Two Motifs in the Translational Repressor PHAS-I Required for Efficient Phosphorylation by Mammalian Target of Rapamycin and for Recognition by Raptor*

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Mammalian target of rapamycin (mTOR) is the central element of a signaling pathway involved in the control of mRNA translation and cell growth. The actions of mTOR are mediated in part through the phosphorylation of the eukaryotic initiation factor 4E-binding protein, PHAS-I. In vitro mTOR phosphorylates PHAS-I in sites that control PHAS-I binding to eukaryotic initiation factor 4E; however, whether mTOR directly phosphorylates PHAS-I in cells has been a point of debate. The Arg-Ala-Ile-Pro (RAIP motif) and Phe-Glu-Met-Asp-Ile (tor signaling motif) sequences found in the NH2- and COOH-terminal regions of PHAS-I, respectively, are required for the efficient phosphorylation of PHAS-I in cells. Here we show that mutations in either motif markedly decreased the phosphorylation of recombinant PHAS-I by mTOR in vitro. Wild-type PHAS-I, but none of the mutant proteins, was communoprecipitated with hemagglutinin-tagged raptor, an mTOR-associated protein, after extracts of cells overexpressing raptor had been supplemented with recombinant PHAS-I proteins. Moreover, raptor overexpression enhanced the phosphorylation of wild-type PHAS-I by mTOR but not the phosphorylation of the mutant proteins. The results not only provide direct evidence that both the RAIP and tor signaling motifs are important for the phosphorylation by mTOR, possibly by allowing PHAS-I binding to raptor, but also support the view that mTOR phosphorylates PHAS-I in cells.

Many hormones, growth factors, and nutrients stimulate protein synthesis by promoting the phosphorylation of PHAS-I (also known as 4E-BP1), the prototypic member of a family of translational repressor proteins (1, 2). Nonphosphorylated PHAS-I binds tightly to the mRNA cap-binding protein, eIF4E, and blocks binding of eIF4E to eIF4G, a scaffolding protein that organizes several other important initiation factors, including eIF3, which links the complex to the 40 S ribosomal subunit (3). When phosphorylated in the appropriate sites, PHAS-I dissociates from eIF4E, allowing formation of the complex needed for the proper positioning of the 40 S ribosome and for efficient scanning of the 5′-untranslated region.

PHAS-I is phosphorylated in five sites (4, 5), all of which conform to a (Ser/Thr)-Pro motif (see Fig. 1). Insulin promotes the phosphorylation of Thr36, Thr45, Ser64, and Thr69 (4, 6, 7). Phosphorylation occurs in an ordered fashion, with phosphorylation of Thr36 and Thr45 preceding that of Thr69 and Ser64 (5, 8). The phosphorylation of Ser62 does not appear to affect eIF4E binding (9), and although phosphorylation of Ser64 markedly decreases binding in vitro, mutating Ser64 to Ala does not significantly change the amount of PHAS-I bound to eIF4E in cells (5). In contrast, Thr to Ala mutation of any of the three Thr-Pro sites increases eIF4E binding (5). Thus, it is clear that the Thr-Pro sites are important in controlling the function of PHAS-I.

Findings with rapamycin provided the first evidence implicating the mammalian target of rapamycin (mTOR) in the control of PHAS-I (6, 10). Rapamycin treatment of cells attenuates the phosphorylation of all four insulin-sensitive sites (4, 6, 7). When presented as a complex with its intracellular receptor, FKBP12, rapamycin binds with high affinity to mTOR (11). mTOR (also known as FRAP or RAFT1) (12–14) is a founding member of a family of Ser/Thr protein kinases that have catalytic domains homologous to that in phosphatidylinositolspecific 3-OH-kinase (15, 16). In reactions conducted in vitro, mTOR phosphorylated purified PHAS-I in Thr36, Thr45, Ser64, and Thr69, suggesting that (Ser/Thr)-Pro was a recognition motif for phosphorylation by mTOR (17, 18).

Although there is general agreement that PHAS-I phosphorylation in vivo is controlled by the mTOR signaling pathway, it has been argued that mTOR does not directly phosphorylate PHAS-I in cells. Uncertainty as to the role of mTOR as a PHAS-I kinase arose from the demonstration that mTOR phosphorylates S6K-1 in Thr389 which is flanked by nonprolyl hydrophobic residues (16). This finding clearly eliminates (Ser/Thr)-Pro as the sole determinant for phosphorylation and indicates that the features of the substrate that allow recognition by mTOR are more complicated than the primary sequence of amino acids surrounding the phosphorylation sites. mTOR was shown recently (18–20) to exist in a complex with raptor, a large (Mw = 150,000) protein possessing a unique NH2-terminal region followed by three HEAT motifs and seven WD-40 domains. Raptor has been shown to bind directly to both PHAS-I and S6K-1, and it has been suggested that raptor presents these proteins to mTOR for phosphorylation (18).

Although the structural motifs in the substrate that allow direct phosphorylation by mTOR have not been fully defined, two sequences in PHAS-I that are required for the efficient phosphorylation of the protein in cells have been described. The first, referred to as the RAIP motif because of the sequence of amino acids involved, is found in the NH2-terminal region of
PHAS-I (21). The second, referred to as the {TOS} motif, is formed by the last five amino acids (Phe-Glu-Met-Asp-Ile) in the protein (22). Disrupting the {TOS} motif by a Phe to Ala point mutation, or removing the {RAIP} motif either by Δ16 NH2-terminal truncation or by mutating Ile-Pro to Ala-Ala, markedly decreased phosphorylation of overexpressed PHAS-I in human embryonic kidney 293 cells (21, 22). In the present study, we have investigated the influence of the {RAIP} and {TOS} motifs on the phosphorylation of PHAS-I by mTOR.

EXPERIMENTAL PROCEDURES

Antibodies—Antibody to the COOH-terminal region of PHAS-I (23), the mTOR antibodies, mTab1 and mTab2 (17), and the phosphospecific antibodies, P-Thr36/45 and P-Thr36/45 (7), were generated as described previously (7, 17, 23). Because the sequences of amino acids immediately surrounding Thr36 and Thr45 are identical, the P-Thr36/45 antibodies bind to PHAS-I phosphorylated in either Thr36 or Thr45 (7).

To generate receptor antibodies, a peptide (CEKEKGSKLQWSRMDK) having a sequence identical to positions 36–53 in human raptor was coupled to keyhole limpet hemocyanin (19), and the peptide-hemocyanin conjugate was used to immunize rabbits as described previously (24). Antibodies were purified using columns containing affinity resins prepared by coupling the peptide to SulfoLink beads (Pierce). Monoclonal antibody 9E10, which recognizes the Myc epitope tag, and 12C5, which recognizes the HA epitope tag, were purified from hybridoma culture medium. Monoclonal antibody to the AU1 epitope tag was from Promega Corporation. Immunoreactivity Antibody Company

Mutations and Generation of PHAS-I Expression Vectors—Rat PHAS-I cDNA in pBluescript (SK+ was used as template for generating mutations (23). CDNA encoding PHAS-I with a Phe113 to Ala point mutation (F113A PHAS-I) was generated by using the PCR and primers of Ile15-Pro16 into Ala15-Ala16 (RAAA PHAS-I), site-directed mutagenesis of 19 amino acids (H9004) for bacterial expression or pCMV-Tag 3a (Stratagene) for expression in mammalian cells. Primers encoding the mutagenic procedures. mTOR antibodies, mTAb1 and mTAb2 (17), and the phosphospecific antibodies, P-Thr36/45 and P-Thr36/45 (7), were generated as described previously (7, 17, 23). Because the sequences of amino acids immediately surrounding Thr36 and Thr45 are identical, the P-Thr36/45 antibodies bind to PHAS-I phosphorylated in either Thr36 or Thr45 (7).

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Frame shifts encoding PHAS-I proteins were excised from pBluescript with BamHI and inserted into the BamHI site of either pET14b (Novagen) for bacterial expression or pCMV-Tag 3a (Stratagene) for expression in mammalian cells. Proper orientation of the inserts was determined by restriction mapping and confirmed by nucleotide sequencing.

Bacterial Expression and Recombinant of PHAS-I Proteins—The pET14b constructs encode NH2-terminal His-tagged proteins, which were expressed in bacteria and purified as described previously (25). Protein content was determined using the method of Smith et al. (26). To assess purity and to confirm concentration, samples (2 μg) of the recombinant proteins were subjected to SDS-PAGE (27). The proteins were found to be single bands of equal Coomassie Blue staining intensity. 2

Raptor Expression Construct—CDNA (4008 bp) encoding HA-tagged human raptor was assembled from three fragments, which were generated by PCR using primers based on the published raptor sequence (18–20). Fragment 1 (bp 1–1043) was amplified from I.M.A.G.E. clone 3635369, fragment 2 (bp 952–1968) was amplified from reverse-transcribed human skeletal muscle cDNA (7175–1; Clontech), and fragment 3 (bp 1963–4008) was generated from I.M.A.G.E. clone 6057826. BamHI sites at bp 1963–1968 were introduced into both fragment 2 and fragment 3. Creating these sites did not change amino acid coding, and it allowed joining of the pieces. All three fragments were inserted into pBluescript, and the three HA tag sequences were added. Next, CDNA encoding the triple HA tag from pHis3 (28) was amplified by PCR and ligated into the SalI and EcoRI sites of pBluescript. Fragment 1 was excised and inserted into the EcoRI and XmaI sites of this construct. Separately, fragment 3 was excised with BamHI and XmaI and inserted between these sites in pBluescript (SK–). Fragment 2 was then removed with XmaI and BamHI and inserted between these sites in the fragment 3/pBluescript construct. The resulting construct was digested with KpnI and XmaI, which cut pBluescript and the 5′ end of fragment 2, respectively. Between these sites was inserted the HA-fragment 1 cDNA that had been excised from pBluescript with KpnI and XmaI, thus generating full-length HA-tagged raptor. The raptor cDNA was excised from pBluescript with KpnI and NdeI and inserted between these sites in pcDNA3. The coding region was sequenced and found to be free of errors.

Overexpression of mTOR, Raptor, and PHAS-I Proteins—293T cells were seeded into plastic tissue culture dishes (2 × 106 cells/cm2; Falcon) and cultured in humidified 5.0% CO2 in air for 24 h in growth medium composed of 10% (v/v) fetal bovine serum in Dulbecco’s modified Eagle’s medium. For overexpression of mTOR, 293T cells were transfected with pcDNA3 alone or pcDNA3 containing an AU1-mTOR insert by using TransIT-LT2 (Mirus Corp., Madison, WI) and 5 μg of DNA/100-mm-diameter dish as described previously (15). Transfections for expressing PHAS-I proteins and HA-raptor were performed in exactly the same manner, except using the expression vectors described in the preceding paragraphs. Cells were used in experiments 18–20 h after transfection.

Immunoprecipitations—For AU1-mTOR and HA-raptor, cells were homogenized in buffer (750 μl/100-mm-diameter dish) containing 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1% Tween 20, 1 mM dithiothreitol, 2.5 mM MgCl2, 0.5 μM microcystin LR, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 0.05% Nonidet P-40, 10 μM sodium pyrophosphate, 50 mM NaF, 1% Nonidet P-40, and 50 mM sodium HEPES, pH 7.4 supplemented with 500 μM microcystin LR, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,000 rpm for 30 min at 4 °C. Supernatants (50 μl) of the supematants were incubated for 1 h with 10 μg/ml of anti-mouse IgG-agarose beads (20 μl packed; ICN Biomedicals). The beads were washed (1 ml buffer/wash) twice with lysis buffer and twice with buffer minus Nonidet P-40. Immune Complex Assay of mTOR Activity—Prior to the kinase assay, washed AU1-mTOR complexes were incubated at 21 °C for 90 min without additions or with 5 μg of mTab1 in 20 μl of Buffer A (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM microcystin LR, 10 mM Na-HEPES, and 50 mM β-glycerophosphate, pH 7.4). The beads were rinsed twice 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-32P]ATP (2000 μCi/mlmole), 20 mM MnCl2, and 40 μg/ml of wild-type mTOR. Reactions were terminated after 5 min by adding SDS sample buffer. Measurements under these conditions reflect the initial rate of phosphorylation, as less than 5% of the available substrates were phosphorylated, and the reactions have been shown to proceed linearly for 60 min (29).

Electrophoretic Analyses—Samples were subjected to SDS-PAGE before proteins were electrophoretically transferred to Immobilon (Millipore) membranes and immunoblotted as described previously (7). The amounts of 32P incorporated into PHAS-I proteins were determined by scintillation counting of gel slices. Relative levels of 32P incorporation were determined by phosphorimaging. Signal intensities of bands in immunoblots were determined by scanning laser densitometry.

RESULTS

Inhibitory Effects of Mutations of the TOS and RAIP Motifs on Phosphorylation of PHAS-I by mTOR—The effects of disrupting the TOS and RAIP motifs on phosphorylation of PHAS-I by mTOR were investigated by performing immune complex kinase assays. mTOR was expressed as an AU1 epitope-tagged protein in 293T cells and immunoprecipitated prior to the assay. After incubating the immunopurified mTOR with the antibody, mTab1, mTOR activity was assessed using

2 K. M. Choi, L. P. McMahon, and J. C. Lawrence, Jr., unpublished observations.
purified recombinant PHAS-I proteins as substrates. mTab1 activates mTOR by binding to an inhibitory regulatory domain located in the COOH-terminal region of mTOR (17, 31). Phosphorylation was assessed by $^{32}$P incorporation from $[\gamma-^{32}\text{P}]$ATP. As the three Thr-Pro sites are the preferred sites in PHAS-I for phosphorylation by mTOR, phosphorylation was also assessed by immunoblotting with P-Thr36/45 and P-Thr69 antibodies.

The mTOR motif, which is formed by the last five COOH-terminal amino acids in PHAS-I (Fig. 1), was disrupted by mutating Phe$^{113}$ to Ala (22). The mutant protein, designated F113A PHAS-I, was phosphorylated by mTOR at only 25% of the rate at which mTOR phosphorylated wild-type PHAS-I (Fig. 2). Disrupting the mTOR motif decreased the phosphorylation of Thr$^{36}$/Thr$^{45}$ and Thr$^{69}$ by 72 and 86%, respectively. In contrast, the Phe$^{113}$ to Ala mutation attenuated the phosphorylation of neither Thr$^{36}$/Thr$^{45}$ nor Thr$^{69}$ by mitogen-activated protein (MAP) kinase.

Two mutant proteins were generated to investigate the influence of the RAIP motif (21). In one the NH$_2$-terminal region containing the motif (Fig. 1) was removed by truncation, yielding Δ16 PHAS-I. The other involved a more conservative approach in which Ile$^{35}$ and Pro$^{46}$ were each mutated to Ala, resulting in RAAA PHAS-I (21). Although neither the truncation nor the double mutation attenuated the ability of mitogen-activated protein kinase to phosphorylate PHAS-I (Fig. 3), both reduced phosphorylation by mTOR (Fig. 2). Δ16 PHAS-I was phosphorylated at only one-third of the rate of the wild-type protein. Interestingly, truncation had a more pronounced effect on the phosphorylation of Thr$^{36}$/Thr$^{45}$ than on phosphorylation of Thr$^{69}$. The effects of the double mutation were somewhat smaller than those of truncation but comparable in the sense that the decrease in Thr$^{36}$/Thr$^{45}$ phosphorylation was greater than the decrease in Thr$^{69}$ phosphorylation.

**Rapamycin Sensitivity of the Phosphorylation of Wild-type and Mutant PHAS-I Proteins by mTOR**—None of the PHAS-I proteins were appreciably phosphorylated when immune complex kinase reactions were conducted with samples from cells transfected with vector alone (Fig. 4), indicating that the phosphorylation reactions required mTOR. Confirmation of this point was provided by results of experiments in which the effects of mTOR activators and inhibitors were investigated. mTab1 increased the overall phosphorylation, as well as the phosphorylation of Thr$^{36}$/Thr$^{45}$ and Thr$^{69}$, in wild-type and all three mutant proteins (Fig. 4). Phosphorylation of all four proteins was abolished by LY294002 (Fig. 4), the phosphatidylinositol 3-OH kinase inhibitor that also inhibits mTOR (29, 32). The phosphorylation of all four proteins was also decreased by rapamycin.

Rapamycin-FKBP12 does not fully inhibit the phosphorylation of PHAS-I by mTOR (29). The phosphorylation of Thr$^{36}$/Thr$^{45}$ and Thr$^{69}$ in PHAS-I by mTOR (29). The phosphorylation of Thr$^{36}$/Thr$^{45}$ and Thr$^{69}$ by 72 and 86%, respectively. In contrast, the Phe$^{113}$ to Ala mutation attenuated the phosphorylation of neither Thr$^{36}$/Thr$^{45}$ nor Thr$^{69}$ by mitogen-activated protein kinase (MAP) kinase (Fig. 3), indicating that phosphorylation sites were not compromised in the recombinant protein.

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even more pronounced when Thr^{36}/Thr^{45} phosphorylation was assessed by immunoblotting (Fig. 5B). Maximal inhibitory concentrations of rapamycin decreased Thr^{36}/Thr^{45} phosphorylation in the wild-type protein by only 30% but decreased phosphorylation of these sites in RAAA PHAS-I and F113A PHAS-I by 90%. The dose response curves for inhibition of $^{32}$P incorporation into the mutant proteins (Fig. 5A), as well as phosphorylation of Thr^{36}/Thr^{45} (Fig. 5B) and Thr^{69} (Fig. 5C), were almost identical, with half-maximal inhibition occurring at 20 nM rapamycin. In contrast to the effects of disrupting the RAIP and TOS motifs on Thr^{36}/Thr^{45} phosphorylation, mutation of these motifs had little, if any, effect on the rapamycin sensitivity of Thr^{69} phosphorylation (Fig. 5C).

Rapamycin-FKBP12 clearly inhibited phosphorylation of the mutant proteins (Fig. 5), indicating that the RAIP and TOS motifs are not absolutely required for recognition by mTOR. These results predict that if mTOR phosphorylates the proteins in vivo, then phosphorylation of the mutant proteins should still be inhibited by rapamycin. To investigate this prediction, wild-type and mutant PHAS-I proteins were expressed in 293T cells. These rapidly proliferating cells are persistently activated, so that even without stimulation, PHAS-I is highly phosphorylated. As shown in Fig. 6, wild-type PHAS-I overexpressed in 293T cells was found in the highly phosphorylated γ form. Relatively little γ forms of F113A and RAAA proteins were detected, suggesting that these proteins were less highly phosphorylated than wild-type. Moreover, rapamycin promoted the dephosphorylation of the mutant proteins, as evidenced by the increased mobility of F113A, RAAA, and Δ16 PHAS-I.


detections disrupted binding of the PHAS-I antibodies (Fig. 3), the interactions between PHAS-I Proteins and Raptor—To investigate the possibility that the RAIP and TOS motifs are involved in binding of PHAS-I to raptor, experiments were conducted in which extracts from HA-raptor overexpressing 293T cells were supplemented with purified PHAS-I proteins before immunoprecipitations were performed with anti-HA antibodies. Wild-type PHAS-I was readily detected in complexes containing raptor (Fig. 7, A and B). The fact that PHAS-I binding persisted during washes of the immune complexes is indicative of a relatively high affinity interaction between raptor and PHAS-I. None of the mutant proteins coimmunoprecipitated with HA-raptor (Fig. 7, A and B). Because none of the mutations disrupted binding of the PHAS-I antibodies (Fig. 3), the results are consistent with the interpretation that the RAIP and TOS motifs are required for the efficient binding of PHAS-I to raptor.

As raptor has been proposed to present PHAS-I to mTOR for phosphorylation (18), we investigated the effect of raptor overexpression on the phosphorylation of PHAS-I proteins by mTOR (Fig. 8). Coexpression of raptor with mTOR resulted in a marked increase in the phosphorylation of wild-type PHAS-I by mTOR (Fig. 8A). In contrast, raptor had relatively little effect on the phosphorylation of F113A PHAS-I, RAAA PHAS-I, or Δ16 PHAS-I proteins.

Observing increased phosphorylation by mTOR after raptor overexpression implies that the mTOR-raptor complex phosphorylates PHAS-I more efficiently than mTOR lacking raptor.
However, it was important to confirm that the overexpressed raptor formed a stable complex with mTOR particularly, because certain nonionic detergents have been shown to disrupt this complex (18). When Triton X-100 was substituted for Tween 20 in the homogenization buffer, a comparable amount of mTOR was recovered, but almost no raptor coimmunoprecipitated (Fig. 9A). These findings confirm the previous observations of Hara et al. (18).

To estimate how much overexpressing raptor increased the mTOR-raptor complex AU1 mTOR was immunoprecipitated, and immunoblots were prepared using polyclonal raptor antibodies. Raptor was detected in AU1 immunoprecipitates from 293T cells transfected with AU1-mTOR alone, indicative of a complex between AU1-mTOR and endogenous raptor (Fig. 9B). Assuming the raptor antibodies react equally well with endogenous and HA-tagged raptor, the results indicate that overexpressing HA-raptor increased the total amount of raptor coimmunoprecipitating with mTOR by ~10-fold.

Raptor overexpression increased phosphorylation of Thr$^{36/45}$Thr$^{69}$ in wild-type PHAS-I by ~5-fold (Fig. 8B). Phosphorylation of Thr$^{36/45}$/Thr$^{69}$ was lower in the three mutant proteins than in wild-type PHAS-I, both without and with raptor overexpression (Fig. 8B). The effects on Thr$^{69}$ phosphorylation were more complicated (Fig. 8C). Without raptor overexpression, mTOR-mediated phosphorylation of Thr$^{69}$ in Δ16 PHAS-I was lower than phosphorylation of this site in wild-type PHAS-I. Under these conditions phosphorylation of this site in neither of the other mutant proteins was less than that in wild-type. Overexpressing raptor increased phosphorylation of Thr$^{69}$ in wild-type PHAS-I by ~5-fold (Fig. 8C). Interestingly, raptor enhanced the phosphorylation of Thr$^{69}$ in both proteins in which the RAIP motif was disrupted. Overexpressing raptor did not increase phosphorylation of Thr$^{69}$ in PHAS-I lacking the TOS motif.

**DISCUSSION**

The major point of this report is that disrupting either the RAIP or TOS motifs markedly inhibits the ability of mTOR to phosphorylate PHAS-I. The findings provide important information concerning the features involved in substrate recognition by mTOR. Moreover, when considered with previous evidence that the RAIP and TOS motifs are needed for the efficient phosphorylation of PHAS-I in cells (21, 22), the present results support the view that mTOR phosphorylates PHAS-I in vivo.

Characterization of a hypophosphorylated PHAS-I fragment that bound tightly to eIF4E in cells undergoing apoptosis led to discovery of the importance of the RAIP motif (21). Amino acid sequencing revealed that the apoptotic fragment was generated by caspase cleavage of the Asp$^{24}$/Gly$^{25}$ bond. Tee and Proud (21) subsequently localized the critical region needed for efficient phosphorylation of PHAS-I in cells to the RAIP motif. The major point of this report is that disrupting either the RAIP or TOS motifs markedly inhibits the ability of mTOR to phosphorylate PHAS-I. The findings provide important information concerning the features involved in substrate recognition by mTOR. Moreover, when considered with previous evidence that the RAIP and TOS motifs are needed for the efficient phosphorylation of PHAS-I in cells (21, 22), the present results support the view that mTOR phosphorylates PHAS-I in vivo.

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Thr45 than on decreasing phosphorylation of Thr69 by mTOR
in intact cells when the TOS motif was removed by truncation
(35).

It is difficult to formally prove that any kinase phosphorylates a particular substrate in a cell, and the fact that rapamycin inhibits PHAS-I phosphorylation does not necessarily mean that mTOR phosphorylates the protein in vivo. For example, in *Saccharomyces cerevisiae* the effects of rapamycin are mediated in part by effects on protein phosphatases (36), and the possibility that phosphatase activation contributes to the effects of rapamycin on decreasing the phosphorylation of PHAS-I cannot be eliminated. However, the findings that disrupting either the RAIP or TOS motif decreased phosphorylation of PHAS-I, both in cells and by mTOR in vitro, contribute to the increasing evidence that mTOR is a major PHAS-I kinase. Included in this evidence are findings that mTOR phosphorylates the same sites in PHAS-I that are phosphorylated in a rapamycin-sensitive manner in cells (7, 17). Rapamycin treatment of cells has a more pronounced effect on inhibiting phosphorylation of Thr69 than the phosphorylation of Thr36/Thr45 (5, 37). Likewise, the phosphorylation of Thr69 by mTOR in vitro is more sensitive to rapamycin-FKBP12 than the phosphorylation of Thr36/Thr45 (7, 29) (Fig. 4). Very recently Haral et al. (18) demonstrated that PHAS-I associates directly with raptor, a binding partner of mTOR, providing a close physical link between PHAS-I and mTOR.

The previous finding that raptor enhanced phosphorylation of PHAS-I by mTOR (18) led us to investigate the hypothesis that the TOS and/or RAIP motifs facilitate PHAS-I binding to raptor. The finding that purified wild-type PHAS-I, but not PHAS-I proteins with disrupted RAIP and TOS motifs, coimmunoprecipitated with HA-tagged raptor (Fig. 7) supports this hypothesis. Also, overexpression of raptor with mTOR increased the phosphorylation of wild-type PHAS-I but not the mutant proteins by mTOR in vitro (Fig. 8). Although we favor the interpretation that the motifs enhance PHAS-I phosphorylation by allowing the interaction of PHAS-I with raptor, we cannot eliminate the possibility that increasing raptor increases the binding of PHAS-I to mTOR or perhaps other proteins associated with the raptor-mTOR complex. Such proteins might become limiting for activity with overexpression of both raptor and mTOR. This could explain why the percentage increase in PHAS-I kinase activity produced by raptor overexpression appeared to be less than the increase in the raptor-mTOR complex (5-fold versus 10-fold) (see Fig. 8B and Fig. 9B).

In *S. cerevisiae* TOR proteins are found in two complexes (20). TORC1 contains TOR1 or TOR2, LST8, a protein with seven WD-40 domains, and KOG1, the yeast counterpart to raptor. TORC1 binds rapamycin with high affinity. Another complex, TORC2, contains TOR2, LST8, and the three proteins, AVQ1, AVQ2, and AVQ3 (20). TORC2 does not bind rapamycin, possibly because of the lack of raptor, or because one or more of the AVQ proteins interferes with the binding site. The evidence for a mammalian TORC1 is strong, as both raptor and mLST8 have been shown to coimmunoprecipitate with mTOR (20). A human AVQ1 homolog, hSIN1, exists and its pattern of expression very closely resembles that of mTOR, although there is no direct evidence of association of mTOR and hSIN1 (20). Thus, whether complexes resembling TORC1 and TORC2 are formed with the single mammalian TOR protein is not known.
Mothe-Satney et al. (2001) and the reference therein. It is interesting that the resistance of Thr36/Thr45 to rapamycin-FKBP12 was largely lost by disrupting either the RAIP or TOS motifs (Fig. 5B), as these results are suggestive of phosphorylation of PHAS-I by two complexes, one sensitive to rapamycin and the other insensitive. However, the two complexes do not necessarily correspond to mTORC1 and mTORC2. An explanation consistent with the present results is that tight binding to raptor, an interaction requiring both the TOS and RAIP motifs, provides a degree of protection from the inhibitory effects of rapamycin on phosphorylation of Thr36/Thr45. The present findings also suggest that optimal phosphorylation of Thr36/Thr45 by mTOR depends on the high affinity interaction between PHAS-I and raptor, as phosphorylation was decreased by disrupting either the TOS or RAIP motifs.

The results involving Thr69 phosphorylation are difficult to reconcile with a simple model. After raptor overexpression, disrupting the TOS and RAIP motifs actually had opposite effects on phosphorylation of this site by mTOR (Fig. 8, B and C). One interpretation is that phosphorylation of Thr69 was mediated by different protein kinases. However, we do not believe that this is the case, as there is very good evidence that the Thr36/Thr45 and Thr69 sites are phosphorylated by mTOR. For example, dose response curves for the inhibition of phosphorylation of Thr36/Thr45 by a rapamycin analog, LY294002, and caffeine were identical to those for inhibition of Thr69 (29). Moreover, mTOR harboring an Asp2338 to Ala mutation in the catalytic domain is capable of phosphorylating either Thr36/Thr45 or Thr69 (7, 29).

Phosphorylation of PHAS-I in cells occurs in a hierarchical manner (5, 8), with the phosphorylation of Thr36 and Thr45 preceding that of Thr69 and Ser84. Inspection of the stoichiometry of phosphorylation and the electrophoretic mobility of PHAS-I phosphorylated in Thr36/Thr45 by mTOR in vitro reveals that the phosphorylation of the protein by mTOR also involves an ordered mechanism. For example, in Fig. 4 note that after incubating mTOR with mTAb1 approximately one-third of the P-Thr36/45 antibody reactivity in wild-type PHAS-I was up-shifted. Because a previous study (1) has established that phosphorylation Thr36 and Thr45 does not significantly retard the electrophoretic mobility of PHAS-I, the up-shift in P-Thr36/Thr45 immunoactivity must have been caused by phosphorylation of another site. In our experiment, 32P incorporation indicated that mTOR introduced a maximum of 0.034 mol of phosphate per mol of PHAS-I. Therefore, if phosphorylation of the site responsible for the gel shift had been equally likely to occur in nonphosphorylated PHAS-I (at least 96.6% of the total) and Thr36/Thr45-phosphorylated protein, then the up-shifted fraction of P-Thr36/45 reactivity should have been negligible (less than 3.4% of the total). The site responsible for the shift is most likely Thr69, whose phosphorylation is known to promote the α to β shift (1). In a previous study, we found that mutating Thr36 and Thr45 to Ala markedly diminished the rate of Thr69 phosphorylation by mTOR in vitro (29), providing further support for the conclusion that phosphorylation of Thr36 and Thr45 is needed for optimal phosphorylation of Thr69.

Regardless of the explanation, the results are consistent with the view that the RAIP motif suppresses Thr69 phosphorylation. As a working hypothesis, we propose that phosphorylation of Thr36/Thr45, or the Δ16 and the Ille15-Pro16 to Ala15-Ala16 mutations, reverse the inhibitory influence of the RAIP motif. We also propose that interaction of PHAS-I with raptor, mediated by the TOS motif, is still required for the phosphorylation of Thr36/Thr45, though the strength of this interaction is insufficient to allow communoprecipitation of PHAS-I with raptor. In this model, disrupting either the TOS or RAIP motifs would be expected to decrease phosphorylation of Thr36 and Thr45, the initial phosphorylation events are enhanced by tight binding to raptor. Disrupting the RAIP motif would obviate the need of Thr36/Thr45 phosphorylation for Thr69 phosphorylation, thereby allowing increased phosphorylation of Thr36 when the raptor-mTOR complex was increased by raptor overexpression. On the other hand, disrupting the TOS motif would be expected to decrease Thr69 phosphorylation by reducing both the interaction of PHAS-I with raptor and the phosphorylation of Thr36/Thr45.

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Two Motifs in the Translational Repressor PHAS-I Required for Efficient Phosphorylation by Mammalian Target of Rapamycin and for Recognition by Raptor

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