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Rheb Binds and Regulates the mTOR Kinase

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Summary

Background: The target of rapamycin (TOR), in complex with the proteins raptor and LST8 (TOR complex 1), phosphorylates the p70S6K and 4E-BP1 to promote mRNA translation. Genetic evidence establishes that TOR complex activity in vivo requires the small GTPase Rheb, and overexpression of Rheb can rescue TOR from inactivation in vivo by amino-acid withdrawal. The Tuberous Sclerosis heterodimer (TSC1/TSC2) functions as a Rheb GTPase activator and inhibits TOR signaling in vivo.

Results: Here, we show that Rheb binds to the TOR complex specifically, independently of its ability to bind TSC2, through separate interactions with the mTOR catalytic domain and with LST8. Rheb binding to the TOR complex in vivo and in vitro does not require Rheb guanyl nucleotide charging but is modulated by GTP and impaired by certain mutations (Ile39Lys) in the switch 1 loop. Nucleotide-deficient Rheb mutants, although capable of binding mTOR in vivo and in vitro, are inhibitory in vivo, and the mTOR polypeptides that associate with nucleotide-deficient Rheb in vivo lack kinase activity in vitro. Reciprocally, mTOR polypeptides bound to Rheb(Gln64Leu), a mutant that is nearly 90% GTP charged, exhibit substantially higher protein kinase specific activity than mTOR bound to wild-type Rheb.

Conclusions: The TOR complex 1 is a direct target of Rheb-GTP, whose binding enables activation of the TOR kinase.

Introduction

Target of rapamycin (TOR) is a giant protein kinase that regulates cell growth and proliferation in *S. cerevisiae* as well as in metazoans [1, 2]. TOR functions in two independent complexes. The TOR complex 1 (TORC1) is comprised of TOR in association with the proteins raptor [3, 4] (KOG1 in *S. cerevisiae* [5]) and LST8 [5] (also called GβL [6]), and its output is inhibited by rapamycin. The TOR complex 2 (TORC2) contains TOR in association with the proteins AVO3/rictor and LST8 [5, 7, 8]. TORC2 controls the actin cytoskeleton; its output is insensitive to rapamycin and will not be considered further here.

A major target of TORC1 regulation in all cells is mRNA translation [9]. In mammalian cells, mTOR stimulates translational initiation through the phosphorylation of 4E-BP1, an inhibitor of the binding of the mRNA-cap binding protein eIF-4E to the eIF-4G scaffold. Phosphorylation of 4E-BP1 promotes its dissociation from eIF-4E, enabling recruitment of the latter into the eIF-4F complex. mTOR also directly phosphorylates and, in collaboration with PDK1, activates the p70 S6 kinase [10]; the latter regulates cell size [11] through incompletely defined mechanisms. The ability of the mTOR kinase to phosphorylate these targets is dependent on the association of TOR with raptor, inasmuch as raptor binds the TOR substrates 4E-BP1 and p70S6K and presents them to mTOR [3]. In fact, the ability of mTOR to phosphorylate 4E-BP1 is nearly eliminated by removal of raptor in vitro [3, 12], or in vivo by mutation of the 4E-BP1 "TOS" motif [13], the 4E-BP1 segment that mediates binding to raptor [12, 14–16]. The ability of TOR to regulate p70S6K is also strongly dependent on the association of p70S6K with raptor. Mutation of the p70S6K TOS motif reduces mTOR-catalyzed phosphorylation of p70S6K in vitro by approximately 75% and renders p70S6K insensitive to inhibition in vivo by rapamycin or to regulation by ambient amino acids [12]. LST8, the third component of the TORC1 complex [5, 6], is a 36 kDa polypeptide whose predicted structure is composed entirely of seven WD repeats, presumably arrayed in a so-called "β propellor" [17]. LST8 plays an important but incompletely defined role in TOR regulation. The polypeptide binds tightly to the TOR catalytic domain and enhances the association of raptor with mTOR [6]; however, other roles remain likely.

In mammalian cells, mTOR output is sensitive to amino-acid (especially leucine and arginine) sufficiency but is also controlled by overall energy supply through the AMP-activated protein kinase (AMPK) and by inputs from cell surface receptors through the PI-3 Kinase (PI3K)-PKB pathway [2]. Recent evidence indicates that the tuberous sclerosis complex (a TSC1/TSC2 heterodimer) acts as a negative regulator upstream of mTOR and is a major target through which PKB, AMPK, and perhaps amino-acid sufficiency control mTOR activity [18–20]. Inactivation of TSC results in constitutive activation of p70S6K that is resistant to inhibition by withdrawal of amino acids but sensitive to inhibition by rapamycin [18, 20, 21]. PKB, whose overexpression indirectly promotes the hyperphosphorylation of 4E-BP1 and activation of p70S6K, phosphorylates TSC2 at several sites and may accelerate the degradation of both TSC2 and TSC1, perhaps by promoting the dissociation of the heterodimer [22–24]. In contrast, AMPK-cata-

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lyzed phosphorylation of TSC2 enhances the ability of the TSC1/TSC2 complex to inhibit TOR [25].

The inhibition of TOR signaling by the TSC complex is attributable to the ability of the TSC complex to act as a specific GTPase-activating protein for the small GTPase Rheb [26–31]; the latter is a positive regulator of cell growth, which, in *Drosophila*, is epistatic to the TSC complex but requires TOR to achieve growth stimulation [32, 33]. The effectors utilized by Rheb to control cell function are as yet unknown. Rheb is reported to bind to the amino terminus of c-Raf1 and B-Raf [34, 35]. Nevertheless, Rheb does not activate Raf [35] and inhibits cell transformation when coexpressed with Ras(Gly12Val) [36].

The present studies were undertaken to elucidate the mechanisms utilized by Rheb to control TOR signaling. Our results demonstrate that Rheb regulation of mTOR signaling occurs, at least in part, through the direct binding of Rheb-GTP to mTOR, an interaction that promotes activation of the mTOR kinase activity.

Results

Rheb Overexpression Rescues p70S6K from Inactivation by Amino-Acid Withdrawal

Coexpression of p70 S6 kinase (p70S6K) with wild-type Rheb in HEK293 cells stimulates the phosphorylation of p70S6K (Thr412; this numbering refers to the 525 amino acid α1 isoform and is equivalent to Thr389 in the 502 amino acid α2 isoform) and almost completely overcomes the inhibitory effect of amino-acid withdrawal (Figure 1A). The effect of Rheb is entirely abolished by rapamycin, indicating a requirement for mTOR (data not shown). Rheb expression has no significant effect on the site-specific phosphorylation of the MAPK activation loop or on PKB Ser473. By comparison, Ras(Gly12Val) strongly stimulates the activity of endogenous MAPK but only slightly stimultes p70S6K Thr412 phosphorylation and doesn't stimulate PKB Ser473 phosphorylation. Rap1b(Gly12Val) has little effect on any of these protein kinases (Figure S1). These results support the now widely held view that Rheb acts as a positive regulator upstream of TOR and downstream of the TSC complex [26, 27, 32, 33]; the latter functions as a Rheb GTPase [26–31] and a negative regulator of TOR [19].

Effects of Rheb Mutations on Signaling

The effect of Rheb mutations on signaling to p70S6K was examined. Rheb Ser20 corresponds to Ras Ser17, whose replacement by Asn inactivates Ras and creates a dominant inhibitory mutant [37]. Replacement of Rheb Ser20 by Asn was shown previously to result in a loss of function in *S. pombe* Rheb [38]. Transfected human Rheb(Ser20Asn) is expressed poorly as compared with wild-type Rheb, and not surprisingly, this mutant is unable to rescue p70S6K from amino-acid deprivation; nevertheless, Rheb(Ser20Asn) causes a marked inhibition of p70S6K Thr412 phosphorylation in the presence of serum and amino acids despite unaltered S6K expression (Figure 1A, compare lane 5 with lanes 1 and 3).

Mutations were introduced into three sites in the Rheb switch 1 segment, Thr38Met (also shown to result in loss of function of *S. pombe* Rheb [38]), Ile39Lys, and Asn41Ala. All three mutants are expressed at levels comparable to wild-type Rheb. Both Thr38Met and Ile39Lys lose the ability to stimulate p70S6K Thr412 phosphorylation in complete medium and to rescue p70S6K Thr412 dephosphorylation induced by amino-acid withdrawal (Figure 1A, lanes 1–4, 7, 8, and 11–16). In addition, Ile39Lys expression causes inhibition of p70S6K Thr412 phosphorylation in complete medium (Figure 1A, compare lane 15 with lane 11). The Asn41Ala mutant retains the ability to stimulate p70S6K phosphorylation in complete medium but is greatly compromised in its ability to rescue p70S6K from dephosphorylation induced by amino-acid withdrawal (Figure 1A, lanes 1–4, 9, and 10).

The Rheb switch 2 mutant Asp60Ile was recently reported to interfere selectively with GTP binding in vitro [39]. This mutant is unable to rescue p70S6K from amino-acid-withdrawal-induced dephosphorylation (Figure 1A, lanes 17, 18, 21, 22) and causes a moderate inhibition of p70S6K Thr412 phosphorylation in complete medium (Figure 1A, compare lane 21 with lane 17). Finally, we find that mutation of the Rheb prenylation site, Cys181Ser, reduces but does not eliminate the ability of Rheb to rescue p70S6K from amino-acid deprivation. The Cys181Ser mutant is still reasonably effective in stimulating p70S6K Thr412 phosphorylation in complete medium (lanes 23–28).

The effects of wild-type and mutant Rheb on the phosphorylation of 4E-BP1 in amino-acid replete and deficient medium parallel those seen with p70S6K (Figure S2). Thus, the domains in Rheb concerned with guanyl nucleotide binding and effector activation in the TOR pathway are homologous to those defined for other Ras-like GTPases.

Effects of Rheb Mutations on Guanyl Nucleotide Binding

Wild-type and mutant Rheb were expressed transiently as GST-fusion proteins in cells labeled with extracellular $^{32}P_i$ (Figure 1B). Recovery of total Rheb-associated ^{32}P-guanyl nucleotide increases in proportion to GST-Rheb expression. At the lowest Rheb expression examined, i.e., at ^{32}P levels bound to GST-Rheb that are only two to four times greater than those recovered with GST, the fraction of total Rheb bound nucleotide that is ^{32}P-GTP varied from 42%–63%. This proportion stabilized near 60% at higher levels of GST-Rheb polypeptide. Similar high levels of GTP charging have been reported previously for recombinant Rheb [35, 40] and have been attributed to saturation of endogenous Rheb-GAP by the overexpressed Rheb polypeptide, together with the very low rate of intrinsic Rheb GTPase activity.

The Rheb switch 1 mutants (Thr38Met and Ile39Lys) exhibited %GTP similar to wild-type, whereas Rheb (Cys181Ser) exhibited a slightly lower %GTP than wild-type (Figure 1B). In contrast, no ^{32}P-guanyl nucleotide was recovered with Rheb(Ser20Asn) or (Asp60Ile), although these mutants were expressed in amounts comparable to those of wild-type Rheb. Therefore, the steady-state binding of GTP + GDP in vivo to recombinant Rheb(Ser20Asn) and (Asp60Ile) is less than 5% that of wild-type Rheb and may be lacking completely.

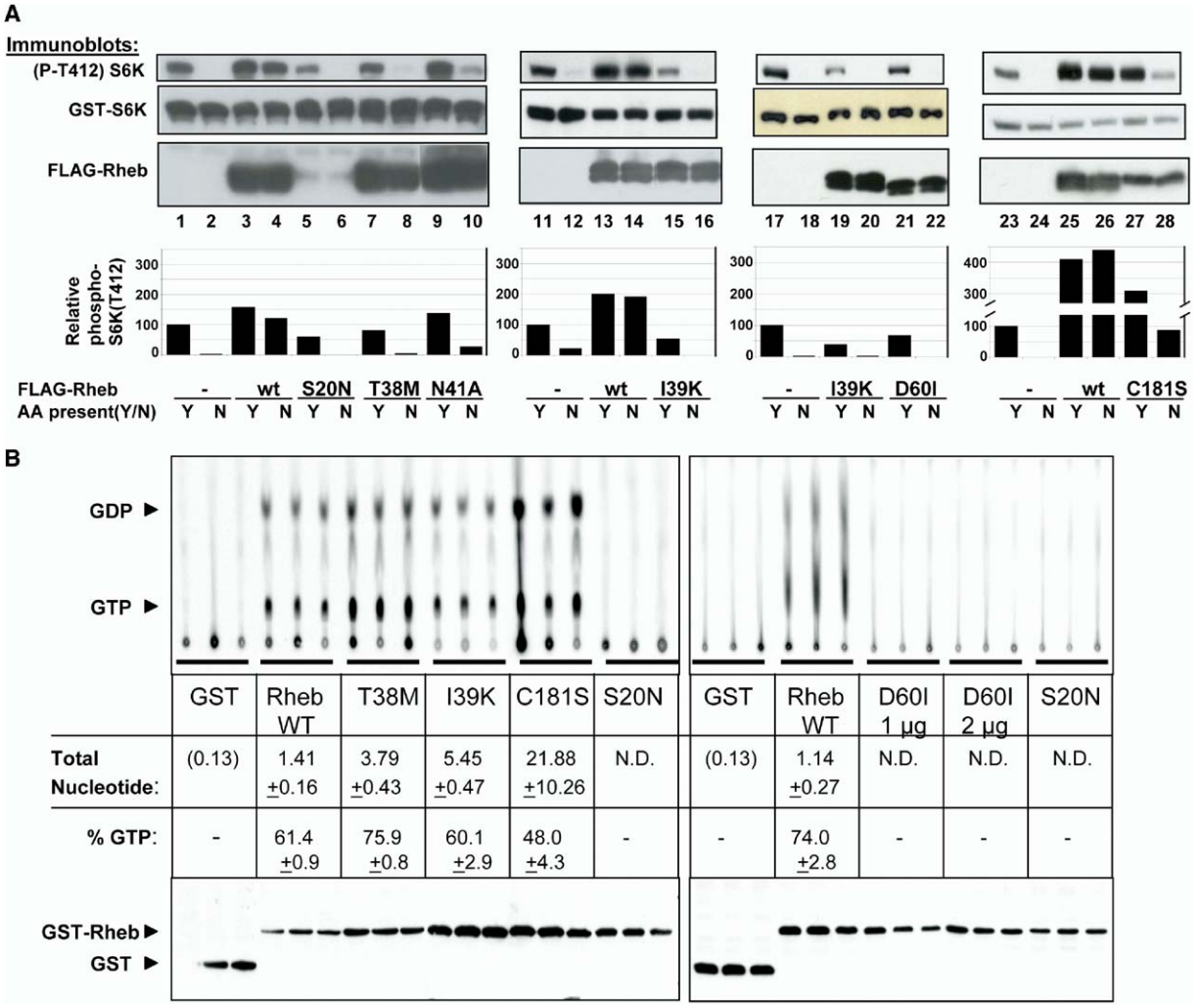

Figure 1. Characterization of Wild-Type and Mutant Rheb

(A) The effect of wild-type and mutant Rheb on the phosphorylation in vivo of p70S6K in amino-acid-replete and -deficient medium. The effect of wild-type and mutant Rheb on the phosphorylation in vivo of p70S6K in amino-acid-replete and -deficient medium. HEK293T cells were transfected with pEBG-p70S6K (lanes 1–28) with pCMV5-FLAG vector (lanes 1, 2, 11, 12, 17, 18, 23, and 24) or with pCMV5-FLAG-Rheb wild-type (lanes 3, 4, 13, 14, 25, and 26) or various mutants as indicated above each lane. 40 hr later, some plates were transferred to D-PBS (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28) and all cells were harvested 2 hr thereafter. Each panel represents a separate experiment. The bar graph is a quantitation of the anti-Thr412-P immunoblot results for the experiments shown with the value of S6K coexpressed with empty vector set to 100. A quantitative summary of the effect of recombinant Rheb on S6K(Thr412P) in replete medium as a % of vector control is: Rheb(wild-type) (0.5 μg/plate, n = 8; not including the experiment in lanes 23 and 25) = 194 ± 49 (= 1 SD)%; Rheb(Asp60Ile) (2.8 μg/plate, n = 3; 3.5 μg, n = 1) 60 ± 16 (= 1 SD)%; Rheb(Ser20Asn), two experiments (0.5 μg/plate) 40.6% and 59.6%.

(B) Guanyl nucleotide binding in vivo by wild-type and mutant Rheb. GST or GST fusions of wild-type or mutant Rheb were transiently expressed in HEK293T cells in triplicate with 0.3 μg of the pEBG vector, except for Ser20Asn (1.0 μg) and Asp60Ile (1.0 or 2.0 μg). 40 hr later, the cells were washed and incubated in phosphate-free DMEM containing 0.2 mCi/ml of [^{32}P] orthophosphate. After 4 hr, the GST and the GST-fusion proteins were extracted and purified by GSH affinity chromatography and washed, and the bound guanyl nucleotides were extracted and separated by thin layer chromatography as described in methods. ^{32}P comigrating with GDP and GTP were quantified by phosphoimager; the total [^{32}P-GDP + GTP] (in arbitrary phosphoimager units) and the percentage of total [^{32}P-GDP + GTP] as GTP are shown, ± one standard deviation. The GST immunoblot shows the relative amounts of purified GST proteins.

We examined the ability of these Rheb mutants to bind guanyl nucleotide in vitro, in comparison to wild-type Rheb. The extent of γ^{35}S-GTP binding in vitro by both Rheb(Ser20Ala) and (Asp60Ile) is less than 10% that of wild-type Rheb, and their binding of ^{3}H-GDP was near background (data not shown). Thus the Ser20Asn and Asp60Ile mutations markedly reduce and may eliminate Rheb binding of guanyl nucleotide.

Rheb Binds Directly to the TOR Complex in a Guanyl-Nucleotide-Independent Manner

Biochemical and genetic evidence establish that the ability of the TSC1/TSC2 heterodimer to act as an inhibitor of TOR signaling is mediated through Rheb. To test the possibility that Rheb acts directly on mTOR, we first examined whether Rheb is capable of binding mTOR. Recombinant GST or a variety of GST-Rheb fusion pro-

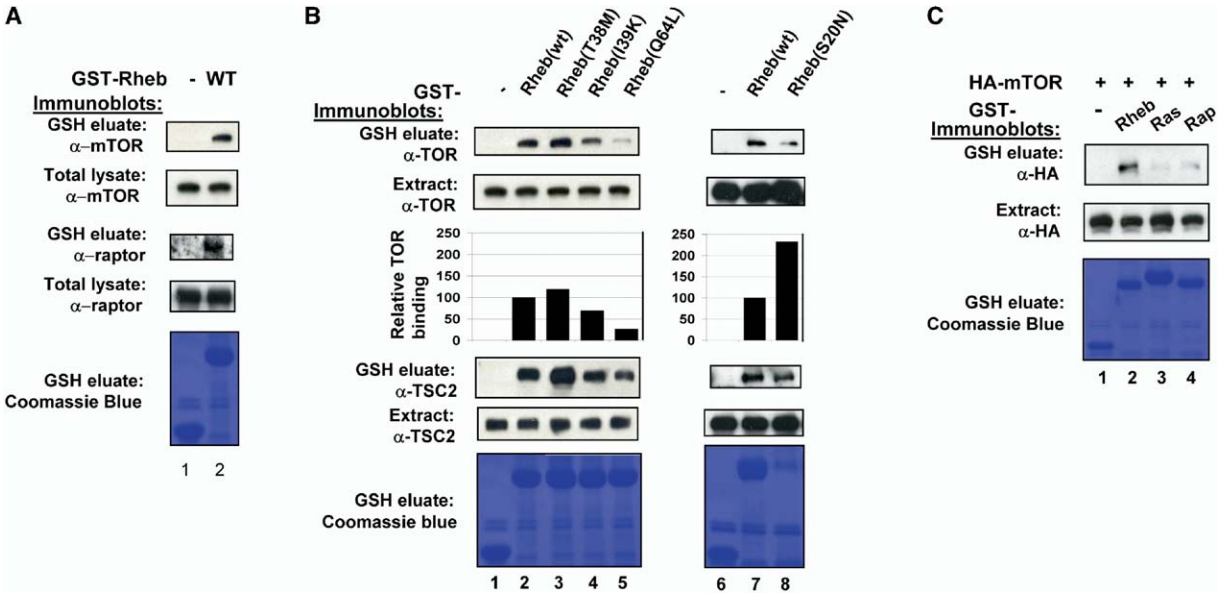

Figure 2. Recombinant Rheb Binds Specifically to Endogenous and Recombinant mTOR

(A) Recombinant Rheb binds to endogenous mTOR and raptor. HEK293T cells were transfected with pEBG (lane 1) or pEBG-Rheb wild-type (lane 2). 40 hr later, cells were extracted, and the GST fusion proteins were purified on GSH-Sepharose. The GSH eluates and aliquots of the extract were analyzed by immunoblot for mTOR (top two panels) and raptor (panels 3 and 4 from top). A Coomassie blue stain of the GSH eluate is in the bottom panel.

(B) Coprecipitation of endogenous mTOR and TSC2 by recombinant wild-type or mutant Rheb. HEK293T cells were transfected with pEBG (lanes 1 and 6) or pEBG-Rheb wild-type (lanes 2 and 7) or pEBG-Rheb mutants (Thr38Met, lane 3; Ile39Lys, lane 4; Gln64Leu, lane 5; Ser20Asn, lane 8). The cells were processed as in Figure 2A. The GSH eluates and aliquots of the extract were analyzed by immunoblot for mTOR (top two panels) and TSC2 (fourth and fifth panels from top). A Coomassie blue stain of the GSH eluate is shown in the bottom panel. The bar graph in the third panel from top shows the ratio of endogenous mTOR recovered relative to GST-Rheb with the value for wild-type Rheb set to 100.

(C) mTOR binds preferentially to Rheb as compared to Ras and Rap1b. HEK293T cells were transfected with pcDNA1-HA-mTOR with pEBG (lane 1), pEBG-Rheb (lane 2), Ha-Ras(Gly12Val) (lane 3), or Rap1b(Gly12Val) (lane 4). 40 hr thereafter, some cells were harvested. The GST fusion proteins purified on GSH-Sepharose were eluted and analyzed by anti-HA immunoblot (top) and Coomassie blue stain (bottom).

teins were transiently expressed in HEK293 cells, and the GSH isolate was probed for the presence of endogenous mTOR. As seen in Figures 2A and 2B, GST-Rheb can coprecipitate endogenous mTOR (Figures 2A and 2B), as well as raptor (Figure 2A) and TSC2 (Figure 2B). Notably, the inactive Rheb switch 1 mutant Thr38Met binds endogenous mTOR in a manner equivalent to wild-type GST-Rheb, whereas mTOR binding to Rheb Ile39Lys and to GST-Rheb Gln64Leu is reduced as compared to wild-type Rheb (Figure 2B). Surprisingly, the nucleotide-free Rheb mutants Ser20Asn (Figure 2B) and Asp60Ile (see below) also bind mTOR and retrieve greater amounts of endogenous (Figure 2B) and recombinant mTOR (see below) than does wild-type Rheb. As a specificity control, the GTPase-deficient forms of the two most closely related small GTPases, GST-Ha-Ras (Gly12Val) and GST-Rap1b(Gly12Val), bind much less HA-mTOR than GST-Rheb in transient coexpression experiments (Figure 2C).

We next sought to identify the region of mTOR responsible for binding Rheb. We coexpressed GST-Rheb with a variety of FLAG-tagged fragments of mTOR. The results are summarized in Figure 3A, and the data is shown in Figure S3. No binding of Rheb to mTOR 1–670 and 1–1967 was observed; however, the mTOR fragments 1967–2191, encompassing the FKBP12-rapamycin binding domain, and 2148–2549, encompassing the kinase catalytic domain to the protein carboxyterminus, each individually exhibit binding to GST-Rheb. Further analysis shows that TOR 1967–2148 does not bind Rheb, indicating that the aminoterminal margin of the Rheb binding site is located between TOR 2148–2191. Carboxyterminal truncation of the mTOR 2148–2549 fragment shows that Rheb binding is unaffected by deletion to mTOR amino acid 2300. The localization of the Rheb binding site on mTOR to the segment between amino acids 2148–2300 achieved through transient coexpression was confirmed by in vitro binding assays with purified GST-Rheb and immunopurified FLAG-tagged mTOR fragments (Figure 3B).

The mTOR segment 2148–2300 encompasses the aminoterminal portion of the mTOR catalytic domain and overlaps the proposed ATP binding site (based on homology to the PI3Kα), which includes the lysine at mTOR amino acid 2187, which is universally conserved in PI-3 kinases and PIKKs and the glycine-rich sequence GLIGW (mTOR amino acids 2235–2239). This region of mTOR is highly conserved within the PI3K-related protein kinase (PIKK) family [41]. We therefore inquired whether Rheb can bind to other members of this family, such as ATM or ATR. We were unable to achieve matching levels of expression of full-length mTOR, ATM, and ATR, so we compared the ability of Rheb to bind to the homologous segments of these

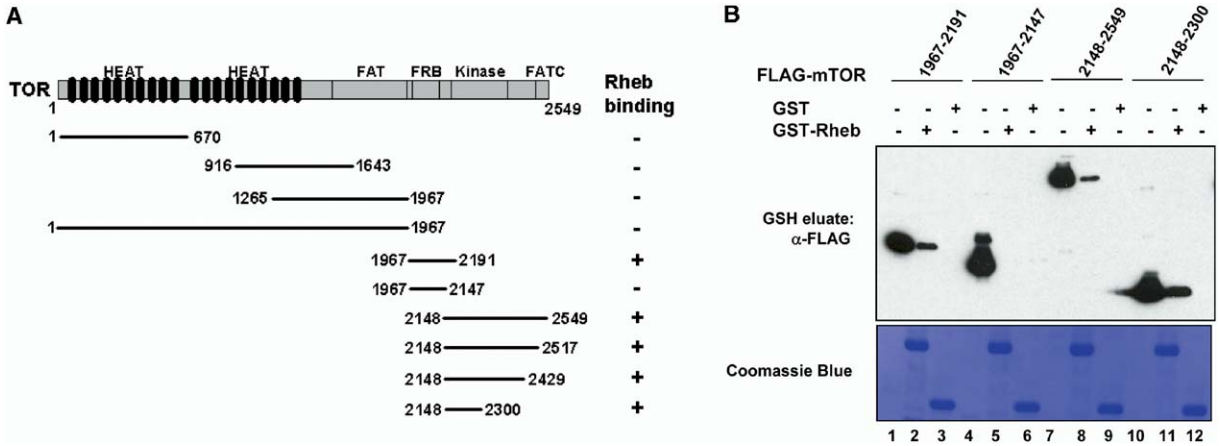

Figure 3. Recombinant Rheb Binds to the mTOR Catalytic Domain In Vivo and In Vitro

(A) GST-Rheb binding to mTOR fragments during transient expression. HEK293T cells were transfected with plasmids encoding FLAG-tagged mTOR fragments and pEBG vector or pEBG-Rheb. 40 hr later, cells were harvested and lysates subjected to GSH-Sepharose affinity purification. The eluates and cell lysates were analyzed by anti-FLAG immunoblot or by Coomassie blue stain. The recovery of the FLAG-tagged mTOR fragments with GST-Rheb is indicated on the right as + or -. The immunoblot data supporting this summary is shown in Figure S3.

(B) GST-Rheb specifically binds purified mTOR 2148–2300 directly in vitro. The FLAG-tagged mTOR fragments 1967–2191, 1967–2147, 2148–2549, and 2148–2300 were each expressed individually in HEK293T cells and eluted after immunopurification on immobilized anti-FLAG monoclonal antibody. Aliquots of each FLAG-mTOR fragment were incubated in vitro with GSH-Sepharose-immobilized GST (lanes 3, 6, 9, and 12) or GST-Rheb (lanes 2, 5, 8, and 11), both also purified after transient expression and charged in vitro with GMPPNP. After being washed, the polypeptides retained on GSH-Sepharose were analyzed by anti-FLAG immunoblot (top) and Coomassie blue stain (bottom). An aliquot representing 10% of the FLAG-polypeptide loaded with the GST proteins is shown in lanes 1, 4, 7, and 10.

polypeptides, i.e., FLAG-tagged mTOR 2148–2549, ATM 2678–3056, and ATR 2288–2644. These PIKK segments exhibited comparable binding to GST-Rheb (Figure 4A, top). In contrast, GST-Rheb does not bind to the catalytic domains of several conventional protein kinases (Figure 4A, bottom). In summary, recombinant Rheb binds to a 153 amino-acid segment of the mTOR polypeptide that encompasses the ATP binding site of the mTOR catalytic domain. Rheb exhibits comparable binding to this region of ATM and ATR but does not bind to the catalytic domain of several subfamilies of conventional protein kinases. The functional significance of the Rheb binding to ATM/ATR is as not known and is under examination.

GST and the GST-Rheb fusion protein were also coexpressed with FLAG-tagged versions of Raptor, mLST8, TSC2, TSC1, and TSC2 together and a carboxy-terminal fragment of TSC2 (1531–1807) encompassing the GAP domain (Figure 4B). Specific binding of Rheb to LST8 and to the TSC2 carboxyterminal fragment is consistently observed. Weak binding of recombinant Rheb to recombinant full-length raptor is usually detectable, whereas Rheb binding to the carboxyterminal (WD propellor) segment of Raptor is consistently observed. Thus, recombinant Rheb is capable of specific binding to the TOR complex at multiple sites.

We examined whether the ability of Rheb to bind to the components of the mTOR complex is dependent on Rheb GDP/GTP charging in in vitro binding assays. In a preliminary experiment, immunopurified FLAG-Rheb and FLAG-Ras(Gly12Val), charged with either GTPγS or GDP, were incubated with GST-c-Raf1 50–150, a prokaryotic recombinant fusion protein encompassing the c-Raf1 "Ras binding domain" (RBD) [42, 43]. As expected, the binding of Ras-GTP was much greater than Ras-GDP. However, Rheb binding, although specific for the presence of the Raf-RBD, was not only much weaker than Ras but largely independent of whether GTP or GDP is bound (Figure S4).

Subsequent experiments indicated that, as observed during transient expression in vivo, the ability of Rheb to bind to the components of the mTOR complex in vitro does not require the presence of any bound guanine nucleotide (Figure 5). The ability of highly immunopurified FLAG-tagged versions of c-Raf1 1–257, mTOR 2148–2549, hLST8, and Raptor 1009–1335 polypeptides to bind in vitro to mammalian recombinant GST-Rheb, either nucleotide free or charged with GDP or GMPPNP, was examined (Figure 5). Each of these FLAG-tagged polypeptides could bind to GST-Rheb in vitro. Binding does not, however, require nucleotide charging with either GTP or GDP. In fact, charging with GTP appears to substantially diminish the binding of Rheb to mTOR 2148–2549 as well as to the c-Raf1 1–257 fragment, as compared to nucleotide-free Rheb. The ability of nucleotide-free Rheb to bind in vitro to purified mTOR 2148–2549 is consistent with the ability of the nucleotide-deficient Rheb mutants Ser20Asn and Asp60Ile to bind full-length mTOR and the mTOR 2148–2549 fragment in vivo more strongly than does wild-type Rheb. Moreover, the diminished binding of mTOR 2148–2549 to GTP-charged Rheb as compared to nucleotide-free or GDP-charged Rheb is also consistent with the lesser ability of recombinant Rheb(Asn64Leu) as compared to wild-type Rheb to bind endogenous (Figure 2B) and recombinant (see below) mTOR. TSC2 1531–1807 fragment was used as a control in these binding assays, as it contains the putative GAP domain

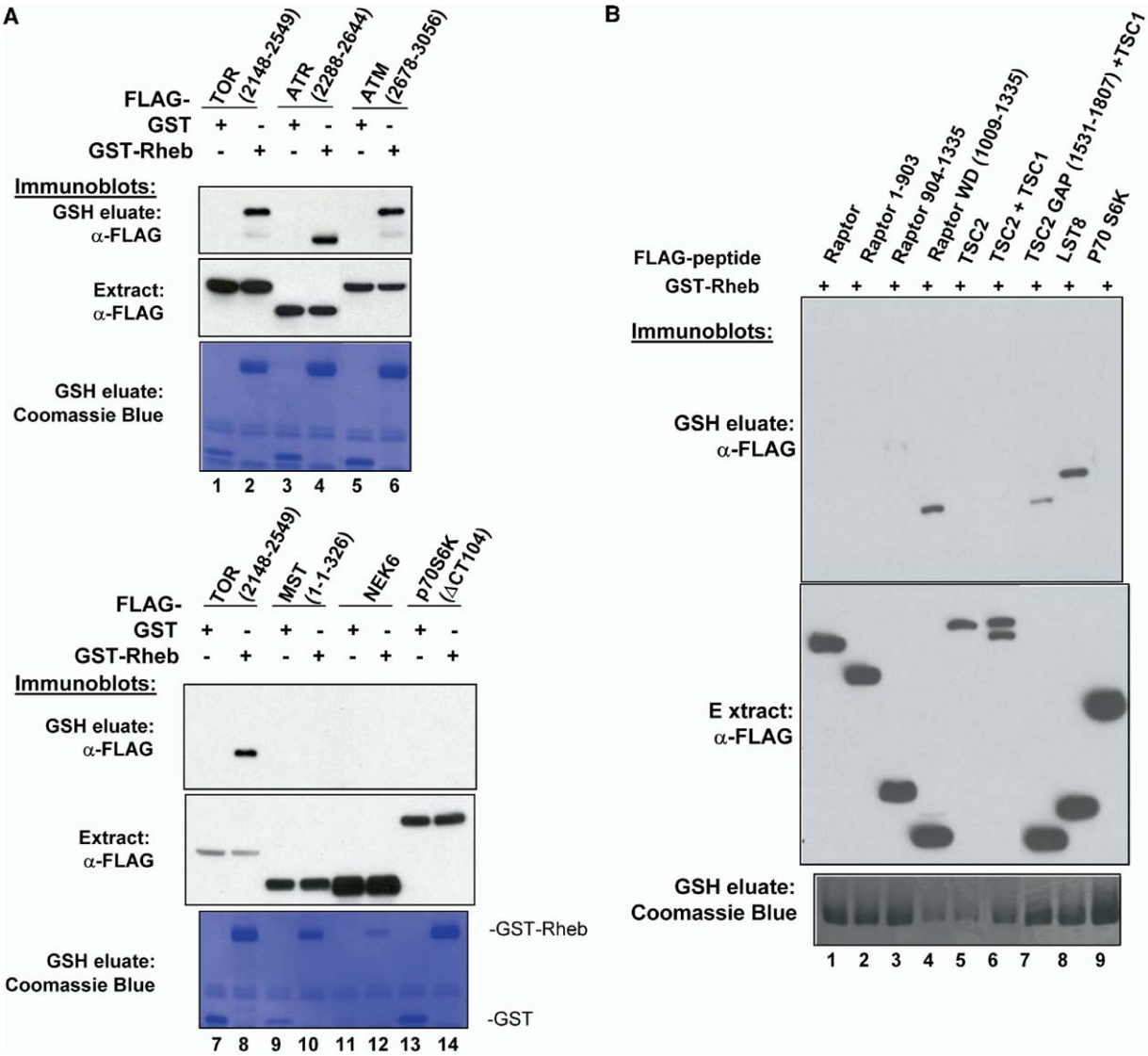

Figure 4. Rheb Binding to Other PIKKs, Conventional Protein Kinases, LST8, and Raptor

(A) GST-Rheb binds to homologous regions of the catalytic domains of the PIKKs, mTOR, ATM, and ATR but not to the catalytic domains of several subfamilies of conventional protein kinases. In the experiment shown in the upper panels, pEBG or pEBG-Rheb was coexpressed with plasmids encoding FLAG-tagged mTOR 2148–2549 (lanes 1 and 2), ATR 2288–2644 (lanes 3 and 4), or ATM 2678–3056 (lanes 5 and 6). A second experiment is shown in the lower panels in which pEBG or pEBG-Rheb was coexpressed with plasmids encoding FLAG-tagged mTOR 2148–2549 (lanes 7 and 8), MST1 1–326 (lanes 9 and 10), Nek6 (lanes 11 and 12), or p70S6KΔCT104 (lanes 13 and 14). The affinity-purified GST fusion proteins were eluted with GSH and examined by anti-FLAG immunoblot and Coomassie blue stain. An anti-FLAG immunoblot of the cell lysates is also shown (middle).

(B) GST-Rheb binds to LST8, the raptor carboxyterminal WD domain, and the TSC2 GAP domain. HEK293T cells were transfected with pEBG (not shown) or pEBG-Rheb and plasmids encoding p70S6K, mTOR complex components, or fragments thereof (as indicated above each lane). 40 hr later, the cells were harvested, and GST-Rheb was processed as in Figure 3A. No FLAG immunoreactivity coprecipitated with GST (not shown).

for Rheb. The TSC2 GAP domain bound GMPPNP-charged Rheb much better than GDP-charged Rheb. Surprisingly, TSC2 GAP domain also bound nucleotide-free Rheb as well as GMPPNP-charged Rheb.

Rheb Regulates TOR Kinase Activity

The ability of Rheb to bind directly to mTOR impelled us to examine whether Rheb directly regulates mTOR kinase activity assayed in vitro. Direct addition in vitro of an excess of mammalian recombinant GST-Rheb, charged with GTP or GDP, does not alter the kinase activity of HA-mTOR, in comparison to the addition of a comparable amount of GST (data not shown). Although difficult to assess quantitatively, the efficiency with which recombinant Rheb associates with HA-mTOR in vivo or in vitro appears to be low. We therefore examined specifically the kinase activity of mTOR polypeptides that are bound to recombinant Rheb. Wild-type

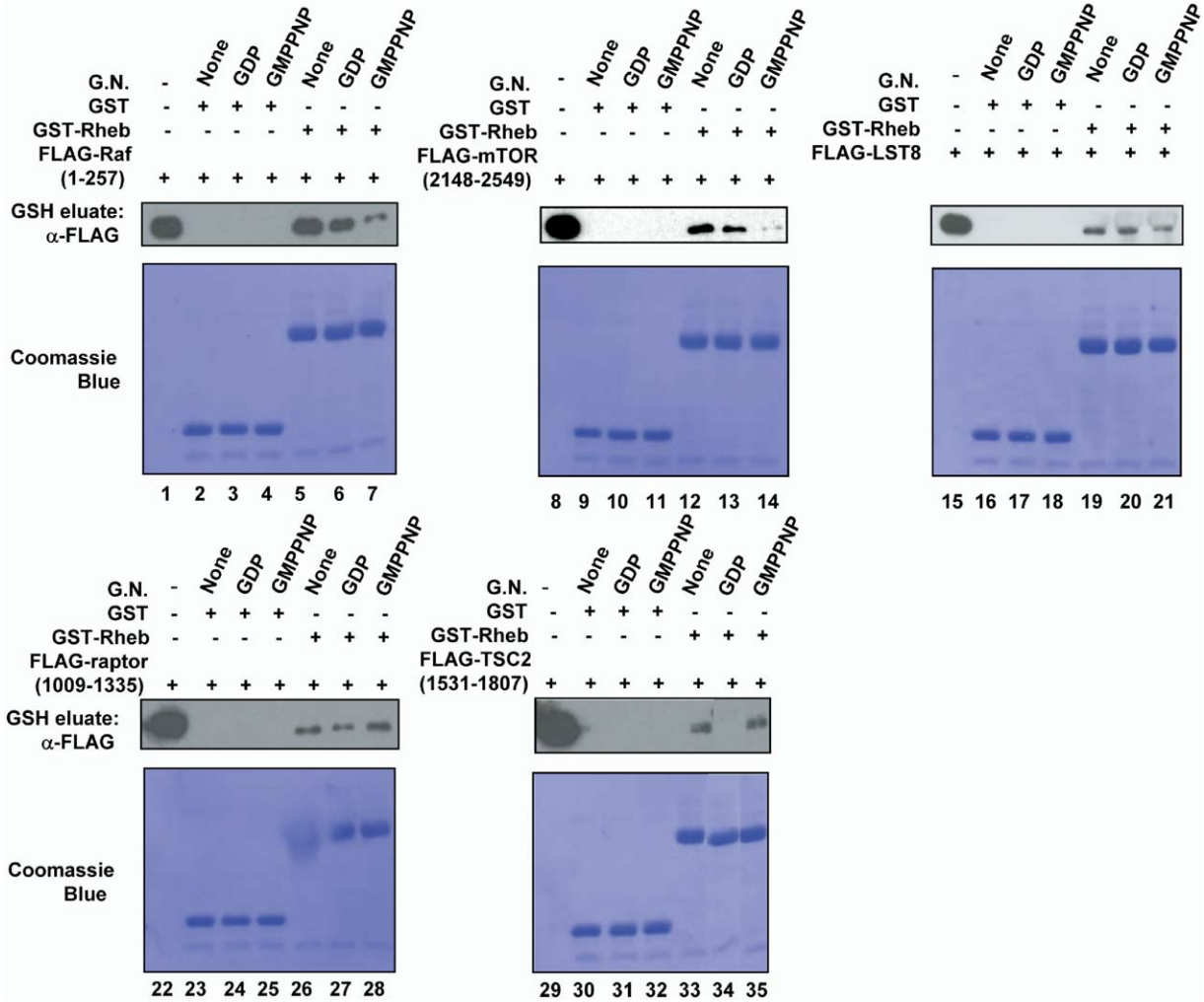

Figure 5. The Effect of Guanyl Nucleotide on Rheb Binding In Vitro to the c-Raf1 Ras Binding Domain, the mTOR Catalytic Domain, LST8, the Raptor WD Domain, and the TSC2 GAP Domain

GST-Rheb, purified on GSH-Sepharose after transient expression in HEK293 cells, was either stripped of endogenous nucleotide with EDTA and washed in nucleotide-free buffer or charged in vitro with GDP or GMPPNP. Recombinant GST was treated in parallel. Each species of GST or GST-Rheb was incubated with immunoaffinity-purified, soluble FLAG-tagged polypeptides: c-Raf1 1–257 (lanes 2–7), mTOR 2148–2549 (lanes 9–14), LST8 (lanes 16–21), raptor 1009–1335 (lanes 23–28), or TSC2 1531–1807 (lanes 30–35). After being washed, the adsorbed polypeptides were subjected to SDS-PAGE and analyzed by anti-FLAG immunoblot and Coomassie blue stain. Lanes 1, 8, 15, 22, and 29 contain 10% of the amount of FLAG-polypeptide added into the binding reaction.

Rheb expressed singly, when purified, exhibits a slight protein kinase activity toward p70S6K/4E-BP1 in vitro, presumably because of a copurifying kinase. Coexpression of Rheb with wild-type HA-mTOR markedly increases the kinase activity recovered with Rheb (Figure 6A), whereas the kinase activity associated with GST-Rheb coexpressed with kinase-dead HA-mTOR(Asn-2343Lys) [44] is very low and similar to that coprecipitating with GST (Figure 6A, right). Moreover, the Rheb-associated kinase activity directed specifically toward p70S6K Thr412 is increased by coexpression with wild-type HA-mTOR to an even greater extent than is the overall Rheb-catalyzed ^{32}P transfer to p70S6K (Figure 6A). Thus, the Rheb-associated p70S6K Thr412 kinase activity is attributable to mTOR.

HA-TOR bound to nucleotide-deficient mutants of Rheb is essentially devoid of kinase activity (Figure 6B). Wild-type HA-mTOR was coexpressed with wild-type GST-Rheb or with the inactive Rheb mutants, GST-Rheb(Ser20Asn) or (Asp60Ile) and the TOR kinase activity recovered with the GSH-Sepharose isolates was assayed. Inasmuch as greater amounts of HA-mTOR coprecipitate with the nucleotide-deficient Rheb mutants than with wild-type Rheb, we used the HA immunoblot to equalize the amount of HA-mTOR introduced into the kinase assays (Figure 6B, bottom two panels). It is evident that the HA-TOR coprecipitating with the mutant, inactive Rheb exhibits little or no p70S6K- and 4E-BP1-kinase activity (Figure 6B, panels 2–4 from top). Thus, although the nucleotide-deficient Rheb mutants bind HA-mTOR more tightly than does wild-type Rheb, the HA-mTOR polypeptides bound to these mutants

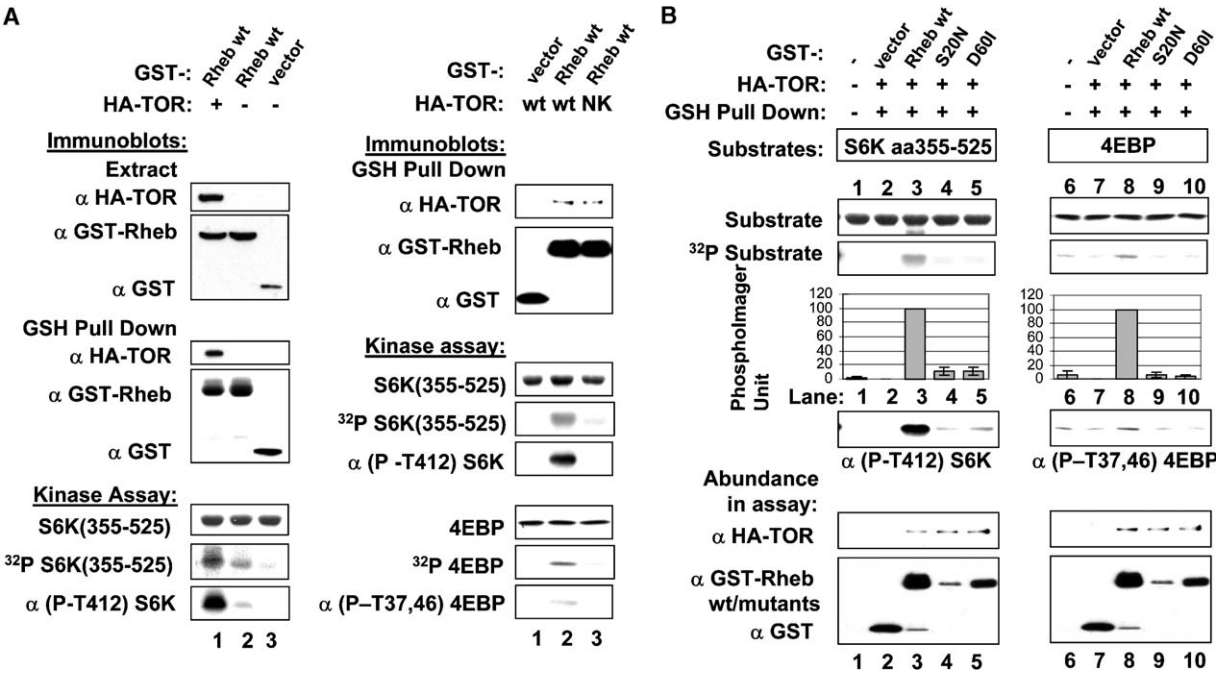

Figure 6. Regulation of TOR Kinase Activity by Rheb

(A) Coexpression of wild-type but not kinase-dead mTOR with GST-Rheb increases the in vitro Rheb-associated kinase activity toward p70S6K and 4E-BP. Left, GST-Rheb expressed alone (lane 2) or with HA-mTOR (lane 1) was purified on GSH-Sepharose and equal amounts of GST-Rheb polypeptide (second panel from top) were assayed for kinase activity (lowest three panels) toward a recombinant p70S6(355–525) polypeptide (third panel from bottom). Substrate phosphorylation in vitro was estimated by overall incorporation of ^{32}P (second panel from bottom) and by anti-p70S6K(Thr412P) immunoblot (bottom). Right, the kinase activity associated with GST (lane 1) or GST-Rheb (lanes 2 and 3) expressed with wild-type HA-mTOR (lane 2) was compared to GST-Rheb expressed with the kinase-inactive mTOR mutant (Asn2343Lys) (lane 3) in assays equalized for identical amounts of mTOR polypeptide (top). The phosphorylation of p70S6K 355–525 (middle three panels) and 4E-BP1 (lowest three panels) was analyzed by overall incorporation of ^{32}P (second and fourth panels from bottom) and by immunoblot against p70S6K(Thr421P) (fourth panel from bottom) and 4E-BP1(Thr37P, Thr46P), respectively. Each experiment was repeated twice with nearly identical results.

(B) mTOR bound to guanyl nucleotide-deficient Rheb mutants exhibits little or no protein kinase activity. HA-mTOR was expressed with GST (lane 2) or with GST fusions to Rheb wild-type (lane 3), Rheb(Ser20Asn) (lane 4), or Rheb(Asp60Ile) (lane 5). After purification on GSH-Sepharose, the bound proteins were eluted, and aliquots containing equal amounts of HA-mTOR polypeptide (second panel from bottom) were assayed for kinase activity toward p70S6K(355–525) (lanes 1–5) and 4E-BP1 (lanes 6–10). Substrate phosphorylation was estimated by overall ^{32}P incorporation, displayed both by a representative autoradiograph (fifth panel from bottom) and quantitatively by phosphoimager (fourth panel from bottom). The bar graphs display the combined results from three experiments, as a percentage (± 1 SD) of the mTOR kinase activity, set to 100, associated with wild-type Rheb. Representative immunoblots with anti-p70S6K(Thr412P) (third panel from bottom, lanes 1–5) and anti-4E-BP1(Thr37P, Thr46P) (third panel from bottom, lanes 6–10) antibodies are shown.

are unable to acquire kinase activity during transient expression. Reciprocally, we examined the protein kinase activity of HA-mTOR polypeptides bound to GST-Rheb(Gln64Leu) as compared to wild-type Rheb (Figure 7). Overexpressed recombinant Rheb(Gln64Leu) is reported to be approximately 90% GTP charged as compared to the 50% GTP charging observed for wild-type Rheb expressed in parallel at comparable levels [40]. Somewhat lesser amounts of HA-mTOR polypeptide are recovered with GST-Rheb(Gln64Leu) as compared with wild-type GST-Rheb (Figure 7B); however, when comparable amounts of Rheb bound HA-mTOR polypeptide are assayed for kinase activity in vitro (Figures 7C and 7D), the HA-mTOR bound to GST-Rheb(Gln64Leu) exhibits substantially higher p70S6K Thr412 kinase activity than that bound to wild-type GST-Rheb. We infer that HA-mTORs bound to wild-type Rheb reflect a mixture of HA-mTORs bound to Rheb-GTP, which have high kinase activity, and (given the 40%–50% GDP charging we observe for wild-type Rheb) a comparable amount of HA-mTOR bound to Rheb-GDP, which we presume to have lower kinase activity. We are unable to demonstrate the latter point directly, inasmuch as we have not identified a Rheb mutant that is locked into the GDP bound form. Nevertheless, the ability of the Rheb-GAP, TSC1/TSC2, to inhibit mTOR signaling in vivo is consistent with the inference that Rheb-GDP provides a less effective stimulus than Rheb-GTP.

Finally, we examined the kinase activity of the small amounts of HA-mTOR that associate with coexpressed GST-Ha-Ras(Gly12Val) and GST-Rap1b(Gly12Val), in comparison to that associated with GST-Rheb and GST-Rheb(Asp60Ile) (Figure S5). When compared at equal HA-mTOR polypeptide levels, the p70S6K Thr412 kinase activity associated with GST-Ha-Ras(Gly12Val) is very low and similar to that recovered with GST-Rheb-(Asp60Ile), whereas the p70S6K Thr412 kinase activity associated with GST-Rap1b(Gly12Val) is about 50%

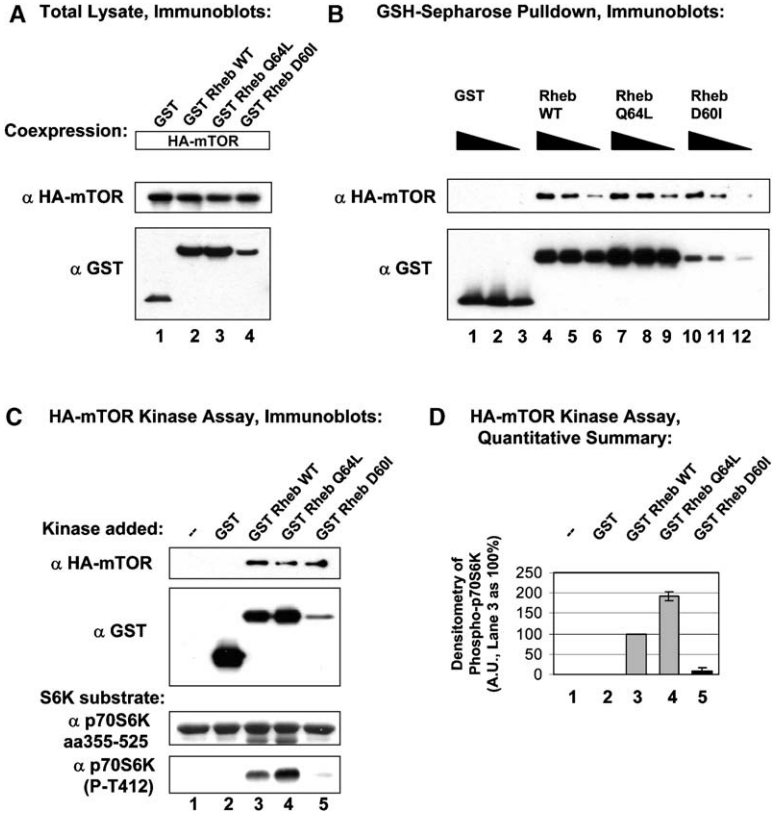

Figure 7. mTOR Bound to Rheb(Gln64Leu) Has Higher Kinase Activity than mTOR Bound to Wild-Type Rheb

(A) Immunoblots of lysates from cells coexpressing HA-mTOR with GST or with GST fusions to Rheb wild-type, Rheb(Gln64Leu), or Rheb(Asp60Ile).

(B) Immunoblots of the GST proteins and copurifying HA-mTOR polypeptides after purification on GSH-Sepharose. Lanes 1–12 demonstrate the GST polypeptides as 4×, 2×, and 1× aliquots (bottom) and associated HA-mTOR (top).

(C) A repesentative kinase assay of the GST bound HA-mTOR polypeptides. After elution with GSH, aliquots containing equal amounts of HA-mTOR polypeptide, as shown by immunoblot (top) were assayed for kinase activity toward p70S6K(355–525) (third panel from top). Substrate phosphorylation was estimated by immunoblot with anti-p70S6K(Thr412P) (bottom).

(D) A summary of three mTOR kinase assays. The bar graph shows results from two experiments. The results are expressed as in Figure 6B.

that associated with GST-Rheb. Thus, in addition to their very much weaker ability than Rheb to bind mTOR, Rap1(Gly12Val) and especially Ras(Gly12Val) exhibit much less ability to support the activation of bound mTOR polypeptides than does wild-type Rheb.

Discussion

The primary aim of the present experiments was to determine whether Rheb interacts with the TOR complex and whether such an interaction has a positive regulatory effect; the results provide affirmative answers to these two questions. Thus, mTOR is a direct target and an "effector" of the Rheb GTPase. The major difference between the mode of action of Ras [45] and that proposed here for Rheb is that in the case of Ras, GTP charging is necessary for the ability of Ras both to bind and activate its effectors, whereas Rheb GTP charging is necessary only for effector activation. Rheb binding to its putative effector mTOR does not require Rheb guanyl nucleotide charging.

Recombinant Rheb binds to the mTOR catalytic domain specifically, in the sense that other small GTPases most closely related to Rheb (i.e., Ras and Rap1) exhibit much weaker binding to mTOR. Moreover, although Rheb binds to the catalytic domains of other PIKKs such as ATM and ATR, Rheb does not bind to any of the several conventional protein kinase domains examined. Surprisingly, the ability of Rheb to bind mTOR does not require Rheb nucleotide charging; in fact, nucleotide-free Rheb binds to mTOR more tightly than does Rheb-GTP, both in vivo and in vitro. Nevertheless, the relevance of Rheb-GTP binding to mTOR function is demonstrated by the finding that the mTOR polypeptides that bind in vivo to the nucleotide-deficient Rheb mutants (Ser20Asn or Asp60Ile) are greatly deficient in protein kinase activity assayed in vitro. Reciprocally, mTOR polypeptides bound to recombinant Rheb(Gln64Leu), a mutant reported to be approximately 90% GTP charged in vivo [40], exhibit a substantially higher kinase specific activity in vitro than does mTOR bound to wild-type Rheb, which is only 50%–60% GTP charged. These results establish the regulatory significance of the Rheb-TOR interaction. The association of mTOR with GTP-charged Rheb increases mTOR kinase activity. Conversely, if mTOR associates with a nucleotide-deficient Rheb polypeptide in vivo, the mTOR is trapped in a kinase-inactive form that is unable to phosphorylate in vitro both raptor-dependent (4E-BP1) and raptor-independent (p70S6K 355–525 fragment) substrates. Based on these results, we propose that the association of mTOR with Rheb-GTP is a necessary step in the physiologic activation of the mTOR kinase. The present data do not establish however whether the mTOR kinase, once activated, requires an association of Rheb-GTP for the maintanence of mTOR kinase activity.

How Rheb-GTP functions within the TOR complex to enable the acquisition of mTOR kinase activity and to overcome the inhibition of TOR signaling caused by amino-acid withdrawal is not yet known. The effort to elucidate the site(s) at which amino-acid deficiency in-

terrupts mTOR signaling and the mechanism of rescue by recombinant Rheb are impeded by the inability as yet to fully reconstitute this regulatory behavior in vitro. Thus, despite their dramatic effects in vivo, neither amino-acid withdrawal [46] or coexpressed recombinant wild-type Rheb, singly or together, alter mTOR kinase activity assayed in vitro (data not shown). This behavior suggests that the effect(s) of amino-acid removal and recombinant Rheb on mTOR are poorly retained on cell disruption and may not be due to stable alterations in any of the components of the TOR complex. Alternatively, the conditions used for mTOR kinase assay in vitro may obscure the functional impact of alterations in the TOR complex. We find that amino-acid withdrawal inhibits the binding of Rheb to mTOR (X.L., S.O.-V., Y.L., and J.A., unpublished data). If the association of endogenous Rheb with mTOR is required for the maintenance as well as for the initiation of TOR kinase activation, then the ability of amino-acid withdrawal to inhibit the Rheb-mTOR interaction may be responsible in part for the concomitant inhibition of mTOR signaling. If, however, the Rheb-mTOR interaction is only necessary for the initiation of mTOR kinase activation, then some additional site of Rheb action is required, within the TOR complex or elsewhere, to explain both the progressive and ultimately complete inhibition of TOR signaling that occurs in the first 2 hr after withdrawal of amino acids as well as Rheb's ability to overcome the inhibitory effect of amino-acid withdrawal.

Any proposal for the mechanism by which amino acids regulate TOR signaling must explain why mutations of the p70S6K TOS motif that eliminate p70S6K binding to raptor [12], although they reduce p70S6K phosphorylation in vivo at Thr412 (the dominant site of mTOR-catalyzed p70S6K phosphorylation), also render this phosphorylation insensitive to amino-acid withdrawal [46]. This behavior suggests that amino-acid deficiency, rather than impairing mTOR catalytic activity per se, interferes with the ability of raptor to present substrates to mTOR, perhaps, for example, by altering raptor binding to either mTOR or to p70S6K/4E-BP1. We have not observed effects of amino-acid withdrawal on p70S6K or 4E-BP1 association with raptor (data not shown). Moreover, Kim et al. [4] reported that amino-acid withdrawal actually increases raptor association with mTOR. Nor have we observed any effect of coexpressed Rheb on the binding of raptor to either mTOR or to p70S6K/4E-BP1 in the presence or absence of amino acids (data not shown). Nevertheless, the possibility remains that overexpressed Rheb-GTP is able to restore effective coupling between raptor and mTOR, perhaps mediated by Rheb's multiple interactions within the TOR complex.

In summary, the current data support the following model for the role of Rheb in the activation of the mTOR kinase: Rheb binds to the TOR catalytic domain and independently to LST8, which also binds nearby to the TOR catalytic domain [6]; Rheb also shows a weak interaction with the raptor carboxyterminal WD domains. Rheb binding to these elements does not require Rheb guanyl nucleotide charging. Nevertheless, the data shown in Figure 5 indicate that the binding of TOR to a nucleotide-free Rheb does not activate but rather impedes TOR kinase activation, whereas binding to Rheb-GTP is accompanied by a high kinase activity. Thus, we propose that association of a wild-type, GTP-charged Rheb with the TOR complex is necessary to initiate the transformation of the complex into a configuration competent to catalyze phosphorylation of physiologic substrates. As with other Ras-like GTPases, binding GTP restructures the Rheb switch 1, switch 2 and perhaps other Rheb segments. Although Rheb GTP charging weakens the association of Rheb with the mTOR catalytic domain, the configuration of GTP-charged Rheb enables the TOR complex to adopt a form that is both catalytically active and capable of productive signaling in vivo. Once mTOR is activated, the current evidence does not specify whether a continued association of Rheb-GTP is required for the maintanence of an active configuration of TORC1. Rheb-GTP may remain loosely associated with the complex, in which case alterations in the activity of the TOR-associated TSC complex may be critical in the regulation of TOR kinase activity. Alternatively, Rheb-GTP may be concerned primarily with the initiation of TOR activation, and subsequent alterations in TORC1 may be necessary to maintain the active state. In either case, the fact that physiologic regulation of the TSC complex (a Rheb-GAP) is reflected relatively quickly by alterations in mTOR signaling suggests that mTOR kinase activity is in a state of continuous turnover in amino-acid-replete cells. The ability of overexpressed Rheb to rescue TOR signaling from amino-acid withdrawal may be attributable to the ability of Rheb-GTP to promote mTOR kinase activity (as shown herein) and perhaps also the coupling between mTOR and raptor; however, direct evidence for the latter action remains to be established.

Supplemental Data

Supplemental Data include five figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/8/702/DC1/.

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