Current Biology, Vol. 13, 1259–1268, August 5, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S0960-9822(03)00506-2

Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-Activating Protein Complex toward Rheb

Andrew R. Tee,[1] Brendan D. Manning,[1,2] Philippe P. Roux,[1] Lewis C. Cantley,[1,2] and John Blenis[1,*]
[1]Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115
[2]Division of Signal Transduction
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Summary

Background: Tuberous Sclerosis Complex (TSC) is a genetic disorder that occurs through the loss of heterozygosity of either *TSC1* or *TSC2*, which encode Hamartin or Tuberin, respectively. Tuberin and Hamartin form a tumor suppressor heterodimer that inhibits the mammalian target of rapamycin (mTOR) nutrient signaling input, but how this occurs is unclear.

Results: We show that the small G protein Rheb (Ras homolog enriched in brain) is a molecular target of TSC1/TSC2 that regulates mTOR signaling. Overexpression of Rheb activates 40S ribosomal protein S6 kinase 1 (S6K1) but not p90 ribosomal S6 kinase 1 (RSK1) or Akt. Furthermore, Rheb induces phosphorylation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and causes 4E-BP1 to dissociate from eIF4E. This dissociation is completely sensitive to rapamycin (an mTOR inhibitor) but not wortmannin (a phosphoinositide 3-kinase [PI3K] inhibitor). Rheb also activates S6K1 during amino acid insufficiency via a rapamycin-sensitive mechanism, suggesting that Rheb participates in nutrient signaling through mTOR. Moreover, Rheb does not activate a S6K1 mutant that is unresponsive to mTOR-mediated signals, confirming that Rheb functions upstream of mTOR. Overexpression of the Tuberin-Hamartin heterodimer inhibits Rheb-mediated S6K1 activation, suggesting that Tuberin functions as a Rheb GTPase activating protein (GAP). Supporting this notion, TSC patient-derived Tuberin GAP domain mutants were unable to inactivate Rheb in vivo. Moreover, in vitro studies reveal that Tuberin, when associated with Hamartin, acts as a Rheb GTPase-activating protein. Finally, we show that membrane localization of Rheb is important for its biological activity because a farnesylation-defective mutant of Rheb stimulated S6K1 activation less efficiently.

Conclusions: We show that Rheb acts as a novel mediator of the nutrient signaling input to mTOR and is the molecular target of TSC1 and TSC2 within mammalian cells.

Introduction

TSC is a human genetic syndrome characterized by the formation of benign tumors in the brain, heart, kidneys, eyes, and skin (for review see [1]). TSC mainly occurs through the loss of heterozygosity of the *TSC1* or *TSC2* gene, encoding for Hamartin and Tuberin protein, respectively [2, 3]. Tuberin and Hamartin form a heterodimer that inhibits cell growth and proliferation [3–6]. As a consequence, the loss of function of either Hamartin or Tuberin can lead to improperly regulated cell growth and tumorigenesis.

Hamartin, a 130 kDa protein, contains a putative transmembrane domain, implying that it is membrane bound, as well as two coiled-coil domains that are necessary for its association with Tuberin [7, 8]. Hamartin also interacts with the ezrin-radixin-moesin family member proteins and activates the small-GTP binding protein Rho [9], suggesting that Hamartin regulates cell adhesion. Hamartin's binding partner, Tuberin (198 kDa), contains a region of limited homology to the catalytic domain of Rap1GAP at its C terminus. Tuberin has been reported to weakly stimulate GTP hydrolysis of Rap1a and Rab5 but not of Rap2, Ras, Rab3a, Rab6, Rac, or Rho in vitro [10, 11]. However, Tuberin has been suggested to function as a RhoGAP in vivo [12].

Both *Drosophila* and mammalian studies demonstrated that Tuberin/Hamartin act upstream of S6K1 and downstream of Akt (also referred to as protein kinase B (PKB)) within the PI3K signaling pathway [13–16]. A number of reports that followed revealed that Tuberin/Hamartin inhibited the nutrient signaling input to mTOR [17–19]. mTOR is a known regulator of both cell growth and cell proliferation, which are coordinated by at least two downstream mTOR targets, S6K1 and 4E-BP1 (also known as PHAS-I) [20]. Loss of either Hamartin or Tuberin expression within cells resulted in constitutive S6K1 activation in the absence of amino acids [18]. Furthermore, overexpression of Hamartin and Tuberin blocked S6K1 activation upon the readdition of amino acids to nutrient-starved cells [18, 19].

It is unclear how mTOR senses nutrients, but it is likely that it senses intracellular rather than extracellular amino acids through an unknown mechanism [21]. The mechanism by which the Tuberin/Hamartin heterodimer inhibits the nutrient-mediated input to mTOR is currently undefined. Mutational analysis of *TSC2* from TSC patients suggested that the GAP domain of Tuberin is critical for its function [22]. For instance, common TSC patient-derived *TSC2* mutations result in the loss of the GAP domain of Tuberin through C-terminal truncations, whereas some of the more infrequent *TSC2* point mutations are clustered within the GAP domain [22]. It is, therefore, possible that Tuberin possesses GAP activity toward a small G protein, which would normally enhance mTOR signaling when in an active GTP bound state during conditions of nutrient sufficiency.

Rheb (Ras homolog enriched in brain) is a likely small G protein candidate that may be involved during nutrient signaling. The loss of Rheb in yeast was shown to mimic nutrient starvation by causing G_0/G_1 arrest [23]. Deletion of Rheb in *S. cerevisiae* also resulted in the increased uptake of both arginine and lysine [24]. Rheb is related

*Correspondence: john_blenis@hms.harvard.edu

to the Ras, Rap, and Ral subfamilies [25] and is farnesylated within a C-terminal CaaX motif (a is an aliphatic amino acid, and X is a serine, alanine, glutamine, cysteine or methionine) [26]. Rheb has also been shown to cooperate synergistically with Raf-1 to transform NIH3T3 cells [26] and to bind and inhibit B-Raf kinase [27]. In this study we reveal that Rheb enhances mTOR-mediated signaling toward S6K1 and 4E-BP1. Overexpression of Rheb was sufficient to enhance S6K1 activity in the absence of nutrients, indicating that Rheb may be a component of the nutrient-sensing machinery of mTOR. We also report that Tuberin-Hamartin heterodimers inhibit Rheb-induced mTOR signaling, which requires an intact GAP domain of Tuberin. Furthermore, we show that Tuberin when associated with Hamartin functions as a GAP toward Rheb.

Results

Rheb Overexpression Activates S6K1 but Not Akt or RSK1

To examine whether Rheb overexpression could modulate S6K1 activity, we coexpressed S6K1 with Rheb at two different expression levels in HEK293E cells and assayed for kinase activity by using GST-S6 as a substrate (Figure 1A). Coexpression of Rheb at either expression level significantly increased the basal and insulin-stimulated activity of S6K1. Higher levels of Rheb expression enhanced the basal and insulin-stimulated activity of S6K1 by 5.6- and 1.7-fold, respectively, and this activity level was more potent than the S6K1 activity observed in the presence of lower levels of Rheb protein. These results indicate that Rheb activates signaling cascades that result in S6K1 activation.

Given that the activity of S6K1 is enhanced upon cell signaling through mTOR- [28], PI3K- (for review see [29]), and mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-mediated pathways [30], we determined which signaling pathway was activated upon Rheb overexpression. To investigate PI3K-mediated signaling, we coexpressed Rheb with Akt, a downstream target of PI3K, within HEK293E cells and assayed for kinase activity by using GST-tagged glycogen synthase kinase-3β (GSK-3β) as a substrate (Figure 1B). Whereas EGF stimulation led to a 4-fold increase in Akt activity, Rheb overexpression did not enhance basal or EGF-stimulated Akt activity. In contrast, Rheb potently activated S6K1 by 11-fold when assayed in parallel. We used wortmannin as a control to show that we were specifically measuring PI3K-mediated activation of Akt upon stimulation with EGF. To examine whether Rheb enhanced MAPK-mediated signaling, we coexpressed Rheb with RSK1, a known downstream signaling component of MAPK, within HEK293E cells (Figure 1C). Although EGF stimulation led to a 12-fold increase in RSK1 activity, Rheb did not augment the basal or EGF-induced activation of RSK1 assayed with GST-S6 as a substrate. In contrast, Rheb overexpression drastically increased S6K1 activity when assayed in parallel. To inhibit activation of ERK (extracellular signal-regulated kinase) through MEK (MAPK/ERK-kinase)-mediated signaling, we treated cells with the U0126 compound to specifically inhibit MEK (as observed by reduced ERK1 and ERK2 phosphorylation and RSK1 activity). These findings suggest that Rheb does not function upstream of either PI3K/Akt or ERK/RSK1 signaling pathways.

Rheb Induces 4E-BP1 Phosphorylation, which Is Rapamycin Sensitive and Wortmannin Insensitive

Because Rheb enhanced the activity of S6K1, a downstream component of mTOR, we investigated the effects of Rheb-mediated signaling on 4E-BP1, another downstream component of mTOR. Dephosphorylated species of 4E-BP1 bind to and inhibit eIF4E-driven cap-dependent translation (for review see [31]). Phosphorylation of 4E-BP1 at multiple Ser/Thr-Pro residues upon mitogenic stimulation leads to the release of 4E-BP1 from eIF4E, which is blocked by both rapamycin and nutrient starvation. Three different phosphorylated species of 4E-BP1 resolve on SDS-PAGE, with γ- and α-isoforms being the most and least phosphorylated species, respectively. To determine whether Rheb activates mTOR- or PI3K-mediated signaling, we coexpressed Rheb with hemagglutinin (HA)-tagged 4E-BP1 in the presence of either rapamycin or wortmannin to inhibit mTOR or PI3K, respectively (Figure 2A). Insulin-induced Akt phosphorylation on Ser473 was blocked by wortmannin, revealing that the concentration of wortmannin used in this study efficiently inhibited PI3K-mediated signaling. Insulin-induced phosphorylation of 4E-BP1 was also blocked by wortmannin, as observed by the reduced mobility shift of 4E-BP1 to the less-phosphorylated isoforms and by decreased Ser65 phosphorylation. Rheb overexpression within serum-starved cells potently enhanced 4E-BP1 phosphorylation, which was still sensitive to rapamycin. In contrast, treatment of cells with wortmannin was modestly effective at reducing 4E-BP1 phosphorylation upon Rheb overexpression, indicating that PI3K signaling is not essential for Rheb-induced 4E-BP1 phosphorylation. These data suggest that Rheb signals by using an mTOR-dependent rather than a PI3K-dependent mechanism. Rheb-induced 4E-BP1 phosphorylation should promote the release of 4E-BP1 from eIF4E. To confirm this, we purified endogenous eIF4E on m^7GTP-Sepharose, which mimics the cap-structure found at the extreme 5′ terminus of most cytoplasmic mRNAs, and examined how much HA-tagged 4E-BP1 was bound to eIF4E (Figure 2B). As expected, 4E-BP1 was released from eIF4E upon Rheb overexpression, implying that Rheb activates cap-dependent translation.

Rheb Overexpression Enhances Nutrient-Mediated Signaling toward S6K1

The above data implies that Rheb may modulate mTOR signaling. Given that the loss of Rheb in yeast mimicked nutrient starvation [23], Rheb overexpression may promote mTOR signaling through a nutrient-regulated signaling pathway. To address this possibility, we investigated whether Rheb promotes S6K1 activation in the absence of amino acids (Figure 3). During conditions of amino acid withdrawal, Rheb overexpression potently activated S6K1 (Figure 3, lane 6), which was completely blocked by rapamycin (Figure 3, lane 7) but only partially

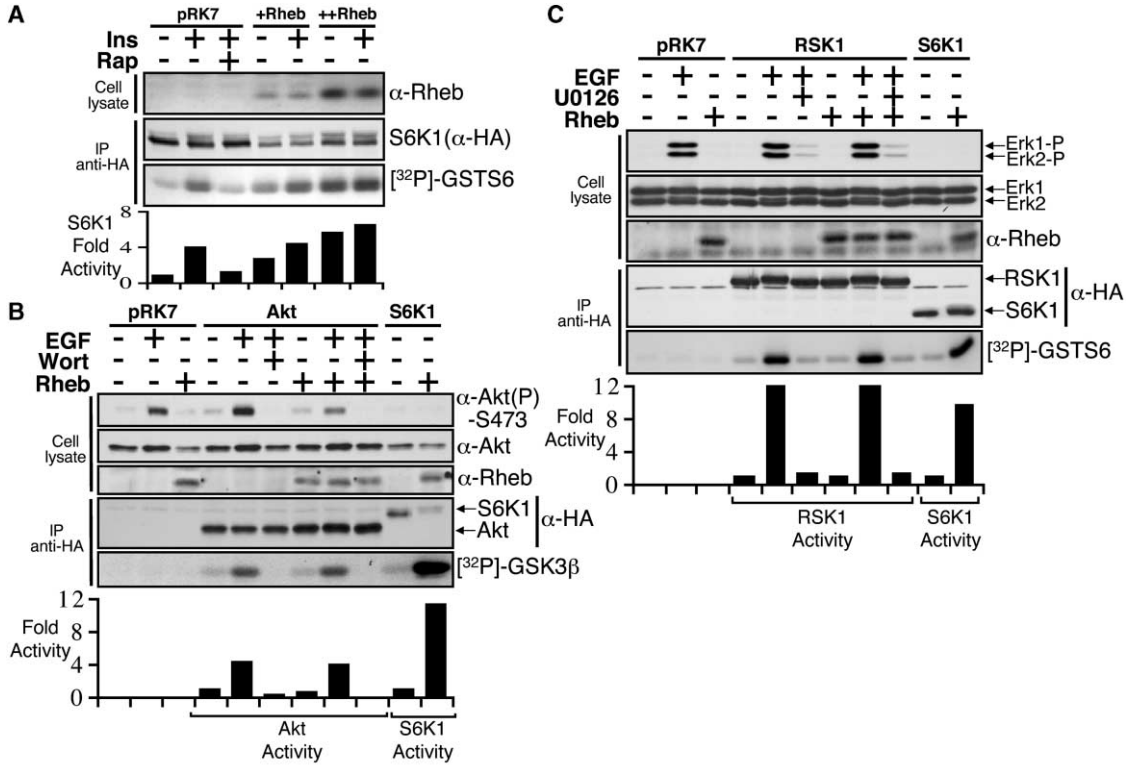

Figure 1. Rheb Overexpression Activates S6K1 and Not RSK1 or Akt

(A) HEK293E cells coexpressing low (+) or high (++) levels of Rheb, where indicated, with HA-tagged S6K1 were serum starved. The cells were pretreated with 25 nM rapamycin for 30 min and stimulated with 100 nM insulin for 30 min, where indicated. The total levels of Rheb and S6K1 are shown. S6K1 activity was measured by incorporation of ^{32}P label into GST-S6 substrate, as described in the Experimental Procedures, and was assessed by autoradiograph.

(B) HEK293E cells transiently transfected with plasmids expressing Rheb and HA-tagged Akt and S6K1, where indicated, were serum starved. These cells were pretreated with 100 nM wortmannin (Wort) for 30 min before being stimulated with 25 ng/ml EGF for 10 min, where stated. Akt and S6K1 activity was measured as described in the Experimental Procedures. The total levels of Akt, Rheb, and S6K1 and Akt phosphorylation on Ser473 are shown.

(C) HEK293E cells expressing Rheb and HA-tagged RSK1 and S6K1, where indicated, were serum starved. These cells were pretreated with 2.5 μM U0126 for 30 min before being stimulated with 25 ng/ml EGF for 10 min, where indicated. RSK1 and S6K1 activity was measured as described in the Experimental Procedures. The total levels of Erk1/2, Rheb, RSK1, and S6K1 are shown as well as Erk1/2 phosphorylation. Fold activation of S6K1, Akt, and RSK1 from untreated conditions is graphed, where applicable.

inhibited by wortmannin (Figure 3, lane 8). Importantly, insulin stimulation of these amino acid-deprived cells potently activated Akt (as observed by Akt phosphorylation on Ser473) but only weakly activated S6K1 (Figure 3, lane 9). Therefore, unlike Rheb-mediated signaling, acute stimulation of PI3K and Akt is not sufficient to fully activate S6K1 during nutrient insufficiency. The modest insulin-induced activation of S6K1 during amino acid insufficiency was blocked by wortmannin (Figure 3, lane 11), revealing that this activation is completely dependent on PI3K. Activation of S6K1 upon readdition of amino acids (Figure 3, lane 12) was enhanced when Rheb was overexpressed or when cells were stimulated with insulin (Figure 3, lanes 13 and 16, respectively). Interestingly, Rheb-induced S6K1 activation upon readdition of amino acids was completely inhibited by rapamycin but only partially inhibited by wortmannin (Figure 3, lanes 14 and 15, respectively). In contrast, insulin-induced S6K1 activity was markedly impaired by both rapamycin and wortmannin. These data convincingly reveal that Rheb potently activates S6K in the absence of nutrients through mTOR rather than PI3K-mediated signaling. Therefore, it is likely that Rheb enhances nutrient-mediated signaling through mTOR.

Rheb Overexpression Specifically Enhances mTOR-Mediated Signaling to S6K1

To decisively determine whether Rheb positively activates mTOR signaling, we made use of a rapamycin-resistant mutant of S6K1 (S6K1-F5A-ΔCT) [32] (Figure 4). Unlike treatment with wortmannin, rapamycin treatment was unable to prevent insulin-induced activation of S6K1-F5A-ΔCT (Figure 4, lane 11), demonstrating that this mutant is responsive to PI3K signaling but not mTOR signaling. As a positive control, we coexpressed PDK1 and PKCζ, which are known to activate S6K1 through a PI3K-dependent input [33]. Overexpression of Rheb potently activated wild-type S6K1 basally (by 11-fold; Figure 4, lane 5) and during insulin stimulation (by 17-fold; Figure 4, lane 5) but did not enhance the activity of the S6K1-F5A-ΔCT mutant (Figure 4, lanes 13 and 14). In contrast, increased PI3K-mediated signaling toward

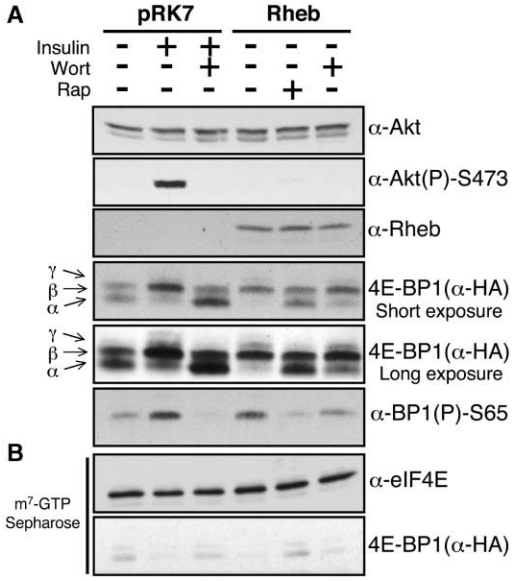

Figure 2. Rheb Induces 4E-BP1 Phosphorylation, which is Rapamycin Sensitive and Wortmannin Insensitive

(A) HEK293E cells expressing Rheb, where indicated, with HA-tagged 4E-BP1 were serum starved. The cells were pretreated with 25 nM rapamycin or 100 nM wortmannin for 30 min and then stimulated with 100 nM insulin for 30 min, where indicated. The phosphorylation of exogenous 4E-BP1 was determined with an anti-HA antibody and phospho-specific antibodies for Ser65 as indicated. The α, β, and γ species of 4E-BP1 are labeled accordingly. Rheb and Akt protein levels as well as Akt phosphorylation on Ser473 were determined.

(B) The cell extracts from Figure 2A were subjected to affinity chromatography on m^{7}GTP-Sepharose, as described in the Experimental Procedures. The protein levels of both endogenous eIF4E and HA-tagged 4E-BP1 were determined.

S6K1 by coexpression of PDK1 and PKCζ resulted in significantly enhanced activation of both wild-type S6K1 (Figure 4, lanes 7 and 8) and S6K1-F5A-ΔCT (Figure 4, lanes 15 and 16). These findings strongly suggest that Rheb induces S6K1 activation via a signaling input that is upstream of mTOR but not PI3K.

The Tuberin/Hamartin Heterodimer Inhibits Rheb and Requires the GAP Domain of Tuberin

Previous work revealed that overexpression of wild-type Rheb led to a significant increase in its activity [27] and implies that the majority of the overexpressed Rheb must exist in the active GTP bound form. If this is true, then the RhebGAP activity must be a limiting factor. If Tuberin possesses RhebGAP activity, overexpression of Tuberin should switch Rheb from an active GTP bound state to an inactive GDP bound state. To indirectly measure Rheb activity, we analyzed Rheb-induced S6K1 activation within nutrient-deprived HEK293E cells (Figure 5A). We observed that coexpression of Hamartin and Tuberin completely blocked Rheb's ability to activate S6K1, implying that Tuberin may function as a RhebGAP.

If the GAP domain of Tuberin is essential for Rheb inactivation, then patient-derived *TSC2* GAP domain point mutants should not block Rheb-induced S6K1 activation. To address this, we generated three Tuberin mutants that mimic patient-derived *TSC2* mutations that occur within the GAP domain and coexpressed these Tuberin mutants with Hamartin and S6K1. Under serum-starved conditions, Rheb potently activated S6K1, which was fully blocked by coexpression of wild-type Tuberin with Hamartin (Figure 5B). In contrast, the three *TSC2* GAP domain point mutants were unable to repress Rheb-induced S6K1 activation, revealing that the GAP domain of Tuberin is critical for Tuberin's ability to repress Rheb-mediated signaling.

Tuberin and Hamartin Act as a GTPase-Activating Protein Complex toward Rheb

Our in vivo overexpression data strongly suggests that the Tuberin-Hamartin heterodimer inhibits Rheb function. In order to test whether this is a direct inhibition due to the GAP activity of Tuberin, we performed in vitro GAP assays on purified Rheb. We expressed Flag-tagged Hamartin and Flag-tagged Tuberin separately or together in HEK293 cells and immunoprecipitated the respective protein(s) for use in Rheb-GAP assays. Interestingly, immunoprecipitated Hamartin or Tuberin displayed nearly identical GAP activity toward Rheb; both

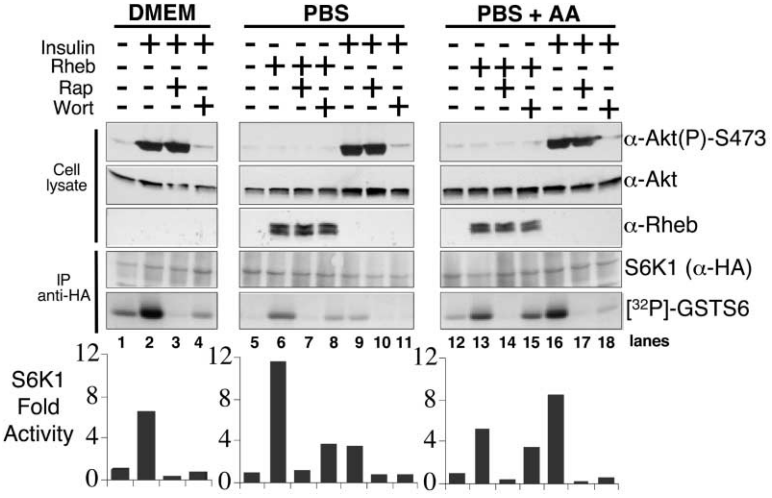

Figure 3. Rheb Overexpression Enhances S6K1 Activity in Nutrient-Deprived HEK293E Cells

HEK293E cells overexpressing Rheb, where indicated, with S6K1 were serum starved and nutrient deprived (labeled as "D-PBS"), as described in the Experimental Procedures, for 1 hr. Cells were pretreated with either 25 nM rapamycin or 100 nM wortmannin for 30 min (where stated) before the readdition of amino acids (labeled as "PBS + AA") for 1 hr in the continued presence of inhibitors. Cells were stimulated with 100 nM insulin for 30 min, where indicated. Protein levels of Rheb and Akt as well as Akt phosphorylation on Ser473 where determined. S6K1 kinase assays were carried out as for Figure 1. The graphs show the activity of S6K1 that is standardized to 1 for the untreated sample for each cell medium used (i.e., DMEM, PBS, and PBS + AA).

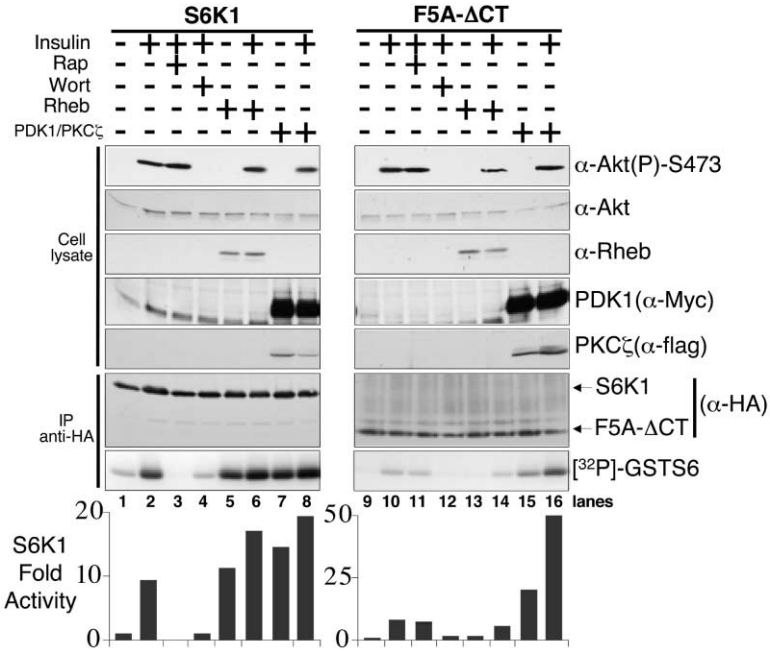

Figure 4. Rheb Overexpression Does Not Activate the mTOR-Insensitive S6K1 Mutant, S6K1-F5A-ΔCT

HEK293E cells coexpressing wild-type S6K1 or S6K1-F5A-ΔCT (F5A-ΔCT) with either Rheb alone or Myc-tagged PDK1 and Flag-tagged PKCζ together were serum starved. These cells were pretreated with either 25 nM rapamycin (Rap) or 100 nM wortmannin (Wort) for 30 min (where stated) prior to being stimulated with 100 nM insulin for 30 min, where indicated. Protein levels of Rheb, PDK1, PKCζ, and Akt as well as Akt phosphorylation on Ser473 were determined. S6K1 kinase assays were carried out as for Figure 1. The activity of wild-type S6K1 and S6K1-F5A-ΔCT are graphed separately. The serum-starved controls are standardized to 1 within the graphs.

enhanced the intrinsic GTPase activity of Rheb (vector) by approximately 2-fold (Figures 6A and 6B). This suggests that a complex between Tuberin and Hamartin is essential for Tuberin's GAP activity toward Rheb and that endogenous levels of coimmunoprecipitating Tuberin or Hamartin are limiting in these reactions. In support of this, coexpression and immunoprecipitation of both Tuberin and Hamartin resulted in immune complexes that enhanced Rheb GTPase activity by more than 100-fold over the activity of either alone and approximately 200-fold over intrinsic Rheb activity (Figures 6A and 6B). This dramatic increase in GAP activity is detected despite no significant difference in the amount of Tuberin immunoprecipitated when expressed alone or with Hamartin (not shown). Therefore, Tuberin and Hamartin together form a GTPase-activating protein

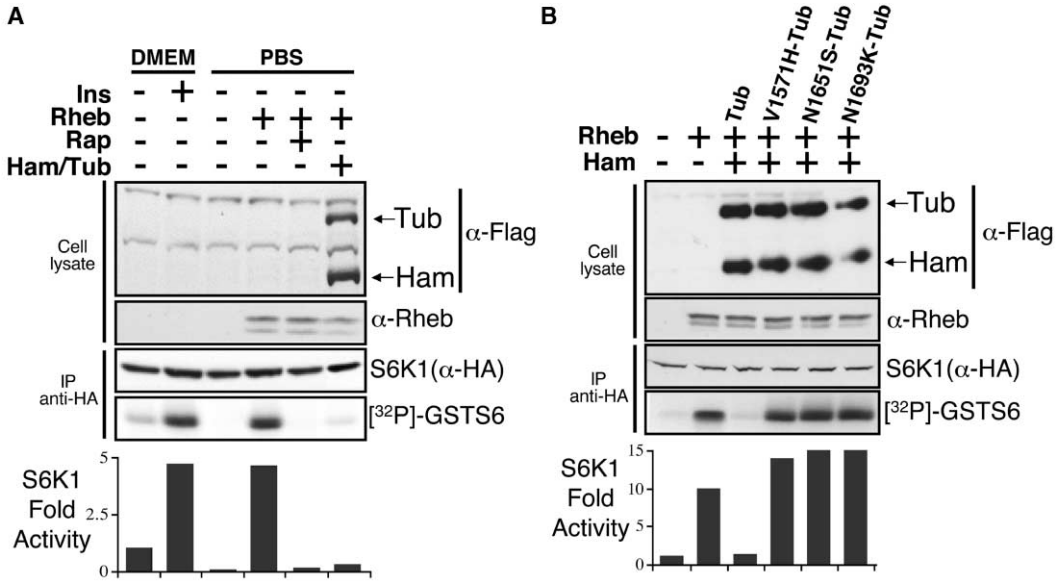

Figure 5. Tuberin-Hamartin Heterodimers Inhibit Rheb and Require the GAP Domain of Tuberin

(A) Rheb was coexpressed in HEK293E cells with HA-tagged S6K1 with or without Hamartin and Tuberin (Ham/Tub), as indicated. These cells were serum starved (DMEM) and then nutrient deprived (D-PBS) for 1 hr, as for Figure 3. Cells were pretreated with 25 nM rapamycin for 30 min, where indicated. S6K1 kinase assays were carried out as for Figure 1, and an autoradiograph of the gel showing ^{32}P label incorporated into GST-S6 is presented. The total levels of Hamartin, Tuberin, Rheb, and S6K1 are shown.

(B) HEK293E cells coexpressing Rheb with or without Hamartin (Ham), Tuberin (Tub), and the TSC patient-derived Tuberin mutants (V1571H-Tub, N1651S-Tub, and N1693K-Tub), as indicated, with HA-tagged S6K1 were serum starved. The samples were analyzed as for Figure 5A.

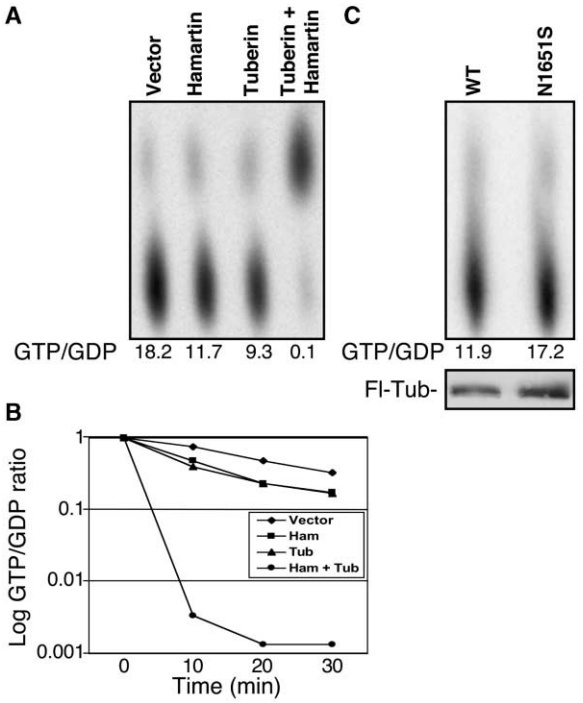

Figure 6. The Tuberin-Hamartin Heterodimer Is a GAP for Rheb

(A) Tuberin and Hamartin together form a functional Rheb GAP complex. Flag-Hamartin, Flag-Tuberin, or both were immunoprecipitated from HEK293 cells and assayed for GAP activity toward purified GST-Rheb (see Experimental Procedures). Shown are [α-^{32}P]GTP and GDP eluted from Rheb after a 10 min incubation with the specified immune complexes and resolved by one-dimensional thin-layer chromatography. The ratio of GTP to GDP was determined by phosphoimager and is given for each assay. GAP activity is seen as a decrease in the GTP-to-GDP ratio relative to vector alone (i.e., intrinsic Rheb GTPase activity).

(B) Time course of GAP activity toward Rheb. Rheb GAP assays were performed as described in (A), and the GTP-to-GDP ratio was determined after 10, 30, and 60 min incubations with the specified immune complexes. The graph is given as the log of the GTP-to-GDP ratio and is normalized to the 0 time point, in which [α-^{32}P]GTP-loaded Rheb was incubated on ice with M2-agarose beads.

(C) A patient-derived GAP domain mutant of Tuberin is defective in Rheb GAP activity. Flag-Tuberin and Flag-Tuberin^{N1651S} were assayed for GAP activity toward purified GST-Rheb as described in (A), and the resulting GTP-to-GDP ratios are given.

complex that greatly enhances the intrinsic GTPase activity of Rheb.

In order to test if this activity is potentially important in the prevention of the TSC disease, we compared the GAP activity of wild-type Tuberin to that of a patient-derived mutant mapped to the Tuberin GAP domain (N1651S). Compared to wild-type Tuberin, the Tuberin(N1651S) mutant was greatly reduced in its ability to enhance Rheb GTPase activity. This suggests that there is a correlation in the ability of Tuberin to act as a GAP toward Rheb and its ability to suppress the TSC disease.

Rheb Farnesylation Is Required for Optimal Activation of S6K1

Previous work has shown that farnesylation of Rheb is required for cell cycle progression of *S. pombe* [34].

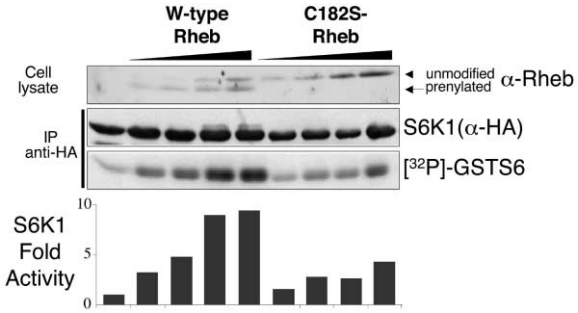

Figure 7. Rheb Farnesylation Is Required for Optimal Activation of S6K1

Wild-type Rheb or a farnesylation-deficient mutant of Rheb (C182S-Rheb) was coexpressed at four different levels with HA-tagged S6K1 within HEK293E cells that were serum starved. Protein levels of Rheb (determined with N19 anti-Rheb antibodies) and S6K1 were determined. S6K1 kinase assays were carried out as for Figure 1. Fold activation of S6K1 from untreated control is graphed.

To investigate whether farnesylation was important for Rheb's ability to activate S6K1, we generated a farnesylation-defective Rheb(C182S) mutant, in which the cysteine within the farnesylation CAAX motif is substituted for a serine. When overexpressed, the Rheb(C182S) mutant was less efficient at enhancing S6K1 activity than wild-type Rheb (Figure 7). The mutant Rheb(C182S) protein migrated as the upper band on SDS-PAGE, which indicates that it is not being prenylated and is consistent with earlier studies showing that prenylated Rheb migrates more quickly on SDS-PAGE [24]. In contrast, the majority of wild-type Rheb resolved as the lower prenylated band. These findings suggest that the membrane localization of Rheb through farnesylation is important for Rheb to efficiently augment mTOR-mediated signaling.

Discussion

In this study we show that Rheb functions upstream of mTOR within the nutrient signaling pathway. We reveal that Rheb specifically activates mTOR-mediated signaling rather than cell signaling through MEK/ERK and PI3K, as shown by Rheb-mediated activation of S6K1 (Figure 1A) but not Akt or RSK1 (Figures 1B and 1C, respectively). Therefore, it is unlikely that Rheb activates PI3K and Raf, two downstream effectors of Ras (for review, see [35]). Rheb has previously been shown to interact with Raf in vitro [26], but our data suggest that Raf is not an effector of Rheb in vivo. Additionally, Rheb overexpression does not increase the activity of the rapamycin-resistant S6K1 mutant (F5A-ΔCT) that is unresponsive to mTOR signaling inputs but is activated in response to PI3K signaling (Figure 4). S6K1 activation is regulated by multiple signaling inputs, one of which is directed by PI3K. Therefore, these findings are important and confirm that Rheb overexpression specifically promotes mTOR rather than PI3K signaling. Furthermore, we show that Rheb-induced 4E-BP1 phosphorylation is completely sensitive to rapamycin but not to wortmannin (Figure 2A), which further strengthens the notion that Rheb acts upstream of mTOR rather than PI3K. 4E-

BP1 dissociates from eIF4E upon Rheb overexpression (Figure 2B), revealing that Rheb-mediated signaling through mTOR promotes cap-dependent translation.

We also provide evidence that Rheb functions within the nutrient signaling cascade upstream of mTOR, as shown by Rheb's ability to potently stimulate S6K1 activity during amino acid insufficiency (Figure 3). During amino acid withdrawal, acute insulin stimulation was still able to elicit high levels of Akt phosphorylation but poorly activated S6K1 (Figure 3), showing that the nutrient-mediated mTOR signaling input is essential for optimal S6K1 activation, as previously reported (for review see [36]). Therefore, Rheb overexpression supersedes the dependency of the nutrient input to mTOR, suggesting that Rheb is an activator of mTOR within the nutrient-signaling pathway. Interestingly, resupplying cells with amino acids further enhanced the activity of S6K1 when Rheb was overexpressed (Figure 3), suggesting that amino acids may promote the activation of Rheb. Our research as well as that of others revealed that the Tuberin-Hamartin heterodimer functioned as an inhibitor of nutrient signaling through mTOR [17–19]. We show that the Tuberin-Hamartin heterodimer inhibits Rheb-induced S6K1 activation during conditions of amino acid withdrawal (Figure 5A). Our work, therefore, extends these earlier studies revealing that inhibition of Rheb is the mechanism by which the Tuberin-Hamartin heterodimer inhibits nutrient-mediated signaling. Importantly, the Rheb-inhibitory function of Tuberin-Hamartin heterodimers depends on an intact Tuberin GAP domain; patient-derived point mutations within the GAP domain of *TSC2* prevented the Tuberin-Hamartin heterodimer from blocking Rheb-induced S6K1 activation (Figure 5B). These data indicate that the GAP activity of Tuberin promotes inactivation of Rheb in vivo, presumably through increasing the intrinsic GTPase activity of Rheb. Confirming this hypothesis, in vitro Rheb GTPase activity assays revealed that Tuberin enhanced the intrinsic GTPase activity of Rheb (Figures 6A and 6B). Interestingly, coexpression of Hamartin with Tuberin markedly enhanced the GTPase activity of Rheb, implying that Hamartin promotes the GAP function of Tuberin toward Rheb. We propose a model whereby Rheb promotes mTOR signaling when it is in an active GTP bound form, whereas the Tuberin-Hamartin heterodimer inhibits Rheb by converting it to an inactive GDP bound state (Figure 8). These findings reveal that the Tuberin-Hamartin heterodimer and Rheb respectively inhibit and activate the nutrient-signaling input to mTOR. Small G proteins are additionally regulated by guanine nucleotide exchange factors (GEFs). In our model we propose that a RhebGEF becomes activated during conditions of nutrient sufficiency, and its activation switches Rheb to an active GTP bound form. Therefore, identifying this Rheb-GEF will be of great importance and may provide new insights into how mTOR senses intracellular amino acids. However, at this point we cannot rule out the possibility that Rheb- and nutrient-mediated signaling may function in parallel pathways upstream of mTOR. Further experiments will be carried out to investigate this possibility.

Upon preparation of this manuscript, reports characterizing Rheb signaling in *D. melanogaster* appeared

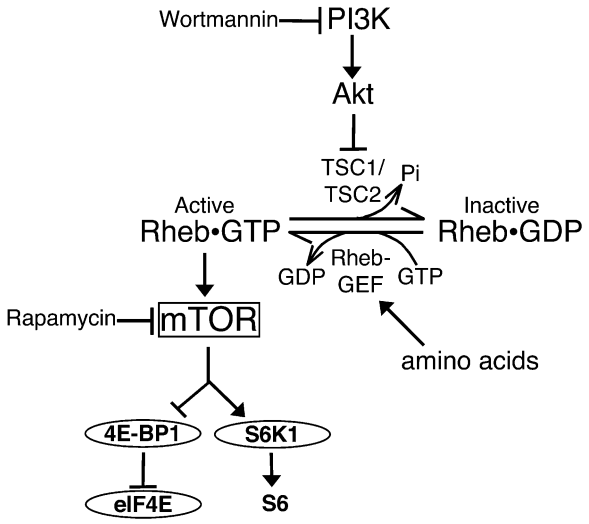

Figure 8. Model that Illustrates Rheb-Mediated Signaling through mTOR, which Is Inhibited by the TSC Tumor Suppressor Complex

When Rheb is in an active GTP bound state, its localization to the membrane stimulates mTOR-mediated signaling to downstream components S6K1 and 4E-BP1. The TSC tumor suppressor complex (TSC1/TSC2) acts as a GAP toward Rheb, which promotes hydrolysis of Rheb•GTP, converting it to an inactive GDP bound state. Amino acid-induced signaling activates RhebGEF, which converts Rheb•GDP to the active Rheb•GTP and promotes mTOR signaling. Akt phosphorylates TSC2, which inactivates the TSC tumor suppressor complex.

[37–39]. Collectively, these papers show that *Drosophila* Rheb promotes cell growth and proliferation by promoting dTOR/dS6K1-mediated signaling and that Rheb is a molecular target of dTSC1/2. Epistasis analysis placed Rheb downstream of dTSC1/2 and upstream of dTOR [37, 38]. In the absence of nutrients, overexpression of Rheb enhanced the growth and proliferation of *D. melanogaster* cells [37] and also potently activated dS6K1 [37, 38]. Work carried out by Zhang et al. also revealed that *TSC2* functioned as a GAP toward Rheb and that patient-derived mutations of *TSC2* within the GAP domain prevented *TSC2* RhebGAP activity [39]. These experiments complement our work revealing that the function of Rheb is conserved between flies and mammals.

We further show that Rheb farnesylation is important for Rheb to optimally function as an activator of mTOR signaling (Figure 7). Supporting our finding, previous reports have shown that Rheb farnesylation is required for cell cycle progression in *S. pombe* [34] and inhibition of arginine uptake in *S. cerevisiae* [24]. Farnesylation mediates the membrane localization of many proteins through increased hydrophobicity [40]. Many signaling components of mTOR are tethered to the membrane. For instance, it is likely that Tuberin-Hamartin heterodimers are localized to the membrane through the transmembrane domain of Hamartin [41]. mTOR is also found within membrane fractions [42]. Therefore, farnesylation of Rheb may recruit Rheb to the mTOR signaling complex located at the membrane. However, the farnesylation-deficient Rheb was still able to activate S6K1 to a lesser degree than the wild-type when it was expressed

at higher levels (Figure 7). One explanation for this observation is that, during our expression studies, a higher proportion of Rheb is sufficiently proximal to the membrane without being farnesylated to activate mTOR signaling. The decreased ability of the farnesylation-deficient Rheb to activate mTOR signaling suggests that farnesylation inhibitors might be effective drugs in the treatment of the TSC disease.

It is likely that tumors within TSC patients possess a higher proportion of the active GTP bound form of Rheb as a consequence of nonfunctional Tuberin-Hamartin heterodimers. It is also possible that TSC is caused through Rheb mutations that lead to its constitutive activation, in a manner analogous to transforming Ras mutations of Gln61 [43]. Given that genetic analysis does not always show loss of heterozygosity of either *TSC1* or *TSC2* within TSC patient lesions [44], it would be important to investigate whether these lesions occur as a consequence of *Rheb* mutations. Rheb's name was derived from the discovery that Rheb was enriched in the brain [25]. The abundance of Rheb within the brain may explain why TSC patients preferentially acquire brain lesions when compared to lesions in other organs (for review see [1]).

Conclusions

This work identifies Rheb as a novel molecular target of *TSC1* and *TSC2* and as an upstream regulator of mTOR-mediated signaling. We show that Rheb overexpression potently enhances the activity of S6K1 during conditions of nutrient withdrawal. Furthermore, we show that Tuberin when associated with Hamartin functions as a RhebGAP. Additional studies will have to be carried out to characterize the mechanism by which Rheb modulates cell signaling through mTOR. Moreover, elucidation of how Rheb functions within the nutrient signaling pathway and identification of the RhebGEF will help determine how mTOR senses the levels of intracellular amino acids. This study reveals Rheb as a suitable molecular drug target for the treatment of TSC as well as a key regulatory component of mTOR signaling.

Experimental Procedures

Chemicals and Materials

Wortmannin and U0126 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA), and insulin was bought from Sigma. Epidermal growth factor (EGF) was purchased from Invitrogen (Carlsbad, CA), and radio-labeled ATPγ[^{32}P] was purchased from NEN Life Science Products (Boston, MA). All other reagents were obtained from VWR Scientific (WestChester, PA), unless otherwise stated.

Plasmids

Human *Rheb* cDNA (bought from ATCC, accession number B1599937) was subcloned into pRK7. The GST-Rheb construct was made by PCR cloning of the human Rheb cDNA in frame into the BamHI and EcoRI sites of pGEX-4T2. pRK7 expressing N-terminal Flag-tagged Hamartin and Tuberin [19] and N-terminal HA-tagged wild-type and F5A-Δct S6K1 were generated as previously described [32]. pRK7 expressing N-terminal HA-tagged RSK1 was generated as previously described [45]. pACTAG2 expressing human HA-tagged 4E-BP1 was a kind gift from N. Sonenberg (McGill University, Montreal, Canada). The Rheb and Tuberin point mutants used in this study were generated by site-directed mutagenesis according to the manufacturer's instructions (QuikChange; Stratagene). pCMV6 expressing N-terminal HA-tagged Akt was obtained from P. Tsichlis (Thomas Jefferson University, Philadelphia, PA).

Mammalian Tissue Culture, Transfection, and Sample Preparation

Human embryonic kidney 293E (HEK293E) cells were cultured (at 37°C within 5% CO_2) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfections were performed by $CaPO_4$ as previously described [32]. Empty pRK7 was used so that all transfection mixes contained 6 μg of total DNA per 6 cm^2 plate. To deprive cells of amino acids, we washed the cells once and incubated them with D-PBS containing 1 mg/ml D-glucose (Gibco BRL). To resupply cells with amino acids, we replaced the media with D-PBS (pH 7.2) (1 mg/ml D-glucose) supplemented with 5$\times$ amino acid solution (MEM, Gibco BRL). eIF4E was purified via affinity chromatography on m^{7}GTP-Sepharose as previously described [20]. After cells were treated, they were washed twice with STE (pH 7.2) buffer and then harvested with lysis buffer (10 mM KPO_4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% NP-40, 0.1% Brij 35, 1 mM sodium orthovanadate, 40 mg/ml phenylsufonyl fluroride, 10 ug/ml leupeptin, and 5 μg pepstatin [pH 7.2]) as previously described [20]. The cell extracts generated were spun at 14,000 rpm for 10 min and then stored at -80°C.

Analysis of Protein Phosphorylation and Association of Translation Factors

For Western blotting, lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and blotted with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibodies. All immunoblots were detected by enhanced chemiluminescence. Anti-Rheb antibodies (N19 and C19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rheb was detected with the C19 antibody unless otherwise stated. Anti-Flag antibodies (M2) were purchased from Eastman Kodak Company, New Haven, CT. Anti-HA antibodies were kindly provided by M. Chou (University of Pennsylvania, Philadelphia, PA). Anti-4E-BP1 and -Akt antibodies were supplied by Cell Signaling Technology. Anti-ERK1/2 antibodies were generated as previously described [46].

Immunoprecipitation and Immune Complex Kinase Assays

For immunoprecipitation studies of HA-tagged S6K1, RSK1, and Akt, cell extracts were immunoprecipitated with an anti-HA antibody bound to protein A-Sepharose (Pharmacia) for 3 hr. Immunoprecipitates were washed as previously described [47]. For determining the kinase activity of S6K1 and RSK1 from the washed immunoprecipitates in vitro, recombinant GST-S6 (32 amino acids of the C terminus) was used as a substrate, as previously described [47]. Akt activity assays were determined in vitro with recombinant GST-tagged GSK-3β that was generated by subcloning of the first 22 amino acids of GSK3β into pGEX-3X (Amersham-Pharmacia) [48]. Quantification of ^{32}P label incorporation was determined with a Bio-Rad PhosphorImager and ImageQuant software. All data presented in this study are representative of at least three experiments.

GTPase-Activating Protein Assay

GST-Rheb expressed from pGEX-4T2-Rheb was purified from *E. coli* BL21 cells after induction with 50 μM isopropyl-β-D-thiogalactopyranoside for 2 hr at 28°C. Bacterial lysates were prepared by freezing and thawing bacterial pellets in 1/50 volume TNE buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA) plus protease inhibitors (10 μg/ml phenymethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml pepstatin) followed by treatment with DNase for 10 min on ice. GST-Rheb was then precipitated with glutathione beads for 1 hr at 4°C, and the beads were washed three times in Rheb wash buffer (50 mM HEPES [pH 7.5], 0.5 M NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 0.005% SDS plus protease inhibitors), then once in PBS plus protease inhibitors. Precipitated GST-Rheb was then eluted off of the beads with an equal volume of 30 mM glutathione in PBS (pH 7.2) for 1 hr at 4°C. Eluted GST-Rheb was then analyzed and quantitated by SDS-PAGE and Coomassie staining of resolved proteins and BSA standards. This procedure resulted in a

concentration of purified GST-Rheb between 0.8 and 1 μg/μl. This GST-Rheb was then used for in vitro GAP assays as described below.

Rheb GAP assays were performed on complexes immunoprecipitated from HEK-293 cells via a modified version of the protocol previously described [49]. Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) were used for transfecting HEK 293 cells with pRK7, pRK7-Flag-TSC1, pRK7-Flag-TSC2, or pRK7-Flag-TSC2(N1651S) on 10-cm culture dishes according to the manufacturer's instructions (4 μg plasmid DNA per construct per plate). Sixteen hours after transfection, cells were treated with 100 nM wortmannin for 15 min before lysis in 1 ml NP-40 lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol [DTT], 50 mM β-glycerophosphate, and 50 mM NaF, plus protease inhibitors). Flag-tagged proteins were then immunoprecipitated for 2 hr with 80 μl of an M2-agarose affinity gel slurry (Sigma-Aldrich Co., St. Louis, MO). Immune complexes on beads were then washed three times in IP wash buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT, 50 mM β-glycerophosphate, and 50 mM NaF, plus protease inhibitors) and once in 1 ml Rheb exchange buffer (50 mM HEPES [pH 7.4], 1 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA, and 1 mM DTT plus protease inhibitors). The washed beads were then separated into four aliquots. Three of these were used for separate GAP assays, and one was resolved by SDS-PAGE and immunoblotted to determine protein levels. GST-Rheb (10 μg) was loaded with 100 μCi [α-^{32}P]GTP by incubation for 5 min at 37°C in 100 μl GTP-loading buffer (50 mM HEPES [pH 7.5], 5 mM EDTA, and 5 mg/ml BSA plus protease inhibitors). After 5 min, 2.5 μl 1 M $MgCl_2$, 100 μl cold 50 mM HEPES (pH 7.4), and 20 μl 10 mM GDP were added to the GTP-loaded Rheb kept on ice. GAP assays were initiated by the addition of 20 μl GTP-loaded Rheb mixture (approximately 1 μg GST-Rheb) to each aliquot of M2-agarose immune complexes described above. Assays were performed at room temperature with constant agitation for 10, 30, or 60 min. Reactions were stopped by the addition of 300 μl Rheb wash buffer containing 1 mg/ml BSA. M2-agarose immune complexes were removed by brief centrifugation, and nucleotide bound GST-Rheb was purified from the supernatant with 20 μl glutathione bead slurry as described above. After three washes with Rheb wash buffer, radiolabeled GTP and GDP were eluted from Rheb with 20 ul Rheb elution buffer (0.5 mM GDP, 0.5 mM GTP, 5 mM DTT, 5 mM EDTA, and 0.2% SDS) at 68°C for 20 min. Aliquots (1 μl) of each eluted reaction were resolved by thin-layer chromatography on PEI cellulose (Sigma) with KH_2PO_4 as the solvent. Relative [α-^{32}P]GTP and GDP levels were then detected and quantitated with a phosphoimager.

Acknowledgments

We thank The Tuberous Sclerosis Alliance and The Rothberg Courage Fund. This work was supported by National Institutes of Health (NIH) grants GM51405 and CA46595 awarded to J.B. and GM56203 and GM41890 awarded to L.C.C. A postdoctoral fellowship ALT2002-463 was awarded by the European Molecular Biology Organization to A.R.T. to fund this research. The Human Frontier Science Program and American Cancer Society funded postdoctoral fellowships to P.P.R. and B.D.M., respectively.

Received: June 4, 2003
Revised: June 23, 2003
Accepted: June 23, 2003
Published online: July 10, 2003

References

1. Cheadle, J.P., Reeve, M.P., Sampson, J.R., and Kwiakowski, D.J. (2000). Molecular genetic advances in tuberous sclerosis. Hum. Genet. *107*, 97–114.
2. Carbonara, C., Longa, L., Grosso, E., Borrone, C., Garre, M.G., Brisigotti, M., and Migone, N. (1994). 9q34 loss of heterozygosity in a tuberous sclerosis astrocytoma suggests a growth suppressor-like activity also for the *TSC1* gene. Hum. Mol. Genet. *3*, 1829–1832.
3. Green, A.J., Smith, M., and Yates, J.R. (1994). Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat. Genet. *6*, 193–196.
4. Green, A.J., Johnson, P.H., and Yates, J.R. (1994). The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum. Mol. Genet. *3*, 1833–1834.
5. Ito, N., and Rubin, G.M. (1999). *Gigas*, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell *96*, 529–539.
6. Onda, H., Lueck, A., Marks, P.W., Warren, H.B., and Kwiatkowski, D.J. (1999). Tsc2(+/−) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. J. Clin. Invest. *104*, 687–695.
7. van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., et al. (1997). Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. Science *277*, 805–808.
8. van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D., and van der Sluijs, P. (1998). Interaction between hamartin and tuberin, the *TSC1* and *TSC2* gene products. Hum. Mol. Genet. *7*, 1053–1057.
9. Lamb, R.F., Roy, C., Diefenbach, T.J., Vinters, H.V., Johnson, M.W., Jay, D.G., and Hall, A. (2000). The *TSC1* tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. Nat. Cell Biol. *2*, 281–287.
10. Wienecke, R., Konig, A., and DeClue, J.E. (1995). Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. J. Biol. Chem. *270*, 16409–16414.
11. Xiao, G.H., Shoarinejad, F., Jin, F., Golemis, E.A., and Yeung, R.S. (1997). The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. J. Biol. Chem. *272*, 6097–6100.
12. Astrinidis, A., Cash, T.P., Hunter, D.S., Walker, C.L., Chernoff, J., and Henske, E.P. (2002). Tuberin, the tuberous sclerosis complex 2 tumor suppressor gene product, regulates Rho activation, cell adhesion and migration. Oncogene *21*, 8470–8476.
13. Potter, C.J., Huang, H., and Xu, T. (2001). *Drosophila Tsc1* functions with *Tsc2* to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell *105*, 357–368.
14. Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev. *15*, 1383–1392.
15. Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., and Neufeld, T.P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev. *14*, 2712–2724.
16. Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell *10*, 151–162.
17. Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648–657.
18. Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R.S., Ru, B., and Pan, D. (2002). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat. Cell Biol. *4*, 699–704.
19. Tee, A.R., Fingar, D.C., Manning, B.D., Kwiatkowski, D.J., Cantley, L.C., and Blenis, J. (2002). Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc. Natl. Acad. Sci. USA *99*, 13571–13576.
20. Fingar, D.C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. *16*, 1472–1487.
21. Beugnet, A., Tee, A.R., Taylor, P.M., and Proud, C.G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. Biochem. J. *372*, 555–566.
22. Niida, Y., Lawrence-Smith, N., Banwell, A., Hammer, E., Lewis, J., Beauchamp, R.L., Sims, K., Ramesh, V., and Ozelius, L. (1999). Analysis of both *TSC1* and *TSC2* for germline mutations

in 126 unrelated patients with tuberous sclerosis. Hum. Mutat. *14*, 412–422.
23. Mach, K.E., Furge, K.A., and Albright, C.F. (2000). Loss of *Rhb1*, a Rheb-related GTPase in fission yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. Genetics *155*, 611–622.
24. Urano, J., Tabancay, A.P., Yang, W., and Tamanoi, F. (2000). The *Saccharomyces cerevisiae* Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. J. Biol. Chem. *275*, 11198–11206.
25. Yamagata, K., Sanders, L.K., Kaufmann, W.E., Yee, W., Barnes, C.A., Nathans, D., and Worley, P.F. (1994). Rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J. Biol. Chem. *269*, 16333–16339.
26. Clark, G.J., Kinch, M.S., Rogers-Graham, K., Sebti, S.M., Hamilton, A.D., and Der, C.J. (1997). The Ras-related protein Rheb is farnesylated and antagonizes Ras signaling and transformation. J. Biol. Chem. *272*, 10608–10615.
27. Im, E., von Lintig, F.C., Chen, J., Zhuang, S., Qui, W., Chowdhury, S., Worley, P.F., Boss, G.R., and Pilz, R.B. (2002). Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. Oncogene *21*, 6356–6365.
28. Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell *69*, 1227–1236.
29. Martin, K.A., and Blenis, J. (2002). Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. Adv. Cancer Res. *86*, 1–39.
30. Blenis, J., and Erikson, R.L. (1986). Stimulation of ribosomal protein S6 kinase activity by pp60v-src or by serum: dissociation from phorbol ester-stimulated activity. Proc. Natl. Acad. Sci. USA *83*, 1733–1737.
31. Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.
32. Schalm, S.S., and Blenis, J. (2002). Identification of a conserved motif required for mTOR signaling. Curr. Biol. *12*, 632–639.
33. Romanelli, A., Dreisbach, V.C., and Blenis, J. (2002). Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J. Biol. Chem. *277*, 40281–40289.
34. Yang, W., Tabancay, A.P., Jr., Urano, J., and Tamanoi, F. (2001). Failure to farnesylate Rheb protein contributes to the enrichment of G0/G1 phase cells in the *Schizosaccharomyces pombe* farnesyltransferase mutant. Mol. Microbiol. *41*, 1339–1347.
35. Wittinghofer, A., and Herrmann, C. (1995). Ras-effector interactions, the problem of specificity. FEBS Lett. *369*, 52–56.
36. Proud, C.G., Wang, X., Patel, J.V., Campbell, L.E., Kleijn, M., Li, W., and Browne, G.J. (2001). Interplay between insulin and nutrients in the regulation of translation factors. Biochem. Soc. Trans. *29*, 541–547.
37. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003). Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. Nat. Cell Biol. *5*, 559–566.
38. Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat. Cell Biol. *5*, 566–571.
39. Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat. Cell Biol. *5*, 578–581.
40. Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. Cell *57*, 1167–1177.
41. Plank, T.L., Yeung, R.S., and Henske, E.P. (1998). Hamartin, the product of the tuberous sclerosis 1 (*TSC1*) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles. Cancer Res. *58*, 4766–4770.
42. Sabatini, D.M., Barrow, R.K., Blackshaw, S., Burnett, P.E., Lai, M.M., Field, M.E., Bahr, B.A., Kirsch, J., Betz, H., and Snyder, S.H. (1999). Interaction of RAFT1 with gephyrin required for rapamycin-sensitive signaling. Science *284*, 1161–1164.
43. Chipperfield, R.G., Jones, S.S., Lo, K.M., and Weinberg, R.A. (1985). Activation of Ha-ras p21 by substitution, deletion, and insertion mutations. Mol. Cell. Biol. *5*, 1809–1813.
44. Niida, Y., Stemmer-Rachamimov, A.O., Logrip, M., Tapon, D., Perez, R., Kwiatkowski, D.J., Sims, K., MacCollin, M., Louis, D.N., and Ramesh, V. (2001). Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. Am. J. Hum. Genet. *69*, 493–503.
45. Richards, S.A., Dreisbach, V.C., Murphy, L.O., and Blenis, J. (2001). Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1. Mol. Cell. Biol. *21*, 7470–7480.
46. Richards, S.A., Fu, J., Romanelli, A., Shimamura, A., and Blenis, J. (1999). Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK. Curr. Biol. *9*, 810–820.
47. Martin, K.A., Schalm, S.S., Richardson, C., Romanelli, A., Keon, K.L., and Blenis, J. (2001). Regulation of ribosomal S6 kinase 2 by effectors of the phosphoinositide 3-kinase pathway. J. Biol. Chem. *276*, 7884–7891.
48. Shimamura, A., Ballif, B.A., Richards, S.A., and Blenis, J. (2000). Rsk1 mediates a MEK-MAP kinase cell survival signal. Curr. Biol. *10*, 127–135.
49. Downward, J. (1995). Measurement of nucleotide exchange and hydrolysis activities in immunoprecipitates. In Methods in Enzymology, vol. 255, W.E. Balch, C.J. Der, and A. Hall, ed. (London: Academic Press), pp. 110–117.