CHAPTER ELEVEN

Monitoring Mammalian Target of Rapamycin (mTOR) Activity

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Abstract

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase implicated in a wide array of cellular processes such as cell growth, proliferation, and survival. Analogous to the situation in yeast, mTOR forms two distinct functional complexes termed mTOR complex 1 and 2 (mTORC1 and mTORC2). mTORC1 activity is inhibited by rapamycin, a specific inhibitor of mTOR, whereas mTORC2 activity is resistant to short-term treatments with rapamycin. In response to growth factors, mTORC2 phosphorylates Akt, an essential kinase involved in cell survival. On the other hand, mTORC1 can be activated by both growth factors and nutrients such as glucose and amino acids. In turn, mTORC1 regulates the activity of the translational machinery by modulating S6 kinase (S6K) activity and eIF4E binding protein 1 (4E-BP1) through direct phosphorylation. Consequently, protein synthesis and cell growth are stimulated in a variety of different cell types. In addition, mTORC1 inhibits autophagy, an essential protein degradation and recycling system, which cells employ to sustain their viability in times of limited availability of nutrients. Recent studies have highlighted the fact that autophagy plays crucial

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roles in many aspects of human health including cancer development, neurodegenerative disease, diabetes, and aging. It is likely that dysregulation of the mTOR-autophagy pathway may contribute at least in part to these human disorders. Therefore, the assessment of mTOR activity is important to understand the status of autophagy in the cells being analyzed and its role in autophagy-related disorders. In this section, we describe methods to monitor mTOR activity both *in vitro* and *in vivo*.

1. Introduction

Mammalian target of rapamycin (mTOR) is a major constituent of the signaling pathways that regulate cell growth because of the control it exerts on translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization, and autophagy (Wullschleger *et al.,* 2006). As its name indicates, mTOR is a target of the drug called rapamycin (sirolimus), a macrolide antibiotics from *Streptomyces hygroscopicus*, which is also an FDA-approved immunosuppressant. To confer the effect of rapamycin on mTOR in cells, rapamycin forms a drug-receptor complex with the cellular protein FKBP12 (immunophilin FK506-binding protein 12) and then binds to mTOR, which prevents mTOR from phosphorylating currently known targets (Schmelzle and Hall 2000). mTOR is evolutionally conserved and it contains a carboxy-terminal amino acid sequence with significant homology to the catalytic domain of phosphoinositide 3-kinase (PI3K) (Abraham 2004). However, similar to other members of the PI3K family such as ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), mTOR is a genuine serine/threonine protein kinase.

mTOR exists in at least two different complexes, mTOR complex 1 and 2, mTORC1 and mTORC2, respectively (Loewith *et al.,* 2002; Sabatini 2006). These two mTOR complexes have distinct physiological functions and are regulated by different mechanisms. Importantly, mTORC1 activity is highly sensitive to inhibition by rapamycin, whereas mTORC2 activity is resistant at least after a relatively short treatment (Sarbassov *et al.,* 2006). mTORC1 exists as a multiprotein complex containing mTOR, Raptor, PRAS40 and mLST8. The mechanism by which rapamycin dominantly inhibits mTORC1 activity is that the rapamycin-FKBP12 complex preferably disrupts the interaction between mTOR and Raptor, an essential scaffolding protein that recruits mTORC1 substrates into the mTORC1 complex (Kim *et al.,* 2002; Oshiro *et al.,* 2004).

In response to growth stimuli, class I PI3K indirectly activates Akt, also known as PKB (protein kinase B). Stimulation of Akt inhibits the GAP (GTPase activating protein) activity of the TSC1/2 heterodimeric complex

by phosphorylating TSC2 of the heterodimeric complex leading to the induction of GTP loading on the Rheb small GTPase (Garami *et al.,* 2003; Inoki *et al.,* 2003; Tee *et al.,* 2003; Zhang *et al.,* 2003). Subsequently, the GTP-Rheb complex activates mTORC1 by an unknown mechanism resulting in the promotion of translation, mRNA maturation, and cell growth via phosphorylating ribosomal S6 Kinase and 4E-BP (Long *et al.,* 2005). Akt also directly phosphorylates PRAS40, a possible negative regulator of the mTORC1 complex and induces the dissociation of PRAS40 from the mTORC1 complex (Sancak *et al.,* 2007; Vander Haar *et al.,* 2007). Recently, it has been proposed that in response to amino acids, the Rag small GTPase complex binds to Raptor, a component of mTORC1, which stimulates mTOR by changing the localization of mTORC1 to endomembrane structures in the perinuclear region within cells where mTORC1 can be activated by Rheb (Sancak *et al.,* 2008). However, whether the Rag-Raptor complex exists as an endogenous preformed complex needs to be further determined.

In spite of the body of the evidence regarding the regulation of the mTORC1 pathway, the regulation of the mTORC2 pathway is still not well understood. mTORC2 consists of mTOR, Rictor, mSin1, mLST8, and PRR5 and phosphorylates Akt Ser473 both *in vivo* and *in vitro* (Sarbassov *et al.,* 2005). A series of the genetic studies convincingly demonstrates that loss of mTOR, Rictor, mSin1, or mLST8 abolishes Akt Ser473 phosphorylation in embryonic fibroblasts, indicating that in addition to mTOR kinase activity, mTORC2 integrity is essential for Akt S473 phosphorylation (Guertin *et al.,* 2006; Jacinto *et al.,* 2006; Shiota *et al.,* 2006). Interestingly, loss of function of mTORC2 selectively attenuates FoxO phosphorylation among the Akt substrates (Jacinto *et al.,* 2006). Although mTORC2 activity is enhanced by serum or growth factors, the molecular mechanism by which mTORC2 is activated by these stimuli remains elusive (Sarbassov *et al.,* 2005).

Analogous to the situation in yeast, rapamycin or nutrient starvation contributes to a stimulation of autophagy in mammalian cells (Mizushima *et al.,* 2008). This indicates that mTORC1 has a negative effect on the autophagic pathway, and when there is a shortage of nutrients, cells stimulate autophagy possibly through inhibiting mTORC1 activity to stay alive. Therefore, the activation of the lysosomal autophagic pathway in response to inhibition of mTORC1 or starvation conditions helps in the production of amino acids and other elements needed for biosynthetic pathways. Interestingly, a recent study has shown that rapamycin-insensitive mTORC2 has a role in the regulation of autophagy through the Akt-FOXO axis (Mammucari *et al.,* 2007); FoxO3 is necessary and sufficient for the induction of autophagy in skeletal muscle. These studies indicate that both mTORC1 and mTORC2 might be involved in the regulation of

autophagy. Therefore, the assessment of mTOR activity is critical to determine its role in the autophagy pathway.

2. Methods

2.1. Cell culture and preparation of cell lysates

The best method to monitor mTOR activity in cells is Western blot analysis using phospho-specific antibodies (antibodies specific to phosphorylated residues of the target proteins) against the mTOR substrates. Growth factors and nutrients such as glucose and amino acids stimulate mTOR activity promoting substrate phosphorylation. Hence, to study the signaling events that are supposed to stimulate mTOR activity, the following general protocol to determine mTOR activity in HEK293 cells is provided (for additional details, see the subsequent protocol for rapamycin treatment):

1. Cells are generally grown in DMEM containing 10% fetal bovine serum (FBS). For serum starvation, cells are washed by exchanging the medium with PBS and then cultured in serum free media for 16 h.
2. Cells are then treated with the appropriate stimuli such as insulin (100 n*M* to 400 n*M*) for 15 min.
3. After stimulation, the cells are lysed for 10 min on ice with occasional shaking by hand.

Note: To avoid additional inputs for mTOR activity during harvesting of the cells, we normally put lysis buffer directly into the well without washing the cells with PBS.

4. The soluble components are then collected into 1.5-ml tubes using a pipette and centrifuged at 13,000 rpm for 10 min at 4 °C.
5. After centrifugation, the supernatant fractions are transferred into new 1.5-ml tubes. If the protein extracts will be resolved by SDS PAGE, the collected supernatant fractions need to be denatured immediately by boiling at 100 °C for 5 min in 1X SDS sample loading buffer containing a reducing reagent such as β-mercaptoethanol or DTT. Otherwise, extracts do not need to be denatured at once and can be used for other experiments such as immunoprecipitation (IP) and pull-down (CoIP) assays.

Following is the detailed protocol for protein extraction from cell culture for determining mTOR activity in the presence or absence of rapamycin treatment.

1. Plate 1×10^6 HEK293T cells into two 3.5-cm dishes a day before rapamycin treatment: one dish will be used for a no treatment control and the other dish will be treated with 5–20 n*M* rapamycin.
2. On the second day, treat cells with or without rapamycin (we generally use 20 n*M*) and incubate them at 37 °C in a humidified 5% CO_2

incubator for 30 min. Rapamycin is available from Sigma, Cell Signaling, or LC laboratory.
3. Place the cell culture dish on ice and completely remove the medium with mild aspiration, then add in 300 μl of cold lysis buffer (40 m*M* HEPES, pH 7.5, 120 m*M* NaCl, 1 m*M* EDTA, 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF, 1.5 m*M* Na_3VO_4, 1% Triton X-100, and EDTA-free protease inhibitors (Roche, 14132300)). Leave on ice for 10 min with occasional shaking by hand.
4. Collect the supernatant fraction using a pipette and transfer into 1.5-ml tubes.
5. Centrifuge at 13,000 rpm for 10 min at 4 °C.
6. Remove the supernatant fractions and transfer into new 1.5-ml tubes. Add SDS sample loading buffer (stock 4X) to 1X working concentration.
7. Denature the proteins by incubating tubes containing the samples at 100 °C for 5 min.
8. Briefly centrifuge the tubes (13,000 rpm, 10 s) and either load the samples onto an SDS-PAGE gel or store at −20 °C for later analysis.

2.2. Preparation of tissue sample

To monitor the mTOR activity in animal tissues by Western blotting, we use the following protocol:

1. The tissue should be immediately lysed in lysis buffer (see section 2.1) to avoid the decrease of mTOR activity during the process of protein extraction from the tissue. The isolated tissues are transferred into cold lysis buffer (200 mg tissue/500 μl lysis buffer).
2. The tissues are homogenized in a Dounce homogenizer by repeated strokes (approximately 20 strokes) until the tissue is torn apart into very small pieces.
3. Centrifuge at 13,000 rpm for 15 min at 4 °C.
4. Transfer the supernatant fractions to new 1.5-ml tubes. It is recommended to avoid any contamination with debris, and especially fat, when the supernatant fractions are transferred into new tubes. Supernatant fractions containing extracted proteins from the tissues can be used for analyses or snap-frozen using liquid nitrogen followed by storage at −80 °C.

2.3. Substrates of mTOR complexes

p70S6K (Thr389), 4E-BP1 (Thr37, Thr46, Ser65, Thr70), and PRAS40 (Ser183) are known to be convincing direct substrates of mTORC1 (Brunn *et al.*, 1997; Burnett *et al.*, 1998; Gingras *et al.*, 1999; Fonseca *et al.*, 2007; Oshiro *et al.*, 2007). The phosphorylation of S6K1 on Thr389 and 4E-BP1

on Thr37, Thr46, and Ser65 are often used as functional readouts of mTORC1 activity, as phosphorylation of these sites by mTORC1 has been confirmed both *in vitro* and *in vivo* and is inhibited by rapamycin treatment. Recently, it has been also reported that Ser183 and Ser221 of PRAS40 are also directly phosphorylated by mTORC1 (Fonseca *et al.*, 2007; Oshiro *et al.*, 2007; Wang *et al.*, 2008). The phosphorylation of 4E-BP1 upon stimulation (growth factors, mitogens, and hormones) occurs at multiple sites in a hierarchical manner (first Thr37 and Thr46, then Thr70, and finally Ser65) (Gingras *et al.*, 2001).

In contrast, Akt (Ser473) is the only substrate shown to be directly phosphorylated by mTORC2 (Sarbassov *et al.*, 2005). The phosphorylation of Ser473 in a hydrophobic motif of Akt has been shown to be mediated by mTORC2 both *in vitro* and *in vivo* (Sarbassov *et al.*, 2005). Although debates still exist as to whether mTORC2 is the sole kinase for Akt on Ser473, the observation that the levels of Akt Ser473 phosphorylation are indeed abolished in Rictor or hSin1 knockout cells allows this site to be employed for monitoring mTORC2 activation. PKCα (S657) is also reported to be regulated by mTORC2. However, the regulation seems to be rather indirect, as direct phosphorylation of PKCα (S657) by mTORC2 has not been successfully demonstrated (Sarbassov *et al.*, 2004).

2.4. Monitoring phosphorylation status of mTOR substrates

To analyze the phosphorylation status of mTORC1 substrates, Western blot analysis using phospho-specific antibodies is performed. Phospho-S6K1 Thr389 (#9234) antibody and phospho-4E-BP1 Thr37/Thr46 (#9459), Ser65 (#9451), and Thr70 (#9455) antibodies are available from Cell Signaling (Fig. 11.1).

To examine the activation status of mTORC2 *in vivo*, Western blot analysis using phospho-Akt Ser473 antibody (#4058, #9271, Cell Signaling) is usually performed. Although mTORC2 enhances phosphorylation of PKCα Ser657 possibly in an indirect manner, it is not ideal to use this phosphorylation as a functional readout for mTORC2 activation, as phosphorylation of the site is quite stable and resistant to at least short-term treatment with stimuli and inhibitors (Ikenoue *et al.*, 2008).

In addition, monitoring reduced mobility of the proteins by SDS PAGE analysis also can be an alternative to phospho-specific antibodies, due to the decrease in protein mobility associated with their posttranslational modifications, especially phosphorylation. To detect the mobility shift of S6K1 (70 kDa) and 4E-BP1 (22 kDa), it is ideal to use 10% and 15% SDS PAGE gels, respectively (Fig. 11.1). Optimal resolution can be achieved by using gels containing a lower concentration of methylene *bis*-acrylamide. The phosphorylation of 4E-BP1 can be often more clearly determined by mobility shift rather than by using a phospho-specific antibody.

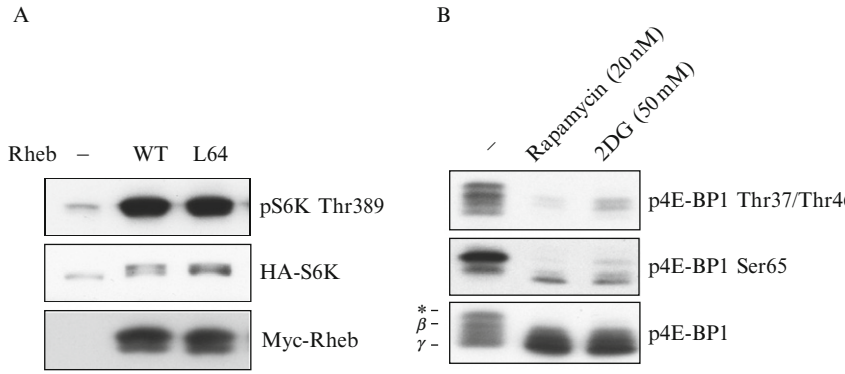

Figure 11.1 Monitoring of mTORC1 activity using phospho-specific antibodies for S6K1 and 4E-BP1 and mobility shifts of S6K1 and 4E-BP1 proteins. (A) HEK293 cells were transfected with HA-S6K1 together with Myc-tagged empty vector, wild-type Rheb (WT), or mutant Rheb (L64). After serum starvation, the cell lysates were analyzed by Western blot analysis using the indicated antibodies. (B) HEK293 cells were treated with the indicated concentration of rapamycin or 2-deoxyglucose (2DG), a non-metabolizable d-glucose, for 15 min. Phosphorylation of endogenous 4E-BP1 proteins were determined by Western blot analysis using the indicated antibodies. ⋆, β, and γ denote hyper-, less-, and nonphosphorylated forms of 4E-BP1 proteins, respectively.

The 4E-BP1 proteins can be resolved into three bands (usually termed ⋆, β, and γ) with different mobilities that correspond to hyper-, less-, and non-phosphorylated forms, respectively (Fig. 11.1B).

To examine the effect of drugs on mTORC1 or mTORC2, phosphorylation of endogenous substrates for either mTOR complex can be used. In contrast, to analyze the effect of proteins of interest on mTORC1 and mTORC2, cotransfection of the plasmids encoding genes of interest and the substrates of mTOR complexes is frequently required, especially in cells with rather low transfection efficiency. For example, cotransfection of Myc-Rheb and HA-S6K1 can be used for assessing the effect of Rheb on mTORC1 activity (Fig. 11.1A).

The following is an example of how to monitor exogenous phospho-S6K1 Thr389 in HEK293T cells.

1. Plate HEK293T cells into a 6-well plate (30-50% confluency) a day before the transfection.
2. For the transfection, add 100 ng of pRK5 HA-S6K1 in a microcentrifuge tube and mix with 150 μl of Opti-MEM I (GIBCO) by vortexing briefly (2 s). Add 3 μl of lipofectamine (Invitrogen) in the tube and mix it with brief vortexing and incubate at room temperature (RT) for 30 min. Meanwhile, add 850 μl of Opti-MEM I after a wash with this medium in a 6-well plate.
3. Add transfection mixture into the well containing 850 μl of Opti-MEM I.

4. Incubate for 4–6 h at 37 °C in a humidified 5% CO_2 incubator and remove Opti-MEM I with the transfection mixture by aspiration. Then, add cell culture medium (10% FBS in DMEM with 1X penicillin/streptomycin).
5. 48-h posttransfection, the transfected cells can be treated with rapamycin (20 n*M*) to inhibit phosphorylation of S6K1 on Thr389. Cells cultured in the 6-well plate are lysed in 300 μl of mild lysis buffer (10 m*M* Tris·HCl, pH 7.5, 100 m*M* NaCl, 1% Nonidet P-40, 50 m*M* NaF, 2 m*M* EDTA, 1 m*M* PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) on ice for 10 min with occasional shaking.
6. Collect the supernatant fraction and centrifuge at 13,000 rpm for 10 min at 4 °C.
7. Transfer the clear supernatant fraction into new tubes and denature the proteins with SDS sample loading buffer.
8. Samples are resolved by 10% SDS PAGE and transferred onto PVDF membrane at 350 mA for 2 h at 4 °C. Before transfer, the PVDF membrane is activated by soaking it in methanol and washed in water briefly, and then washed in transfer buffer for 5 min.
9. Block the membrane with 5% nonfat dry milk in Tris-buffered saline (100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl) containing 0.05% Tween 20 (TBST) for 1 h and incubate the membrane with phospho-S6K1 Thr389 antibody (#9234, Cell Signaling) overnight at 4 °C with gentle shaking, or use antibody against HA to see the mobility shift due to phosphorylation.
10. After several washes in TBST for a total of 30 min, the membrane is incubated with secondary antibody conjugated with horseradish peroxidase (NA934, GE Healthcare) for 1 h at RT with gentle shaking.
11. Following several washes with TBST for a total of 40 min, the membrane is treated briefly with ECL reagent and exposed to X-ray film until clear bands are observed after developing the film.

Rapamycin treatment is supposed to inhibit phosphorylation of S6K1 on Thr389 even in the presence of serum. Therefore, it is rare to see phospho-S6K1 bands in rapamycin-treated samples. If there are residual bands corresponding to S6K1 or phospho-S6K1 even after rapamycin treatment, approximately 2 h of treatment with rapamycin might be necessary to obtain complete inhibition of mTOR.

2.5. mTOR activity *in vitro*

2.5.1. Preparation of substrates for *in vitro* kinase assay

Recombinant S6K1 and 4E-BP1 proteins can be used as substrates for an *in vitro* mTORC1 kinase assay, whereas recombinant Akt can be used for the mTORC2 kinase assay. Recombinant proteins produced from bacteria,

baculovirus, or mammalian cells can be used as substrates; however, those from baculovirus or mammalian cells work better as substrates in our experience. To prepare the substrates for mTORC1 or mTORC2 from mammalian cells, we usually transfect GST-S6K1 or GST-Akt expression vectors into HEK293T cells, treat the cells with rapamycin or PI3 kinase inhibitors such as LY294002 (Sigma) for 30 min to induce dephosphorylation of S6K1 on Thr398 or Akt on Ser473.

The following is a standard procedure to purify the GST-fusion proteins from cultured mammalian cells in a 10-cm dish.

1. HEK293T cells (50% confluence) are transfected with a mammalian expression GST-S6K1 or GST-Akt construct (2 μg DNA/10 cm dish) using Lipofectamine (Invitrogen).
2. 48 h posttransfection, the transfected cells are treated with rapamycin (20 n*M* for S6K1) or LY294002 (20 μ*M* for S6K1 and Akt) for 30 min to induce dephosphorylation of the substrate proteins.
3. Cells are washed with ice-cold PBS and lysed with 1 ml of PBST buffer (PBS containing 1 m*M* EDTA, 0.1% β-mercaptoethanol, 1% TritonX-100, protease inhibitor mixture; Roche).
4. Collect the lysates into 1.5-ml tubes and centrifuge at 13,000 rpm for 15 min at 4 °C.
5. Transfer the clear supernatant fraction into new tubes and rotate with 50 μl of glutathione sepharose beads (Amersham Biosciences) for 4 h at 4 °C.
6. After incubation, GST-protein bound beads are washed 3 times with 1 ml of PBST and then once with glutathione-free GST buffer (50 m*M* Tris, pH 8.0, 150 m*M* NaCl, 1 m*M* DTT, 5 m*M* $MgCl_2$).
7. Elute the GST-proteins with 50 μl of GST buffer containing 10 m*M* reduced glutathione (Sigma) for 20 min at 4 °C. Repeat the elution at least twice.
8. Collected eluents are dialysed in at least 100 times volume of dialysis buffer (10 m*M* Tris, pH 7.5, 50 m*M* NaCl, 0.05% β-mercaptorthanol, 0.5 m*M* EDTA) for 4 h at 4 °C. The dialyzed solution is concentrated by microcon centrifugation (YM100, Millipore).
9. The purified protein and different concentrations of bovine serum albumin (BSA) are subjected to SDS-PAGE and stained with coomassie blue to determine the concentration of GST-protein. To obtain a sufficient amount of purified GST-fusion proteins for the mTOR kinase assay, we normally harvest the cells from at least ten 10-cm dishes.

2.5.2. Purification of mTOR complexes

Recent, landmark studies reveal that Raptor plays a critical role in the mTORC1 complex for recruiting mTOR substrates such as S6K1 and 4E-BP1 to mTORC1 (Hara *et al.*, 2002; Kim *et al.*, 2002; Schalm and

Blenis 2002; Schalm *et al.*, 2003). The interaction between mTOR and Raptor is detergent sensitive. For instance, nonionic detergents such as NP-40 or Triton-X100 dissociate the mTOR-Raptor complex suggesting that hydrophobicity of the residues at the interface of these proteins plays a role in complex formation. Consistent with this idea, the interaction of these proteins can be well sustained in ionic detergent buffers such as CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)-containing buffer (Hara *et al.*, 2002; Kim *et al.*, 2002). Interestingly, variations in the association of Raptor and mTOR can be seen when using CHAPS buffer for lysis and wash. For example, amino acid withdrawal (nutrient deprivation) enhances Raptor's binding to mTOR, whereas rapamycin treatment reduces the binding (Kim *et al.*, 2002). Considering that both conditions impair phosphorylation of downstream substrates such as S6K1 on Thr389, it is notable that rapamycin and nutrient deprivation inhibit mTORC1 by distinct mechanisms.

In addition to the detergent conditions, the salt concentration in the wash buffer is also important. High-salt washes during mTORC1 purification increase the basal activity of mTORC1 (Sancak *et al.*, 2007). In addition, PRAS40 can be dissociated from TORC1 by high-salt washes leading to an increase in the activity of mTORC1. However, recent studies have shown that PRAS40 can be directly phosphorylated by mTOR, suggestive of PRAS40 as a substrate of mTORC1 (Fonseca *et al.*, 2007; Oshiro *et al.*, 2007). This evokes the question of whether it is appropriate to retain PRAS40 in the purified mTORC1 complex to measure native mTORC1 kinase activity.

The mTOR-Rictor interaction in the mTORC2 complex is also diminished in 1% NP-40 containing buffer, but not in 0.3% CHAPS-containing buffer (Sarbassov *et al.*, 2004). Although Rictor was originally identified as a component of the rapamycin-insensitive mTOR complex, long-term rapamycin treatment disrupts the binding of Rictor to mTOR in a cell type-specific manner (Sarbassov *et al.*, 2006).

For the purification of mTORC1 and mTORC2, we use the following general protocol (see subsequently for further details):

1. Subconfluent cells are lysed in 1 ml of CHAPS lysis buffer (40 m*M* HEPES, pH 7.4, 120 m*M* NaCl, 2 m*M* EDTA, 0.3% CHAPS, 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF, EDTA-free protease) containing protease and phosphatase inhibitors (Kim *et al.*, 2002; Sarbassov and Sabatini 2005).
2. After collecting the lysates, 30 μl is saved for Western blot analysis as input controls.
3. The remaining lysates are used for the immunoprecipitation (IP). We usually incubate the lysates with antibody for 1–2 h at 4 °C with rocking. Anti-FRAP (mTOR) antibody (N-19; Santa Cruz) can be used for

immunoprecipitation of endogenous mTOR complexes and does not interfere with the kinase reaction.
4. To recover the antibody-antigen complex, 10 μl of a 50% slurry of protein G-sepharose (17–0618-01 GE Healthcare) are added and incubated for 2 h at 4 °C with rocking.
5. Immunoprecipitates are centrifuged at 10,000 rpm for 5 s and then washed with CHAPS lysis buffer 3 times without protease and phosphatase inhibitors.
6. After the final wash, the supernatant fractions are completely removed and the immunoprecipitates are subjected to the kinase reaction.

2.5.3. Reaction of mTOR kinase assay

The kinase assays are performed according to a modified protocol based on previous reports (Kim *et al.*, 2002; Sarbassov *et al.*, 2005; Yang *et al.*, 2006) described briefly here and in detail subsequently.

1. For kinase reactions, 200 ng of GST-S6K1 and 50 ng of His-Akt (#14-279, Upstate) can be used for the mTORC1 and mTORC2 kinase assay, respectively.
2. 250-500 μ*M* ATP with or without 1 μCi of [$\gamma-^{32}$ P] ATP (for detection by autoradiography) is added in the kinase assay buffer (described subsequently) 10 min prior to the start of the kinase reaction.
3. The kinase reactions are stopped by adding 5 μl of 4X SDS sample buffer and resolved in 10% and 15% SDS-PAGE gels for S6K1 and Akt and for 4E-BP1, respectively, and transferred to PVDF membranes followed by autoradiography.

Immunoblotting using phospho-specific antibodies for mTOR-dependent sites in S6K1, 4E-BP1, and Akt described earlier can be performed to determine the kinase activity of mTOR in each immunoprecipitate instead of using radioactivity (Fig. 11.2). A summary of the purification of the TOR complexes with low-salt washes (using exogenously expressed mTOR components) and kinase assays is the following:

1. HEK293T cells are cultured in 10-cm plates.
2. When the cell density reaches 3×10^6 cells per plate, cells are transfected with either mTORC1 components (MYC-mTOR, HA-Raptor, and/or MYC-mLST8) or mTORC2 components (MYC-mTOR, HA-Rictor, MYC-Sin1, and/or MYC-mLST8). After 48 h transfection, the cells are lysed in CHAPS lysis buffer (40 m*M* HEPES, pH 7.4, 120 m*M* NaCl, 2 m*M* EDTA, 0.3% CHAPS, 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF, EDTA-free protease) on ice.
3. To immunoprecipitate mTORC1 or mTORC2, one μg of anti-HA (for immunoprecipitation of HA-Raptor or HA-Rictor) antibody is added to

Figure 11.2 **Determination of mTORC2 activity *in vitro*.HEK293 cells were grown in 10-cm dishes and lysed using CHAPS Lysis buffer (containing 0.3% CHAPS). The lysates were immunoprecipitated with Rictor or mTOR antibody or without antibody. The immunoprecipitates were subjected to an *in vitro* kinase assay using His-Akt (full-length) as a substrate. Phosphorylation of Akt Ser473 was analyzed using phospho-specific antibody. The amounts of mTOR and Rictor in each immunoprecipitate were also determined by immunoblotting.**

each of the cellular lysates and incubated at 4 °C for 120 min with gentle rocking.

4. 10 μl of protein G Sepharose slurry (50%) are added to the lysates and incubated for another hour.
5. The sepharose beads are centrifuged for 15 s at 10,000 rpm. The immunoprecipitates are washed 3 times in low-salt wash buffer (40 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 0.3% CHAPS, 10 m*M* pyrophosphate, 10 m*M* glycerophosphate, 50 m*M* NaF).
6. The immunoprecipitates are washed twice in kinase wash buffer (25 m*M* HEPES-KOH, pH 7.4, 20 m*M* KCl). The mTORC1 kinase assays, are performed for 30 min at 30 °C in a final volume of 15 μl consisting of the mTORC1 kinase buffer (25 m*M* HEPES-KOH, pH 7.4, 50 m*M* KCl, 10 m*M* $MgCl_2$, and 250 μM ATP). For the mTORC2 kinase assay, reactions are performed in the mTORC2 Kinase Buffer (25 m*M* HEPES-KOH, pH 7.4, 100 m*M* potassium acetate, 1 m*M* $MgCl_2$, and 500 μM ATP).
7. To stop the reaction, 5 μl of 4x SDS sample buffer are added to each reaction, which are then boiled for 5 min and the reactions are resolved by SDS PAGE and then visualized by autoradiography or Western blot analysis with phospho-specific antibodies for substrates used in the kinase assay.

2.5.4. Monitoring mTOR activity in tissues

To study mTOR activity in animal tissues by immunohistochemistry (IHC), the tissue samples are prepared as either a frozen or a paraffinized section. Because the mTOR activity is very sensitive to the availabilities of both growth factors and nutrients, the preparation for tissue samples should be done as promptly as possible. To obtain a paraffinized sample, the animals are

first perfused with 4% paraformaldehyde, and fixed tissue are paraffinized, and embedded in paraffin. Sections of 2–10-μm thickness of sections can be cut using a microtome and stained with antibodies against phospho-S6K1, S6, Akt and mTOR. The following is a standard procedure for immunostaining the paraffin embedded sample using DAB (diaminobenzidine) detection.

1. Deparaffinize and hydrate sections by sequential washes in the following: xylene 3 min (3 times), 100% ethanol 1 min (twice), 95% and 80% ethanol 1 min each, and then wash in distilled water 2 min once. Rock gently in each solution.
2. Warm 300 ml of 10 m*M* sodium citrate, pH 6.0 to 95 °C–100 °C in a Coplin staining jar.
3. Quickly immerse slides into the hot citrate buffer and incubate for 20–40 min. The optimal incubation time needs to be determined empirically.
4. Cool the slides on the bench in citrate solution at RT for 30 min.
5. Rinse sections in PBS for 2 min twice.
6. Using a hydrophobic pen (S2002, Dako), surround the area to be stained.
7. Block sections in 2% BSA in PBS for 60 min at RT in a humidified chamber to prevent drying.
8. Remove the blocking solution.
9. Add primary antibody (IHC available p-S6 [#4857 Cell Signaling], p-Akt [#3787, #9266 Cell Signaling], or p-mTOR [#2976 Cell Signaling]) diluted in 1% BSA, 30–50 μl per section at the appropriate dilution as indicated by the supplier and incubate for 1 h at RT or overnight at 4 °C in a humidified chamber with gentle agitation.
10. Rinse with PBS for 3 min twice.
11. Block sections with peroxidase blocking solution (3% hydrogen peroxide) for 10 min.
12. Rinse in PBS for 5 min 3 times.
13. Add 20 μl of HRP-conjugated antirabbit secondary antibody (K4002 Dako) per section and incubate 1 h at RT.
14. Rinse in PBS for 10 min 3 times.
15. Add 20 μl of DAB solution (substrate buffer:chromogen = 50:1 [K3466, Dako]). DAB is oxidized and forms a stable brown end product at the site of the target antigen.

3. Concluding Remarks

The mTOR complexes are part of a major pathway that transduces growth factor signaling, leading to the induction of anabolic biosynthesis. Furthermore, regulation of mTOR also affects the autophagic pathway,

which plays a major role in cell maintenance in the absence of nutrients. Recent studies have elucidated that both mTORC1 and mTORC2 are involved in the regulation of autophagy. We have introduced methods to evaluate the activity of mTORC1 and mTORC2 *in vivo* and *in vitro*. *In vivo* activities of mTORC1 and mTORC2 can be measured by the phosphorylation status of their downstream targets using Western blot analysis with cell lysates. On the other hand, *in vitro* kinase assays using immunoprecipitated mTORC1 and mTORC2 and their substrates are used for assessing their direct activities on targets *in vitro*. Given that mTOR is the critical regulator of the autophagic pathway, it is useful to establish the methods to measure the activity of mTOR in response to various stimuli, which possibly affect the autophagic pathway. This approach for monitoring mTOR activity will be helpful to resolve the role of proteins that might be involved in the mTOR-involved autophagic pathway. Although the mTOR pathway plays a critical role in the regulation of autophagy in a wide array of organisms, it is noteworthy that mTOR-independent regulation of the autophagy pathway has been postulated (Sarkar *et al.,* 2007; Williams *et al.,* 2008). Furthermore, recent studies also suggest that autophagy can be upstream of mTORC1 activity. For instance, the autophagy-related gene 1 (*ATG1*), an essential kinase for vesicle formation during autophagy, inhibits mTOR1 activity, indicative of another direction in the cross talk between mTORC1 and autophagy (Neufeld 2007). It is certain that current studies that address the molecular mechanism of regulation between the mTOR pathway and autophagy will reveal the detailed mechanism that controls this complex pathway in the mammalian system.

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