



Regulation of eIF-4E BP1 Phosphorylation by mTOR

Citation

Hara, Kenta, Kazuyoshi Yonezawa, Mark T. Kozlowski, Tadanori Sugimoto, Khurshid Andrabi, Qing-Ping Weng, Masato Kasuga, Ikuo Nishimoto, and Joseph Avruch. 1997. "Regulation of eIF-4E BP1 Phosphorylation by mTOR." *Journal of Biological Chemistry* 272 (42): 26457–63. <https://doi.org/10.1074/jbc.272.42.26457>.

Published version

<https://doi.org/10.1074/jbc.272.42.26457>

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:41483018>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)

Regulation of eIF-4E BP1 Phosphorylation by mTOR*

(Received for publication, April 22, 1997, and in revised form, July 9, 1997)

Kenta Hara^{‡§}, Kazuyoshi Yonezawa[¶], Mark T. Kozlowski[‡], Tadanori Sugimoto^{**},
Khurshid Andrabi[‡], Qing-Ping Weng[‡], Masato Kasuga^{**}, Ikuo Nishimoto^{‡‡},
and Joseph Avruch^{‡§§}

From the [‡]Diabetes Unit and Medical Services, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02129, [¶]Biosignal Research Center, Kobe University, Kobe 657, Japan, ^{**}The Second Department of Internal Medicine, Kobe University School of Medicine, Kobe 657, Japan, and ^{‡‡}Department of Pharmacology and Neurosciences, Keio University School of Medicine, Tokyo 160, Japan

The proteins eIF-4E BP1 and p70 S6 kinase each undergo an insulin/mitogen-stimulated phosphorylation *in situ* that is partially inhibited by rapamycin. Previous work has established that the protein known as mTOR/RAFT-1/FRAP is the target through which the rapamycin-FKBP12 complex acts to dephosphorylate/deactivate the p70 S6 kinase; thus, some mTOR mutants that have lost the ability to bind to the rapamycin-FKBP12 complex *in vitro* can protect the p70 S6 kinase against rapamycin-induced dephosphorylation/deactivation *in situ*. We show herein that such mTOR mutants also protect eIF-4E BP1 against rapamycin-induced dephosphorylation, and for both p70 S6 kinase and eIF-4E BP1, such protection requires that the rapamycin-resistant mTOR variant retains an active catalytic domain. In contrast, mutants of p70 S6 kinase rendered intrinsically resistant to inhibition by rapamycin *in situ* are not able to protect coexpressed eIF-4E BP1 from rapamycin-induced dephosphorylation. We conclude that mTOR is an upstream regulator of eIF-4E BP1 as well as the p70 S6 kinase; moreover, these two mTOR targets are regulated in a parallel rather than sequential manner.

Rapamycin is an immunosuppressive macrolide whose major cellular receptor is the cytosolic 12-kDa FK506-binding protein (FKBP12)¹ (1, 2). Rapamycin binds to FKBP12 at a single site, identical to the site bound by the structurally related drug FK506, and both agents, acting *in situ* as a drug-protein complex, are immunosuppressive through inhibition of T-cell proliferation. Despite these similarities, the two drugs operate through distinct mechanisms. The FK506-FKBP12 complex blocks T-cell receptor signal transduction by directly inhibiting

protein phosphatase 2B/calcineurin. The rapamycin-FKBP12 complex does not inhibit T-cell receptor signal transduction or calcineurin activity but rather inhibits interleukin-2-stimulated signal transduction, concomitant with a potent (>95%) and selective inhibition *in situ* of the p70 S6 kinase (3, 4), an enzyme critical for the G₁ to S transition, at least in some cells (5, 6). Rapamycin, acting indirectly *in situ*, causes a partial dephosphorylation and deactivation of p70; the direct target of rapamycin-FKBP12 complexes *in situ* relevant to the rapamycin-inhibition of the p70 S6 kinase is the protein known variously as RAFT-1/FRAP/RAPT-1 or mTOR.

The TOR proteins were first identified in *Saccharomyces cerevisiae*, where rapamycin (but not FK506) is growth-inhibitory. The TOR proteins are the product of one class of mutant genes that confer resistance to rapamycin-induced growth inhibition as a dominant phenotype (7, 8). The yeast (7–9) and mammalian TOR proteins (specifically, FRAP (10); RAFT-1 (11); RAPT-1 (12); and mTOR (13)) are >250-kDa polypeptides that contain at their carboxyl terminus a protein and/or lipid kinase catalytic domain, most closely related to those of the DNA protein kinase and the ATM, MEC1, and Tel1 checkpoint gene products, and somewhat more distantly related to the PI-3 kinases (14). The rapamycin/FKBP12 complex binds directly to TOR, and the TOR mutations that confer rapamycin resistance *in situ* result in a loss of rapamycin/FKBP12 binding (15–17). The role of TOR as the rapamycin target responsible for inhibition of p70 S6 kinase has been established by the work of Brown *et al.* (17), who showed that coexpression of p70 with certain mutant TORs, which lack the ability to bind rapamycin/FKBP12 complexes, confers partial resistance to the rapamycin-induced inhibition of coexpressed p70; a further mutation that inactivates the TOR catalytic domain abrogates this rescue of p70.

PHAS-1/eIF-4E BP1 is another rapamycin-sensitive protein; this 12-kDa polypeptide binds to the 7-methylguanosine cap-binding protein, eIF-4E, and prevents eIF-4E binding to p220/eIF-4G (18). The assembly of eIF-4E with the RNA helicase eIF-4A and the RNA binding protein eIF-4B on the p220/eIF-4G polypeptide creates a functional eIF-4F complex. Mitogens stimulate the phosphorylation of eIF-4E BP1 resulting in its disassociation from eIF-4E; the latter can then interact with p220/eIF-4G (19). Mitogen-stimulated phosphorylation eIF-4E BP1 is potentially inhibited by rapamycin (20–23); inasmuch as eIF-4F activity is limiting for the translation of certain mitogen-sensitive mRNAs (*e.g.* ornithine decarboxylase), it is likely that TOR controls the expression of these mRNA at least in part by regulating eIF-4E BP1 phosphorylation (19). The identity of the kinases operating upstream of eIF-4E BP1 *in situ* is not known. Although eIF-4E BP1 is phosphorylated by p42 mitogen-activated protein kinase *in vitro*, the MEK inhibitor

* This work was supported in part by National Institutes of Health Grant DK17776. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a fellowship from JDFI. Present address: Biosignal Research Center, Kobe University, Kobe Japan.

¶ Supported by a fellowship from JDFI.

§§ To whom correspondence should be addressed: Diabetes Unit, Massachusetts General Hospital, 149 13th St., Charlestown, MA 02129. Tel.: 617-726-6909; Fax: 617-726-5649; E-mail: avruch@helix.mgh.harvard.edu.

¹ The abbreviations used are: FKBP12, 12-kDa FK506-binding protein; PI, phosphatidylinositol; PCR, polymerase chain reaction; bp, base pair(s); HA, hemagglutinin; GST, glutathione S-transferase; CHO-IR, Chinese hamster ovary cells overexpressing human insulin receptors; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; DMEM, Dulbecco's modified Eagle's medium.

PD098059 does not inhibit mitogen-stimulated eIF-4E BP1 phosphorylation. Recombinant eIF-4E BP1 is not a substrate for the p70 S6 kinase *in vitro*; however, the activity of p70 S6 kinase and the phosphorylation of eIF-4E BP1 respond in a parallel fashion to a variety of inhibitors (wortmannin, SQ20006, cAMP congeners) (20–23). We therefore inquired whether 1) TOR is the rapamycin-sensitive element that regulates eIF-4E BP1 phosphorylation, and if so, whether 2) TOR signals to eIF-4E BP1 through the p70 kinase. We find that, as with p70, a rapamycin-resistant mutant of mTOR (S2035T) substantially overcomes the ability of rapamycin to inhibit the phosphorylation of eIF-4E BP1; in contrast, rapamycin-resistant variants of p70 are unable to rescue eIF-4E BP1 phosphorylation from inhibition by rapamycin. We conclude that TOR regulates the phosphorylation of both eIF-4E BP1 and the p70 S6 kinase; however, p70 is not upstream of eIF-4E BP1 but is regulated in parallel.

MATERIALS AND METHODS

Dulbecco's modified Eagle's minimal essential medium and fetal calf serum were purchased from Sigma. Protein G-agarose was from Life Technologies, Inc. Radioisotopes were obtained from NEN Life Science Products, and ECL reagents from Amersham Corp. The antibodies used were: 12CA5, a monoclonal antibody against influenza virus hemagglutinin (24); a monoclonal anti-FLAG antibody (M2) (Eastman Kodak Co.); and a polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 337–352 of p70 α 1 S6 kinase (25).

cDNA Cloning and Constructions—A cDNA fragment encoding mammalian TOR was isolated by PCR, using first strand cDNA from mouse brain and degenerate oligonucleotide 5'-GAIGA(C/T)ITI(A/C)GICA(A/G)GA and 5'-ICC(A/G)AAA/GTCIAT(A/G)TG based on two short sequences that are well conserved in the catalytic domains of bovine p110 PI-3 kinase and yeast TOR-1 and TOR-2 ((E/D)D(L/I)RQD and HIDFG). Two major DNA products, 500 and 600 bp, were generated, purified, subcloned into pUC vectors, and subjected to DNA sequencing. The 500-bp product was found to be identical to the kinase domain of human p110 β (26), and the 600-bp product was 76% identical to the kinase domain of yeast TOR2. The 600-bp PCR product was used to screen a λ Zap rat brain cDNA library (a gift from Dr. Ivan Gout, Ludwig Institute for Cancer Research, London). A set of overlapping clones were obtained of which the two largest clones contained an open reading frame of 2549 amino acids (289 kDa), 43% identical to yeast TOR-2 and identical in DNA sequence to RAFT-1 (11). To facilitate recovery after transient expression in mammalian cells, the mTOR cDNA was modified by insertion of epitopes at its amino terminus; a 9-amino acid epitope from the influenza virus hemagglutinin (HA) (24) was introduced immediately after the initiator methionine using PCR, and the HA-tagged mTOR was ligated into the vector pCDNA1 (Invitrogen) to give pCDNA1-HA-mTOR. The mTOR cDNA was also ligated into vector pCMV-FLAG so as to introduce a FLAG epitope at the amino terminus (pCMV-FLAG-mTOR). Mutants of mTOR were constructed by insertion of a *KpnI*-*XbaI* fragment, encoding bp 5897–8434, into the M13 vector; site-directed mutagenesis was performed by the Kunkel (27) method. Mutagenesis primers for each mutant were as follows: 5'-GTACAAGC-GAGTGGCCTCTTC-3' for Ser²⁰³⁵ → Thr, 5'-GTACAAGCAGCGGCC-TCTTC-3' for Ser²⁰³⁵ → Ala, 5'-CCCCAAAGTACAAGCGACGGGCC-TCTTC-3' for Ser²⁰³⁵ → Ala, 5'-GGTCCAGCATAAGCTTGGATGGGTGC-3' for Asn²³⁴³ → Lys. After performing mutagenesis, the *KpnI*-*XbaI* fragment of pCDNA1-HA-mTOR and pCMV-FLAG-mTOR was replaced by the mutant *KpnI*-*XbaI* fragment from M13. Each mutation was verified by DNA sequence analysis.

A cDNA encoding PHAS-1/eIF-4E BP1 was isolated by PCR using a human B cell cDNA library as template and the oligonucleotides: 5'-CGCAATCCATGTCCGGGGCAGCAGC (forward) and 5'-TGCTCTAGATTAATGTCCATCTC (reverse). The eIF-4E BP1 cDNA was verified by DNA sequence analysis and inserted into pCMV-FLAG.

The cDNA encoding the wild-type α 1 p70 S6 kinase and the Δ 2–46/ Δ CT104 mutant was described in Weng *et al.* (28), tagged at the amino terminus with an HA or FLAG epitope, and introduced into the vector pMT2. A cDNA encoding FKBP12 (a gift from G. Crabtree) was ligated into the pGEX vector and expressed in *Escherichia coli* as a GST fusion protein.

Cell Culture and cDNA Expression—Chinese hamster ovary cells overexpressing human insulin receptors (CHO-IR) (29), HEK293, and COS7 cells were maintained and cultured as described previously (28).

Transient expression of the recombinant proteins in mammalian cells (COS7, HEK293, or CHO-IR cells) employed transfection of plasmids by the lipofection method using LipofectAMINE (Life Technologies, Inc.). When multiple plasmids were co-transfected and subjected to different treatments prior to harvest, the cells were split 24 h after transfection, replated on the number of plates appropriate to that experiment, and harvested 24–48 h later.

Immunoprecipitation and Autophosphorylation—Cells were lysed in ice-cold buffer A (50 mM Tris/HCl (pH 8.0), 1% Nonidet P-40, 120 mM NaCl, 20 mM NaF, 1 mM benzamide, 1 mM EDTA, 6 mM EGTA, 20 mM β -glycerophosphate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin), and the extracts were centrifuged at 10,000 $\times$ g for 20 min. Aliquots of the supernatants were subjected to immunoprecipitation with monoclonal antibodies. The immunoprecipitates were adsorbed to protein G-agarose beads, washed twice with buffer A containing 0.5 M NaCl, and twice with the buffer consisting of 25 mM Hepes (pH 7.6) and 0.1% Nonidet P-40. The autophosphorylation reactions were initiated by addition of 25 mM Hepes (pH 7.6), 50 mM KCl, 10 mM MgCl₂, 0.1% Nonidet P-40, 20% glycerol, 1 mM DTT, and 100 μ M ATP (6 μ Ci of [γ -³²P]ATP), incubated for 15 min at 30 °C, and terminated by adding SDS sample buffer. The phosphorylated proteins were analyzed by SDS-PAGE and subsequent autoradiography.

Immunoblot and p70 S6 Kinase Assay—p70 S6 kinase activity was determined in immunoprecipitates by using 40 S ribosomal subunits (25, 28) or a synthetic S6 peptide KRRRLSSLRASTKSSESSQK as substrates. The immobilized immunoprecipitates were washed twice with buffer A containing 0.5 M NaCl, twice with 20 mM MOPS (pH 7.2), 10 mM β -glycerophosphate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride. The S6 peptide kinase reaction was initiated by adding the reaction mixture I (25 mM MOPS (pH 7.2), 12 mM MgCl₂, 2 mM EGTA, 0.5 mM DTT, 10 mM β -glycerophosphate, 0.5 μ M protein kinase inhibitor, 100 μ M ATP (2 μ Ci of [γ -³²P]ATP), and 0.1 mM S6 peptide). The 40 S S6 protein kinase assay used reaction mixture II (50 mM MOPS (pH 7.2), 12 mM MgCl₂, 2 mM EGTA, 1 mM DTT, 10 mM β -glycerophosphate, 0.5 μ M protein kinase inhibitor, 0.5 A₂₆₀ units of 40 S ribosomal subunits, and 60 μ M ATP (5 μ Ci of [γ -³²P]ATP). The reaction was continued for 20 min at 30 °C. The S6 peptide kinase assay was terminated by addition of 20 mM EDTA and 1.5 mM adenosine. The reaction mixtures were spotted on P81 phosphocellulose paper, followed by washes in 0.5% phosphoric acid; ³²P-labeled peptides were measured by Cerenkov counting. The 40 S kinase assay was terminated by adding SDS sample buffer followed by SDS-PAGE and autoradiography. Immunoblot was performed using the ECL method as described by the manufacturer (Amersham Corp.).

In Vitro Binding of mTOR to GST-FKBP12—Prokaryotic recombinant GST or GST-FKBP12 (15 μ g) were incubated with 15 μ l of glutathione-Sepharose beads (60 min at 4 °C), washed three times with 50 mM Hepes (pH 7.6), 0.5 M NaCl, and 0.1% Triton X-100, once with PBS. Immobilized GST fusion proteins were incubated in 1 ml of PBS with or without 500 ng/ml rapamycin; after 1 h at 4 °C, the PBS was discarded, and the cell lysate expressing mTOR was added, with or without 200 ng/ml rapamycin, as indicated in Fig. 2A. In Fig. 2B, all GST proteins and lysates contained rapamycin at the concentrations indicated above. COS7 cells or HEK293 cells expressing FLAG- or HA-tagged wild-type or mutant mTOR were extracted, 72 h after transfection, with buffer B (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 1% Triton X-100, 300 μ l/10-cm plate) and centrifuged at 10,000 $\times$ g for 20 min. The supernatants were diluted by adding 700 μ l of buffer B without detergent; aliquots from each extract were subjected to immunoprecipitation and immunoblot with anti-FLAG or anti-HA antibody to verify the expression of mTOR variants. The remaining supernatant was incubated with the immobilized GST proteins at 4 °C for 3 h. The beads were washed three times with buffer B containing 0.3% Triton X-100 and 0.5 M NaCl, once with 50 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% Triton X-100. In Fig. 2A, the bound proteins were eluted directly into SDS sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with monoclonal anti-FLAG antibody. The GST proteins were identified by Coomassie Blue stain. Alternatively, GST fusion proteins were eluted twice by the incubation in buffer that contained 100 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT, and 20 mM glutathione. One-quarter volume of the eluate was subjected to SDS-PAGE on a 14% acrylamide gel, followed by Coomassie Blue staining to verify that the eluted GST proteins were almost equal. Three-quarters volume of the eluates were separated by SDS-PAGE on 6% polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with anti-HA antibody to detect retained HA-TOR polypeptides.

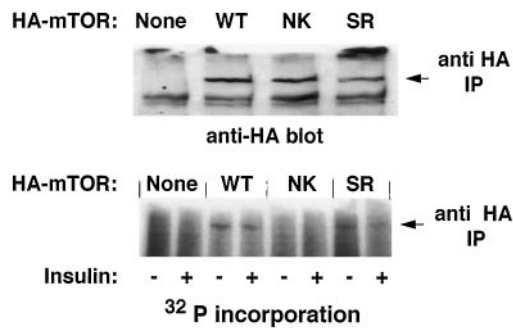


FIG. 1. Expression and *in vitro* autophosphorylation of mTOR variants. CHO-IR cells were grown in 100-mm dishes to 80% confluence and transfected with 12 μ g of pCDNA vector encoding HA-tagged mTOR wild type (WT), Asn²³⁴³ $\rightarrow$ Lys (NK), Ser²⁰³⁵ $\rightarrow$ Arg (SR), or pCDNA1 vector alone; 24 h later, each plate was split into three 60-mm dishes. At 60 h after transfection, media were replaced with Ham's F-12 medium without fetal calf serum. 72 h after transfection, cells were treated with carrier or insulin (10^{-7} M) for 15 min as indicated. Cell extracts were prepared, and immunoprecipitation was carried out with the anti-HA antibody, 12CA5. One set of immunoprecipitates was subjected to anti-HA immunoblot to verify mTOR expression (upper panel). The immunoprecipitates from control and insulin treated cells were subjected to *in vitro* autophosphorylation as described under "Materials and Methods"; the 32 P-labeled immunoprecipitates were subjected to autoradiography after SDS-PAGE (lower panel).

RESULTS

The mTOR cDNA was tagged at its amino terminus with an HA or FLAG epitope and expressed transiently after transfection into CHO-IR and COS cells. Anti-epitope immunoblot of anti-HA immunoprecipitates revealed a recombinant polypeptide of the expected size (Fig. 1, upper panel). Incubation of the immunoprecipitated HA-mTOR with Mg^{2+} and [γ - 32 P]ATP was accompanied by 32 P incorporation into the 290-kDa polypeptide (Fig. 1, lower panel, WT). The CHO-IR cells express approximately 10^6 recombinant human insulin receptors per cell; pretreatment of the cells with insulin (10^{-7} M) for 15 min prior to harvest did not alter the extent of *in vitro* phosphorylation of HA-mTOR (Fig. 1, lower panel). Evidence that the 32 P incorporation into mTOR *in vitro* reflected mTOR autophosphorylation was obtained by mutation of Asn²³⁴³ in the mTOR catalytic domain to Lys. This mutation corresponds in location to one previously reported to cause inactivation of the yeast VPS34 PI kinase (30). The mTOR mutant N2343K, which is expressed at levels comparable to wild-type mTOR (Fig. 1, upper panel, compare WT to NK), exhibits a complete loss of 32 P incorporation from [γ - 32 P]ATP into the recombinant polypeptide *in vitro*, whereas mutation of Ser²⁰³⁵ $\rightarrow$ Arg, which corresponds to the mutation which confers rapamycin resistance to yeast TOR2 (7, 8), does not significantly affect mTOR autophosphorylation *in vitro* (Fig. 1, lower panel, SR). These results demonstrate that the recombinant wild-type mTOR is expressed and is catalytically active. In agreement with Sabatini *et al.* (31), we observe that the recombinant wild-type mTOR, despite an ability to catalyze autophosphorylation, did not catalyze phosphatidylinositol phosphorylation *in vitro*. In contrast, an antiserum raised to an amino-terminal 100-amino acid fragment of mTOR, which precipitates endogenous mTOR, coprecipitated PI kinase activity (data not shown).

The ability of recombinant TOR to associate *in vitro* with GST-FKBP12 was determined. COS cell extracts containing recombinant FLAG-mTOR were incubated *in vitro* with purified prokaryotic recombinant GST or GST-FKBP12 immobilized on GSH-Sepharose. Little or no binding of FLAG-mTOR to GST-FKBP12 is detected, unless rapamycin (200 ng/ml) is present (Fig. 2A). Thus recombinant mTOR binds to FKBP12 *in vitro* in a rapamycin-dependent way. Previous

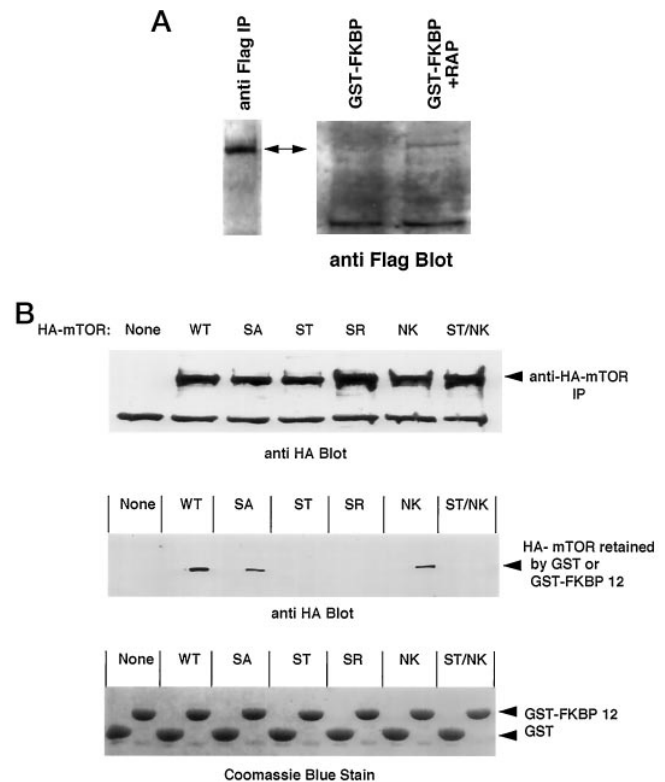
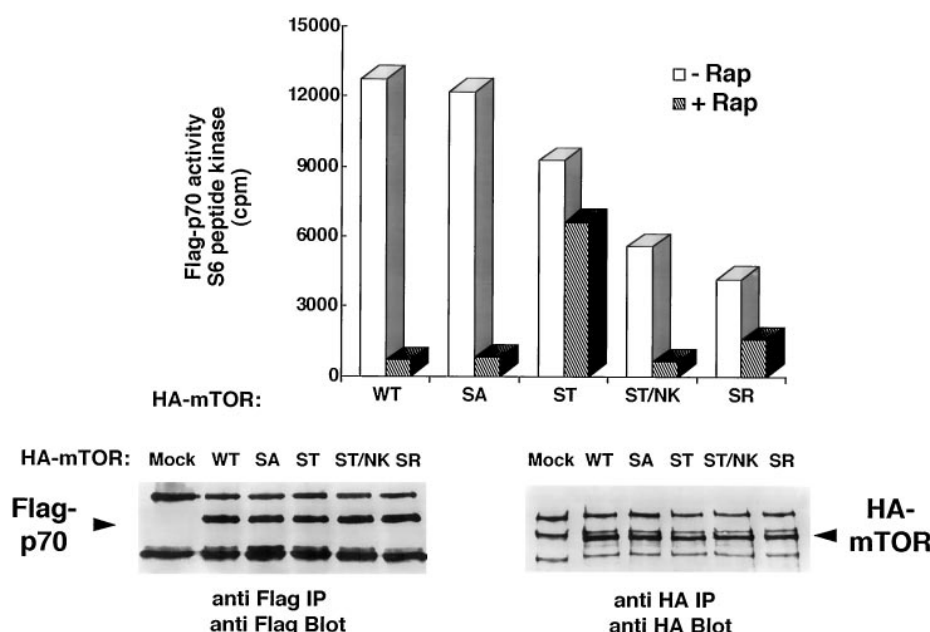


FIG. 2. Binding *in vitro* of mTOR variants to FKBP12/rapamycin. A, aliquots containing 15 μ g of prokaryotic recombinant GST-FKBP12 were immobilized on glutathione-Sepharose beads. Lysates prepared from COS7 cells transfected with plasmids encoding wild-type FLAG-mTOR were divided into three aliquots. One was subjected to anti-FLAG immunoprecipitation and immunoblot to verify mTOR expression. Rapamycin (final concentration, 200 ng/ml) or carrier was added to the remaining aliquots, which were incubated with the immobilized GST-FKBP12 for 3 h at 4 $^{\circ}$ C. The beads were washed and eluted into SDS sample buffer. The mTOR proteins retained by the GST-FKBP12 in the presence and absence of rapamycin were analyzed by anti-FLAG immunoblot. B, recombinant GST or GST-FKBP12 (15 μ g/aliquot) were immobilized on glutathione-Sepharose and prebound with rapamycin (500 ng/ml) for 1 h at 4 $^{\circ}$ C. Extracts prepared from 293 cells expressing recombinant HA-tagged mTOR variants (WT, wild type; SA, Ser²⁰³⁵ $\rightarrow$ Ala; ST, Ser²⁰³⁵ $\rightarrow$ Thr; SR, Ser²⁰³⁵ $\rightarrow$ Arg; NK, Asn²³⁴³ $\rightarrow$ Lys; NK/ST, double mutant) were divided into three aliquots. One was subjected to anti-HA immunoprecipitation and anti-HA immunoblot (top panel); the other two were brought to 200 ng/ml rapamycin and incubated with the immobilized GST or GST-FKBP12 at 4 $^{\circ}$ C for 3 h. The beads were washed and eluted twice with 0.1 M Tris (pH 8.0), 0.15 M NaCl, 1 mM DTT, and 20 mM glutathione. Three-quarters volume of the samples was separated by SDS-gel electrophoresis on a 6% polyacrylamide gel, transferred to PVDF membranes, and immunoblotted with anti-HA antibody (middle panel); a quarter volume of each eluate was run on a 14% polyacrylamide gel, followed by Coomassie staining (bottom panel), which showed that the eluted GST proteins were almost equal (~ 3 μ g).

work has defined the FKBP12 binding domain in yeast and mammalian TOR and shown that most mutations at Ser²⁰³⁵ (Ser^{1972/1975} in yeast TOR 1/2) can block the binding of mTOR to FKBP12/rapamycin (15–17, 32). We observe, consistent with previous reports (17, 32), that substitution of mTOR Ser²⁰³⁵ by Arg or Thr (Fig. 2B) results in the complete loss of binding of COS recombinant mTOR to GST-FKBP12- γ -rapamycin *in vitro*, whereas conversion of mTOR Ser²⁰³⁵ $\rightarrow$ Ala does not cause detectable impairment of mTOR binding to GST-FKBP12- γ -rapamycin. The catalytically inactive HA-mTOR N2343K mutant also binds to the GST-FKBP12- γ -rapamycin complex to an extent comparable to wild-type HA-mTOR.

Inasmuch as the biochemical function of mTOR relevant to its biologic role(s) is not known, we attempted to establish the

FIG. 3. Rescue of p70 kinase from rapamycin inhibition by mTOR variants. HEK293 cells grown to 80% confluence in 100-mm dishes were cotransfected with cDNAs encoding FLAG-p70(α I) (1 μ g) and HA-mTOR variants (12 μ g) as indicated (abbreviations as in Fig. 2B), or with empty vectors only (*mock*). At 24 h after transfection, each plate was split into five 60-mm dishes; at 72 h after transfection, duplicate plates were treated with carrier or rapamycin (200 nM, 30 min at 37 °C). After extraction, anti-HA immunoprecipitates were subjected to S6 peptide kinase assay (*upper panel*; each bar is the average of kinase assays from duplicate plates). The fifth plate from each set was subjected to immunoprecipitation and immunoblot with anti-FLAG antibody (*lower left panel*) and anti-HA antibody (*lower right panel*) to verify expression of p70 and the mTOR variants, respectively.



functionality of recombinant mTOR *in situ* by its ability to confer resistance toward the rapamycin inhibition of p70 kinase *in situ*. The activity of recombinant, wild-type HAp70 kinase toward an S6 peptide substrate (and 40 S subunits, see Fig. 5), assayed *in vitro* after transient expression in COS cells, is inhibited >95% by pretreatment of cells with rapamycin prior to extraction (>20 nM, 15 min). Half-maximal p70 inhibition is observed at approximately 2 nM (3, 28). Coexpression of p70 with wild-type FLAG-mTOR does not alter p70 activity *in vitro* as compared with FLAG vector control,² nor does the coexpressed wild-type mTOR alter the 95% inhibition induced by a supramaximal concentration of rapamycin (Fig. 3). The mTOR mutant S2035A, which is unimpaired in its binding to GST-FKBP12-rapamycin, like wild-type mTOR, is also unable to protect p70 against rapamycin inhibition. In contrast FLAG-mTOR S2035T, despite comparable polypeptide expression to wild-type and S2035A mTOR, does provide substantial protection against rapamycin inhibition of p70 kinase activity. The ability of mTOR S2035T to protect p70 from rapamycin inhibition requires a functional TOR catalytic domain; mutation of mTOR Asn²³⁴³ → Lys, which abolishes mTOR autophosphorylation *in vitro* (Fig. 1), also abolishes the ability of mTOR S2035T to rescue p70 from rapamycin inhibition (Fig. 3). Surprisingly, the mTOR S2035R mutant, which is capable of autophosphorylation *in vitro* (*i.e.* is catalytically active), and like S2035T, is unable to bind GST-FKBP12-rapamycin *in vitro* (Fig. 2B), nevertheless provides little or no protection for p70 against rapamycin inhibition *in situ*. Thus the S2035R mutation, in addition to preventing mTOR association with GST-FKBP12 rapamycin may also disable an mTOR function critical for regulation of p70 S6 kinase.

Recombinant FLAG-tagged eIF-4E BP-1, expressed transiently in 293 cells, migrates on SDS-PAGE as a ladder of polypeptide bands similar to that reported (22, 23) for endogenous PHAS/4E-BPs. Readdition of serum to deprived cells rapidly shifts a portion of the eIF-4E BP1 polypeptide to a slower mobility on SDS-PAGE, reflecting further phosphorylation of the eIF-4E BP1 polypeptide (Fig. 4A). Conversely, treatment of cells with rapamycin results in the disappearance of the most

slowly migrating eIF-4E BP1 polypeptides, reflecting partial dephosphorylation (Fig. 4B), similar to the response of p70 to rapamycin (3, 28). Coexpression of eIF-4E BP1 with mTOR S2035T greatly attenuates the rapamycin-induced dephosphorylation/downshift in mobility of eIF-4E BP1 (Fig. 4, B and C), whereas wild-type mTOR (Fig. 4, B and C) and mTOR S2035A (Fig. 4C) are without effect. As with p70, the ability of mTOR S2035T to protect 4E-BP1 against rapamycin induced-dephosphorylation is abolished by inactivation of the mTOR catalytic domain through an N2343K mutation (Fig. 4C).

The parallel changes in behavior of p70 S6 kinase and eIF-4E-BP1 in response to rapamycin and mTOR mutants could be due to regulation of both mTOR targets by common upstream element, or to the regulation of eIF-4E-BP1 by the p70 kinase itself (directly or indirectly). These two possibilities can be distinguished by use of recombinant versions of p70 that have been mutated to a form that is resistant to inhibition *in situ* by rapamycin. Deletion of a p70 amino-terminal noncatalytic segment (p70 α I amino acids 2–46 or 29–46) together with deletion of the p70 carboxyl-terminal 104 amino acids results in a p70 polypeptide that exhibits a serum-stimulated 40 S kinase that is approximately 20–50% that of wild-type p70 when both are expressed in 293 cells. Most importantly, the p70 Δ 2–46/ Δ CT104 variant is completely resistant to inhibition by rapamycin (28). To ascertain whether this rapamycin-resistant p70 variant can protect eIF-4E BP1 from rapamycin-induced dephosphorylation, 293 cells were cotransfected with expression vectors encoding Flag-eIF-4B-P1 with wild-type HA p70 or the rapamycin-resistant HA p70 Δ 2–46/ Δ CT104 or vector (Fig. 5). The mobility of recombinant eIF-4E BP1 and the activity of the recombinant p70 variants were examined with and without pretreatment with rapamycin. In the cells transfected with wild-type p70 (or empty vector), rapamycin induced a marked deactivation of p70 S6 kinase, as well as a downshift in the SDS-PAGE mobility of recombinant eIF-4E BP1. By contrast, the activity of the recombinant p70 Δ 2–46/ Δ CT104 was not inhibited by rapamycin. Despite the persistent activity of p70 Δ 2–46/ Δ CT104 in the presence of drug, rapamycin still caused a marked downshift in the mobility of eIF-4E BP1. This provides good evidence that p70 is *not* situated upstream of eIF-4E BP1 phosphorylation *in situ*.

² In five experiments, the activity of p70 coexpressed with wild-type FLAG-mTOR was $93.4 \pm 13\%$ that of p70 coexpressed with FLAG vector.

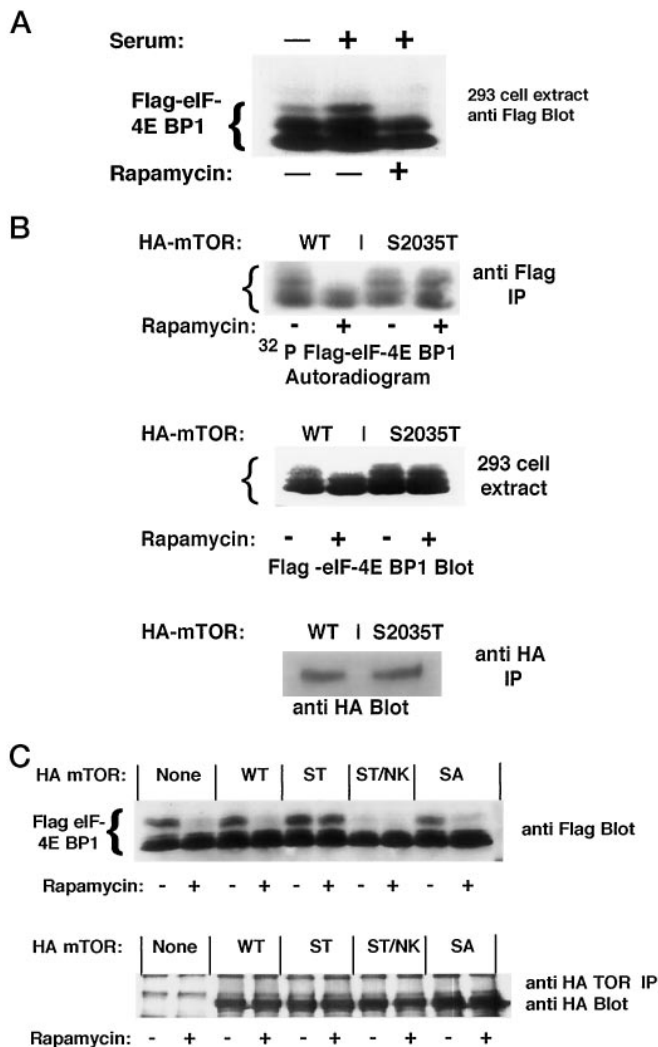


FIG. 4. Regulation of eIF-4E BP1 phosphorylation by rapamycin and mTOR. A, regulation of the phosphorylation of recombinant eIF-4E BP1 by serum and rapamycin. HEK293 cells were transfected with cDNA encoding FLAG-eIF-4E BP1 (1 μ g). At 36 h after transfection, the medium was replaced with serum-free DMEM. At 48 h after transfection, carrier or rapamycin (50 ng/ml) were introduced as indicated, and 15 min later, fetal calf serum (to a final concentration of 15%, v/v) or additional DMEM was added. Thirty minutes later, cells were rinsed and extracted, and aliquots of the extracts were subjected to SDS-PAGE and anti-FLAG immunoblot. B, mTOR S2035T prevents rapamycin-induced dephosphorylation of eIF-4E BP1. cDNA encoding FLAG-eIF-4E BP1 (1 μ g) and HA-mTOR, either wild-type (WT) or the Ser²⁰³⁵ → Thr mutant S2035T (12 μ g) were cotransfected into HEK293 cells. At 24 h after transfection, each 10-cm plate was split into four 6-cm dishes; 60 h after transfection, the medium was replaced with serum-free DMEM. Two dishes from each set of transfected cells were washed once with 2 ml of phosphate-free DMEM medium containing 0.1% bovine serum albumin and 20 mM Hepes and incubated in 2 ml of the same medium containing 0.4 mCi of [³²P]orthophosphate. After 90 min, cells were treated with or without 200 nM rapamycin for 30 min, then cells were stimulated with 15% fetal calf serum for 20 min. Cells were rinsed once with ice-cold PBS and extracted with buffer A. Lysates were centrifuged at 10,000 $\times g$ for 20 min, and the supernatants were divided into two aliquots. One aliquot was subjected to immunoprecipitation with anti-FLAG antibody, the immunoprecipitates were separated by SDS-PAGE, and the phosphorylated proteins were analyzed by autoradiography (top panel). The second aliquot was boiled for 5 min, cooled on ice, and centrifuged at 10,000 $\times g$ for 20 min. The heat-stable proteins remaining in the supernatant were separated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-FLAG antibody (middle panel). The remaining dishes of each transfection were extracted directly and subjected to anti-HA immunoprecipitation and immunoblot to verify the expression of mTOR variants (bottom panel). C, mTOR rescue of eIF-4E BP1 from rapamycin-induced dephosphorylation requires an intact mTOR kinase domain. HEK293 cells grown to 80% confluence in 60-mm dishes were cotransfected with cDNA encod-

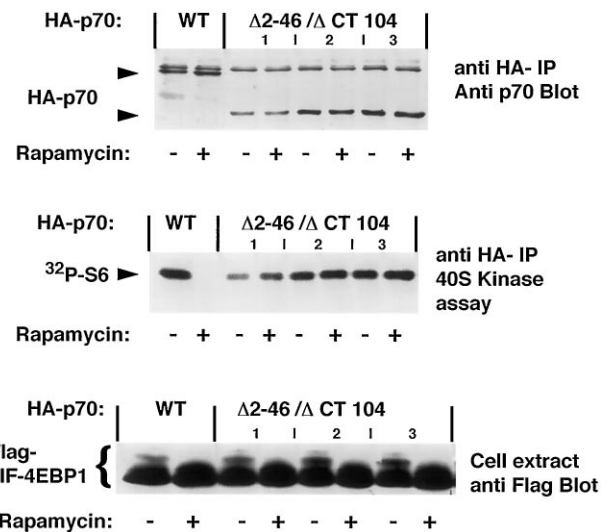


FIG. 5. A rapamycin-resistant p70 S6 kinase does not protect eIF-4E BP1 from rapamycin-induced dephosphorylation. HEK293 cells were transfected with cDNA encoding FLAG-eIF-4E BP1 (0.1 μ g) and either wild-type HA-p70 (WT, 0.15 μ g) or a rapamycin-resistant p70 variant, HA-p70 (Δ 2-46/ Δ CT104, lane 1, 0.4 μ g; lane 2, 0.6 μ g; lane 3, 0.8 μ g). At 30 h after transfection, the cells were deprived of serum. At 48 h after transfection, carrier or rapamycin (200 nM, final concentration) was added, followed after 30 min by the readdition of fetal calf serum to 10%, v/v. The cells were extracted into 0.3 ml of buffer A, and the supernatants were divided into two aliquots. One was subjected to immunoprecipitation with anti-HA antibody and followed by assay of 40 S S6 kinase. The kinase reaction mixtures were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blotted with polyclonal anti-p70 peptide antibody (top panel) or subjected to autoradiography (middle panel). The second aliquot of supernatant was boiled for 5 min and cooled, and the soluble heat-stable proteins were analyzed by anti-FLAG immunoblot (bottom panel).

DISCUSSION

The present results confirm many of the reported biochemical features of recombinant mammalian mTOR, the ability of recombinant mTOR to catalyze autophosphorylation *in vitro* (17), its inability to catalyze PI phosphorylation whereas immunoprecipitated endogenous TOR can catalyze this reaction (31), the ability of mTOR to bind GST-FKBP12 in a rapamycin-dependent fashion *in vitro*, and the effects of mTOR mutations at Ser²⁰³⁵ on this *in vitro* interaction (17, 32). In addition we confirm that the mTOR S2035T mutant, which is unable to bind GST-FKBP12-rapamycin *in vitro*, will partially protect the activity of recombinant p70 S6 kinase from inhibition by rapamycin when coexpressed with the p70 S6 kinase, and this protection requires an active mTOR catalytic domain (17).

The present results provide several new findings relating primarily to eIF-4E BP1. First, we show that the rapamycin-induced dephosphorylation of eIF-4E BP1, like that of p70, can also be rescued by mTOR S2035T, but not mTOR wild type, S2035A (Fig. 4), or S2035R (not shown). Thus, as with p70 S6 kinase, the rapamycin-sensitive element regulating the acti-

ing FLAG-eIF-4E BP1 (0.1 μ g) and HA-mTOR variants (4 μ g; abbreviations as in Fig. 2B). After 30 h, cells were deprived of serum. At 48 h after transfection, two dishes from each set were exposed to rapamycin (200 nM) or carrier as indicated. Thirty minutes later, serum was reintroduced to all plates to a final concentration of 10%, v/v. After a further 20 min of incubation, the cells were extracted into buffer A, cleared by centrifugation (10,000 $\times g$ for 20 min), and the supernatants were divided into two aliquots. One aliquot was boiled for 5 min and cooled, and the soluble heat-stable proteins were separated by SDS-PAGE and analyzed by anti-FLAG immunoblot (upper panel). The second aliquot was subjected to anti-HA immunoprecipitation and immunoblot to verify the expression of mTOR variants (lower panel).

vating phosphorylation of eIF-4E BP1 is mTOR. In addition, we demonstrate that coexpression of eIF-4E BP1 with a catalytically active, rapamycin-resistant mutant of p70 is not able to prevent rapamycin-induced dephosphorylation of eIF-4E BP1, indicating that the rapamycin-induced decrease in phosphorylation of eIF-4E is not the consequence of the inhibition of p70. Thus, whereas mTOR is upstream of eIF-4E BP1 as well as the p70 S6 kinase, p70 is not upstream of eIF-4E BP1. It is not yet clear whether TOR signals to a common element that controls the phosphorylation of both p70 and eIF-4E BP1, or whether separate pathways arise from directly from mTOR, each concerned with p70 or eIF-4E BP1.

The data in Figs. 3 and 4C clearly demonstrate that the ability of mTOR (S2035T) to protect both p70 and eIF-4E BP1 from rapamycin-induced dephosphorylation *in situ* is abolished by mutation of mTOR Asn²³⁴³ → Lys, a mutation that eliminates mTOR autophosphorylating activity (Fig. 1). Brown *et al.* (17) showed previously that the mTOR mutations D2338A and D2357E, which also abolish the *in vitro* mTOR autophosphorylation, abrogate mTOR S2035T or S2035I protection of p70 from rapamycin inhibition. Moreover, they showed that the rapamycin-FKBP12 complex added *in vitro* directly inhibits mTOR autophosphorylation. Thus it is clear that an active mTOR catalytic domain is required to maintain the phosphorylation state of the p70 kinase and eIF-4E BP1 *in situ*, and the rapamycin-FKBP12 complex, on binding to mTOR, interferes with its catalytic activity. Nevertheless, the manner by which mTOR phosphotransferase activity contributes to the phosphorylation of p70 and eIF-4E BP1 is not known. In preliminary experiments, we have not detected any ability of immunoprecipitated endogenous mTOR or recombinant baculovirally expressed mTOR to catalyze phosphate transfer directly from [γ -³²P]ATP to recombinant p70 S6 kinase or eIF-4E BP1 polypeptides. Nevertheless, it will be necessary to examine mTOR phosphotransferase activity toward p70 and eIF-4E BP1 under a variety of conditions before the possibility that mTOR directly catalyzes the phosphorylation of these proteins can be discarded. An alternative hypothesis is that mTOR acts on p70/eIF-4E BP1 indirectly, by controlling a set of protein kinases and/or protein phosphatases that regulate p70 and eIF-4E BP1. Moreover, we speculate that mTOR regulation of p70/eIF-4E BP1 phosphatase(s) is the more likely alternative, based on the observation that activation of (the rapamycin-resistant) p70 Δ 2–46/ Δ CT104 polypeptide *in situ* still requires a mitogen-activated, multisite phosphorylation that can be inhibited by wortmannin (at concentrations that inhibit PI-3 kinase), but is not impaired by rapamycin. Thus rapamycin inhibition of mTOR does not appear to inhibit the activating kinase(s) that control p70 Δ 2–46/ Δ CT104, pointing to the alternative explanation, *i.e.* negative regulation of an eIF-4E BP1/p70 phosphatase by the mTOR phosphotransferase. The role of the PI-4 kinase activity that coprecipitates with endogenous mTOR is unclear. We, like Sabatini *et al.* (31), have not detected PI kinase activity in association with recombinant wild-type mTOR, expressed in either mammalian or insect cells. Consequently, it is not clear whether PI kinase activity is an intrinsic property of the mTOR catalytic domain, or an associated activity that, like p70/eIF-4E BP1, requires the mTOR activity.

The inhibitory activity of the mTOR S2035R mutant toward p70 was unexpected. An S to R mutation at the corresponding site was found in a mutant yeast TOR-2 that conferred resistance to rapamycin-induced growth inhibition (7, 8). Consistent with this phenotype, the mTOR S to R mutation abrogates binding of the FKBP12-rapamycin complex; in addition, however, the yeast phenotype also implies that the S to R mutation

enables retention of sufficient TOR-2 function to permit continued yeast growth despite the rapamycin inhibition of the wild-type TOR-1 polypeptide. We therefore anticipated that mTOR S2035R, like mTOR S2035T, would rescue p70 from rapamycin-induced inhibition. The coexpression of mTOR S2035R with p70, however, results in a marked inhibition of p70 activity averaging $57 \pm 15\%$ inhibition in four experiments ($p < 0.05$). Inhibition of p70 is not observed on coexpression with mTOR wild-type,² S2035T, or S2035A. As suggested by the results in Fig. 3, it appears that mTOR S2035R does confer some protection of p70 from inhibition by rapamycin, although the protection is clearly less than that provided by mTOR S2035T, which preserves about 50–60% of initial p70 activity. Consequently, the inhibitory effect of mTOR S2035R itself, combined with the partial inhibition imposed by rapamycin, conspires to reduce p70 activity to levels perhaps 10–20% of control, making it difficult to distinguish the residual p70 activity seen after rapamycin addition in the presence of mTOR S2035R to that seen after rapamycin addition in the absence of recombinant mTOR.

The explanation for the inhibitory action of mTOR S2035R is not known. An attractive hypothesis is that mTOR S2035R, unlike S2035A, S2035T, or S2035I, although still capable of autophosphorylation (Fig. 1), is (in contrast to S2035A, S2035T, or S2035I) substantially impaired in its ability to signal to the p70 S6 kinase, but is able to interact nonproductively with one or more of the endogenous elements utilized by mTOR to signal to p70; mTOR S2035R thereby competes with endogenous mTOR for these effectors, and when overexpressed is able to sequester these mTOR effectors, and thus act as a dominant inhibitor of endogenous mTOR function. Consistent with this hypothesis, the mTOR N2343K mutation is also associated with a 50% inhibition of p70 S6 kinase activity (*e.g.* see Fig. 3) and an inhibition of eIF-4E BP1 phosphorylation (see Fig. 4C). Although these observations do not allow any firm conclusions concerning the biochemical mode of action of mTOR or the nature of its downstream effectors (33), they point to a strategy for the identification of the mTOR targets relevant to p70 and eIF-4E BP1. The identification of proteins that interact with mTOR, and further information concerning the kinases and phosphatases that regulate p70 and eIF-4E BP1 will enable the elucidation of the pathways downstream of mTOR.

Note Added in Proof—Brunn *et al.* (Brunn, G. J., Hudson, C. C., Sekulić, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr., and Abraham, R. T. (1997) *Science* **277**, 99–101) reported that immunoprecipitated endogenous and recombinant mTOR can catalyze the phosphorylation *in vitro* of eIF-4E-BP1; we have confirmed this result.

REFERENCES

- Kunz, J., and Hall, M. N. (1993) *Trends Biochem. Sci.* **18**, 334–338
- Schreiber, S. L. (1991) *Science* **251**, 283–287
- Price, D. J., Grove, J. R., Calvo, V., Avruch, J., and Bierer, B. E. (1992) *Science* **257**, 973–977
- Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992) *Nature* **358**, 70–73
- Lane, H. A., Fernandez, A., Lamb, N. J., and Thomas G. (1993) *Nature* **363**, 170–172
- Reinhard, G., Fernandez, A., Lamb, N. J. C., and Thomas, G. (1994) *EMBO J.* **13**, 1557–1565
- Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R., and Hall, M. N. (1993) *Cell* **73**, 585–596
- Cafferkey, R., Young, P. R., McLaughlin, M. M., Bergsma, D. J., Koltin, Y., Sathe, G. M., Faucette, L., Eng, W. K., Johnson, R. K., and Livi, G. P. (1993) *Mol. Cell. Biol.* **13**, 6012–6023
- Helliwell, S. B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R., and Hall, M. N. (1994) *Mol. Cell. Biol.* **14**, 105–118
- Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) *Nature* **369**, 756–758
- Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) *Cell* **78**, 35–43
- Chiu, M. I., Katz, H., and Berlin, V. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12574–12578

13. Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrecht, G., and Abraham, R. T. (1995) *J. Biol. Chem.* **270**, 815–822
14. Keith, C. T., and Schreiber, S. L. (1995) *Science* **270**, 50–51
15. Cardenas, M. E., and Heitman, J. (1995) *EMBO J.* **14**, 5892–5907
16. Stan, R., McLaughlin, M. M., Cafferkey, R., Johnson, R. K., Rosenberg, M., and Livi, G. P. (1994) *J. Biol. Chem.* **269**, 32027–32030
17. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) *Nature* **377**, 441–446
18. Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995) *EMBO J.* **14**, 5701–5709
19. Sonenberg, N. (1996) in *Translational Control* (Hershey, W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 245–269, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Alarcon, C. M., Cardenas, M. E., and Heitman, J. (1996) *Genes Dev.* **10**, 279–288
21. Beretta, L., Gingras, A.-C., Suitkeim, Y. V., Hall, M. N., and Sonenberg, N. (1996) *EMBO J.* **15**, 658–664
22. Azipiazu, I., Saltiel, A. R., DePaoli-Roach, A. A., and Lawrence, J. C., Jr. (1996) *J. Biol. Chem.* **271**, 5033–5039
23. Diggle, T. A., Moule, S. K., Avison, M. B., Flynn, A., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1996) *Biochem J.* **315**, 146–162
24. Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rogers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) *Mol. Cell. Biol.* **8**, 2159–2165
25. Grove, J. R., Banerjee, P., Balasubramanyam, A., Coffey, P. J., Price, D. J., Avruch, J., and Woodgett, J. R. (1991) *Mol. Cell. Biol.* **11**, 5541–5550
26. Hu, P., Mondino, A., Skolnik, E. Y., and Schlessinger, J. (1993) *Mol. Cell. Biol.* **13**, 7677–7688
27. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492
28. Weng Q.-P., Andrabi, K., Kozlowski, M. T., Grove, J. R., and Avruch, J. (1995) *Mol. Cell. Biol.* **15**, 2333–2340
29. Yonezawa, K., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuba, H., Shii, K., Yokono, K., Fukui, Y., Calas, B., Grigorescu, F., Dhand, R., Gout, I., Otsu, M., Waterfield, M. D., and Kasuga, M. (1992) *J. Biol. Chem.* **267**, 25958–25966
30. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) *Science* **260**, 88–91
31. Sabatini, D. M., Pierchala, B. A., Barrow, R. K., Schell, M. J., and Snyder, S. H. (1995) *J. Biol. Chem.* **270**, 20875–20878
32. Chen, J., Zheng, X.-f., Brown, E. J., and Schreiber, S. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4947–4951
33. Schmidt, A., Bickle, M., Beck, T., and Hall, M. N. (1997) *Cell* **88**, 531–542

Regulation of eIF-4E BP1 Phosphorylation by mTOR

Kenta Hara, Kazuyoshi Yonezawa, Mark T. Kozlowski, Tadanori Sugimoto, Khurshid Andrabi, Qing-Ping Weng, Masato Kasuga, Ikuo Nishimoto and Joseph Avruch

J. Biol. Chem. 1997, 272:26457-26463.

doi: 10.1074/jbc.272.42.26457

Access the most updated version of this article at <http://www.jbc.org/content/272/42/26457>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 32 references, 18 of which can be accessed free at <http://www.jbc.org/content/272/42/26457.full.html#ref-list-1>