The mammalian target of rapamycin (mTOR) plays a pivotal role in the regulation of cell growth in response to a variety of signals such as nutrients and growth factors. mTOR forms two distinct complexes in vivo. mTORC1 (mTOR complex 1) is rapamycin-sensitive and regulates the rate of protein synthesis in part by phosphorylating two well established effectors, S6K1 (p70 ribosomal S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1). mTORC2 is rapamycin-insensitive and likely regulates actin organization and activates Akt/protein kinase B. Here, we show that mTOR forms a multimer via its N-terminal HEAT repeat region in mammalian cells. mTOR multimerization is promoted by amino acid sufficiency, although the state of multimerization does not directly correlate with the phosphorylation state of S6K1. mTOR multimerization was insensitive to rapamycin treatment but hindered by butanol treatment, which inhibits phosphatidic acid production by phospholipase D. We also found that mTOR forms a multimer in both mTORC1 and mTORC2. In addition, Saccharomyces cerevisiae TOR proteins Tor1p and Tor2p also exist as homo-multimers. These results suggest that mTOR multimerization is a conserved mechanism for TOR functioning.

The mammalian target of rapamycin (TOR) is a highly conserved Ser/Thr protein kinase that regulates cell growth from yeast to mammals (1, 2). This protein was originally identified by the analysis of mutants that show resistance to rapamycin in Saccharomyces cerevisiae (3). Although two highly homologous genes (TOR1 and TOR2) exist in yeast, only one TOR gene is known in higher eukaryotes (1, 2). Mammalian TOR (mTOR) is a large protein with a molecular mass of 289 kDa and belongs to the phosphatidylinositol-3-phosphate kinase-related kinase (PIKK) family, which includes ATM, ATR, and DNA-dependent protein kinase (1). PIKK family proteins have characteristic domains: at their N-terminal halves, tandem HEAT (Huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, and TOR1) repeats (4); and at their C-terminal regions, FAT, kinase catalytic, and FATC domains (5). In the case of mTOR, there are ~20 tandem HEAT repeats, which are believed to mediate protein-protein interaction. Indeed, several proteins that interact with this region have been identified (6–9). Between FAT and kinase catalytic domains, mTOR has the FRB domain, to which rapamycin, a specific inhibitor of mTOR, binds in complex with FKBP12 (FK506-binding protein 12) (1).

To date, both in yeast and mammalian cells, TOR is known to form two distinct complexes (TORC1 and TORC2), each of which has different roles (10–13). In mammalian cells, mTORC1, which consists of mTOR, mLST8 (also named GBL), and raptor, is sensitive to rapamycin and regulates the phosphorylation of two well established effectors, S6K1 (p70 ribosomal S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1), leading to the promotion of protein synthesis (7, 14, 15). In the other complex known as mTORC2, mTOR is associated with mLST8 and rictor (also named mAVO3). This complex is insensitive to rapamycin and appears to regulate actin organization (12, 13). In addition, it was reported recently that mTORC2 is responsible for the phosphorylation of Akt/protein kinase B at Ser473 (16). Although rapamycin has been used as a specific inhibitor of mTOR activity, it has been revealed that rapamycin binds to mTOR in mTORC1 but not in mTORC2 (12, 13), suggesting that mTOR may contribute to more cellular functions than previously thought.

The activity of mTORC1 is regulated by a variety of signals such as amino acid availability, growth factors, and energy status. Recently, it was revealed that tuberous sclerosis tumor suppressor proteins TSC1 and TSC2 act as upstream negative regulators of mTORC1 in signaling from growth factors and energy status (1, 2). TSC2 exerts its negative regulation on mTOR through activity as a GTPase-activating protein on the
mTOR Multimerization via the HEAT Repeat Region

small GTP-binding protein Rheb (17−19), which directly interacts with and positively regulates mTOR (20). Insulin stimulation leads to Akt/protein kinase B activation, which is thought to inactivate the GTPase-activating protein activity of TSC2 by undefined mechanisms. Under energy starvation conditions, AMP-activated protein kinase is activated and then enhances TSC2 activity (21). On the other hand, the mechanism by which amino acid availability leads to mTOR activation is less understood. Although the regulation of mTOR by amino acid availability is unlikely to require TSC2 (22), Rheb overexpression could prevent S6K1 dephosphorylation caused by amino acid withdrawal (17−19), suggesting that amino acid availability may lead to the modulation of Rheb independently of the TSC1/2 complex. Recent studies also indicated the involvement of human VPS34 in amino acid-mediated signaling to mTOR independently of the TSC-Rheb pathway (23, 24). In contrast to mTORC1, no signal leading to mTORC2 activation has been identified.

Multimerization is a well documented general mechanism for the activation of protein kinases. ATM, a member of the PIKK family, forms a dimer, and dimerization is important for its regulation (25). In this study, we show that mTOR exists as a multimer via its N-terminal HEAT repeat region in mammalian cells and that this multimerization is promoted by amino acid sufficiency but hindered by butanol treatment, which inhibits cells and that this multimerization is promoted by amino acid availability leads to mTOR activation is less understood. Although the regulation of mTOR by amino acid availability is unlikely to require TSC2 (22), Rheb overexpression could prevent S6K1 dephosphorylation caused by amino acid withdrawal (17−19), suggesting that amino acid availability may lead to the modulation of Rheb independently of the TSC1/2 complex. Recent studies also indicated the involvement of human VPS34 in amino acid-mediated signaling to mTOR independently of the TSC-Rheb pathway (23, 24). In contrast to mTORC1, no signal leading to mTORC2 activation has been identified.

Multimerization is a well documented general mechanism for the activation of protein kinases. ATM, a member of the PIKK family, forms a dimer, and dimerization is important for its regulation (25). In this study, we show that mTOR exists as a multimer via its N-terminal HEAT repeat region in mammalian cells and that this multimerization is promoted by amino acid sufficiency but hindered by butanol treatment, which inhibits cells and that this multimerization is promoted by amino acid availability leads to mTOR activation is less understood. Although the regulation of mTOR by amino acid availability is unlikely to require TSC2 (22), Rheb overexpression could prevent S6K1 dephosphorylation caused by amino acid withdrawal (17−19), suggesting that amino acid availability may lead to the modulation of Rheb independently of the TSC1/2 complex. Recent studies also indicated the involvement of human VPS34 in amino acid-mediated signaling to mTOR independently of the TSC-Rheb pathway (23, 24). In contrast to mTORC1, no signal leading to mTORC2 activation has been identified.

EXPERIMENTAL PROCEDURES

Materials—Anti-Myc (9E10) and anti-hemagglutinin (HA; 12CA5) ascites fluids were kind gifts from Dr. Yukata Hoshikawa. Anti-FLAG antibody M2, anti-FLAG M2 beads, 3X FLAG® peptide, and anti-Myc beads were purchased from Sigma. Anti-Xpress antibody was from Invitrogen. Anti-mTOR and anti-phospho-S6K1 Thr^389 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA), and anti-S6K1 antibody was Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for raptor (BL888) and rictor (BL2181) were from Bethyl Laboratories, Inc. (Montgomery, TX). Insulin, coumermycin, and rapamycin were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Construction of Plasmids—To create expression plasmids for Myc-mTOR (pcDNA3.1-Myc-mTOR) and FLAG-mTOR (pcDNA3.1-FLAG-mTOR), pcDNA1-HA-mTOR (27) was digested with AccIII and XbaI, and the resultant fragment containing a full-length mTOR open reading frame was inserted into the BamHI and XbaI sites of pcDNA3.1/N-Myc and pcDNA3.1/N-FLAG,3 respectively. Expression plasmids for mTOR deletion mutants were produced by digestion with the appropriate restriction enzymes, blunt ending, and self-ligation of pcDNA3.1-Myc-mTOR or pcDNA3.1-FLAG-mTOR. To generate the expression plasmid for Xpress-tagged raptor (pcDNA3.1-Xpress-raptor), a PCR fragment of human raptor cDNA obtained using primers Kpn-raptor-F (AAGGTAC-CAATGGAGTCCGGAAATGCTCAATGCCG) and raptor-Sph-R (CACATTCTGTGCAAACCGAGCGCAG) was digested with KpnI and SphI and then inserted into the KpnI and NotI sites of the pcDNA3.1/His plasmid (Invitrogen) together with an SphI-NotI fragment derived from the KIAA1303 cDNA clone (a generous gift from the Kajita DNA Research Institute) (28). The pcDNA3.1/His plasmid possesses both the His, sequence and the Xpress epitope. Plasmids for HA-mTOR(N2343K) (pcDNA1-HA-mTOR(N2343K)) and FLAG-mTOR(N2343K) (pCMV-FLAG-mTOR(N2343K)) were described previously (27). The expression plasmids for HA-S6K1 (pRK5-HA-S6K1) and FLAG-ATR (pBJ-FLAG-ATR) were kindly provided by Dr. Akio Yamakawa and Karlene Cimprichs, respectively.

To generate a plasmid used to construct the triple HA epitope (HA3)-tagged version of TOR1 or TOR2 (pFA6a-HIS3MX6-PTOR1–3HA or pFA6a-HIS3MX6-PTOR2–3HA, respectively), the GAL1 promoter region in pFA6a-HIS3MX6-PGAL1–3HA (29) was replaced with a region upstream of TOR1 (~600 to +3 relative to the translational start site of TOR1) or TOR2 (~600 to +3 relative to the translational start site of TOR2). To generate a plasmid used to construct triple FLAG epitope (FLAG3)-tagged TOR1 or TOR2 (pFA6a-HIS3MX6-PTOR1–3FLAG or pFA6a-HIS3MX6-PTOR2–3FLAG, respectively), a fragment encoding the HA3 epitope tag in pFA6a-HIS3MX6-PTOR1–3HA or pFA6a-HIS3MX6-PTOR2–3HA was replaced with a sequence encoding the FLAG3 tag. All plasmids were verified by DNA sequencing.

Mammalian Cell Culture and Transfection—COS-7, HEK293T, and HeLa cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) with 1000 or 4500 mg/liter glucose (Sigma) supplemented with 10% fetal bovine serum under 5% CO2 atmosphere. Transient transfection of the indicated plasmids was performed with PolyFect reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. In these assays, the transfection efficiencies were estimated to 30−40%, and the total expression levels of recombinant mTOR proteins were usually <3-fold compared with those of endogenous mTOR. For amino acid starvation, cells were washed once with Dulbecco’s phosphate-buffered saline (D-PBS) containing 100 mg/liter each CaCl2 and MgCl2 and 1000 or 4500 mg/liter glucose (according to DMEM used for cell cultures) and incubated in the same saline for the indicated times. In experiments for the re-addition of amino acids, an amino acid mixture was added at the following final concentrations: l-Arg, 84 mg/liter; l-Cys, 48 mg/liter; l-His, 84 mg/liter; l-Ile, 105 mg/liter; l-Leu, 105 mg/liter; l-Lys, 145 mg/liter; l-Met, 30 mg/liter; l-Phe, 66 mg/liter; l-Thr, 95 mg/liter; l-Trp, 20 mg/liter; l-Tyr, 72 mg/liter; l-Val, 94 mg/liter; and l-Gln, 584 mg/liter.

Cell Lysate Preparation and Immunoprecipitation—After 24−48 h of transfection, cells grown on 6-well plates were

3 T. Maeda, unpublished data.
mTOR Multimerization via the HEAT Repeat Region

Yeast Cell Culture and Immunoprecipitation—Yeast strains were grown to \( \Delta_{600} = 1.0 \) at 30 °C in YPD medium (yeast extract/peptone/dextrose). For nitrogen or carbon starvation, cells were washed once with water and cultured in synthetic dextrose medium without ammonium sulfate and supplemented with leucine and uracil or in synthetic complete medium without glucose, respectively. For other treatments, each reagent was added directly to YPD medium. Cells were harvested by centrifugation at 1500 \( \times g \) for 5 min and disrupted in yeast lysis buffer (D-PBS, 10% glycerol, 0.5% Tween 20, 50 mM NaF, 10 mM β-glycerophosphate, 1.5 mM Na\(_3\)VO\(_4\), 40 μg/ml aprotinin, and 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) using 5 nm filter ceramics beads and a Multi-Beads Shocker (Yasui Kikai Corp., Osaka) 10 times for each 30-s pulse. After centrifugation at 13,000 \( \times g \) for 10 min, the supernatants were incubated with anti-FLAG M2 beads for 2 h at 4 °C. Immune complexes were washed three times with yeast lysis buffer and resolved by SDS-PAGE.

RESULTS

mTOR Exists as a Multimer—To examine whether mTOR exists as a multimer, Myc epitope-tagged mTOR and FLAG epitope-tagged mTOR were transiently coexpressed in mammalian cells. As shown in Fig. 1A, Myc-mTOR was coimmunoprecipitated with FLAG-mTOR, indicating that mTOR exists as a multimer in the cell. The endogenous mTOR-associating proteins raptor, rictor, and mLST8 (7, 12–15) were also coimmunoprecipitated with FLAG-mTOR (data not shown). Similar to mTOR-raptor interaction (7, 14), mTOR-mTOR interaction was also disrupted when we used lysis buffer containing Triton X-100 as a detergent (Fig. 1A). To determine the region involved in the multimerization of mTOR, a series of truncation mutants of mTOR with a FLAG epitope tag were coexpressed with Myc-mTOR in mammalian cells, and their ability to interact with Myc-mTOR was examined by coimmunoprecipitation analysis (Fig. 1, B and C). A FLAG-tagged N-terminal region (fragment 1–1484) of mTOR was able to

TABLE 1

S. cerevisiae strains used in this study

| Strain         | Genotype                        |
|----------------|---------------------------------|
| TM141          | MATa ura3 leu2 his3 trp1        |
| TM225          | MATa ura3 leu2 his3 lys2        |
| KS014          | MATa ura3 leu2 his3 trp1 His3MX6-P\(_{TOR1}\)-3HA-TOR1 |
| KS015          | MATa ura3 leu2 his3 trp1 His3MX6-P\(_{TOR2}\)-3HA-TOR2 |
| T1-001         | MATa ura3 leu2 his3 lys2 His3MX6-P\(_{TOR1}\)–3FLAG-TOR2 |
| T1-005         | MATa ura3 leu2 his3 lys2 His3MX6-P\(_{TOR2}\)–3FLAG-TOR1 |
| T2-004         | MATa/TMa ura3/ura3 leu2/leu2 his3/3his trp1/+ lys2/+ His3MX6-P\(_{TOR1}\)-3HA-TOR2/His3MX6-P\(_{TOR2}\)-3FLAG-TOR2 |
| T2-007         | MATa/TMa ura3/ura3 leu2/leu2 his3/3his trp1/+ lys2/+ His3MX6-P\(_{TOR1}\)-3HA-TOR2/His3MX6-P\(_{TOR2}\)-3FLAG-TOR2 |
| T2-012         | MATa/TMa ura3/ura3 leu2/leu2 his3/3his trp1/+ lys2/+ His3MX6-P\(_{TOR1}\)-3FLAG-TOR1/His3MX6-P\(_{TOR2}\)-3FLAG-TOR2 |
| T2-013         | MATa/TMa ura3/ura3 leu2/leu2 his3/3his trp1/+ lys2/+ His3MX6-P\(_{TOR1}\)-3HA-TOR1/His3MX6-P\(_{TOR2}\)-3FLAG-TOR1 |

mTOR Multimerization via the HEAT Repeat Region

Mammalian cells were transfected with plasmids expressing FLAG\(_{3}\)-Tor1p (T1-005) and FLAG\(_{3}\)-Tor2p (T1-001) were similarly constructed using pFA6a-HIS3MX6-P\(_{TOR1}\)-3FLAG and pFA6a-HIS3MX6-P\(_{TOR2}\)-3FLAG, respectively, in the TM225 strain background. All strains were confirmed by DNA sequencing of their genomic DNA for correct insertion of the intended tag into TOR1 or TOR2. Diploid strains were constructed by mating appropriate strains.
interact with Myc-tagged full-length mTOR. The remaining region (fragment 1484–2549) of mTOR tagged with FLAG only marginally interacted with Myc-tagged full-length mTOR, and shorter deletion mutants (fragments 1879–2549 and 2106–2549) were not able to interact with Myc-mTOR (Fig. 1C). On the other hand, shorter deletion mutants of the N-terminal region (fragments 1–1380, 1–670, and 575–1380) still interacted with recombinant full-length mTOR (Fig. 1C). Reciprocal immunoprecipitation with Myc-mTOR showed similar results (Fig. 1D). Another PIKK protein containing the HEAT repeat region, ATR (31), did not interact with Myc-mTOR (Fig. 1E), indicating that the interaction is specific. These results suggest that mTOR is able to multimerize through its N-terminal HEAT repeat region.

**mTOR Multimerization Is Affected by Amino Acid Sufficiency and Butanol Treatment**—We next investigated whether mTOR multimerization is affected by amino acid starvation. Cells transiently transfected with expression plasmids for both Myc-mTOR and FLAG-mTOR were first maintained in DMEM containing 10% serum, and then the medium was replaced with D-PBS for 150 min before harvesting. The amount of Myc-mTOR co-immunoprecipitated with FLAG-mTOR was greatly reduced by this treatment (Fig. 2A, lanes 1 and 6). The addition of amino acids to D-PBS restored the interaction (lane 2). These results suggest that mTOR multimerization is modulated by extracellular amino acid sufficiency. Insulin is another known input leading to S6K1 phosphorylation at Thr389 through the activation of mTORC1 signaling, although this action of insulin requires extracellular amino acids (32, 33). Consistent with the action of insulin for S6K1 phosphorylation, insulin alone did not enhance mTOR multimerization (lane 3). Rapamycin treatment was found to have no effect on mTOR multimerization at the concentration inhibiting S6K1 phosphorylation (lanes 4 and 5).

Similar but more marked changes were observed using N-terminal fragment 1–1380, for which multimerization was considerably more robust compared with full-length mTOR (Fig. 2B). Kinase-dead mTOR (27) also responded to amino acid sufficiency (Fig. 2C). Thus, the N-terminal HEAT repeat region is sufficient for modulating multimerization in response to amino acid sufficiency, and the kinase activity of mTOR is not required for the modulation. Both FLAG-tagged fragments 1–670 and 575–1380 were able to interact with Myc-tagged fragment 1–1380, but these interactions were less sensitive to amino acid

---

**FIGURE 1.** mTOR forms a multimer via the HEAT repeat domain. A, COS-7 cells were cotransfected with the expression plasmids for Myc-mTOR and FLAG-mTOR. After 24 h, the cells were lysed with lysis buffer containing 0.3% CHAPS or 1% Triton X-100. FLAG-mTOR was immunoprecipitated (IP), and the immune complexes were analyzed by immunoblotting using the indicated antibodies. B, shown is a schematic representation of domains in mTOR. The regions of mTOR fragments used in C and D are shown below. C, COS-7 cells were transfected with the expression plasmids for FLAG-tagged mTOR fragments and Myc-tagged full-length mTOR. After the recovery of cell lysates, the interaction between FLAG-tagged fragments and Myc-mTOR was analyzed as described for A. D, HEK293T cells were transfected with the expression plasmids for Myc-tagged mTOR fragments and FLAG-tagged full-length mTOR. After the recovery of cell lysates, the interaction between Myc-tagged fragments and FLAG-mTOR was analyzed as described for A, except that the antibodies for immunoprecipitation and immunoblotting were switched. E, COS-7 cells were transfected with the expression plasmid for Myc-mTOR or FLAG-ATR alone or together and analyzed as described for A. The results from control immunoprecipitation with cell lysates that had been transiently transfected with Myc-mTOR and FLAG-mTOR are also shown.
mTOR Multimerization via the HEAT Repeat Region

To identify other conditions affecting mTOR multimerization, we tested several treatments that were already reported to reduce S6K1 and 4E-BP1 phosphorylation (18, 21, 26, 34). The addition of 2-deoxyglucose, which leads to AMP-activated protein kinase activation, did not significantly affect mTOR multimerization (Fig. 3A, lane 5). On the other hand, the addition of 1-butanol significantly and that of 2-butanol weakly reduced mTOR multimerization (Fig. 3A, lanes 6 and 7). The different effects of 1- and 2-butanol on mTOR multimerization appear to be physiologically relevant because 1-butanol more strongly inhibits PA production compared with 2-butanol (26). Again, similar results were observed for mTOR N-terminal fragment 1–1380 (Fig. 3B). Although PA has been shown to bind directly to the FRB domain of mTOR and to activate mTOR signaling (26), our results suggest the possibility that PA also exerts a regulatory effect on mTOR through other mechanisms.

The State of mTOR Multimerization Does Not Directly Correlate with the Phosphorylation State of S6K1—To address the correlation between mTOR multimerization and S6K1 phosphorylation at Thr^{389}, we examined the time courses of both events in response to changes in amino acid sufficiency. Phosphorylation of S6K1 at Thr^{389} has been extensively used as a readout of mTORC1 activity both in vivo and in vitro (7, 32). Amino acid withdrawal gradually decreased Myc-mTOR co-immunoprecipitation with FLAG-mTOR over 90 min, whereas S6K1 phosphorylation at Thr^{389} was almost completely diminished after 30 min (Fig. 4A). Conversely, mTOR multimerization increased up to 60 min after changing the medium from D-PBS to DMEM containing 10% serum, although S6K1 phosphorylation occurred as quickly as in 5 min and peaked after 30 min (Fig. 4B). Thus, S6K1 phosphorylation by amino acid stimulation occurs prior to the induction of mTOR multimerization. In addition, using the coumermycin-GyrB (gyrase B)-induced dimerization system (35, 36), we artificially induced dimerization of GyrB-mTOR fusion protein independently of amino acid availability. However, dimerization of GyrB-mTOR did not stimulate Thr^{389} phosphorylation of S6K1 under amino acid-deprived conditions (Fig. 4C). Taken together, these results suggest that the state of mTOR multimerization does not directly correlate.

FIGURE 2. mTOR multimerization is modulated by amino acid sufficiency. A, COS-7 cells were cotransfected with the expression plasmids for Myc-mTOR and FLAG-mTOR. After 40 h, the cells were transferred into D-PBS and incubated for 90 min (lanes 1–5) or left in DMEM containing 10% serum (DMEM + S; lane 6). Cells treated with D-PBS were then left in D-PBS (lane 1) or transferred into D-PBS containing amino acids (AA; lane 2), 100 nM insulin (Ins; lane 3), or both amino acids and 100 nM insulin alone (AA + Ins; lane 3) or with 200 nM rapamycin (AA + Ins + Rap; lane 5) for 60 min prior to harvest. Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads and then analyzed by immunoblotting with the indicated antibodies. anti-S6K-P (Thr^{389}), anti-phospho-S6K1 Thr^{389} antibody. B, HEK293T cells transiently expressing Myc-mTOR-(1–1380) and FLAG-mTOR-(1–1380) were transferred into D-PBS for 90 min. Cells were then left in D-PBS (lane 1); transferred into D-PBS containing amino acids (lane 2), 100 nM insulin (lane 3); or both amino acids and 100 nM insulin alone (lane 4) or with 200 nM rapamycin (lane 2); or transferred into DMEM containing 10% serum (lane 6) for 60 min prior to harvesting. Immunoprecipitation assays were performed as described for A. C, HEK293T cells were cotransfected with the expression plasmid for FLAG-tagged kinase-dead mTOR (FLAG-mTOR(N2343K)) (lane 1), HA-tagged kinase-dead mTOR (HA-mTOR[N2343K]) (lane 2), or both (lanes 3–5). After 40 h, the cells were left in DMEM containing 10% serum (lanes 1–3) or transferred into D-PBS (lanes 4 and 5). After 90 min, the cells treated with D-PBS were left in D-PBS (lane 5) or transferred into D-PBS containing amino acids (lane 4). Immunoprecipitation assays were performed as described for A. D, FLAG-tagged mTOR fragments were transiently expressed together with Myc-mTOR-(1–1380) in COS-7 cells. After 40 h, the cells were transferred into D-PBS for 90 min and then left in D-PBS (lanes 1, 4, and 7), treated with amino acids in D-PBS (lanes 2, 5, and 8), or transferred into DMEM containing 10% serum (lanes 3, 6, and 9) for 60 min before harvesting. Immunoprecipitation assays were performed as described for A.
with the Thr\textsuperscript{389} phosphorylation state of S6K1, although amino acid sufficiency promotes both events.

**Multimeric mTOR Exists in Both mTORC1 and mTORC2**

To examine whether mTOR in mTORC1 or mTORC2 is in a multimeric state, we performed two sequential immunoprecipitations with cell lysates from COS-7 cells transiently expressing FLAG-mTOR with and without Myc-mTOR. First, FLAG-mTOR was immunoprecipitated using anti-FLAG M2 beads, and then the immunoprecipitated complex released from the anti-FLAG M2 beads was further immunoprecipitated with anti-Myc beads. After these sequential immunoprecipitations, endogenous raptor and rictor were still recovered with Myc-mTOR (Fig. 5A), suggesting the formation of ternary complexes composed of FLAG-mTOR, Myc-mTOR, and raptor or rictor. Similar results were also observed in HEK293T cells (data not shown). Thus, mTOR appears to form a multimer in both mTORC1 and mTORC2.

We also examined the effect of raptor overexpression on mTOR multimerization. The overexpression of Xpress-raptor together with Myc-mTOR and FLAG-mTOR had only a marginal effect on the multimerization of mTOR under amino acid-replete conditions (Fig. 5B, lanes 2, 4, and 6). This result indicates that raptor itself does not likely mediate the formation of multimeric mTOR. On the other hand, the overexpression of Xpress-raptor, which would increase the ratio of the raptor-associated mTOR fraction to the rictor-associated one, led to mTOR multimerization being less sensitive to amino acid starvation in a dose-dependent manner (lanes 1, 3, and 5), suggesting that mTOR multimerization in the rictor-associated fraction, viz. mTORC1, might be insensitive to amino acid sufficiency.

**Gel Filtration Analysis of mTOR**—To investigate the change in the multimerization of endogenous mTOR in response to amino acid sufficiency and to determine which of the two complexes (mTORC1 or mTORC2) contributes to the change, we analyzed lysates from two representative cell lines by gel filtration chromatography: HEK293T and HeLa cells, which are abundant in mTORC1 and mTORC2, respectively (7, 12). Fig. 6A shows the elution profiles of endogenous mTOR, raptor, and rictor in HEK293T cells. Endogenous mTOR was distributed mainly into three peaks: fractions 4 and 5 (\(\sim 2\) MDa; peak A), fractions 11–13 (\(\sim 1\) MDa; peak B), and fractions 17 and 18 (\(\sim 0.5\) MDa; peak C). Raptor distributed to peak B, and rictor distributed to peaks A and B as well as to some lower ranges (fractions 14 and 15), indicating that mTOR multimerization in the rictor-associated fraction, viz. mTORC1, might be insensitive to amino acid sufficiency.

To determine which of these peaks corresponds to a multimeric form, we used lysates from cells transiently expressing both Myc-mTOR and FLAG-mTOR. The elution profiles of both recombinant mTOR proteins were similar to that of endogenous mTOR (Fig. 6C). Co-immunoprecipitation analysis with three peak fractions revealed that multimeric mTOR was distributed mainly in peak A, to a lesser extent in peak B, and barely in peak C (Fig. 6D).

In response to amino acid starvation, the relative amount of mTOR in peak A consistently decreased in lysates from HeLa cells, a much larger amount of mTOR distributed to peak A and only a marginal amount to peak C. Raptor again distributed only to peak B, and rictor distributed mainly to peak A. The difference is consistent with previous observations that HEK293T cells contain more mTORC1 compared with HeLa cells and that HeLa cells have more mTORC2 compared with HEK293T cells (7, 12).
results suggest that it is mTORC2 that is subjected to modulation in multimerization by amino acid sufficiency, which is consistent with the above observation indicating that mTOR multimerization in mTORC1 is insensitive to amino acid sufficiency. We also noticed increases in the relative amounts of mTOR at fractions 16–18 and of raptor at fractions 18–21 in DMEM for both HEK293T and HeLa cells lysates, although the relevance to mTOR multimerization remains unclear at present.

**TOR Multimerization Is Conserved in Yeast**—To investigate whether TOR multimerization is conserved through evolution, we constructed *S. cerevisiae* diploid strains expressing differently tagged TOR proteins at the N termini from each allele. Upon immunoprecipitation with anti-FLAG M2 beads, an association between HA3-Tor1p and FLAG5-Tor1p was detected (Fig. 7, lane 5), and Tor2p-Tor2p interaction was similarly detected (Fig. 7B, lane 5). On the other hand, as reported previously (10, 11), no interaction was observed between Tor1p and Tor2p (Fig. 7, A and B, lanes 1). Thus, TOR multimerization is also conserved in yeast, and multimerization is restricted to homomultimerization, i.e. Tor1p-Tor1p or Tor2p-Tor2p. As Tor1p is able to form TORC1 but not TORC2 in yeast (10, 11), the TOR protein in TORC1 is also multimeric in yeast.

We next examined whether TOR multimerization in yeast is also affected by changing culture conditions. As in mammalian cells, nutrient starvation has been demonstrated to inhibit TORC1 signaling in yeast (37); however, nitrogen or carbon starvation did not affect either Tor1p-Tor1p or Tor2p-Tor2p interaction (Fig. 7, A and B, lanes 5 and 6). Rapamycin treatment also did not change multimerization, as in mammalian cells (Fig. 7, A and B, lanes 7). We also tested the effects of 1- and 2-butanol on yeast TOR multimerization, although PA involvement in TOR signaling in yeast has not been demonstrated. These treatments did not show significant effects on either Tor1p-Tor1p or Tor2p-

---

**FIGURE 4.** The multimerization state of mTOR does not parallel the phosphorylation state of S6K1. A and B, COS-7 cells were transiently transfected with the expression plasmids for Myc-mTOR and FLAG-mTOR. After 40 h, the cells were transferred into D-PBS for the indicated times (A) or were transferred into D-PBS for 90 min and then incubated in DMEM containing 10% serum (DMEM + S) for the indicated times (B). Cell lysates were used for immunoprecipitation (IP) with anti-FLAG M2 beads and analyzed by immunoblotting with the indicated antibodies. Anti-S6K-P (Thr389) antibody. C, HEK293T cells were transiently transfected with plasmids for GyrB-Myc-mTOR and GyrB-FLAG-mTOR. After 40 h, the cells were transferred into D-PBS for 90 min and then treated with increasing concentrations of coumermycin (0 (lane 1), 0.1 (lane 2), 1.0 (lane 3), and 10 μM (lane 4)) or with D-PBS containing amino acids (+AA/PBS; lane 5) for 60 min. Cell lysates were subjected to immunoprecipitation with anti-FLAG M2 beads and analyzed by immunoblotting with the indicated antibodies.

**FIGURE 5.** mTOR forms a multimer in both mTORC1 and mTORC2. A, COS-7 cells were transiently transfected with the expression plasmid for FLAG-mTOR with (+) or without (−) Myc-mTOR. After 40 h, the cells were lysed with lysis buffer. Cleared cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads. After washing, the immune complex was eluted with the 3X FLAG peptide. The eluates were then immunoprecipitated with anti-Myc beads. Cell lysates, eluates with 3X FLAG peptides, and immunoprecipitates with anti-MyC beads were analyzed by immunoblotting with the indicated antibodies. B, COS-7 cells were transiently transfected with the expression plasmids for Myc-mTOR and FLAG-mTOR and analyzed by immunoblotting with the indicated antibodies. Anti-S6K-P (Thr389) antibody. C, HEK293T cells were transiently transfected with plasmids for GyrB-Myc-mTOR and GyrB-FLAG-mTOR. After 40 h, the cells were transferred into D-PBS for 90 min and then treated with increasing concentrations of coumermycin (0 (lane 1), 0.1 (lane 2), 1.0 (lane 3), and 10 μM (lane 4)) or with D-PBS containing amino acids (+AA/PBS; lane 5) for 60 min. Cell lysates were subjected to immunoprecipitation with anti-FLAG M2 beads and analyzed by immunoblotting with the indicated antibodies.
Tor2p interaction (Fig. 7, A and B, lanes 6 and 7), although such treatments substantially affected mTOR multimerization. Thus, in contrast to mTOR, we observed no change in TOR multimerization in yeast.

DISCUSSION

We have demonstrated that mTOR forms a multimer in vivo via the N-terminal HEAT repeat domain. mTOR multimerization occurs in both mTORC1 and mTORC2 (Fig. 5A). Our study has also revealed that TOR multimerization is conserved in yeast Tor1p and Tor2p. Because Tor1p exists solely in TORC1 (10, 11, 40), this observation indicates that the multimerization occurs in TORC1 (Fig. 7A). Consistent with our results, it was reported recently that Tor2p in yeast and TOR in Drosophila also exist as multimers in vivo and that TORC2 is in an oligomeric state in yeast (38, 39). Thus, multimerization of TOR is evolutionally conserved from yeast to mammals, occurs in both TORC1 and TORC2, and therefore appears to be important for TOR functioning.

FIGURE 7. Multimerization of TOR is conserved in yeast. A and B, diploid yeast cells expressing HA-Tor1p and FLAG2-Tor1p (T2-013), FLAG2-Tor1p and HA-Tor2p (T2-012), HA-Tor1p and FLAG2-Tor2p (T2-007), or HA-Tor2p and FLAG2-Tor2p (T2-004) were grown in YPD medium for 3 h and then starved of nitrogen (synthetic dextrose medium without ammonium sulfate (SD-N) or carbon (synthetic complete medium without glucose (SC-Glc)) or treated with 200 nM rapamycin (Rap), 0.5% 1-butanol (1-B), or 0.5% 2-butanol (2-B) for 60 min. Cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads, and the immune complexes were analyzed by immunoblotting using anti-HA and anti-FLAG antibodies.
mTOR Multimerization via the HEAT Repeat Region

filtration analysis showed that multimeric mTOR formed two major complexes with large molecular masses of >2 MDa (peak A) and 1 MDa (peak B) (Fig. 6). Because raptor and rictor also migrated into the corresponding fractions, respectively, it was suggested that peak A represents multimeric mTORC2, whereas peak B represents multimeric mTORC1.

In response to amino acid sufficiency, only a few changes in the formation of protein complexes containing mTOR have been reported thus far (7, 41). We have demonstrated here, for the first time to our knowledge, that mTOR multimerization is sensitive to amino acid deprivation as well as to butanol treatment. As these two conditions have already been reported to reduce S6K1 and 4E-BP1 phosphorylation (1, 7, 14, 26), it is conceivable that mTOR multimerization regulates mTORC1 activity or mTOR functioning in general. However, careful comparison between the time courses of mTOR multimerization and S6K1 phosphorylation in response to changes in amino acid sufficiency revealed that changes in the latter precede changes in the former. This difference in time courses may be due to S6K1 phosphorylation being regulated both positively by a protein kinase, possibly mTOR itself, and negatively by a protein phosphatase, both of which are under reciprocal control by a protein kinase, possibly mTOR itself, and negatively by a protein phosphatase, both of which are under reciprocal control by a nutrient-sensitive mechanism may regulate both mTOR multimerization and mTORC1 activity independently. As mTOR multimerization in mTORC1 appeared to be refractory to amino acid deprivation (Fig. 5B), amino acid-mediated change in mTOR multimerization may occur solely in mTORC2. Consistent with this idea, formation of a complex of >2 MDa (peak A) containing mTOR and rictor was sensitive to amino acid deprivation in HeLa cells (Fig. 6D). If this is the case, the output regulated by mTOR multimerization appears to differ from Akt phosphorylation at Ser473, which was recently identified as a target of mTORC2 (16), because it is not modulated by amino acid sufficiency (data not shown) (33). The observation that artificially dimerized GyrB-mTOR did not promote the phosphorylation of Akt at Ser473 is consistent with this idea (data not shown). Instead, it is possible that mTOR multimerization might play a role in the regulation of a different subset of mTORC2 outputs. In line with this argument, it has been reported that amino acid sufficiency affects the activation of some mTORC2 outputs such as RhoA and Rac1 (13).

At present, it remains to be determined whether mTOR-mTOR interaction is direct or not. Raptor will not mediate the multimerization of mTOR because mTOR-raptor interaction is reduced by rapamycin treatment (data not shown) (7, 43), whereas mTOR-mTOR interaction is not. Moreover, butanol treatment reduced mTOR-mTOR interaction but not mTOR-raptor interaction (Fig. 3 and data not shown). The overexpression of Xpress-raptor consistently had only a marginal effect on the multimerization of mTOR under amino acid-replete conditions (Fig. 5B).

Unlike mTOR, the multimerization of TOR proteins in yeast was not affected by nutrient deprivation. This may be because TOR multimerization is regulated only in TORC2, as mentioned above for mTORC2, and the tested conditions affect only TORC1. Otherwise, the regulation of mTOR multimerization may be an evolutionarily new mechanism.

In conclusion, we found that TOR exists as a multimer in vivo. As the state of multimeric mTOR is sensitive to amino acid sufficiency, elucidation of the role and regulatory mechanism of multimerization will provide an important clue to understanding mTOR signaling.

Acknowledgments—We thank Drs. Yutaka Hoshikawa, Akio Yamakawa, Fred Winston, Mark S. Longtime, Kenji Takehana, Karlene Cimprichs, Akira Matsuura, Roger M. Pelmutter, and Yukiko Gotoh for various materials; Katsuyuki Seki for constructing yeast strains; and Suguru Koyama for helping with gel filtration analysis. The KIAA1303 cDNA clone was a generous gift from the Kazusa DNA Research Institute. We also thank all of the members of the Maeda laboratory for help, advice, and discussion.

REFERENCES

1. Harris, T. E., and Lawrence, J. C. (2003) Sci. STKE 212, RE15
2. Martin, D. E., and Hall, M. N. (2005) Curr. Opin. Cell Biol. 17, 158–166
3. Heitman, J., Movva, N. R., and Hall, M. N. (1991) Science 253, 905–909
4. Andrade, M. A., and Bork, P. (1995) Nat. Genet. 11, 115–116
5. Bosotti, R., Isacchi, A., and Sonnhammer, E. L. (2000) Trends Biochem. Sci. 25, 225–227
6. Sabatini, D. M., Barrow, R. K., Blackshaw, S., Burnett, P. E., Lai, M. M., Field, M. E., Bahr, B. A., Kirsch, J., Betz, H., and Snyder, S. H. (1999) Science 284, 1161–1164
7. Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002) Cell 110, 163–175
8. Wu, S., Mikhailov, A., Kallo-Hosein, H., Hara, K., Yonezawa, K., and Avruch, J. (2002) Biochim. Biophys. Acta 1542, 41–56
9. Choi, J. H., Bertram, P. G., Drenan, R., Carvalho, J., Zhou, H. H., and Zheng, X. F. S. (2002) EMBO Rep. 3, 988–994
10. Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M. N. (2002) Mol. Cell 10, 457–468
11. Wedaman, K. P., Reinke, A., Anderson, S., Yates, J., McCaffery, J. M., and Powers, T. (2003) Mol. Biol. Cell 14, 1204–1220
12. Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) Curr. Biol. 14, 1296–1302
13. Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M. A., Hall, A., and Hall, M. N. (2004) Nat. Cell Biol. 6, 1122–1128
14. Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidaya, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002) Cell 110, 177–189
15. Kim, D. H., Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2003) Mol. Cell 11, 895–904
16. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Science 307, 1098–1101
17. Garami, A., Zwartkruis, F. J. T., Nobukuni, T., Joaquín, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003) Mol. Cell 11, 1457–1466
18. Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003) Genes Dev. 17, 1829–1834
19. Lee, A. H., Rodan, Y., Zaidi, A., Siggia, E. D., and Brake, A. R. (2003) Mol. Cell 13, 1259–1268
20. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005) Curr. Biol. 15, 702–713
21. Inoki, K., Zhu, T., and Guan, K. L. (2003) Cell 115, 577–590
22. Smith, E. M., Finn, S. G., Tee, A. R., Browne, G. J., and Proud, C. G. (2005) J. Biol. Chem. 280, 18717–18727
23. Nobukuni, T., Joaquín, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J. T., and

SEPTEMBER 29, 2006•VOLUME 281•NUMBER 39 JOURNAL OF BIOLOGICAL CHEMISTRY 28613
mTOR Multimerization via the HEAT Repeat Region

Thomas, G. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14238–14243
24. Byfield, M. P., Murray, J. T., and Backer, J. M. (2005) J. Biol. Chem. 280, 33076–33082
25. Bakkenist, C. J., and Kastan, M. B. (2003) Nature 421, 499–506
26. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) Science 294, 1942–1945
27. Hara, K., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andrabi, K., Weng, Q. P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997) J. Biol. Chem. 272, 26457–26463
28. Nagase, T., Kikuno, R., Ishikawa, K. I., Hiroswa, M., and Ohara, O. (2000) DNA Res. 7, 65–73
29. Longtine, M. S., McKenzie, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philipp, P., and Pringle, J. R. (1998) Yeast 14, 953–961
30. Gietz, R. D., and Woods, R. A. (2002) Methods Enzymol. 350, 87–96
31. Perry, J., and Klechner, N. (2003) Cell 112, 151–155
32. Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998) J. Biol. Chem. 273, 14484–14494
33. Wang, X., Campbell, L. E., Miller, C. M., and Proud, C. G. (1998) Biochem. J. 334, 261–267
34. Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, R., Kozma, S. C., and Thomas, G. (2001) Science 294, 1102–1105
35. Farrar, M. A., Olson, S. H., and Perlmutter, R. M. (2000) Methods Enzymol. 327, 421–429
36. Farrar, M. A., Alberola-Lla, J., and Perlmutter, R. M. (1996) Nature 383, 178–181
37. Crespo, J. L., and Hall, M. N. (2002) Microbiol. Mol. Biol. Rev. 66, 579–591
38. Wullschleger, S., Loewith, R., Oppliiger, W., and Hall, M. N. (2005) J. Biol. Chem. 280, 30697–30704
39. Zhang, Y., Billington, C. J., Jr., Pan, D., and Neufeld, T. P. (2005) Genetics 172, 355–362
40. Reinke, A., Anderson, S., McCaffery, J. M., Yates, J., Aronova, S., Chu, S., Fairclough, S., Iveson, C., Wedaman, K. P., and Powers, T. (2004) J. Biol. Chem. 279, 14752–14762
41. Long, X., Ortiz-Vega, S., Lin, Y., and Avruch, J. (2005) J. Biol. Chem. 280, 23433–23436
42. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4438–4442
43. Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Har, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004) Genes Cells 9, 359–366