The Rapamycin-Binding Domain Governs Substrate Selectivity by the Mammalian Target of Rapamycin

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The mammalian target of rapamycin (mTOR) is a Ser/Thr (S/T) protein kinase, which controls mRNA translation initiation by modulating phosphorylation of the translational regulators PHAS-I and p70^{S6K}. Here we show that in vitro mTOR is able to phosphorylate these two regulators at comparable rates. Both (S/T)P sites, such as Thr36, Thr45, and Thr69 in PHAS-I and the h(S/T)h site (where h is a hydrophobic amino acid) Thr389 in p70^{S6K}, were phosphorylated. Rapamycin-FKBP12 inhibited mTOR activity. Surprisingly, the extent of inhibition depended on the substrate. Moreover, mutating Ser2035 in the rapamycin-binding domain (FRB) not only decreased rapamycin sensitivity as expected but also dramatically affected the sites phosphorylated by mTOR. The results demonstrate that mutations in Ser2035 are not silent with respect to mTOR activity and implicate the FRB in substrate recognition. The findings also impose new limitations on interpreting results from experiments in which rapamycin and/or rapamycin-resistant forms of mTOR are used to investigate mTOR function in cells.

The mammalian target of rapamycin (mTOR, also known as FRAP or RAFT1) is a Ser/Thr (S/T) protein kinase that functions in a signaling pathway that has been implicated in the control of a wide variety of metabolic and transcriptional processes that lead to cell growth and/or proliferation (18, 36). Defining processes in cells that are mediated by mTOR has been greatly facilitated by the highly specific inhibitor rapamycin, a lipophilic macrolide originally isolated from a strain of *Streptomyces hygroscopicus* (1). Rapamycin binds mTOR with high affinity when presented in a complex with the FK506 binding protein with a molecular weight of 12,000 (FKBP12). The FKBP12-rapamycin binding domain (FRB) is located upstream of the catalytic domain between residues 2025 and 2114 (18, 36). This domain is essential for the protein kinase activity of mTOR, and mTOR lacking the FRB is not capable of supporting G₁ progression in cells (43). Ser2035 is critical for rapamycin binding, as mutation of this residue to any amino acid larger than Ala markedly reduces the binding affinity of the isolated FRB for FKBP12-rapamycin (12).

Studies with rapamycin provided the first evidence that mTOR was involved in the control of mRNA translation (6). Rapamycin blocks the activation of p70^{S6K} by growth factors or nutrient stimuli (13, 32), an action that has been proposed to decrease the translation of mRNAs containing a polypyrimidine motif (22). Rapamycin also promotes the dephosphorylation of PHAS-I (also known as 4E-BP1), thereby increasing PHAS-I binding to eIF4E and decreasing cap-dependent mRNA translation (3, 26). Both p70^{S6K} and PHAS-I are controlled by multisite phosphorylation. Of the eight phosphorylation sites in p70^{S6K}, Thr229, Ser371, Thr389, Ser404, and

Ser411 are sensitive to rapamycin (2, 15). These sites conform to one of two motifs. Thr229, Thr389, and Ser404 are flanked by hydrophobic residues. Ser371 and Ser411 are followed by proline residues, as are three other sites located in the auto-inhibitory domain in the COOH terminal region of the kinase. Both Thr229 in the activation loop and Thr389 must be phosphorylated for the kinase to be active (2, 15). The remaining sites have less critical modulatory roles. PHAS-I is phosphorylated in the following five sites, all of which conform to an (S/T)P motif: Thr36, Thr45, Ser64, Thr69, and Ser82 (16, 28). Except for Ser82, all are sensitive to rapamycin, although in some cells, the phosphorylation of Thr36 and Thr45 is inhibited less by rapamycin than the phosphorylation of Ser64 and Thr69 (17).

mTOR phosphorylates both PHAS-I and p70^{S6K} in vitro (7, 8, 10, 17, 21). The rate of PHAS-I phosphorylation is markedly enhanced by the mTOR antibody mTab1, whose epitope is located near the COOH terminus of mTOR (7). Deleting the mTab1 epitope also increases the PHAS-I kinase activity of mTOR, suggesting that the epitope is located in an inhibitory regulatory domain (RD) (39). The effects of mTab1 or of deleting the inhibitory domain on the phosphorylation of p70^{S6K} by mTOR have not been investigated. There are conflicting reports concerning the relative rates of phosphorylation and the sites phosphorylated in the two substrates. Under some assay conditions p70^{S6K} is phosphorylated much more rapidly than PHAS-I by mTOR (10), an observation that has led to the argument that mTOR phosphorylates p70^{S6K} in cells but not PHAS-I. Some investigators have concluded that Thr36 and Thr45 are the only sites in PHAS-I phosphorylated by mTOR (10, 17), whereas we have detected significant phosphorylation of Ser64 and Thr69 as well (7, 27). The lack of agreement with respect to the sites phosphorylated by mTOR is worrisome since there is little precedent for phosphorylation by a single protein kinase of sites as different as those con-

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FIG. 1. Phosphorylation of wild-type and mutant PHAS-I proteins by mTOR. 293T cells were transfected with pcDNA3 containing an AU1-mTOR insert. After 18 h extracts were prepared and immunoprecipitations were conducted with anti-AU1 antibody bound to protein G-agarose beads. After extensive washing, immune complexes were incubated for 90 min at 22°C without additions (open symbols) or with 0.25 mg of mTab1 (filled symbols)/ml before the beads were rinsed and suspended in 20 μl of buffer A. The kinase reactions were initiated by adding 20 μl of buffer A supplemented with 0.2 mM [γ - ^{32}P]ATP (2,000 mCi/mmol), 20 mM MnCl_2 , and 40 μg of wild-type PHAS-I, T36-PHAS-I, T45-PHAS-I, T69-PHAS-I, or 5A-PHAS-I per ml. After increasing times of incubation at 30°C, the reactions were terminated by adding SDS sample buffer. Samples were subjected to SDS-PAGE. (A) ^{32}P incorporation into the proteins was determined by scintillation counting of gel slices. The small amounts of ^{32}P incorporated into 5A-PHAS-I were subtracted from the values presented. The results for ^{32}P incorporation are expressed relative to the phosphorylation of wild-type PHAS-I that had been incubated for 60 min with mTab1-activated mTOR. At this time the substrate was phosphorylated to a stoichiometry of 0.08 mol/mol. Immunoblots were prepared with P-Thr36/45 antibodies (B) or P-Thr69 antibodies (C). The results represent signal intensities determined by laser densitometry and are expressed relative to the intensity of the wild-type PHAS-I signal after the 60-min incubation.

forming to the hydrophobic and proline motifs in p70^{S6K} and PHAS-I (23).

The present study addresses issues relating to the phosphorylation site specificity of mTOR. The findings are consistent with a model in which substrate selectivity is controlled by the FRB and the COOH-terminal region of mTOR.

MATERIALS AND METHODS

Antibodies. mTab1 was generated by immunizing rabbits with keyhole limpet hemocyanin conjugated to the NH₂-terminal cysteine of a peptide having a sequence identical to that of residues 2431 to 2451 in mTOR (7). A monoclonal antibody to the AU1 epitope was purchased from Berkeley Antibody Company. The phospho-specific antibodies, P-Thr36/45 and P-Thr69, were prepared as described previously (27). The sequence of amino acids immediately surrounding Thr36 and Thr45 is identical (20). Consequently, P-Thr36/45 binds to PHAS-I phosphorylated in either Thr36 or Thr45 (27). Phospho-specific antibody to the Thr389 site in p70^{S6K} was purchased from New England Biolabs.

Preparation of recombinant proteins. PHAS-I, T36-PHAS-I, T45-PHAS-I, T69-PHAS-I, and 5A-PHAS-I were expressed in bacteria and purified as described previously (28). These proteins lack an epitope tag and were only used in experiments for Fig. 1.

Histidine-tagged PHAS-I ([H⁶]PHAS-I) was used in most experiments. This protein (M_r , ~15,000) was purified as described previously (19). In experiments with wortmannin and fluorosulfonylbenzoyl adenosine (FSBA) (described later), [H⁶]PHAS-I was reduced and alkylated before use. This was accomplished by incubating the protein for 30 min at 37°C with 1 mM dithiothreitol (DTT), followed by a 30-min incubation with 3 mM *N*-ethylmaleimide (NEM) and exhaustive dialysis.

To prepare a histidine-tagged carboxyl-terminal fragment of p70^{S6K} (CT-p70^{S6K}), cDNA encoding amino acids from 332 to 502 of p70^{S6K} α -II (accession no. M60725) was inserted between the *Eco*RI and *Xho*I sites in pProEX HT (Invitrogen). *Escherichia coli* [BL21(DE3) containing pLysS] was transformed with pProEX HT-CT-p70^{S6K} and grown to an optical density at 600 nm of 0.8 before expression of CT-p70^{S6K} was induced by incubating the bacteria with 0.2 mM isopropyl- β -D-thiogalactopyranoside for 4 h at 37°C. The recombinant protein (M_r , ~22,600), which was recovered in inclusion bodies, was dissolved in 8 M urea and applied to a column containing Ni²⁺-nitrilotriacetic acid resin (Qia-gen). After stepwise removal of urea, CT-p70^{S6K} was eluted with 200 mM imidazole and dialyzed against 100 mM NaCl and 50 mM HEPES, pH 7.4.

Glutathione *S*-transferase (GST)-FKBP12 was expressed in bacteria and purified as described previously (19, 34).

mTOR expression constructs and site-directed mutagenesis of Ser2035. cDNAs encoding mTOR proteins with NH₂-terminal AU1 epitope tags were inserted into pcDNA3. The constructs used to express wild-type mTOR (wt), Ser2035→Ile mTOR (SI), Asp2338→Ala mTOR (KD), Δ (2433-2451) mTOR (Δ), Δ (2433-2451), Ser2035→Ile mTOR (SI/ Δ), Δ (2433-2451), Asp2338→Ala mTOR (KD/ Δ), Ser2448→Glu mTOR, and Ser2448→Glu, Ser2035→Ile mTOR were generated as described previously (previous designations are in parentheses) (39).

To generate cDNA encoding additional mutant mTOR proteins, the *Kpn*I-*Xho*I fragment was excised from wild-type pcDNA3-mTOR and inserted between the *Kpn*I and *Xho*I sites in pBluescript SK(-). This plasmid was used as a template for oligonucleotide-directed mutagenesis (Transformer Site-Directed Mutagenesis Kit; Clontech). Appropriately designed mutant oligonucleotides were used to convert the Ser2035 codon into Ala, Arg, Asp, Glu, Thr, and Trp codons and to convert the Cys2546 codon into Ala. In each case, an oligonucleotide that destroyed the unique *Sca*I site in pBluescript was used for selection. After mutagenesis the mTOR fragments were sequenced to confirm that the correct mutations were present and that no unexpected changes had been introduced. The mutant fragments were excised with *Kpn*I and *Not*I, which cuts just downstream of *Xho*I in pBluescript, and were used to replace the corresponding *Kpn*I-*Not*I fragment in wild-type pcDNA3-mTOR.

Cell culture and transfections. 293T cells were seeded into plastic tissue culture dishes (2×10^4 cells/cm²; Falcon) and cultured in a humidified atmosphere of 5.5% CO₂ in air for 24 h in growth medium composed of 10% (vol/vol) fetal bovine serum in Dulbecco's modified Eagle medium. The cells were transfected using TransIT-LT2 polyamine transfection reagent (Mirus, Madison, Wis.) and 5 μg of DNA per 100-mm-diameter dish.

Measurements of mTOR kinase activities. After culturing of cells for 18 h, extracts were prepared and AU1-mTOR proteins were immunoprecipitated as described previously by using anti-AU1 antibody bound to protein G-agarose beads (27). Prior to the kinase assay, the exhaustively washed beads were incubated at 22°C for 90 min without additions or with 2 to 5 μg of mTab1 in 20 μl of buffer A (50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM microcystin LR, 10 mM Na-HEPES, and 50 mM β -glycerophosphate, pH 7.4) and were then rinsed twice. In experiments with wortmannin and FSBA, DTT was omitted from the reaction mixture. Unless otherwise indicated, the beads were suspended in 20 μl of buffer A, and the kinase reactions were initiated by adding 20 μl of buffer A supplemented with 0.2 mM [γ - ^{32}P]ATP (2,000 mCi/mmol), 20 mM MnCl_2 , and

40 μ g of PHAS-I protein or CT-p70^{S6K} per ml. The reactions were terminated by adding sodium dodecyl sulfate (SDS) sample buffer.

Electrophoretic analyses. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) by using the method of Laemmli (25). Typically, 25% of each reaction (250 ng of substrate) was loaded. Relative amounts of ³²P incorporated into proteins were determined by phosphorimaging, and absolute amounts were determined by scintillation counting of gel slices. Immunoblots were prepared to detect mTOR and phosphorylated proteins as described previously (8, 27). Proteins were electrophoretically transferred to Immobilon (Millipore) membranes. Alkaline phosphatase-conjugated secondary antibodies and the Tropix Western-Star Chemiluminescence Kit were used to detect binding of the primary antibodies. Relative signal intensities were determined by scanning laser densitometry.

Other materials. cDNA encoding hemagglutinin (HA)-tagged p70^{S6K} in pRK7 was a generous gift of John Blenis. Rapamycin, wortmannin, and LY294002 were from Calbiochem-Novabiochem International. Caffeine, DTT, NEM, and FSBA were from Sigma Chemical Co. [γ -³²P]ATP was from Perkin-Elmer Life Science.

RESULTS

Phosphorylation of sites in PHAS-I by mTOR. To investigate the phosphorylation of sites in PHAS-I by mTOR, AU1-tagged mTOR was overexpressed in 293T cells and immunopurified with anti-AU1 antibodies. The washed immune complexes were incubated without or with mTab-1 before initiating the protein kinase reactions by adding Mn:[γ -³²P]ATP and wild-type PHAS-I, T36-PHAS-I, T45-PHAS-I, or T69-PHAS-I. The latter three proteins retain Thr36, Thr45, or Thr69, respectively, but have Ser/Thr→Ala in the other four (S/T)P sites. Thus, ³²P incorporation into the mutant proteins should provide an index of the phosphorylation of Thr36, Thr45, and Thr69. mTab-1 increased the rate of phosphorylation of all four PHAS-I substrates, determined both by ³²P labeling (Fig. 1A) and by immunoblotting with phospho-specific antibodies (Fig. 1B and C).

The reactivity of wild-type PHAS-I, T36-PHAS-I, and T45-PHAS-I with the P-Thr36/45 antibodies (Fig. 1B) and that of wild-type and T69-PHAS-I with the P-Thr69 antibodies (Fig. 1C) increased linearly with time when the proteins were phosphorylated with mTab1-activated mTOR. The ratio of the immunoblotting signals obtained with T36-PHAS-I and T45-PHAS-I was very similar to the ratio of ³²P incorporation into the two proteins, indicating that the P-Thr36/45 antibody provides a reliable index of the relative level of phosphorylation of Thr36 and Thr45. The amounts of the P-Thr36/45 antibody, as well as P-Thr69 antibody, bound decreased in a linear manner with dilution of phosphorylated PHAS-I (results not presented), supplying additional evidence that binding of the phospho-specific antibodies is proportional to the amount of the phosphorylated sites.

The absolute rates of phosphorylation of the PHAS-I proteins assessed by ³²P incorporation differed considerably, with phosphorylation of wild-type PHAS-I > T45-PHAS-I > T36-PHAS-I $\gg$ T69 PHAS-I (Fig. 1A). The very low rate of ³²P incorporation into T69-PHAS-I underestimates the phosphorylation of Thr69 in the wild-type protein, as immunoblotting with P-Thr69 antibodies indicated that phosphorylation of Thr69 was approximately eightfold higher in wild-type PHAS-I than in T69-PHAS-I (Fig. 1C).

Rapamycin sensitivity of the phosphorylation of PHAS-I and CT-p70^{S6K} by mTOR. Increasing concentrations of rapamycin, in the presence of 10 μ M GST-FKBP12, inhibited the phosphorylation of PHAS-I by mTOR; however, the maximum

effect represented a decrease of only about 50% (Fig. 2A and B). Surprisingly, sites in PHAS-I differed markedly with respect to the extent of inhibition produced by rapamycin (Fig. 2C and D). In this experiment, Thr36/45 phosphorylation was decreased by only 30%, even at the highest concentrations of rapamycin tested. Due to the relatively small inhibitory response, it was difficult to determine precisely the 50% inhibitory concentration (IC₅₀) for inhibition of Thr36/45 phosphorylation, but significant inhibition occurred in the low-nanomolar range of rapamycin concentrations. Thr69 phosphorylation was almost completely inhibited by concentrations of rapamycin above 1 μ M.

mTOR activity was also assessed by using a fragment of p70^{S6K}. CT-p70^{S6K} lacks the protein kinase domain but contains three sites (Ser371, Thr389, and Ser404) that are phosphorylated in a rapamycin-dependent manner in cells (2). Burnett et al. (10) demonstrated that a GST-tagged form of this fragment of p70^{S6K} was phosphorylated as efficiently as full-length p70^{S6K} by mTOR. When incubations were conducted without mTab1, CT-p70^{S6K} was phosphorylated much more heavily than PHAS-I (Fig. 2A). mTab1 increased the ³²P incorporation into CT-p70^{S6K} and the phosphorylation of Thr389, but the effects were much smaller than those on PHAS-I (Fig. 2A). The relatively small effect of mTab1 on CT-p70^{S6K} phosphorylation was not due to lack of available phosphorylation sites. Control experiments indicated that the phosphorylation reaction proceeded in a linear manner for at least 30 min and that only a small fraction of the CT-p70^{S6K} protein was phosphorylated (0.05 mol of ³²P/mol of CT-p70^{S6K} in Fig. 2A).

Increasing concentrations of rapamycin progressively decreased CT-p70^{S6K} phosphorylation (Fig. 2A and B). The maximum effect represented almost complete inhibition of phosphorylation. Moreover, the dose-response curve for inhibition of CT-p70^{S6K} phosphorylation (IC₅₀ $\approx$ 20 nM) was almost superimposable on that for inhibition Thr69 phosphorylation (IC₅₀ $\approx$ 25 nM) (Fig. 2B and D), supporting the conclusion that mTOR mediates the phosphorylation of Thr69 and CT-p70^{S6K}.

Effects of inhibitors on mTOR activity. Because Thr36/45 phosphorylation was only partially inhibited by rapamycin, we considered the possibility that a portion of Thr36/45 phosphorylation was catalyzed by a kinase other than mTOR. If this were the case, then phosphorylation of PHAS-I would be expected to be partly resistant to inhibition by other mTOR inhibitors.

Wortmannin is an active site inhibitor that has been shown to abolish mTOR autophosphorylation (9, 31). Another active site inhibitor is FSBA, an ATP analogue that binds covalently in the active site of many kinases and inhibits kinase activity (41). Both wortmannin and FSBA are unstable in the presence of sulfhydryl-reducing agents (9, 41). For this reason it was necessary to omit DTT from the reaction mixtures in experiments with these inhibitors. Interestingly, this omission markedly decreased PHAS-I phosphorylation. Reduction followed by alkylation of sulfhydryls in PHAS-I with *N*-ethylmaleimide obviated the need for DTT (results not presented), indicating that the effect of the DTT was primarily on the substrate. Thus, it was possible to investigate the effects of increasing concentrations of wortmannin and FSBA on mTOR activity by using

FIG. 2. Inhibitory effects of increasing concentrations of rapamycin on the phosphorylation of PHAS-I and CT-p70^{S6K} by mTOR. AU1-mTOR was overexpressed, immunoprecipitated, and incubated without and with mTab1 as described in the legend to Fig. 1. The samples were rinsed twice and incubated in 20 μ l of buffer containing no additions or 10 μ M GST-FKBP12 and increasing concentrations of rapamycin. Control experiments verified that, in the absence of GST-FKBP12, rapamycin was without effect on the phosphorylation of either [³H]PHAS-I or CT-p70^{S6K}. After 20 min 20 μ l of reaction mixtures containing 200 μ M [γ -³²P]ATP and 50 μ g of either [³H]PHAS-I or CT-p70^{S6K} per ml was added, and the samples were incubated for either 30 or 10 min, respectively, before the reactions were terminated by adding SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblots were prepared with phospho-specific antibodies to sites in PHAS-I. (A) Autoradiograms of ³²P-labeled [³H]PHAS-I and p70^{S6K} are presented. (B) The relative amounts of ³²P incorporated into [³H]PHAS-I and CT-p70^{S6K} were determined by using a phosphorimager. The results are expressed as percentages of the respective amounts of ³²P incorporated in the absence of rapamycin. The stoichiometries of phosphorylation of [³H]PHAS-I and CT-p70^{S6K} after incubation with mTab-1-treated mTOR in the absence of rapamycin were 0.12 and 0.05 mol/mol, respectively. (C) P-Thr36/45 and P-Thr69 immunoblots are shown. (D) The relative intensities of the bands in the immunoblots were determined by optical density scanning. The results are expressed as percentages of intensities of the bands from samples that had been incubated in the absence of rapamycin.

NEM-treated PHAS-I as the substrate. At concentrations above 1 μ M, wortmannin abolished the phosphorylation of both PHAS-I and CT-p70^{S6K} (Fig. 3A). Half-maximal inhibition of the phosphorylation of both PHAS-I and CT-p70^{S6K} (Fig. 3A), as well as inhibition of phosphorylation of Thr36/45 and Thr69 in PHAS-I (Fig. 3B) and Thr389 in CT-p70^{S6K} (results not presented), occurred at 200 nM, which is the concentration of the drug previously shown to inhibit half maximally mTOR autophosphorylation (9). FSBA did not fully inhibit the phosphorylation of either PHAS-I or CT-p70^{S6K} (Fig. 3A). The dose-response curves for inhibition of ³²P incorporation into PHAS-I with wortmannin and FSBA were almost identical to those for inhibition of the overall phosphorylation of CT-p70^{S6K} by the respective inhibitors.

The effects of increasing concentrations of two other mTOR inhibitors, LY294002 (9) and caffeine (38), on the phosphorylation of sites in PHAS-I were also assessed by immunoblotting with phospho-specific antibodies (Fig. 3B). Above concentrations of 10 μ M and 1 mM, respectively, LY294002 and caffeine abolished the phosphorylation of both Thr36/45 and Thr69. Dose-response curves for inhibition of Thr36/45 and Thr69 by LY294002 were essentially identical. Likewise, the phosphorylation of Thr36/45 and Thr69 exhibited nearly identical sen-

sitivity to caffeine. The half-maximal inhibition of phosphorylation by LY294002 occurred at a concentration of 2 μ M. Caffeine was approximately 100 times less potent than LY294002. The nearly identical sensitivity of Thr36/45 and Thr69 phosphorylation to the various inhibitors supports the conclusion that these sites are phosphorylated by mTOR.

Effect of mutations in the FRB and RD on mTOR activity. To investigate features in mTOR responsible for the quantitatively different effects of rapamycin and mTab1 on the phosphorylation of sites in PHAS-I, we compared the kinase activity of wild-type mTOR with the activities of several mutant mTOR proteins (Fig. 4). These mutations include removal of the mTab1 epitope and the following point mutations: Asp2338→Ala, Ser2448→Glu, and Ser2035→Ile. An Asp in the position corresponding to residue 2338 in mTOR is highly conserved in protein kinases, and it is essential for kinase activity (5, 8). Ser2448 is phosphorylated in response to insulin (29, 33, 37, 39), and the negative charge supplied by Glu is sometimes able to mimic the effect of phosphorylation. The Ser2035→Ile point mutation decreases markedly the affinity for rapamycin-FKBP12 (5) but has been otherwise assumed to be silent with respect to mTOR function. All of the mTOR proteins had NH₂ terminal AU1 epitope tags, and after ex-

FIG. 3. Effect of increasing concentrations of mTOR inhibitors on the phosphorylation of PHAS-I and CT-p70^{S6K}. (A) After immunoprecipitation of AU1-mTOR, immune complexes were incubated with 0.25 mg of mTab1/ml for 90 min. Samples were rinsed twice to remove DTT and were incubated in 20 μ l with increasing concentrations of wortmannin or FSBA, which were assessed in separate experiments. Reaction mixtures (20 μ l) containing 200 μ M [γ -³²P]ATP and 40 μ g of either NEM-alkylated [3 H]PHAS-I or CT-p70^{S6K} per ml were added. After 10 min the reactions were terminated by adding SDS sample buffer. The amounts of ³²P incorporated into [3 H]PHAS-I and CT-p70^{S6K} are expressed as percentages of the amount of ³²P incorporated into the respective proteins in the absence of inhibitors. (B) mTOR immune complexes were incubated with 2.5 mg of mTab1/ml before the samples were rinsed twice to remove DTT and were incubated with increasing concentrations of inhibitors. Separate experiments were performed to assess the effects of wortmannin, LY294002, or caffeine. After termination of the reactions, immunoblots were prepared using P-Thr36 and P-Thr69 antibodies. The relative intensities of the bands in immunoblots prepared with P-Thr36/45 and P-Thr69 antibodies were determined by optical density scanning. The results are expressed as percentages of intensities of the bands from samples that had been incubated in the absence of inhibitors.

pression in 293T cells, approximately equal amounts of the proteins were recovered with anti-AU1 antibody (Fig. 4). Table 1 summarizes results from four experiments in which relative levels of ³²P incorporation and phosphorylation of Thr36/45 and Thr69 were corrected for the slight differences in mTOR recovery.

Deleting the mTab1 epitope markedly increased the phosphorylation of PHAS-I (Fig. 4; Table 1). When incubated without mTab1, the rates of phosphorylation of Thr36/45 and Thr69 by Δ (2433-2451) mTOR were approximately 4- and 10-fold higher, respectively, than the phosphorylation of the sites by wild-type mTOR (Table 1). However, removal of the mTab1 epitope did not fully activate mTOR, since the rates of Thr36/45 and Thr69 phosphorylation by Δ (2433-2451) mTOR were still only 60 and 10%, respectively, of the rates at which these sites were phosphorylated by wild-type mTOR after incubation with mTab1. As expected, mTab1 was without effect on PHAS-I phosphorylation by Δ (2433-2451) mTOR, which lacks the mTab1 epitope (Table 1).

mTOR harboring an Asp2338 $\rightarrow$ Ala mutation exhibited little, if any, PHAS-I kinase activity, even after incubation with the activating antibody (Table 1). Likewise, introducing the Asp2338 $\rightarrow$ Ala mutation essentially abolished the kinase activ-

FIG. 4. Relative rates of phosphorylation of sites in PHAS-I by wild-type and mutant mTOR proteins. 293T cells were transfected with the pcDNA3 vector alone (Vector) or with pcDNA3 containing inserts for expression of the following AU1-tagged mTOR proteins: wild type, Ser2035 $\rightarrow$ Ile mTOR (Ser2035 $\rightarrow$ Ile), Asp2338 $\rightarrow$ Ala mTOR (D2338 $\rightarrow$ A), Δ (2433-2451) mTOR, Δ (2433-2451) forms of mTOR having an additional mutation in either Ser2035 [Δ (2433-2451), S2035 $\rightarrow$ I] or Asp2338 [Δ (2433-2451), D2338 $\rightarrow$ A]. After preparation of extracts, the mTOR proteins were immunoprecipitated with AU1 antibody and were then incubated at 22°C without or with mTab1 for 90 min before [3 H]PHAS-I phosphorylation was assessed. An autoradiogram of ³²P-labeled [3 H]PHAS-I is presented (³²P). Also shown are immunoblots prepared with P-Thr36/45 or P-Thr69 antibodies.

ity of Δ (2433-2451) mTOR. That a point mutation in the catalytic domain of mTOR abolishes activity provides independent confirmation that Thr36/45 and Thr69 sites are both phosphorylated by mTOR. Mutating Ser2448 to Glu had relatively little effect on the phosphorylation of either Thr36/45 or Thr69 in PHAS-I.

The rate of phosphorylation of PHAS-I by Ser2035 $\rightarrow$ Ile mTOR was much less than that by wild-type mTOR (Fig. 4; Table 1). Strikingly, almost no phosphorylation of Thr36/45 was detected by the rapamycin-resistant form of mTOR, even after it had been incubated with mTab1. The phosphorylation of Thr36/45 by Δ (2433-2451) mTOR was also markedly decreased by the Ile substitution. This point mutation did not simply cripple the kinase, as Thr69 phosphorylation was still observed after wild-type mTOR was incubated with mTab1 and as introducing the Ser2035 $\rightarrow$ Ile mutation into either Δ (2433-2451) mTOR or Ser2448 $\rightarrow$ Glu mTOR actually enhanced Thr69 phosphorylation (Table 1).

Effect of mutating residue 2035 on the phosphorylation of sites in PHAS-I and CT-p70^{S6K}. Because of the striking effect that mutating Ser2035 $\rightarrow$ Ile had on inhibiting the phosphorylation of Thr36/45 in PHAS-I, we investigated the effect of other amino acid substitutions at this position on phosphorylation of sites in PHAS-I and CT-p70^{S6K} (Fig. 5). Phosphorylation of sites in PHAS-I by Ser2035 $\rightarrow$ Ala mTOR was indistinguishable from that by wild-type mTOR (Fig. 5A, C, and D). CT-p70^{S6K} phosphorylation assessed by ³²P incorporation from [γ -³²P]ATP was somewhat higher in the Ala2035 mTOR incubated without the activating antibody (Fig. 5B); however, there was no difference between the rates of phosphorylation of CT-p70^{S6K} by the mutant and wild-type mTORs after incubation with mTab1. The Ala mutation did not prevent inhibition by rapamycin-FKBP12. This was not unexpected, since the

TABLE 1. Phosphorylation of Thr36/45 and Thr69 by mutant mTORs^a

Mutation in AU1 mTOR	Values for:					
	³² P incorporation		Thr36/45 phosphorylation		Thr69 phosphorylation	
	– mTab1	+ mTab1	– mTab1	+ mTab1	– mTab1	+ mTab1
None (wild type)	11.9 ± 2.1	100 ± 0.0	13.6 ± 5.2	100 ± 0.0	1.1 ± 0.1	100 ± 0.0
S2035→I	8.8 ± 2.3	20.7 ± 5.9	1.1 ± 0.1	4.9 ± 3.1	9.5 ± 6.7	65.5 ± 27.4
D2338→A	1.1 ± 0.5	1.9 ± 0.3	5.6 ± 5.5	7.3 ± 5.4	0.0 ± 0.0	0.0 ± 0.0
Δ(2433–2451)	52.4 ± 13.7	58.3 ± 15.8	59.3 ± 14.0	62.7 ± 19.3	11.1 ± 7.3	8.8 ± 4.7
Δ(2433–2451), S2035→I	17.6 ± 5.7	16.7 ± 5.2	9.7 ± 4.4	8.1 ± 4.3	43.6 ± 28.0	52.1 ± 23.6
Δ(2433–2451), D2338→A	0.5 ± 0.3	0.4 ± 0.4	0.9 ± 1.6	0.8 ± 0.6	0.4 ± 0.3	0.1 ± 0.1
S2448→E	15.2 ± 5.2	89.0 ± 18.1	9.0 ± 3.4	92.8 ± 9.1	6.0 ± 2.7	118.6 ± 31.8
S2448→E, S2035→I	10.4 ± 1.8	21.6 ± 4.8	4.3 ± 1.7	9.1 ± 3.5	24.5 ± 10.0	129.6 ± 38.8

^a Phosphorylation reactions were conducted as described in the legend to Fig. 6. The relative amounts of ³²P incorporated into [H⁶]PHAS-I were determined by phosphorimaging. Phosphorylation of Thr36/45 and Thr69 was assessed by immunoblotting with phospho-specific antibodies. The results are expressed relative to the respective signals obtained with wild-type AU1-mTOR that had been incubated with mTab1 and are mean values ± standard errors from four experiments.

Ala substitution did not significantly decrease the affinity of the isolated FRB fragment for rapamycin (12).

Mutating Ser2035 to Glu decreased the mTab1-stimulated phosphorylation of PHAS-I, although the phosphorylation that was observed was resistant to inhibition by rapamycin (Fig. 5A). The decrease in phosphorylation was not simply a matter of an acidic substitution, since an Asp substitution did not decrease Thr36/45 phosphorylation. The acidic substitutions inhibited the overall phosphorylation of CT-p70^{S6K} by 80 to 90% (Fig. 5B). The effects of an Ser2035→Arg mutation on both PHAS-I and CT-p70^{S6K} were comparable to those produced by the Glu mutation. The relatively conservative Ser2035→Thr mutation decreased the mTab1-stimulated phosphorylation of Thr36/45 in PHAS-I (Fig. 5C). The Ser2035→Thr mutation also decreased the phosphorylation of CT-p70^{S6K}, although the effect was less than that produced by the acidic and basic substitutions.

The Ser2035→Ile mutation substantially inhibited the ability of mTOR to phosphorylate CT-p70^{S6K} (Fig. 5B), but it had a much more dramatic effect on decreasing Thr36/45 phosphorylation (Fig. 5C). The marked inhibitory effect on Thr36/45 could not be attributed to a bulky hydrophobic residue, since substituting Trp for Ser2035 had little, if any, effect on PHAS-I phosphorylation when incubations were conducted without rapamycin (Fig. 5A, C, and D). Interestingly, the Trp substitution had a relatively modest inhibitory effect on the phosphorylation of CT-p70^{S6K} (Fig. 5B), while conferring marked rapamycin resistance to the phosphorylation of both PHAS-I and CT-p70^{S6K}. Thus, of the rapamycin-resistant mTORs, the activity spectrum of Ser2035→Trp protein was closest to that of wild-type mTOR incubated without rapamycin.

Effect of Ser2035 mutations on mTOR activity in vivo. To determine whether the in vitro measurements of mTOR activities were of value in predicting activities of mTOR in vivo, wild-type and mutant mTOR proteins were coexpressed with HA-tagged, full-length p70^{S6K} in 293T cells. The cells were then treated with rapamycin to inhibit endogenous mTOR before the HA-tagged kinase was immunoprecipitated and before the phosphorylation of Thr389 was assessed by immunoblotting (Fig. 6A). Rapamycin abolished the phosphorylation of Thr389 in cells transfected with wild-type mTOR. The range of protection to inhibition by rapamycin in vivo ranged from almost none with Ser2035→Ala mTOR to over 60% protec-

tion with the Ser2035→Trp mTOR. In Fig. 6B, the rapamycin-resistant Thr389-phosphorylating activity of the different rapamycin-resistant mTOR proteins in vitro is plotted against the relative level of Thr389 phosphorylation detected in cells. Linear regression analysis revealed a strong positive correlation ($r = 0.97$) between the in vivo and in vitro measurements (Fig. 6B).

DISCUSSION

Although it is well established that mTOR has a central role in the control of cell growth, the mechanisms involved in regulating the activity of this protein kinase are not well understood. The present study yielded several novel and unexpected findings that provide mechanistic insight into substrate recognition by mTOR and/or that have important practical implications for studies of mTOR function in cells.

Nature of the phosphorylation sites. After incubation with mTab1, mTOR phosphorylated PHAS-I and CT-p70^{S6K} at comparable rates (Fig. 2, for example). The fact that all of the sites known to be phosphorylated by mTOR conform to either an (S/T)P motif, such as those in PHAS-I, or an h(S/T)h motif (where h = a hydrophobic residue), such as Thr389 in p70^{S6K}, is consistent with the interpretation that the amino acid sequence context provides one of the determinants for phosphorylation by mTOR. Many Ser/Thr protein kinases also utilize primary structure in selecting a particular Ser or Thr for phosphorylation (23). However, few, if any, of these kinases recognize two motifs as different as those represented by the mTOR phosphorylation sites. The kinase domain of mTOR is more closely related to the lipid kinase phosphatidylinositol (PI) 3-kinase than to the larger family of Ser/Thr protein kinases (18, 36). Perhaps it should not be surprising that mTOR exhibits more flexibility in its substrate recognition than the typical protein kinase, since PI 3-kinase is able to phosphorylate both lipid and protein (4). Such flexibility does not appear to extend to other members of the PI 3-kinase-related protein kinases, which exhibit a strong preference for (S/T)Q sites (24).

Contributing to the complexity of the problem of substrate recognition by mTOR is evidence that prior phosphorylation of the TP sites in PHAS-I increases the rate of Ser64 phosphorylation by mTOR (17, 28). The low rate of phosphorylation by mTOR of Thr69 in T69-PHAS-I (Fig. 1C), which lacks

FIG. 5. Effect of introducing different mutations at position 2035 on the phosphorylation of sites in PHAS-I and CT-p70^{S6K}. Wild-type and mutant AU1-mTOR proteins were immunoprecipitated before being incubated for 90 min without or with 0.25 mg of mTAB1/ml and were then rinsed twice and incubated with or without 10 μM FKBP12 and 1 μM rapamycin for 20 min. Samples were then incubated for 10 min with [γ - ^{32}P]ATP and either [H]PHAS-I or CT-p70^{S6K}. The reactions were terminated, and samples were subjected to SDS-PAGE. Autoradiograms were prepared to allow detection of ^{32}P -labeled PHAS-I (A) and CT-p70^{S6K} (B). Immunoblots were prepared with P-Thr36/45 (C) and P-Thr69 (D) antibodies. The results are expressed relative to the respective signals obtained with AU1-mTOR that had been incubated with mTAB1 and are mean values + standard errors from three experiments.

Thr36 and Thr45, relative to that of Thr69 in wild-type mTOR is consistent with the hypothesis that prior phosphorylation also enhances Thr69 phosphorylation. However, prior phosphorylation of Thr36 and Thr45 cannot be strictly required for the phosphorylation of Thr69, since T69 PHAS-I was phosphorylated by mTOR. Also, mutating Ser2035 to Ile markedly decreased Thr36 and Thr45 phosphorylation while only modestly decreasing Thr69 phosphorylation (Fig. 5).

Mutations in Ser2035 affect more than sensitivity to rapamycin. mTOR proteins harboring different mutations in Ser2035 in the FRB exhibited a spectrum of activities with respect to phosphorylation of sites in PHAS-I and p70^{S6K} (Fig.

5). In a previous study, introducing a Trp2027→Phe mutation just upstream of Ser2035 abolished the phosphorylation of PHAS-I by mTOR (43). Thus, it is clear that certain mutations in the FRB are not silent with respect to mTOR function. The dramatic inhibition of Thr36/45 phosphorylation resulting from the Ser2035→Ile mutation was the most striking example of a switch in substrate selectivity. However, there were many other instances in which mutations at position 2035 exerted more pronounced effects on the phosphorylation of one substrate than on that of another. For example, mutating Ser2035→Arg markedly decreased the phosphorylation of Thr389 in p70^{S6K}, both in vitro and in vivo, but had relatively

FIG. 6. Effect of Ser2035 mutations in mTOR on the phosphorylation of p70^{S6K} in cells. 293T cells were cotransfected with cDNA encoding HA-p70^{S6K} and either wild-type AU1-mTOR (Ser) or with mTOR proteins harboring different mutations at position 2035. After 18 h the cells were incubated with 200 nM rapamycin for 1 h before extracts were prepared. HA-p70^{S6K} was immunoprecipitated with antibodies to the epitope. (A) Immunoblots prepared with antibodies to P-Thr389 or to the HA and AU1 epitopes. (B) The relative band intensities from the Thr389 blots were determined and expressed relative to the signal observed with wild-type mTOR from cells incubated without rapamycin. After correction for levels of expression of the different AU1-mTOR proteins, these values were plotted versus those obtained from P-Thr389 immunoblots of CT-p70^{S6K} samples that had been phosphorylated by different mTOR proteins in the presence of FKBP12 and rapamycin as described in the legend to Fig. 5. The results are means $\pm$ standard errors of three in vitro kinase experiments and means $\pm$ one-half the range of two experiments involving overexpression of HA-p70^{S6K}. The line and correlation coefficient (r) were determined by linear regression analysis.

little effect on the phosphorylation of PHAS-I (Fig. 5 and 6). There is precedence for differential effects of Arg mutations at the highly conserved Ser2035 equivalents in target of rapamycin proteins in yeasts. Mutating Ser1834 to Arg in *tor1*⁺ in *Schizosaccharomyces pombe* essentially abolished the function of *tor1*⁺ in sexual development (45), but *Saccharomyces cerevisiae* *tor1p* with such a mutation retains at least part of its functionality. Indeed, Ser1972 $\rightarrow$ Arg was one of the original mutations in *tor1p* that led to the discovery of the target of rapamycin proteins by allowing the budding yeast to grow in the presence of rapamycin (11).

Initial studies of the mTOR kinase demonstrated that autophosphorylation, now known to occur on Ser2481 (31), was not fully inhibited by rapamycin (9). Similarly, rapamycin had a modest inhibitory effect on the phosphorylation of Thr36/45 in

PHAS-I by mTOR (Fig. 2C and D). In contrast, the phosphorylation of CT-p70^{S6K} and Thr69 in PHAS-I was almost completely inhibited by sufficiently high concentrations of the drug (Fig. 2A and B). Because the rates of phosphorylation of the different sites were not affected equally, it is clear that rapamycin does not inhibit the catalytic center of mTOR. Rapamycin could potentially inhibit activity either by competitively inhibiting substrate binding or by inducing conformational changes that reduce substrate affinity.

Role of the FRB: a working hypothesis. The findings with rapamycin, as well as results of experiments with mTOR having mutations within the FRB, suggest that the FRB participates in substrate recognition. This hypothesis would account for the differential effects of Ser2035 mutations and rapamycin on the phosphorylation of sites in PHAS-I and CT-p70^{S6K}. It is unlikely that the FRB recognizes the proline adjacent to Thr36/45 and Thr69, since the sensitivities of these sites to rapamycin and Ser2035 mutations differ greatly. Instead, we propose that the FRB functions as an initial substrate discriminator. This domain might recognize motifs in p70^{S6K} and PHAS-I that directly facilitate binding of substrates to mTOR. Alternatively, the FRB may bind protein cofactors, such as those postulated by Nishiuma et al. (30), that present substrate to mTOR. The substrate or cofactor binding motifs are predicted to be distinct from the (S/T)P and h(S/T)h determinants. A correlative prediction of our working hypothesis is that interactions with the FRB position the substrates for phosphorylation by the catalytic domain, which phosphorylates those sites having the appropriate proline or hydrophobic determinants.

Recent reports describe two motifs, well removed from the phosphorylation sites, which facilitate the phosphorylation of PHAS-I and/or p70^{S6K} in cells. Tee and Proud (42) demonstrated that mutations of residues 13 to 16 in PHAS-I (Arg, Ala, Ile, and Pro, designated the RAIP motif) markedly decreased phosphorylation of the protein in cells. The RAIP motif is also present in PHAS-II but not in p70^{S6K}. Schalm and Blenis (35) identified a motif, designated TOS (for TOR signaling), which is found at the COOH termini of all PHAS isoforms and at the NH₂ terminus of p70^{S6K}. Mutating the TOS motif in p70^{S6K} significantly inhibited the activation of the kinase in cells and prevented the inhibitory effect of p70^{S6K} overexpression on PHAS-I phosphorylation. von Manteuffel et al. (44) had previously found that overexpressing either wild-type or kinase-dead forms of p70^{S6K} inhibited PHAS-I phosphorylation, an observation suggesting that the two proteins were phosphorylated by the same kinase, possibly mTOR. The TOS motif cannot be strictly required for phosphorylation of Thr389, since mTOR phosphorylated CT-p70^{S6K} (Fig. 2 and 7) which lacks the motif. Nevertheless, it will be interesting to determine how mutations to the RAIP and TOS motifs affect the phosphorylation of substrates by mTOR in vitro.

Role of the COOH-terminal RD. Binding of mTab1 or deletion of the mTab1 epitope had much more pronounced effects on increasing the phosphorylation of sites in PHAS-I than on sites in p70^{S6K}. Our results and earlier findings by Sekulić et al. (39) suggest that the mTab1 epitope is found within an inhibitory regulatory domain, which inhibits substrate interactions with mTOR. Binding of mTab1 is proposed to change the conformation, removing the barrier to substrate

FIG. 7. Model for control of mTOR activity. Details are described in the text.

binding, as does deletion of the mTab1 epitope (Fig. 7). Presumably, binding of $p70^{S6K}$ is less restricted by RD in the basal state than binding of PHAS-I, since mTab1 has a relatively small effect on the phosphorylation of $p70^{S6K}$. Because of the relatively low activity in the absence of mTab1, we have been unable to determine whether the antibody decreases the K_m of mTOR for PHAS-I.

PI 3-kinase-related kinases contain a region of homology referred to as the FAT domain (for FRAP, ATM, and TRRAP), which is located between amino acids 1382 and 1982 in mTOR (18). All of these kinases also contain a short, very highly conserved, COOH-terminal domain, designated FATC (FAT COOH-terminal domain), which is critical for the function of mTOR (40). It has been suggested that, because the FAT and FATC domains are always found together, molecular interactions occur between the two (18). Since the FAT domain is adjacent to the FRB, such interactions could be accommodated by slight modification of the working hypothesis in Fig. 7. However, the barrier to phosphorylation of sites in PHAS-I by the RD need not be the COOH terminus itself, as drawn in Fig. 7, nor must the RD directly interact with the substrate recognition motif, since the effects of an interaction with other regions of mTOR could in principle be propagated via conformation changes to the catalytic domain or to other regions of mTOR involved in substrate recognition.

Implications for studies of the phosphorylation of mTOR.

Ser2035 in the FRB represents a potential phosphorylation site. The inhibitory effects of acidic substitutions at this position predict that phosphorylation would inhibit activity towards $p70^{S6K}$. The finding that the Ser2035→Ala mTOR immunoprecipitated from 293T cells exhibited somewhat higher activity towards CT- $p70^{S6K}$ would be consistent with such an action (Fig. 5B). While there is no direct evidence that Ser2035 is phosphorylated, several studies have now demonstrated that Ser2448 may be phosphorylated by PKB in vitro and/or in response to PKB activation in cells (29, 37, 39). As phosphorylation occurs within the mTab1 epitope, it has been postu-

lated that phosphorylation mimics the effect of the activating antibody. If this were true, then our findings indicate that phosphorylation of Ser2448 should have a much larger effect on PHAS-I than on $p70^{S6K}$ (Fig. 1). In this connection, it is interesting that PHAS-I phosphorylation is very sensitive to changes in PKB activity, whereas $p70^{S6K}$ activity is affected only by membrane-targeted forms of PKB, which exhibit abnormally high activities (14).

Ser2448→Glu mTOR exhibited little, if any, difference in activity from wild-type mTOR (Table 1). This negative finding does not eliminate the possibility that phosphorylation of Ser2448 activates mTOR, since acidic substitutions are only sometimes able to substitute for phosphorylation in proteins. Because of the inability to efficiently phosphorylate Ser2448 in vitro, it has not been feasible to determine the effect of directly phosphorylating Ser2448 on mTOR activity. The potential role of Ser2448 phosphorylation on $p70^{S6K}$ and PHAS-I in cells has been investigated by comparing the effects of Ser2035→Ile mTOR and an Ser2035→Ile mTOR with an Ser2448→Ala mutation (39). After expression in HEK293 cells, which were incubated with rapamycin to inhibit endogenous mTOR, the effects of the two mTOR proteins on PHAS-I phosphorylation and $p70^{S6K}$ activity were indistinguishable. These results argue against a role of Ser2448 in controlling PHAS-I and $p70^{S6K}$, but there are potential complications that weaken this argument. For instance, whether it is reasonable to expect to observe much of an effect of preventing Ser2448 phosphorylation on the activity of the severely crippled Ser2035→Ile mTOR towards Thr36/45 is debatable.

Practical implications for studies of mTOR in cells. Since mTOR is the only known target affected by low-nanomolar concentrations of rapamycin in cells, inhibition of a process by such concentrations of the drug is believed to be strong evidence for an input from mTOR. The present results indicate that the converse is not true. Rapamycin is a far more subtle and/or selective inhibitor of mTOR function than would be predicted for a direct inhibitor of the mTOR kinase domain.

Thus, rapamycin treatment is not the equivalent of an mTOR knockout in mammalian cells, and the failure of rapamycin to block a process does not prove that mTOR is not involved.

The present findings with Ser2035 mutants also raise a cautionary note on the approach in which mTOR function is investigated by overexpressing a rapamycin-resistant mTOR, such as Ser2035→Ile mTOR. The presumption has been that rapamycin treatment abolishes the effect of endogenous mTOR and that activity of the rapamycin-resistant mTOR is representative of that of wild-type mTOR. The relative lack of effect of rapamycin on the phosphorylation of Thr36/45 in PHAS-I by mTOR (Fig. 2D) and the relative inability of Ser2035→Ile mTOR to phosphorylate Thr36/45 (Fig. 4 and 5; Table 1) indicate that neither assumption is safe. We believe that Ser2035→Trp mTOR, whose kinase activity spectrum most closely resembled that of wild-type mTOR (Fig. 5A to D), will be a better option than Ser2035→Ile mTOR for investigating mTOR in cells.

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REFERENCES

1. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* **14**:483–510.
2. Avruch, J., C. Belham, Q. Weng, K. Hara, and K. Yonezawa. 2001. The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog. Mol. Subcell. Biol.* **26**:115–154.
3. Beretta, L., A.-C. Gingras, Y. V. Vitkin, M. N. Hall, and N. Sonenberg. 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **15**:658–664.
4. Bondeva, T., L. Pirola, G. Bulgarelli-Leva, I. Rubio, R. Wetzker, and M. P. Wymann. 1998. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science* **282**:293–296.
5. Brown, E. J., P. A. Beal, C. T. Keith, J. Chen, T. B. Shin, and S. L. Schreiber. 1995. Control of p70 S6 kinase by kinase activity of FRAP *in vivo*. *Nature* **377**:441–446.
6. Brown, E. J., and S. L. Schreiber. 1996. A signaling pathway to translational control. *Cell* **86**:517–520.
7. Brunn, G. J., P. Fadden, T. A. J. Haystead, and J. C. Lawrence, Jr. 1997. The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH-terminus. *J. Biol. Chem.* **272**:32547–32550.
8. Brunn, G. J., C. C. Hudson, A. Sekulić, J. M. Williams, H. Hosoi, P. J. Houghton, J. C. Lawrence, Jr., and R. T. Abraham. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* **277**:99–101.
9. Brunn, G. J., J. Williams, C. Sabers, G. Wiederrecht, J. C. Lawrence, Jr., and R. T. Abraham. 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J.* **15**:5256–5267.
10. Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. USA* **95**:1432–1437.
11. Cafferkey, R., P. R. Young, M. M. McLaughlin, D. J. Bergsma, Y. Koltin, G. M. Sathe, L. Faucette, W. K. Eng, R. K. Johnson, and G. P. Livi. 1993. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol. Cell. Biol.* **13**:6012–6023.
12. Chen, J., X. F. Xheng, E. J. Brown, and S. L. Schreiber. 1995. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289 kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc. Natl. Acad. Sci. USA* **92**:4947–4951.
13. Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69**:1227–1236.
14. Dufner, A., M. Andjelkovic, B. M. Burgering, B. A. Hemmings, and G. Thomas. 1999. Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol. Cell. Biol.* **19**:4525–4534.
15. Dufner, A., and G. Thomas. 1999. Ribosomal S6 kinase signaling and the control of translation. *Exp. Cell Res.* **253**:100–109.
16. Fadden, P., T. A. J. Haystead, and J. C. Lawrence, Jr. 1997. Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. *J. Biol. Chem.* **272**:10240–10247.
17. Gingras, A.-C., S. P. Gygi, B. Raught, R. D. Polakiewicz, R. T. Abraham, M. F. Hoekstra, R. Aebersold, and N. Sonenberg. 1999. Regulation of 4E-BP1 phosphorylation: a novel two step mechanism. *Genes Dev.* **13**:1422–1437.
18. Gingras, A. C., B. Raught, and N. Sonenberg. 2001. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* **15**:807–826.
19. Haystead, T. A. J., C. M. M. Haystead, C. Hu, T.-A. Lin, and J. C. Lawrence, Jr. 1994. Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase. Identification of a site phosphorylated by MAP kinase *in vitro* and in response to insulin in adipocytes. *J. Biol. Chem.* **269**:23185–23191.
20. Hu, C., S. Pang, X. Kong, M. Velleca, and J. C. Lawrence, Jr. 1994. Molecular cloning and tissue distribution of PHAS-I, an intracellular target for insulin and growth factors. *Proc. Natl. Acad. Sci. USA* **91**:3730–3734.
21. Isotani, S., K. Hara, C. Tokunaga, H. Inoue, J. Avruch, and K. Yonezawa. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase *in vitro*. *J. Biol. Chem.* **274**:34493–34498.
22. Jefferies, H. B. J., C. Reinhard, S. C. Kozma, and G. Thomas. 1994. Rapamycin selectively represses translation of the “polypyrimidine tract” mRNA family. *Proc. Natl. Acad. Sci. USA* **91**:4441–4445.
23. Kennelly, P. J., and E. G. Krebs. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**:15555–15558.
24. Kim, S. T., D.-S. Lim, C. E. Canman, and M. B. Kastan. 1999. Substrate specificities and identification of putative substrates of ATM kinase family members. *J. Biol. Chem.* **274**:37538–37543.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
26. Lin, T.-A., X. Kong, A. R. Saltiel, P. J. Blackshear, and J. C. Lawrence, Jr. 1995. Control of PHAS-I by insulin in 3T3-L1 adipocytes: synthesis, degradation, and phosphorylation by a rapamycin-sensitive and MAP kinase-independent pathway. *J. Biol. Chem.* **270**:18531–18538.
27. Mothe-Satney, I., G. J. Brunn, L. P. McMahon, C. T. Capaldo, R. T. Abraham, and J. C. Lawrence, Jr. 2000. Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. *J. Biol. Chem.* **275**:33836–33843.
28. Mothe-Satney, I., D. Yang, P. Fadden, T. A. J. Haystead, and J. C. Lawrence, Jr. 2000. Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. *Mol. Cell. Biol.* **20**:3558–3567.
29. Navé, B., M. Ouwers, D. J. Withers, D. R. Alessi, and P. R. Shepherd. 1999. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino acid deficiency on protein translation. *Biochem. J.* **344**:427–431.
30. Nishiuma, T., K. Hara, Y. Tsujishita, K. Kaneko, K. Shii, and K. Yonezawa. 1998. Characterization of the phosphoproteins and protein kinase activity in mTOR immunoprecipitates. *Biochem. Biophys. Res. Commun.* **252**:440–444.
31. Peterson, R. T., P. A. Beal, M. J. Comb, and S. L. Schreiber. 2000. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.* **275**:7416–7423.
32. Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* **257**:973–977.
33. Reynolds, T. H., S. Bodine, and J. C. Lawrence, Jr. 2002. Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. *J. Biol. Chem.* **277**:17657–17662.
34. Sabers, C. J., M. M. Martin, G. J. Brunn, J. M. Williams, F. J. Dumont, G. Wiederrecht, and R. T. Abraham. 1995. Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270**:815–822.
35. Schalm, S. S., and J. Blenis. 2002. Identification of a conserved motif required for mTOR signaling. *Curr. Biol.* **12**:632–639.
36. Schmelzle, T., and M. N. Hall. 2000. TOR, a central controller of cell growth. *Cell* **103**:253–262.
37. Scott, P. H., G. J. Brunn, A. D. Kohn, R. A. Roth, and J. C. Lawrence, Jr. 1998. Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **95**:7772–7777.
38. Scott, P. H., and J. C. Lawrence, Jr. 1998. Attenuation of mammalian target of rapamycin activity by increased cAMP in 3T3-L1 adipocytes. *J. Biol. Chem.* **273**:34496–34501.
39. Sekulić, A., C. C. Hudson, J. L. Homme, P. Yin, D. M. Otterness, L. M. Karnitz, and R. T. Abraham. 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin (mTOR) in mitogen-stimulated and transformed cells. *Cancer Res.* **60**:3504–3513.

40. Takahashi, T., K. Hara, H. Inoue, Y. Kawa, C. Tokunaga, S. Hidayat, K. Yoshino, Y. Kuroda, and K. Yonezawa. 2000. Carboxyl-terminal region conserved among phosphoinositide-kinase-related kinases is indispensable for mTOR function *in vivo* and *in vitro*. *Genes Cells* **5**:765–775.
41. Taylor, S. S., A. R. Kerlavage, and M. J. Zoller. 1983. Affinity-labeling of cAMP-dependent protein kinases. *Methods Enzymol.* **99**:140–153.
42. Tee, A. R., and C. G. Proud. 2002. Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol. Cell. Biol.* **22**:1674–1683.
43. Vilella-Bach, M., P. Nuzzi, Y. Fang, and J. Chen. 1999. The FKBP12-rapamycin-binding domain is required for FKBP12-rapamycin-associated protein kinase activity and G1 progression. *J. Biol. Chem.* **274**:4266–4272.
44. von Manteuffel, S. R., P. B. Dennis, N. Pullen, A.-C. Gingras, N. Sonenberg, and G. Thomas. 1997. The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70^{s6k}. *Mol. Cell. Biol.* **17**:5426–5436.
45. Weisman, R., and M. Choder. 2001. The fission yeast TOR homolog, *tor1*⁺, is required for the response to starvation and other stresses via a conserved serine. *J. Biol. Chem.* **276**:7027–7032.