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Microtubule-associated Protein/Microtubule Affinity-regulating Kinase 4 (MARK4) Is a Negative Regulator of the Mammalian Target of Rapamycin Complex 1 (mTORC1)*

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Background: mTORC1 is regulated by various energy, nutrition, and stress conditions.
Results: In this study, we reported that MARK4 negatively regulates mTORC1 activity.
Conclusion: Our data demonstrate MARK4 as a new negative regulator of mTORC1.
Significance: We propose that Raptor 722/792 phosphorylation might serve as an integration point to receive different upstream signals to modulate mTORC1 activity.

The mammalian target of rapamycin (mTOR) is a central cell growth regulator. It resides in two protein complexes, which in mammals are referred to as mTORC1 and mTORC2. mTORC1, which is directly inhibited by rapamycin, promotes cell growth by stimulating protein synthesis and inhibiting autophagy. A wide range of extra and intracellular signals, including growth factors, nutrients, energy levels, and various stress conditions, regulates mTORC1. Dysregulation of mTORC1 contributes to many human diseases, including cancer, cardiovascular disease, autoimmunity, and metabolic disorder. In this study, we identified MARK4, an AMP-activated kinase-related kinase, as a negative regulator of mTORC1. In *Drosophila* S2 cells and mammalian cells, knockdown of MARK family member increased mTORC1 activity, whereas overexpression of MARK4 in mammalian cells significantly inhibited mTORC1 activity. Interestingly, MARK4 selectively inhibits mTORC1 activation by Rag GTPases, which are involved in amino acid signaling, but does not inhibit the effect of Rheb, which directly binds to and activates mTORC1. In addition, we found that MARK4 phosphorylates Raptor, a key component of mTORC1, and this phosphorylation may interfere with Raptor-Rag interaction. Our data demonstrate MARK4 as a new negative regulator of mTORC1.

The target of rapamycin is an evolutionary conserved protein kinase that regulates a wide span of cellular processes (1). It forms two structurally and functionally distinct complexes. mTORC1 consists of mTOR,[2] Raptor, mLST8, PRAS40, and Deptor and can phosphorylate S6K, 4EBP1, and ULK1 (2, 3). mTORC2 consists of mTOR, Rictor, mLST8, sin1, and Deptor. Known substrates of mTORC2 include Akt, serum/glucocorticoid regulated kinase (SGK), and PKC (4).

mTORC1 integrates various signals including nutrient, growth factors, stress, and energy to regulate cell growth and metabolism (1). A large number of efforts have been put into exploring mTORC1 regulation by growth factors and cellular energy levels, going through the key upstream regulator TSC1 and TSC2 tumor suppressors. Growth factors activate PI3K-AKT pathway (1). The activated Akt phosphorylates TSC2 and inhibits the GTPase activating protein activity of TSC1/2 toward Rheb (5, 6). Additionally, AKT phosphorylates PRAS40, an mTORC1 component. The phosphorylated PRAS40 dissociates from mTORC1 and relieves its inhibition on mTORC1 (7, 8). The energy level regulates mTORC1 through AMPK (1). AMPK senses low energy status and phosphorylates TSC2 and activates TSC complex GAP activity (9). AMPK also phosphorylates Raptor, posing another regulation node on mTORC1 (10). Recently, studies have identified Rag GTPases as important mediators that relay amino acid signals to mTORC1 activation (11, 12). It was shown that through interaction with Raptor, Rag GTPases affect the lysosomal localization and hence the activation of mTORC1 (12).

Although significant emphasis has been placed on how mTORC1 activity is set, there are still a lot of unanswered questions. Considerable gaps remain on how different signals regulate mTORC1. For example, in addition to Rag GTPases, studies also show that VPS34, MAP4K3, and RalA have important roles in mTORC1 activation in response to amino acids, but the exact mechanism remains unknown (13–16). Rab and Arf GTPases have also been shown to regulate mTORC1, possibly through regulating the trafficking of the pathway components (17, 18). In addition, many signals are known to affect mTORC1 activity, although the exact mechanisms remain unknown. For example, it has been known for decades that osmotic stress induced by sorbitol treatment decreases S6K phosphorylation. However, the underlying biochemical mechanism is still largely unknown. Here, we report that MARK4 is a negative regulator of mTORC1. Our data suggest a possible role of MARK4 in sorbitol-induced mTORC1 inhibition. Mechanistically, MARK4 increases Raptor Ser-792 phosphorylation, an inhibitory site, to inhibit mTORC1.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Chemicals—Anti-*Drosophila* S6 kinase antibody was provided by Mary Stewart (North Dakota State University, Fargo, ND). Anti-phospho-*Drosophila* S6K, anti-S6K, anti-phospho-S6K, anti-Akt, anti-phospho-Akt, and

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[2] The abbreviations used are: mTOR, mammalian target of rapamycin; mTORC, mammalian target of rapamycin complex; MARK4, microtubule affinity-regulating kinase 4; AMPK, AMP-activated kinase; S6K, S6 protein kinase; dS6K, *Drosophila* S6K; MEF, mouse embryonic fibroblast; TSC, tuberous sclerosis complex.

anti-phospho-4EBP1 antibodies were from Cell Signaling. Anti-Myc, Anti-HA and anti-FLAG antibodies were from Santa Cruz Biotechnology, Covance, and Sigma, respectively. RagA/C constructs were made as described previously (11). HA-Raptor S722A/792A was made using the Stratagene mutagenesis kit. All other DNA constructs, including HA-MARK4, HA-S6K, Myc-4EBP1, GST-Akt, and Myc-Rheb, were from laboratory stock. Insulin and brefeldin A were obtained from Sigma. siRNA targeting human MARK4 was obtained from Dharmacon. Sorbitol was obtained from sigma. Phos-tag-conjugated acrylamide was purchased from Wako Chemicals.

Cell Culture—Drosophila S2 cells (Invitrogen) were cultured in *Drosophila* serum-free medium (Invitrogen) supplemented with 18 mM L-glutamine and maintained at 28 °C. MEF, HEK293, and HeLa cells were cultured in DMEM supplemented with 10% FBS. Amino acid-containing (SDMK) or -free (SDMK-AA) medium used for *Drosophila* S2 cells was made using Schneider's *Drosophila* medium (Invitrogen) formulation as described previously (11). Amino acid-containing (DMEMK) or amino acid-free (DMEMK-AA) medium used for HEK293 and HeLa cells was made using DMEM medium (Invitrogen, catalog number 12430) formulation.

RNA Interference—Drosophila RNA interference (RNAi) experiments were performed as described previously (19).

Transfection and Cell Lysis—Transfection was performed in serum-free conditions using Lipofectamine reagent (Invitrogen) as described by the manufacturer. Cells were lysed in SDS lysis buffer (1% SDS, 0.1 M Tris, pH 7.5).

Immunoprecipitation—HEK293 cells transfected with the indicated plasmids were lysed in ice-cold lysis buffer (40 mM HEPES (pH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, and 0.3% CHAPS and one tablet of EDTA-free protease inhibitors (Roche Applied Science) per 10.5 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,200 rpm for 10 min by centrifugation in a microcentrifuge. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 h at 4 °C. 10 μl of 50% slurry of protein G-Sepharose was then added, and the incubation continued for an additional 1 h. Immunoprecipitates were washed four times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 min, resolved by SDS-PAGE, and analyzed by immunoblotting as described.

Kinase Assay—For the MARK4 kinase assays, HEK293 cells were transfected with HA-MARK4 or HA-AMPKα, Myc-AMPKβ, and Myc-AMPKγ. 48 h after transfection, cells were lysed with lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, protease inhibitor mixture (Roche Applied Science), 1 mM DTT, 1 mM PMSF) and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were washed three times with lysis buffer, once with wash buffer (40 mM HEPES, 200 mM NaCl), and once with kinase assay buffer (30 mM HEPES, 50 mM potassium acetate, 5 mM $MgCl_2$). The immunoprecipitated proteins were subjected to a kinase assay in the presence of 500 μM cold ATP, 10 μCi of [γ-^{32}P]ATP, and 1 μg of GST-Raptor fragment expressed and purified from *Escherichia coli* as substrate. The reaction mixtures were incubated at 30 °C for 30 min, terminated with SDS sample buffer, and subjected to SDS-PAGE and autoradiography. The same procedure was used for mTOR kinase assay except that different kinases and substrates were used.

RESULTS AND DISCUSSION

Knockdown of Par-1 Increases TORC1 Activity in Drosophila S2 Cells—Kinases have profound effects in almost every cellular process. They are particularly important in signal transduction because they are highly regulated and because of their ability to amplify signal cascade. In TOR signaling pathway, several kinases are involved in regulating pathway activity including AMPK and AKT. We hypothesized that there might be more protein kinases involved in TORC1 regulation. Based on this assumption, we performed an RNAi screen of 251 annotated *Drosophila* kinases using *Drosophila* S2 cells. We searched for kinases whose silencing might affect the phosphorylation of dS6K in S2 cells and identified Par-1 as a potential negative regulator on dS6K phosphorylation. As shown in Fig. 1*A*, in an amino acid deprivation condition, which has low basal S6K phosphorylation, knockdown of Par-1 significantly increased dS6K phosphorylation to the same extent as PTEN (phosphatase and tensin homolog), which is a known upstream negative regulator of TORC1.

Par-1 was first identified in *Caenorhabditis elegans* as one of the six Par genes essential for the asymmetric division of zygotes (20). In *Drosophila*, Par-1 is reported to have a similar function in regulating cell polarity as in *C. elegans* (21). The Par-1 mutant showed defective anterior-posterior axes of the oocyte and embryo and problems with oocyte fate determination and maintenance. In addition, PAR-1 regulates the microtubule cytoskeleton in *Drosophila* and is also shown to be implicated in both the canonical and the noncanonical Wnt signaling pathways (22). There is no previous implication of Par-1 function in TORC1 regulation, but a Par-1 family member, AMPK, is a well known upstream negative regulator of TORC1.

Mammalian Par-1 Homolog MARK4 Regulates mTORC1—We next examined the function of Par-1 homologs in mammalian cells. Humans have four Par-1 homologs, MARK1, MARK2, MARK3, and MARK4. Each showed ~30–40% sequence identity with *Drosophila* Par-1. Knockdown of MARK1, MARK2, and MARK3 does not have a dramatic effect on S6K phosphorylation, whereas knockdown of MARK4 significantly increased S6K phosphorylation in normal culture condition as shown by both phospho-antibody and gel mobility shift using Phos-tag-containing SDS-PAGE gels (Fig. 1*B*), indicating that the effect of Par-1 on TORC1 is conserved from *Drosophila* to human. Human MARK proteins are known to phosphorylate microtubule-associated kinase Tau, MAP2, and MAP4. The phosphorylated proteins disassociate from microtubule, and the activities of these microtubule-associated proteins are important for microtubule dynamics.

Because knockdown of MARK4 showed the most dramatic effect on S6K phosphorylation, we chose MARK4 to further explore its function on mTOR pathway. MARK4 is shown to be localized to the microtubule network and centrosomes. Expression of wild type MARK4 and phospho-mimic mutant MARK4

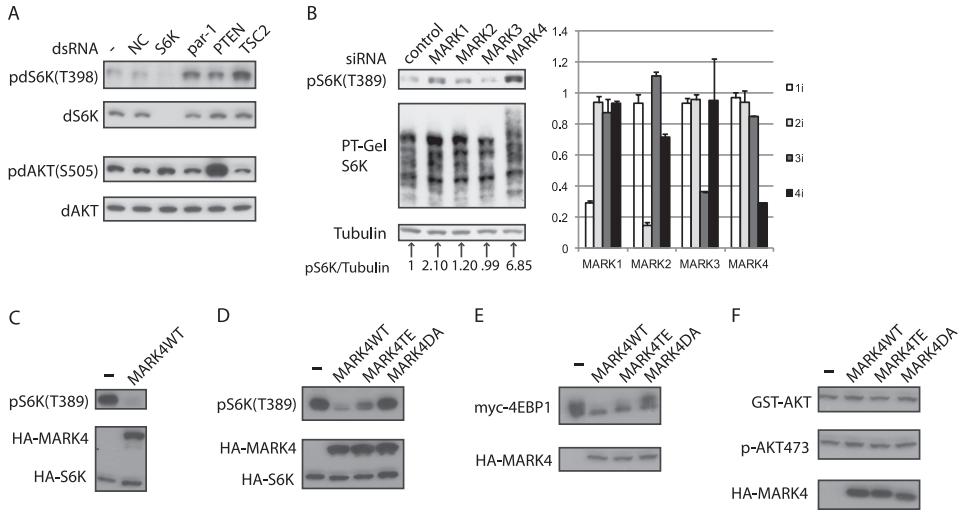

FIGURE 1. **MARK and its homolog regulate TORC1 activity in *Drosophila* and mammalian cells.** *A*, Par-1 negatively regulates TORC1 activity in *Drosophila* S2 cells. D*rosophila* S2 cells untreated (*NC*, *lane 1*) or treated with the double-stranded RNA against individual genes (as indicated above) were starved of amino acids for 1 h before lysis. Phosphorylation (*p*) and protein levels of dS6K were determined by immunoblotting with the indicated antibodies. *PTEN*, phosphatase and tensin homolog. *B*, knockdown of MARK4 proteins increases S6K phosphorylation. Each MARK siRNA pool was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. Phos-tag-containing SDS-PAGE gels (*PT-Gel*) were used as indicated. Knockdown efficiency of individual MARK siRNA was determined by quantitative RT-PCR (*right panel*). *Error bars* indicate S.D. *C*, MARK4 inhibits S6K phosphorylation. MARK4 was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. *D–F*, MARK4WT, not the MARK4 kinase dead mutant, regulates TORC1 but not TORC2 activity. Each indicated MARK protein construct was co-transfected with HA-S6K, Myc-4EBP1, or GST-Akt. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

T214E significantly decreased S6K phosphorylation, whereas the kinase dead mutant MARK4D199A showed no inhibitory effect on S6K phosphorylation (Fig. 1, *C* and *D*), indicating that the effect of MARK4 on S6K phosphorylation is dependent on its kinase activity. To further confirm the role of MARK4 on mTORC1 regulation, we determined the phosphorylation status of another well characterized mTORC1 substrate, 4EBP1. As shown in Fig. 1*E*, wild type MARK4 significantly decreased 4EBP1 phosphorylation, whereas the kinase dead mutant showed no effect. On the other hand, MARK4 had little effect on Akt Ser-473 phosphorylation, a TORC2 substrate (Fig. 1*F*). These results support the notion that MARK4 specifically inhibits mTORC1 but not mTORC2.

MARK4 Specifically Inhibits Rag-induced but Not Rheb-induced mTORC1 Activation—To investigate the role of MARK4 in mTORC1 regulation, we tested whether MARK4 overexpression specifically inhibits a subgroup of activation signal. We observed that MARK4 blocks amino acid-, glucose-, and insulin-induced mTORC1 activation (Fig. 2, *A–C*). In addition, co-expression of MARK4 strongly inhibited the active RagA/C heterodimer (RagAQL+RagCSN)-induced mTORC1 activation, suggesting that the position of MARK4 in the pathway either is parallel to these different signals or has a direct effect on mTORC1 complex activity. On the other hand, MARK4 had no effect on S6K phosphorylation in the presence of active RhebS16H mutant, consistent with the function of Rheb as direct activator of mTOR (Fig. 2*D*).

MARK4 Phosphorylates Raptor on Ser-722/792—Similar to other MARK family members, MARK4 proteins are known to phosphorylate the microtubule-associated proteins Tau, MAP2, and MAP4. Thus, we tested whether MARK4 affects mTORC1 through its regulation of microtubule function. Nocodazole, a drug that has a similar function as MARK4 in destabilizing microtubule, also showed an inhibitory effect on S6K phosphorylation, which is consistent with the MARK4 effect on mTORC1 via microtubule (data not shown). However, when the cells were treated with Taxol, a drug that stabilizes microtubules, which should show an opposite effect to MARK4, we still observed an inhibitory effect of Taxol on S6K phosphorylation (data not shown). These results suggest that disruption of microtubule dynamics, by either stabilizing or destabilizing the microtubule, inhibits TORC1 activity, which is consistent with the recent publications demonstrating that TORC1 activity is largely affected by the trafficking pathway (17, 18, 23). Moreover, it is possible that the inhibitory effect of MARK4 on mTORC1 may be mediated through other mechanisms but not directly due to disruption of microtubule.

Until now, little was known about the functional redundancy and specificity of different MARK proteins. However, there is evidence suggesting that different MARK isoforms may have specific functions in various signaling pathways in addition to their fundamental role in cell polarity and microtubule stabilization. For example, MARK3 has been shown to regulate cell cycle through phosphorylating Cdc25 phosphatase and Pim-1. It has also been shown to phosphorylate kinase suppressor of Raf-1 (KSR1) to regulate the Ras-MAPK pathway (24). Less is known about MARK4.

Recently, AMPK was shown to directly phosphorylate Raptor on two well conserved serine residues in response to energy stress (10). The phosphorylation of Raptor induces 14-3-3 binding to Raptor and inhibits mTORC1 kinase activity (10). In AMPKα1/α2 double knock-out MEFs, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide- or 2-deoxy-D-glucose-induced Raptor phosphorylation is compromised, but sorbitol-induced Raptor phosphorylation persists, suggesting that

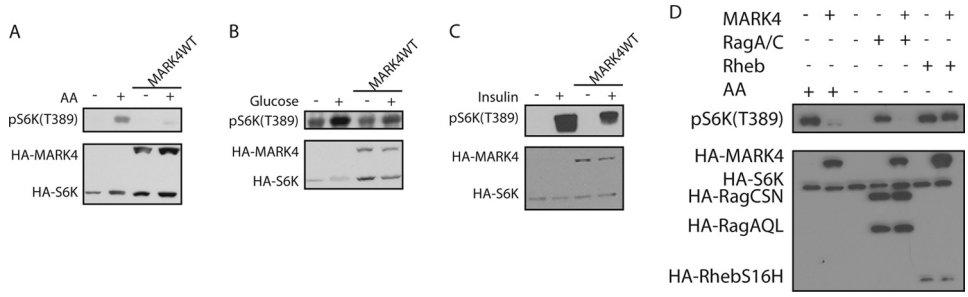

FIGURE 2. **MARK4 blocks Rag-stimulated but not Rheb-induced mTORC1 activation.** *A*, MARK4 inhibits mTORC1 activity induced by amino acids (*AA*). MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were starved for amino acids for 1 h followed by amino acid stimulation for 30 min before harvesting. Phosphorylation (*p*) and protein levels were determined by immunoblotting with the indicated antibodies. *B*, MARK4 inhibits mTORC1 activity induced by glucose. MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived of glucose for 2 h followed by glucose stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. *C*, MARK4 inhibits mTORC1 activity induced by insulin. MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived of serum for 5 h followed by insulin stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. *D*, MARK4 blocks Rag- but not Rheb-induced S6K phosphorylation. MARK4 was transfected into HEK293 cells with or without RagA/C or Rheb construct as indicated. S6K was included in the co-transfection. Phosphorylation and protein levels of the transfected proteins were determined by immunoblotting with the indicated antibodies.

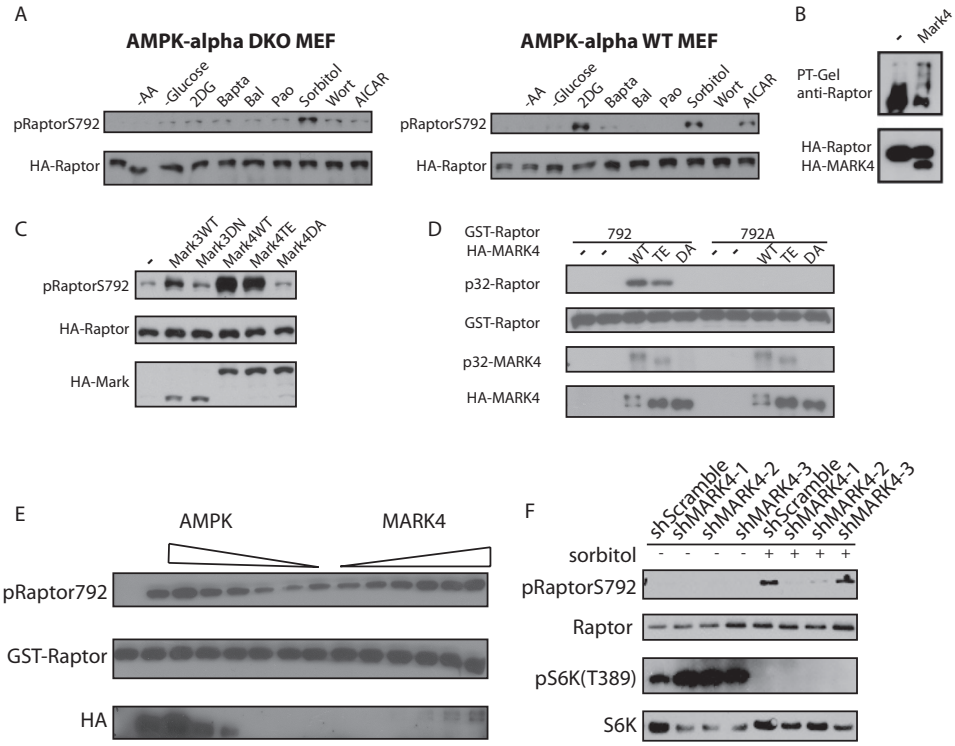

FIGURE 3. **MARK4 phosphorylates Raptor *in vivo* and *in vitro*.** *A*, sorbitol induces Raptor 792 phosphorylation in AMPK double knock-out MEFs. AMPK WT and double knock-out MEFs were treated with the indicated drugs. Phosphorylation (*p*) and protein levels were determined by immunoblotting with the indicated antibodies. *AA*, amino acids; *2DG*, 2-deoxy-D-glucose; *Bapta*, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; *Wort*, wortmannin; *AICAR*, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; *Bal,* 2, 3-dimercapto-1-propanol; *Pao,* polyamine oxidase. *B*, MARK4 phosphorylates Raptor. MARK4 construct was co-transfected with HA-Raptor into HEK293 cells. Phosphorylation was determined through mobility shift, and protein levels were determined by immunoblotting with the indicated antibodies. Phos-tag-containing SDS-PAGE gels (*PT-Gel*) were used as indicated. *C*, MARK4 phosphorylates Raptor on Ser-792. A different MARK construct was co-transfected with HA-Raptor into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. *D*, MARK4 phosphorylates Raptor on Ser-792 *in vitro*. HA-MARK4 was immunoprecipitated from transfected HEK293 cells. An *in vitro* kinase assay was performed using purified GST-Raptor fragment containing Ser-792 as a substrate in the presence of [^{32}P]ATP. GST-Raptor 792A mutant was used as a negative control. *WT*, wild type; *TE*, T214E; *DA*, D199A. *E*, MARK4 phosphorylates Raptor as potently as AMPK. HA-MARK4 or HA-AMPK was immunoprecipitated from transfected HEK293 cells. An *in vitro* kinase assay was performed using purified GST-Raptor fragment containing Ser-792 as a substrate in the presence of cold ATP. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. *F*, MARK4 knockdown decreased sorbitol-induced Raptor phosphorylation. AMPKα1/α2 double knock-out MEF cells were treated with control or three different MARK4 shRNA and then treated with sorbitol as indicated. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

another kinase can phosphorylate Raptor on Ser-792 under osmotic stress conditions (Fig. 3*A*).

Given the fact that MARK4 is a member of the AMPK subfamily, we tested whether MARK4 may phosphorylate Raptor. Overexpression of MARK4 strongly induced Raptor phosphorylation as indicated by both gel mobility shift assay using Phos-tag-containing SDS-PAGE gels and phospho-antibody detection of Ser-792 specific antibody (Fig. 3, *B* and *C*). MARK3 overexpression induced a much weaker Raptor Ser-792 phosphorylation, whereas the kinase inactive mutant of MARK4 did

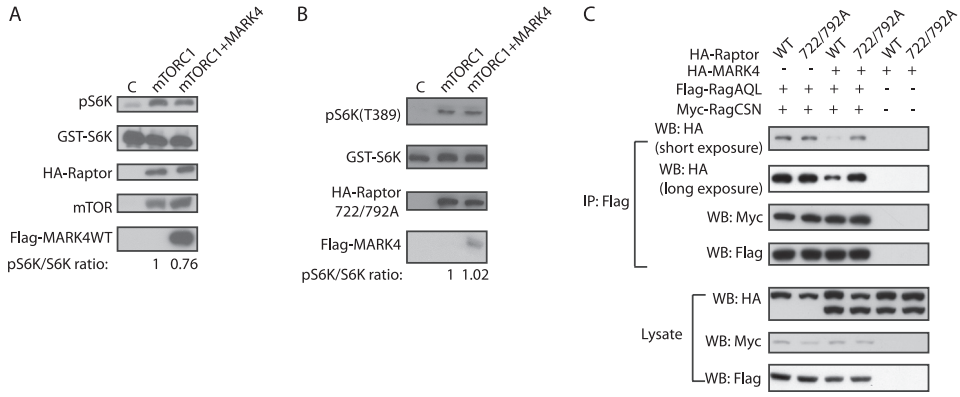

FIGURE 4. **Regulation of TORC1 by MARK4.** *A*, MARK4 overexpression slightly decreases mTORC1 kinase activity. Raptor was immunoprecipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate. IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr-389 S6K1 antibody as well as for level of immunoprecipitated Raptor, mTOR. *B*, MARK4 overexpression does not affect the mTORC1 kinase activity containing Raptor 722/792 mutant. Raptor 722A/792A was immunoprecipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate. IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr-389 S6K1 antibody as well as for level of immunoprecipitated Raptor, mTOR. *C*, MARK4 decreases Raptor-Rag interaction. RagA/C and Raptor were co-transfected into HEK293 cells with or without MARK4. The interaction was determined by co-immunoprecipitation with short exposure and long exposure.

not increase Raptor phosphorylation. An *in vitro* kinase assay using a fragment of Raptor containing Ser-792 residue and flanking region (which did not contain Ser-922) as a substrate showed that MARK4 could phosphorylate the Raptor fragment as potently as AMPK (Fig. 3, *D* and *E*). Moreover, MARK4 specifically phosphorylated Ser-792 in Raptor as the S792A mutant was not phosphorylated by MARK4 *in vitro* (Fig. 3*D*). MARK4 knockdown slightly decreased the basal Raptor phosphorylation level (data not shown). AMPK is known to contribute to Raptor phosphorylation. We performed MARK4 knockdown in AMPK$^{-/-}$ MEF and found that MARK4 knockdown strongly blocked Raptor phosphorylation in response to sorbitol (Fig. 3*F*). All of these data indicate that MARK4 can phosphorylate Raptor on Ser-792 *in vivo* and *in vitro*.

Raptor 722/792 Phosphorylation Affects mTORC1 Activity—Phosphorylation of Raptor on Ser-722/792 inhibits mTORC1 kinase activity (10). We tested whether the inhibitory effect of MARK4 on mTORC1 is mediated through Raptor phosphorylation. We immunoprecipitated mTORC1 from cells expressing vector control or MARK4. An *in vitro* kinase assay showed that Mark4 overexpression only slightly decreased mTORC1 activity in an *in vitro* kinase assay (Fig. 4*A*), which is significantly less dramatic than the effect of MARK4 overexpression on S6K phosphorylation *in vivo*. A similar *in vitro* kinase assay using Raptor 722A/792A mutant showed no decrease of mTORC1 kinase activity under MARK4 overexpression condition (Fig. 4*B*). These data suggest that MARK4 might have other mechanisms to regulate mTORC1 besides directly affecting its kinase activity.

It has been reported that active RagA/C heterodimer (RagAQL+RagCSN) strongly interacts with Raptor (12). In response to amino acids, Raptor/mTOR moves to the lysosome compartment where Rheb is localized and thus is activated by Rheb (12, 23). This lysosomal localization requires the Rag GTPases. We decided to test whether Raptor 722/792 phosphorylation affects its binding with RagA/C. We found that the interaction between RagA/C and wild type Raptor was decreased upon MARK4 overexpression, whereas the interaction between RagA/C and the Raptor S722A/792A mutant was not affected by MARK4 overexpression (Fig. 4*C*). These data suggest that MARK4 could inhibit mTORC1 activation by disrupting the interaction between Raptor and Rag.

Raptor Ser-722/792 are known to be phosphorylated by AMPK. It appears that Ser-722/792 of Raptor can be phosphorylated by different kinases to inhibit mTORC1 activation. When MARK4 is knocked down, sorbitol-induced Raptor 792 phosphorylation is greatly compromised, yet the effect of sorbitol on mTORC1 activity, as indicated by S6K phosphorylation, still remains, suggesting that the effect of sorbitol on mTORC1 does not only come from MARK4. In addition, in AMPK-deficient MEFs, MARK4 knockdown showed no effect on sorbitol-induced mTORC1 inhibition, yet it decreased sorbitol-induced Raptor phosphorylation (Fig. 3*F*). We propose that Raptor 722/792 phosphorylation might serve as an integration point to receive different upstream signals to modulate mTORC1 activity. Our study has identified MARK4 as a novel regulator of mTORC1. We suggest that MARK4 may mediate inhibitory signals to suppress mTORC. MARK4 phosphorylates Raptor and disrupts the interaction with Rag as a possible mechanism in mTORC1 inhibition. This conclusion is also consistent with the observation that MARK4 inhibits Rag-induced but not Rheb-induced mTORC1 activation because Rheb can directly bind to and activate mTORC1.

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