Isolation of Hyperactive Mutants of Mammalian Target of Rapamycin*  

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Yoichiro Ohneh, Terunao Takahara1, Riko Hatakeyama3, Tomoko Matsuzaki3, Makoto Noda3, Noboru Mizushima3, and Tatsuya Maeda4,2  

From the 1Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan, 4Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan, and 5Department of Physiology and Cell Biology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan  

The target of rapamycin (TOR)3 is a Ser/Thr kinase highly conserved from yeast to mammals and which plays central roles in the regulation of cell growth (1, 2). In the yeast Saccharomyces cerevisiae there exist two homologous genes (TOR1 and TOR2) encoding TOR, whereas only one TOR gene exists in higher eukaryotes. TOR belongs to the family of phosphatidylinositol kinase-related kinases which shares characteristic domains including tandem HEAT repeats, FAT, kinase, and FATC domains (3). In addition, TOR family members also possess the FRB domain located between the FAT and the kinase domains, which directly binds to rapamycin, a potent inhibitor of TOR, in complex with FK506-binding protein 12 (FKBP12).  

TOR forms two functionally distinct complexes termed the TOR complex 1 (TORC1) and 2 (TORC2). Yeast TORC1 consists of Kog1, Lst8, and either Tor1 or Tor2, whereas TORC2 consists of Avo1, Avo2, Tsc11/Avo3, Lst8, and Tor2 (4). The components of the two complexes are also evolutionarily conserved in mammals. Mammalian TORC1 (mTORC1) contains the Kog1 homolog Raptor (5, 6) and the Lst8 homolog mLst8 (7), whereas mTORC2 contains the Avo3 homolog Rictor (8, 9), the Avo1 homolog mSin1 (10–12), and mLst8. Both TORC1 and TORC2 exist in homomultimeric forms in vivo (13–16). Several additional components of either TORC1 or TORC2 have also been identified recently in yeast as well as in mammals. The functions of the individual components of the two complexes are mostly unknown, although biochemical analyses in mammalian cultured cells suggest that Raptor seems to be required for the recognition of mTORC1 substrates (5), and mLst8 enhances mTOR kinase activity through the binding to the kinase domain (7). The rapamycin-FKBP12 complex inhibits mTORC1 activity in part by promoting dissociation of Raptor from mTOR (6, 17). In contrast, the rapamycin-FKBP12 complex is unable to bind to mTORC2 and thus, fails to inhibit the mTORC2 activity, although a recent study indicates prolonged rapamycin treatment can also inhibit mTORC2 assembly and function (18).  

Early studies on the physiological functions of mTOR solely relied on the pharmacological effect of rapamycin and, thus, were confined to mTORC1, although subsequent mutational or RNAi-mediated inactivation of the components of the two mTOR complexes has revealed consistent as well as new...
insights. mTORC1 has been implicated in the regulation of a wide array of growth-related processes such as protein translation, cell sizing, ribosomal biogenesis, and autophagy. Although direct targets of mTORC1 leading to those cellular processes are poorly identified, the phosphorylation of ribosomal S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4EBP1) by mTORC1 coordinately leads to activation of protein translation and an increase in cell size (19). In contrast, mTOR antagonizes autophagy, the bulk degradation process of intracellular components, to provide energy and nutrient sources. Although yeast TORC1 controls the early activation step of autophagy through the phosphorylation of Atg13 (20), a similar regulatory mechanism in mammalian cells has not been elucidated. mTORC2 was previously sought PDK2, which directly phosphorylates Ser-473 in the hydrophobic motif of Akt1 (21, and is involved in phosphatidylinositol-3-phosphate kinase-Akt signaling. In addition, mTORC2 regulates the organization of actin cytoskeleton in mammals (9) as in yeast (22), although detailed mechanisms remain unclear.

As described above, the physiological roles of mTOR have been defined from loss-of-function analyses both pharmacologically as well as (molecular)genetically. Because cellular responses caused by mTORC1 inactivation are similar to those induced by growth factors or nutrient deprivation, mTORC1 activity is believed to be regulated by signals from these factors. However, it has been elusive whether and how amino acid deprivation causes down-regulation of mTORC1 activity, which in turn induces these cellular responses. Toward elucidating whether modulation in the mTORC1 activity directs these cellular responses and understanding the roles of mTOR in cellular and physiological contexts, most important clues will be available from analysis using gain-of-function mutants of mTOR. Furthermore, although several lines of evidence suggest that some types of cancer are accompanied by hyperactivation of mTOR (23), it remains unclear whether mTOR hyperactivation is a primary cause. The gain-of-function analysis would also provide an understanding of how mTOR hyperactivation links to tumorigenesis. In this report we have developed a yeast screening system for the isolation of hyperactive mutants of mTOR. This system allowed us to identify mTOR mutants with enhanced kinase activities. We have also investigated the effects of the isolated mTOR mutants on the cellular processes that have been associated with the mTOR activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-hemagglutinin (HA) ascites fluid (12CA5) was a kind gift from Dr. Yutaka Hoshikawa. Anti-FLAG antibody M2, anti-FLAG M2 beads, and puromycin dihydrochloride were purchased from Sigma–Aldrich. Antibodies for phospho-S6K1 Thr-389, phospho-4EBP1 Thr-37/46, phospho-Akt Ser-473, Raptor, mLst8, and 4EBP1 were from Cell Signaling Technology (Beverly, MA). Anti-S6K1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Rictor antibody was from Bethyl Laboratories (Montgomery, TX). Anti-LC3 antibody (PM036) was from Medical & Biological Laboratories (Nagoya, Japan). Anti-actin antibody was from MP Biomedicals (Irvine, CA). Recombinant Akt1 (inactive) was from Millipore (Billerica, MA). The GST fusion protein of human 4EBP1 (GST-4EBP1) was purified from the Escherichia coli BL21(DE3) strain carrying the pGEX-6P-1–4EBP1 plasmid.

**Screening for Hyperactive mTOR**—The TOR2-mTOR chimera construct was designed based on a previous report (24). To construct the TOR2ΔN-pRS426 plasmid (pYO189), which contains the Tor2 coding region (amino acid residues 1–1689), the Smal-BamHI fragment from pJK4 (25) was inserted into the Smal-BamHI sites of pRS426 (26). A DNA fragment corresponding to the C-terminal one-third of mTOR (amino acid residues 1721–2549) flanked by additional sequences homologous to TOR2 at the 5′-terminal region and to the vector at the 3′-terminal region for homologous recombination was obtained by PCR with pcDNA1-HA-mTOR (27) as a template using primers mTOR0422-F (TGAAGCAATTTAAAAATTTCAACTTAGATGGCTATGATTTAGGTTTGAGCCT-Ggtgcagaccatgcagcag; capital letters indicate homologous sequence to TOR2) and mTOR0422-R (GAAAGCTGGAGCGCAACACTCGATTCCAGCGTGGCCGCGCAGATGACTAGTGGAT-CCcttagtggtgcttcattata; capital letters indicate homologous sequence to the vector). The PCR fragment and pYO189 linearized with BamHI were co-transformed into a wild-type yeast strain, and the resultant gap-repaired TOR2-mTOR-pRS426 plasmid, pYO203, was recovered from uracil-prototroph transformants. To generate the acceptor plasmid pYO207, the region corresponding to the kinase domain of mTOR (residues 2186–2422) in the pYO203 was replaced by a fragment consisting of HindIII and Xhol recognition sites using two-step overlap extension PCR. First PCR reactions were separately performed with pYO203 as a template using primers oYO112 (ctcgagacagtggAGGAGAAAAAAACTCTGACC) and oYO101 (GCTATGACCATGATTACGCCG) or oYO111 (aagctccgctcgagTATAGCCCTCTGTAATGGTAGTCCcttagtggtgcttcattata) and oYO113 (ctcgagctggaggaag). The first PCR products were used as overlapping templates for the second PCR reaction using primers oYO101 and oYO113. The second PCR products were digested with EcoNI and Spel and inserted between the EcoNI and Spel sites of pYO203.

The DNA region corresponding to the mTOR kinase domain (amino acid residues 2161–2451) was randomly mutagenized by error-prone PCR using GeneMorph II random mutagenesis kit (Stratagene, La Jolla, CA) with primers GapMutF (gcaagt-tcatcggaagaataggagtctgtc) and GapMutR (ctgcagaataggagtctgtc) according to the manufacturer’s instruction. The amplified DNA fragment was purified by phenol extraction followed by ethanol precipitation and was introduced directly into the pLs8a strain TM478 (MATα ura3-52 lst8Δ) together with pYO207 digested with HindIII and Xhol. The molar ratio of the mutagenized PCR product to the acceptor plasmid was 5 to 1. Transformation was conducted essentially as described by Gietz and Woods (28). Transformed yeast strains were plated on synthetic complete without uracil (SC-U) plates and incubated at 25 °C. Sixteen hours later, culture temperature was shifted to 37 °C, and plates were incubated for 7 days. Candidate plasmids were isolated from the colonies grown at 37 °C.

**Mammalian Expression Plasmids**—The expression plasmid for FLAG-mTOR (pcDNA3.1-FLAG-mTOR) was described previously (13). To generate pcDNA3.1-FLAG-mTOR<sup>S104</sup> plasmid (pYO223), the BsrGI-Xbal fragment from TOR2-
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mTORSL1-pRS426 was inserted between BsrG1 and XbaI sites of pcDNA3.1-FLAG-mTOR. To construct expression plasmids for FLAG-mTOR12017T and FLAG-mTOR12020V, I2017T or A2020V mutation was first introduced into pTB178, which contains the KpnI-XbaI fragment of mTOR, by site-directed mutagenesis using primers RV-N (TGTGAATTTGAGCGG) and oTB52 (GGCCATGATGATGAGACTGTAACGCTTGAATGCCATCCG) for I2017T or oTB53 (GGCCATGATGATGAGACTGTAACGCTTGAATGCCATCCG) for A2020V, to generate pTB182 or pTB183, respectively. Then, the pcDNA1-Amp-HA-mTOR12017T plasmid (pYO279) was constructed by ligation of three pieces of DNA fragments; they are the KpnI-XbaI fragment from pTB182, the HindIII-KpnI fragment from pcDNA1-HA-mTOR, and the HindIII-XbaI fragment from pcDNA1-Amp. Finally, to construct the pcDNA3.1-FLAG-mTOR12017T plasmid (pYO290), the XhoI-PmaCl fragment derived from the pYO279 was inserted between the XhoI and PmaCl sites of the pcDNA3.1-FLAG-mTOR. The pcDNA3.1-FLAG-mTOR12017T plasmid (pYO293) was constructed similarly by using pYO223 instead of the pcDNA3.1-FLAG-mTOR plasmid. The pcDNA3.1-FLAG-mTOR12020V (pYO291) and the pcDNA3.1-FLAG-mTOR12017T12020V (pYO295) plasmids were also generated similarly. To construct pIRES-puro3-FLAG-mTOR (pYO265), the Pmel-SspI fragment from the pcDNA3.1-FLAG-mTOR plasmid was inserted into the EcoRV site of pIRES-puro3 (Clontech, Mountain View, CA). pIRES-puro3-FLAG-mTORSL1 (pYO268), pIRES-puro3-FLAG-mTOR12017T (pYO304), and pIRES-puro3-FLAG-mTORSL112017T (pYO302) were also generated similarly by using pYO223, pYO290, and pYO293 plasmids instead of pcDNA3.1-FLAG-mTOR plasmid. The expression plasmids for HA-S6K1 (pRK5-HA-S6K1) and Akt1-HA (pCS2-hAkt1-HA) were kindly provided by Drs. Akio Yamakawa and Yukiko Gotoh, respectively.

Mammalian Cell Culture and Transfection—HeLa and HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) under 5% CO2 atmosphere. Transient transfection was performed with LipofectAmine2000 (Invitrogen) according to the manufacturer’s instructions. For amino acid deprivation, cells were washed once with DMEM (Invitrogen) according to the manufacturer’s instructions. For transient transfection was performed with LipofectAmine2000 after transfection cells were trypsinized and re-seeded in DMEM. For transient transfection with puromycin selection experiments, 24 h after transfection cells were trypsinized and re-seeded in DMEM + 10% FBS containing 1.5 μg/ml puromycin and grown for 24 h. Twenty-four hours after transfection the cells were trypsinized and split to re-plate into two dishes and cultured in DMEM + 10% FBS containing 1.5 μg/ml puromycin. After 24 h of selection, the cells were transferred into DMEM + 10% FBS or modified DMEM containing an amino acid mixture at 25% in DMEM and cultured for another 24 h. Then the cells were trypsinized, suspended in ice-cold phosphate-buffered saline containing 1% FBS, and washed once with ice-cold phosphate-buffered saline. The cells were diluted with Isoton 2 (Beckman Coulter, Fullerton, CA) and subjected to a Coulter counter (Multisizer 3, Beckman Coulter). The experiments were repeated 5 times, and at least 10,000 cells were analyzed at each experiment.

Transformation Assay—NIH/3T3 cells were transfected with empty vector or expression plasmids for wild-type FLAG-mTOR, FLAG-mTOR12017T12020V, or an oncogenic form of RAS (H-RAS12V) by calcium phosphate coprecipitation. Transfected cells were trypsinized and reseeded in DMEM + 10% FBS with or without 1 mg/ml G418. Focus formation efficiency was measured by counting foci, and transfection efficiency was evaluated by counting G418-resistant colonies.

RESULTS

Screening for Hyperactive mTOR Mutants—We have previously obtained active mutants of Tor2 that exhibit increased in vitro kinase activity because of a point mutation within the kinase domain. Importantly, these mutants were also able to partially suppress the temperature-sensitive growth of an lst8 strain, TM478. To obtain mTOR mutants with enhanced kinase activity, we utilized the finding that the kinase domain of mTOR is interchangeable with that of Tor2 in S. cerevisiae (24) (Fig. 1A). To this end, Tor2-mTOR chimera mutants were screened for those that could rescue growth of TM478 at the restrictive temperature by virtue of enhanced activity of their mTOR kinase domain. The screening scheme is summarized in

4 M. Yoneyama and T. Maeda, unpublished information.
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A schematic representation of the Tor2-mTOR chimera protein. The regions of each domain (HEAT, FAT, FRB, kinase) are depicted in the wild-type Tor2, wild-type mTOR, and the Tor2-mTOR chimera. The dotted line in each structure indicates the junction for creating the Tor2-mTOR chimera. The resultant Tor2-mTOR chimera was randomly mutagenized through error-prone PCR, and the resultant product was introduced into TM478 together with the acceptor plasmid. By screening about 31864 million transformants, we obtained three clones of mTOR mutants with enhanced kinase activity. The DNA fragments corresponding to the kinase domain of mTOR was randomly mutagenized with error-prone PCR and introduced together with the acceptor plasmid into the 
mTOR chimera. The resultant Tor2-mTOR chimera with 2518 amino acid residues is composed of the N-terminal two-thirds of Tor2 segment (residues 1–1689, shaded bar) and the C-terminal one-third of mTOR segment (residues 1721–2549, open bar). B, the scheme of screening for the Tor2-mTOR chimera harboring random mutations in the kinase domain. The transformants that grew under nonpermissive temperature on a selective plate were selected as candidates.

Fig. 1B. A cDNA fragment encompassing the mTOR kinase domain was randomly mutagenized through error-prone PCR, and the resultant product was introduced into TM478 together with the acceptor plasmid, which lacks the region corresponding to the kinase domain of Tor2-mTOR. By screening about 4 million transformants, we obtained three clones of Tor2-mTORSL1 (suppression of lst8ts) mutants (TOR2-mTORSL1, TOR2-mTORSL2, and TOR2-mTORSL3) that partially suppressed temperature-sensitive growth of the TM478 (data not shown). Each mutant has the following mutations: three amino acid substitutions in Tor2-mTORSL1 (V2198A, L2216H, and L2260P), two in Tor2-mTORSL2 (A2290V, K2440R), and one in Tor2-mTORSL3 (L2302Q). Because Tor2-mTORSL1 most potently suppressed temperature sensitivity of TM478, we focused on this mutant in the following experiments.

mTOR Mutants Exhibit Enhanced Kinase Activity in Vitro—To investigate whether three amino acid substitutions in Tor2-mTORSL1 indeed enhanced kinase activity of mTOR, we conducted an in vitro kinase assay of the mTOR mutants using GST-4EBP1 as a substrate. The FLAG-tagged mTOR mutant carrying three mutations (V2198A, L2216H, and L2260P; FLAG-mTORSL1) was transiently expressed in HeLa cells and then purified by immunoprecipitation using anti-FLAG beads. The kinase reaction was conducted as described under “Experimental Procedures.” The phosphorylation levels and the amounts of GST-4EBP1 were examined by immunoblotting using an anti-phospho-4EBP1(Thr37/46) and anti-FLAG antibodies. The phosphorylation levels of wild-type FLAG-mTOR and its variants were immunoprecipitated with anti-FLAG beads. The kinase reaction was performed as described under “Experimental Procedures.” The phosphorylation levels and the amounts of GST-4EBP1 were examined by immunoblotting using an anti-phospho-4EBP1(Thr37/46) and by Coomassie Brilliant Blue (CBB) staining, respectively.

Previously, we also isolated two Tor2 mutants with a point mutation within the FRB domain (I1954T or A1957V) that conferred weak ramycin resistance on S. cerevisiae. Interestingly, Tor1 with a mutation at the homologous site (I1954V or A1957V), was recently identified as conferring caffeine resistance on S. cerevisiae and displaying enhanced kinase activity (29). Thus, we attempted to measure kinase activity of the mTOR mutants carrying the homologous mutation (I2017T or A2020V). FLAG-mTORST17T and FLAG-mTORST2020V mutants exhibited higher activity than wild-type FLAG-mTOR or FLAG-mTORSL1 (Fig. 2, lanes 2 and 4, and data not shown). Remarkably, introduction of both SL1 and I2017T mutations into FLAG-mTOR (FLAG-mTORSL1+IT) further increased the kinase activity (Fig. 2, lane 5). These results indicate that mutations in the kinase domain (V2198A, L2216H, and L2260P) and in the FRB domain (I2017T or A2020V) enhance the kinase activity of mTOR independently and, thus, had an additive effect on the kinase activity of mTOR.

5 T. Umeda and T. Maeda, unpublished information.
The mTOR Mutants Hyperactivate the mTORC1 Pathway—Next, we investigated the effect of expressing these mTOR mutants on the mTORC1 pathway in mammalian cells. We co-expressed HA-S6K1 as a reporter for the mTORC1 activity together with various FLAG-mTOR mutants in HeLa cells. The phosphorylation status of HA-S6K1 during amino acid starvation was examined using an antibody specific to phospho-Thr-389 of S6K1. Phosphorylation levels of Thr-389 was rapidly decreased in response to amino acid starvation in cells expressing FLAG-mTOR variants as in Fig. 2. After 48 h cells were transferred into fresh growth medium for 4 h and then incubated in DMEM deprived of amino acids (AA–) for indicated times (A, B, and C) or in DMEM containing 100 nM rapamycin together with lysis buffer, and lysate was analyzed by immunoblotting using the indicated antibodies. D, the effect of mTOR mutants on endogenous mTORC1 substrates. HeLa cells were transfected with pIRES-puro3-FLAG-mTOR variants as in Fig. 2. After 24 h, cells were trypsinized and re-seeded in puromycin-containing growth medium and then incubated for additional 24 h. Puromycin-resistant cells were transferred into fresh growth medium for 4 h and then incubated in DMEM deprived of amino acids for the indicated times.

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The effect of expressing the mTOR mutants on endogenous mTORC1 substrates was also investigated. We used pIRES-puro3-FLAG-mTOR, in which both mTOR and puromycin-N-acetyltransferase were translated bicistronically from a single mRNA using an internal ribosomal entry site (IRES). Thus, cells transiently expressing FLAG-mTOR could be selected by puromycin. HeLa cells were transfected with pIRES-puro3-FLAG-mTOR or its mutants variants and grown in a puromycin-containing medium. Selected transfectants were then incubated in an amino acid-deprived medium. During amino acid starvation, the phosphorylation levels of endogenous S6K and 4EBP1 were maintained higher in FLAG-mTOR mutants expressing cells as compared with that in wild-type FLAG-mTOR-expressing cells (Fig. 3D, lanes 1–4 versus 5–8, 9–12, and 13–16). Especially in FLAG-mTORSL1+IT-expressing cells, phospho-Thr-37/46 of 4EBP1 appeared to be retained nearly completely 60 min after amino acid deprivation (Fig. 3D, lanes 13–16). Furthermore, even in the amino acid-repleted condition (time 0), phosphorylation level of S6K was higher in FLAG-mTORSL1+IT-expressing cells than that in wild-type FLAG-mTOR-expressing cells (Fig. 3D, lanes 1 and 13). Taken together, our mTOR mutants could maintain phosphorylation of endogenous mTORC1 substrates as well as exogenously expressed HA-S6K1 under the amino acid-starved condition.

Most recently, Tamanoi and co-workers (30) have reported mTOR mutants that exhibit constitutive activation under nutrient-starved conditions. One of these mutants, carrying the E2419K mutation in the kinase domain, exhibited remarkable activity under the amino acid-starved condition in vitro and in vivo. To compare the mutant with our mutants, we introduced
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**A**

| FLAG-mTOR variants: | WT | SL1+IT | Buffer |
|---------------------|----|--------|--------|
| **Reaction time (min)** | 0  | 30 | 0  |
| anti-p-Akt (Ser473) | 1  | 3 | 5  |
| anti-Akt | 1  | 2 | 3  |
| Silver stain (FLAG-mTOR) | 1  | 2 | 4  |

**B**

| FLAG-mTOR variants: | WT | SL1+IT |
|---------------------|----|--------|
| **Serum- (h)** | 4  | 0  |
| anti-FLAG | 1  | 3  |
| anti-p-Akt (Ser473) | 1  | 2  |
| anti-Akt | 1  | 4  |
| anti-p-S6K (Thr389) | 1  | 3  |
| anti-S6K | 1  | 2  |

**C**

| FLAG-mTOR variants: | WT | SL1+IT |
|---------------------|----|--------|
| **IP:** anti-FLAG |    |        |
| anti-Rictor | 1  | 2  |
| anti-Raptor | 1  | 4  |
| anti-mLST8 | 1  | 3  |
| Lysate | 1  | 2  |

**D**

| FLAG-mTOR variants: | WT | SL1+IT |
|---------------------|----|--------|
| **Serum- (h)** | 4  | 0  |
| anti-FLAG | 1  | 3  |
| anti-p-Akt (Ser473) | 1  | 2  |
| anti-HA (Akt1-HA) | 1  | 4  |

**FIGURE 4.** Hyperactive mTOR exhibits modestly enhanced kinase activity toward Akt1 in vitro, whereas it exerts a limited effect on mTORC2 pathway in cells. A. *In vitro* kinase activity of the active mTOR mutant toward Akt1. HeLa cells were transfected with the expression plasmids for wild-type FLAG-mTOR (WT) or FLAG-mTORSL1+IT (SL1+IT). After 48 h, an *in vitro* kinase assay was performed as in Fig. 2 and under “Experimental Procedures.” As a negative control, kinase reaction was performed with anti-FLAG beads only (Buffer). The phosphorylation level of Akt1 was examined by using anti-phospho-Akt(Ser-473) antibody. IP, immunoprecipitation. B. The effect of the active mTOR mutant on endogenous Akt. HeLa cells were transfected with pIRES-puro3-FLAG-mTOR (WT) or pIRES-puro3-FLAG-mTORSL1+IT (SL1+IT) and selected by puromycin as described in Fig. 3D. Next, we expressed FLAG-mTORSL1+IT in HeLa cells and assessed phosphorylation of endogenous Akt under a serum-starved condition. Unexpectedly, in FLAG-mTORSL1+IT-expressing cells, the phosphorylation of endogenous S6K stayed higher in FLAG-mTORSL1+IT-expressing cells as compared with that in wild-type FLAG-mTOR-expressing cells (Fig. 4B, lanes 1–3 versus 4–6). Thus, the effect of hyperactive mTOR is limited to the mTORC1 pathway, although hyperactive mTOR exhibited enhanced kinase activity toward Ser-473 of Akt1 in vitro. One possible explanation for the different effects of the active mutant on mTORC1 and mTORC2 activities is that the disparity in activation of mTORC1 and mTORC2 by the active mutant cannot be attributed to different effects on assembly of two mTOR complexes of this mutant. To address this possibility, complex formation of FLAG-mTORSL1+IT was compared with that of wild-type FLAG-mTOR. Immunoprecipitates of FLAG-mTORSL1+IT and wild-type FLAG-mTOR contained similar amount of Raptor, Rictor, and mLst8 (Fig. 4C), indicating that the disparity in activation of mTORC1 and mTORC2 by the active mutant cannot be attributed to different effects on assembly of two mTOR complexes. Another possible explanation for the limited activation of Akt is that even if mTORC2 activity is enhanced, decreased plasma membrane phosphatidylinositol 1,4,5-trisphosphate caused by serum starvation renders Akt unavailable for phosphorylation by mTORC2. Consistently, when Akt1 was overexpressed, under the condition where restriction in E2419K amino acid substitution into FLAG-mTOR (FLAG-mTOR\textsuperscript{E2419K}) and examined the activity toward the phosphorylation of co-expressed HA-S6K1. As reported previously, in FLAG-mTOR\textsuperscript{E2419K} expressing cells, HA-S6K1 showed resistance to dephosphorylation under the amino acid-starved condition. However, in our assay condition, the phosphorylation level of HA-S6K1 was gradually decreased during amino acid starvation, similar to our mutants (Fig. 3E, lanes 13–15), and thus, mTOR\textsuperscript{E2419K} was by no means constitutively active. In addition, like our FLAG-mTOR\textsuperscript{SL1+IT}, FLAG-mTOR\textsuperscript{EK+IT}, which possessed both E2419K and I2017T mutations, also exhibited higher activity than the original mutants (Fig. 3E, lanes 16–18). Importantly, FLAG-mTOR\textsuperscript{SL1+IT} showed higher activity toward the phosphorylation of HA-S6K1 than FLAG-mTOR\textsuperscript{E2419K} or FLAG-mTOR\textsuperscript{EK+IT}. Thus, mTOR\textsuperscript{SL1+IT} would be more desirable to investigate the impact of mTORC1 hyperactivation on cellular functions.
availability of Akt1 for phosphorylation could be relieved, its phosphorylation was significantly sustained in cells expressing the active mutant even after serum starvation (Fig. 4D).

Expression of the Hyperactive mTORSL1+IT Mutant Increases Cell Size—The observation that the mTORSL1+IT mutant hyperactivates the mTORC1 pathway prompted us to evaluate the roles of mTOR in the regulation of cellular functions using gain-of-function analyses. First, we examined the cell size of hyperactive mTOR-expressing cells under nutrient-repleted conditions. HeLa cells were transfected with pIRES-puro3-FLAG-mTORSL1+IT and grown in the puromycin-containing growth medium as in Fig. 3D. The puromycin-resistant cells were transferred into growth medium (FBS+) or DMEM-containing amino acid mixture at 25% that in DMEM (AA 25%) and incubated for additional 24 h. Cells were subjected to cell size determination using Coulter Counter. Cell size distribution of FLAG-mTOR-expressing cells in FBS+ (gray dotted line) or in 25%AA (gray line) and that of FLAG-mTORSL1+IT expressing cells in FBS+ (black dotted line) or in 25%AA (black line) in a representative experiment is shown (upper panel). The mean cell diameters ± S.E. of five experiments are shown in a bar graph (lower panel). The increase in cell size in FLAG-mTORSL1+IT-expressing cells is significant as assessed by t tests (p < 0.05). The cell size reduction in FLAG-mTORSL1+IT-expressing cells is significantly lower compared with that in FLAG-mTOR-expressing cells (p < 0.05).

FIGURE 5. Hyperactive mTOR mutant increases cell size. HeLa cells were transfected with pIRES-puro3-FLAG-mTOR (WT) or pIRES-puro3-FLAG-mTORSL1+IT (SL1+IT) and grown in the puromycin-containing growth medium as in Fig. 3D. Puromycin-resistant cells were transferred into amino acid-deprived DMEM containing (AA−) in the presence or absence of protease inhibitors (10 μg/ml pepstatin A and 10 μg/ml E64-d) and incubated for indicated times. The amount of endogenous LC3-I and LC3-II was examined by immunoblotting using anti-LC3 antibody.
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| TABLE 1 | Transformation assay for hyperactive mTOR |
|---------|-----------------------------------------|
| NIH 3T3 cells were transfected with the indicated expression plasmids. |
| G418-resistant colonies (per µg) | Foci (per µg) |
| Vector | 4227 | 8 |
| Wild-type mTOR | 3720 | 2 |
| mTORSL1+IT | 3467 | 8 |
| H-RAS12V | 3520 | 82 |

In contrast, in FLAG-mTORSL1+IT-expressing cells, accumulation of LC3-II was less obvious (Fig. 6, lanes 10–12). Thus, mTOR hyperactivation under the starvation condition rendered cells resistant to autophagy induction, demonstrating that autophagy was induced by starvation at least partly through mTOR inactivation.

Hyperactive mTOR Does Not Induce Cellular Transformation in NIH/3T3 Cells—To determine whether mTOR hyperactivation could contribute to tumorigenesis, we performed a transformation assay using NIH/3T3 cells. NIH/3T3 cells were transfected with expression plasmids for wild-type FLAG-mTOR and FLAG-mTORSL1+IT. We also transfected the cells with an empty vector and an expression plasmid for the oncogenic mutant of RAS (H-RAS12V) as a negative and a positive control, respectively. The number of foci of transformed cells in NIH/3T3 cells was counted, and the transformation efficiency for each expression plasmid was determined by counting G418-resistant colonies. The plasmid expressing H-RAS12V induced a reasonable number of transformed foci (Table 1). In contrast, the plasmid expressing either wild-type FLAG-mTOR or FLAG-mTORSL1+IT failed to induce conspicuous foci above the level found in the cells transfected with the empty vector. Because transfection efficiencies were in a similar range among the expression plasmids, this result suggests that hyperactive mTOR itself is not transforming to NIH/3T3 cells.

DISCUSSION

In this report we have isolated hyperactive mutants of mTOR (mTORSL1, mTORSL2, and mTORSL3) through screening for Tor2-mTOR chimera mutants that suppress temperature-sensitive growth of an lst8+ strain. We also revealed that I2017T and A2020V substitutions in the FRB domain enhance mTOR kinase activity, whose homologous mutations were previously isolated from independent screening by Powers et al. in Tor1 (29) and by our group in Tor2.5 Remarkably, we successfully obtained a potent activating mutant of mTOR by the combinatorial introduction of SL1 and I2017T mutations.

Interestingly, yeast mutants with homologous mutations are able to overcome TORC1 inactivation caused by a wide array of means. Mutations in Tor1 or Tor2 at sites homologous to Ile-2017 and Ala-2020 of mTOR confer S. cerevisiae cells resistance to both caffeine and rapamycin, and that to Val-2198, one of the mutation sites in the mTORSL1 mutant, also corresponds to the site in a Tor2 mutant allowing Rhb1-independent growth in Schizosaccharomyces pombe (30). We propose that enhanced kinase activity of TOR contributes in common to overcome TORC1 inactivation by divergent causes, although direct evidence has to be demonstrated.

What, then, is the mechanism underlying the enhanced kinase activity in our mTOR mutants? In the case of mTORSL1, we found that two mutations (V2198A and L2216H) are important for the enhanced kinase activity (data not shown). Interestingly, these two mutations were located on the same αC helix when the three-dimensional structure of the mTOR kinase domain was modeled based on the similarity to phosphatidylinositol-3-phosphate kinase γ (not shown). This α-helix corresponds to the αC helix conserved in the structures of kinase domains, which is known to be an important mediator of conformational changes in a kinase catalytic center (36). In the fibroblast growth factor receptor 2 (FGFR2) kinase, a naturally occurring activating mutation (K526E) located on αC leads to kinase activation by disrupting the “molecular brake” in the kinase hinge region as revealed by a structural analysis (37). Thus, the mutations located on the α-helix of mTOR corresponding to αC might contribute to the enhanced kinase activity in a similar manner. A few activating mutations on the same putative α-helix were also isolated in Tor2 of S. pombe, confirming the importance of this region on the kinase activity of TOR (30). Although it also resides in the kinase domain, the E2419K mutation seems to activate mTOR in a different way. The E2419K mutation is just adjacent to the repressor domain and, thus, was proposed to relief repression of the kinase activity mediated by this domain (30). In contrast to these mutations in the kinase domain, the I2017T mutation is in the FRB domain. Reinke et al. reported that a mutation at the homologous site in Tor1 increased Kog1 binding (29). However, in our coprecipitation experiment, the interaction between Raptor and FLAG-mTOR2017T or FLAG-mTORSL1+IT was similar to wild-type FLAG-mTOR (Fig. 4C and data not shown). Furthermore, we found that the kinase activity of FLAG-mTOR2017T was higher than wild-type FLAG-mTOR even if Raptor was removed from the mTOR complex by washing immunoprecipitated mTOR with a Triton X-100-containing buffer (data not shown). Because Ile-2017 is located within the binding region to FKBP38 (38), it is possible that the association with FKBP38 might be changed in this mTOR mutant. Further investigation is needed to clarify the mechanism by which the I2017T mutation causes the enhanced kinase activity.

Irrespective of the mode of action, our hyperactive mTOR mutants would be a useful tool for investigating the direct biological function of mTOR because of its potent ability to activate the mTORC1 pathway as evidenced by the prolonged phosphorylation of S6K and 4EBP1 under the amino acid-starved condition (Fig. 3). We re-evaluated the involvement of mTOR activity in previously attributed cellular functions from gain-of-function aspects. In hyperactive mTOR-expressing cells, cell size was enlarged, which is consistent with a previous report that overexpression of S6K1 or eIF4E resulted in cell size enlargement (39). Notably, hyperactivation of the mTORC1 pathway partially interfered with cell size reduction caused by amino acid starvation, suggesting that inactivation of mTORC1 is a requisite for cell size reduction. These observations confirmed that the cell controls its own size in response to environmental nutrient availability through the mTORC1 activation and inactivation and further emphasized the importance of mTOR as the primary regulator of cell size.

It was uncertain whether starvation-induced autophagy is promoted through mTOR so far. Rapamycin treatment
induced autophagy in COS-7 cells (40), whereas leucine-deprivation and rapamycin treatment has an additive effect on autophagy induction in C2C12 cells (32). Furthermore, Kanazawa et al. (33) reported that rapamycin had no effect on the suppression of autophagy by amino acid addition in isolated hepatocytes. Our result from the gain-of-function approach argues that inactivation of mTOR is essential for autophagy induced by amino acid starvation. Slight accumulation of LC3-II observed in hyperactive mTOR expressing cells might suggest that another pathway also controls autophagy in parallel with the mTOR pathway, although it might also be due to slight inactivation of mTORC1 activity in hyperactive mTOR expressing cells.

mTOR activation has been implicated in tumorigenesis because rapamycin effectively prevents cellular transformation caused by hyperactivation of the phosphatidylinositol-3-phosphate kinase-Akt pathway (41). Overexpression of eIF4E, which acts as a downstream effector of mTORC1 and plays a key role in mRNA translation, also caused cellular transformation (42). Furthermore, the tumor suppressors TSC1/2 and LKB1 negatively regulate mTORC1 activity (43–46). These observations suggest that mTORC1 up-regulation is sufficient to induce cellular transformation by itself. However, our observation that the hyperactive mTOR mutant did not induce transformation of NIH/3T3 cells argues that concomitant activation of mTOR with additional pathways such as the Akt-dependent survival pathway rather than the sole activation of mTOR is required for cellular transformation. One possible reason for this is the feedback inhibition of the phosphatidylinositol-3-phosphate kinase-Akt pathway by hyperactivation of mTORC1. In fact, it has been previously demonstrated that hyperactivation of mTORC1 in the TSC-related tumors showed only a modest phenotype but resulted in more severe tumor if reactivation of the phosphatidylinositol-3-phosphate kinase-Akt pathway occurred by additional mutation in PTEN (47).

Unexpectedly, the hyperactive mTOR mutant was not able to fully activate the mTORC2 pathway. In hyperactive mTOR mutant expressing cells, endogenous Akt was dephosphorylated under the serum-starved condition in a similar manner as in wild-type mTOR-expressing cells, although in vitro kinase activity toward Akt1 was increased in hyperactive mTOR compared with wild-type mTOR. In contrast, endogenous S6K was resistant to the dephosphorylation under the same conditions, indicating that mTORC1 and mTORC2 substrates are differentially regulated and the mechanisms to dephosphorylate Akt1 are able to overcome enhanced kinase activity of mTORC2. Recently, Sabatini and co-workers demonstrated that in vitro kinase activity of mTORC2 was enhanced by insulin treatment, suggesting that the modification of kinase activity might control the Akt phosphorylation (21). In contrast, earlier reports indicated that phosphorylation of Ser-473 of Akt1 was dominantly controlled by the membrane-translocation of Akt1 through the production of phosphatidylinositol 3,4,5-trisphosphate, and thus, the kinase responsible for the phosphorylation of Ser-473 of Akt1 appears to be localized at the plasma membrane and constitutively active (48). Our result is consistent with the latter observation. Because Akt1 dissociates from the plasma membrane by a decrease in phosphatidylinositol 1,4,5-trisphosphate during serum starvation regardless of the kinase activity of mTORC2, Ser-473 of Akt1 could, thus, be dephosphorylated in the hyperactive mTOR-expressing cells. It remains possible, however, that hyperactive mTOR did not have enough activity to maintain the phosphorylation of Akt under serum-starved conditions, because we observed that the enhancement of the kinase activity of hyperactive mTOR toward Akt1 was much smaller than that toward GST-4EBP1. It is also possible that alteration of kinase activity of mTORC2 could selectively regulate the phosphorylation of substrates other than Akt. The effect of hyperactive mTOR on the other mTORC2 substrates including PKCα (8) is to be examined in a future study.

In conclusion, we obtained a hyperactive mTOR mutant that is able to activate the mTORC1 pathway more potently than the activated mTOR mutants previously reported (Fig. 3E) (30). This mutant proved to be a useful tool for re-evaluation of mTOR function from the gain-of-function approaches as well as to probe pathway epistasis and identify novel effectors of the mTOR pathway. In addition, we are now generating transgenic animals that express the hyperactive mTOR. The analyses of the animals will provide crucial knowledge on the role of mTOR in the nutrient response of the whole organism as well as that in tumorigenesis.

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