Amino Acids (2010) 38:223–228
DOI 10.1007/s00726-008-0230-7

ORIGINAL ARTICLE

mTOR phosphorylated at S2448 binds to raptor and rictor

M. Rosner · N. Siegel · A. Valli · C. Fuchs · M. Hengstschläger

Received: 9 December 2008 / Accepted: 22 December 2008 / Published online: 15 January 2009

Abstract In mammalian cells, the mammalian target of rapamycin (mTOR) forms an enzyme complex with raptor (together with other proteins) named mTOR complex 1 (mTORC1), of which a major target is the p70 ribosomal protein S6 kinase (p70S6K). A second enzyme complex, mTOR complex 2 (mTORC2), contains mTOR and rictor and regulates the Akt kinase. Both mTORC1 and mTORC2 are regulated by phosphorylation, complex formation and localization. So far, the role of p70S6K-mediated mTOR S2448 phosphorylation has not been investigated in detail. Here, we report that endogenous mTOR phosphorylated at S2448 binds to both, raptor and rictor. Experiments with chemical inhibitors of the mTOR kinase and of the phosphatidylinositol-3-kinase revealed that downregulation of mTOR S2448 phosphorylation correlates with decreased mTORC1 activity but can occur decoupled of effects on mTORC2 activity. In addition, we found that the correlation of the mTOR S2448 phosphorylation status with mTORC1 activity is not a consequence of effects on the assembly of mTOR protein and raptor. Our data allow new insights into the role of mTOR phosphorylation for the regulation of its kinase activity.

Keywords mTOR · Phosphorylation · Raptor · Rictor

M. Rosner · N. Siegel · A. Valli · C. Fuchs · M. Hengstschläger (✉)
Medical Genetics, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria
e-mail: markus.hengstschlaeger@meduniwien.ac.at

Introduction

The so called insulin signalling pathway is centrally involved in growth regulation, proliferation control and cancer cell metabolism. Activated receptor tyrosine kinases activate the phosphatidylinositol-3-kinase (PI3K) through phosphorylation of adaptors, such as the insulin receptor substrate 1 (IRS1). Phosphorylation of the membrane lipid phosphatidylinositol-4,5-biphosphate (PIP_2) by PI3K produces the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3) in a reaction that can be reversed by the phosphatase phosphatase and tensin homolog (PTEN). Phosphoinositide-dependent kinase-1 (PDK1) and the oncogenic kinase Akt (also known as protein kinase B) bind to PIP_3 at the plasma membrane, and PDK1 phosphorylates Akt at T308. Akt-mediated phosphorylation at S939 and T1462 downregulates the GTPase activating (GAP) potential of tuberin, the gene product of the tuberous sclerosis gene 2, toward Ras homolog enriched in brain (Rheb), which is a potent regulator of the mammalian target of rapamycin (mTOR) (Guertin and Sabatini 2007; Yang and Guan 2007; Dann et al. 2007; Rosner et al. 2008).

In mammalian cells two structurally and functionally distinct mTOR-containing complexes have been identified. mTORC1 contains regulatory associated protein of mTOR (raptor), mLST8 (also known as GβL) and proline-rich Akt substrate 40 kDa (PRAS40). Whereas the function of mLST8 is not really clarified, raptor regulates mTORC1 functioning as a scaffold for recruiting TORC1 substrates. PRAS40 is phosphorylated by Akt at T246 releasing its inhibitory effects on mTORC1. One of the major substrates of mTORC1 known so far is the p70 ribosomal protein S6 kinase (p70S6K), a regulator of mRNA translation. mTORC1 phosphorylates and activates p70S6K at T389 to

activate the ribosomal protein S6 via phosphorylation at S235/236 and S240/244. In addition, p70S6K also phosphorylates mTOR protein at S2448, which is assumed to be an additional level of mTOR regulation although its role has not been fully clarified so far (Rosner et al. 2004; Guertin and Sabatini 2007; Yang and Guan 2007; Chiang and Abraham 2007; Dann et al. 2007; Bhaskar and Hay 2007).

mTORC2 consists of the mLST8 protein, rapamycin-insensitive companion of mTOR (rictor) and stress-activated protein kinase-interacting protein 1 (sin1). The latter appear to stabilize each other through binding, building the structural foundation for mTORC2. mTORC2 additionally contains protein observed with rictor (protor), but its role for mTORC2 activity still remains elusive. mTORC2 phosphorylates Akt at S473, what in conjunction with the PDK1-mediated phosphorylation of Akt at T308 (described above) drives full activation of Akt. Akt is known to regulate a wide variety of different targets (Guertin and Sabatini 2007; Yang and Guan 2007; Chiang and Abraham 2007; Bhaskar and Hay 2007).

In this study we further investigated the role of mTOR S2448 phosphorylation. (1) Immunoprecipitations of endogenous proteins revealed that S2448 phosphorylated mTOR binds to both, the mTORC1 component raptor and the mTORC2 component rictor. (2) Using chemical inhibitors of the mTOR kinase and of PI3K we found that downregulation of mTOR S2448 phosphorylation correlates with decreased mTORC1 activity (represented by the S6 S235/236 phosphorylation status) but can occur decoupled of effects on mTORC2 activity (represented by the phosphorylation of Akt S473). (3) Downregulation of mTOR S2448 phosphorylation correlates with decreased mTORC1 activity, but this correlation is not a consequence of effects on the assembly of mTOR protein and raptor. In this report, the relevance of these new findings is discussed in the context of the existing literature.

Materials and methods

Cell culture

HEK293 (adenovirus transformed human embryonic kidney) cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM) at 4.5 g/l glucose, supplemented with 10% calf serum and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate) at 37°C and 5% CO_2 and were routinely screened for mycoplasma.

For short-term treatments involving the pharmacological inhibitors of PI3K (wortmannin, purchased from Upstate) and mTOR (rapamycin, purchased from Calbiochem) cells were grown in medium containing 0.1% serum for 16–18 h and subsequently stimulated with 10% serum or insulin at 2 μg/ml for 20 min. Thirty minutes prior to restimulation, wortmannin or rapamycin was added at a final concentration of 100 nM.

Protein extraction

Extracts of cellular total protein were prepared by physical disruption of cell membranes by repeated freeze and thaw cycles. Briefly, cells were washed with PBS and harvested by scraping. Pellets were lysed in buffer A containing 20-mM Hepes, pH 7.9, 0.4-M NaCl, 2.5% glycerol, 1-mM EDTA, 0.5-mM DTT, 1-mM PMSF, 0.5-mM NaF, 0.5-mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0,3 μg/ml benzamidinchloride, 10 μg/ml trypsininhibitor by freezing and thawing. Supernatants were collected by centrifugation at 15,000 rpm for 20 min at 4°C and stored at −80°C (Rosner et al. 2007).

For immunoprecipitation of mTOR complexes total cellular protein was extracted as described in (Sarbassov et al. 2005) to achieve maximum mTOR complex recovery. Therefore, cells were harvested by trypsin-EDTA, rinsed twice with cold PBS and lysed on ice for 20 min in buffer containing 40-mM Hepes pH 7.5, 120-mM NaCl, 1-mM EDTA, 10-mM 2-glycerophosphate, 50-mM NaF, 0.5-mM Na_3VO_4 and 0.3% CHAPS supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchloride, 10 μg/ml trypsininhibitor. Soluble fractions of lysates were isolated by centrifugation at 15,000 rpm for 20 min at 4°C.

Immunoprecipitation

For immunoprecipitation of endogenous mTORC1 and mTORC2 complexes total lysates were prepared in CHAPS-containing lysis buffer as outlined above. Immunoprecipitations were carried out as described in (Sarbassov et al. 2005).

Briefly, crude cell extracts (200–500 μg) were diluted in CHAPS-containing total cell lysis buffer and precleared with 30-μl protein A/G-Sepharose beads (Pierce) for 30–60 min at 4°C. Indicated primary antibodies were added to the cleared lysates and incubated with constant rotation for 12–16 h (over night) at 4°C. Thirty microlitres of a 50% slurry of protein A/G-sepharose were then added and the incubation continued for another 90 min at 4°C. So captured immunoprecipitations were washed four times with lysis buffer containing 40-mM Hepes pH 7.5, 120-mM NaCl, 1-mM EDTA, 10-mM 2-glycerophosphate, 50-mM NaF, 0.5-mM Na_3VO_4 and 0.3% CHAPS supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0,3 μg/ml benzamidinchloride, 10 μg/ml trypsininhibitor. The final

washing step was carried out in wash buffer containing 50-mM Hepes pH 7.5, 40-mM NaCl and 2-mM EDTA. Immunoprecipitated proteins were then denatured and separated from the sepharose beads by adding SDS-sample buffer and boiling for 5 min. For immunoprecipitation antibodies specific for the following proteins were used: raptor (Bethyl Laboratories, #A300-553A) and rictor (Bethyl Laboratories, #A300-458A).

To avoid interference in the detection of mTOR and mTOR phosphorylated at S2448 (both detected by rabbit polyclonal antibodies) on raptor- or rictor-containing immunoprecipitates, denatured precipitates were divided into equal amounts (50:50 ratio), loaded and detected separately with antibodies against mTOR and phosphorylated mTOR.

Immunoblotting

Samples prepared from total lysates and immunoprecipitated proteins were resolved by 7–10% SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein.

For immunodetection, antibodies specific for the following proteins were used: mTOR (Cell Signaling, #2972), phospho-mTOR S2448 (Cell Signaling, #2971), raptor (Bethyl Laboratories, #A300-506A), rictor (Bethyl Laboratories, #A300-459A), phospho-Akt S473 (Cell Signaling, #4058), phospho-S6 S235/236 (Cell Signaling, #4856) and α-tubulin (DM1A, Calbiochem, #CP06). Rabbit polyclonal and monoclonal antibodies were detected using anti-rabbit IgG, a HRP-linked heavy and light chain antibody from goat (A120-101P, Bethyl Laboratories); mouse monoclonal antibodies were detected using anti-mouse IgG, a HRP-linked heavy and light chain antibody from goat (A90-116P, Bethyl Laboratories). Signals were detected using the enhanced chemiluminescence method (Pierce).

Results and discussion

As already described above, the p70S6K is a downstream target of mTORC1. mTORC1 phosphorylates and activates p70S6K at T389 to activate the ribosomal protein S6 via phosphorylation at S235/236 and S240/244 (Chiang and Abraham 2007; Dann et al. 2007). Accordingly, analysing endogenous levels of phosphorylated S6 as a functional readout of p70S6K activity, as performed in the here presented study, is a widely used approach allowing conclusions on endogenous mTORC1 activity. On the other hand, since the major target of mTORC2 is Akt, analysing the phosphorylation status of Akt S473 is widely used as an endogenous functional readout of mTORC2 kinase activity. Both, mTORC1 and mTORC2 are regulated on several levels, including complex formation, phosphorylation or intracellular localization (Rosner et al. 2004; Guertin and Sabatini 2007; Yang and Guan 2007; Rosner and Hengstschläger 2007; Rosner and Hengstschläger 2008). Recently, it was shown that the phosphorylation of mTOR protein at S2448 is mediated by p70S6K. These reports come to the conclusion that mTOR S2448 phosphorylation is not controlled directly by Akt but rather occurs in a feedback fashion catalyzed by the downstream target of mTOR, p70S6K (Chiang and Abraham 2005; Holz and Blenis 2005). The fact that the p70S6K-mediated phosphorylation affects sites in the so called "mTOR repressor domain" suggests a functional importance of this phosphorylation, although its relevance for mTOR regulation, especially with respect to the two different mTOR complexes, mTORC1 and mTORC2, remains elusive.

First, we investigated whether mTOR phosphorylated at S2448 can be found in mTORC1, mTORC2 or both. We chose the approach of immunoprecipitating endogenous raptor (specific for mTORC1) and endogenous rictor (specific for mTORC2) using the same cells under the same experimental conditions. As proven by cell doubling studies and flowcytometric DNA analyses (data not shown) the cells were growing logarithmically at the time point of protein extraction. Western blot analyses revealed that endogenous total mTOR protein and endogenous phospho-mTOR S2448 co-immunoprecipitated with both proteins, raptor and rictor (Fig. 1). These data provide evidence that in mammalian cells mTOR phosphorylated at S2448 via p70S6K is a component of both mTOR complexes, mTORC1 and mTORC2.

The finding that mTOR protein phosphorylated at S2448 is part of both mTOR complexes prompted us to investigate whether the mTOR S2448 phosphorylation status correlates with the activities of both, mTORC1 and mTORC2. For such an investigation we chose the approach to stimulate endogenous mTORC1 activity (here represented by the intracellular S6 phosphorylation status) and endogenous mTORC2 activity (represented by the Akt phosphorylation status) by serum restimulation or by insulin. Downregulation of mTOR activity was triggered via treatment with the mTOR inhibitor rapamycin, known to specifically downregulate mTORC1 activity in short-term experiments, and via treatment with the PI3K inhibitor wortmannin, known to negatively affect both, mTORC1 and mTORC2 activity. After determination of the protein concentration using the Bio-Rad protein assay with bovine serum albumin as the standard, for each point exactly the same amount of total protein was run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. Ponceau-S staining proved that the amount of protein loaded was equal in the Western blot presented in Fig. 2. Besides, we

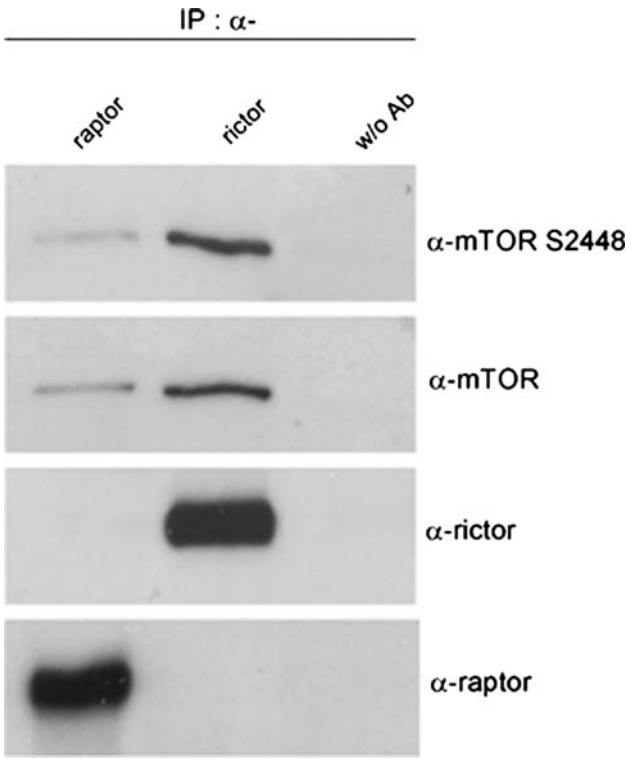

Fig. 1 mTOR phosphorylated at S2448 can be found in a complex with both, raptor and rictor. Total lysates of logarithmically growing HEK293 cells were prepared and used for immunoprecipitation of endogenous raptor or rictor. Precipitated proteins were analysed for (co-) immunoprecipitated mTOR phosphorylated at S2448, mTOR total protein, raptor and rictor. As a negative control, reactions were included, in which the immunoprecipitating antibody was omitted from the procedure and which were analysed in parallel

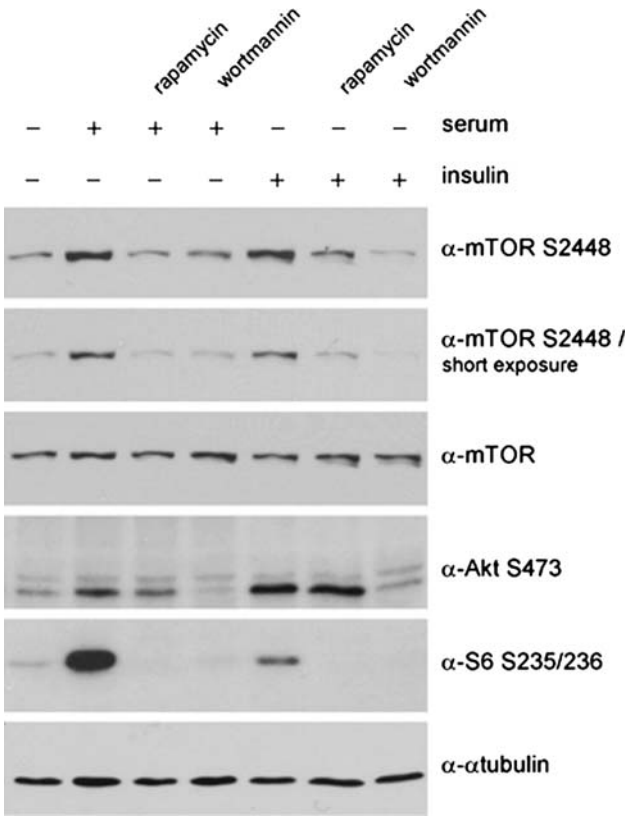

Fig. 2 The regulation of mTOR S2448 phosphorylation correlates with mTORC1 but not mTORC2 activity. Logarithmically growing HEK293 cells were deprived of serum for 16–18 h in medium containing 0.1% serum and treated with either 10% serum or 2 μg/ml insulin for 20 min. Where indicated, serum starved cells were pretreated with rapamycin (100 nM) or wortmannin (100 nM) for 30 min. Total lysates of so treated cells were analysed for levels of mTOR phosphorylated at S2448, mTOR total protein, Akt phosphorylated at S473 and S6 phosphorylated at S235/236 via immunoblotting. Detection of α-tubulin was included to prove equal loading of lysates

co-analysed α-tubulin expression as an additional loading control. Whereas mTOR protein itself is not regulated, it is known that serum restimulation and insulin treatment induces both, mTORC1 and mTORC2 activity (reviewed in Wullschleger et al. 2006). By studying mTOR protein levels, the phosphorylation status of S6 (mTORC1 readout) and the phosphorylation status of Akt (mTORC2 readout) these data have been confirmed under the here chosen experimental conditions (Fig. 2). In addition, we also found phospho-mTOR S2448 to be induced upon serum restimulation and insulin (Fig. 2). Treatment with both chemical kinase inhibitors, rapamycin or wortmannin, blocked the serum-induced as well as the insulin-triggered upregulation of mTOR phosphorylation at S2448. Whereas wortmannin inhibited the upregulation mTORC1 and mTORC2 activity via serum and via insulin under the here chosen experimental conditions, rapamycin exclusively blocked mTORC1 activity without effects on mTORC2 activity (Fig. 2). To our best knowledge, the findings presented here show for the first time that although S2448 phosphorylated mTOR is bound to raptor and rictor, the regulation of this mTOR phosphorylation can be decoupled from mTORC2 activity. We found short-term rapamycin incubation to mediate significant negative effects on mTOR S2448 phosphorylation without affecting mTORC2 activity studied by analysing endogenous phospho-Akt S473 (Fig. 2).

Rapamycin bound to the protein FKBP12 generates a drug–receptor complex that binds and inhibits mTORC1. FKBP12-rapamycin suppresses the assembly of mTOR/raptor. Since it does not bind to preformed mTORC2, rapamycin was originally thought to only inhibit mTORC1. However, recently it was shown that whereas short-term incubation with rapamycin (as performed in this study) is without effects on mTORC2, long-term treatment also suppresses the function of mTORC2 (Sarbassov et al. 2006; Rosner and Hengstschläger 2008). Taken together, our data presented so far demonstrate that mTOR protein phosphorylated at S2448 is part of both, mTORC1 and mTORC2, but this phosphorylation status only correlates

with mTORC1 activity. Since the latter findings were observed in rapamycin experiments and since this drug blocks the protein assembly of mTOR and raptor, it was important to answer the question whether the phosphorylation status of mTOR S2448 correlates with mTORC1 assembly. Immunoprecipitating endogenous raptor, we found that both, the mTOR inhibitor rapamycin and the PI3K inhibitor wortmannin, trigger a downregulation of the levels of phospho-mTOR S2448 bound to raptor. But interestingly, whereas rapamycin affects the assembly of mTOR and raptor, wortmannin does not influence this protein assembly (Fig. 3).

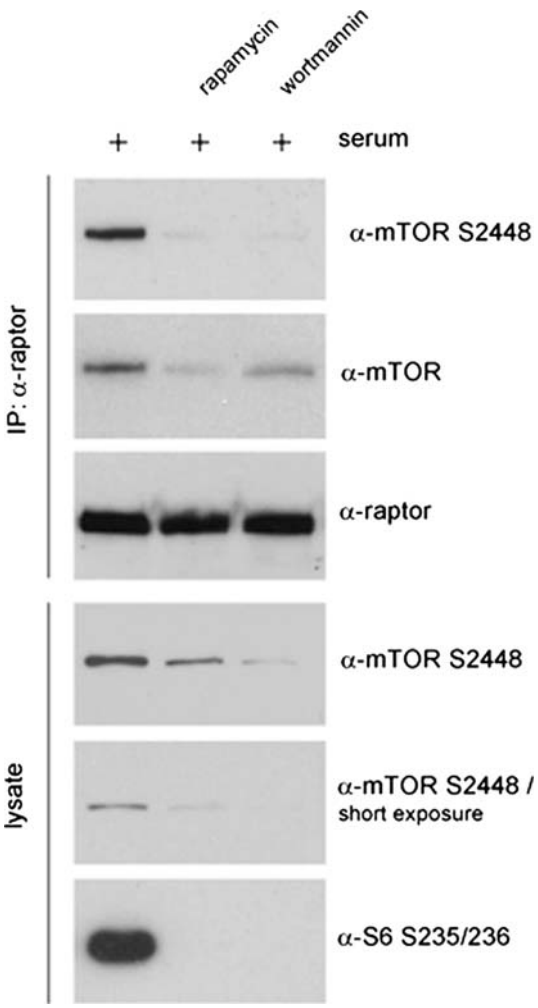

Fig. 3 mTORC1 complex formation is not dependent on mTOR phosphorylation at S2448. Cells were starved and serum restimulated under indicated conditions as described for Fig. 2. Total cell extracts were used for immunoprecipitation of endogenous raptor and precipitates were analysed for protein levels of co-immunoprecipitated mTOR and phospho-mTOR (mTOR S2448) via immunoblotting. Successful immunoprecipitation was proven by co-analysing raptor (*upper panel*). In addition total lysates were examined for phospho-mTOR (mTOR S2448) and phospho-S6 (S6 S235/236) (*lower panel*)

In summary, the findings presented here provide evidence that phospho-mTOR S2448 protein can be found in mTORC1 and in mTORC2, whereas the status of this mTOR phosphorylation only correlates with mTORC1 activity under the here chosen experimental conditions. In addition, we found that downregulation of mTOR phosphorylation at S2448 is not necessarily accompanied by decreased assembly of mTOR and raptor. These data strongly suggest that phosphorylation of mTOR at this site provides an additional level of mTOR regulation besides, e.g. mTOR complex assembly or localization, which should be studied in more detail in future. On the other hand, the study presented here also highlights that analysing endogenous levels of phospho-mTOR S2448 does not represent a complete picture of total active mTOR in the cell, since, e.g. this phosphorylation status does not essentially correlate with mTORC2 activity as proven under the here used experimental conditions. This notion is further supported by our recent finding that in different non-transformed and tumour cell lines the endogenous levels of this mTOR phosphorylation do not necessarily reflect mTOR activity (Burgstaller et al. 2008).

Acknowledgments Research in our laboratory is supported by the FWF Austrian Science Fund (P18894-B12), by the Marie Curie Research Network of the European Community (FP6 036097-2) and by the Herzfelder′sche Familienstiftung.

References

Bhaskar PT, Hay N (2007) The two TORCs and Akt. Dev Cell 12:487–502. doi:10.1016/j.devcel.2007.03.020

Burgstaller S, Rosner M, Lindengrün C, Hanneder M, Siegel N, Valli A, Fuchs C, Hengstschläger M (2008) Tuberin, p27 and mTOR in different cells. Amino Acids. doi:10.10007/100726-008-0066-1

Chiang GG, Abraham RT (2005) Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. J Biol Chem 280:25485–25490. doi:10.1074/jbc.M501707200

Chiang GG, Abraham RT (2007) Targeting the mTOR signaling network in cancer. Trends Mol Med 13:433–442. doi:10.1016/j.molmed.2007.08.001

Dann SG, Selvaraj A, Thomas G (2007) mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med 13:252–259. doi:10.1016/j.molmed.2007.04.002

Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. Cancer Cell 12:9–22. doi:10.1016/j.ccr.2007.05.008

Holz MK, Blenis J (2005) Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. J Biol Chem 280:26089–26093. doi:10.1074/jbc.M504045200

Rosner M, Hengstschläger M (2007) Cytoplasmic/nuclear localization of tuberin in different cell lines. Amino Acids 33:575–579. doi:10.1007/s00726-007-0541-0

Rosner M, Hengstschläger M (2008) Cytoplasmic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components rictor and sin1. Hum Mol Genet 17:2934–2948. doi:10.1093/hmg/ddn192

Rosner M, Freilinger A, Hengstschläger M (2004) Proteins interacting with the tuberous sclerosis gene products. Amino Acids 27:119–128. doi:10.1007/s00726-004-0119-z

Rosner M, Freilinger A, Hanneder M, Fujita N, Lubec G, Tsuruo T, Hengstschläger M (2007) p27Kip1 localization depends on the tumor suppressor protein tuberin. Hum Mol Genet 16:1541–1556. doi:10.1093/hmg/ddm103

Rosner M, Hanneder M, Siegel N, Valli A, Fuchs C, Hengstschläger M (2008) The mTOR pathway and its role in human genetic diseases. Mutat Res 659:284–292. doi:10.1016/j.mrrev.2008.06.001

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101. doi:10.1126/science.1106148

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 22:159–168. doi:10.1016/j.molcel.2006.03.029

Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. Cell 124:471–484. doi:10.1016/j.cell.2006.01.016

Yang Q, Guan K-L (2007) Expanding mTOR signaling. Cell Res 17:666–681. doi:10.1038/cr.2007.64