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Specific Activation of mTORC1 by Rheb G-protein *in Vitro* Involves Enhanced Recruitment of Its Substrate Protein*[S]

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Rheb G-protein plays critical roles in the TSC/Rheb/mTOR signaling pathway by activating mTORC1. The activation of mTORC1 by Rheb can be faithfully reproduced *in vitro* by using mTORC1 immunoprecipitated by the use of anti-raptor antibody from mammalian cells starved for nutrients. The low *in vitro* kinase activity against 4E-BP1 of this mTORC1 preparation is dramatically increased by the addition of recombinant Rheb. On the other hand, the addition of Rheb does not activate mTORC2 immunoprecipitated from mammalian cells by the use of anti-rictor antibody. The activation of mTORC1 is specific to Rheb, because other G-proteins such as KRas, RalA/B, and Cdc42 did not activate mTORC1. Both Rheb1 and Rheb2 activate mTORC1. In addition, the activation is dependent on the presence of bound GTP. We also find that the effector domain of Rheb is required for the mTORC1 activation. FKBP38, a recently proposed mediator of Rheb action, appears not to be involved in the Rheb-dependent activation of mTORC1 *in vitro*, because the preparation of mTORC1 that is devoid of FKBP38 is still activated by Rheb. The addition of Rheb results in a significant increase of binding of the substrate protein 4E-BP1 to mTORC1. PRAS40, a TOR signaling (TOS) motif-containing protein that competes with the binding of 4EBP1 to mTORC1, inhibits Rheb-induced activation of mTORC1. A preparation of mTORC1 that is devoid of raptor is not activated by Rheb. Rheb does not induce autophosphorylation of mTOR. These results suggest that Rheb induces alteration in the binding of 4E-BP1 with mTORC1 to regulate mTORC1 activation.

Rheb defines a unique member of the Ras superfamily G-proteins (1). We have shown that Rheb proteins are conserved and are found from yeast to human (2). Although yeast and fruit fly have one Rheb, mouse and human have two Rheb proteins termed Rheb1 (or simply Rheb) and Rheb2 (RhebL1) (2). Structurally, these proteins contain G1-G5 boxes, short stretches of amino acids that define the function of the Ras superfamily G-proteins including guanine nucleotide binding (1, 3, 4). Rheb proteins have a conserved arginine at residue 15 that corresponds to residue 12 of Ras (1). The effector domain required for the binding with downstream effectors encompasses the G2 box and its adjacent sequences (1, 5). Structural analysis by x-ray crystallography further shows that the effector domain is exposed to solvent, is located close to the phosphates of GTP especially at residues 35–38, and undergoes conformational change during GTP/GDP exchange (6). In addition, all Rheb proteins end with the CAA*X* (C is cysteine, A is an aliphatic amino acid, and *X* is the C-terminal amino acid) motif that signals farnesylation. In fact, we as well as others have shown that these proteins are farnesylated (7–9).

Rheb plays critical roles in the TSC/Rheb/mTOR signaling, a signaling pathway that plays central roles in regulating protein synthesis and growth in response to nutrient, energy, and growth conditions (10–14). Rheb is down-regulated by a TSC1·TSC2 complex that acts as a GTPase-activating protein for Rheb (15–19). Recent studies established that the GAP domain of TSC2 defines the functional domain for the down-regulation of Rheb (20). Mutations in the *Tsc1* or *Tsc2* gene lead to tuberous sclerosis whose symptoms include the appearance of benign tumors called hamartomas at different parts of the body as well as neurological symptoms (21, 22). Overexpression of Rheb results in constitutive activation of mTOR even in the absence of nutrients (15, 16). Two mTOR complexes, mTORC1 and mTORC2, have been identified (23, 24). Whereas mTORC1 is involved in protein synthesis activation mediated by S6K and 4EBP1, mTORC2 is involved in the phosphorylation of Akt in response to insulin. It has been suggested that Rheb is involved in the activation of mTORC1 but not mTORC2 (25).

Although Rheb is clearly involved in the activation of mTOR, the mechanism of activation has not been established. We as well as others have suggested a model that involves the interaction of Rheb with the TOR complex (26–28). Rheb activation of mTOR kinase activity using immunoprecipitated mTORC1 was reported (29). Rheb has been shown to interact with mTOR (27, 30), and this may involve direct interaction of Rheb with the kinase domain of mTOR (27). However, this Rheb/mTOR interaction is a weak interaction and is not dependent on the presence of GTP bound to Rheb (27, 28). Recently, a different model proposing that FKBP38 (FK506-binding protein 38) mediates the activation of mTORC1 by Rheb was proposed (31, 32). In this model, FKBP38 binds mTOR and negatively regulates mTOR activity, and this negative regulation is blocked by the binding of Rheb to FKBP38. However, recent reports dispute this idea (33).

To further characterize Rheb activation of mTOR, we have utilized an *in vitro* system that reproduces activation of mTORC1 by the addition of recombinant Rheb. We used mTORC1 immunoprecipitated from nutrient-starved cells

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using anti-raptor antibody and have shown that its kinase activity against 4E-BP1 is dramatically increased by the addition of recombinant Rheb. Importantly, the activation of mTORC1 is specific to Rheb and is dependent on the presence of bound GTP as well as an intact effector domain. FKBP38 is not detected in our preparation and further investigation suggests that FKBP38 is not an essential component for the activation of mTORC1 by Rheb. Our study revealed that Rheb enhances the binding of a substrate 4E-BP1 with mTORC1 rather than increasing the kinase activity of mTOR.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1x penicillin/streptomycin at 37 °C and 5% CO_2. To assess the activity of mTOR, cells were serum-starved in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin overnight and then cultured in phosphate-buffered saline containing Mg^{2+} and Ca^{2+} (Invitrogen) for 1 h. Transfection of plasmids was performed by using Polyfect (Qiagen) according to the manufacturer's instructions. Transfection of siRNAs[2] was carried out by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Reagents—Anti-FKBP38 antibody was provided by Keiichi Nakayama (Kyushu University). Antibodies against S6, phospho-S6 (Ser$^{235/236}$), 4E-BP1, phospho-4E-BP1 (Thr$^{37/46}$), phospho-4E-BP1 (Thr^{70}), Akt, and phospho-Akt (Ser^{473}) were obtained from Cell Signaling. Anti-FLAG M2 antibody was obtained from Sigma. Anti-mTOR antibody was obtained from Cell Signaling and Santa Cruz Biotechnology. Anti-raptor antibody was obtained from Cell Signaling and BETHYL Lab. Anti-rictor and Anti-GβL antibodies were obtained from BETHYL Lab. Recombinant 4E-BP1 and Akt/PKB (inactive) were obtained from Stratagene and Millipore, respectively. Recombinant human proline-rich Akt substrate (PRAS40) was obtained from BIOSOURCE. [^{35}S]GTPγS and [γ-^{32}P]ATP were obtained from PerkinElmer Life Sciences.

Plasmids and siRNA—p3×FLAG-HA-h-FKBP38 (amino acids 1–413) was kindly provided by Keiichi Nakayama. pCDNA3-FLAG-Rheb was produced as described previously (34). T38A, N41A, and Y54A mutations were introduced into Rheb by QuikChange site-directed mutagenesis kit (Stratagene). Plasmids expressing His_6-tagged Rheb was produced by inserting NdeI/BamHI fragment of pRPUmychsRheb (9) into the same site of pET28a(+) (Novagen). Small interference RNA targeting AGAGTGGCTGGACATTCTGG in the cording sequence of FKBP38 and scrambled siRNA coding AAGCGCGCUUUGUAGGAUUC as a control siRNA were purchased from Invitrogen.

Protein Purification—pET28 constructs carrying small GTPases such as Rheb1 and Rheb2 were transformed into *Escherichia coli* BL21(DE3)-Star (Invitrogen) and the proteins induced for 4 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were lysed in the buffer (20 mM $NaPO_4$, pH 7.4, 0.5 M NaCl, 0.5 M $MgCl_2$, 1× Complete Protease Inhibitor Mixture (EDTA-Free) (Roche Applied Science), 0.1 mg ml^{-1} lysozyme] by sonication. The lysates were cleared and applied to nickel-nitrilotriacetic acid-agarose (Qiagen). After binding, the beads were washed three times with wash buffer A (20 mM $NaPO_4$ pH 6.0, 0.5 M NaCl) supplemented with 0.5 mM $MgCl_2$. The proteins were then eluted using wash buffer supplemented with 350 mM imidazole and stored in 50% glycerol at −20 °C until use. For guanine nucleotide binding on purified Rheb GTPases, the proteins were incubated in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA) with 0.1 mM GTPγS or 1 mM GDP at 37 °C for 10 min. For GTP binding on other Ras family G-proteins, buffer B (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol) was used instead of buffer A. After incubation, the proteins were added with 20 mM $MgCl_2$ and stored at −20 °C until use.

Guanine Nucleotide Binding Assays—Guanine nucleotide binding was carried out in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 0.5% bovine serum albumin, 2 mM ATP, 0.2 μCi of [^{35}S]GTPγS, 5 mM cold GTP). 0.5 μg of purified protein was added, and binding was performed at 37 °C for 10 min. The binding reaction was then applied to a 0.22-μm GSWP membrane (Millipore) and washed three times with wash buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 25 mM $MgCl_2$) using a vacuum manifold. Radioactivity was measured with a liquid scintillation counter.

Immunoprecipitation and in Vitro Kinase Assay—Immunoprecipitation of mTORC1 or mTORC2, and subsequent *in vitro* kinase assay were carried out essentially as described in Ref. 35. Briefly, the cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% CHAPS, and 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4) supplemented with 1× Complete Protease Inhibitor Mixture from Roche Applied Science. The supernatant from the centrifugation at 15,000 × *g* for 20 min at 4 °C was immunoprecipitated using indicated antibodies and protein G- or protein A-Sepharose 4FF beads (Amersham Biosciences). To detect the FKBP38 bound to mTORC1, the cells were lysed with the lysis buffer containing 25 mM NaCl, and the concentration of NaCl was adjusted to 25–150 mM after centrifugation. The immunoprecipitates were washed three times with the lysis buffer. For *in vitro* kinase assay, the immunoprecipitates were further washed with wash buffer B (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$). The immunoprecipitates were then mixed with 0.5 μg of recombinant 4E-BP1 for mTORC1 or Akt for mTORC2 in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mM ATP) and incubated for 20 min at 37 °C. To observe the effect of proteins such as Rheb on mTOR complexes, the proteins were added before incubation. To see the activation of mTOR by Mn^{2+}, 10 mM of $MnCl_2$ was supplemented in the kinase buffer instead of 10 mM $MgCl_2$ before reaction. The kinase reaction was stopped by the addition of 1× SDS sample buffer (3% SDS, 5% glycerol, 62 mM Tris-HCl, pH 6.7) and subsequent incubation at 95 °C for 5 min. The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Rheb and Rheb2 were visualized by

[2] The abbreviations used are: siRNA, small interfering RNA; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

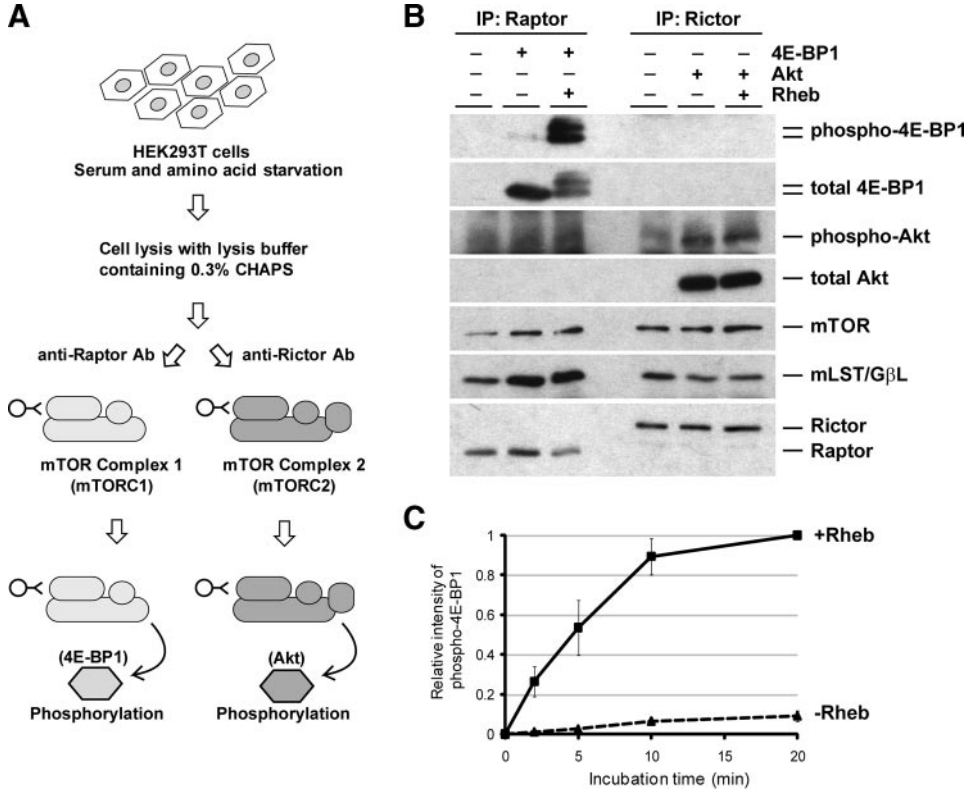

FIGURE 1. **Recombinant Rheb activates mTORC1 but not mTORC2 *in vitro*.** *A*, a schematic procedure of mTOR *in vitro* kinase assay is outlined. The cells are first starved for serum and amino acids to shut down mTOR signaling. The cells are then lysed, and mTORC1 or mTORC2 is immunopurified using anti-raptor or -rictor antibody, respectively. In the raptor complex, mTOR and mLST8/GβL are contained together with raptor, whereas the rictor complex has mTOR, mLST8/GβL, rictor and Sin1. Each preparation is mixed with a specific substrate, 4E-BP1 for mTORC1 or Akt for mTORC2, in the kinase buffer supplemented with 0.2 mM ATP and 10 mM $MgCl_2$, and incubated for 2–20 min at 37 °C. The kinase activities are estimated by the phosphorylation level of the substrates. *B*, *in vitro* kinase assay of mTOR complexes was performed in the absence or presence of recombinant Rheb loaded with GTPγS at 37 °C for 20 min. The proteins were resolved by SDS-PAGE, and the indicated protein bands were detected by Western blotting. *C*, time course analysis of mTORC1 activity was performed in the presence (*squares*) or absence (*triangles*) of Rheb-GTPγS. Phosphorylation of 4E-BP1 at $Thr^{37/46}$ was detected by Western blotting. The relative band intensities were measured using Scion Image. The standard deviation was derived from three independent experiments. *IP*, immunoprecipitation.

staining with 0.2% Ponceau S in 3% trichloroacetic acid. Other proteins were detected by Western blotting.

4E-BP1 Binding Assay—The cells were lysed with the lysis buffer as described above. The supernatant from the centrifugation at 15,000 × *g* for 20 min at 4 °C was incubated with anti-raptor antibody and protein A-Sepharose 4FF beads at 4 °C for 4 h. The immunoprecipitates were washed three times with the lysis buffer and once with washing buffer. The immunoprecipitates were mixed with recombinant 4E-BP1 in the buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl) and incubated at 37 °C for 20 min. The samples were washed three times with the lysis buffer and incubated at 95 °C for 5 min in 1× SDS sample buffer. 4E-BP1 bound to mTORC1 was detected by Western blot.

Silver Staining—After immunoprecipitating mTORC1, the proteins were eluted in 1× SDS sample buffer at 95 °C for 5 min. Protein bands were resolved by SDS-PAGE using NuPAGE 4–12% Bis-Tris Gel (Invitrogen). The proteins were detected by SilverQuest silver staining kit (Invitrogen) according to the manufacturer's instructions.

RESULTS

Rheb Activates mTORC1 but Not mTORC2 in the in Vitro Assay—A variety of studies support the idea that Rheb activates mTOR (1, 9–18). However, the mechanism by which Rheb regulates mTOR has not been established. We have used *in vitro* reactions in a series of experiments to examine the direct effect of Rheb on mTOR complexes. Fig. 1*A* shows the scheme for how this is done. HEK293T cells were first starved for nutrients to shut down mTOR activity. Two different complexes of mTOR are present in the cell; mTORC1 that contains raptor as an associated protein and mTORC2 that contains rictor. Therefore, each complex can be isolated selectively by using antibodies against the specific associated proteins. mTORC1 and mTORC2 were isolated, and the kinase activities were examined by using 4E-BP1 and Akt as a substrate, respectively. These complexes exhibit minimal kinase activity as examined by the phosphorylation of each substrate protein. However, the addition of recombinant Rheb loaded with GTP to mTORC1 dramatically enhanced the phosphorylation of 4E-BP1 (Fig. 1, *B* and *C*). On the other hand, mTORC2 activity as detected by the phosphorylation of Akt was not increased by the addition of Rheb-GTP. In the presence of Rheb, the phosphorylation of 4E-BP1 by mTORC1 was observed after 2.5 min of incubation and increased over 20 min, whereas in the absence of Rheb, the phosphorylation remained at a low level even after 20 min (Fig. 1*C*). These data show that Rheb strongly enhances mTORC1 activity *in vitro*.

Activation of mTORC1 Is Specific to Rheb—Rheb belongs to the Ras superfamily G-proteins. Within the Ras family, there are many members as shown in Fig. 2*A*. We picked representative members, K-Ras, RalA, RalB, R-Ras, and Rad proteins, which are quite distinct from Rheb (indicated by *dots* in Fig. 2*A*), and examined whether the mTORC1 activation is specific to Rheb proteins. These small G-proteins tagged with six histidine residues were purified by expressing them in *E. coli* and bound with GTP, and then we tested their ability to activate mTORC1. As can be seen in Fig. 2*B*, Rheb was the only G-protein capable of activating mTORC1. The *lower panels* of this figure confirm that the amount of mTOR and raptor used was similar for each G-protein. We have also tested Rho family small G-proteins, Cdc42 and Rac1, in a

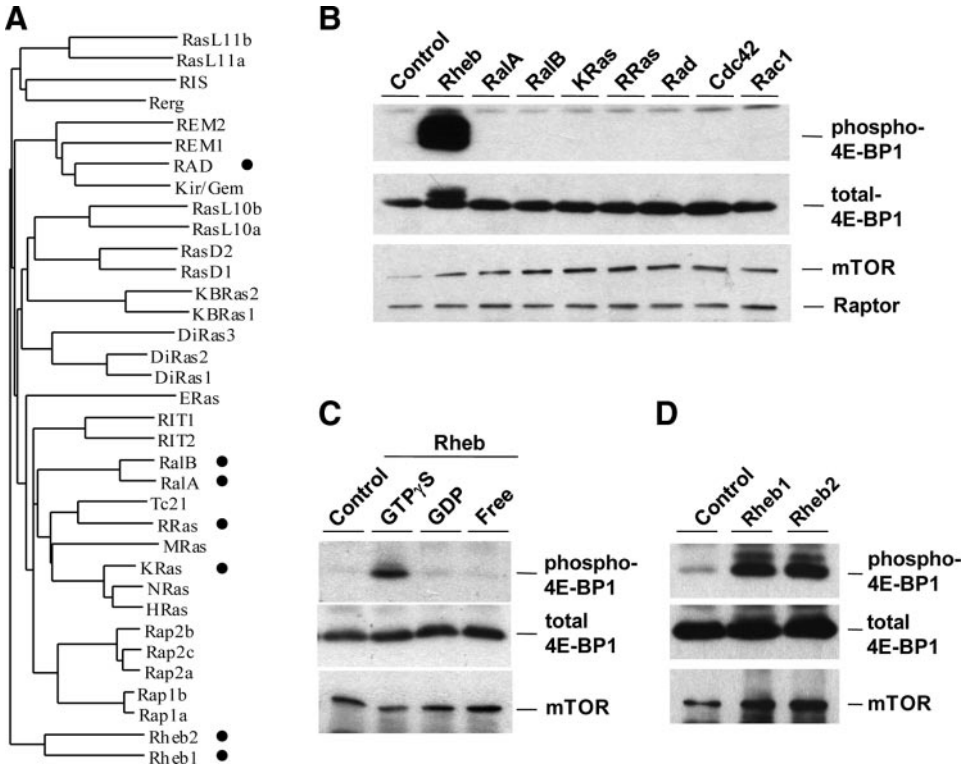

FIGURE 2. **Rheb G-protein specifically activates mTORC1.** *A*, the phylogenetic tree of Ras superfamily was generated using ClustalW program. The proteins used in the following experiments were indicated by *dots*. *B*, G-proteins indicated were purified from *E. coli*, loaded with GTPγS, and used for mTORC1 kinase assay *in vitro*. The proteins were resolved by SDS-PAGE, and phospho-4E-BP1 at $Thr^{37/46}$ was detected using phospho-specific antibody. *C*, GTPγS-bound Rheb (*GTPγS*), GDP-bound Rheb (*GDP*), and the nucleotide free form of Rheb (Free) were prepared and used for the *in vitro* mTORC1 kinase assay. The proteins were resolved by SDS-PAGE, and the indicated protein bands were detected by Western blotting. *D*, *in vitro* kinase activity of mTORC1 was compared in the absence (*Control*) or in the presence of recombinant Rheb (*Rheb1*) or recombinant Rheb2 loaded with GTPγS. Phospho-4E-BP1 at $Thr^{37/46}$ was detected by Western blotting.

similar way, but they did not activate mTORC1 (Fig. 2*B*, *right two lanes*). We then tested GTP dependence of Rheb for mTORC1 activation. Rheb bound with GTP dramatically activated mTORC1, whereas Rheb bound with GDP or nucleotide-free Rheb did not activate mTORC1 (Fig. 2*C*), pointing to the stringent dependence of bound GTP on Rheb for mTORC1 activation.

Mammalian cells express two Rheb proteins called Rheb (Rheb1) and Rheb2 (RhebL1) (2). Although they have similar size, they differ significantly in their amino acid sequence, and the identity between the two proteins is ~53%. Although the N-terminal region of these proteins is highly conserved, the differences exist in their C-terminal region. Thus, it was of interest to examine their ability to activate mTORC1. Both Rheb1 and Rheb2 were purified and tested for their ability to activate mTORC1. As can be seen in Fig. 2*D*, we observed mTORC1 activation by both Rheb1 and Rheb2 and that the extent of activation was comparable between the two.

The Residues at 36–41 within the Effector Domain of Rheb Are Required for the Rheb -dependent Activation of mTORC1—The effector domain which overlaps the switch I region is a critical region for small G-proteins to bind and/or activate their downstream effectors (5). Mutations within this domain abolish the effector activation without affecting intrinsic properties such as the binding of guanine nucleotides. In the case of Rheb, the switch I region and the effector domain are believed to encompass residues 33–41 and 35–43, respectively. To gain further insight into the Rheb domain important for mTORC1 activation in the *in vitro* assay, we prepared recombinant Rheb proteins that have alanine substitution at the region encompassing the switch I and the effector domain. This region is well conserved among Rheb1, Rheb2, and Ras. These mutant proteins were purified and tested for their ability to bind GTP and to activate mTORC1 *in vitro*. As shown in Fig. 3*A*, alanine substitution at residue 33, 36, 37, 38, or 41 did not significantly change guanine nucleotide binding ability, whereas a mutation at residue 35, 39, 40, 42, or 43 reduced the amount of nucleotide bound to Rheb. When their ability to activate mTOR *in vitro* was examined, there was a dramatic decrease in these mutants except D33A (Fig. 3*B*). The D33A mutant still bound GTP and was capable of activating mTORC1. These results, especially with D36A, P37A, T38A, and N41A, clearly show that the mutations in the effector domain decrease mTOR activation without significantly affecting GTP binding activity.

FKBP38 Does Not Play a Role in the Activation of mTORC1 by Rheb—Our results that Rheb can activate mTORC1 *in vitro* raise the possibility that activation of mTORC1 by Rheb is due to a direct action. In recent reports (31, 32), it is proposed that Rheb binds mitochondrial FK506-binding protein 38, FKBP38, and relieves negative regulation of mTOR by FKBP38, resulting in the activation of mTORC1. This model is inconsistent with our results, because our mTORC1 preparation appears not to contain FKBP38. In Fig. 4*A*, we examined whether FKBP38 can be detected in the immunopurified mTORC1 preparation we used. The band of FKBP38 was hardly detected in our preparation even when the concentration of sodium chloride was cut down to 25 mM (Fig. 4*A*). On the other hand, FKBP38 was easily detected in the lysate. FKBP38 was not detected by silver staining of the mTORC1 preparation, whereas mTOR and raptor were detected (supplemental Fig. S1). To further exclude the possibility that a little or an undetectable amount of proteins is present in the mTORC1 preparation, and this may be sufficient to mediate Rheb activation of mTORC1, we used siRNA against FKBP38 to decrease the intracellular amount of FKBP38. mTORC1 was prepared from these cells and was tested for its responsiveness to Rheb in the *in vitro* assay. Fig. 4*B* left shows that the treatment of HEK293T cells with FKBP38-specific siRNA substantially reduced the expression of FKBP38.

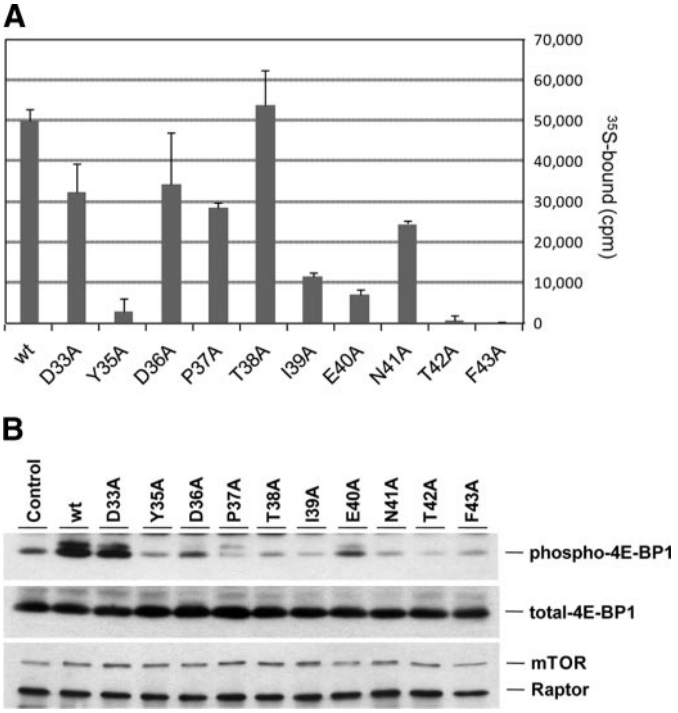

FIGURE 3. **Characterization of Rheb mutants.** *A*, 0.5 μg of each protein was incubated in the buffer containing 0.2 μCi of [^{35}S]GTPγS for 10 min at 37 °C to examine GTP binding. *B*, wild type (*wt*) and mutant Rheb proteins were purified from *E. coli* and were loaded with GTPγS. Each protein was mixed with mTORC1 immunoprecipitates, and mTORC1 activity was evaluated *in vitro*. The proteins were resolved by SDS-PAGE, and phospho-4E-BP1 at Thr$^{37/46}$ was detected by Western blotting.

Although there was a slight increase in the phosphorylation of ribosomal protein S6, phosphor-S6K was not detected, and no changes in the shift of 4E-BP1 band were detected. We next immunopurified mTORC1, and the kinase activity was measured *in vitro*. As shown in the *right panel* of Fig. 4*B*, the amount of phospho-4E-BP1 obtained was low using mTORC1 from both knockdown cells and control cells, and the addition of Rheb significantly increased the phosphorylation of 4E-BP1 in both samples. Thus, reducing the amount of FKBP38 had little effect on the ability of Rheb to activate mTORC1.

Effects of FKBP38 overexpression in cells also support the conclusion that FKBP38 is not a major regulator of mTOR. Fig. 4*C* shows the results of experiment where we transfected the plasmid encoding FKBP38 in HEK293T cells and examined whether inhibitory effect on mTORC1 can be observed by detecting S6 phosphorylation. In the absence of amino acids, phospho-S6 was hardly detected, indicating that mTORC1 is shut down by the nutrient starvation (*lane 3*). However, reactivation of mTORC1 by the addition of amino acids increased the amount of phosphorylated S6 (*lane 1*). Overexpression of FKBP38 did not have a significant effect on the phosphorylation of S6 (*lane 2*). To further examine the possible effects of FKBP38 on Rheb-induced activation of mTOR, mTORC1 was stimulated by transfecting Rheb after amino acid starvation. The expression of FLAG-Rheb increased S6 phosphorylation even in amino acid-starved cells (*lane 4*). However, the phosphorylation of S6 remained high despite the similar level of coexpression of Rheb and FKBP38 (*lane 5*).

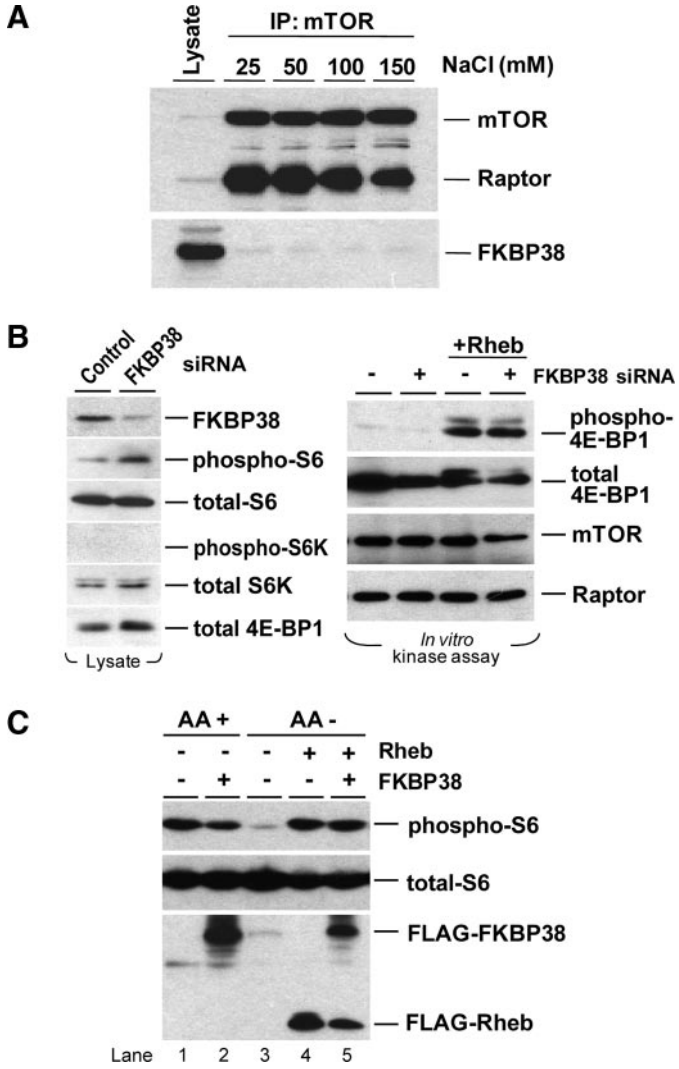

FIGURE 4. **Rheb activation of mTORC1 is independent of FKBP38.** *A*, cells were starved for serum and amino acids and lysed with lysis buffer containing 25 mM NaCl (*Lysate*). The concentration of sodium chloride was then adjusted as indicated and used for mTORC1 immunoprecipitation (*IP*). The amount of mTORC1 components and FKBP38 in the lysate or in immunopurified mTORC1 was detected by Western blotting. *B*, small interference RNA against FKBP38 (FKBP38 siRNA) was transfected in HEK293T cells. Scramble siRNA transfected cells were used as control cells (control siRNA). After culturing for 3 days, the cells were starved for nutrients and lysed with lysis buffer as described under "Experimental Procedures," and the amount of FKBP38, phospho-S6 at Ser$^{235/236}$, and total S6 was examined by Western blotting (*left panel*). From these lysates, mTORC1 was immunopurified, and the *in vitro* activity was measured in the presence or absence of recombinant Rheb-GTPγS (*right panel*). The proteins were resolved by SDS-PAGE, and phospho-4E-BP1 at Thr$^{37/46}$ was analyzed by Western blotting. *C*, cells were transfected with pCDNA3 (control), FLAG-FKBP38 and/or FLAG-Rheb, respectively. The cells were starved for serum and amino acids (*AA* −) or restimulated with amino acid mixture after amino acid starvation (*AA* +). The proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Activation of mTORC1 by Rheb Involves Enhanced Binding of 4E-BP1—Phosphorylation of 4E-BP1 by mTORC1 involves recruitment of the substrate protein to mTORC1 followed by the phosphorylation by mTOR kinase activity. The association of mTORC1 with its substrate proteins such as 4E-BP1 and S6 kinase is mediated by raptor (36–40). Overexpression of raptor results in the stimulation of the mTORC1 pathway, whereas the mTOR inhibitor rapamycin is believed to inhibit mTORC1 activation through the disruption of mTOR-raptor binding (40,

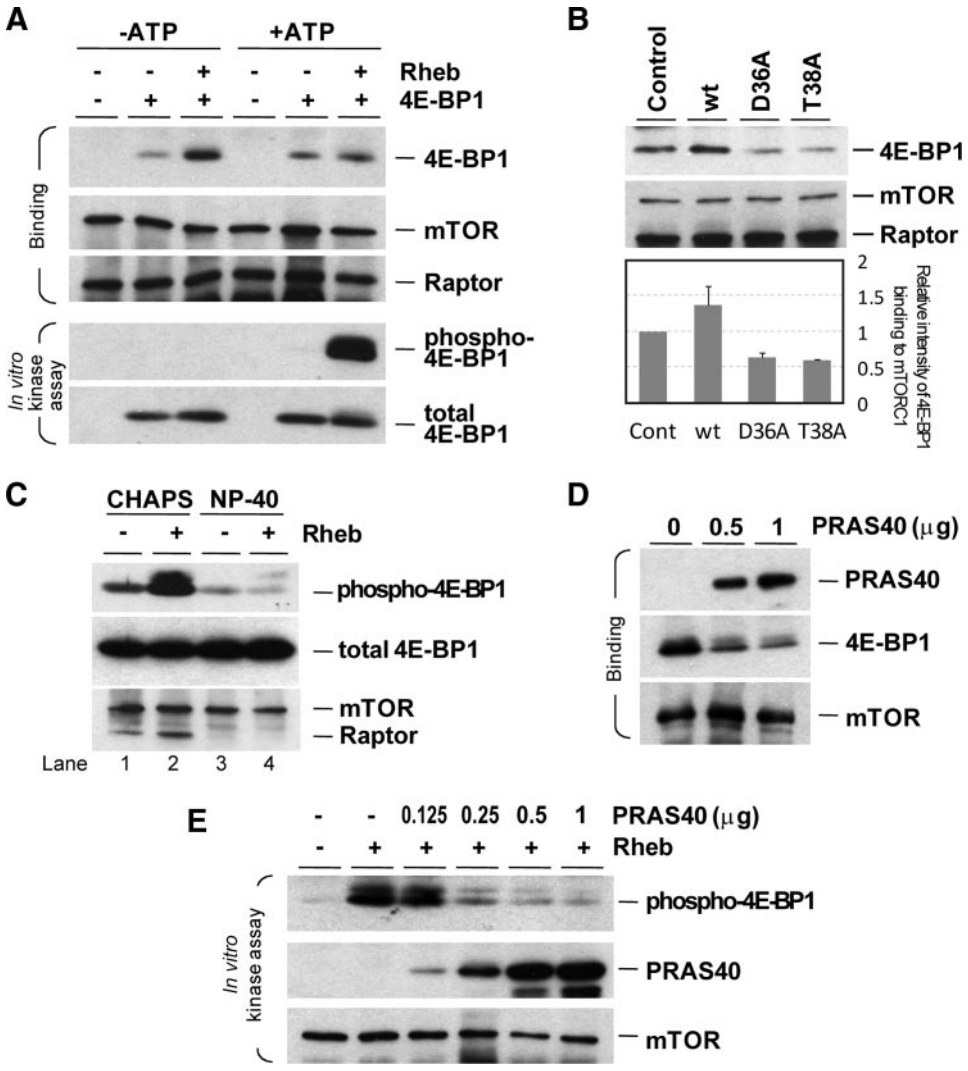

FIGURE 5. **Rheb enhances the binding of 4E-BP1 to mTORC1.** *A*, amino acid-starved cells were collected, and mTORC1 was immunoprecipitated using anti-raptor antibody. The immunoprecipitated mTORC1 was then mixed with 1 μg of recombinant 4E-BP1 in the presence or absence of Rheb, and 4E-BP1 binding assay was performed in the buffer with or without ATP at 37 °C for 20 min. After washing, bound 4E-BP1 was detected by Western blot. *B*, wild type (*wt*) or mutant Rheb proteins bound with GTPγS were added to the immunopurified mTORC1, and the amount of 4E-BP1 bound to mTORC1 was measured by Scion Image after Western blot analysis using specific antibody. The standard deviation was derived from three independent experiments. *C*, amino acid starved cells were lysed in the lysis buffer supplemented with 0.3% CHAPS or with 1% Nonidet P-40. mTOR was immunopurified from each cell lysates using anti-mTOR antibody, and the kinase activity was measured in the presence or absence of Rheb-GTPγS. Proteins were resolved by SDS-PAGE, and phospho-4E-BP1 at $Thr^{37/46}$ was detected by Western blotting. *D*, 4E-BP1 bound to mTORC1 was measured in the presence of indicated amount of PRAS40. After washing, the amount of 4E-BP1 or PRAS40 bound to mTORC1 was detected by Western blotting. *E*, the kinase assay of mTORC1 was performed *in vitro*. 1 μg of recombinant Rheb-GTPγS and/or described amount of recombinant PRAS40 was added before incubation at 37 °C for 20 min. After reaction, the proteins were resolved by SDS-PAGE, and phospho-4E-BP1 at $Thr^{37/46}$ was detected by Western blot.

41). In addition, it was reported that insulin-dependent phosphorylation of 4E-BP1 is associated with the enhanced binding of 4E-BP1 to raptor (42). Therefore, we were interested in examining whether Rheb affects binding of mTORC1 with 4E-BP1. To detect the binding of mTORC1 with 4E-BP1, the mTORC1 preparation used for the *in vitro* kinase assay was incubated with 4E-BP1 in the presence or absence of Rheb. After washing, the amount of 4E-BP1 bound to mTORC1 was estimated by Western blot. As seen in the *left three lanes* of Fig. 5*A*, 4E-BP1 bound to mTORC1 was detected when 4E-BP1 was added to mTORC1. This binding was significantly increased by the addition of Rheb. On the other hand, in the presence of ATP, the augmentation of 4E-BP1 binding to mTORC1 by Rheb was not detected (Fig. 5*A*, *right three lanes*). We confirmed that the phosphorylation of 4E-BP1 was detected only in the sample supplemented with 4E-BP1 and ATP (Fig. 5*A*, *lower panels*). This result is in agreement with the previous report that the amount of 4E-BP1 binding to raptor is reduced by mTOR-dependent phosphorylation of 4E-BP1 (43). To further examine the effect of Rheb on mTORC1 binding to 4E-BP1, we used D36A and T38A effector domain mutants that bind GTP but lack mTOR activation ability. As shown in Fig. 5*B*, D36A and T38A did not increase 4E-BP1 binding to mTORC1, although wild type Rheb increased the amount of 4E-BP1 interacting with mTORC1.

Raptor Plays an Important Role in the Activation of mTORC1 by Rheb—We next investigated whether Rheb activation of mTORC1 is dependent on the raptor bound to the complex. To address this issue, mTOR was immunoprecipitated using the lysis buffer supplemented with 1% Nonidet P-40 in which raptor but not $G_{\beta}L$ is washed out from mTORC1 (39, 40, 44). On the other hand, immunoprecipitated mTOR, which is prepared by using the lysis buffer containing 0.3% CHAPS, was used as a positive control because the interaction between mTOR and raptor is not affected by 0.3% CHAPS (39, 40). As observed in Fig. 5*C*, raptor was detected in the mTOR precipitates prepared by using the lysis buffer with 0.3% CHAPS but not from that with 1% Nonidet P-40. The *in vitro* kinase assay showed that mTOR without raptor phosphorylated 4E-BP1, but the amount was much less than that obtained with mTOR bound to raptor (Fig. 5*C*, *lanes 1* and *3*). The addition of Rheb significantly increased the phosphorylation of 4E-BP1 in the samples containing raptor, but Rheb did not significantly increase 4E-BP1 phosphorylation in the samples without raptor (Fig. 5*C*, *lanes 2* and *4*).

The proline-rich Akt substrate of 40 kDa (PRAS40), which has been postulated as a negative regulator of mTOR, has a TOS motif essential for the binding to raptor and therefore competes with 4E-BP1 for raptor binding leading to inhibition of the

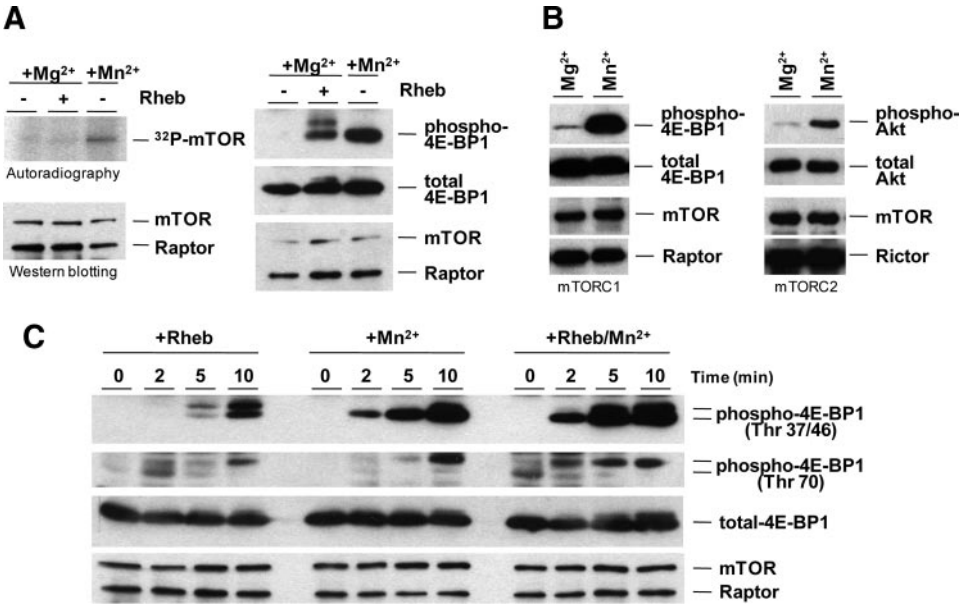

FIGURE 6. **Rheb does not induce autophosphorylation of mTOR.** *A*, HEK293T cells were lysed and mTORC1 was immunoprecipitated using anti-raptor antibody. The *in vitro* kinase assay of mTORC1 was started in the buffer containing 10 mM Mg^{2+}, 10 mM Mg^{2+}, and 1 μg recombinant Rheb loaded with GTPγS or 10 mM Mn^{2+} instead of Mg^{2+}. 1 μg of 4E-BP1 or 10 μCi of [γ-^{32}P]ATP was added to examine phosphorylation of 4E-BP1 or autophosphorylation of mTOR. After 20 min reaction at 37 °C, the samples were resolved by SDS-PAGE, and the phosphorylation of mTOR was detected by autoradiography. Other proteins were detected by Western blotting. *B*, the immunopurified mTORC1 and mTORC2 were isolated as described in Fig. 1*A*. The kinase assay was performed in the kinase buffer containing 10 mM Mg^{2+} or 10 mM Mn^{2+} for 20 min at 37 °C. Phospho-4E-BP1 at $Thr^{37/46}$ and phospho-Akt at Ser^{473} were detected by Western blotting. *C*, HEK293T cells were lysed, and mTORC1 was immunoprecipitated using anti-raptor antibody. The *in vitro* kinase assay of mTORC1 was started in the kinase buffer containing 1 μg of Rheb-GTPγS and 10 mM Mg^{2+}, 10 mM Mn^{2+} or the combination of 1 μg recombinant Rheb-GTPγS and 10 mM Mn^{2+}. The kinase reaction was stopped at indicated time points. Sample proteins were resolved by SDS-PAGE, and phosphorylation of 4E-BP1 at $Thr^{37/46}$ or Thr^{70} was detected by Western blotting using specific antibodies, respectively.

mTOR signaling pathway (29, 45–49). Accordingly, PRAS40 is expected to inhibit the activation of mTORC1 by Rheb if Rheb enhances the binding of 4E-BP1 to mTORC1 mediated by the recognition of the TOS motif. We first examined whether recombinant PRAS40 competes with 4E-BP1 for mTORC1 binding *in vitro*. As can be seen in Fig. 5*D*, the binding of PRAS40 to mTORC1 was increased in a dose-dependent manner, whereas 4E-BP1 binding to mTORC1 was markedly reduced. Thus, we expected that the Rheb induced mTORC1 activation would be inhibited by PRAS40. This was what we observed; Rheb-induced activation of mTORC1 in the *in vitro* assay was suppressed by the addition of PRAS40 (Fig. 5*E*).

Rheb Does Not Induce Autophosphorylation of mTOR—Previous reports have shown that TOR proteins exhibit intrinsic kinase activity that is greatly enhanced by the addition of Mn^{2+} (50–52). This is confirmed in the experiments shown in Fig. 6. In the experiment shown in Fig. 6*A* (*left panel*), mTORC1 preparation was incubated for 20 min with γ-^{32}P-labeled ATP, and the proteins were resolved by SDS-PAGE, and the phosphorylation of mTOR was detected by autoradiography. A clear band was detected when Mn^{2+} was used, whereas a weak band was detected in the presence of Mg^{2+}. We asked whether the addition of Rheb can induce this autophosphorylation in the presence of Mg^{2+}. The results show that Rheb is incapable of inducing autophosphorylation. Fig. 6*A* (*right panel*) shows that Rheb is capable of phosphorylating 4E-BP1 in the presence of Mg^{2+}. Interestingly, when Mg^{2+} was replaced with Mn^{2+}, phosphorylation of 4E-BP1 can proceed without Rheb. We also found that Mn^{2+} can activate not only mTORC1 but also mTORC2 (Fig. 6*B*). Phosphorylation of Akt by mTORC2 is readily observed in the presence of Mn^{2+}. These results support the idea that although Mn^{2+} can activate kinase activity of mTOR, Rheb does not directly activate the kinase activity of mTOR.

Interestingly, Rheb and Mn^{2+} appear to act differently on the phosphorylation of 4E-BP1. Although two bands of 4E-BP1 were detected when mTORC1 was activated by Rheb in the presence of Mg^{2+}, only one band was detected when mTORC1 was activated by Mn^{2+} (Fig. 6*A*). Because 4E-BP1 is phosphorylated at multiple sites such as Thr^{37}, Thr^{46}, and Thr^{70} by mTOR (53–55), we thought that this difference reflects the different ways 4E-BP1 is phosphorylated. To further examine the phosphorylation state of 4E-BP1, we examined separately the phosphorylation at threonine 70 as well as at threonine 37 and 46 after the *in vitro* mTORC1 kinase assay with Rheb, Mn^{2+}, or both Rheb and Mn^{2+} (Fig. 6*C*). In the presence of Rheb, phosphorylation of 4E-BP1 at Thr^{70} was clearly detected at 2 min as double bands, whereas phospho-4E-BP1 at $Thr^{37/46}$ was detected weakly at 2 min and increased over 10 min (Fig. 6*C*, *+Rheb*). On the other hand, in the presence of Mn^{2+}, the bands of phospho-4E-BP1 at Thr^{70} were weak at 2 min and gradually increased over 10 min (Fig. 6*C*, *+Mn^{2+}*). Correlated with the slow phosphorylation of 4E-BP1 at Thr^{70}, phospho-4E-BP1 at $Thr^{37/46}$ was largely detected as a single band at lower position even after 10 min, although the band was clearly detected from 2 min. Mixture of Rheb and Mn^{2+} quickly increased the phosphorylation of 4E-BP1 both at $Thr^{37/46}$ and Thr^{70} (Fig. 6*C*, *+Rheb/Mn^{2+}*). These results suggest that both Rheb and Mn^{2+} can cause mTORC1 activation but that the mechanism of activation differs between the two.

DISCUSSION

Our results using an *in vitro* system strongly support the idea that Rheb activates mTORC1 directly leading to increased phosphorylation of its substrate proteins. GTP-bound Rheb significantly activates mTORC1 but has no effect on mTORC2. Remarkable specificity for the requirement of Rheb G-protein was observed, because other members of the Ras family G-proteins such as KRas, RalA/B, Rras, and Rad did not activate mTORC1. Rho family G-proteins such as Cdc42 and Rac1 also did not activate mTORC1. The activation of mTORC1 was

observed with both Rheb1 and Rheb2. This result is in agreement with our previous study showing that both Rheb1 and Rheb2 can activate the mTOR signaling *in vivo* (14) and is also consistent with the fact that both these Rheb proteins have similar effector domain sequence (2). Rheb1 and Rheb2 exhibit different tissue expression profiles; Rheb1 is ubiquitously expressed, whereas Rheb2 has more limited tissue expression (56, 57). Thus, it appears that these two Rheb proteins exhibit similar function but that their tissue expression differs.

The *in vitro* activation of mTORC1 by Rheb is dependent on the presence of an intact effector domain. Alanine substitution at residue 36, 37, 38, or 41 led to a dramatic decrease in Rheb's ability to activate mTORC1 *in vitro*, although they still retain sufficient ability to bind GTP. These results are in agreement with *in vivo* studies that used mutant Rheb proteins having alanine substitutions in and around the effector domain (30, 58). Significance of the effector domain for the function of Rheb was also reported with the fission yeast protein (26). In addition, analysis of Rheb three-dimensional structure suggested that a major conformational change during GTP/GDP cycling occurs at the switch I region, especially at residues 35–39 (6). Thr^{35}, which shields the phosphates of GTP, is thought to be important in maintaining binding of GTP, because alanine substitution of Thr^{35} drastically reduced the amount of [^{35}S]GTP bound to Rheb as seen in Fig. 3. Deletion of the side chains of Asp^{36} and Thr^{38}, which are movable during GTP/GDP cycling, had little effect on GTP binding but caused the loss of mTORC1 activation, suggesting that these residues play important roles in the interaction with mTORC1. P37A and N41A mutants also failed to activate mTORC1 despite maintaining sufficient GTP binding ability, also pointing to the significance of the effector domain.

Our results do not support the recent proposal that Rheb activates mTORC1 by interacting with FKBP38 (32). According to this model, Rheb interacts with FKBP38 and prevents negative regulation of mTOR by FKBP38 with mTOR. However, we have shown that the mTORC1 preparation that is devoid of FKBP38 can be activated by Rheb. In fact, Rheb activates mTORC1 prepared from cells treated with siRNA to decrease FKBP38 expression. In another experiment, we depleted FKBP38 from the lysate using anti-FKBP38 antibody. Again, mTORC1 prepared from the FKBP38-depleted cells was activated by Rheb.[3] These results clearly suggest that Rheb does not require FKBP38 to activate mTORC1. In our cell experiments, phosphorylation of 4E-BP1 and S6K1 was not detected when FKBP38 knockdown cells were starved for amino acids. A slight increase in S6 phosphorylation was observed. Overexpression of FKBP38 did not suppress mTOR activation induced by the expression of Rheb or stimulated by amino acids. Similar lack of FKBP38 effects on mTOR signaling was recently reported (33). On the other hand, recombinant FKBP38 inhibited 4E-BP1 phosphorylation in the *in vitro* mTORC1 kinase assay.[3] Taken together, these results support the idea that FKBP38 is not a major contributor to the mTORC1 signaling. It is, however, not excluded that FKBP38 has a role under some particular conditions.

[3] T. Sato and F. Tamanoi, unpublished observation.

Our study revealed that Rheb has a significant enhancing effect on the binding of 4E-BP1 to mTORC1. This effect was captured when the binding of 4E-BP1 to mTORC1 was examined in the absence of kinase reaction. Importantly, we showed that the Rheb effector domain mutants D36A and T38A do not enhance this 4E-BP1/mTORC1 binding. The significance of raptor in the Rheb-induced activation of mTORC1 is suggested from the lack of mTOR activation by Rheb when mTOR preparation that is devoid of raptor was used. These results agree with the important role raptor plays in the binding of 4E-BP1 to mTORC1 through its interaction with the TOS motif. We also observed that PRAS40, a TOS motif-containing protein that competes with 4E-BP1 for binding to mTORC1, inhibits Rheb-induced activation of mTORC1. This is correlated with the loss of 4E-BP1 binding to mTORC1 by PRAS40. Although Rheb effects are mediated by raptor, we do not think Rheb interacts directly with raptor. We can detect binding of Rheb to mTOR but not to raptor.[3] Thus, our current working hypothesis is that Rheb binds mTOR and influences the interaction of raptor with the substrate protein leading to the production of phospho-4E-BP1. This idea is consistent with the finding (42) that insulin activation of mTORC1 involves enhancement of the binding of 4E-BP1 with mTORC1. Insulin activates Rheb through Akt-dependent phosphorylation of Tsc2, leading to the activation of mTORC1 pathway.

We have also shown that Rheb does not induce autophosphorylation of mTOR, suggesting that Rheb does not activate the catalytic activity of mTOR. Although autophosphorylation can be readily detected in the presence of Mn^{2+}, the addition of Rheb did not result in the phosphorylation of mTOR. In addition, we found that Mn^{2+} can activate mTORC1 even in the absence of Rheb and Mg^{2+}. This is reminiscent of previous observations concerning activation of yeast Ras effector proteins (59, 60); the requirement for Ras to activate yeast adenylate cyclase can be bypassed when Mg^{2+} is replaced with Mn^{2+}. We also observed differences in the way that 4E-BP1 is phosphorylated between Mn^{2+} and Rheb. Rheb quickly enhanced the phosphorylation of 4E-BP1 at Thr^{70}, whereas the phosphorylation of 4E-BP1 at Thr^{37} and Thr^{40} was quickly observed when Mn^{2+} was used. Rheb may stimulate the access of threonine 70 of 4E-BP1 to mTORC1. Further experiments are needed to fully understand how Rheb activates mTOR.

It is notable that the activation of mTOR was observed by the use of recombinant Rheb protein purified after expression in *E. coli*. Because farnesylation does not occur in *E. coli*, lipid modification is not required for the ability of Rheb to activate mTORC1. To further investigate this point, we have used farnesylated Rheb protein obtained by using baculovirus-infected cells in our *in vitro* system. The results showed that the ability of the farnesylated Rheb protein was comparable or even less that than seen with the unmodified Rheb protein.[3] Thus, farensylation is not required for the ability of Rheb to activate mTORC1. On the other hand, we as well as others have previously shown that farnesylation is important for Rheb function *in vivo* (8, 9, 16, 19). These results suggest that farnesylation is important for the proper cellular localization of Rheb but not for direct activation of mTORC1.

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