TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity

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Target of rapamycin (TOR) plays a central role in cell growth regulation by integrating signals from growth factors, nutrients, and cellular energy levels. TOR forms two distinct physical and functional complexes, termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1, which is sensitive to rapamycin, regulates translation and cell growth, whereas TORC2, which is insensitive to rapamycin, regulates cell morphology and cell growth. The Ras homology enriched in brain (Rheb) small GTPase is known to be a key upstream activator of TORC1, although the mechanism of Rheb in TORC1 activation remains to be determined. However, the function of Rheb in the TORC2 regulation has not been elucidated. By measuring Akt and S6K phosphorylation as a functional assay for TORC1 and -2, here, we report that dRheb has an inhibitory effect on dTORC2 activity in *Drosophila* S2 cells. This negative effect of dRheb on dTORC2 is possibly due to a feedback mechanism involving dTORC1 and dS6K. We also observed that Rheb does not activate TORC2 in human embryonic kidney 293 cells, although it potently stimulates TORC1. Furthermore, tuberous sclerosis complex 1 (TSC1) and TSC2, which are negative regulators of Rheb, have negative and positive effects on TORC1 and -2, respectively. Our observations suggest that TSC1/2 and Rheb have different effects on the activity of TORC1 and -2, further supporting the complexity of TOR regulation.

Akt | S6K | target of rapamycin | tuberous sclerosis complex | *Drosophila*

Target of rapamycin (TOR) is a protein kinase that belongs to a phosphatidylinositol kinase-related kinase family and is highly conserved in all eukaryotes (1). The TOR signaling pathway has a wide array of functions to regulate cell growth, proliferation, apoptosis, and autophagy by integrating multiple signals from growth factors, energy, and nutrients. Emerging evidence suggests that TOR exists in two distinct physical and functional complexes, termed TOR complex 1 (TORC1) and TORC2. TORC1 and -2 were initially identified in the budding yeast *Saccharomyces cerevisiae*, which has two TOR genes (2). Recently, TORC1 and -2 have also been characterized in mammalian and *Drosophila* cells that have a single TOR gene, termed *mTOR* and *dTOR*, respectively (3–6). Both of the mammalian TOR complexes contain *mTOR* and mammalian homologue of LST8 (*mLST8*)/*Gβ*-like (*GβL*) (2, 7). However, regulatory associated protein of *mTOR* (Raptor) and rapamycin-insensitive companion of *mTOR* (Rictor)/*mAVO3* are exclusively associated with TORC1 and -2, respectively (5, 6). Importantly, only TORC1, but not TORC2, is sensitive to inhibition by rapamycin (2, 5). The prevailing view is that TORC1 mainly regulates cell growth and protein synthesis by phosphorylating two key translational regulators eukaryote initiation factor 4E-binding protein (4EBP1) and S6K1 (8).

The function of TORC2 is much less well characterized. However, it has been reported that TORC2 regulates the actin cytoskeleton by modulating protein kinase C and Rho-family small GTPases in a rapamycin-insensitive manner (5, 6, 9, 10). One of the exciting findings about TORC2 is that it directly phosphorylates Akt/PKB at the hydrophobic motif site Ser-473, which is the phosphorylation site of a presumed PDK2, an enzyme not identified until recently (11). Akt/PKB plays essential roles in many

aspects of cellular regulation, including cell growth and cell survival (12). The demonstration that TORC2 has PDK2 activity places TORC2 at the center stage of cellular signaling and multiple cellular processes. TORC2 does not phosphorylate the wild-type S6K1 under normal physiological conditions. But, interestingly, TORC2 can phosphorylate a mutant S6K1 that has a deletion of the C-terminal region (Fig. 1A) (13). Both Akt and S6K1 belong to the AGC kinase family. Many AGC kinases, such as Akt, do not contain the C-terminal extension present in S6K1. Therefore, it is speculated that TORC2 may also phosphorylate other members of the AGC kinases, such as PKC and SGK (5, 14).

It has been well established that TOR activity is positively regulated by the phosphatidylinositol 3-kinase (PI3K) signaling in higher eukaryotes, such as *Drosophila* and mammals (8, 15). Furthermore, recent studies have identified that the tumor suppressors tuberous sclerosis complex 1 (TSC1) and TSC2 function as key negative regulators of TOR in both *Drosophila* and mammals (16, 17). Mutation of either the *TSC1* or *TSC2* gene results in TSC (17). TSC1 and -2 form a physical complex that is important for their physiological functions (18). Phosphorylation of S6K1 and eukaryote initiation factor 4E-binding protein is highly elevated in TSC tumor cells, indicating constitutive activation of TORC1 in *TSC1*^{−/−} or *TSC2*^{−/−} cells (8, 17). These results demonstrate that TSC1 and -2 negatively regulate TORC1 activity. The connection between TOR and PI3K is revealed by the observation that Akt, which is activated by the PI3K pathway, phosphorylates and inhibits TSC2 (19–21). Furthermore, the understanding of the molecular function of TSC1/2 in regulating TORC1 was significantly advanced when Ras homology enriched in brain (Rheb) was identified as a key component acting downstream of TSC1/2 and upstream of TOR (22–27). TSC2 inhibits Rheb activity by functioning as a GTPase-activating protein toward Rheb. In addition, Rheb stimulates phosphorylation of S6K1 and eukaryote initiation factor 4E-binding protein in a TOR-dependent manner. Rheb may directly bind to and stimulate TORC1 function (28). Therefore, a signaling pathway of PI3K-Akt-TSC2-Rheb-TORC1 has been proposed. Interestingly, active S6K1, in turn, suppresses the PI3K-Akt pathway by inactivating insulin receptor substrate (IRS) (29, 30). S6K1 has been shown to directly phosphorylate IRS1 and, thereby, attenuates PI3K activation in response to growth factors, such as insulin. Therefore, S6K1 is a key component in the feedback regulation of PI3K by TORC1.

The function of TSC1/2 and Rheb in TORC1 regulation is well established. However, no conclusion has been reached as to whether and how TSC1/2 and Rheb regulate TORC2. Here, we show that, in *Drosophila* S2 cells, knockdown of dRheb expression

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Abbreviations: *GβL*, *Gβ*-like; HEK, human embryonic kidney; IRS, insulin receptor substrate; MEF, mouse embryonic fibroblast; *mLST8*, mammalian homologue of LST8; *mTOR*, mammalian TOR; PI3K, phosphatidylinositol 3-kinase; Raptor, regulatory associated protein of TOR; Rheb, Ras homology enriched in brain; Rictor, rapamycin-insensitive companion of TOR; RNAi, RNA interference; TOR, target of rapamycin; TORC, TOR complex; TSC, tuberous sclerosis complex; HA, hemagglutinin.

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Fig. 1. Rheb and TSC1/2 show opposite effects on TORC1 and -2 in *Drosophila* S2 cells. (A) Schematic illustration of S6K1 and Akt as functional readouts of TORC1 and -2, respectively. S6K1 was used as a functional readout of TORC1 activity, whereas S6K1 3A/ΔC and Akt were used as functional indicators of TORC2 activity. Phosphospecific antibodies recognizing Ser-473 of Akt and Thr-389 of S6K1 were used to detect Akt and S6K1 activity, respectively. (B) dRheb RNAi promotes dAkt phosphorylation and inhibits dS6K phosphorylation. *Drosophila* S2 cells were treated with 4 μg of dRheb dsRNA as indicated. Insulin stimulation (600 ng/ml insulin for 30 min) is indicated. The phosphorylation of dAkt and dS6K were detected by the phosphospecific antibodies recognizing mammalian Akt Ser-473 and S6K1 Thr-389, respectively. To avoid confusion, we kept the labeling as p-dAkt(S505) and p-dS6K(T398) for *Drosophila* proteins and p-Akt(S473) and p-S6K1(T389) for mammalian proteins in all figures, although the same phosphospecific antibodies were used. dS6K protein was detected by *Drosophila* anti-dS6K antibody, and dAkt protein was monitored by mammalian anti-Akt antibody. The two Akt isoforms generated by alternative splicing are indicated by arrows. The phospho-Akt antibody also detected a nonspecific band between the two Akt splicing forms. It should be noted that the phospho-Akt Western blot of the samples treated with insulin was exposed for a much shorter time than the corresponding Western blot of samples without insulin treatment. (C) Knockdown of dTSC1 or -2 by RNAi decreased dAkt phosphorylation but enhanced dS6K phosphorylation. Experiments were performed similarly to those described in B.

by RNA interference (RNAi) ablates the phosphorylation of dS6K1 but enhances the phosphorylation of dAkt. Knockdown of other components of the TORC1 signaling pathway, including dRaptor and dS6K1, also increases dAkt phosphorylation. In contrast, knockdown of dTSC1 or -2 enhances dS6K1 phosphorylation but inhibits dAkt phosphorylation, suggesting that dRheb positively regulates dTORC1 while negatively affecting dTORC2. Consistent with these observations, S6K1 phosphorylation is elevated, whereas Akt phosphorylation is inhibited in TSC1^{-/-} and TSC2^{-/-} mammalian cells. Furthermore, Rheb activates TORC1 kinase activity but not TORC2, as determined by *in vitro* kinase assays. Together, our data demonstrate that TSC1/2 and Rheb have different effects on TORC1 and -2.

Results

dRheb, dTSC1, and dTSC2 Have Opposite Effects on the Phosphorylation of dAkt and dS6K. TORC1 and -2 phosphorylate different substrates and have distinct cellular functions. However, TORC1 and -2 share two common components, TOR and LST8/GβL. TORC1 has been demonstrated to be activated by Rheb in both

Drosophila and mammalian cells (22–25, 31). However, it is not clear whether Rheb directly activates TORC1 or requires intermediate molecules. To determine whether Rheb activates TORC2, we used RNAi in cultured *Drosophila* S2 cells to knock down *Drosophila* Rheb (dRheb) expression. TORC2 activity is examined by measuring the phosphorylation of the hydrophobic motif site, Ser-505, of *Drosophila* Akt (dAkt), which is a direct physiological substrate of TORC2 (Fig. 1A) (11). In parallel, TORC1 activity is measured by the phosphorylation of the hydrophobic motif site, Thr-398, of *Drosophila* S6K (dS6K). Knockdown of dRheb expression with dsRNAs promoted the phosphorylation of dAkt and inhibited the phosphorylation of dS6K (Fig. 1B). In addition, knockdown of dRheb enhanced insulin-stimulated dAkt phosphorylation but blocked dS6K phosphorylation (Fig. 1B). These data show that dRheb displays opposite effects on dTORC1 and -2. dRheb plays a positive role in the phosphorylation of the TORC1 substrate dS6K and a negative role in the phosphorylation of the TORC2 substrate dAkt.

As expected, knockdown of dTSC1 or -2 significantly increased dS6K phosphorylation. Interestingly, knockdown of either dTSC1 or -2 expression decreased dAkt phosphorylation, especially in response to insulin stimulation (Fig. 1C), indicating that dTSC1 and -2 stimulate dTORC2. The above observations are consistent with data obtained with dRheb knockdown, therefore further supporting the notion that dRheb activates dTORC1 and inhibits dTORC2. However, the above data cannot distinguish whether dRheb inhibits dTORC2 directly or indirectly.

TORC2 Mediates the Inhibitory Effect of dRheb on dAkt Phosphorylation. We examined the effects of components in the TOR signaling pathway on phosphorylation of dAkt and dS6K. Knockdown of dS6K increased dAkt phosphorylation; however, knockdown of dAkt did not significantly inhibit dS6K phosphorylation (Fig. 2A). These results are consistent with two reports that dAkt does not play a major role in dS6K phosphorylation in *Drosophila* cells (11, 32). However, Lizcano *et al.* (33) reported that knockdown of dAkt inhibited the phosphorylation of dS6K. The reasons for this discrepancy are not obvious; therefore, further studies are needed to clarify the function of dAkt in dTORC1 regulation in *Drosophila* S2 cells.

As expected, knockdown of dRaptor decreased dS6K phosphorylation but increased dAkt phosphorylation (Fig. 2A). These results are consistent with the current model that Raptor is a key component present in only TORC1 (3–6). Furthermore, knockdown of dRictor decreased dAkt phosphorylation and reproducibly caused a moderate increase in dS6K phosphorylation. Moreover, knockdown of dTOR decreased phosphorylation of both dAkt and dS6K, consistent with the role of dTOR in both dTORC1 and -2. It should be noted that knockdown of dRictor and dTOR caused only partial reductions in dAkt phosphorylation, although the results are readily reproducible (Fig. 2A). These results suggest the presence of an additional source of PDK2 activity in S2 cells or an incomplete knockdown by RNAi experiments. Consistent with the later possibility, knockdown of dTOR did not completely eliminate phosphorylation of dS6K (Fig. 2A), whereas rapamycin completely eliminated dS6K phosphorylation under the same conditions (data not shown). We also noticed that dLST8 RNAi did not inhibit dS6K phosphorylation, whereas mLST8 RNAi inhibits S6K1 phosphorylation in human embryonic kidney 293T (HEK293T) cells (7). A reasonable explanation could be that dLST8 is not as potent for dTORC1 function in *Drosophila* S2 cells as it is in HEK293T cells. Knockdown of dPTEN increased phosphorylation of both dAkt and dS6K (Fig. 2A), supporting the fact that both TORC1 and -2 are stimulated by PI3K signaling.

PDK1 phosphorylates the activation loop of S6K1 and Akt (34, 35). It has been reported that knockout of PDK1 eliminates phosphorylation of S6K1 on both the activation loop Thr-229 and the hydrophobic motif site Thr-389 in mammalian cells (36),

A possible mechanism for Rheb to inhibit TORC2 is the feedback inhibition of IRS by S6K1. Rheb activates TORC1, which then activates S6K1, which, in turn, phosphorylates and inhibits IRS. We examined the effect of the *Drosophila* IRS gene, *Chico*. When *Chico* dsRNA was combined with dRheb, dRaptor, dS6K, dPDK1, or dPTEN, the phosphorylation of dAkt was inhibited (Fig. 2D). Our results obtained in *Drosophila* cells suggest that dRheb regulates TORC1 and -2 oppositely. It is worth noting that knockdown of either dRheb or dRaptor produced a stronger dAkt phosphorylation than the knockdown of dS6K. These data indicate that a

negative feedback inhibition by dS6K cannot be the sole factor mediating the inhibitory effect of dRheb on dAkt (Fig. 2D). dRheb may affect the balance between the two TOR complexes, thereby exerting its positive effect on dTORC1 and negative effect on dTORC2.

TORC1 is known to be regulated by nutrients, such as amino acids (8, 38). We observed that removal of amino acids induced a dramatic dephosphorylation of dS6K (Fig. 2E). Interestingly, amino acid starvation increased dAkt phosphorylation. Addition of amino acids stimulated dS6K and, at the same time, reversed the nutrient starvation-induced dAkt phosphorylation. It is worth noting that the effects of amino acids on dS6K and dAkt can be readily observed within 10 minutes. Rapamycin effectively blocked the effects of amino acids on the phosphorylation of both dS6K and dAkt (Fig. 5, which is published as supporting information on the PNAS web site). These results demonstrate that TORC1 and -2 are regulated differently by amino acids.

Rheb Stimulates Phosphorylation of the TORC1 Substrate S6K1 but Not the TORC2 Substrates Akt and S6K1 3A/ΔC. To examine the regulation of the TOR complexes by Rheb in mammalian cells, we used a S6K1 mutant containing both a TOS motif mutation and a C-terminal deletion (S6K1 3A/ΔC) (13, 39). Mutation of the TOS motif renders this S6K1 unphosphorylatable by TORC1 (Fig. 1A). As a result, phosphorylation of S6K1 3A/ΔC on Thr-389 would be resistant to rapamycin inhibition. Interestingly, S6K1 3A/ΔC can still be phosphorylated by TORC2 as a result of the deletion of the C-terminal extension. We used the phosphorylation of S6K1 3A/ΔC and Akt as the indicators of TORC2 activity (Fig. 1A). As expected, rapamycin inhibited phosphorylation of S6K1 but not Akt or S6K1 3A/ΔC in HEK293 cells (Fig. 3A). Rheb stimulated S6K1 phosphorylation (Fig. 3A), and the stimulatory effect of Rheb on S6K1 was completely inhibited by rapamycin. Rheb, however, also slightly activated S6K1 3A/ΔC. We suspected that the modest increase in phosphorylation of S6K1 3A/ΔC was due to a slight responsiveness of S6K1 3A/ΔC to TORC1. Therefore, we treated the cells with rapamycin to exclude the TORC1 effect. Indeed, the phosphorylation of S6K1 3A/ΔC reduced to the basal level after rapamycin treatment. In agreement with the results obtained with S6K1 3A/ΔC, Akt phosphorylation was not stimulated by Rheb (Fig. 3A). In addition, LY294002, a PI3K inhibitor, inhibited the phosphorylation of wild-type S6K1, S6K1 3A/ΔC, and Akt (Fig. 3A). These results are consistent with the notion that both TORC1 and -2 are regulated by the PI3K pathway. The above data show that Rheb activates TORC1 but not TORC2 *in vivo*, as determined by the phosphorylation of S6K1, S6K1 3A/ΔC, and Akt. A noticeable difference from the *Drosophila* S2 results is that Rheb does not inhibit TORC2 in HEK293 cells (Fig. 3A).

We further tested the effect of TSC1/2. Expression of TSC1/2 inhibited phosphorylation of the cotransfected S6K1 but not S6K1 3A/ΔC (Fig. 3B). In fact, TSC1/2 slightly increased the phosphorylation of S6K1 3A/ΔC, indicating that TSC1/2 regulate TORC1 and -2 oppositely. To further demonstrate the differential regulation of TORC1 and -2, the effects of various stimulation conditions on the phosphorylation of TORC1 and -2 substrates were examined. We found that phosphorylation of S6K1 3A/ΔC and Akt are similarly regulated by various extracellular stimuli. Consistent with our earlier results, phosphorylation of S6K1 was regulated differently from Akt and S6K1 3A/ΔC (Fig. 6, which is published as supporting information on the PNAS web site). For example, energy starvation induced by 2-deoxy-glucose strongly inhibited phosphorylation of S6K1 but had little effect on the phosphorylation of S6K1 3A/ΔC and Akt. Collectively, our data indicate that TORC1 and -2 are regulated differently by TSC1/2 and intracellular energy levels.

In TSC mutant mouse embryonic fibroblast (MEF) cells, both Rheb and TORC1 are constitutively active, as determined by the

Fig. 3. Regulation of S6K1, Akt, and S6K1 3A/ΔC phosphorylation by Rheb and TSC1/2. (A) Rheb stimulates only the phosphorylation of S6K1 but not Akt or the rapamycin-resistant phosphorylation of S6K1 3A/ΔC. HA-S6K1, GST-Akt, and HA-S6K1 3A/ΔC were transfected into HEK293 cells in the presence or absence of MYC-Rheb (50 ng). The phosphorylation of S6K1 and S6K1 3A/ΔC were detected by the phospho-specific antibody recognizing S6K1 Thr-389, whereas the phosphorylation of Akt was detected by the phospho-Akt antibody, pAkt (Ser-473). Protein levels were determined by using anti-HA (S6K1 and S6K1 3A/ΔC), anti-GST (transfected GST-Akt), anti-Akt (endogenous Akt), and anti-MYC (Rheb) antibodies. Cells were treated with either 50 μ M LY294002 or 25 nM rapamycin for 30 min as indicated. (B) TSC1/2 inhibit only S6K1 phosphorylation but not S6K1 3A/ΔC. HEK293 cells were transfected with HA-S6K1 and HA-S6K1 3A/ΔC. MYC-Rheb (25 ng) or MYC-TSC1 (250 ng)/HA-TSC2 (250 ng) was cotransfected where indicated. The phosphorylation was detected by pS6K1 (Thr-389) antibody, and the protein level was determined by either anti-HA or anti-MYC antibody.

high S6K1 phosphorylation (Fig. 4A and B). Both TSC1^{-/-} and TSC2^{-/-} MEF cells have higher basal S6K1 phosphorylation and lower Akt phosphorylation than the wild-type MEF cells. These results suggest that TSC1/2 negatively regulates TORC1 and positively regulates TORC2. It has been reported that constitutively active S6K1 causes an inhibition of IRS (29, 30). The inhibition of IRS by S6K1 leads to a decreased response to insulin stimulation and, therefore, decreases TORC2 activity. Prolonged rapamycin treatment, which inhibits S6K1 activity, should restore the insulin

inhibition of Akt phosphorylation in HEK293 cells. In these cells, PTEN activity is relatively low, and TORC2 activity is high. Therefore, the effect of overexpressed Rheb on TORC2 may be overridden by the low PTEN activity in HEK293 cells. Consistent with the hypothesis that Rheb has an inhibitory effect on TORC2, TSC1/2 have a positive effect on the phosphorylation of Akt, as determined in both the knockout and overexpression cells (29, 30). Our data support the argument that Rheb activates TORC1 in a more direct manner, possibly by a direct interaction. However, we do not suggest that Rheb directly inhibits TORC2. On the contrary, the inhibitory effect of Rheb on TORC2 may be rather indirect, certainly including a feedback inhibition involving TORC1 and downstream targets.

We observed that the TORC1-specific component Raptor has an inhibitory effect on TORC2, whereas the TORC2-specific component Rictor, reciprocally, has an inhibitory effect on TORC1. A possible molecular basis for these mutually inhibitory effects is that Raptor and Rictor may compete for common components of the TOR complexes, such as mTOR and mLST8. Rheb may activate TORC1 and inhibit TORC2 by increasing the ratio of TORC1 to TORC2 in cells. In addition to the negative feedback regulation, the above model suggests another mechanism by which Rheb inhibits TORC2. In conclusion, our data from both *Drosophila* S2 and mammalian cells support a model in which the effect of TSC1/2 and Rheb on TORC1 and -2 are different.

Materials and Methods

***Drosophila* RNAi.** *Drosophila* RNAi experiments were performed as described in ref. 40, with minor modification (see *Supporting Materials and Methods* and Table 1, which are published as supporting information on the PNAS web site). *Drosophila* S2 cells were cultured in 12-well plates for 4 days, with a starting density of 2×10^5 cells per well. On days 1 and 3, 4 μ g of dsRNA targeting the gene of interest was added directly to the cells. Cells were lysed at the end of day 4, with 150 μ l per well of the mild lysis buffer (10

mM Tris-HCl, pH 7.5/100 mM NaCl/1% Nonidet P-40/50 mM NaF/2 mM EDTA/1 mM DTT/1 mM PMSF/10 μ g/ml leupeptin/10 μ g/ml aprotinin). Cell lysates were analyzed by Western blot.

Immunoprecipitations and Kinase Assay. HEK293 cells were cultured in 10-cm plates. When the cell density reached 3×10^6 cells per plate, cells were transfected with either TORC1 components [MYC-mTOR, hemagglutinin (HA)-Raptor, and MYC-mLST8/G β L] or TORC2 components (MYC-mTOR, HA-Rictor/mAVO3, and MYC-mLST8/G β L); MYC-Rheb (200 ng) was cotransfected where indicated. Cells were lysed in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer as described in ref. 3. To immunoprecipitate TORC1 or -2, 1 μ g of anti-HA (HA-Raptor or HA-Rictor/mAVO3) antibody was added to each of the cellular lysates and incubated at 4°C for 90 min. Then 20 μ l of protein G Sepharose slurry (50%) was added to the lysates and incubated for another hour. Immunoprecipitates were washed four times in the lysis buffer. At the last wash, the beads were divided equally for parallel experiments. For kinase assay, the immunoprecipitate was first washed once with kinase buffer and then incubated in 15 μ l of kinase assay reaction mix at 37°C for 30 min. Kinase assay reactions were designed as reported in refs. 3 and 11. To stop the reaction, 5 μ l of 4 $\times$ SDS sample buffer was added to each reaction, which was then boiled for 5 min.

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