

## RESEARCH COMMUNICATION

# Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling

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**Tuberous sclerosis complex (TSC) is a genetic disease caused by mutation in either *TSC1* or *TSC2*. The *TSC1* and *TSC2* gene products form a functional complex and inhibit phosphorylation of S6K and 4EBP1. These functions of *TSC1/TSC2* are likely mediated by mTOR. Here we report that *TSC2* is a GTPase-activating protein (GAP) toward Rheb, a Ras family GTPase. Rheb stimulates phosphorylation of S6K and 4EBP1. This function of Rheb is blocked by rapamycin and dominant-negative mTOR. Rheb stimulates the phosphorylation of mTOR and plays an essential role in regulation of S6K and 4EBP1 in response to nutrients and cellular energy status. Our data demonstrate that Rheb acts downstream of *TSC1/TSC2* and upstream of mTOR to regulate cell growth.**

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Tuberous sclerosis complex (TSC) is characterized by hamartoma formation in a wide range of tissues and is a genetic disorder caused by mutations in either the *TSC1* or the *TSC2* gene (Kwiatkowski 2003). The most serious clinical complications are mental retardation, epilepsy, and autism caused by tumor growth in the brain (Gomez 1991). Other symptoms include renal dysfunction, dermatological abnormalities, and heart problems. Heterozygous deletions of either *TSC1* or *TSC2* produce similar phenotypes and increase tumor incidence particularly in the kidney, whereas homozygous deletion of either gene is embryonic lethal (Au et al. 1998; Kobayashi et al. 2001). Genetic data from *Drosophila* show that *TSC1* and *TSC2* negatively regulate cell growth and cell size (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001).

Recent studies have established that *TSC1/TSC2* inhibits phosphorylation of the ribosomal S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding protein (4EBP1), two key regulators of translation (Goncharova et al. 2002; Inoki et al. 2002; Manning et al. 2002; Tee et al. 2002). Phosphorylation of S6K and 4EBP1 enhances translation (Gingras et al. 1999). How *TSC1/TSC2* regu-

lates the phosphorylation of S6K and 4EBP1 is a key question yet to be answered. The mammalian target of rapamycin (mTOR) is directly responsible for phosphorylation of both S6K and 4EBP1 (Brown et al. 1995; Hara et al. 1998). Recent studies have suggested that *TSC1/TSC2* acts through mTOR to regulate the phosphorylation of S6K and 4EBP1 (Gao et al. 2002; Inoki et al. 2002; Tee et al. 2002). Consistent with this model is that *TSC1* and *TSC2* are important for cellular nutrient response, which requires the function of mTOR (Gao et al. 2002). However, it is unclear how *TSC1/TSC2* inhibits mTOR activity.

The C-terminal region of *TSC2* displays significant homology to the Rap GTPase-activating protein (GAP; The European Chromosome 16 Tuberous Sclerosis Consortium 1993). In fact, GAP activity of *TSC2* toward Rap1 and Rab5 had previously been reported (Wienecke et al. 1995; Xiao et al. 1997). However, the reported GAP activity was extremely low, and the functional significance is unclear. Rheb is a small GTPase initially isolated as a Ras homolog enriched in brain (Yamagata et al. 1994) and is widely expressed. Rheb shares higher sequence identity with Ras than with Rho family members. The biological function of mammalian Rheb is unclear. Conflicting studies report that Rheb both inhibits and activates the Raf-MAP kinase pathway (Clark et al. 1997; Yee and Worley 1997). Interestingly, mutation of the *Rheb* gene in *Schizosaccharomyces pombe* produces a phenotype similar to nutrient starvation, indicating that Rheb is possibly involved in nutrient signaling (Mach et al. 2000).

In this report, we provide direct biochemical data demonstrating that *TSC2* has GAP activity toward Rheb in vitro. Furthermore, we show that *TSC2* regulates Rheb-GTP levels in vivo. We show that one of the roles of Rheb is to stimulate phosphorylation of S6K and 4EBP1, two of the best characterized cellular downstream targets of *TSC1/TSC2*. Both the effector domain and the GTP binding are essential for Rheb function. The ability of Rheb to stimulate S6K phosphorylation requires the function of mTOR, indicating that mTOR acts downstream of Rheb. Consistent with this is that Rheb stimulates the phosphorylation of mTOR on serine residue 2448. Furthermore, our data support that Rheb plays an important role in cellular responses to energy limitation and nutrient starvation. Together, this study provides a model that Rheb is a direct downstream target of *TSC2* and acts upstream of mTOR to regulate translation and cell growth.

## Results and Discussion

### *TSC2 stimulates GTP hydrolysis of Rheb*

The C-terminal region of *TSC2* contains a putative GAP domain with significant homology to RapGAP (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Scheffzek et al. 1998). However, the precise physiological and biochemical functions of the putative *TSC2* GAP domain have not been demonstrated. Interestingly, a high frequency of *TSC*-associated mutations occurs in the C-terminal putative GAP domain of *TSC2*, indicating the GAP domain may be important for *TSC2* function (Jin et al. 1996; Momose et al. 2002; Kwiatkowski

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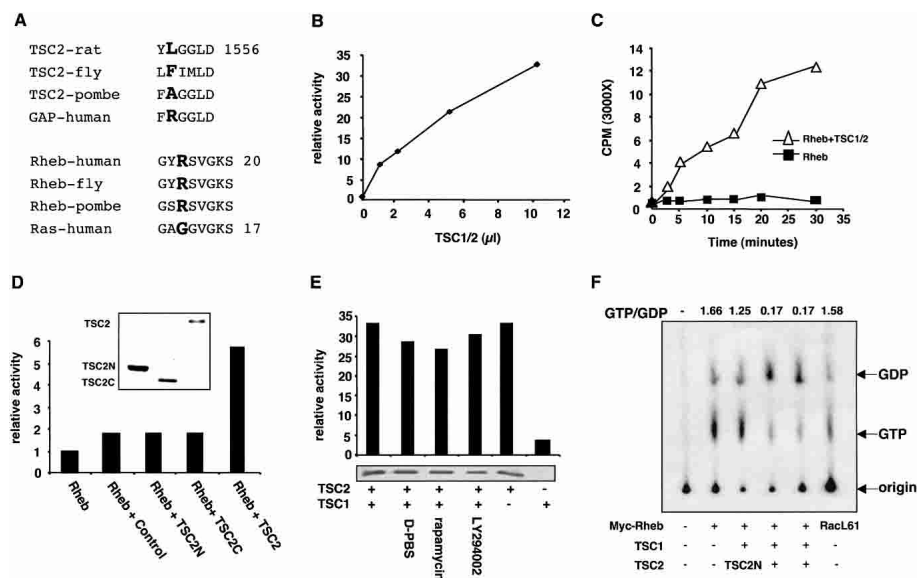
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2003). To study the biochemical functions of TSC2, we expressed and purified the GAP domain of TSC2 in *Escherichia coli* and tested for GAP activity toward the Ras subfamily GTPases (Ras, Rap, TC21, and Rheb) and the Rho family GTPases (Rac and Cdc42). Our *in vitro* assays failed to detect significant GAP activity, whereas the positive control of RasGAP1 showed activity toward Ras (data not shown). It is worth noting that the catalytic arginine residue essential for GAP activity in the Rap GAP family is not conserved in TSC2 (Fig. 1A), suggesting that TSC2 may have no GAP activity (Scheffzek et al. 1998). It is also possible that TSC2 has GAP activity, but a different arginine or a completely different catalytic mechanism may be used.

To further test for GAP activity of TSC2, we expressed TSC2 together with TSC1 in HEK293 cells. The TSC1/TSC2 complex was purified by immunoprecipitation, and *in vitro* GAP activity was determined using various small GTPases. We made particular efforts on Rheb because deletion of Rheb in *S. pombe* produces phenotypes similar to nutrient starvation (Mach et al. 2000). We found that the TSC1/TSC2 complex stimulated the GTP hydrolysis of Rheb (Fig. 1B,C) but not Ras. The GAP activity of TSC2 requires the full-length protein because neither the N-terminal nor the C-terminal region, which contains the GAP homology domain, displayed activity toward Rheb (Fig. 1D). To exclude the possibility that the GAP activity detected in the TSC1/TSC2 immunoprecipitation is due to another coprecipitated GAP, we

made TSC2-R1701Q and TSC2-R1703Q mutants, which are found in TSC patients, and detected no GAP activity (data not shown). Therefore, we conclude that TSC2 is an Rheb GAP. The lack of GAP activity of TSC2C could be due to the requirement of residues in the N-terminal region of TSC2 for GAP activity or incorrect folding of TSC2C. The ability of TSC2 to stimulate GTPase activity of Rheb is very interesting because TSC2 does not have the conserved active-site arginine found in other related RapGAPs and Rheb is an unusual GTPase (Fig. 1A). Almost all Ras family GTPases contain a conserved glycine residue at the position equivalent to codon 12 in Ras (Lowy and Willumsen 1993). Mutation of this glycine to almost any residue, including valine found in many cancers, activates Ras family GTPases. The RasV12 mutant is known to be resistant to down-regulation by RasGAP (Trahey and McCormick 1987). Interestingly, Rheb has an arginine residue at the corresponding position codon 12 and has a much lower basal GTPase activity than Ras (data not shown). However, the GTPase activity of Rheb can be effectively stimulated by TSC1/TSC2. Therefore, Rheb and TSC2 are unusual GTPases and GAPs, respectively.

To determine whether TSC1 is required for the GAP activity of TSC2, HA-TSC2 alone was expressed in HEK293 cells and immunoprecipitated. The precipitated TSC2, which may also coimmunoprecipitate a small amount of endogenous TSC1, showed significant GAP activity toward Rheb (Fig. 1E). In contrast, immunopre-



**Figure 1.** TSC2 has GAP activity toward Rheb. (A) Rheb and TSC2 are an unusual GTPase and GAP. The catalytic active-site arginine (the residue in bold) in RapGAP is not conserved in TSC2. Rheb contains an arginine residue (the residue in bold) at the position corresponding to codon 12 of Ras, which has a glycine. Numbers indicate positions of the last residues. (B) Immunoprecipitated TSC1/TSC2 stimulates GTP hydrolysis of Rheb. Increasing amounts (in microliters) of immunoprecipitated TSC1/TSC2 were incubated with GST-Rheb at room temperature for 20 min. Release of free  $^{32}$ P-phosphate was measured by radioactive counting. Background activity of a control immunoprecipitation was subtracted. The basal GTPase activity of Rheb was arbitrarily set as 1. (C) Time-dependent GTP hydrolysis of Rheb stimulated by TSC1/TSC2. GTP hydrolysis was determined in the absence (■) and presence (△) of TSC1/TSC2. (D) Neither the N-terminal nor the C-terminal region of TSC2 has GAP activity toward Rheb. Two truncated TSC2 constructs (TSC2N, amino acids 1–1007; TSC2C, amino acids 1008–1765) were coexpressed with TSC1 and immunoprecipitated and assayed for GAP activity *in vitro*. The amount of HA-TSC2 used in this assay is equivalent to 1  $\mu$ L in B. The expression levels of these proteins were determined by Western blot. (E) TSC2, but not TSC1, has GAP activity. Transfected HEK293 cells were untreated or treated with D-PBS, rapamycin (20 nM), or LY294002 (50  $\mu$ M) for 30 min as indicated. The relative amount of TSC2 used in the GAP assay was determined by Western blot and is shown *below* each bar. (F) TSC1/TSC2 decreases the Rheb-GTP levels *in vivo*. Myc-Rheb was transfected in HEK293 cells and labeled with  $^{32}$ P-phosphate. Myc-Rheb was immunoprecipitated, and the bound nucleotides were eluted and resolved on a cellulose plate. Cotransfection with TSC1 and TSC2 are indicated. Myc-RacL61 was included as a control. The ratio of GTP/GDP was calculated by the formula GTP counts/3 divided by GDP counts/2, and indicated on *top* of each lane.

precipitated TSC1 showed a very low level of GAP activity toward Rheb possibly due to coprecipitation of endogenous TSC2 protein. These observations indicate that TSC1 stabilizes TSC2 *in vivo*, but is not directly required for GAP activity. TSC1/TSC2 was also immunoprecipitated under various conditions, including treatments with rapamycin, LY294002, and D-PBS, which are all known to inhibit phosphorylation of S6K and 4EBP1. We observed that these treatments did not directly affect the GAP activity of immunoprecipitated TSC1/TSC2 (Fig. 1E).

The effect of TSC2 on Rheb GTP levels was also determined *in vivo*. The nucleotide-binding status of Rheb was determined by *in vivo* labeling with  $^{32}\text{P}$ -phosphate. Interestingly, Rheb contains a high basal GTP level (Fig. 1F). The high GTP level of wild-type Rheb is consistent with it having an arginine at a position equivalent to codon 12 of Ras (Fig. 1A). Expression of TSC1/TSC2 decreased the GTP/GDP ratio of cotransfected Rheb ~10-fold (Fig. 1F). As a control, expression of the N-terminal domain of TSC2 had no significant effect on the GTP level of Rheb. These data provide *in vivo* evidence that TSC2 is an Rheb GAP.

#### *Rheb stimulates the phosphorylation of S6K and 4EBP1*

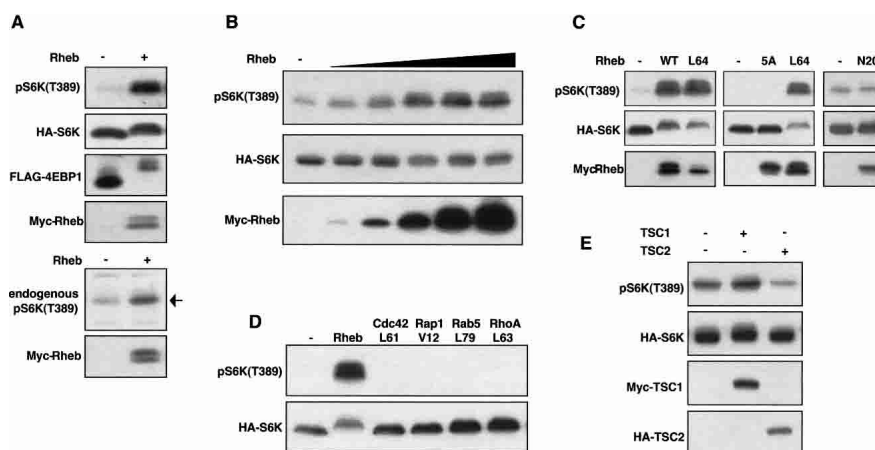
If Rheb is a key downstream target of TSC2, Rheb should stimulate the phosphorylation of S6K and 4EBP1. Expression of the wild-type Rheb induced a dramatic increase in S6K phosphorylation and mobility shift of 4EBP1 (Fig. 2A). Rheb also increased the phosphorylation of endogenous S6K in transfected HEK293 cells. In contrast, Rheb did not stimulate ERK phosphorylation (data not shown). The high activity of wild-type Rheb is consistent with it being predominantly GTP bound (Fig. 1F). Rheb also stimulates S6K phosphorylation in a dose-dependent manner (Fig. 2B).

Five residues between 38 and 42 in the effector domain of Rheb were mutated to alanines to produce Rheb5A. We found that mutation of the effector domain completely abolished the ability of Rheb to stimulate S6K phosphorylation (Fig. 2C). We also tested constitutively active Rheb (RhebL64) and observed that RhebL64 is approximately two to three times more active than the wild type (Fig. 2C). In an attempt to create a dominant-negative mutant, we made an RhebN20. Surprisingly, RhebN20 does not show any dominant-negative effect nor does it stimulate S6K (Fig. 2C). A similar dominant-negative mutant of RasN17 is effective in blocking Ras function through sequestration of the Ras guanine nucleotide exchange factor (GEF; Lowy and Willumsen 1993). This observation suggests that Rheb may not need a GEF because of its high basal GTP level and that GTP binding is required to stimulate S6K phosphorylation.

To determine the specificity of Rheb, several other GTPases were examined, including Cdc42, Rap1, RhoA, and Rab5. Constitutively active mutants of these GTPases fail to stimulate S6K phosphorylation (Fig. 2D). These results demonstrate that Rheb is specific to stimulate S6K phosphorylation. We tested the effect of TSC1 or TSC2 alone on S6K phosphorylation. Expression of TSC2, but not TSC1, significantly decreased the basal phosphorylation of cotransfected S6K (Fig. 3E), consistent with the fact that TSC2 alone has GAP activity. The ability of TSC2 but not TSC1 to inhibit S6K indicates that TSC2 is the active component of the TSC1/TSC2 complex, whereas TSC1 may have regulatory functions, such as localization and stabilization. Furthermore, these observations suggest that inhibition of endogenous Rheb by TSC2 decreases S6K phosphorylation, thus supporting the importance of Rheb in S6K regulation.

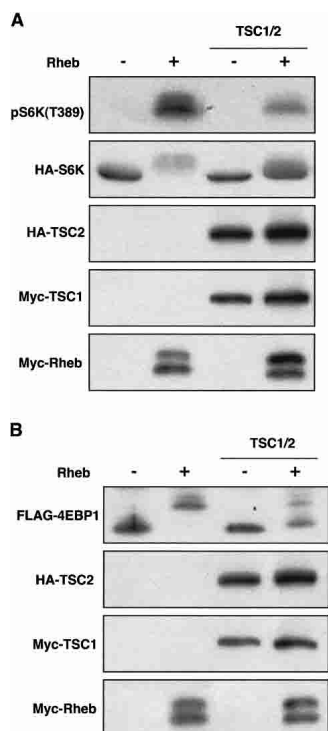
#### *TSC1/TSC2 inhibits Rheb function*

The functional relationship between TSC1/TSC2 and Rheb was examined *in vivo*. Cotransfection of TSC1/



**Figure 2.** Rheb stimulates phosphorylation of S6K and 4EBP1. (A) Rheb stimulates phosphorylation of S6K and 4EBP1. HA-S6K and Flag-4EBP1 were transfected into HEK293 cells in the presence or absence of Myc-Rheb (100 ng). Phosphorylation of S6K was determined by phosphospecific antibody, pS6K(T389), whereas phosphorylation of 4EBP1 was determined by mobility shift (*upper panels*). Phosphorylation of endogenous S6K was also determined (*lower panels*). (B) Rheb stimulates phosphorylation of S6K in a dose-dependent manner. HA-S6K (15 ng) was cotransfected with 0, 2, 5, 20, 50, and 100 ng of Myc-Rheb. (C) The effector domain of Rheb is required to stimulate S6K phosphorylation. HA-S6K was cotransfected with wild-type Rheb (100 ng), RhebL64 (40 ng), Rheb5A (200 ng), and RhebN20 (300 ng). The relative expression of Rheb mutants is also shown. (D) Stimulation of S6K phosphorylation by different GTPases. Constitutively active mutants (100 ng for each) of Cdc42, Rap1, Rab5, RhoA, and the wild-type Rheb (40 ng) were used to stimulate phosphorylation of S6K. (E) TSC2, but not TSC1, inhibits S6K phosphorylation. HA-S6K was coexpressed with either Myc-TSC1 or HA-TSC2. Phosphorylation of S6K was determined.

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**Figure 3.** TSC1/TSC2 inhibits Rheb function in vivo. (A) TSC1/TSC2 inhibits the ability of Rheb to induce S6K phosphorylation. (B) TSC1/TSC2 inhibits the ability of Rheb to induce 4EBP1 phosphorylation.

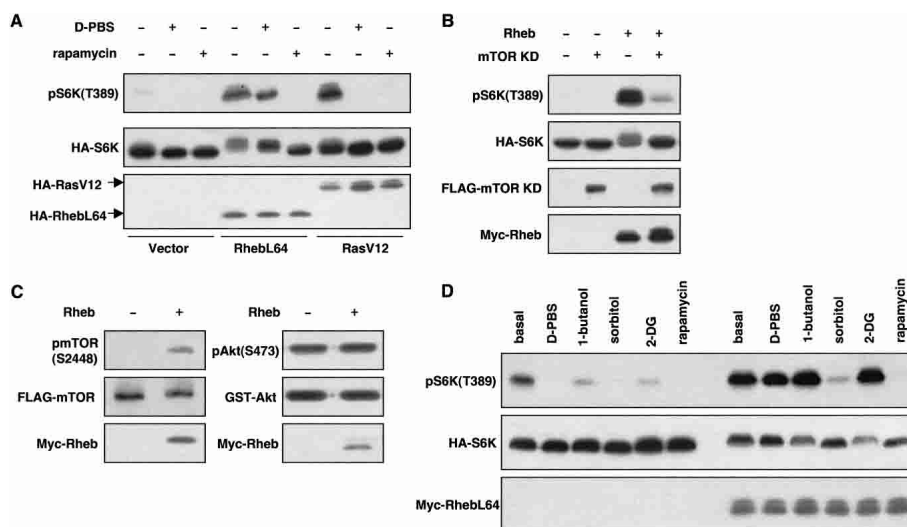
TSC2 suppressed the ability of Rheb to stimulate S6K (Fig. 3A). Similar results were observed with 4EBP1 (Fig. 3B). These data further support the idea that TSC2 acts as a GAP toward Rheb.

#### *mTOR acts downstream of Rheb*

Previous studies have suggested that mTOR acts downstream of TSC2 (Gao et al. 2002; Inoki et al. 2002; Tee et al. 2002). To determine the relationship between Rheb and mTOR, the effect of rapamycin was investigated. Treatment of cells with rapamycin completely blocked the stimulatory effect of Rheb on S6K phosphorylation (Fig. 4A), indicating that Rheb functions upstream of mTOR. To further test the functional relationship between Rheb and mTOR, a kinase-inactive mutant of mTOR was coexpressed with Rheb. We found that the dominant-negative mTOR-KD significantly blocked the phosphorylation of S6K by Rheb (Fig. 4B). We had previously observed that TSC1/TSC2 inhibits the phosphorylation of S2448 of mTOR (Inoki et al. 2002; Kenerson et al. 2002). Coexpression of Rheb caused a significant increase of mTOR S2448 phosphorylation, whereas phosphorylation of Akt was not affected (Fig. 4C), indicating that Rheb activates mTOR.

#### *Rheb is involved in multiple signaling pathways that regulate S6K*

Phosphorylation of S6K is regulated by numerous cellular signals including nutrients (Proud 2002). The functional significance of Rheb in nutrient signaling was analyzed. In Rheb-transfected HEK293 cells, nutrient starvation (treatment with D-PBS that contains glucose, but not amino acids) induced a minor dephosphorylation of S6K (Fig. 4A). In contrast, nutrient starvation inhibited S6K phosphorylation in RasV12-transfected cells (Fig. 4A). These results demonstrate that Rheb plays an important role in the nutrient signaling pathway, but Ras is not directly involved in nutrient signaling. We also examined the effect of osmotic stress by sorbitol, energy depletion by 2-deoxyglucose (2-DG), and inhibition of phospholipase D by 1-butanol. These treatments are



**Figure 4.** Rheb functions upstream of mTOR and is involved in response to various signals. (A) Rheb acts downstream of nutrient signals and upstream of mTOR. HA-S6K was cotransfected with RhebL64 or RasV12 as indicated. Cells were treated with rapamycin or D-PBS for 30 min. Phosphorylation of S6K was determined. (B) Kinase inactive mTOR KD blocks Rheb-induced S6K phosphorylation. (C) Rheb stimulates the phosphorylation of mTOR. Flag-mTOR or GST-Akt was cotransfected with Rheb and immunoprecipitated. (Left) Phosphorylation of mTOR was detected by the anti-phospho mTOR (S2448) antibody. (Right) Phosphorylation of Akt was monitored by the anti-phospho Akt (S473) antibody. (D) Role of Rheb in S6K regulation by various signaling pathways. HA-S6K was transfected alone (left half) or together with RhebL64 (right half). Cells were treated with D-PBS, 1-butanol (0.3%), sorbitol (600 mM), 2-DG (25 mM), or rapamycin (20 nM) for 30 min as indicated.

known to induce S6K dephosphorylation (Fig. 4D; Dennis et al. 2001; Fang et al. 2001; Desai et al. 2002). Rheb significantly blocked the effect of 2-DG and 1-butanol, but had little effect on S6K dephosphorylation induced by sorbitol (Fig. 4D). These data suggest that Rheb acts in the cellular energy-sensing pathway and the phospholipase D pathway, but is not directly involved in the osmotic stress pathway. Consistently, TSC1/TSC2 also plays an important role in regulation of S6K in response to cellular energy depletion (data not shown).

The importance of TSC1/TSC2 in cell growth regulation has been well established (Potter and Xu 2001). The major cellular function of TSC1/TSC2 is to inhibit the phosphorylation of S6K and 4EBP1, likely through mTOR. This study provides a significant advance in the understanding of the molecular mechanism of how TSC1/TSC2 regulates the phosphorylation of S6K and 4EBP-1. Based on both *in vitro* and *in vivo* biochemical experiments, we conclude that TSC2 is an Rheb-specific GAP and Rheb is a key downstream target of TSC1/TSC2 (Fig. 5). Rheb is closely related to Ras and is an unusual GTPase in that it is predominantly GTP bound in cells. The biological function of Rheb has not been clearly defined. Our studies support that Rheb promotes cell growth by stimulating phosphorylation of S6K and 4EBP1, but has no role in ERK activation.

Inhibition of Rheb by TSC1/TSC2 is likely to be critical for the function of these two tumor suppressor proteins because Rheb potently regulates the phosphorylation of S6K and 4EBP1. Interestingly, mutation of the Rheb ortholog in *S. pombe* displays phenotypes characteristic of nutrient starvation (Mach et al. 2000). Our data are consistent with the genetic observations made in *S. pombe* and support the notion that Rheb plays an essential role in nutrient signaling. *S. pombe* also contains TSC1- and TSC2-related molecules (Matsumoto et al. 2002). We have also shown that TSC2 is an unusual GAP because it lacks the conserved arginine found in other Rap family GAPs. Yet it has very high and selective GAP activity toward Rheb. Future studies to elucidate the catalytic mechanism of TSC2 on Rheb-GTP hydrolysis will provide new insights into how this unusual GTPase and GAP work.

Our studies present a model in which Rheb is involved in S6K regulation in response to nutrients and energy levels (Fig. 5). In contrast, osmotic stress regulates S6K independently of Rheb. A major function of Rheb is to stimulate mTOR; however, we did not detect a significant association between Rheb and mTOR, suggesting that Rheb may activate mTOR indirectly. Future efforts

will focus on how Rheb stimulates mTOR. Our model predicts that TSC2 acts through Rheb to regulate translation (Fig. 5). It suggests that Rheb positively regulates cell growth and plays an important role in the development of TSC. Therefore, inhibition of Rheb could be a potential target for the treatment of TSC and cancer.

## Materials and methods

### Antibodies and plasmids

Anti-phospho S6K (T389), anti-mTOR, and anti-phospho mTOR (S2448) antibodies were from Cell Signaling Inc., and anti-Myc antibodies were from Santa Cruz Biotechnology. Anti-HA and anti-Flag were purchased from Covance and Sigma, respectively. TSC1 and TSC2 constructs were described previously (Inoki et al. 2002). The HA-tagged S6K1 ( $\alpha$  II) construct was generously provided by J. Blenis (Harvard University, Cambridge, MA) and Flag-tagged mTOR and kinase inactive mTOR (mTOR-KD) were from S. Schreiber (Harvard University, Cambridge, MA). Rab5 is a gift of G. Li (University of Oklahoma, Health Sciences Center, Oklahoma City, OK). All other DNA constructs including Ras, Rheb, Rap1, Rac, Cdc42, RhoA, 4E-BP1, Akt, and ERK2 were laboratory stocks. All mutant constructs of TSC2 and Rheb were created by PCR mutagenesis and verified by DNA sequencing.

### Cell culture, transfection, and immunoprecipitation

HEK293 cells were seeded and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Transfection was performed using Lipofectamine Reagent (Invitrogen) following the manufacturer's instructions. Cells were lysed in lysis buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1% NP-40, 1% Triton X-100, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, 10  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL aprotinin) and immunoprecipitated with the indicated antibodies and protein G-Sepharose beads. Immunocomplexes were subjected to SDS-PAGE.

### *In vitro* GTPase assay

GST-Rheb, GST-Ras, GST-Rac, and GST-Rap were expressed in the bacterial strain BL21 and purified using glutathione-Sepharose 4B beads (Sigma) as described. Small GTPases were loaded with  $^{32}$ P- $\gamma$ -GTP before GAP assays. Here, 10  $\mu$ g of small GTPase protein bound on glutathione beads was incubated with 50  $\mu$ Ci of  $^{32}$ P- $\gamma$ -GTP in loading buffer (20 mM Tris at pH 8, 5 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA) in a volume of 20  $\mu$ L. The loading reaction was stopped by addition of MgCl<sub>2</sub> to 10 mM. Then the small GTPase protein was eluted.

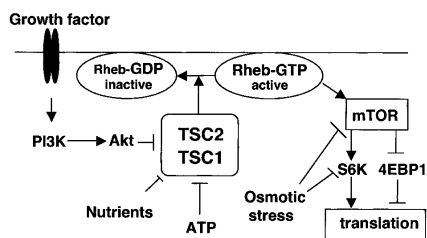
HA-TSC2 was transfected to HEK293 cells and immunoprecipitated by anti-HA antibody. The immune complexes were washed three times with wash buffer (20 mM Tris at pH 7.4, 800 mM NaCl, 2 mM EDTA, 1% NP-40) and two times with GAP assay wash buffer (20 mM Tris at pH 8, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT). The final GAP assays included 0.5  $\mu$ g of  $^{32}$ P- $\gamma$ -GTP loaded small GTPase, and immunoprecipitated TSC2 from  $1 \times 10^7$  transfected to HEK293 cells. Reactions were carried out in 40  $\mu$ L of GAP assay buffer (20 mM Tris at pH 8, 10 mM MgCl<sub>2</sub>, and 4 mM DTT), at room temperature for 20 min. The reaction was stopped by 300  $\mu$ L of charcoal buffer (5% charcoal, 20 mM phosphoric acid, 0.6 M HCl), then vortexed for 10 min and spun at 13,000 rpm for 10 min. Then 100  $\mu$ L of supernatant was taken, and the  $^{32}$ P release was determined by scintillation counting.

### *In vivo* labeling of Rheb

The cells were washed once with phosphate-free DMEM and incubated with 0.5 mCi/mL  $^{32}$ P-orthophosphate (ICN) for 4 h. The cells were lysed with labeling lysis buffer (1% Triton X-100, 50 mM HEPES at pH 7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mg/mL BSA, 1 mM DTT, 1 mM PMSF, 10  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL aprotinin). Myc-tagged Rheb was immunoprecipitated. The Rheb-bound nucleotides were eluted with elution buffer (2 mM EDTA, 0.2% SDS, 1 mM GDP, 1 mM GTP) at 68°C for 20 min. Then the eluted nucleotides were subjected to thin layer chromatography using PEI cellulose plates (Baker-flex) in 0.75 M KH<sub>2</sub>PO<sub>4</sub> (pH 3.4).

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**Figure 5.** A proposed model of Rheb functions downstream of TSC1/TSC2 and upstream of mTOR. TSC2 acts as a GAP to inactivate Rheb by directly stimulating GTP hydrolysis. Rheb stimulates mTOR. Nutrient and cellular energy status signals through Rheb, whereas osmotic stress signals independently of Rheb.

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