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

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The eukaryotic initiation factor 4E (eIF4E)-binding protein, PHAS-I, was phosphorylated rapidly and stoichiometrically when incubated with [γ-32P]ATP and the mammalian target of rapamycin (mTOR) that had been immunoprecipitated with an antibody, mTAb1, directed against a region near the COOH terminus of mTOR. PHAS-I was phosphorylated more slowly by mTOR obtained either by immunoprecipitation with other antibodies or by affinity purification using a rapamycin/FKBP12 resin. Adding mTAb1 to either of these preparations of mTOR increased PHAS-I phosphorylation severalfold, indicating that mTAb1 activates the mTOR protein kinase. mTAb1-activated mTOR phosphorylation increased PHAS-I phosphorylation severalfold, indicating that mTAb1 activates the mTOR protein kinase. mTAb1-activated mTOR phosphorylated Thr36, Thr45, Ser64, Thr69, and Ser82 in PHAS-I.

The mammalian target of rapamycin, mTOR (1) (also known as FRAP (2) or RAFT1 (3)), is a homolog of the Tor1p and Tor2p proteins that are required for cell cycle progression in Saccharomyces cerevisiae (4). Like the yeast proteins, mTOR has a COOH-terminal catalytic domain that is homologous to those found in yeast and mammalian phosphatidylinositol 3-OH kinases, although none of the TOR proteins have been shown to possess intrinsic lipid kinase activity (5). The function of mTOR is inhibited by incubating cells with rapamycin, a potent immunosuppressant and antiproliferative agent (5). The mammalian target of rapamycin (mTOR) that had been prepared by coupling peptides to Sulfolink beads (Pierce).

Antibodies—Peptides having NH2-terminal C residues followed by sequences identical to positions 2433–2450 (DTNAGKNSRRTTDSYS) and 1272–1290 (ARRSVKDDWLRLRLSE) in mTOR (1) were coupled to keyhole limpet hemocyanin, and the peptide-hemocyanin conjugates were used to immunize rabbits by using the procedures described previously (20). Antibodies, designated mTAb1 and mTAb2, respectively, were purified by using columns containing affinity resins prepared by coupling peptides to Sulfolink beads (Pierce).

EXPERIMENTAL PROCEDURES

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Measurements of PHAS-I Kinase Activity After Affinity Purification and Immunoprecipitation of mTOR—Rat brains were rinsed and homogenized (1 g of tissue/ml of buffer) by using a Polytron tissue dis-rupter (setting of 4 for 10 s, then setting of 8 for 10 s). Homogenization buffer contained solution A (100 mM NaCl, 10% glycerol, and 50 mM Tris/HCl, pH 7.4) supplemented with 2 mM β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 14,500 × g for 30 min at 4 °C, and the supernatant was retained for purification of mTOR.

His6PHAS-I (21) and GST-FKBP12 (1) were expressed in bacteria and purified as described previously. To purify mTOR, GST-FKBP12 (100 μg) was incubated with 25 μl glutathione-agarose (Sigma) in Tris-buffered saline (100 mM NaCl and 50 mM Tris/HCl, pH 7.4). After 1 h at 21 °C, the resin was washed 3 times (1 ml wash) with Tris-buffered saline, then suspended in homogenization buffer, and incubated (1 ml final volume) at 4 °C with extract (3 mg of protein), 200 mM microcystin-LR, and 10 μM rapamycin (Calbiochem). Control incubations were conducted with FK506 (Pfuijsawa Pharmaceuticals). After 1 h the beads were washed twice (1 ml wash) with solution A plus 1 mM dithiothreitol, then washed twice with solution B (50 mM NaCl, 1 mM dithiothreitol, and 10 mM Na-HEPES, pH 7.4). For immunoprecipitations, antibodies (5 μg) were incubated at 21 °C with protein A-agarose
**RESULTS AND DISCUSSION**

**Immunoprecipitation of mTOR with mTAb1 and mTAb2**

mTOR was readily detected by immunoblotting after subjecting samples of rat brain extracts to SDS-PAGE (Fig. 1). The apparent $M_r$ of the mTOR estimated by SDS-PAGE was 240,000, which is similar to the $M_r$ of 289,000 predicted from the nucleotide sequence of the mTOR cDNA. The protein was recognized by either of the antibodies, mTAb1 and mTAb2, which were directed against two different regions of the mTOR molecule. As expected, the antibodies recognized mTOR that had been affinity-purified using rapamycin/FKBP12. No mTOR was detected if the affinity-purification procedures were conducted in an identical manner except using FK506-FKBP12, which does not bind mTOR. mTOR could be immunoprecipitated using either mTAb1 or mTAb2. In this case, the recoveries of mTOR were blocked by the respective antigenic peptides supporting the specificity of the antibodies.

**Phosphorylation of PHAS-I by mTOR—**When incubated in a reaction mixture containing $Mn^{2+}$ and [γ-32P]ATP, [His$^6$]PHAS-I was phosphorylated by mTOR that had been immunoprecipitated with either mTAb1 or mTAb2. The rate of phosphorylation was much higher with mTAb1 than with mTAb2. mTAb1 was somewhat more efficient in immunoprecipitating mTOR than mTAb2 (Fig. 1); however, the higher rate of kinase activity could not be explained by the larger amount of mTOR present in the mTAb1 immunoprecipitates.

To investigate the possible stimulatory effect of mTAb1, mTOR was immunoprecipitated with mTAb2, then incubated with mTAb1 before assessing kinase activity (Fig. 2). Under these conditions, the rate of phosphorylation of PHAS-I was increased approximately 3-fold by mTAb1. In contrast, adding mTAb2 to mTOR that had been immunoprecipitated with mTAb1 decreased the rate of incorporation of $^{32}$P into PHAS-I by approximately 50%. Previous studies demonstrated that mTOR autophosphorylation, although markedly diminished, still occurred when the protein was bound to rapamycin/FKBP12, indicating that rapamycin/FKBP12 does not fully inhibit mTOR (24). When purified by rapamycin/FKBP12, indicating that rapamycin/FKBP12 does not fully inhibit mTOR (24). When purified by rapamycin/FKBP12, indicating that rapamycin/FKBP12 does not fully inhibit mTOR (24). When purified by rapamycin/FKBP12, indicating that rapamycin/FKBP12 does not fully inhibit mTOR (24). When purified by rapamycin/FKBP12, indicating that rapamycin/FKBP12 does not fully inhibit mTOR (24).
known standard (bovine serum albumin) on silver stained polyacrylamide gels, we estimate that an mTOR immunoprecipitate from 3 mg of brain extract protein contained approximately 0.05 $\mu$g of mTOR protein (data not shown). This amount of mTOR phosphorylated PHAS-I at a rate comparable to that of 0.1 $\mu$g recombinant MAP kinase, which is by far the best of the kinases that have been reported to phosphorylate PHAS-I in vitro (21). The sensitivity of mTOR and MAP kinase to wortmannin and rapamycin/FKBP12 were distinctly different (Fig. 3). PHAS-I phosphorylation by mTOR was abolished by 1 $\mu$M wortmannin, and the activity was markedly decreased by rapamycin/FKBP12. These agents had little if any effect on PHAS-I phosphorylation by MAP kinase.

Identification of Phosphorylation Sites—To investigate the specificity of mTOR, the sites in PHAS-I phosphorylated by mTOR were identified. [His$_6$]PHAS-I was phosphorylated using 0.05 $\mu$g mTOR and either [γ-32P]ATP or 0.1 $\mu$g recombinant MAP kinase (B). Samples were then subjected to SDS-PAGE, and the amounts of 32P incorporated into [His$_6$]PHAS-I were determined by scintillation counting of gel slices containing the protein. The insets show autoradiograms of $\beta$-labeled [His$_6$]PHAS-I after incubation for 40 min as described above without other additions or with the following: 10 $\mu$M rapamycin plus 200 $\mu$g/ml FKBP12 (Rap/FKBP), 10 $\mu$M FK506 plus 200 $\mu$g/ml FKBP12 (FK506/FKBP), 0.1 $\mu$M wortmannin (Wort), or 1 $\mu$M wortmannin. Note that no phosphorylation of [His$_6$]PHAS-I was observed if immunoprecipitations were conducted using nonimmune IgG (NI-IgG) or if buffer was substituted for MAP kinase (MAPK).
resolved by reverse phase HPLC (Fig. 4). mTOR phosphorylated sites that were recovered in phosphopeptides that eluted in peaks found in the same positions as LE-P1, LE-P2, LE-P3, and LE-P4, the four peaks previously characterized in analyses of PHAS-I phosphorylation by MAP kinase (19). LE-P1, LE-P2, and LE-P3 contain peptides in which Thr<sup>69</sup>, Ser<sup>64</sup>, and Ser<sup>82</sup> respectively, are phosphorylated (19). LE-P4 contains both Thr<sup>36</sup> and Thr<sup>35</sup>. These sites may be resolved by digesting LE-P4 with chymotrypsin, which generates peptides that elute in two peaks, designated CT-P1 and CT-P2 (19), containing Thr<sup>45</sup> and Thr<sup>36</sup> respectively. Digesting the pooled LE-P4 fractions from mTOR-phosphorylated [His<sup>6</sup>]PHAS-I with chymotrypsin-generated <sup>32</sup>P-labeled peptides that eluted from the reverse phase column in precisely the same positions as CT-P1 and CT-P2 (Fig. 4).

The finding that phosphopeptides derived from the mTOR-phosphorylated sample eluted identically from the reverse phase column as those from the MAP kinase-phosphorylated protein suggested that mTOR phosphorylated the same sites as MAP kinase. However, because the peptides contain multiple Ser/Thr residues, it was necessary to determine the position of the phosphorylated residue in each peptide to confirm the site assignment. Subjecting LE-P1, LE-P2, LE-P3, CT-P1, and CT-P2 from mTOR-phosphorylated samples to sequential Edman degradation resulted in release of <sup>32</sup>P in cycles 1, 8, 10, 3, and 3, indicative of phosphorylation of Thr<sup>69</sup>, Ser<sup>64</sup>, Ser<sup>82</sup>, Thr<sup>45</sup>, and Thr<sup>36</sup>, respectively (Fig. 5).

Although it facilitated the phosphorylation site analyses, the finding that mTOR phosphorylated the same sites in PHAS-I as MAP kinase was unexpected in view of the dissimilar catalytic domains of the two enzymes (25, 26). An initial concern was that the PHAS-I phosphorylation observed with mTOR immunoprecipitates might be due to contamination with MAP kinase. Both biochemical and pharmacological evidence indicates that this was not the case. mTOR phosphorylated Thr<sup>69</sup> much more rapidly than this site was phosphorylated by MAP kinase (19). Phosphorylation by mTOR was markedly inhibited by rapamycin/FKBP12, which was without effect on the phosphorylation of PHAS-I by MAP kinase (Fig. 3); and, rather, which binds and covalently modifies mTOR, abolished phosphorylation by mTOR but not phosphorylation by MAP kinase (Fig. 3). Moreover, the finding that the PHAS-I kinase associated with affinity-purified mTOR was increased by mTab1 (Fig. 2) provides strong evidence that PHAS-I phosphorylation is mediated by mTOR itself.

The similarity of the kinetics and stoichiometry of phosphorylation of PHAS-I by mTOR and MAP kinase was also unexpected, although subtle but potentially important differences in the activities of these enzymes toward PHAS-I are apparent. Comparing the phosphate incorporated into a single phosphorylation site relative to the total phosphate in all of the sites, mTOR reproducibly phosphorylated Thr<sup>69</sup> more rapidly than did MAP kinase (Fig. 4 and Ref. 19). Additionally, the stoichiometry of phosphorylation of PHAS-I by either enzyme appears to plateau at just over 1 mol of phosphate/mol of PHAS-I (Fig. 3) indicating that mono-phosphorylated PHAS-I may not be a preferred substrate for further phosphorylation. Taken together these findings indicate that relative site preferences of PHAS-I kinase(s) may play a role in regulating PHAS-I function. It will be interesting to determine the relative role each phosphorylation site or combination of sites plays in determining the affinity of PHAS-I for eIF4E.

All of the sites phosphorylated by mTOR conform to a (Ser/Thr)-Pro motif. An implication is that Pro on the COOH-terminal side of a Ser/Thr residue might be an important determinant in the specificity of mTOR. The sites phosphorylated by mTOR in vitro are phosphorylated in response to insulin in adipocytes (19), and their phosphorylation markedly decreases the ability of PHAS-I to bind to eIF4E (9). Moreover, treating adipocytes with rapamycin has been shown to decrease phosphorylation of the sites. These findings support the conclusion that the sites in PHAS-I phosphorylated by mTOR are physiologically relevant and that mTOR contributes to the phosphorylation of PHAS-I in cells.

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