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Mammalian Target of Rapamycin Complex 1 (mTORC1) Activity Is Associated with Phosphorylation of Raptor by mTOR*[S]

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Lifu Wang[1], John C. Lawrence, Jr.[†], Thomas W. Sturgill, and Thurl E. Harris

From the Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908

mTORC1 contains multiple proteins and plays a central role in cell growth and metabolism. Raptor (regulatory-associated protein of mammalian target of rapamycin (mTOR)), a constitutively binding protein of mTORC1, is essential for mTORC1 activity and critical for the regulation of mTORC1 activity in response to insulin signaling and nutrient and energy sufficiency. Herein we demonstrate that mTOR phosphorylates raptor *in vitro* and *in vivo*. The phosphorylated residues were identified by using phosphopeptide mapping and mutagenesis. The phosphorylation of raptor is stimulated by insulin and inhibited by rapamycin. Importantly, the site-directed mutation of raptor at one phosphorylation site, Ser^{863}, reduced mTORC1 activity both *in vitro* and *in vivo*. Moreover, the Ser^{863} mutant prevented small GTP-binding protein Rheb from enhancing the phosphorylation of S6 kinase (S6K) in cells. Therefore, our findings indicate that mTOR-mediated raptor phosphorylation plays an important role on activation of mTORC1.

Mammalian target of rapamycin (mTOR)[2] has been shown to function as a critical controller in cellular growth, survival, metabolism, and development (1). mTOR, a highly conserved Ser-Thr phosphatidylinositol 3-kinase-related protein kinase, structurally forms two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each of which catalyzes the phosphorylation of different substrates (1). The best characterized substrates for mTORC1 are eIF4E-binding protein (4E-BP, also known as PHAS) and p70 S6 kinase (S6K) (1), whereas mTORC2 phosphorylates the hydrophobic and turn motifs of protein kinase B (Akt/protein kinase B) (2) and protein kinase C (3, 4). mTORC1 constitutively consists of mTOR, raptor, and mLst8/GβL (1), whereas the proline-rich Akt substrate of 40 kDa (PRAS40) is a regulatory component of mTORC1 that disassociates after growth factor stimulation (5, 6). Raptor is essential for mTORC1 activity by providing a substrate binding function (7) but also plays a regulatory role on mTORC1 with stimuli of growth factors and nutrients (8). In response to insulin, raptor binding to substrates is elevated through the release of the competitive inhibitor PRAS40 from mTORC1 (9, 10) because PRAS40 and the substrates of mTORC1 (4E-BP and S6K) appear to bind raptor through a consensus sequence, the TOR signaling (TOS) motif (10–14). In response to amino acid sufficiency, raptor directly interacts with a heterodimer of Rag GTPases and promotes mTORC1 localization to the Rheb-containing vesicular compartment (15).

mTORC1 integrates signaling pathways from growth factors, nutrients, energy, and stress, all of which generally converge on the tuberous sclerosis complex (TSC1-TSC2) through the phosphorylation of TSC2 (1). Growth factors inhibit the GTPase-activating protein activity of TSC2 toward the small GTPase Rheb via the PI3K/Akt pathway (16, 17), whereas energy depletion activates TSC2 GTPase-activating protein activity by stimulating AMP-activated protein kinase (AMPK) (18). Rheb binds directly to mTOR, albeit with very low affinity (19), and upon charging with GTP, Rheb functions as an mTORC1 activator (6). mTORC1 complexes isolated from growth factor-stimulated cells show increased kinase activity yet do not contain detectable levels of associated Rheb. Therefore, how Rheb-GTP binding to mTOR leads to an increase in mTORC1 activity toward substrates, and what the role of raptor is in this activation is currently unknown. More recently, the AMPK and p90 ribosomal S6 kinase (RSK) have been reported to directly phosphorylate raptor and regulate mTORC1 activity. The phosphorylation of raptor directly by AMPK reduced mTORC1 activity, suggesting an alternative regulation mechanism independent of TSC2 in response to energy supply (20). RSK-mediated raptor phosphorylation enhances mTORC1 activity and provides a mechanism whereby stress may activate mTORC1 independent of the PI3K/Akt pathway (21). Therefore, the phosphorylation status of raptor can be critical for the regulation of mTORC1 activity.

In this study, we investigated phosphorylation sites in raptor catalyzed by mTOR. Using two-dimensional phosphopeptide mapping, we found that Ser^{863} and Ser^{859} in raptor were phosphorylated by mTOR both *in vivo* and *in vitro*. mTORC1 activity *in vitro* and *in vivo* is associated with the phosphorylation of Ser^{863} in raptor.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to raptor (9), HA (9), and phosphospecific antibodies to the Thr^{36} and Thr^{45} sites (22) have been described previously. Phosphospecific antibodies to the Thr^{389} site in S6K1 were from Cell Signaling Technology, Inc. FLAG antibodies were from Sigma-Aldrich. Recombinant human insulin (Novolin R) was from Novo Nordisk. Rapamycin was from Calbiochem-Novabiochem. Tween 20 was from Fisher, and Triton X-100 was

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† Deceased December 19, 2006.

[1] To whom correspondence should be addressed: Dept. of Pharmacology, University of Virginia Health System, P. O. Box 800735, 1300 Jefferson Park Ave., Charlottesville, VA 22908. Tel.: 434-924-1582; Fax: 434-982-3878; E-mail: lw6j@virginia.edu.

[2] The abbreviations used are: mTOR, mammalian target of rapamycin; raptor, regulatory associated protein of mTOR; 4E-BP, eIF4E-binding protein; FKBP12, FK506-binding protein of *M*r = 12,000; PI3K, phosphatidylinositol 3-kinase; Rheb, Ras homolog enriched in brain; TOS, TOR signaling; AMPK, AMP-activated protein kinase; RSK, ribosomal S6 kinase; S6K, S6 kinase; HA, hemagglutinin.

from Sigma-Aldrich. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. Cellulose TLC plates were from EMD Chemicals Inc.

Cell Culture, Treatment, and Extract Preparation—3T3-L1 adipocytes were differentiated as described previously (9) and were used in 10–12 days. HEK293, HEK293E, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and used for transfection experiments. HEK293T cells were used to perform ^{32}P labeling and phosphopeptide mapping experiments. When compared with HEK293 and HEK293T cells, there are low basal levels of the phosphorylation of mTOR targets in HEK293E cells, and thus, HEK293E cells were used for insulin signaling studies. For amino acid starvation, the culture medium was replaced with a solution containing 145 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, 0.2 mM sodium phosphate, and 10 mM HEPES, pH 7.4 for 1 h. To terminate the incubation, cells were rinsed once with chilled phosphate-buffered saline and then homogenized with a syringe with a 20-gauge needle in lysis buffer as described previously (9). Homogenates were centrifuged at 12,000 × *g* for 10 min, and the supernatants were retained for analyses.

Transfection and Immunoprecipitation—Cells were seeded in 6-cm dishes or 6-well plates. 24 h later, plasmids were transfected using Lipofectamine 2000 (Invitrogen) at 1:1 ratio (w/v). The cells were harvested and analyzed at 36 h after transfection. Cell extracts were incubated with antibodies (2 μg) bound to protein A- or G-agarose beads at 4 °C for 2 h with constant mixing. The beads were then washed four times.

In Vitro Kinase Assay—As described (9), immune complex beads were rinsed with 1 ml of kinase buffer (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 μM microcystin LR, 10 mM HEPES, and 50 mM β-glycerophosphate, pH 7.4) and suspended in 60 μl of kinase buffer. The kinase reactions were initiated by adding to 20 μl of the suspension 5 μl of kinase buffer supplemented with 0.5 mM [γ-^{32}P]ATP (PerkinElmer Life Sciences, 1,000 mCi/mmol), 50 mM $MnCl_2$, and 0.25 μg of 4E-BP1 as substrates or no substrate for the phosphorylation of raptor-mTOR complex. Reactions were terminated after 30 min at 30 °C by adding SDS sample buffer. The relative amounts of ^{32}P incorporation were determined by phosphorimaging after SDS-PAGE.

In Vivo ^{32}P Radiolabeling and Two-dimensional Phosphopeptide Mapping—In vivo ^{32}P radiolabeling and two-dimensional phosphopeptide mapping were performed as described previously (23).

RESULTS AND DISCUSSION

Phosphorylation of Raptor by mTOR in Vitro—When conducting mTORC1 kinase assays *in vitro* with substrates such as 4E-BP1, S6K1, and PRAS40, we observed that both mTOR and raptor, isolated in the mTORC1 immune complex, also incorporated [γ-^{32}P]ATP. mTOR has been previously observed to undergo autophosphorylation at Ser^{2481} (24). Therefore, we decided to investigate whether the phosphorylation of raptor is mediated by mTOR and to reveal, if possible, the functional consequences of raptor phosphorylation by mTOR. Triton X-100 causes disassociation of the mTOR-raptor complex, whereas nonionic detergents of Tween 20 do not (7, 8). Therefore, cell extracts

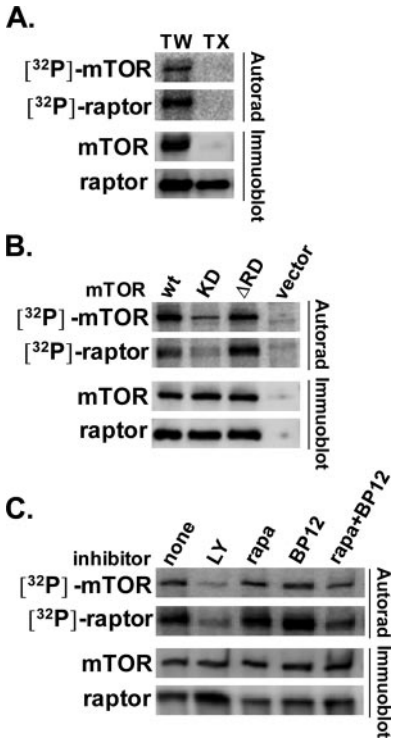

FIGURE 1. **Phosphorylation of raptor by mTOR *in vitro*.** *A,* FLAG-tagged raptor and Myc-tagged mTOR were co-expressed in HEK293T cells, and immunoprecipitation with antibody to FLAG tag was conducted in the presence of 0.2% Tween 20 (*TW*) or 0.2% Triton X-100 (*TX*). The immune complexes were mixed with [γ-^{32}P]ATP, and *in vitro* phosphorylation was performed. Autoradiography (*Autorad*) was visualized by phosphorimaging, and levels of mTOR and raptor were detected with immunoblots. *B*, mTOR wild type (*wt*), kinase dead (S2338A, *KD*), and constitutive active (deleting 2433–2451, Δ*RD*) mutants were co-expressed with raptor in HEK293T cells. The mTOR-raptor complex was isolated by immunoprecipitation of FLAG-tagged mTOR, and then *in vitro* phosphorylation was performed by incubating with [γ-^{32}P]ATP. *C*, the mTOR-raptor immune complex was obtained as described in *A* with Tween 20. Washed immune complexes were incubated for 20 min with no additions (*none*) or the following: 10 μM LY294002 (*LY*), 5 μM rapamycin (*rapa*), 5 μM GST-FKBP12 (*BP12*), or 5 μM GST-FKBP12 plus 5 μM rapamycin (*rapa*+*BP12*), and then *in vitro* phosphorylation was measured by adding [γ-^{32}P]ATP.

and immunoprecipitations were prepared in Tween 20 or Triton X-100 to isolate raptor immune complexes with or without mTOR. After incubation of the raptor immune complexes prepared in the presence of Tween 20 *in vitro*, raptor was phosphorylated, whereas incubation with [γ-^{32}P]ATP/Mn^{2+} did not result in raptor phosphorylation when cell extracts were prepared with Triton X-100 (Fig. 1*A*). To confirm that the phosphorylation of raptor *in vitro* depends on mTOR activity, mTOR kinase dead (S2338A, KD) and constitutive active (deleting 2433–2451, ΔRD) mutants were utilized in the mTORC1 kinase assay. With equal amounts of raptor recovered by mTOR, when compared with wild type, the mTOR KD mutant largely abolished kinase activity toward raptor, whereas mTOR ΔRD substantially enhanced the phosphorylation of raptor (Fig. 1*B*). This suggests that the phosphorylation of raptor was dependent on mTOR kinase activity and inhibited by an intact regulatory domain of mTOR, as are the phosphorylations of mTORC1 substrates (4E-BP1, S6K1, and PRAS40). Of note, mTOR KD reduced the autophosphorylation of mTOR *in vitro*, but mTOR ΔRD did not affect autophosphorylation (Fig. 1*B*). The mechanism by which the inhibitory domain of mTOR controls kinase activity is unknown. To investigate further,

we asked whether raptor phosphorylation is inhibited by rapamycin, the mTORC1-specific inhibitor. The mTORC1 immune complex was incubated with rapamycin and FKBP12. Incubating mTORC1 with rapamycin or FKBP12 alone was without effect on raptor phosphorylation; however, the combination of rapamycin and FKBP12 reduced the phosphorylation of raptor (Fig. 1*C*). Consistently, LY294002, which is also an inhibitor of PI3K/mTOR but by a different mechanism, abolished raptor phosphorylation as well. Taken together, the *in vitro* phosphorylation of raptor in mTORC1 is mediated by mTOR with similar characteristics as the other substrates of mTORC1 such as 4E-BP1, S6K1, and PRAS40.

Phosphorylation of Raptor by mTOR in Vivo Maps to Ser^{859} and Ser^{863} and Is Sensitive to Rapamycin—The phosphorylation of raptor in cells was studied by expressing raptor in HEK293T cells labeled with [^{32}P]orthophosphate. ^{32}P-labeled recombinant raptor was immunoprecipitated by FLAG tag antibody and resolved by SDS-PAGE. A ^{32}P-labeled raptor band with increased ^{32}P suitable for mapping was obtained by overexpression of raptor (Fig. 2*A*). The raptor band was excised and digested by trypsin and then subjected to two-dimensional phosphopeptide mapping analysis. Raptor phosphorylated by mTOR *in vitro*, prepared as described in the legend for Fig. 1 (*panel A*), was digested by trypsin and mixed with raptor peptides phosphorylated *in vivo* to compare raptor phosphorylation sites catalyzed by mTOR *in vitro* with those obtained *in vivo*. The major phosphorylated peptides *in vivo* were obtained as indicated by spots of *a–d* and *in vitro* as indicated by spots of *1–5* (Fig. 2*B*). Phosphopeptides of *b*, *c*, and *d* from raptor phosphorylated *in vivo* co-migrated with spots of *1*, *2*, and *4* from *in vitro* mTOR-phosphorylated peptides (Fig. 2*B*, *last panel*), suggesting that these peptides (*spots b*, *c*, and *d*) could be phosphorylated by mTOR *in vivo*. We noticed that some peptides, such as spot *3* and *5*, were phosphorylated by mTOR *in vitro* but were not present in the *in vivo* raptor phosphopeptide map. The additional phosphopeptides arising from the *in vitro* kinase reaction is not unusual and could be from several sources. In addition to kinases *in vitro* being more promiscuous than *in vivo*, it is also possible that the dephosphorylation of some sites occurs *more* rapidly *in vivo*. Alternatively, there could be a loss of some proteins during the immunoprecipitation of mTORC1 that could change the phosphorylation of raptor by mTOR *in vitro* when compared *in vivo*.

Mass spectrometry has become a powerful technology for phosphoproteomic studies, providing a starting point for biologic studies. Olsen *et al.* (25) defined a phosphoproteome in HeLa cells after stimulation of epidermal growth factor. In this phosphopeptide library (Ref. 25, document S2 in supplemental data) raptor phosphorylations at Ser^{859}, Ser^{863}, and Ser^{884} were detected. We mutated these serine residues to alanine and tested whether they are phosphorylated in cells by using two-dimensional phosphopeptide mapping. As shown in Fig. 2*C*, Ser^{863} and Ser^{859} were phosphorylated *in vivo* because the mutations of Ser to Ala at 863 and 859 eliminated phosphopeptides represented at spots *b* and *c*. Interestingly, mutation of Ser^{863} to Ala (S863A) caused the disappearance of both spot *b* and spot *c*, whereas mutation of Ser^{859} to Ala (S859A) only eliminated spot *c* (Fig. 2*C*). Because Ser^{859} and Ser^{863} reside in the same tryptic peptide of ^{850}VLDTSSLTQ***S***APA***S***PTNK^{868}, the spot *b* and spot *c* likely represent different phosphorylation patterns within this single peptide. Spot *c* migrated slower in the first dimension and was less hydrophobic in the second dimension than spot *b*, suggesting that spot *c* has more phosphorylation than spot *b*. Thus, spot *c* represents the phosphorylation of both Ser^{863} and Ser^{859}, and spot *b* represents a single phosphorylation. As only S863A but not S859A caused the disappearance of spot *b*, spot *b* most likely represents phosphorylation at Ser^{863}. This suggests that Ser^{859} is phosphorylated only when Ser^{863} is phosphorylated first. The phosphopeptide mapping of mutation at Ser^{884} (S884A) remained unaltered when compared with wild type (Fig. 2*C*). Furthermore, we mutated Ser^{863} to Glu and Thr. As predicted, mutation of Ser^{863} to Glu eliminated phosphorylation at this site similar to the Ala mutant (supplemental Fig. S1*A*). Because *in vitro* mTORC1-mediated raptor phosphopeptides co-migrated with *in vivo* phosphopeptides containing Ser^{863} and Ser^{859} (spot *1* → *b* and spot *2* → *c*, Fig. 2*B*) and mutations of Ser^{863} and Ser^{859} to Ala eliminated spot *1* and *2* (data not shown), mTOR phosphorylates raptor at Ser^{863} and Ser^{859} *in vitro* as well. We next investigated whether the phosphorylation of Ser^{863} and Ser^{859} were regulated by growth factor

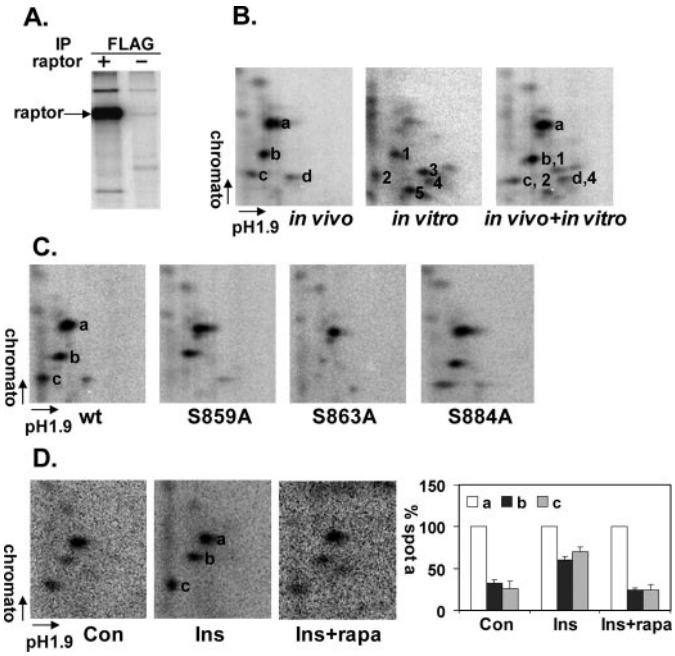

FIGURE 2. **Ser^{863} and Ser^{859} in raptor are phosphorylated by mTOR both *in vivo* and *in vitro*.** *A*, FLAG-tagged raptor were transfected together with mTOR in HEK293T cells, and cells were labeled with [^{32}P]orthophosphate for 3 h followed by insulin treatment (200 nM) for 30 min. ^{32}P-labeled raptor (indicated by *arrow*) was immunoprecipitated (*IP*) with FLAG antibody and visualized by phosphorimaging after SDS-PAGE when compared with the control of immunoprecipitation of cell extract without FLAG-raptor expression. *B*, ^{32}P-labeled raptor phosphorylated by mTOR *in vitro* was prepared as described in the legend for Fig. 1 (for *panel A*). The *in vivo* and *in vitro* ^{32}P-labeled raptor was excised and digested by trypsin. The trypsin-digested peptides of raptor phosphorylated *in vivo*, *in vitro*, and in a mixture of *in vivo* and *in vitro* (*in vivo*+*in vitro*) were resolved by two-dimensional phosphopeptide mapping with electrophoresis at pH 1.9 (*pH 1.9*) as the first dimension and chromatography as the second dimension (*chromato*). Major peptide spots are indicated by *a–d* (*in vivo*), *1–5* (*in vitro*), and *a–d*, *1–5* (co-migration). *C*, raptor wild type (*wt*) and mutants of Ser to Ala at 859, 863, and 884 (*S859A, S863A*, and *S884A*) were used for two-dimensional phosphopeptide mapping. *D*, 3T3-L1 adipocytes were labeled with [^{32}P]orthophosphate for 3 h and treated without or with rapamycin (20 nM) for 20 min before insulin treatment (20 nM) for 30 min. Antibodies toward raptor were used to immunoprecipitate endogenous raptor complex. The trypsin-digested phosphopeptide maps of ^{32}P-labeled raptor were analyzed. The major phosphopeptides are indicated as *a–c*, and the densities of the spots were quantified from three experiments (mean ± S.E. (*error bars*)) and expressed as the percentage of the density of spot *a*. *Con*, control; *Ins*, insulin; Ins+*rapa*, insulin plus rapamycin.

stimulation and rapamycin. Because they have a low level of signaling and a robust response to insulin, 3T3-L1 adipocytes were utilized to analyze inducible raptor phosphorylation. After cells were labeled by [^{32}P]orthophosphate, the mTORC1 complex was immunoprecipitated with raptor antibodies. In multiple experiments, the phosphorylation of the peptide represented in spot *a* appeared not to be affected by insulin and rapamycin treatments. In response to insulin stimulation, the phosphorylation of Ser^{863} and Ser^{859}, represented in spot *b* and *c*, increased as a ratio when compared with spot *a*, and rapamycin treatment prior to insulin stimulation reduced the insulin-induced phosphorylation of Ser^{863} and Ser^{859} (Fig. 2*D*). Taking the phosphorylation of Ser^{863} and Ser^{859} by mTOR directly *in vitro* and their inhibition by rapamycin *in vivo* together, our data demonstrate that the phosphorylation of Ser^{863} and Ser^{859} is mediated by mTOR.

When comparing the sequences of phosphorylation sites catalyzed by mTORC1 (4E-BP1, S6K1, and PRAS40) and sites catalyzed by mTORC2 (Akt1 and protein kinase C-α), there is not a high degree of selectivity (supplemental Fig. S1*B*). This may arise from the fact that the substrate specificity is largely dependent on the specific companion proteins of the complex (raptor for mTORC1 and rictor and SIN1 for mTORC2). For mTORC1, raptor binds to substrates, and the substrate specificity is mediated through the TOS motif within the substrates themselves (10–14). However, the phosphorylation sites characterized as catalyzed by mTOR have two distinct categories: sites directed by proline at +1 or +2 such as Thr^{36}, Thr^{45}, Thr^{69}, and Ser^{64} in 4E-BP1, Ser^{183} in PRAS40, Ser^{450} in Akt1, and Ser^{637} in protein kinase C-α and sites with surrounding hydrophobic residues such as Thr^{389} in S6K1, Ser^{473} in Akt1, and Ser^{656} in protein kinase C-α. Ser^{863} and Ser^{859} in raptor both contain a proline at +1/+2, similar to the mTORC1-mediated phosphorylation sites found in the 4E-BPs and PRAS40. Further comparing the proline-directed phosphorylation sites catalyzed by mTOR reveals that the −3 position shows a bias for hydrophobic residues.

Phosphorylation of Raptor at Ser^{863} Regulates mTORC1 Activity—To determine whether phosphorylations of raptor modulate mTOR function, mTORC1 activity *in vitro* and *in vivo* were compared for cells co-expressing mTOR with raptor *versus* mutants (S859A, S863A, and S884A). mTORC1 immune complexes were isolated by immunoprecipitation of raptor with FLAG antibody. The mTORC1 activity *in vitro* was measured with 4E-BP1 as substrate. When compared with wild type raptor, the mutation of Ser^{863}, but not Ser^{884}, substantially reduced the phosphorylation of 4E-BP1 catalyzed by mTORC1 *in vitro* (Fig. 3*A*). The mutation of Ser^{859} alone did not produce a significant decrease of mTORC1 activity. Because activation of mTORC1 activity is associated with an increase of 4E-BP1 binding to raptor (9), we examined 4E-BP1 binding to raptor phosphorylation mutants by using recombinant 4E-BP1 coupled to CNBr-activated Sepharose. As shown in supplemental Fig. S2*A*, the raptor mutants of S859A, S863A, and S884A did not produce a significant change in binding to 4E-BP1 when compared with raptor wild type. This indicates that raptor phosphorylation does not affect 4E-BP1 binding to raptor and that the mechanism by which the phosphorylation of raptor promotes mTORC1 activity is not due to an increase in substrate binding.

To measure the *in vivo* kinase activity of the expressed mTORC1, the mTOR rapamycin-resistant mutant (S2035W) was

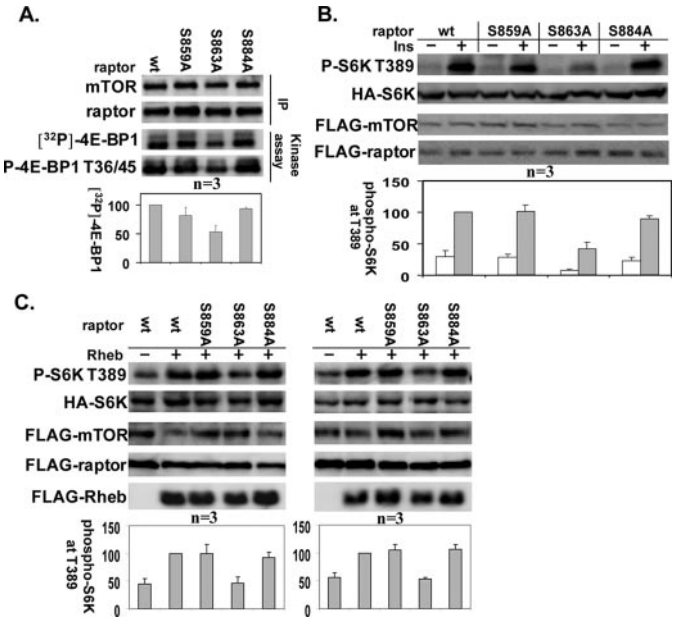

FIGURE 3. **Phosphorylation of raptor at Ser^{863} is important for activation of mTORC1 toward substrates of 4E-BP1 and S6K.** *A*, FLAG-tagged raptor wild type (*wt*) and mutants as indicated were transfected with Myc-mTOR into HEK293 cells. Cell extracts were prepared and immunoprecipitated (*IP*) with FLAG antibody. mTORC1 kinase activity *in vitro* was measured with [γ-^{32}P]ATP and recombinant 4E-BP1 as substrate and visualized with ^{32}P incorporation into 4E-BP1 and phospho-specific antibodies targeting Thr^{36}/Thr^{45} of 4E-BP1. The phosphorylation of 4E-BP1 expressed as ^{32}P incorporation (corrected for recovery of mTOR) was quantified from three experiments (mean ± S.E. (*error bars*)). *B*, raptor wild type and mutants as indicated were transfected with mTOR rapamycin-resistant mutant S2035W and S6K into HEK293E cells. After 36 h, followed by serum starvation of cells in Dulbecco's modified Eagle's medium overnight, cells were treated with rapamycin (20 nM) for 20 min and then insulin (*Ins*, 200 nM) for 30 min. Phospho-Thr^{389} of S6K1 and expression levels of HA-S6K, FLAG-mTOR, and FLAG-raptor were detected by immunoblotting. Phospho-Thr^{389} of S6K1 normalized by the total level of HA-S6K was quantified from three experiments (mean ± S.E. (*error bars*)). *C*, raptor wild type and mutants as indicated were transfected with S6K, Rheb, and mTOR rapamycin-resistant mutant S2035W (*left panel*) or wild type (*right panel*) into HEK293 cells. 36 h later, cell extracts were prepared after rapamycin treatment (20 nM) for 20 min (*left panel*) and starvation of amino acids in a salt-balanced buffer as described under "Experimental Procedures" for 1 h (*right panel*). Phospho-Thr^{389} of S6K1 and total expression levels of HA-S6K, FLAG-mTOR, FLAG-raptor, and FLAG-Rheb were detected by immunoblotting. Phospho-Thr^{389} of S6K1 normalized by total level of HA-S6K was quantified from three experiments (mean ± S.E. (*error bars*)).

utilized, and endogenous mTORC1 activity was inhibited by rapamycin pretreatment. The mutant mTOR S2035W and S6K1 were expressed together with either raptor wild type or mutants in HEK293E cells, treated with rapamycin to block endogenous mTORC1 kinase activity, and then stimulated with insulin. Consistent with the mTORC1 activity *in vitro*, mutation of Ser^{863} to Ala decreased insulin-stimulated mTORC1 activity in *vivo* as shown by decreased phosphorylation of S6K at Thr^{389} (Fig. 3*B*), whereas mutation of Ser^{859} and Ser^{884} did not alter the phosphorylation of S6K at Thr^{389}. Interestingly, when compared with the Ala mutant, mutation of Ser^{863} to Glu appears to mimic phosphorylation, as seen in the recovery of mTORC1 activity toward S6K at Thr^{389} after insulin treatment (supplemental Fig. S2*B*). However, we did not observe that the Ser-Glu mutant lead to increased basal mTORC1 activity (supplemental Fig. S2*B*). We discuss this in the below.

Because overexpression of GTP-binding protein Rheb promotes mTORC1 activity (19), we tested whether the activation of mTORC1 by Rheb requires raptor phosphorylation. Rheb was co-expressed with rapamycin-resistant mTOR, raptor, and S6K1, and

background of endogenous mTORC1 activity was knocked down with rapamycin pretreatment. Overexpression of Rheb enhanced the phosphorylation of S6K1 at Thr^{389}, as expected. However, expression of raptor containing the Ala mutation at Ser^{863}, but not Ser^{859} and Ser^{884}, reduced the phosphorylation of S6K at Thr^{389} activated by Rheb expression (Fig. 3*C*, *left panel*). As reported before (19), overexpression of Rheb overcomes the inhibition of the phosphorylation of S6K1 by amino acid depletion in cell culture medium. When Rheb was overexpressed with raptor wild type and mutants and amino acids were withdrawn from the cell culture medium, the raptor Ser^{863} to Ala mutant suppressed the Rheb-mediated rescue of S6K1 phosphorylation (Fig. 3*C*, *right panel*).

Therefore, the phosphorylation of raptor at Ser^{863} appears to be associated with mTORC1 activity and plays a critical role on the activation of mTORC1 in response to insulin. We propose that in response to growth factors, Rheb provides an initial stimulation of mTOR kinase activity, and the activation of mTOR results in raptor phosphorylation at Ser^{863} and Ser^{859}. Consequently, as an essential component of mTORC1, raptor phosphorylation leads to a stable activation of mTORC1 kinase activity toward downstream substrates such as S6K1 and 4E-BP1. It is possible that Rheb binding to mTOR initiates a conformational change in the mTOR complex. The subsequent phosphorylation of Ser^{863} stabilizes the proposed Rheb-mediated conformational change and leads to an activation of mTORC1 kinase activity that persists in the absence of Rheb association. However, merely mimicking phosphorylation at Ser^{863} by mutation to Glu is insufficient to initiate a conformational change in the absence of Rheb. Thus, *in vivo* the mutation of Ser^{863} to Glu does not increase basal mTORC1 kinase activity in the absence of Rheb activation. In addition, mTORC1 regulation consists of several relatively separate mechanisms. Raptor phosphorylation may modulate an increase in mTOR kinase activity toward substrates that have been bound by raptor, whereas substrate binding to raptor, which is essentially regulated by PRAS40 in response to insulin (10), is independent of raptor phosphorylation.

According to raptor phosphopeptide mapping analysis, mutation of Ser^{863} prevents the phosphorylation of both Ser^{863} and Ser^{859}. It is possible that mTORC1 activity needs phosphorylation at both sites. Recently, Ser^{722} and Ser^{792} in raptor were identified as AMPK-mediated phosphorylation sites and required for the inhibition of mTORC1 induced by energy stress (20), and Ser^{719}, Ser^{721}, and Ser^{722} are primary phosphorylation sites mediated by RSK, which are required for mTORC1 activation by mitogen stimulation (21). All these sites are located in the region between the N-terminal conserved region and the C-terminal seven WD repeats. The raptor C terminus tightly interacts with mTOR (8, 26), and the N-terminal conserved region is suggested to bind to the TOS motif in substrates such as S6K1 and 4E-BP1 (27). Mutation of raptor at Ser^{863} did not change raptor binding to 4E-BP1 as measured by interaction with 4E-BP1 recombinant protein-coupled beads. Similarly, RSK-mediated phosphorylation does not change raptor interaction with 4E-BP1 (21). Therefore, although 4E-BP1 binding to raptor does increase after insulin-stimulated dissociation of PRAS40 (9, 10), this does not involve raptor phosphorylation. Rather, the phosphorylation of raptor may promote the access of substrates bound to raptor to the mTOR kinase active site, probably due to a conformational change in mTORC1. As shown in Fig. 3*A*, mutation of Ser^{863} did not change raptor interaction with mTOR. However, because mTOR and raptor interactions are mediated by multiple sites, raptor phosphorylation might cause different directional interaction changes and thus leads to an undetectable association change *in vitro*. Finally, it cannot be excluded that the effects of raptor phosphorylation involve additional proteins or a phosphatase that modify mTORC1 activity. Because it contains several activity-modifying phosphorylation sites, the region between the N and C terminus in raptor is functionally important and worth further investigating.

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