Published in final edited form as:
Methods Enzymol. 2008 ; 438: 307–320. doi:10.1016/S0076-6879(07)38021-X.

Characterization of the Rheb-mTOR Signaling Pathway in Mammalian Cells: Constitutive Active Mutants of Rheb and mTOR

Tatsuhiro Sato, **Akiko Umetsu**, and **Fuyuhiko Tamanoi**
Department of Microbiology, Immunology and Molecular Genetics, Jonsson Comprehensive Cancer Center, Molecular Biology Institute, University of California, Los Angeles, California

Abstract

Rheb (Ras homolog enriched in brain) is a GTPase conserved from yeast to human and belongs to a unique family within the Ras superfamily of GTPases. Rheb plays critical roles in the activation of mTOR, a serine/threonine kinase that is involved in the activation of protein synthesis and growth. mTOR forms two distinct complexes, mTORC1 and mTORC2. While mTORC1 is implicated in the regulation of cell growth, proliferation, and cell size in response to amino acids and growth factors, mTORC2 is involved in actin organization. However, the mechanism of activation is not fully understood. Therefore, studies to elucidate the Rheb-mTOR signaling pathway are of great importance. Here we describe methods to characterize this pathway and to evaluate constitutive active mutants of Rheb and mTOR that we recently identified. Constitutive activity of the mutants can be demonstrated by the phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) both *in vivo* and *in vitro* after starving cells for amino acids and growth factors. In addition, formation and activity of mTORC1 and mTORC2 can be measured by immunoprecipitating these complexes and carrying out *in vitro* kinase assays. We also describe a protocol for rapamycin treatment, which directly inhibits mTOR and can be used to investigate the mTOR signaling pathway in cell growth, cell size, etc.

1. Introduction

Small GTPases bind guanine nucleotides and serve as a molecular switch to regulate a number of physiological processes such as cell growth and morphology (Bourne *et al*., 1990). Rheb, a small GTPase that belongs to a unique family within the Ras superfamily of GTPases, controls cell growth and proliferation as well as cell size (Aspuria and Tamanoi, 2004; Patel *et al*., 2003; Yamagata *et al*., 1994; Yu *et al*., 2005). Unlike most small GTPases that are predominantly in an inactive GDP bound state, Rheb exists in a high activated state (Im *et al*., 2002), presumably due to a low intrinsic GTPase activity as well as to a limiting amount of Tsc1/Tsc2 GAP protein inside the cell.

Regulation of Rheb is catalyzed by tuberous sclerosis 2 (Tsc2), which acts as a GTPase activating protein (GAP) that enhances the hydrolysis of GTP to GDP in Rheb (Castro *et al*., 2003; Garami *et al*., 2003; Inoki *et al*., 2003; Tee *et al*., 2003; Zhang *et al*., 2003). Tsc2 forms a complex with tuberous sclerosis 1 (Tsc1) and directly inhibits the Rheb activation. It has been found that the negative regulation of Rheb by the Tsc1/2 complex is controlled by insulin. Insulin binding to its receptor triggers the activation of the class I PI3-kinase/Akt pathway. The activated Akt then increases Tsc2 phosphorylation at serine 939 and 981 (Cai *et al*., 2006), leading to the dissociation of the Tsc1/2 complex.

Recent studies suggest that Rheb is involved in the activation of mTOR, a serine/threonine kinase that belongs to the family of PI3-kinase–related kinases. This family of kinases shares common features that include the presence of the HEAT domain, FAT domain, kinase domain,

and FATC domain (Abraham, 2004). In addition, TOR kinases contain the FRB domain where FKBP/rapamycin complex binds. mTOR acts as a central protein that controls cell growth and proliferation through transcriptional and translational mechanisms in response to amino acids and growth factors such as insulin. However, amino acids and insulin use two distinct pathways to activate mTOR. Vps34, a class III PI3-kinase, but not class I PI3-kinase, is activated by amino acid stimulation (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005). On the other hand, growth factors activate the class I PI3-kinase/Akt signaling pathway, which then inactivates Rheb GAP, Tsc1/2, as described previously (Gao and Pan, 2001; Inoki *et al.*, 2002). It has been reported that mTOR forms two distinct complexes, which respond to amino acids or insulin (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). mTOR complex 1 (mTORC1) is rapamycin sensitive and contains Raptor, GβL/mlST8, and PRAS40 (Hara *et al.*, 2002; Sabatini, 2006). This complex phosphorylates S6K1 and 4E-BP1, and plays an essential role in the regulation of cell growth and proliferation (Kim *et al.*, 2002, 2003). Within the complex, Raptor acts as a scaffold protein that connects mTOR to its substrates. PRAS40 is a negative regulator of mTOR that is affected by insulin (Haar *et al.*, 2007; Sancak *et al.*, 2007). GβL is also involved in mTORC1 activity, but its mechanism remains to be elucidated (Kim *et al.*, 2003). On the other hand, mTOR complex 2 (mTORC2) is relatively rapamycin insensitive and contains Rictor, Sin1, and GβL (Frias *et al.*, 2006; Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004; Yang *et al.*, 2006). mTORC2 is involved in actin organization and cell survival, and mediates insulin signal to Akt by the phosphorylation at serine 473 (Hresko and Mueckler, 2005; Jacinto *et al.*, 2006; Sarbassov *et al.*, 2004; Sarbassov *et al.*, 2005; Yang *et al.*, 2006). Interestingly, inhibition of mTORC2 decreases the phosphorylation level of Akt substrates, forkhead transcription factor, FOXO1/3 proteins, but not other Akt substrates such as Tsc2 and GSK3β, suggesting that mTORC2 preferentially affects downstream events mediated by Akt (Guertin *et al.*, 2006; Jacinto *et al.*, 2006).

Our genetic analysis of the Tsc/Rheb/TOR signaling pathway in fission yeast led to the identification of novel Rheb and TOR mutants. In the case of Rheb, we first developed screening assays to identify active Rheb mutants in yeast. Screening of a random mutant library of Rheb identified a number of yeast Rheb mutants that showed phenotypes similar to those exhibited by the cells lacking the Tsc1/2 complex, which negatively regulates Rheb (Urano *et al.*, 2005). Comparison of Rheb sequences from different organisms led to the identification of other active mutants of human Rheb (Yan *et al.*, 2006). These mutants will be valuable in elucidating Rheb function and the activation mechanism for the Rheb-mTOR signaling. Constitutive active mutants of Tor2p have also been identified from the analysis of fission yeast signaling (Urano *et al.*, 2007). Altogether, 22 single amino acid changes have been identified in Tor2p. Introduction of some of these mutations to mTOR conferred nutrient-independent activation of mTOR.

In this chapter, we present methods to characterize the constitutive active mutants of Rheb and mTOR. We also describe methods to detect activation of mTOR and to characterize mTOR complexes. Finally, rapamycin sensitivity will be examined.

2. Methods

2.1. Detection of mTOR activation by examining phospho-S6K1 or phospho-4E-BP1

Activation of mTOR is detected by examining phosphorylation of downstream proteins. We usually examine phosphorylation of S6K1 at Ser 389 and/or phosphorylation of 4E-BP1 at Thr 37/46 or Thr 70 for this experiment. To enhance the sensitivity of detection, genes encoding these proteins are transfected. Figure 21.1 shows an example of detecting mTOR activation after amino acid addition. Briefly, cells are transfected with FLAG-tagged S6K1 and then starved for both serum and amino acids. Then amino acid mixture containing glucose is added and incubated for 30 min. Cells are collected and the level of phospho-S6K1 is examined by

using an antibody specific for phosphorylated S6K1. The total level of S6K1 is examined by using anti-FLAG antibody. Phosphorylation of S6K1 is shut down after serum and amino acid starvation (PBS lane). However, the addition of amino acids and glucose leads to the appearance of phospho-S6K1 band (+glucose +AA lane). We cannot detect the increased phosphorylation of S6K1 in HEK293 cells when treated with glucose (+glucose lane) or amino acid mixture only (not shown).

2.1.1. Cell culture and transfection—HEK293 and HeLa cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum and cultured at 37° in a 5% CO_2 incubator. Transfection in these cells can be performed either by calcium phosphate method or lipofection method. Here we show the former method. The latter method requires reagents that are commercially available from several sources and transfection is performed according to the manufacturer's protocol.

1. Prepare following reagents for the calcium phosphate transfection.

   0.25 *M* $CaCl_2$: Dissolve $CaCl_2$ in water to the concentration of 0.25 *M*, and filter through a 0.45-μm membrane filter.

   2× BBS: Make a 2× BBS solution (50 m*M* BES (N, N-bis [2-hydro-xyethyl]-2-aminoethane sulfonic acid), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4), adjust the pH to 6.95 with NaOH, and filter through a 0.45-μm membrane filter.

   Cell: Plate 1×10^6 HEK293 cells in a 3.5-cm dish the day before transfection. Growth medium is replaced before adding transfection mixture.

2. Add 20 μg of plasmid DNA in 500 μl of 0.25 *M* $CaCl_2$ and vortex well.
3. Add 500 μl of 2× BBS, vortex well, and incubate for 20 min at room temperature.
4. Pour this transfection mixture directly to each dish, mix gently, and incubate cells at 37°.
5. Replace transfection medium with fresh medium at 18 to 24 h after transfection.
6. Incubate at 37° for a total of 48 to 72 h until target proteins are expressed.

2.1.2. Serum and amino acid starvation

1. Incubate cells in DMEM containing 0.1% bovine serum albumin at 37° in a 5% CO_2 incubator for 24 h.
2. Remove medium and wash cells two times with Dulbecco's Phosphate-Buffered Saline (D-PBS, Invitrogen) containing 100 mg/l each $CaCl_2$ and $MgCl_2$. Incubate them in D-PBS for 1 h.

2.1.3. Preparation and addition of amino acid mixture—To make amino acid mixture, add several amino acids in D-PBS at the following concentrations: L-Arg, 84 mg/l; L-Cys, 48 mg/l; L-Glu, 584 mg/l; L-His, 42 mg/l; L-Ile, 105 mg/l; L-Leu, 105 mg/l; L-Lys, 146 mg/l; L-Met, 30 mg/l; L-Phe, 66 mg/l; L-Thr, 95 mg/l; L-Trp, 16 mg/l; L-Tyr, 72 mg/l; L-Val, 94 mg/l. Stir until dissolved and then filter through 0.22-μm membrane filter. To activate mTOR, 4.5 g/l of glucose is mixed in this mixture.

For mTOR stimulation, cells are incubated in this amino acid mixture containing 4.5 g/l of glucose for 30 min at 37° in 5% CO2 after serum and amino acid starvation.

2.1.4. Detection of phospho-S6K1 and phospho-4E-BP1—Lyse the cells with lysis buffer (1% Triton X-100, 20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 1 m*M* EDTA, 50 m*M* β-

glycerophosphate, 1× protease inhibitor cocktail from Roche. After protein quantification, add 1 volume of 2× SDS sample buffer (6% SDS, 10% glycerol, 124.7 m*M* Tris-HCl) (pH 6.7), 2% 2-mercaptoethanol, 0.02% bromophenol blue), and incubate them at 95° for 5 min. These samples are resolved by 10% polyacrylamide gel for S6K1 detection or 14% polyacrylamide gel for 4E-BP1 detection, and analyzed by Western blot. The anti-phospho-S6K at Ser 389, anti-phospho-4E-BP1 at Thr 37/46, and anti-phospho-4E-BP1 at Thr 70 antibodies are available from several sources. In addition, since hyperphosphorylated 4E-BP1 is separated from less- or non-phosphorylated 4E-BP1 in SDS-polyacrylamide gel electrophoresis, total 4E-BP1 can be resolved into three bands; α, β, and γ from the top. Transiently expressed S6K1 and 4E-BP1 are used because the expression and phosphorylation levels of endogenous S6K1 and 4E-BP1 are low in HEK293 cells.

2.2. Overexpression of wildtype Rheb or constitutive active mTOR mutants confer mTOR activation in the absence of nutrients

Rheb activates mTOR and its downstream proteins in the presence of amino acids. Therefore, as described above, no activation is observed after nutrient starvation. However, overexpression of the wildtype or active mutant Rheb, Rheb-N153T, can induce the activation of mTOR that is identified by the high phosphorylation level of mTOR substrates, S6K1 or 4E-BP1, even in the absence of amino acids (Fig. 21.2).

It is important to note that a similar level of mTOR activation is observed with the wildtype and with the mutant Rheb. A possible reason why one can observe mTOR activation after the overexpression of the wildtype Rheb is that the overexpressed wildtype Rheb contains a high GTP level. Presumably, Rheb GAP activity is limiting in some types of cells, including HEK293. In fact, it is reported that the level of GTP bound to Rheb increases as more Rheb DNA is transfected (Im *et al.*, 2002). Therefore, the overexpressed wildtype Rheb contains a high GTP level to begin with, and the differences between the wildtype and the mutant Rheb are not observed in this setting. However, if Tsc1/2 is overexpressed, clear differences are observed (Yan *et al.*, 2006). In this case, mTOR activation is observed only with the mutant Rheb.

The situation is different with mTOR. Activation of mTOR signaling in the absence of nutrients can only be observed when constitutive active mTOR mutants are overexpressed (Fig. 21.3). In contrast, little activation is observed with the wildtype mTOR. This result is consistent with the fact that mTOR has strict requirement for amino acids to be active.

Protocols for the experiments are similar to those described above except that the Rheb or mTOR construct is cotransfected with S6K1 or 4E-BP1 construct.

2.2.1. Rheb mutants—A variety of novel mutations in Rheb that confer constitutive activity have been identified (Urano *et al.*, 2005, 2007; Yan *et al.*, 2006). We commonly use Rheb-N153T as an active mutant. This mutant shows a low GTP-binding activity and higher GTP-bound level than those of the wildtype protein in mammalian cells. Rheb-K120R is also reported to show constitutive activity, but it is unstable in mammalian cells and its expression level is low. Lamb and colleagues found the new active mutants, Rheb-S16N and -S16H, that show high GTP-bound levels and mTOR activation even in the cells overexpressing Tsc1/2 (Yan *et al.*, 2006). These mutants are expected to facilitate analysis of Rheb structure and elucidation of the activation mechanism of mTOR by Rheb. While the Rheb-Q64L mutant, analogous to the H-Ras Q61L, is reported to display a high basal GTP level (Inoki *et al.*, 2003; Li *et al.*, 2004), it is unclear whether Rheb-Q64L is an active mutant, as Rheb-Q64L is sensitive to Tsc2GAP, and its GTP-bound level is decreased by the overexpression of Tsc1/2 (Li *et al.*, 2004).

2.2.2. mTOR mutants—We recently found a number of active mutants of yeast Tor2p (Urano *et al.*, 2007). Introducing these mutations to mTOR enabled identification of two mammalian TOR mutants, E2419K and L1460P. They exhibit mTORC1 activity in HEK293 cells starved for serum and amino acids. On the other hand, these mutants do not confer constitutive activity of mTORC2 toward Akt in HEK293 cells, since comparable amounts of phospho-Akt at Ser 473 are observed with the wildtype and mutant proteins. Interestingly, most of the active mutations identified in yeast Tor2p occur at residues conserved between yeast and human proteins. Further analysis may provide insight into the activation mechanism of mTOR. In addition, the finding that mTOR-activating mutants can be identified gives rise to the possibility that mutations in mTOR are involved in the uncontrolled growth of cancer cells.

2.3. Analysis of mTOR complexes and their *in vitro* kinase assay

The components of mTOR complexes can be examined by immunoprecipitating mTOR and performing Western blot analysis. Figure 21.4A shows detection of mTOR binding proteins; Raptor, a mTORC1 component, and Rictor, a mTORC2 component. Since mTOR complexes are unstable and disrupted in the presence of some detergents such as 1% NP-40 or Triton X-100, mTOR binding proteins cannot be observed in the mTOR immunoprecipitates containing these detergents (see Fig. 21.4A). Therefore, we commonly use lysis buffer containing CHAPS detergent to lyse cells and detect mTOR complexes. Endogenous mTOR can also be immunoprecipitated with anti-mTOR antibody.

mTOR complexes exist as a dimer or multimer in mammalian cells, and dimeric mTOR is reported to be the major form that responds to insulin (Takahara *et al.*, 2006; Wang *et al.*, 2006). The mTOR dimer is observed by the expression of two distinct epitope-tagged mTOR proteins. One epitope is used to immunoprecipitate mTOR, and then the other epitope is detected by the Western blot analysis of immunoprecipitates.

To directly assess the activity of mTOR complexes, *in vitro* kinase assay is performed. The *in vitro* kinase activity of wildtype mTOR, E2419K, and L1460P mutants are shown in Fig. 21.4B. This *in vitro* kinase assay is examined using mTOR immunoprecipitates and recombinant proteins, 4E-BP1, and Akt, which are used as substrates for mTORC1 and mTORC2, respectively. Active mutants of mTOR show higher activity with 4E-BP1 as the substrate compared with the wildtype mTOR in HEK293 cells under nutrient-starved condition. On the other hand, mTORC2 activities of mTOR mutant proteins are similar to that of wildtype mTOR protein. Alternatively, we can measure *in vitro* kinase activity after immunoprecipitation of mTORC1 or mTORC2, separately. Immunoprecipitation with anti-Raptor and anti-Rictor antibodies can isolate mTORC1 and mTORC2, respectively.

2.3.1. Immunoprecipitation of mTOR complex

1. Lyse 1×10^7 HEK293 cells with lysis buffer for immunoprecipitation (0.4% CHAPS, 50 m*M* Tris-HCl [pH 7.4], 150 m*M* NaCl, 10 m*M* $MgCl_2$, 50 m*M* β-glycerophosphate, 1× protease inhibitor). These lysates are incubated for 30 min at 4° and then centrifuged at 15,000 rpm for 15 min at 4°.
2. Put the supernatants into new tubes and add 5 μg of anti-mTOR antibody (Santa Cruz, N-19), or other antibodies that recognize epitope-tagged mTOR as well as 30 μl of protein-G sepharose 4 fast-flow beads (GE Healthcare).
3. Rotate the samples at 4° for 2 h.
4. Centrifuge at 3000 rpm for 2 min at 4°, and then wash the beads three times with 1 ml of washing buffer (0.4% CHAPS, 50 m*M* Tris-HCl [pH 7.4], 150 m*M* NaCl, 10 m*M* $MgCl_2$, 50 m*M* β-glycerophosphate). After the wash, add 30 μl of 2 × SDS sample

buffer (6% SDS, 10% glycerol, 124.7 m*M* Tris-HCl (pH 6.7), 2% 2-mercaptoethanol), and incubate them at 95° for 5 min. These samples are resolved by 8% polyacrylamide gel to detect mTOR, Raptor, and Rictor, and 13% gel to detect GβL and PRAS40. The antibodies for Western blot analysis can be obtained from the following vendors: anti-mTOR and anti-Raptor antibody, Cell Signaling; anti-GβL antibody, BETHYL Lab; and anti-PRAS40 antibody, Biosource.

2.3.2. Dimer formation—To examine the formation of dimeric mTOR, two distinct epitope-tagged mTORs are coexpressed in HEK 293 cells. After one epitope-tagged mTOR is immunoprecipitated using antibody against its epitope, the presence of the other epitope-tagged mTOR is analyzed by Western blot. Coimmunoprecipitation of the two epitope-tagged mTORs indicate the dimer formation. The protocols for transfection and immunoprecipitation are same as above.

2.3.3. *In vitro* mTORC1 and mTORC2 kinase assay

1. Immunoprecipitate mTOR complex using anti-Raptor antibody (BETHYL Lab) for mTORC1 or anti-Rictor antibody (BETHYL Lab) for mTORC2.
2. After the wash, immunoprecipitates are washed one time with 1 × kinase buffer (20 m*M* Tris-HCl [pH 7.4], 10 m*M* $MgCl_2$, 0.2 m*M* ATP)
3. Add 8 μl of 5× kinase buffer and 1 μg of recombinant 4E-BP1 for mTORC1 kinase assay or 1 μg of recombinant unactive Akt (Millipore) for mTORC2 kinase assay, and bring the total volume to 40 μl with sterile water.
4. Incubate immunoprecipitates at 37° for 30 min. To stop the reaction, immediately add 40 μl of 2 × SDS sample buffer and incubate at 95° for 5 min.
5. These samples are resolved by 10% polyacrylamide gel for Akt detection or 14% polyacrylamide gel for 4E-BP1 detection, and analyzed by Western blot. The anti-phospho-Akt at Ser 473, anti-Akt, anti-phospho-4E-BP1 at Thr 37/46, and anti-4E-BP1 antibodies are available from several sources. Phosphorylation of 4E-BP1 at Thr 70 is not detected in this assay.

2.4. Rapamycin treatment

Rapamycin suppresses mTOR kinase activity by the formation of a ternary complex with FKBP12 and mTOR (Chen *et al.*, 1995; Choi *et al.*, 1996). It has been suggested that rapamycin inhibits mTORC1 activity, but does not affect mTORC2 activity. However, recent studies show that a prolonged treatment of rapamycin can suppress mTORC2 as well as mTORC1 in some cells such as PC3 and Jurkat cells (Sarbassov *et al.*, 2006). The mechanism by which rapamycin inhibits mTOR activity is still unknown, but the rapamycin binding to mTOR may perturb the mTOR complexes and lead to the disruption of mTOR interaction with its substrates.

Before treating with rapamycin, cells are cultured in DMEM containing 0.1% bovine serum albumin at 37° in a 5% CO_2 incubator for 24 h. The cells are then treated with 20 n*M* rapamicin in DMEM containing 0.1% bovine serum albumin. Although mTORC1 kinase activity is suppressed by 1 h of treatment, 24 h of treatment are required for the inhibition of mTORC2 kinase activity. Decreased phosphorylation levels of S6K1 and 4E-BP1 are observed in these cells.

3. Conclusion

The Rheb-mTOR signaling pathway has been extensively studied from yeast to human. This pathway plays a pivotal role in the regulation of cell growth, proliferation, cell size, etc. We

presented here the protocols commonly used to examine the Rheb-mTOR signaling pathway. In addition, we showed protocols to evaluate Rheb and mTOR mutants that we recently identified as constitutively active mutants (Urano *et al.*, 2005, 2007). The findings of these activating mutants are significant as it raises the possibility that one mutation in these genes may disrupt homeostasis such as contact inhibition in mammalian cells and cause tumor progression. In fact, activation of the mTOR pathway is implicated in a number of human diseases associated with benign tumors, including tuberous sclerosis. Inhibitors of mTOR, including rapamycin and its derivatives, are under clinical evaluation as anticancer drugs. Further studies on the Rheb-mTOR signaling pathway may provide important insights into the activation mechanism of the Rheb-mTOR signaling pathway.

REFERENCES

Abraham RT. PI 3-kinase related kinases: “Big” players in stress-induced signaling pathways. DNA Repair (Amst.) 2004;3:883–887. [PubMed: 15279773]

Aspuria PJ, Tamanoi F. The Rheb family of GTP-binding proteins. Cell Signal 2004;16:1105–1112. [PubMed: 15240005]

Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: A conserved switch for diverse cell functions. Nature 1990;348:125–132. [PubMed: 2122258]

Byfield MP, Murray JT, Backer JM. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. J. Biol. Chem 2005;280:33076–33082. [PubMed: 16049009]

Cai SL, Tee AR, Short JD, Bergeron JM, Kim J, Shen J, Guo R, Johnson CL, Kiguchi K, Walker CL. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J. Cell Biol 2006;173:279–289. [PubMed: 16636147]

Castro AF, Rebhun JF, Clark GJ, Quilliam LA. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. J. Biol. Chem 2003;278:32493–32496. [PubMed: 12842888]

Chen J, Zheng XF, Brown EJ, Schreiber SL. Identification of an 11-kDa FKBP12-rapamycin–binding domain within the 289-kDa FKBP12-rapamycin–associated protein and characterization of a critical serine residue. Proc. Natl. Acad. Sci. USA 1995;92:4947–4951. [PubMed: 7539137]

Choi J, Chen J, Schreiber SL, Clardy J. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science 1996;273:239–242. [PubMed: 8662507]

Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA, Sabatini DM. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr. Biol 2006;16:1865–1870. [PubMed: 16919458]

Gao X, Pan D. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev 2001;15:1383–1392. [PubMed: 11390358]

Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol. Cell 2003;11:1457–1466. [PubMed: 12820960]

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mlST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev. Cell 2006;11:859–871. [PubMed: 17141160]

Haar EV, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol 2007;9:316–323. [PubMed: 17277771]

Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 2002;110:177–189. [PubMed: 12150926]

Hresko RC, Mueckler M. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem 2005;280:40406–40416. [PubMed: 16221682]

Im E, von Lintig FC, Chen J, Zhuang S, Qui W, Chowdhury S, Worley PF, Boss GR, Pilz RB. Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. Oncogene 2002;21:6356–6365. [PubMed: 12214276]

Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol 2002;4:648–657. [PubMed: 12172553]

Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 2003;17:1829–1834. [PubMed: 12869586]

Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol 2004;6:1122–1128. [PubMed: 15467718]

Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 2006;127:125–137. [PubMed: 16962653]

Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 2002;110:163–175. [PubMed: 12150925]

Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol. Cell 2003;11:895–904. [PubMed: 12718876]

Li Y, Inoki K, Guan KL. Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity. Mol. Cell. Biol 2004;24:7965–7975. [PubMed: 15340059]

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc. Natl. Acad. Sci. USA 2005;102:14238–14243. [PubMed: 16176982]

Patel PH, Thapar N, Guo L, Martinez M, Maris J, Gau CL, Lengyel JA, Tamanoi F. Drosophila Rheb GTPase is required for cell cycle progression and cell growth. J. Cell Sci 2003;116:3601–3610. [PubMed: 12893813]

Sabatini DM. mTOR and cancer: Insights into a complex relationship. Nat. Rev. Cancer 2006;6:729–734. [PubMed: 16915295]

Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol. Cell 2007;25:903–915. [PubMed: 17386266]

Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol 2004;14:1296–1302. [PubMed: 15268862]

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–1101. [PubMed: 15718470]

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell 2006;22:159–168. [PubMed: 16603397]

Takahara T, Hara K, Yonezawa K, Sorimachi H, Maeda T. Nutrient-dependent multimerization of the mammalian target of rapamycin through the N-terminal HEAT repeat region. J. Biol. Chem 2006;281:28605–28614. [PubMed: 16870609]

Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr. Biol 2003;13:1259–1268. [PubMed: 12906785]

Urano J, Comiso MJ, Guo L, Aspuria PJ, Deniskin R, Tabancay AP Jr, Kato-Stankiewicz J, Tamanoi F. Identification of novel single amino acid changes that result in hyperactivation of the unique GTPase, Rheb, in fission yeast. Mol. Microbiol 2005;58:1074–1086. [PubMed: 16262791]

Urano J, Sato T, Matsuo T, Otsubo Y, Yamamoto M, Tamanoi F. Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. Proc. Natl. Acad. Sci. USA 2007;104:3514–3519. [PubMed: 17360675]

Wang L, Rhodes CJ, Lawrence JC Jr. Activation of mammalian target of rapamycin (mTOR) by insulin is associated with stimulation of 4EBP1 binding to dimeric mTOR complex 1. J. Biol. Chem 2006;281:24293–24303. [PubMed: 16798736]

Yamagata K, Sanders LK, Kaufmann WE, Yee W, Barnes CA, Nathans D, Worley PF. rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J. Biol. Chem 1994;269:16333–16339. [PubMed: 8206940]

Yan L, Findlay GM, Jones R, Procter J, Cao Y, Lamb RF. Hyper-activation of mammalian target of rapamycin (mTOR) signaling by a gain-of-function mutant of the Rheb GTPase. J. Biol. Chem 2006;281:19793–19797. [PubMed: 16728407]

Yang Q, Inoki K, Ikenoue T, Guan KL. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev 2006;20:2820–2832. [PubMed: 17043309]

Yu Y, Li S, Xu X, Li Y, Guan K, Arnold E, Ding J. Structural basis for the unique biological function of small GTPase RHEB. J. Biol. Chem 2005;280:17093–17100. [PubMed: 15728574]

Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat. Cell Biol 2003;5:578–581. [PubMed: 12771962]

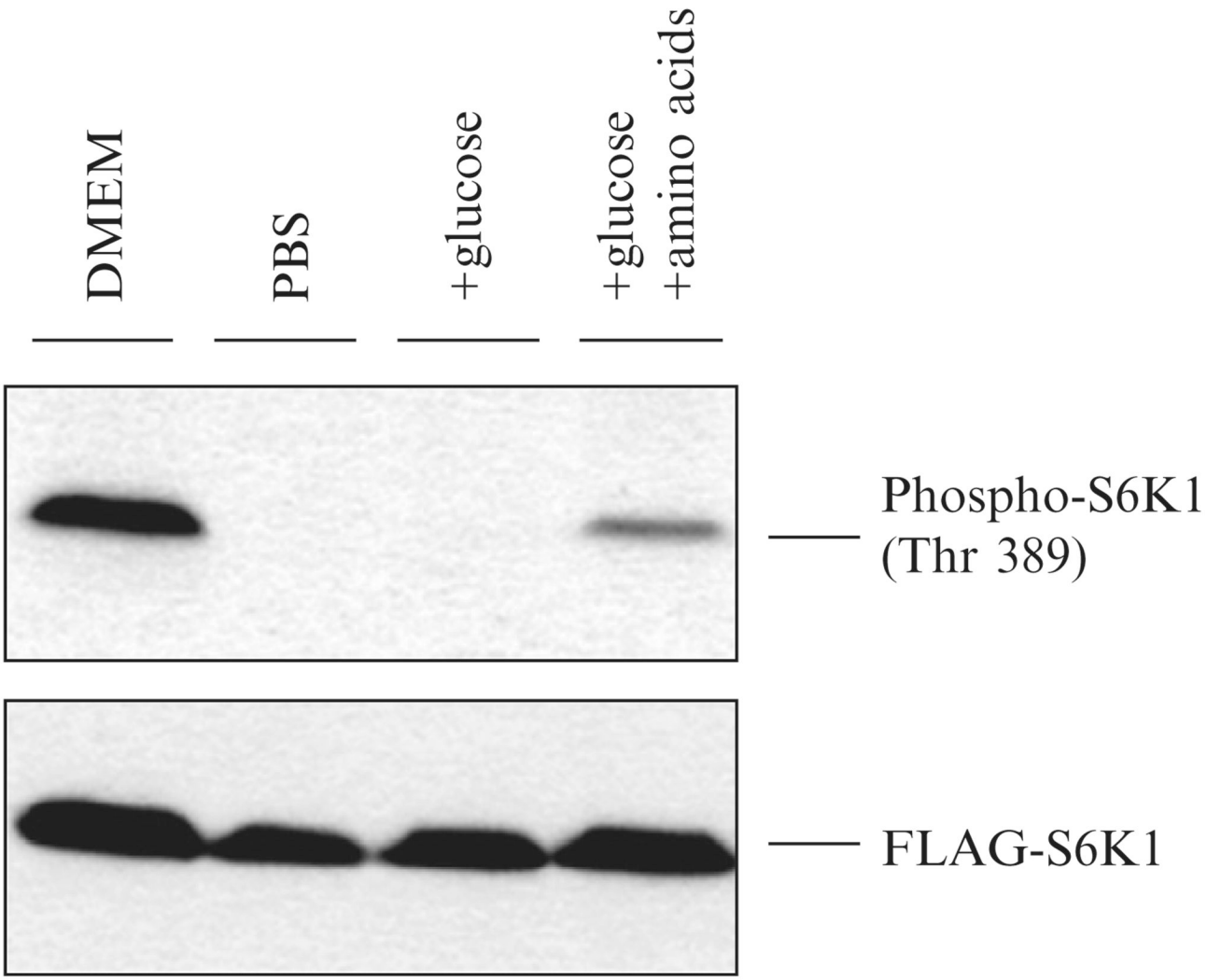

Figure 21.1.
Activation of mTOR by the addition of amino acids. HEK293 cells were transfected with FLAG-S6K1 and starved for serum for 24 h (DMEM). These cells were then cultured in D-PBS for 1 h (PBS) and further incubated in D-PBS containing 4.5 g/l of glucose (+glucose) or 4.5 g/l of glucose and 1× amino acid mixture (+glucose, +amino acids) for 30 min. The amount of total or phosphorylated S6K1 was analyzed by Western blot.

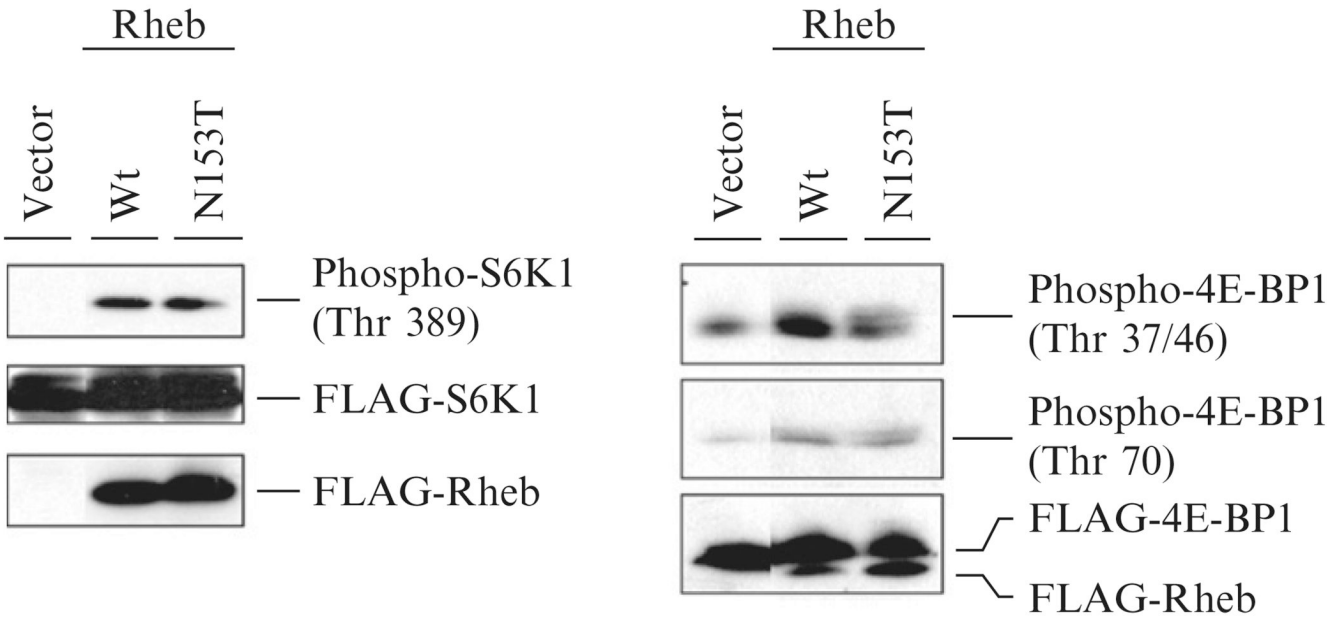

Figure 21.2.

Rheb overexpression confers phosphorylation of mTOR substrates in the absence of nutrients. HEK293 cells were transfected with pcDNA3 as a control (vector) , Rheb wildtype (wt), or N153Tactive mutant. To detect the phosphorylation of S6K1or 4E-BP1, FLAG-S6K1 or FLAG-4E-BP1was cotransfected. After serum and amino acid starvation, the cell lysates were analyzed by Western blot. (Adapted from Urano, J., Sato, T., Matsuo, T., Otsubo, Y., Yamamoto, M., and Tamanoi, F. (2007). Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling inmammalian cells. *Proc. Natl. Acad. Sci. USA* **104**, 3514–3519.)

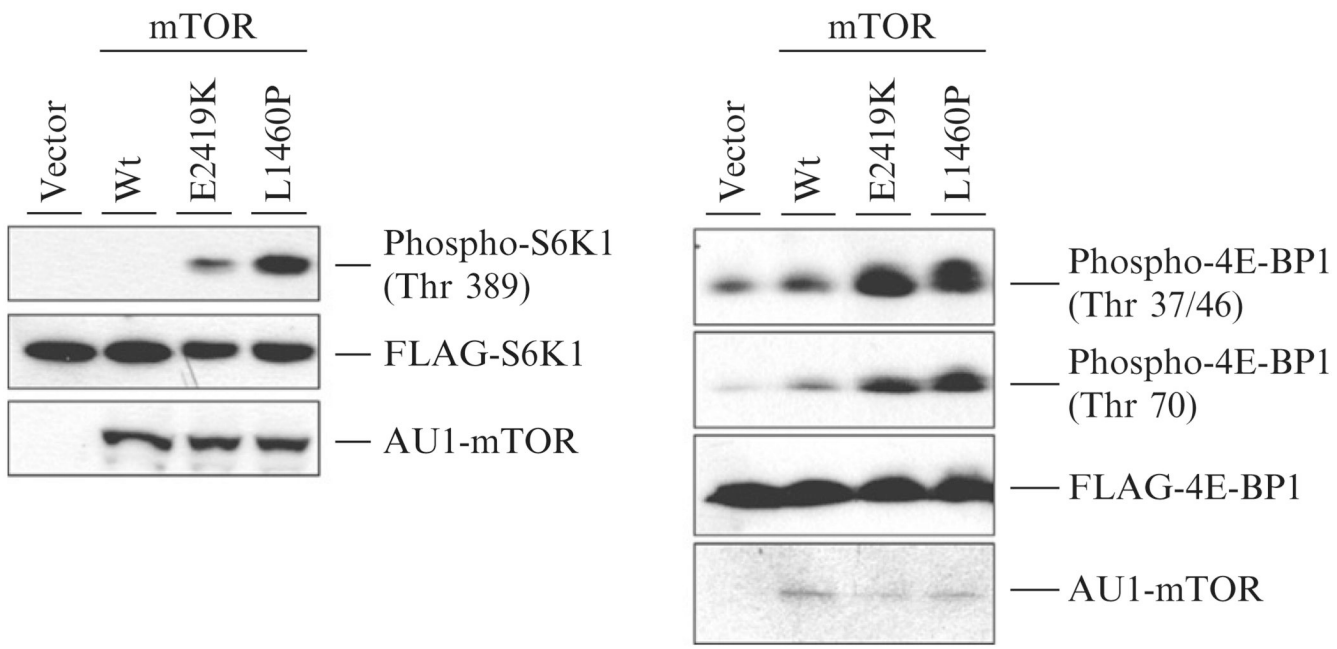

Figure 21.3.

Overexpression of mTOR mutants but not mTOR wildtype confers phosphorylation of mTOR substrates in the absence of nutrients. HEK293 cells were transfected with pcDNA3 as a control (vector), mTOR wildtype, E2419K active mutant, or L1460P active mutant. To detect the phosphorylation of S6K1 or 4E-BP1, FLAG-S6K1 or FLAG-4E-BP1was cotransfected. After serum and amino acid starvation, the cell lysates were analyzed by Western blot. (Adapted from Urano, J., Sato, T., Matsuo, T., Otsubo, Y., Yamamoto, M., and Tamanoi, F. (2007). Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* **104**, 3 51 4–3 519.)

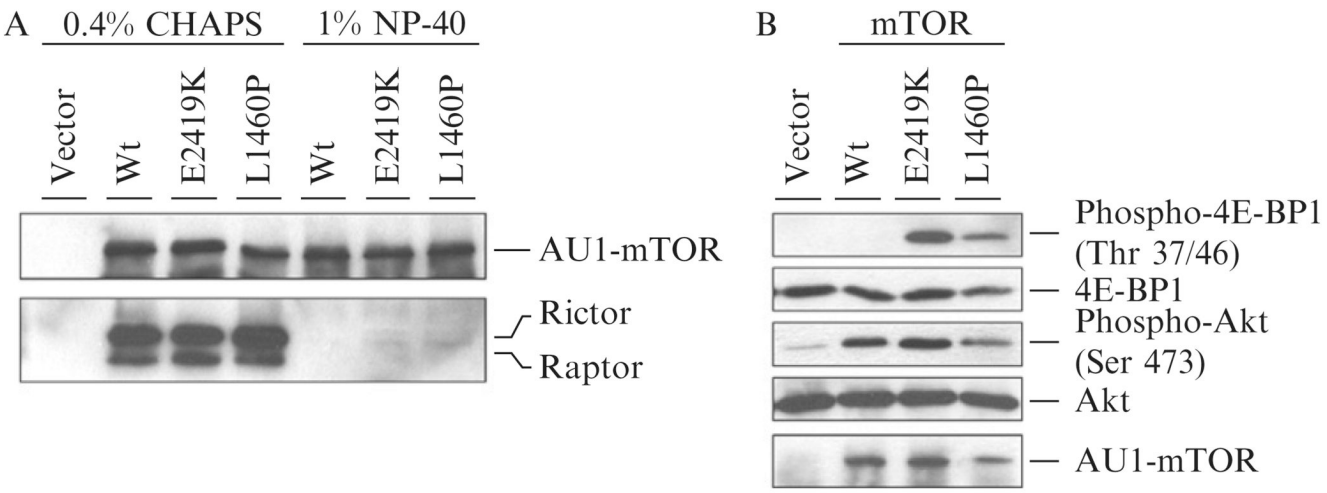

Figure 21.4.

Immunoprecipitation and *in vitro* kinase activity of mTOR complexes. (A) HEK293 cells were transfected with pcDNA3 as a control (vector), mTOR wildtype (wt), E2419K active mutant, or L1460P active mutant. After serum and amino acid starvation, mTOR complexes were immunoprecipitated using anti-AU1 antibody from each cell lysates containing 0.4% CHAPSor1%NP-40 as indicated and the immuno-precipitates were analyzed by Western blot. (B) mTOR immunoprecipitates were divided in two, and used for *in vitro* kinase assay with 4E-BP1as amTORC1substrate, and Akt as a mTORC2 substrate. Phosphorylation of substrates was analyzed by Western blot. (Adapted from Urano, J., Sato, T., Matsuo, T., Otsubo, Y., Yamamoto, M., and Tamanoi, F. (2007). Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* **104**, 3514–3519.)