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 elf4E-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/G8L (1), whereas the proline-rich Akt substrate of 40 kDa (PRAS40) is a regulatory component of mTORC1 that dissociates 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 GTases 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 vitro and in vivo. 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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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 Ser2481 (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 and immunoprecipitations were prepared in Triton X-200 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 Triton 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. 1A). 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. 1B). 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. 1B). 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. 1C). 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 Ser859 and Ser863 and Is Sensitive to Rapamycin—The phosphorylation of raptor in cells was studied by expressing raptor in HEK293T cells labeled with [32P]orthophosphate. 32P-labeled recombinant raptor was immunoprecipitated by FLAG tag antibody and resolved by SDS-PAGE. A 32P-labeled raptor band with increased 32P suitable for mapping was obtained by overexpression of raptor (Fig. 2A). 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. 2B). 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. 2B, 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 bioinformatics 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 Ser859, Ser863, and Ser884 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. 2C, Ser863 and Ser859 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 Ser863 to Ala (S863A) caused the disappearance of both spot b and spot c, whereas mutation of Ser859 to Ala (S859A) only eliminated spot c (Fig. 2C). Because Ser859 and Ser863 reside in the same tryptic peptide of 850VLDTSSLTQSAPASPTN868, 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 Ser863 and Ser859, 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 Ser863. This suggests that Ser859 is phosphorylated only when Ser863 is phosphorylated first. The phosphopeptide mapping of mutation at Ser863 (S884A) remained unaltered when compared with wild type (Fig. 2C). Furthermore, we mutated Ser863 to Glu and Thr. As predicted, mutation of Ser863 to Glu eliminated phosphorylation at this site similar to the Ala mutant (supplemental Fig. S1A). Because in vitro mTORC1-mediated raptor phosphopeptides co-migrated with in vivo phosphopeptides containing Ser863 and Ser859 (spot 1→b and spot 2→c, Fig. 2B) and mutations of Ser863 and Ser859 to Ala eliminated spot 1 and 2 (data not shown), mTOR phosphorylates raptor at Ser863 and Ser859 in vitro as well. We next investigated whether the phosphorylation of Ser863 and Ser859 were regulated by growth factor...
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 with [32P]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 Ser863 and Ser859, 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 Ser863 and Ser859 (Fig. 2D). Taking the phosphorylation of Ser863 and Ser859 by mTOR directly in vitro and their inhibition by rapamycin in vivo together, our data demonstrate that the phosphorylation of Ser863 and Ser859 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. S1B). 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 Thr36, Thr45, Thr69, and Ser64 in 4E-BP1, Ser183 in PRAS40, Ser490 in Akt1, and Ser637 in protein kinase C-α and sites with surrounding hydrophobic residues such as Thr389 in S6K1, Ser473 in Akt1, and Ser656 in protein kinase C-α. Ser863 and Ser859 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 Ser863 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 Ser863, but not Ser884, substantially reduced the phosphorylation of 4E-BP1 catalyzed by mTORC1 in vitro (Fig. 3A).

The mutation of Ser859 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. S24, 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 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 HEK293 cells, treated with rapamycin to block endogenous mTORC1 kinase activity, and then stimulated with insulin. Consistent with the mTORC1 activity in vitro, mutation of Ser863 to Ala decreased insulin-stimulated mTORC1 activity as shown by decreased phosphorylation of S6K at Thr389 (Fig. 3B), whereas mutation of Ser859 and Ser884 did not alter the phosphorylation of S6K at Thr389. Interestingly, when compared with the Ala mutant, mutation of Ser863 to Glu appears to mimic phosphorylation, as seen in the recovery of mTORC1 activity toward S6K at Thr389 after insulin treatment (supplemental Fig. S2B). However, we did not observe that the Ser-Glu mutant lead to increased basal mTORC1 activity (supplemental Fig. S2B). 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

**FIGURE 3. Phosphorylation of raptor at Ser863 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-32P]ATP and recombinant 4E-BP1 as substrate and visualized with 32P incorporation into 4E-BP1 and phospho-specific antibodies targeting Thr389/Thr45 of 4E-BP1. The phosphorylation of 4E-BP1 expressed as 32P 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 HEK293 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 (100 nM) for 30 min. Phospho-Thr389 of S6K1 and expression levels of HA-S6K, FLAG-mTOR, and FLAG-raptor were detected by immunoblotting. Phospho-Thr389 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. After 36 h, cells were treated 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 6 h (right panel). Phospho-Thr389 of S6K1 and total expression levels of HA-S6K, FLAG-mTOR, FLAG-raptor, and FLAG-Rheb were detected by immunoblotting. Phospho-Thr389 of S6K1 normalized by total level of HA-S6K was quantified from three experiments (mean ± S.E. (error bars)).
background of endogenous mTORC1 activity was knocked down with rapamycin pretreatment. Overexpression of Rheb enhances the phosphorylation of S6K1 at Thr389, as expected. However, expression of raptor containing the Ala mutation at Ser863, but not Ser859 and Ser864, reduced the phosphorylation of S6K1 at Thr389 activated by Rheb expression (Fig. 3C, 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 Ser863 to Ala mutant suppressed the Rheb-mediated rescue of S6K1 phosphorylation (Fig. 3C, right panel).

Therefore, the phosphorylation of raptor at Ser863 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 rapamycin interaction with mTOR, thus excluding 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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