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# Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2

## Abstract

Tumor suppressor genes evolved as negative effectors of mitogen and nutrient signaling pathways, such that mutations in these genes can lead to pathological states of growth. Tuberous sclerosis (TSC) is a potentially devastating disease associated with mutations in two tumor suppressor genes, TSC1 and 2, that function as a complex to suppress signaling in the mTOR/S6K/4E-BP pathway. However, the inhibitory target of TSC1/2 and the mechanism by which it acts are unknown. Here we provide evidence that TSC1/2 is a GAP for the small GTPase Rheb and that insulin-mediated Rheb activation is PI3K dependent. Moreover, Rheb overexpression induces S6K1 phosphorylation and inhibits PKB phosphorylation, as do loss-of-function mutations in TSC1/2, but contrary to earlier reports Rheb has no effect on MAPK phosphorylation. Finally, coexpression of a human TSC2 cDNA harboring a disease-associated point mutation in the GAP domain, failed to stimulate Rheb GTPase activity or block Rheb activation of S6K1.

# Insulin Activation of Rheb, a Mediator of mTOR/S6K/4E-BP Signaling, Is Inhibited by TSC1 and 2

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## Summary

Tumor suppressor genes evolved as negative effectors of mitogen and nutrient signaling pathways, such that mutations in these genes can lead to pathological states of growth. Tuberous sclerosis (TSC) is a potentially devastating disease associated with mutations in two tumor suppressor genes, *TSC1* and 2, that function as a complex to suppress signaling in the mTOR/S6K/4E-BP pathway. However, the inhibitory target of TSC1/2 and the mechanism by which it acts are unknown. Here we provide evidence that TSC1/2 is a GAP for the small GTPase Rheb and that insulin-mediated Rheb activation is PI3K dependent. Moreover, Rheb overexpression induces S6K1 phosphorylation and inhibits PKB phosphorylation, as do loss-of-function mutations in TSC1/2, but contrary to earlier reports Rheb has no effect on MAPK phosphorylation. Finally, coexpression of a human TSC2 cDNA harboring a disease-associated point mutation in the GAP domain, failed to stimulate Rheb GTPase activity or block Rheb activation of S6K1.

## Introduction

Tuberous sclerosis (TSC) syndrome is an autosomal-dominant genetic disorder, characterized by mutations in either the *TSC1* or *TSC2* gene, whose protein products hamartin (TSC1) and tuberlin (TSC2) form a putative tumor suppressor complex (Montagne et al., 2001; Sparagana and Roach, 2000). TSC can cause severe pathological consequences, including mental retardation, epilepsy, and autism as well as leading to cardiac, pulmonary, and renal failure (Montagne et al., 2001; Sparagana and Roach, 2000). These pathological states arise from hamartomas, largely manifested as benign tumors, but

which in rare cases progress to renal cell carcinomas (Young and Povey, 1998). The contribution of *TSC1/2* mutations to cancer progression may be much more prevalent than noted, as one in six thousand individuals is born with a mutation in either *TSC1* or 2 (Montagne et al., 2001; Sparagana and Roach, 2000). Consistent with this, studies in *Drosophila* and in mammals demonstrate that TSC1/2 acts to constrain both mitogen- and nutrient-induced activation of the mTOR/S6K/4E-BP signal transduction pathway (Marygold and Leever, 2002; McManus and Alessi, 2002), which has been implicated in the progression of a number of solid tumors (Abraham, 2002; Hidalgo and Rowinsky, 2000; Sekulic et al., 2000). At least in part, TSC1/2 repression of the mitogen-induced segment of the mammalian mTOR/S6K/4E-BP signaling pathway appears to be relieved by protein kinase B (PKB) phosphorylation of TSC2, a response mediated by insulin-induced activation of phosphatidylinositide-3OH kinase (PI3K) (Goncharova et al., 2002; Inoki et al., 2002; Jaeschke et al., 2002; Manning et al., 2002). Moreover, hamartomatous lesions are also observed in patients with one of three related autosomal-dominant disorders associated with germline mutations in *PTEN*, a negative effector of PI3K: Cowden disease (CD), Lhermitte-Duclos disease (LDD), and Bannayan-Zonana syndrome (BZS) (Liaw et al., 1997). However, unlike the case in *PTEN*, the exact mechanism by which TSC1/2 represses mTOR/S6K/4E-BP signaling is unknown, as is the identity of the inhibitory target.

The severest forms of TSC syndrome are associated with mutations that map to the GTPase activating domain of TSC2 (Maheshwar et al., 1997 and D. Franz, personal communication). Consistent with TSC2 functioning as a GTPase activating protein (GAP), it had been reported that the GTPase activity of Rap1, a member of the Ras superfamily of small GTPases, is stimulated in vitro by TSC2 (Wienecke et al., 1995). However, we previously found that insulin had no effect on Rap1 activity in cells overexpressing the insulin receptor (Zwartkruis et al., 1998), while potently activating S6K1 in these same cells (Ming et al., 1994). To identify potential GTPases as targets of TSC2 GAP, we set out to determine whether the activity of any Ras superfamily member (Bos, 1997) was elevated in *TSC2*<sup>-/-</sup> versus *TSC2*<sup>+/+</sup> mouse embryo fibroblasts (MEFs). From such studies, Rheb (*Ras* homologue enriched in brain) emerged as a possible candidate. In parallel, a role for Rheb in the mTOR/S6K/4E-BP signaling pathway became apparent from genetic studies in *Drosophila* (see Discussion). Rheb was first identified in a differential screen of mRNAs induced in neurons by agents that provoke seizures (Yamagata et al., 1994) and is ubiquitously expressed, but particularly abundant, in muscle and brain (Yamagata et al., 1994). Moreover, Rheb was shown to have intrinsic GTPase activity (Yamagata et al., 1994) and to be as efficient as wild-type Raf-1 or H-Ras in inducing transformation in NIH3T3 cells (Yee and Worley, 1997). Importantly, in *S. pombe* deletion of the Rheb ortholog, *Rhb1*, affects amino acid transport and nutrient sensing (Mach et al., 2000), as has been recently

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shown for the deletion of the *S. pombe* TSC1/2 orthologs *ts1* and *2* (Matsumoto et al., 2002). Moreover, recent studies in *S. cerevisiae* have potentially created a link between Rheb and Batten disease, an autosomal-recessive neurodegenerative disorder that is associated in many cases with deletions in the human *CLN3* gene, a transmembrane protein of unknown function (see Cooper, 2003). In brief, earlier studies showed that deletion of the *CLN3* ortholog, *BTN1*, in *S. cerevisiae* leads to the upregulation of only two genes, *HSP30* and *BTN2*, thought to be involved in regulating vacuolar pH (Pearce et al., 1999). Recently, *BTN2* was employed as bait in a yeast two-hybrid screen, which led to the identification of the *S. cerevisiae* ortholog of Rheb, *RSG1*, as a positive interactor (Chattopadhyay et al., 2003). More importantly, it was shown that the two protein products of the *BTN2* and *RSG1* genes, Btn2p and Rsg1p, interact biochemically and genetically to control amino acid uptake (Chattopadhyay et al., 2003). Given the role of amino acids and nutrients in mTOR/S6K/4E-BP signaling (Dennis et al., 1999; Schmelzle and Hall, 2000) and the association of *TSC1/2* mutations in neurological disorders, we were further prompted to query whether Rheb could serve as the inhibitory target of TSC1/2.

Here, we identified Rheb as a potential GTPase target of TSC1/2-GAP by screening Ras superfamily members for elevated GTPase activity in *TSC2*<sup>-/-</sup> MEFs. Next, we determined whether TSC1/2 functions as a Rheb-GAP and whether insulin activates Rheb. In parallel, we tested whether ectopic expression of Rheb induces S6K1 activation and whether insulin-induced S6K1 stimulation is dependent on Rheb. Finally, we analyzed whether Rheb mediated activation of S6K1 is affected by a pathologic mutation in human *TSC2*, which fails to inhibit S6K1 activation.

## Results

If the effects of TSC1/2 loss of function mutations are mediated by an increase in the GTP bound form of a Ras small GTPase superfamily member, then a higher proportion of this form should be detected in *TSC2*<sup>-/-</sup> MEFs, as compared to *TSC2*<sup>+/+</sup> MEFs. To test this possibility, we screened extracts from serum-deprived *TSC2*<sup>+/+</sup> and *TSC2*<sup>-/-</sup> MEFs employing activation-specific probes, in which GTP bound Ras-like GTPases are specifically isolated using GST-fusion proteins containing the downstream GTPase target proteins (van Triest et al., 2001). The results of such an assay show that there was no difference in the level of Ras, Rap2, or Ral bound GTP in the two cell types (Figure 1A). In parallel, we also attempted to pull down Rheb employing these same probes, including a GST-fusion protein that contains the Ras binding domain of Raf1 (GST-RBD). However, these findings were inconclusive, most likely due to the low affinity of Rheb for the various fusion proteins as a consequence of the markedly divergent effector region of Rheb (Bos, 1997). Therefore, Rheb was immunoprecipitated from either [<sup>32</sup>P]-labeled, serum-starved *TSC2*<sup>+/+</sup> MEFs or *TSC2*<sup>-/-</sup> MEFs, and the ratio of GTP to GDP bound Rheb was determined by one-dimensional thin-layer chromatography. The result

showed a striking elevation in the levels of GTP bound Rheb in extracts of *TSC2*<sup>-/-</sup> as compared to *TSC2*<sup>+/+</sup> MEFs, in contrast to what was observed for the other Ras-like GTPases (Figure 1B). These findings would fit a model whereby TSC1/2 functions as a GAP for Rheb. To test this possibility further, myc epitope-tagged Rheb was ectopically expressed in COS cells with either HA epitope-tagged TSC1 or HA epitope-tagged TSC2 alone or with both tumor suppressors together, and the ratio of GTP to GDP bound Rheb was determined. The results show that coexpression of TSC1 had no effect on the amount of Rheb in the GTP bound form, whereas coexpression of TSC2 alone lowered these levels, an effect which was strongly potentiated by TSC1 (Figure 1C). It should be noted that basal ratio of GTP to GDP was always high when ectopically expressing Rheb, consistent with the findings reported by Im et al. (Im et al., 2002 and see Discussion). The specificity of TSC1/2-GAP was controlled by coexpression of HA epitope-tagged Rap1-GAP (RG), a known GAP for Rap GTPase family members, which had no effect on the Rheb GTPase activity (Figure 1D). Likewise, coexpression of TSC1/2-GAP with HA epitope-tagged Rap2 led to no measurable change in GTP bound Rap2, whereas coexpression of RG with Rap2 greatly lowered these levels (Figure 1D). Taken together, these findings support a model whereby TSC1/2 functions as a GAP for Rheb, such that loss of TSC1/2-GAP function leads to increased levels of activated Rheb.

Insulin-induced activation of PI3K relieves TSC1/2 repression of the mTOR/S6K/4E-BP signaling pathway (Jaeschke et al., 2002). If the inhibitory target of TSC1/2 is Rheb, then insulin treatment should increase the amount of GTP bound Rheb, which should be blocked by the PI3K inhibitor wortmannin, but resistant to the mTOR inhibitor rapamycin. To examine this possibility, Rheb was immunoprecipitated from [<sup>32</sup>P]-labeled, serum-starved A14 NIH 3T3 cells that were stimulated for 30 min with insulin alone or in the presence of either wortmannin or rapamycin. The ratio of GTP to GDP bound Rheb was then determined by one-dimensional thin-layer chromatography. As a control, the activation of Ras was followed in parallel. The results show that insulin induces an approximate 2-fold increase in the amount of GTP bound Ras and Rheb (Figure 2A), with the fold increase in GTP bound Rheb being  $1.8 \pm 0.11$  SEM, averaged over three independent experiments. Moreover, the insulin-induced increase in GTP bound Rheb, but not that of Ras, is blocked by wortmannin, whereas both increases are resistant to rapamycin (Figure 2A). That the effects of wortmannin are through PI3K and that rapamycin blocks mTOR signaling are supported by the fact that in parallel cultures treated in the identical manner, except for the omission of [<sup>32</sup>P]-orthophosphate, insulin-induced PKB S473 and S6K1 T389 phosphorylation are blocked by the PI3K inhibitor, whereas only rapamycin inhibits S6K1 T389 phosphorylation (Figure 2B). Furthermore, neither wortmannin nor rapamycin affects MAPK phosphorylation (Figure 2B), consistent with the fact that neither inhibitor has an effect on Ras activation (Figure 2A). These data are compatible with insulin-induced activation of PI3K relieving TSC1/2-GAP inhibition of Rheb, which in turn leads to activation of the mTOR/S6K/4E-BP signaling

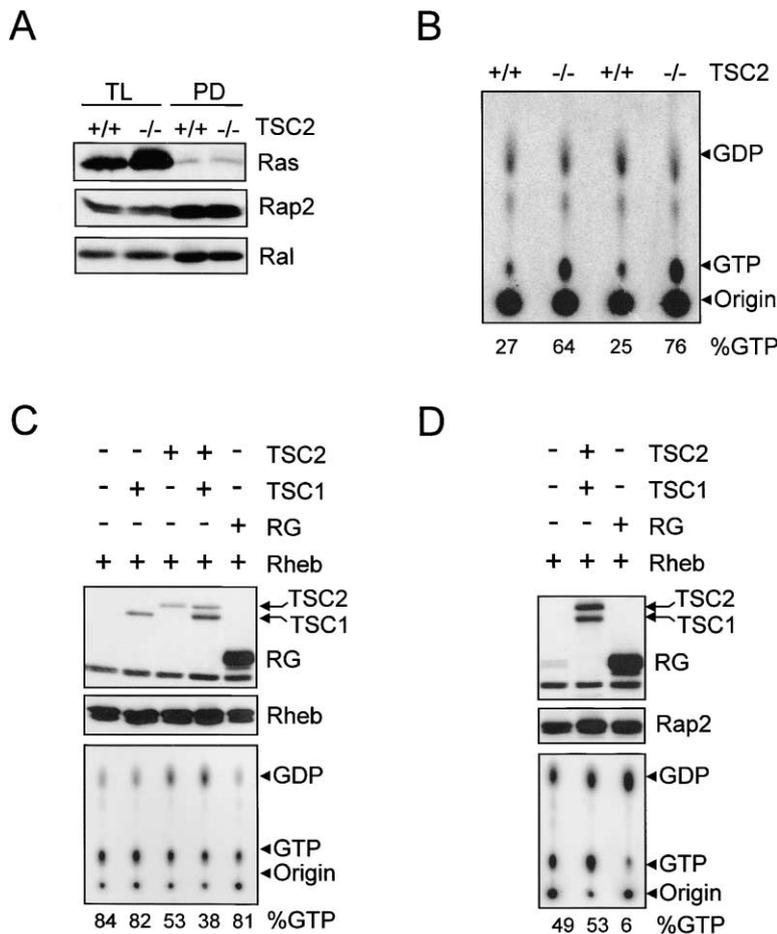


Figure 1. TSC1/2 Exhibit GAP Activity toward the Ras-like Small GTPase Rheb

(A) TSC2 does not affect the activity of the Ras superfamily member Ras, Rap2, and Ral small GTPases. The active, GTP bound form of the indicated endogenous small GTPase was specifically pulled down from serum-deprived *TSC2*<sup>+/+</sup> and *TSC2*<sup>-/-</sup> MEFs and visualized by immunoblotting. TL, total lysate; PD, pull-down.

(B) Loss of TSC2 function leads to elevated levels of GTP bound Rheb. Endogenous Rheb from serum-deprived [<sup>32</sup>P]-orthophosphate labeled *TSC2*<sup>+/+</sup> and *TSC2*<sup>-/-</sup> MEFs was immunoprecipitated, and the ratio of GTP to GDP bound Rheb was determined by PhosphorImager analysis following one-dimensional thin-layer chromatography.

(C) TSC1/2 exhibit GAP activity toward Rheb. COS-7 cells were transfected with 2  $\mu$ g of TSC1, TSC2, Rap1GAP (RG), and Rheb as indicated, and after immunoprecipitation the amount of GTP versus GDP was measured as in (B).

(D) The GTPase activity of Rap2 is not stimulated by TSC2. The experiment was performed as in (C), except that Rap2 was transfected and assayed.

pathway. If Rheb acts downstream of insulin, then Rheb overexpression alone might be expected to activate the mTOR/S6K/4E-BP signaling pathway, especially as under these conditions it is largely in the activated GTP bound form (Figure 1C and data not shown). To test this possibility, myc epitope-tagged S6K1 was coexpressed with Rheb, in the absence or presence of insulin. Coexpression of Rheb alone induced S6K1 activation to a higher extent than that achieved by insulin stimulation, as determined by increased S6K1-T389 phosphorylation (Figure 2C). Likewise, coexpression of Rheb alone also induced S6K2 activation in the absence of mitogen stimulation (Figure 2D). Thus, consistent with the model and TSC1/2 loss of function mutations (Jaeschke et al., 2002), overexpression of Rheb drives constitutive activation of S6K1 as well as S6K2.

Despite the observations above, recent studies have implicated Rheb as a negative effector of Ras signaling through competing with Ras for binding to B Raf, blocking subsequent activation of MEK and MAPK (Im et al., 2002). However, loss of TSC1/2 function has no apparent effect on MAPK activation (Jaeschke et al., 2002). To evaluate the effect of Rheb on Ras/MAPK signaling, HA epitope-tagged p44 MAPK was coexpressed with myc epitope-tagged Rheb in HeLa cells. The results show that expression of Rheb alone or in the presence of serum had no apparent effect on p44

MAPK activation, as judged with a phosphospecific antibody (Figure 3A). This finding is consistent with our inability to pull down Rheb with the Ras binding domain from Raf1 in the activation-specific probe assay and with insulin-induced MAPK activation being independent of the GTP bound status of Rheb (Figures 2A and 2B). Loss of TSC1/2 function also leads to constitutive 4E-BP1 phosphorylation and the suppression of both basal and insulin-stimulated PKB activation (Jaeschke et al., 2002). As 4E-BP1 is a second target of mTOR kinase (Brunn et al., 1997; Gingras et al., 1998), it led to the hypothesis that TSC1/2 functions through inhibiting mTOR (see Discussion), whereas the effects on PKB may be mediated by S6K1 through a negative feedback loop, as recently demonstrated in *Drosophila* (Radimerski et al., 2002). To examine the effect of Rheb on 4E-BP1 and PKB, both proteins were ectopically expressed with an HA epitope-tag. Coexpression of Rheb with 4E-BP1 led to constitutive phosphorylation of 4E-BP1 in the absence of insulin, as judged by 4E-BP1 T70 phosphorylation, an effect not enhanced by insulin (Figure 3B). In parallel, coexpression of Rheb effectively lowers both basal- and insulin-induced PKB S473 phosphorylation, without affecting PKB/Akt protein levels (Figure 3C). These findings support the hypothesis that insulin-induced activation of the mTOR/S6K/4E-BP signaling pathway is mediated by Rheb. To test this possibility,

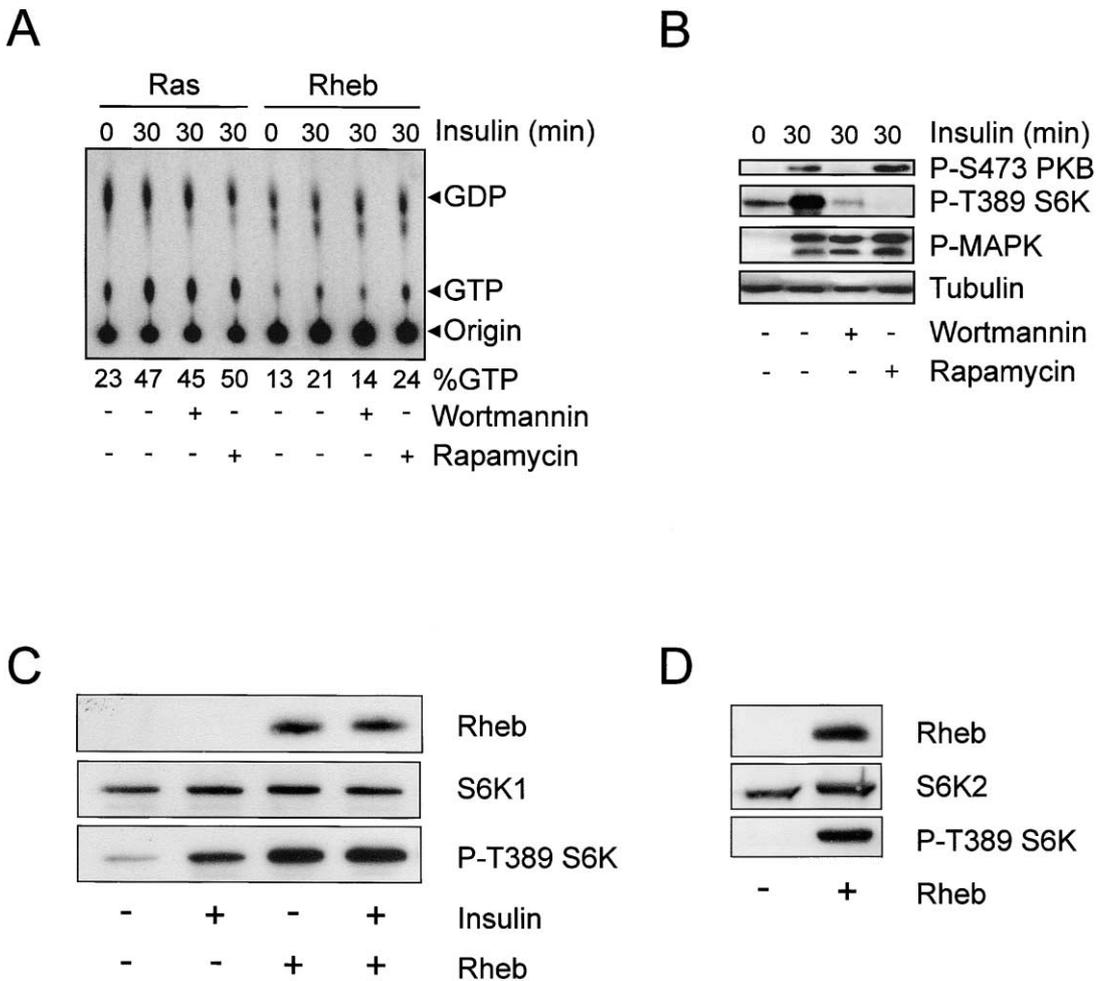


Figure 2. Insulin Induces Rheb Activation, and Ectopic Expression of Rheb Activates S6K1 and 2

(A) Insulin-induced activation of PI3K relieves TSC1/2 GAP mediated inhibition of Rheb. Serum-deprived A14 NIH 3T3 cells were incubated with [<sup>32</sup>P]-orthophosphate for 4–6 hr and then treated with 200 nM insulin alone or in the presence of 100 nM wortmannin or 20 nM rapamycin as indicated. Endogenous Rheb or Ras were pulled down and assayed as in Figure 1B.

(B) Insulin-induced phosphorylation of PKB, S6K1, and MAPK. Cells were treated as in (A), except for the omission of [<sup>32</sup>P]-orthophosphate, and the phosphorylation of PKB, S6K1, and MAPK was followed with phosphospecific antibodies to each protein.

(C) Ectopic expression of Rheb activates S6K1. Myc-S6K1-GST was transiently expressed in HeLa cells alone or together with myc-Rheb. After overnight serum deprivation cells were stimulated with 200 nM insulin as indicated, and phosphorylation of the kinase was detected with a phosphospecific antibody.

(D) Rheb constitutively activates S6K2 in the absence of insulin. HeLa cells were transfected with S6K2-GST alone or together with myc-Rheb. S6K2 activity was assayed as described in (C).

siRNAs were generated against Rheb and tested for their ability to suppress insulin-induced activation of S6K1. The results of such an experiment show that two different siRNAs against Rheb inhibit S6K1 T389 phosphorylation to approximately the same extent to which they lower Rheb expression levels, whereas a control siRNA, known to have no effect on Rheb levels, has no effect on S6K1 activation (Figure 3D). Thus, Rheb appears to mediate insulin-induced activation of the mTOR/S6K/4E-BP signaling pathway, rather than acting in the Ras/MAPK pathway.

As compared to *TSC2*<sup>+/+</sup> MEFs, *TSC2*<sup>-/-</sup> MEFs exhibit constitutive S6K1 activity, which is largely resistant to inhibition by wortmannin, but which remains rapamycin sensitive (Figure 4A and Jaeschke et al., 2002). The ef-

fects of wortmannin were shown to go through PI3K, as dominant interfering alleles of the p85 and p110 subunits of PI3K blocked S6 phosphorylation in *TSC2*<sup>+/+</sup> MEFs, but not in *TSC2*<sup>-/-</sup> MEFs (Jaeschke et al., 2002). These effects have been argued to go through PKB, although not all agonists which activate S6K1 activate PKB (Radimerski and Thomas, 2003). If the effects of wortmannin and rapamycin on the *TSC2* loss-of-function phenotype are mediated by Rheb, similar effects of the two inhibitors would be expected on Rheb-induced S6K1 activation. The results of this experiment reveal that wortmannin has little effect on Rheb-induced S6K1 activation, whereas it totally abolishes the insulin response (Figure 4A). In contrast, rapamycin ablates both Rheb- and insulin-induced S6K1 activation (Figure 4A), similar to what

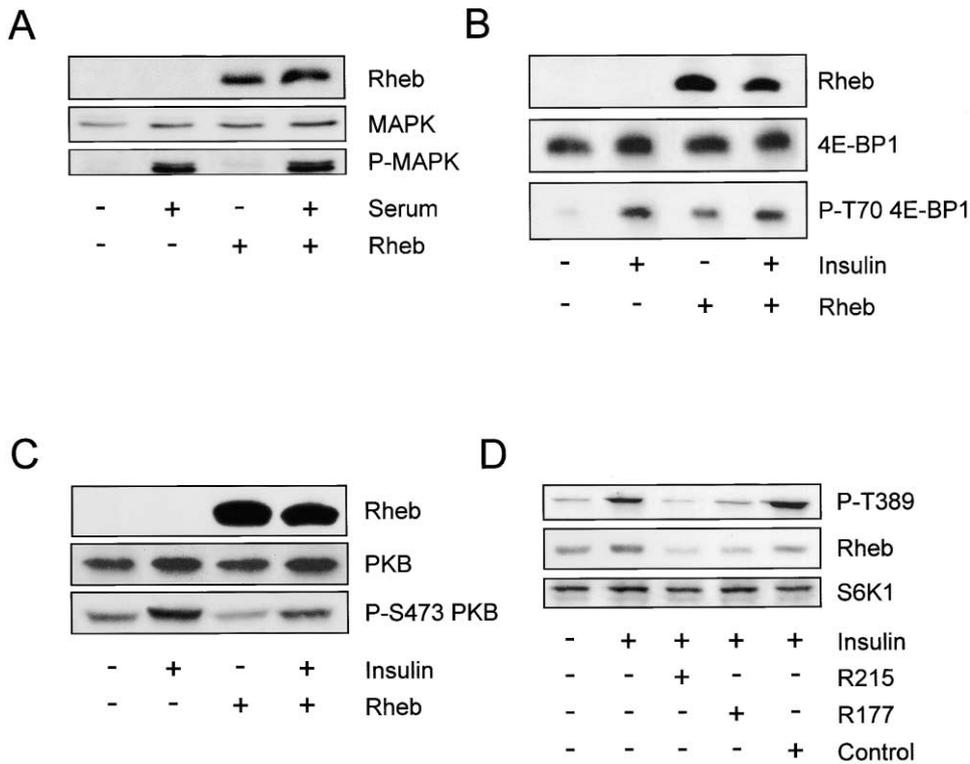


Figure 3. Effect of Ectopic Expression of Rheb and Rheb siRNA on Insulin-Induced Signaling Components

(A) Rheb has no effect on MAPK activity. HeLa cells were transfected with HA epitope-tagged p44 MAPK alone or together with myc epitope-tagged Rheb. Serum-deprived cells were stimulated with 10% fetal calf serum for 30 min as indicated, and p44 MAPK phosphorylation was detected with a phosphospecific antibody.

(B) Rheb induces constitutive 4E-BP1 phosphorylation in the absence of insulin. HA-4E-BP1 was expressed alone in HEK 293 cells alone or together with myc-Rheb, and the extent of 4E-BP1 phosphorylation was monitored with a phosphospecific antibody following no treatment or following stimulation with 200 nM insulin as indicated.

(C) Basal- and insulin-stimulated PKB phosphorylation is inhibited by Rheb. HA-PKB was expressed in HEK 293 cells alone or together with myc-Rheb, and the extent of PKB phosphorylation was monitored with a phosphospecific antibody following no treatment or following stimulation with 200 nM insulin as indicated.

(D) Insulin-induced activation of S6K1 is inhibited in HeLa cells when Rheb levels are decreased by RNAi. Cells were cotransfected with a myc-S6K1-GST reporter alone or together with the indicated Rheb siRNAs (R215 or R177) or with a control siRNA. After stimulation with 200 nM insulin, S6K1 phosphorylation was monitored with a phosphospecific antibody.

was previously observed in *TSC2*<sup>-/-</sup> MEFs (Jaeschke et al., 2002). These findings are consistent with the repressive effects of TSC1/2 on S6K1 activation being relieved by insulin-induced activation of PI3K. It has also been demonstrated that S6K1 activation in TSC1/2-deficient cells is resistant to amino acid deprivation. Both amino acids and ATP affect the mTOR pathway, with the effects of amino acids being indirect and those of ATP being direct on mTOR (Dennis et al., 2001). Consistent with these findings, amino acid withdrawal has no negative impact on Rheb-induced S6K1 activation; indeed, it consistently leads to a slight stimulation, whereas insulin-induced activation is totally abolished (Figure 4A). Consistent with the inhibitory effects of 2-deoxyglucose (2-DG) on mTOR being attributed to its lowering of intracellular ATP levels (Dennis et al., 2001), which act directly on mTOR, 2-DG almost totally abolishes Rheb-induced S6K1 activation, almost to the same extent as rapamycin (Figures 4A). The results support a model whereby insulin-induced PI3K activation is in part responsible for mediating S6K1 activation through

relieving TSC1/2 inhibition of Rheb, and that the effects of amino acid and energy on mTOR function are regulated by distinct mechanisms.

If the severest forms of TSC syndrome are associated with mutations that affect the TSC2 GAP domain, then mutations in this domain associated with the disease should be ineffective in suppressing both Rheb activation and Rheb-induced activation of the mTOR/S6K/4E-BP signaling pathway. To test this possibility, we analyzed the effect of one such human mutation harboring a single amino acid change in the TSC2 GAP domain, N1643K, on both responses. Although, coexpression of wild-type TSC1/2-GAP lowers the amount of GTP bound Rheb, similar to the result depicted in Figure 1C, a TSC1/2-GAP mutant harboring a point mutation in the GAP domain, N1643K, had no effect on the levels of GTP bound Rheb (Figure 4B). Likewise, coexpression of the wild-type TSC2, but not the TSC2 GAP mutant, blocked Rheb-induced S6K1 activation (Figure 4C). These findings are consistent with loss of TSC1/2-GAP function leading to increase in GTP bound Rheb and driving pro-

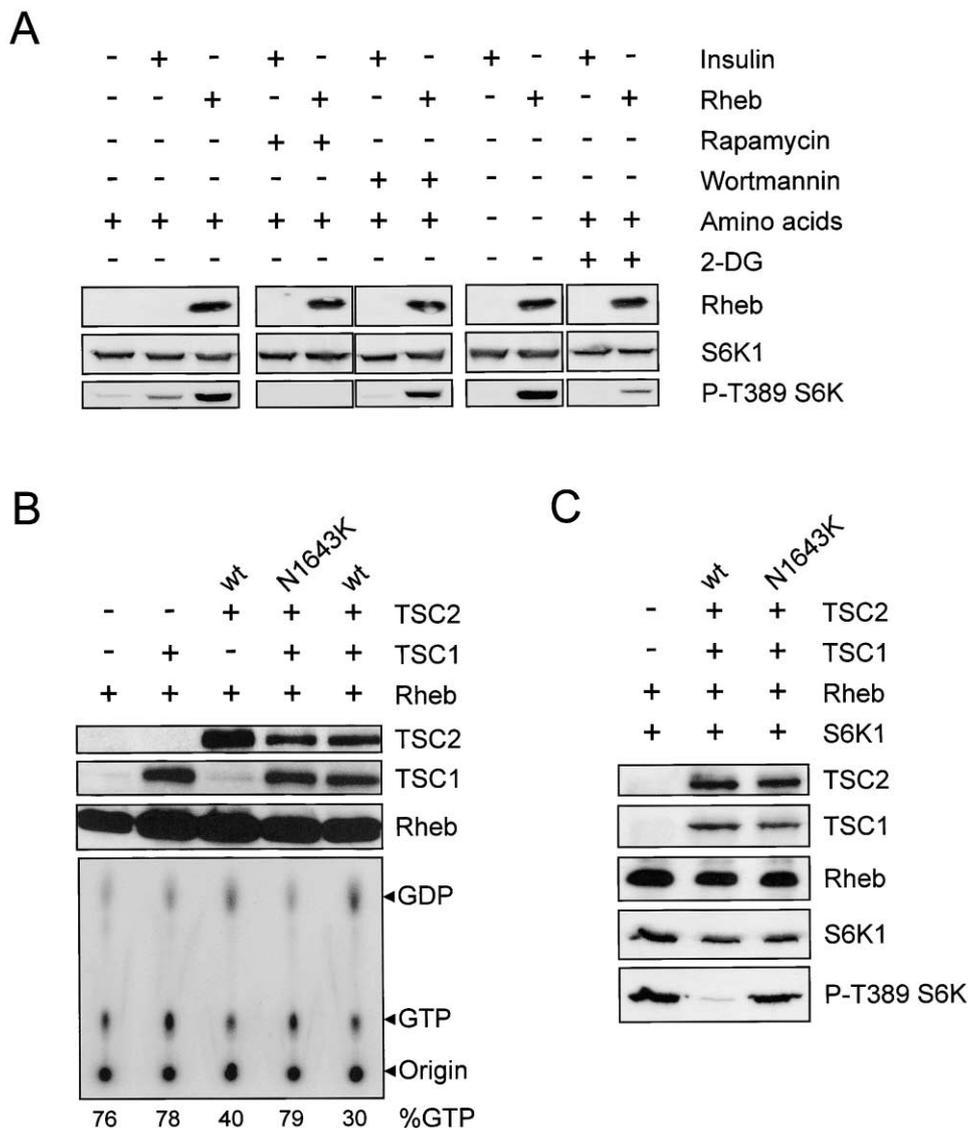


Figure 4. Effect of Pharmacological Agents, Amino Acid Deprivation, and a TSC2 Mutant on Rheb-Induced S6K1 Activation

(A) HeLa cells were transfected with myc-S6K1-GST alone or together with myc epitope-tagged Rheb and then deprived of serum overnight. Cells were then pretreated with either 100 nM wortmannin, 20 nM rapamycin, or 100 mM 2-DG for 15 min or deprived of amino acids for 15 min and then stimulated with 200 nM insulin for 30 min. S6K1 phosphorylation was monitored with a phosphospecific antibody.

(B) A TSC syndrome-related GAP mutant of TSC2 fails to inhibit Rheb activity. Myc-Rheb was transfected alone into COS-7 cells or together with HA-TSC1 and either wild-type Flag-TSC2 or Flag-TSC2 harboring a point mutation, N1643K, in the GAP domain.

(C) A TSC syndrome-related GAP mutant of TSC2 fails to inhibit Rheb-induced S6K1 phosphorylation. Transfections were performed as in (B), except that myc-S6K1-GST was used as a reporter and the experiment was conducted in HeLa cells. S6K1 phosphorylation was monitored with a phosphospecific antibody.

liferation through activation of the mTOR/S6K/4E-BP signal transduction pathway.

## Discussion

The finding that TSC1/2 is a negative effector of the mTOR/S6K/4E-BP signal transduction pathway emerged from studies in *Drosophila*, demonstrating that dS6K was epistatic to the dTsc1/2, the *Drosophila* orthologs of TSC1/2 (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Recent biochemical studies have confirmed the epistatic relationship; overexpression of dTsc1/2 in

larvae represses dS6K activity, whereas larvae harboring mutations in dTsc1 or 2 or *Drosophila* cultured cells treated with dTsc1 or 2 double-stranded RNAi have increased dS6K activity (Gao et al., 2002; Radimerski et al., 2002). In parallel, similar results were found in mammalian systems (Goncharova et al., 2002; Inoki et al., 2002; Jaeschke et al., 2002; Manning et al., 2002; Tee et al., 2002). Here we show that in mammalian cells the target of TSC1/2 is the small GTPase Rheb. During the time these studies were being carried out, we also identified mutations in the *Drosophila* ortholog of Rheb, dRheb, while carrying out a genome-wide screen in

search of new effectors of growth. Such mutants were found to strongly suppress growth and to totally abolish the overgrowth phenotype induced by dTsc1/2 loss-of-function mutations. In contrast, overexpression of dRheb induces overgrowth, an effect that is strongly suppressed in a dS6K-deficient background. Consistent with the findings reported here, larvae harboring mutations in dRheb or larvae overexpressing dRheb have either reduced or elevated dS6K activity, respectively (Stocker et al., 2003). Here we show in mammalian cells, as is presumably the case in *Drosophila*, that these effects are controlled through modulating Rheb GTPase activity. Our results are fully consistent with TSC2 acting as a GAP for Rheb, although in vitro experiments with bacterially produced proteins are required to confirm this. Moreover, we show that insulin-induced activation of Rheb mediates activation of the mTOR/S6K/4E-BP signal transduction pathway, in a PI3K-dependent manner (Figures 2A and 3D).

The question that arises from these studies is the mechanism by which GTP bound Rheb stimulates the mTOR/S6K/4E-BP signal transduction pathway. The fact that dTsc2 and dTOR were found to coimmunoprecipitate with one another when overexpressed in *Drosophila*-cultured cells (Gao et al., 2002) and that coexpression of TSC1/2 with mTOR in mammalian cells was found to block mTOR kinase activity in an immune-complex assay in vitro employing S6K1 as a substrate (Inoki et al., 2002) suggested that the target of TSC1/2 inhibition was mTOR. This conclusion was also supported by the fact that a second kinase target of mTOR, 4E-BP1 (Brunn et al., 1997; Gingras et al., 1998), is also constitutively phosphorylated in *TSC2*<sup>-/-</sup> cells (Jaeschke et al., 2002), and overexpression of TSC1/2 inhibited this response (Inoki et al., 2002). Most importantly, insulin activation of an S6K1 variant, which is decoupled from mTOR, was not suppressed by coexpression of TSC1/2 (Inoki et al., 2002). This variant lacks both the carboxy autoinhibitory domain and the acidic amino terminus and is rapamycin resistant (Cheatham et al., 1995; Dennis et al., 1996; Weng et al., 1995). Such mutants have been exploited to show that amino acids (Hara et al., 1998) or ATP (Dennis et al., 2001) act either upstream of or directly on mTOR, respectively. The studies presented here would support a model whereby the inhibitory effects of TSC1/2 on mTOR are mediated through inhibition of Rheb, which in turn acts as an activator of mTOR.

However, despite the observations above, the point at which TSC1/2 acts in the mTOR pathway is controversial (McManus and Alessi, 2002). First, activation of a second S6K1 variant, which is also decoupled from mTOR, is still suppressed by coexpression of TSC1/2 (Jaeschke et al., 2002). This variant also lacks the carboxy autoinhibitory domain but has glutathione transferase fused to its intact amino terminus and is also resistant to rapamycin (Dennis et al., 2001). Moreover, the ability to detect changes in mTOR activity in vitro following mitogen treatment has been difficult to demonstrate (Dennis et al., 2001; Gingras et al., 2001; Kim et al., 2002). Indeed, we reported earlier, employing the same assay as Inoki et al. (2002), that we were unable to detect changes in mTOR activity as a function of either stimulation of cells with insulin or by ectopic expression of activated alleles of PI3K, despite the fact that both treatments have a

pronounced effect on S6K1 activity (Dennis et al., 2001). In subsequent studies, we have not been able to detect any effects of coexpressing TSC1/2 on mTOR in vitro kinase activity, although such treatment blocks reporter S6K1 activation (S.Y. Kim and G.T., unpublished data). In addition, recent studies have shown that mTOR exists in multienzyme complexes and directly interacts with at least two additional proteins, raptor and mLST8, which mediate its ability to signal to downstream substrates such as S6K1 and 4E-BP1 (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Nojima et al., 2003). This raises the possibility that Rheb, rather than being a direct activator of mTOR, may have a more permissive role, acting instead on one of the proteins, which interact with mTOR. The identification of the downstream Rheb effector will be essential in further elucidating the molecular mechanisms by which this pathway is controlled.

The finding that Rheb is largely in the GTP bound active state in TSC2-deficient cells is consistent with Rheb mediating the pathological effects caused by mutations in *TSC1* and *2*. In this respect, it should be noted that the *Rheb* gene has been mapped to chromosome 7q36 (Mizuki et al., 1996), a locus that has been implicated through positional cloning to contain one or more susceptibility genes for autism (International Molecular Genetic Study of Autism Consortium, 1998). Moreover, 20%–30% of patients who present TSC syndrome have no mutations in *TSC1* or *2* (D. Franz, personal communication), raising the possibility that mutations in Rheb or its overexpression could be responsible for the disease in these patients. Ras family members contain five regions involved in GTP binding, denoted G1 through G5 (Bourne et al., 1990). These regions contain the highest shared homologies between Ras and Rheb, and are consistent with the intrinsic GTPase activity of Rheb (Yamagata et al., 1994). One of the most striking features of Rheb is the G1 box, or the GX<sub>4</sub>GKS/T motif, where Rheb encodes arginine and serine at positions 15 and 16, rather than the glycines that are present at the homologous sites in the other Ras family members (Bos, 1997; Reuther and Der, 2000). The arginine may be particularly important, since an equivalent mutation in Ras results in low intrinsic GTPase activity and resistance to the GTPase-activating effects of Ras-GAP (Bos, 1997; Reuther and Der, 2000). However, the results presented here argue that TSC1/2 can stimulate the GTPase activity of Rheb. In this respect it is interesting to note that TSC2-GAP is a close relative of Rap1-GAP, which induces GTPase activity of Rap1 by a different mechanism than Ras-GAP induces the GTPase activity of Ras (Brinkmann et al., 2002). Similar to Rheb, Rap1 has a T rather than the common Q in the DTAGQE motif of the G3 box, resulting in very low intrinsic GTPase activity. In fact, a similar mutation in Ras makes it resistant to Ras-GAP (Brinkmann et al., 2002).

Rheb is in a high activation state when it is overexpressed at low levels (Im et al., 2002; data not shown), possibly overcoming endogenous levels of TSC1/2-GAP. In agreement with this notion, we did not find any substantial regulation of transfected Rheb by insulin, amino acid depletion, serum starvation, or wortmannin treatment (data not shown). Notably, Rheb overexpression has been detected in a number of tumor cell lines (Gromov et al., 1995), and overexpression of wild-type Rheb

is as efficient as wild-type Raf-1 or H-Ras in inducing transformation in NIH3T3 cells (Yee and Worley, 1997). Furthermore Rheb strongly synergizes with Raf-1, to the same extent as Raf-1 synergizes with wild-type H-Ras, in the NIH 3T3 transformation assay (Yee and Worley, 1997). A similar effect as overexpression can be expected from mutations that render Rheb insensitive to TSC2, like the F64A mutation in Rap1 that makes Rap1 resistant to Rap1GAP (Brinkmann et al., 2002). However, such mutations have not been reported in patients. At this point it will be important to determine whether (1) Rheb is overexpressed or contains mutations in TSC1 or 2, (2) it represents a susceptibility locus for autism, and (3) overexpression of Rheb induces cancer in a model system, such as the mouse.

## Experimental Procedures

### Materials

Reagents were obtained from the following sources: 2-deoxy-D-glucose, insulin, wortmannin, and rapamycin from Sigma-Aldrich Co.; anti-Rheb (C19) antibody from Santa Cruz Biotechnology; antibodies against Ras, Rap2, and Ral were from Transduction Laboratories; antiphosphoprotein (phospho-T389-S6K, phospho-S473-PKB, phospho-T202/Y204-p44/42 MAPK, phospho-T70-4E-BP1) antibodies from Cell Signaling Technology; and all HRP-coupled secondary antibodies from DakoCytomation. 12CA5 and 9E10 antibodies were used for detection of HA and myc epitope-tagged proteins, respectively.

### Cell Cultures and Transfections

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, except for HeLa cells, which contained in addition 4 mM glutamine. HeLa, COS 7, and HEK 293 cells were transfected with either FuGENE (Roche) or Lipofectamine 2000 (Invitrogen) by calcium phosphate precipitation. For RNAi experiments, transfection of siRNAs and reporter constructs was carried out with TransIT-TKO (Mirus Corporation). In all transfections the manufacturer's instructions were followed.

### Constructs and siRNAs

A pcDNA3-Rheb construct encoding human Rheb was used as template for myc tagging of Rheb. The EQKLISEEDL tag was introduced by PCR giving a BamHI-myc-Rheb-NotI fragment, which was cloned into BamHI and NotI cut pRK5 eukaryotic expression vector. All other constructs used in transient expressions were as previously described (Dennis et al., 2001; Jaeschke et al., 2002). siRNAs targeting Rheb were designed for the Rheb coding sequence corresponding to amino acids 177–183 and 215–221.

### Cell Lysates and Immunoblotting

Where applicable, following overnight serum deprivation, cells were either lysed directly or stimulated with insulin or serum for the indicated times. Equal amounts of cleared lysates were analyzed by Western blot analyses with the indicated antibodies as previously described (Dennis et al., 2001).

### Nonradioactive Activation Assays for Small GTPases

The GTP bound form of small GTPases was specifically pulled down as described previously (van Triest et al., 2001). In brief, cells were lysed in 1% NP-40, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM PMSF, 1 mM leupeptin, 0.1 mM aprotinin, 10 mg/ml trypsin inhibitor. Cleared lysates were incubated with GST-tagged forms of the Ras binding domain from Raf1, RalGDS, or Ral binding domain of RLIP76 for pull-down of ras, Rap2, or Ral, respectively. GTPases were visualized by immunoblotting following the transfer to PVDF membranes.

### Radioactive Activation Assay for Small GTPases

Measurements of GTP/GDP-charged state of transfected epitope-tagged small GTPases were performed as described earlier (Wolthuis et al., 1997), except that overexpressed Rheb was pulled down with anti-myc antibody. For measurements of endogenous Rheb and Ras activities, the proteins were pulled down by either the C19 or Y13-259 antibody, respectively.

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