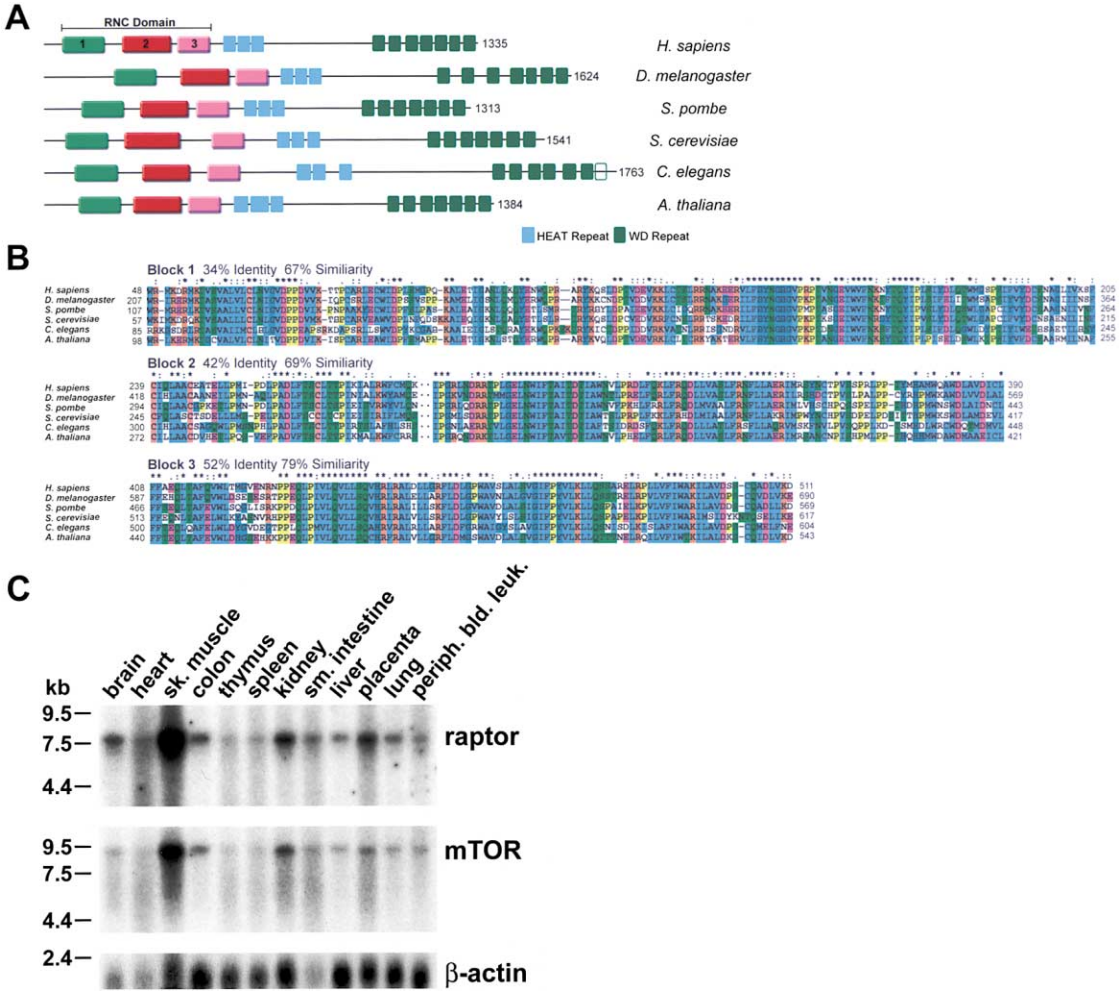

Figure 2. Raptor Is an Evolutionarily Conserved Protein and Is Widely Expressed in Human Tissues in a Similar Pattern to mTOR

(A) Schematic representation of structural features of raptor and its homologs. The seventh WD40 repeat of *C. elegans* raptor is indicated with an empty green box because it lacks the prototypical Trp/Asp pattern. The accession numbers for the raptor homologs are: *D. melanogaster* (AAF46122), *S. pombe* (P87141), *S. cerevisiae* (P38873), *C. elegans* (T19183), and *A. thaliana* (NP_187497).

(B) Amino acid sequence alignment of the RNC domains from the species in (A) shows identical (asterisk), conserved (double dot), and partially conserved (single dot) residues. Amino acid characteristics are depicted with a ClustalX color scheme (Thompson et al., 1997).

(C) Northern blot analysis of a multiple human tissue blot (Clontech) for raptor, mTOR, and β-actin.

mRNAs found in skeletal muscle, brain, kidney, and placenta (Figure 2C).

Specific mTOR-Raptor Interaction In Vivo

To characterize the mTOR-raptor interaction, we generated a polyclonal antibody that specifically recognizes amino acids 985 to 1001 of human raptor. This antibody detected raptor in immunoprecipitates prepared from DSP-treated cell lysates using two different anti-mTOR antibodies but not in immunoprecipitates obtained with control antibodies (Figure 3A). To enable a study of the physiological regulation of the mTOR-raptor interaction, we searched for lysis conditions that might preserve the complex in the absence of the chemical crosslinker. We achieved this by avoiding lysis buffers with Triton X-100, a detergent that eliminated the interaction at all concentrations tested (Figure 3B). Lysis of cells in buffers containing the detergent CHAPS instead of Triton X-100 preserved the interaction with the greatest amount of coprecipitating raptor obtained at 0.3% CHAPS. Interestingly, the majority of in vitro studies on mTOR function have been performed on mTOR isolated from cells lysed with Triton X-100 or the related detergent NP-40, conditions that we find completely disassociate raptor from mTOR. Unless otherwise stated, further experiments were performed using a lysis buffer (Buffer B, see Experimental Procedures) that allowed the recovery of a complex containing a ratio of about 1.0 mTOR to 0.7 raptor (data not shown). In addition to HEK293T cells, the mTOR-raptor complex was detected in all other human cell lines tested, including the uterine cancer HeLa-, B cell lymphoma BJAB-, neuroblastoma SK-N-MC-, and lung cancer A549-derived cell lines (Figure 3C), suggesting that the association likely occurs in most cell types. In transfected HEK293T cells, we also detected the interaction of epitope-tagged raptor with endogenous mTOR (Figure 3D) as well as between epitope-tagged versions of both proteins (Figure 3E).

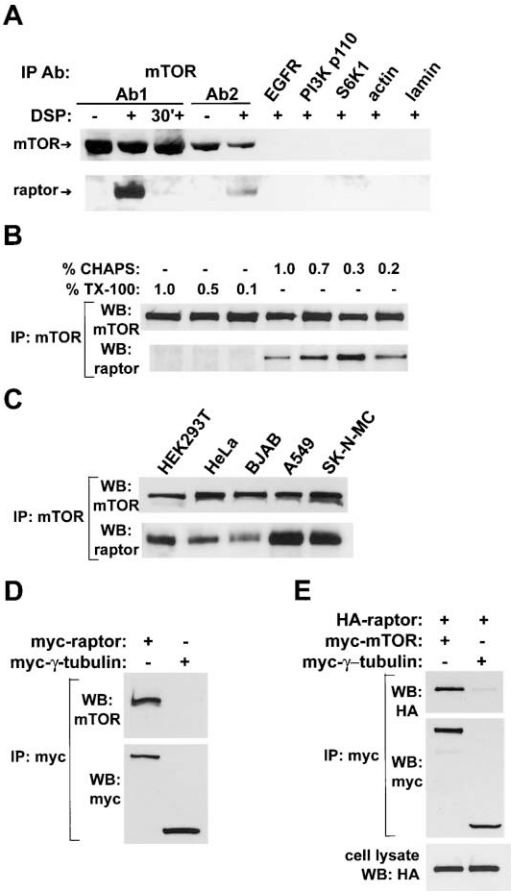

Figure 3. In Vivo Interactions between Endogenous Raptor and mTOR and Recombinant Versions of Both Proteins

(A) Levels of raptor and mTOR in immunoprecipitates prepared with indicated antibodies. The mTOR Ab1 and Ab2 antibodies recognize residues 1–18 and 221–237, respectively, of human mTOR.

(B) Levels of raptor and mTOR in mTOR immunoprecipitates from cells lysed in buffers with the indicated concentrations of Triton X-100 or CHAPS.

(C) mTOR immunoprecipitates prepared from lysates (1 mg total protein) of indicated cell types were analyzed as in (B).

(D) Endogenous mTOR (top) interacts with recombinant raptor but not tubulin (bottom). Anti-myc immunoprecipitates were prepared from HEK293T cells transfected with 100 ng of either a myc-raptor- or a myc-γ-tubulin-encoding plasmid.

(E) Recombinant mTOR and raptor interact in transfected HEK293T cells. Shown are levels of HA-raptor (top) and myc-mTOR and myc-γ-tubulin (middle) in anti-myc immunoprecipitates prepared from HEK293T cells transfected with 100 ng of a plasmid encoding HA-raptor and 100 ng of one encoding either myc-mTOR or myc-γ-tubulin.

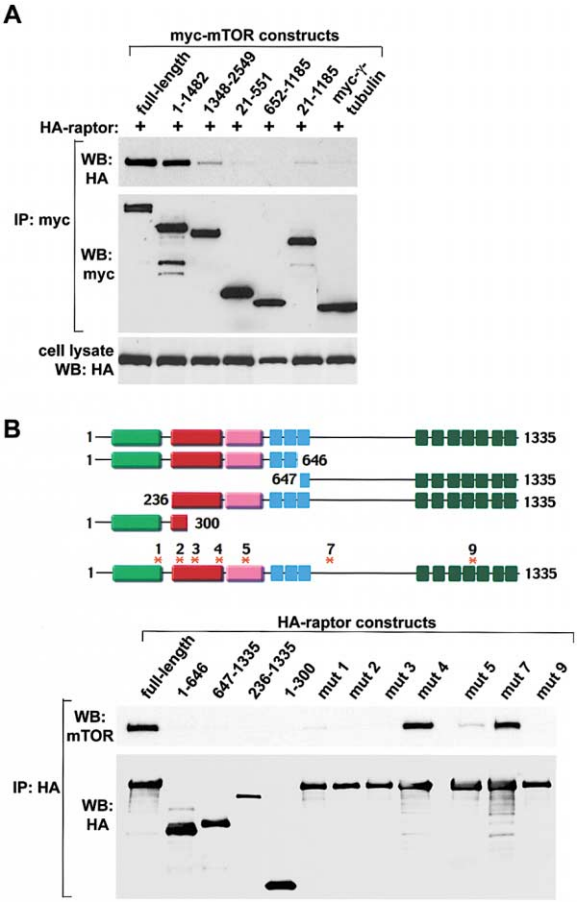

Figure 4. Protein Domains Involved in the mTOR-Raptor Interaction

(A) Myc-tagged mTOR, its indicated fragments, or γ-tubulin were coexpressed in HEK293T cells with HA-raptor (bottom), and anti-myc immunoprecipitates were analyzed by anti-HA (top) and anti-myc (middle) immunoblotting.

(B) Immunoblot analysis shows levels of mTOR (top) in anti-HA immunoprecipitates prepared from HEK293T cells transfected with 1 μg of plasmids encoding HA-tagged raptor, its fragments, or the mutants shown in the schematic. Anti-HA immunoblot shows levels of full-length raptor or fragments in the immunoprecipitates (bottom).

The mTOR-Raptor Interaction Requires the mTOR HEAT Repeats and Multiple Sites on Raptor

To identify which region(s) of mTOR interacts with raptor, we expressed in HEK293T cells several myc-tagged mTOR fragments together with the full-length HA-tagged raptor protein. The N-terminal region of mTOR (amino acids 1–1482) containing all the HEAT motifs interacted with raptor almost as strongly as the full-length protein, whereas the C-terminal region (amino acids 1348–2549) exhibited a weak but greater than background interaction (Figure 4A). Further shortening of the N-terminal region of mTOR by 297 residues severely weakened the interaction, and fragments of the N-terminal region of mTOR (amino acids 21–551 or 652–1185) were incapable of interacting with raptor. These results suggest that the overall structure of the N-terminal portion of mTOR is required for the association with raptor and that the C-terminal half of mTOR contains a weaker interaction site.

None of the individual domains of recombinant HA-tagged raptor expressed in HEK293T cells could interact with the endogenous mTOR (Figure 4B). Several mutations in the RNC or WD40 domains of raptor, generated by changing evolutionarily conserved residues, eliminated the interaction with mTOR, whereas another RNC domain mutation (mut 4), as well as a mutation in the region between the HEAT and WD40 repeats (mut 7), did not affect it. These observations indicate that the mTOR-raptor interaction may involve multiple sites in

raptor and a large region of mTOR and suggest that the proteins make extensive contacts with each other. Alternatively, the mutations in raptor may destabilize its entire structure and disturb the mTOR-raptor association without directly being part of the mTOR interaction site. Interestingly, ATR, a protein which like mTOR is a member of the PIK-related family of kinases, makes extensive contacts with ATRIP (*ATR-i*nteracting *p*rotein), a recently discovered interacting partner of ATR (Cortez et al., 2001).

Raptor Participates in Nutrient Signaling and Maintenance of Cell Size

A major role of the mTOR pathway is to coordinate the synthesis of ribosomal proteins with the levels of available amino acids. This is accomplished by controlling the translational regulator S6K1, a kinase whose phosphorylation state and in vivo activity are regulated by amino acid levels (Fox et al., 1998; Hara et al., 1998) and is phosphorylated in vitro by mTOR (Burnett et al., 1998; Isotani et al., 1999). To investigate the role of raptor in mTOR-mediated signaling, we used small interfering RNA (siRNA) (Elbashir et al., 2001) to decrease endogenous levels of raptor in HEK293T cells and measured the phosphorylation state of S6K1 in response to stimulation with increasing concentrations of leucine. Consistent with a critical role for raptor in nutrient signaling to S6K1, a decreased level of raptor attenuated leucine-stimulated phosphorylation of S6K1 to a similar extent as a decreased level of mTOR achieved with an siRNA specific to mTOR (Figures 5A and 5B). Reductions in the levels of raptor or mTOR did not significantly affect the amounts of S6K1 and ATM, or the phosphorylation state or amount of PKB/Akt, a downstream effector of PI 3-Kinase. Interestingly, mTOR and raptor expression appear to be coordinately regulated because reduced levels of either protein induced by its specific siRNA also decreased the level of the other without affecting the amount of its mRNA (data not shown). The inhibitory effect on S6K1 signaling of the raptor-targeted siRNA is not simply due to its decrease of mTOR levels. In control experiments using the mTOR-targeted siRNA, we found that to observe any effect on S6K1 phosphorylation, mTOR had to be reduced to less than 25% its normal level (data not shown), a far higher reduction than caused by the raptor-targeted siRNA (Figure 5B). The mutual dependence of expression we observed between mTOR and raptor is another similarity the partners share with the ATR-ATRIP interacting pair (Cortez et al., 2001).

In *Drosophila*, the TOR pathway is a major regulator of cell growth and, thus, cell size (Oldham et al., 2000; Zhang et al., 2000). A large part of this regulation is exerted through S6K1, and, in *Drosophila* and mice, loss of function mutations in S6K1 lead to smaller animals with smaller cells (Montagne et al., 1999; Shima et al., 1998). A role for mTOR in regulating cell size can also be demonstrated in tissue culture, as inhibition of the pathway with rapamycin treatment reduces the size of many mammalian cell types, including HEK293Ts, during all phases of the cell cycle (S.M.A. and D.M.S., unpublished data and Figure 5C). Consistent with a role for raptor in size control, we found that actively dividing cells transfected with siRNAs targeting raptor or mTOR underwent comparable reductions in size and that these correlated with a decrease in the phosphorylation state of S6K1 (Figure 5C). The siRNA-mediated reductions in cell size are significant ($p < 0.001$) but less than the reduction caused by treatment of cells with rapamycin for 48 hr. This is expected because rapamycin completely inhibits S6K1 phosphorylation in all of the cells, while the raptor and mTOR siRNAs partially inhibit S6K1 only in the transfected cells.

Raptor Participates in Regulating Cell Size

HEK293T cells grown to confluence in a tissue culture dish become smaller than actively growing cells and, after dilution and plating into fresh media, regain their normal mean size over a period of 3 days (D.-H.K. and D.M.S., unpublished data). As cells transfected with the lamin siRNA emerged from confluence, they gradually increased in mean size over a period of 3 days, an effect that was inhibited in the cells transfected with the mTOR or raptor siRNAs and dramatically reduced by rapamycin (Figure 5D).

We also examined the effects of reducing raptor and mTOR levels on the capacity of cells, in the absence of proliferation, to increase in size after a reduction in size caused by prolonged nutrient deprivation. Cells were transfected with siRNAs targeting lamin, mTOR, or raptor and, 24 hr after transfection, cells were incubated in leucine-free media for an additional 24 hr. During this period of leucine deprivation, the cells ceased to divide and became extremely small so that, irrespective of transfected siRNA, the mean cell volume in all the samples was about 67% of the mean volume of cells in leucine-containing media (Figure 5E). Leucine was then added to the media and cell size measured at 6 and 10 hr after leucine addition. The growth of cells transfected with the mTOR or raptor siRNAs or treated with rapamycin at the time of leucine addition was impaired as these cells increased in size significantly less than those transfected with the lamin siRNA. During this period of growth, the cells did not divide and resumption of cell proliferation did not begin until 15–20 hr after leucine addition (data not shown).

As described above, reductions in raptor and mTOR protein levels have similar effects on nutrient-stimulated increases in S6K1 phosphorylation and cell size and on the maintenance of cell size. In addition, we find that mTOR and raptor exist in a near stoichiometric complex (Figure 1). A parsimonious explanation for these findings is that raptor and mTOR are part of the same signaling system that regulates S6K1 phosphorylation. If this were not the case, we would expect that reducing both raptor and mTOR levels in the same cells would have additive inhibitory effects on the nutrient-stimulated S6K1 phosphorylation because two independent pathways would be affected. However, we find that in cells cotransfected with siRNAs targeting both raptor and mTOR, S6K1 phosphorylation is reduced to a similar extent as in cells transfected with either siRNA alone (see Supplemental Figure S2 at http://www.cell.com/cgi/content/full/110/2/163/DC1), supporting our suggestion that mTOR and raptor are part of the same pathway.

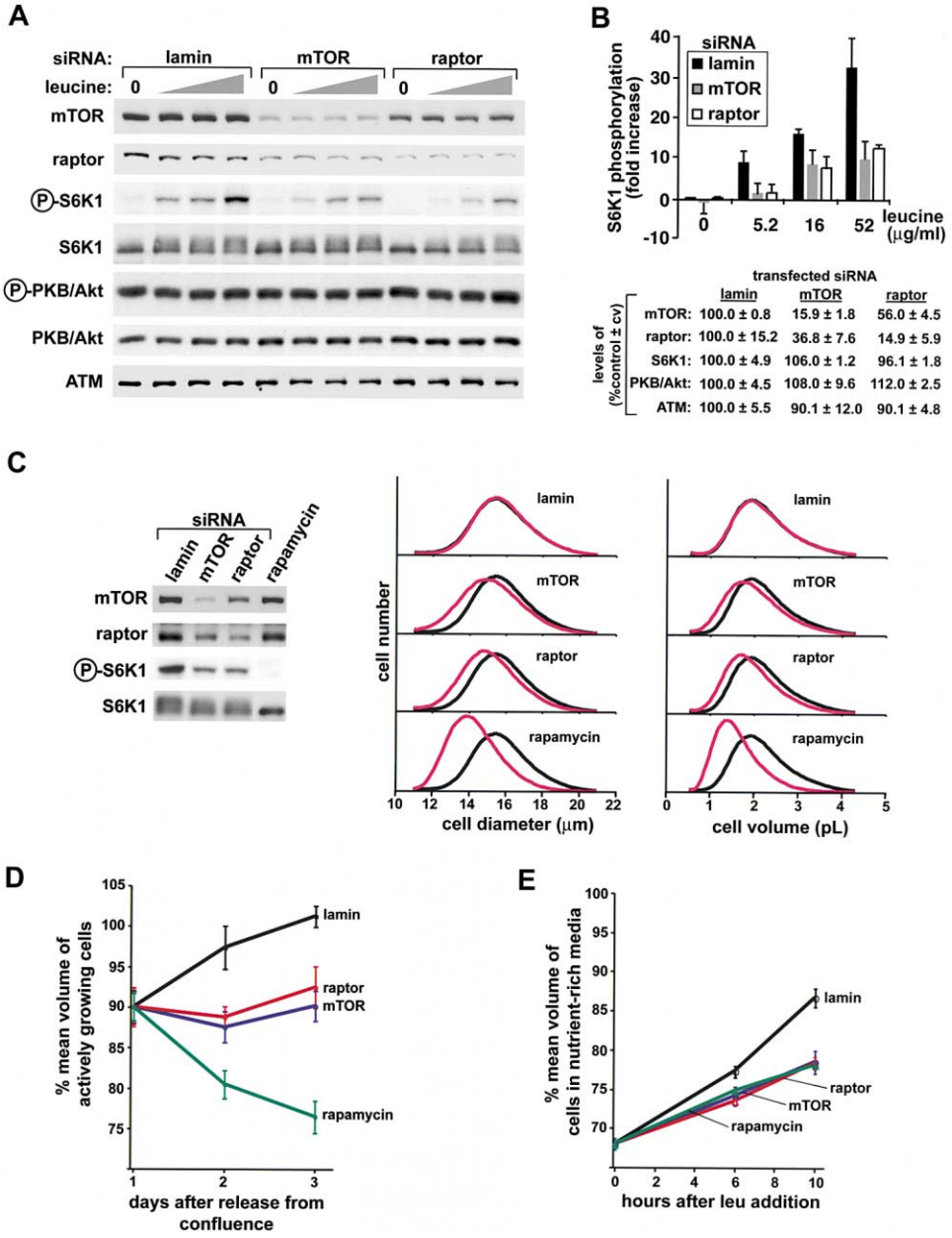

Figure 5. Raptor Participates in Nutrient Signaling to S6K1 and Maintenance and Regulation of Cell Size

(A) HEK293T cells transfected with indicated siRNAs and deprived of leucine for 50 min were stimulated for 10 min with increasing amounts of leucine (0, 5.2, 16, and 52 μg/ml), and lysates (20 μg protein) were analyzed by immunoblotting for indicated proteins and phosphorylation states.

(B) Graph of means ± SD (n = 3) for S6K1 phosphorylation level from experiments performed as in (A) and quantitated by densitometry. Table of means ± SD (n = 4) of levels of indicated proteins determined by densitometry of immunoblots in (A).

(C) Cells transfected with siRNAs were analyzed by immunoblotting for indicated proteins, and cell diameters and volumes were determined 3 days after transfection. Shown are size distributions of nontransfected cells (black line) and of cells transfected with the indicated siRNAs or treated with 20 nM rapamycin for 48 hr (red lines). The mean ± SD cell diameters (μm) and volumes (pL) are, respectively: nontransfected cells, 15.92 ± 0.11 and 2111 ± 44 (n = 5); lamin siRNA, 15.89 ± 0.10 and 2099 ± 39 (n = 5); mTOR siRNA, 15.43 ± 0.09 and 1922 ± 33 (n = 6); raptor siRNA, 15.45 ± 0.07 and 1930 ± 26 (n = 6); and rapamycin-treated cells, 14.61 ± 0.10 and 1632 ± 33 (n = 5). A least 10,000 cells were analyzed per trial.

(D) Cells transfected with the indicated siRNAs were grown to confluence and induced into active growth by dilution into fresh media, and cell volumes were determined at 1, 2, and 3 days after dilution (n = 3). For comparison, nontransfected cells were treated with 20 nM rapamycin at 1 day after dilution. Size reductions caused by raptor and mTOR siRNAs are significant to $p < 0.01$ and 0.05 at the 2 and 3 day time points, respectively.

(E) Cells were transfected with the indicated siRNAs and manipulated as described in the text (n = 3). Size reductions caused by the raptor and mTOR siRNAs and by rapamycin at 10 hr are significant to $p < 0.05$.

Nutrients and Mitochondrial Function Regulate the Stability of the mTOR-Raptor Complex and the mTOR Kinase Activity

To determine if the mTOR-raptor interaction is regulated by conditions that are known to affect the activity of downstream effectors of mTOR, we tested the effects of different nutrient conditions on the stability of the mTOR-raptor complex. The amount of raptor recovered bound to mTOR was markedly increased when HEK293T cells were incubated in amino acid-deprived medium, an effect that was mimicked by the removal of just leucine from the culture medium (Figures 6A and 6B). Importantly, a 10 min stimulation with leucine, which activates the phosphorylation of S6K1 and 4E-BP1 (Figures 5, 6A, and 6B), reversed the effect of leucine deprivation and restored the interaction to the level observed in cells grown in nutrient-rich medium. Furthermore, the in vitro kinase activity of mTOR toward S6K1 and 4E-BP1 inversely correlated with the amount of raptor recovered with mTOR. Greater activity was observed in raptor-deprived complexes obtained after nutrient stimulation. Conversely, reduced activity was observed in raptor-enriched complexes obtained after nutrient starvation (Figures 6A and 6B). We also found that glucose deprivation and readdition affected the mTOR-raptor interaction and mTOR activity in a similar fashion, as did changes in leucine levels.

Although we used a mild buffer to prepare the mTOR immunoprecipitates (in order to preserve the raptor-mTOR association), control kinase assays confirm that the observed activity is that of mTOR and not of a contaminating kinase that also might be capable of phosphorylating S6K1 (Figure 6C). The kinase activity in the mTOR immunoprecipitates depends on the presence of mTOR and is sensitive to LY294002, a known inhibitor of mTOR (Brunn et al., 1996), but not to high concentrations of other kinase inhibitors. Moreover, the kinase activity of recombinant mTOR isolated under the same conditions depends on a wild-type mTOR kinase domain and is sensitive to LY294002. Thus, these findings support the conclusion that, when mTOR is isolated under conditions designed to preserve its interaction with raptor, the activity of the isolated kinase does change in concert with the in vivo effects of stimuli that regulate the pathway.

The identity of the intracellular messengers that signal nutrient availability to mTOR is unknown, but several lines of evidence suggest that the mitochondrial metabolism of nutrients is necessary to activate the pathway (Dennis et al., 2001; Xu et al., 2001; D.D.S., D.-H.K., and D.M.S., unpublished data). We found that valinomycin, a mitochondrial uncoupler (Bernard and Cockrell, 1979), antimycin A, an electron transport inhibitor (Wolvetang et al., 1994), and 2-deoxyglucose, a glycolytic inhibitor, stabilized the mTOR-raptor interaction and inhibited mTOR kinase activity in a fashion similar to nutrient deprivation (Figure 6D). Other cell-stressing conditions known to inhibit S6K1 in vivo, such as the oxidative stress caused by H_2O_2 treatment, which affects mitochondrial function (Majumder et al., 2001), also stabilized the interaction and inhibited the mTOR kinase. On the other hand, a sucrose-induced osmotic shock decreased the phosphorylation state of S6K1 but had only a modest effect on complex stability and kinase activity. In addition to nutrients and cell stress, growth factors such as insulin are also known to regulate downstream components of the mTOR pathway, like S6K1 (Lawrence and Brunn, 2001). However, we found that while treatment of serum-starved cells with insulin increased the phosphorylation state of S6K1, it did not affect the raptor-mTOR interaction (see Supplemental Figure S3 at http://www.cell.com/cgi/content/full/110/2/163/DC1), suggesting that its regulation might be independent of growth factor signaling. Furthermore, in serum-starved cells, the mTOR-raptor interaction was still regulated by nutrients (data not shown).

Increasing the Amount of Raptor Bound to mTOR Leads to an Inhibition of the mTOR Kinase Activity

The inverse correlation we observed between the stability of the mTOR-raptor interaction and the kinase activity of mTOR suggests that a strong association between raptor and mTOR leads to an inhibition of the mTOR catalytic activity. To substantiate this correlation, we compared the activity of mTOR isolated under cell lysis conditions that differentially affect the mTOR-raptor association (see Supplemental Figure S4). A small increase in salt concentration in the lysis buffer slightly reduced the amount of raptor bound to mTOR, and this correlated with a corresponding increase in mTOR activity. Strikingly, the addition of Triton X-100 to the lysis buffer eliminated the interaction and also strongly activated in vitro mTOR activity.

To exclude the possibility that the different isolation conditions directly affect mTOR activity, we determined whether an increase in the intracellular concentration of raptor could drive, even in cells growing in nutrient-rich conditions, the formation of stable mTOR-raptor complexes and inhibit mTOR kinase activity. Overexpression of wild-type raptor, but not of a mutant that cannot interact with mTOR (mut 1 in Figure 4), resulted in an increased amount of raptor bound to mTOR and a decrease in its in vitro kinase activity (Figure 6E). In addition, the overexpression of wild-type but not mutant raptor decreased the in vivo phosphorylation state of S6K1 and increased the amount of 4E-BP1 bound to eIF-4E (Figures 6E and 6F), providing in vivo correlates of the inhibitory effects of raptor on the in vitro mTOR kinase activity. These results strongly indicate that a tightly bound raptor leads to a decrease in mTOR kinase activity and that raptor overexpression can circumvent the normal nutrient-regulated mechanism(s) that control the strength of the interaction.

Evidence that the mTOR-Raptor Complex Exists in Two Binding States

Despite the negative role of raptor in regulating the mTOR kinase activity, the experiments using siRNA indicate that in vivo raptor also has a positive function in the mTOR pathway as decreased levels of raptor reduce S6K1 phosphorylation, cell size, rate of cell growth, and mTOR expression (Figure 5). Moreover, when a cross-linker was present during the cell lysis, raptor was isolated in a stoichiometric complex with mTOR (Figure 1). Thus, it seemed unlikely that the decreased amount of raptor recovered with mTOR isolated from cells grown

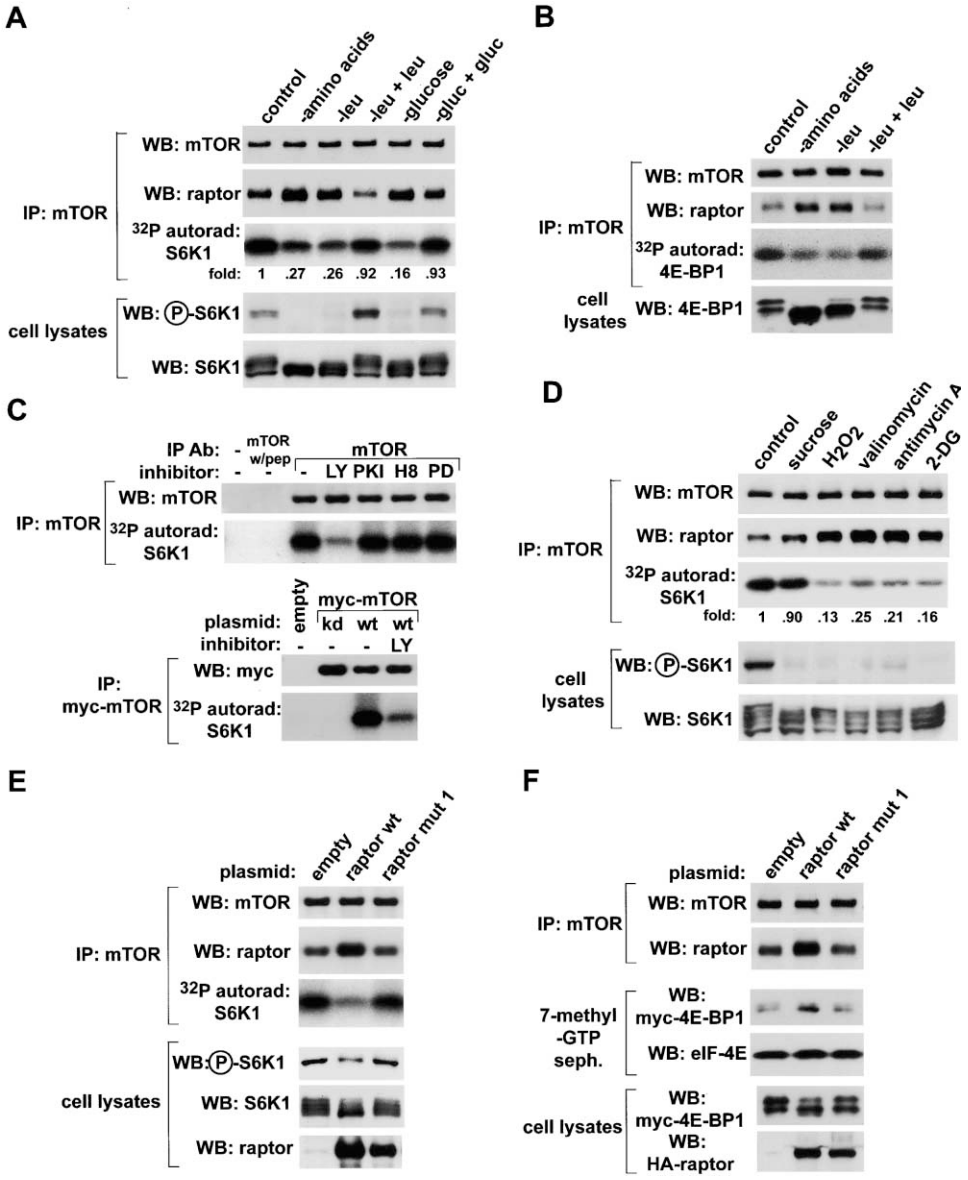

Figure 6. Nutrients, Mitochondrial Function, and Glycolysis Regulate the mTOR-Raptor Interaction and mTOR Kinase Activity

(A) HEK293T cells were not treated (control), deprived of the indicated nutrients for 50 min, or deprived for 50 min and restimulated for 10 min with 52 μg/ml leucine (−leu + leu) or 11 mM glucose (−gluc + gluc). mTOR kinase activity toward GST-S6K1 was determined in anti-mTOR immunoprecipitates (third gel from top). Immunoblots show levels of mTOR and raptor in the kinase reactions and the phosphorylation state and gel mobility of S6K1 (bottom two gels).

(B) Experiment was performed as in (A) except that 4E-BP1 was used a substrate.

(C) The kinase activity in anti-mTOR immunoprecipitates depends on the presence of mTOR and is sensitive to 20 μM LY294002 (LY) but not to 40 nM protein kinase A inhibitor (PKI), 20 μM H-8 (an inhibitor of PKA, PKG, and PKC), and 20 μM PD98059 (PD) (an inhibitor of MEK). The activity of recombinant, myc-tagged mTOR (wt) is absent in the D2357E mutant (kd) and sensitive to 20 μM LY (bottom gels).

(D) Cells treated for 10 min with 500 mM sucrose, 1 mM H_2O_2, 1 μM valinomycin, 5 μM antimycin A, or 100 mM 2-deoxyglucose (2-DG) or not treated (control) were analyzed as in (A).

(E) HEK293T cells transfected with 5 μg of the mammalian expression vector prk5 (empty), the vector encoding raptor (raptor wt), or a mutant incapable of interacting with mTOR (raptor mut 1) were analyzed as in (A).

(F) Experiments were performed as in (E) except that cells were transfected with 50 ng of a plasmid encoding myc-4E-BP1. 7-methyl-GTP affinity chromatography and eIF-4E Western blotting were performed as described (Burnett et al., 1998).

in nutrient-rich conditions (Figures 6A and 6B) reflects a dissociation of the mTOR-raptor complexes in vivo. This was proven to be the case, as the amounts of raptor recovered with mTOR isolated from leucine-deprived or -stimulated cells were similar when cells were lysed in the presence of the crosslinker (Figure 7A).

These findings show that in vivo raptor and mTOR physically interact under both leucine-rich and -poor conditions and suggest that mTOR-raptor complexes exist in at least two nutrient-determined states with differential stability: an unstable complex that does not survive in vitro isolation and a stable complex that does.

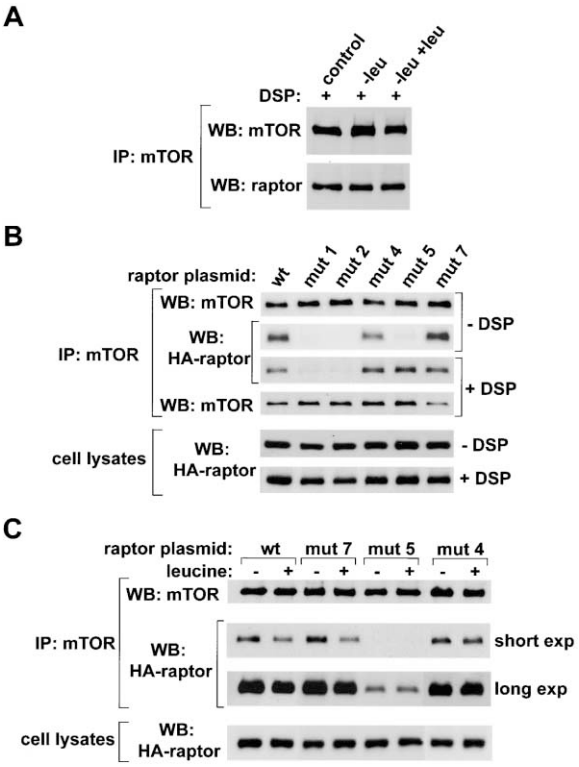

Figure 7. The mTOR-Raptor Complex Exists in Two Binding States

(A) Raptor amounts in mTOR immunoprecipitates prepared from cells grown in leucine-rich or -poor conditions and lysed in the presence of DSP.

(B) Raptor mutant 5 forms an unstable complex with mTOR. mTOR immunoprecipitates were prepared from cells transfected with 10 ng of the indicated raptor plasmids and lysed in the absence or presence of DSP. Immunoblots show amounts of mTOR and indicated HA-raptors in mTOR immunoprecipitates and expression levels of the raptor mutants.

(C) The interactions of raptor mutant 4 with mTOR is not regulated by nutrients. HEK293T cells transfected with 10 ng of the indicated raptor plasmids were incubated for 50 min in leucine-free (−) or -rich (+) media, and amounts of mTOR and HA-raptor in the mTOR immunoprecipitates were analyzed as in (B) (short exp). Leucine levels do not regulate the residual interaction between raptor mutant 5 and mTOR (long exp).

As nutrient-rich conditions decreased the amount of raptor recovered with mTOR, it is likely that nutrients lead to the formation of the unstable complex. We sought evidence for these two binding states by asking if any of the raptor mutants we identified formed complexes with mTOR that were permanently in the unstable or stable state. We searched for a mutant that forms an unstable complex with mTOR by screening the mutants for those that are recovered with mTOR only when cells are lysed in the presence of a crosslinker (Figure 7B). Only one mutant (mut 5) had this characteristic, indicating that in vivo mut 5 does associate with mTOR but that the interaction does not survive the in vitro isolation conditions. As expected, the small residual interaction of mut 5 with mTOR was no longer sensitive to levels of leucine in the media (Figure 7C). Next, we searched for a mutant that forms a stable complex with mTOR irrespective of nutrient conditions. Of the two raptor mutants (muts 4 and 7) that associate with mTOR in the absence of a crosslinker (Figure 7B), the interaction of mut 4 was not sensitive to leucine levels, while that of mut 7 was still regulated (Figure 7C). These findings indicate that mutants 5 and 4 form complexes similar to those found under nutrient-rich and -poor conditions, respectively. Complexes containing mutant 5 are in the unstable state characteristic of nutrient-rich conditions, while those containing mutant 4 are in the stable state found under nutrient-poor conditions.

Rapamycin Severely Weakens the mTOR-Raptor Interaction

In vivo, rapamycin, like nutrient deprivation, inhibits the activation of downstream effectors of mTOR, but exactly how the drug perturbs mTOR function is unknown. We found that treatment of HEK293T cells with rapamycin or the addition of the drug to cell lysates significantly destabilized the mTOR-raptor complex (Figure 8A). The effect was specific to rapamycin, as FK506, an immunosuppressant that also binds FKBP12 but does not target mTOR, had no effect on the interaction, nor did ethanol, the vehicle used for both drugs. However, when lysates were prepared in the presence of the crosslinker DSP, almost normal levels of raptor were recovered with mTOR. Therefore, as with nutrient stimulation and raptor mutant 5, rapamycin destabilizes the raptor-mTOR complex but does not abolish it in vivo.

We also examined the effect of increasing concentrations of rapamycin on the mTOR-raptor interaction in cells deprived of or stimulated with leucine. Interestingly, irrespective of nutrient conditions, rapamycin dominantly destabilized the raptor-mTOR complex, and the order of rapamycin addition and leucine deprivation did not matter (Figure 8B). In addition, leucine-rich conditions enhanced the destabilizing effects of rapamycin, decreasing its EC50 for mTOR-raptor dissociation by about 3-fold (from 5 nM to 1.5 nM) when compared to leucine-poor conditions. A simple explanation for this latter result might be that leucine-poor conditions lead to a decrease in the affinity between mTOR and FKBP12-rapamycin, perhaps by inducing a conformational change in the FKBP12-rapamycin binding site in mTOR. Alternatively, leucine deprivation might increase the affinity between raptor and mTOR so that the complex is less susceptible to the destabilizing effects of FKBP12-rapamycin. The finding that the unstable and stable complexes found in nutrient-rich and -poor conditions, respectively, are differentially sensitive to rapamycin provides further evidence that the raptor-mTOR complex can exist in two binding states.

Discussion

Raptor Forms a Nutrient-Sensitive Complex (NSC) with mTOR

We have identified raptor, a 149 kDa protein that participates in the mTOR pathway and associates in a near stoichiometric ratio with mTOR to form a nutrient-sensitive complex (NSC). Raptor and mTOR associate under all nutrient conditions, but the stability of the complex changes with the activity of the pathway. Under nutrient-poor conditions, the mTOR-raptor association is stabilized and high levels of the complexes are recovered even when cell lysates are prepared without the cross-

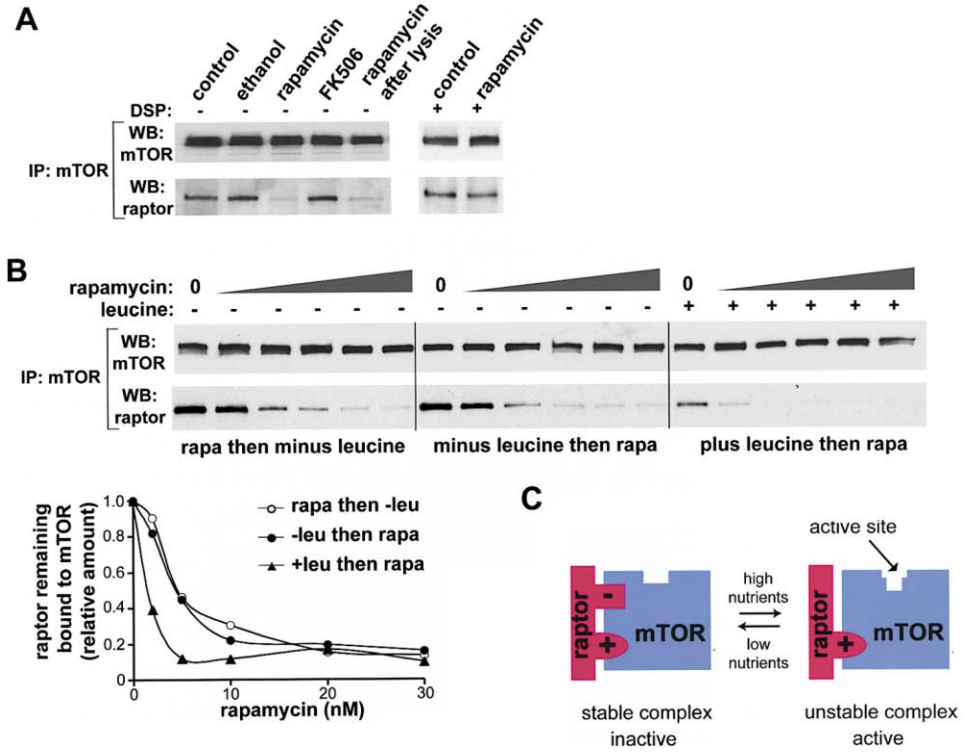

Figure 8. Rapamycin Destabilizes the mTOR-Raptor Interaction under Leucine-Rich or -Poor Conditions

(A) mTOR immunoprecipitates isolated from HEK293T cells treated for 10 min with 10 nM rapamycin, 10 nM FK506, or ethanol or from cell lysates treated with 10 nM rapamycin were analyzed by immunoblotting for mTOR and raptor.

(B) HEK293T cells treated with increasing concentrations of rapamycin (0, 2, 5, 10, 20, or 30 nM) for 20 min before (left) or after (middle) incubation of cells for 30 min in leucine-free RPMI or after incubation for 30 min in leucine-rich RPMI (right). The amount of raptor coimmunoprecipitating with mTOR was quantitated, normalized to the amount obtained in the absence of rapamycin (first lane in each group), and plotted against the rapamycin concentration (graph).

(C) Model to explain effects of nutrients on mTOR-raptor complex and the mTOR activity.

linker. On the other hand, under nutrient-rich conditions, the association is weaker and most of the complex readily falls apart in the absence of the crosslinker. These findings are consistent with a model in which mTOR and raptor are held together in a constitutive, easily disrupted association, which, under nutrient-poor conditions, is strengthened by an additional interaction(s) that also lead to a repression of the kinase activity of mTOR. Thus, we propose that at least two interactions exist between raptor and mTOR: a "constitutive" interaction that is required for in vivo mTOR function and a "nutrient-sensitive" interaction that forms in the absence of nutrients and leads to the inhibition of the mTOR kinase activity (Figure 8C). In support of this model, we found two raptor mutants that associate with mTOR in ways that suggest they have selective defects in the nutrient-sensitive interaction postulated above. In addition to changes in mTOR kinase activity, other mechanisms are also likely involved in controlling downstream effectors of mTOR, such as mTOR regulation of a phosphatase(s) that inhibits S6K1.

The molecular mechanisms by which raptor regulates mTOR are unknown. The nutrient-sensitive mTOR-raptor interaction might decrease the catalytic activity of mTOR by inducing a conformational change in the mTOR kinase domain (mechanism shown in Figure 8C) or by sterically preventing substrates from accessing the mTOR active site. On the other hand, the constitutive interaction is clearly not required for in vitro kinase activity as we found that raptor-depleted mTOR strongly phosphorylates S6K1. Previous studies have shown that to function in vivo mTOR requires more than its kinase activity because truncation and point mutants of mTOR that retain wild-type kinase activity cannot signal to S6K1 within mammalian cells (Brown et al., 1995; Sabatini et al., 1999). Our work suggests that raptor makes extensive contacts with mTOR regions that are far from its kinase domain. Thus, it is likely that in vivo the constitutive mTOR-raptor interaction is required for a function other than mTOR kinase activity. A simple possibility, supported by the finding that a reduction in raptor levels also reduces mTOR levels, is that raptor is required for the proper folding and/or stability of mTOR. On the other hand, raptor could also serve as an adaptor that brings substrates to the mTOR kinase domain and/or may be a determinant of the proper subcellular localization of mTOR.

Rapamycin Destabilizes the NSC Irrespective of Nutrient Conditions

Although rapamycin and nutrient deprivation similarly inhibit the activity of downstream components of the mTOR pathway, such as S6K and 4E-BP1, we find that

they have opposite effects on the mTOR-raptor interaction. Rapamycin destabilizes the interaction regardless of nutrient availability, and its potency for dissociation is increased under nutrient-rich conditions.

Several mechanisms can be proposed to account for the effects of FKBP12-rapamycin on the stability of the complex and to explain how the drug inhibits the pathway. Of the many possible models, two appealing ones will be considered here. In the first, FKBP12-rapamycin dislodges raptor from its nutrient-sensitive binding site on mTOR because it binds to mTOR at or near that site. By replacing raptor at this site, FKBP12-rapamycin would mimic raptor's inhibitory effect on the mTOR kinase that is manifested under nutrient-poor conditions. A prediction of this model is that in vitro FKBP12-rapamycin should inhibit mTOR kinase activity, a result seen in many studies (Brown et al., 1995; Brunn et al., 1996, 1997; Burnett et al., 1998; Isotani et al., 1999). In the second model, FKBP12-rapamycin does not affect the nutrient-sensitive interaction but interferes with the constitutive mTOR-raptor interaction. Because this is a positive interaction required for the in vivo function of mTOR, its interference by FKBP12-rapamycin inhibits the pathway. Of course, it is also possible that rapamycin exerts its negative effects on the pathway independently of its perturbation of the mTOR-raptor complex.

Diverse Signals Converge on the Regulation of the NSC

How might changes in levels of diverse nutrients regulate the strength of the NSC association? Certainly, mTOR and/or raptor could be phosphorylated or modified by upstream "nutrient sensors" that regulate their interaction. We favor a hypothesis in which the mTOR-raptor complex is itself the nutrient sensor. In this scenario, one or more intracellular molecules increase in concentration in nutrient-rich conditions and bind to raptor and/or mTOR, destabilizing the NSC and relieving raptor inhibition of the mTOR kinase (Figure 8C). If a single molecular species sufficed to destabilize the interaction, its concentration would have to reflect the availability of both leucine and glucose, as well as the state of mitochondrial metabolism. Alternatively, destabilization could require several molecular species that are derived from distinct nutrients and act on multiple independent sites on the NSC. Because raptor and mTOR are large proteins with high potentials for small molecule-induced allosteric and conformational changes that may affect protein-protein interactions, the NSC appears well equipped for sensing multiple growth signals.

It is worth noting that the interaction is exquisitely sensitive to the detergent Triton X-100. This may indicate that a hydrophobic molecule, such as a lipid, plays a role in maintaining the interaction, or that Triton X-100 mimics a molecule that normally weakens it in vivo. Consistent with this possibility, mTOR is found in association with membrane fractions (Sabatini et al., 1999).

Polypeptide growth factors such as insulin regulate S6K1 and mammalian cell growth (Efstratiadis, 1998; Taha and Klip, 1999), and mTOR is considered a mediator of insulin signaling (Lawrence and Brunn, 2001). However, insulin does not appear to regulate the mTOR-raptor interaction, suggesting that it signals to S6K1 through other mechanisms. Perhaps growth factors regulate currently unexplored properties of raptor, such as its phosphorylation state, or signal to S6K1 through mechanisms that do not involve this protein. As conserved raptor and mTOR homologs are also found in yeast, it is likely that the TOR pathway evolved to sense nutrient-derived signals that drive cell-autonomous growth. Systems that respond to the polypeptide factors that coordinate the growth of groups of cells found in mammalian organs likely evolved later and became integrated into prior nutrient-sensing pathways in ways that are still not understood.

We have demonstrated that in vivo mTOR interacts with raptor, a protein that functions positively in the mTOR pathway. Under nutrient-deprived conditions, raptor also serves as a negative regulator of mTOR kinase activity. We propose that the regulation of the mTOR-raptor interaction is a critical mechanism by which eukaryotic cells coordinate the rate of cell growth with different environmental conditions. Perhaps small molecules can be designed that perturb the mTOR-raptor association in subtler ways than rapamycin, allowing a finer pharmacological control of the TOR pathway than is currently possible.

Experimental Procedures

Materials

Reagents were obtained from the following sources: DSP and protein G-sepharose from Pierce; L-[^{35}S]methionine and [γ-^{32}P]ATP from NEN; mTOR, EGFR, S6K1, actin, lamin, and PI3-K antibodies and HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; Phospho-T389 S6K1 and Phospho-S473 PKB/Akt antibodies from Cell Signaling; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; myc rabbit polyclonal antibody from Upstate Biotechnology; eIF-4E antibody from Transduction Laboratories; DMEM, leucine, glucose, RPMI, and RPMI without amino acids, leucine, or glucose from Life Technologies; and rapamycin, FK506, valinomycin, antimycin A, PKI, H-8, PD98059, LY294002, and 2-deoxyglucose from Calbiochem. Rabbit polyclonal anti-peptide antibodies recognizing human mTOR and raptor were produced using the regular antibody service from Zymed.

Crosslinking Assay and Immunoprecipitations

Three million HEK293T cells growing in 6 cm dishes in DMEM with 10% dialyzed fetal calf serum were metabolically labeled by the addition of 0.4 mCi of [^{35}S]methionine for 2 hr. Cells were rinsed once with PBS, lysed in 300 μl of ice-cold Buffer A (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, 1% Triton X-100, and one tablet EDTA-free protease inhibitors [Roche] per 10 ml) with or without 2.5 mg/ml DSP, and incubated for 30 min on ice. Crosslinking reactions were quenched by adding 75 μl 1 M Tris-HCl (pH 7.4) followed by an additional 30 min incubation. After clearing, 30 μl of a 50% slurry of protein G-sepharose and 4 μg of the anti-mTOR antibody was added to the supernatant and rotated for 3 hr at 4°C. Immunoprecipitates were washed once each with Tris-buffered saline containing 0.05% Tween 20, Wash Buffer 1 (50 mM HEPES [pH 7.5], 40 mM NaCl, and 2 mM EDTA) with 1% Triton X-100, Wash Buffer 1 with 500 mM LiCl and 0.5% Triton X-100, Wash Buffer 1 with 500 mM LiCl, and Wash Buffer 2 (50 mM HEPES [pH 7.5] and 150 mM NaCl). 5$\times$ sample buffer (0.242 M Tris, 10% SDS, 25% glycerol, 0.5 M dithiothreitol, and bromophenol blue) was added to washed immunoprecipitates and incubated for 1 hr at 37°C to reduce the DSP crosslinking. Samples were resolved by 3%–8% SDS-PAGE, proteins were transferred to (poly(vinylidene difluoride)) PVDF, and the blot was exposed to film.

In the absence of the crosslinker, cells were lysed in ice-cold

Buffer B (Buffer A with 0.3% CHAPS instead of Triton X-100), and mTOR immunoprecipitates were washed four times in Buffer B and twice in Wash Buffer 2.

Mass Spectrometric Analysis

mTOR immunoprecipitates prepared from 250 million HEK293T cells were resolved by SDS-PAGE, and the stained protein band corresponding to raptor was digested with trypsin and batch-fractionated on a Poros 50 R2 RP micro-tip (Erdjument-Bromage et al., 1998), and peptide pools were analyzed by matrix-assisted laser-desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (Geromanos et al., 2000). Selected mass values from the MALDI-TOF spectra were used to search the human segment of a nonredundant protein database (NR; NCBI, Bethesda, MD) using PeptideSearch (Matthias Mann, University of Southern Denmark).

Cloning of the Raptor cDNA, DNA Manipulations, and Mutagenesis

The cDNA for the KIAA1303 protein encodes amino acids 200–1335 of raptor downstream of 126 bases of unknown origin that are not present in the EST databases and likely represent unspliced intronic sequence. Using bases 127–500 of KIAA1303, we identified EST cDNAs that extended the sequence in the 5′ direction. The 5′ sequences of these cDNAs were used to search for cDNAs that further extended the 5′ end of the raptor mRNA, and this process was repeated until no additional cDNAs were found. The sequences obtained in this fashion allowed us to amplify PCR products containing the 5′ end of the raptor mRNA using human first-strand cDNA. To create the cDNA for full-length raptor, the PCR fragments and KIAA1303 were assembled in pBluescript SK-II(+) using restriction sites in overlapping regions. The raptor cDNA in pBluescript SK-II(+) was mutagenized using the QuikChange mutagenesis kit (Stratagene) and subcloned into the Sal 1 and Not 1 sites of HA-prK5. Mutants are mut 1 ($_{194}$YDC$_{196}$-AAA), mut 2 ($_{261}$DLF$_{263}$-AAA), mut 3 ($_{313}$NWIF$_{316}$-AAAA), mut 4 ($_{391}$SQ$_{392}$-PA), mut 5 ($_{473}$FPY$_{475}$-AAA), mut 7 ($_{738}$SLQN$_{741}$-PAAA), and mut 9 ($_{1191}$RVYDRR$_{1196}$-DAAADD).

Sequence Analysis and Alignments

The MEME server (Bailey and Elkan, 1994) at http://meme.sdsc.edu/meme/website was used to identify blocks of similar sequence between the raptor homologs. The sequences were aligned with ClustalX (v1.81) (Thompson et al., 1997) using the Gonnet series weight matrix with pairwise gap opening and gap extension penalties set at 10.00 and 0.10, respectively. Three HEAT-like and seven WD40-like repeats were found in each protein using Pfam v6.6 fragment search (Sonnhammer et al., 1997) at http://pfam.wustl.edu.

Plasmid and siRNA Transfections

Three million HEK293T cells in 6 cm dishes were transfected using the Lipofectamine 2000 transfection reagent (Life Technology). For the siRNA experiments, 21 nucleotide complementary RNAs with symmetrical 2 nucleotide overhangs were obtained from Dharmacon to the following regions: bases 1531–1551 or 3374–3394 of raptor, 2241–2261 of human mTOR, and 608–630 of human lamin. Annealed oligonucleotides were transfected at 40 nM with Effectene (Qiagen) into HEK293T cells cultured in Optimem media (Life Technology) without serum. Forty-eight hours after adding siRNAs, the medium was replaced with DMEM containing 10% serum, and cells were cultured for a further 24 hr before use in experiments.

Cell Size Determinations

To examine regrowth after confluence, HEK293T cells were transfected with siRNAs, grown to confluence in a 6 cm culture dish for 24 hr, harvested, diluted 1:10 or 1:20, and replated in fresh media. The cells were harvested by trypsinization in 2 ml media and diluted 1:20 with counting solution (Isoflow Sheath Fluid, Coulter Corp.), and cell diameters and volumes were determined using a particle size counter (Coulter Multisizer II). For the leucine-induced growth, the siRNA-transfected cells were diluted into fresh media 1 day after transfection, and 24 hr after dilution the cells were deprived of leucine for a further 24 hr and then fed with leucine (52 μg/ml).

In Vitro Kinase Assay for mTOR Activity

Kinase assays were performed in 10 μl at 30°C for 20 min and contained 1/4 of the washed mTOR immunoprecipitates from 10 million HEK293T cells grown on a 10 cm dish, 200 ng of a GST-S6K1 fusion protein (amino acids 332–502) or 1 μg of 4E-BP1, 1 μCi of [γ-^{32} P]ATP, 25 mM HEPES-KOH [pH 7.4], 50 mM KCl, 20% glycerol, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 1 mM DTT, and 50 μM unlabeled ATP. When used, inhibitors were added 10 min prior to the start of the kinase assay. Reactions stopped by adding 5 μl of 2× sample buffer were analyzed by 8% or 16% SDS-PAGE and transferred to PVDF, and phosphorylated proteins were detected with autoradiography. Kinase assays using recombinant mTOR were as described (Burnett et al., 1998) except that immunoprecipitates were washed as above.

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Accession Numbers

The GenBank accession number for the human raptor cDNA sequence is AY090663.