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Isotani, Shuji, Kenta Hara, Chiharu Tokunaga, Hitomi Inoue, Joseph Avruch, and Kazuyoshi Yonezawa. 1999. “Immunopurified Mammalian Target of Rapamycin Phosphorylates and Activates p70 S6 Kinase A in Vitro.” Journal of Biological Chemistry 274 (48): 34493–98. https://doi.org/10.1074/jbc.274.48.34493.

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Immunopurified Mammalian Target of Rapamycin Phosphorylates and Activates p70 S6 Kinase α in Vitro*

(Received for publication, July 30, 1999, and in revised form, September 27, 1999)

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p70 S6 kinase α (p70α),1 whose major substrate is the 40 S ribosomal protein S6, plays a critical role in the translation of a subclass of mRNAs that contain a short oligopyrimidine sequence immediately following the transcriptional start site (1). p70α is activated in response to insulin/mitogens in vivo through a multisite phosphorylation of serine and threonine residues (2). Several sets of independently regulated p70α phosphorylation sites have been identified (3–6); one set consists of Ser/Thr-Pro motifs, five of which are clustered in a pseudosubstrate autoinhibitory domain in the noncatalytic carboxyl-terminal tail (Ser-434, Ser-441, Ser-447, Ser-452, and Thr-444 in p70α), and two others, Thr-390 and Ser-394, are located in a 65-amino acid segment immediately carboxyl-terminal to the kinase catalytic domain. A second set of regulated phosphorylation sites, Thr-412 and Ser-427, exhibit the sequence motif Phe-Ser/Thr-Phe/Tyr. Thr-252, located on the activation loop in catalytic subdomain VIII, is the site at which 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates p70α (7, 8). Among these, the phosphorylation of Thr-252, Ser-394, and Thr-412 is necessary for the activation of p70α kinase catalytic function; the attainment of physiologic levels of p70α activity results from a strongly synergistic, positive site-site interaction between the phosphorylated Thr-252 and Thr-412 residues (7).

In addition to its regulation by insulin and mitogens through PI-3 kinase-dependent pathways, p70α can also be activated by increasing concentrations of extracellular amino acids in the absence of serum or mitogens to the level attained by maximal mitogen stimulation (9–11). Moreover, a threshold level of cellular amino acids is necessary for p70α to be susceptible to activation by mitogens. Withdrawal of amino acids from the nutrient medium results in a rapid, selective deactivation of p70α, which becomes unresponsive to mitogens; readdition of amino acids restores the mitogen responsiveness of p70α (9).

The immunosuppressant drug rapamycin inhibits p70α in vivo (12, 13). This is achieved indirectly by the ability of a rapamycin-FKBP12 complex to bind to the mTOR polypeptide and inhibit mTOR kinase activity; mTOR mutants unable to bind the rapamycin-FKBP12 complex can rescue p70α from rapamycin-induced dephosphorylation and inhibition but only if the mTOR catalytic domain is intact (14, 15). As for the biochemical steps by which the mTOR kinase controls p70α phosphorylation and activity, evidence is available in support of two independent, but nonexclusive mechanisms. The possibility that mTOR inhibits an inactivating p70α-phosphatase is supported by both indirect and direct experiments. Thus, a doubly deleted p70α mutant (p70α-D2–46/ΔCT104) can be activated by mitogens and inhibited by low concentrations of wortmannin but is insensitive to inhibition by rapamycin (3) or amino acid withdrawal (9); these features are most readily explained if mitogens and PI-3 kinase control p70α-kinases, whereas amino acid sufficiency and mTOR negatively regulate a p70α-phosphatase. A recent report provides direct evidence implicating protein phosphatase 2A in this role (16). Conversely, Burnett et al. (17) reported that mTOR can directly phosphorylate prokaryotic recombinant fragments of p70α in vitro at sites important to activation, including Thr-412. The latter finding was surprising, inasmuch as all sites of mTOR-
catalyzed phosphorylation on the eukaryotic initiation fac-
tor-4E binding protein 1 (eIF-4E BP1) reside in Ser/Thr-Pro motifs (18, 19). We therefore inquired whether mTOR can phosphorylate and/or activate a previously isolated full-length p70α polypeptide expressed in mammalian cells and dephospho-
rylated and inactivated in vivo by the pretreatment with rapamycin.

**EXPERIMENTAL PROCEDURES**

**Antibodies and cDNAs—** The anti-mTOR antibody was described pre-
viously (3, 7, 15). The anti-phosphopeptide antibodies against Thr-412, Ser-421, and of GST-PDK1 were described previously (3, 7, 15). Site-specific

Cell Lysis and Immunoprecipitation—Recombiant p70α was pre-
pared from cells that were transfected with wild-type (W-mTOR) and the kinase-negative mutant (NK-mTOR) of mTOR, of the wild-type p70α and the mutant p70α (p70α-ΔCT1104), and of GST-PDK1 were described previously (3, 7, 15). Site-specific

The kinase reaction was started by washing the beads twice with the high salt wash buffer containing 1% Nonidet P-40 or the RIPA buffer, compared with that prepared after washing with the high salt wash buffer without detergent. The result was unexpected, as we have previously shown that washing of mTOR immunoprecipitates with 1% Nonidet P-40 reduces greatly the ability of mTOR to catalyze eIF-4E BP1 phosphorylation (20). These differences in mTOR-catalyzed p70α phosphorylation are not because of differ-
ences in the recovery of mTOR polypeptide, as demonstrated by the immuno blot with the anti-mTOR antibody (Fig. 1A).

mTOR-catalyzed 32P incorporation into p70α is detectable within 5 min after initiation of the kinase reaction and increases over 30 min (Fig. 1B); no 32P incorporation into the wild-type p70α substrate (i.e. p70α autophosphorylation) is detectable in the absence of mTOR (Fig. 1B, lanes 6 and 7). Moreover, mTOR-catalyzed 32P incorporation into the kinase-
inactive, ATP binding site mutant of p70α (p70α-Lys-123 → Met) is similar in extent to that seen with wild-type p70α substrate (data not shown). These results show that the 32P incorporation into p70α occurring in the presence of mTOR is catalyzed by a mTOR-associated kinase and is not because of a stimulation of p70α autophosphorylation.

To confirm that the phosphorylation of p70α by mTOR is dependent on the intrinsic kinase activity of mTOR, we compared the ability of a recombinant wild-type or kinase-negative mutant of mTOR to phosphorylate p70α in vitro (Fig. 1C). In contrast to the robust 32P incorporation into p70α catalyzed by recombinant wild-type mTOR, no phosphorylation of p70α is detectable on incubation with the kinase-negative mutant of mTOR. We next examined the effects of mTOR kinase inhibi-
tors on mTOR-catalyzed p70α phosphorylation in vitro (Fig. 1D). Inhibition of mTOR immunoprecipitates with a rapamycin-PKB complex severely inhibits mTOR autophosphoryla-
tion, as well as the phosphorylation of p70α, whereas neither rapamycin or PKB singly have any effect. Wortmannin, at a concentration previously shown to inhibit mTOR kinase toward eIF-4E BP in vitro also inhibits mTOR-catalyzed p70α phos-
phorylation. These results indicate that the phosphorylation of p70α in vitro requires the intrinsic kinase activity of mTOR. To examine the effects of stimulation by mitogens and depletion of

RESULTS AND DISCUSSION

To obtain a precisely folded full-length p70α polypeptide that was expressed in mammalian cells and dephosphorylated and inactivated in vivo, HEK293 cells were transiently transfected with a full-length HA-tagged p70α, and cells were deprived of serum and treated with rapamycin (0.2 μM) for 30 min prior to harvest. HA-tagged p70α was immunopurified on protein G-Sepharose beads, together with either a control immunoglobulin or an anti-mTOR immunocomplex; each were prepared sepa-

Average mTOR was extracted from HEK293 cells, and recombinant mTOR

Moreover, mTOR-catalyzed 32P incorporation into p70α is substantially enhanced when the immunoprecipitate is washed with the high salt wash buffer containing 1% Nonidet P-40 or the RIPA buffer, compared with that prepared after washing with the high salt wash buffer without detergent. The result was unexpected, as we have previously shown that washing of mTOR immunoprecipitates with 1% Nonidet P-40 reduces greatly the ability of mTOR to catalyze eIF-4E BP1 phosphorylation (20). These differences in mTOR-catalyzed p70α phosphorylation are not because of differ-
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phorylation. These results indicate that the phosphorylation of p70α in vitro requires the intrinsic kinase activity of mTOR. To examine the effects of stimulation by mitogens and depletion of
amino acids on the mTOR kinase activity, serum-deprived cells were treated with or without 10% serum in the presence of amino acids or incubated for up to 2 h in the amino acid-free buffer; mTOR was immunoprecipitated and assayed for kinase activity toward p70α. No significant alterations in the mTOR kinase toward p70α resulted from these treatments (data not shown); thus the effects of these perturbations on mTOR kinase activity toward p70α, if any, do not survive immunoprecipitation and washing.

We employed a panel of anti-p70α phosphopeptide antibodies to test whether p70α is phosphorylated in vitro by mTOR. The results are shown in Figure 1. In panel A, HA-tagged wild-type p70α was immunoprecipitated with (+) or without (−) endogenous mTOR. After washing the beads with the high salt wash buffer containing 1% Nonidet P-40 twice, the samples were subjected to the in vitro kinase assay for the indicated times and analyzed by autoradiography (top) or immunoblot with the indicated antibodies (bottom). In panel B, FLAG-tagged wild-type p70α was immunoprecipitated without (−) or with (+) either HA-tagged wild-type (W) or NK-mTOR. After washing the immunoprecipitates with the high salt wash buffer containing 1% Nonidet P-40, the samples were subjected to the in vitro kinase assay for the indicated time and analyzed by autoradiography (top) or immunoblot with the indicated antibodies (bottom). In panel C, FLAG-tagged wild-type p70α was immunoprecipitated without (−) or with (+) 1 μM wortmannin (lane 3), 5 μg of GST-FKBP and 1 μM rapamycin (lane 4), 5 μg of GST and 1 μM rapamycin (lane 5), 5 μg of GST-FKBP alone (lane 6), 1 μM rapamycin alone (lane 7), or vehicles (lanes 1 and 2). The samples were separated on SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and analyzed by autoradiography (top) or immunoblot (bottom) with (i) the anti-FLAG Ab or (ii) the anti-HA Ab. In panel D, FLAG-tagged wild-type p70α was quantified by BAS 2000 in arbitrary units: lane 1, 63.2; lane 2, 2080; lane 3, 5280; lane 4, 6860; lane 5, 7860; lane 6, 294; lane 7, 694; lane 8, 461. C, FLAG-tagged wild-type p70α was immunoprecipitated without (−) or with (+) either HA-tagged wild-type (W) or NK-mTOR. 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ies (21) to examine whether mTOR catalyzed the phosphorylation in vitro of sites on p70α known to be phosphorylated in vivo. The phospho-specific immunoreactivity at all sites examined appeared to be increased by mTOR; however, this was most unmistakable with Thr-412 (Fig. 1, A and B), which exhibits no phospho-specific immunoreactivity prior to incubation with mTOR. Overall phospho-specific immunoreactivity at Thr-444/Ser-447 is also substantially increased over the initial level, whereas the response at Ser-434 is equivocal in that the modest apparent increase in overall phospho-specific immunoreactivity at Ser-434 may be entirely attributable to the upshift of a fraction of p70α polypeptides resulting from mTOR-catalyzed phosphorylation at other sites with a consequent spreading of Ser-434-P immunoreactivity over a greater area.

In view of the limitation of immunoblot for quantitative analysis, we compared the extent of mTOR-catalyzed 32P incorporation into wild-type p70α with that observed using equal amounts of several p70α mutants as substrates. The p70α mutants examined were (i) p70α-5A in which five Ser/Thr-Pro sites (Ser-434, Ser-441, Thr-444, Ser-447, and Ser-452) in the carboxyl-terminal autoinhibitory domain are substituted by Ala; (ii) p70α-Thr-412 → Ala; (iii) p70α-ΔCT104 in which the carboxyl-terminal 104 amino acids are deleted and the protein terminates after Ser-421; (iv) p70α-ΔCT104/Thr-412 → Ala. The quantitative importance of Thr-412 as a site of mTOR-catalyzed p70α phosphorylation is clearly evident in Fig. 2; mutation of p70α Thr-412 to Ala reduces mTOR-catalyzed 32P incorporation into full-length p70α by about 80% and into p70α-ΔCT104 by a similar extent; the mTOR-catalyzed 32P incorporation into the p70α-ΔCT104/Thr-412 → Ala mutant is less than 10% of that seen with full-length p70α wild-type (Fig. 2B). Thus, Thr-412 is a dominant site of mTOR-catalyzed p70α phosphorylation in vitro. Overall mTOR-catalyzed 32P incorporation into the p70α-5A mutant is diminished by about 25% compared with p70α wild-type, whereas 32P incorporation into the ΔCT104 mutant is diminished by 50–60%. These results indicate that a portion of mTOR-catalyzed p70α phosphorylation is directed to the carboxyl-terminal tail, at least half of which is into the Ser/Thr-Pro sites mutated in the 5A variant. This is consistent with the results of the anti-Thr-444/Ser-447-P immunoblots (Fig. 1, A and B, and Fig. 2A). The lesser total 32P incorporation into p70α-ΔCT104 as compared with p70α-5A suggests that there might be phosphorylation site(s) other than the five Ser/Thr-Pro sites within the carboxyl-terminal 104 amino acids. One such site may be Ser-427, located in a Phe-Ser-Phe motif similar to that surrounding Thr-412. Another possible explanation is that the absence of the carboxy-terminal 104 amino acids may impair the ability of mTOR to phosphorylate Thr-412.

The phosphorylation of p70α Thr-412 is known to be critical for its S6 kinase activity and substitution of this residue with an acidic amino acid results in a substantial increase in "basal" S6 kinase activity (5, 21). We therefore inquired whether the p70α kinase activity is increased by mTOR-catalyzed phosphorylation in vitro. As shown in Fig. 3, the p70α kinase activity shows a time-dependent increase on incubation with mTOR (Fig. 3B), which parallels the extent of mTOR-catalyzed phosphorylation at Thr-412 and Thr-444/Ser-447 detected by immunoblot (Fig. 3A). In contrast, no S6 kinase activity is detectable in the absence of mTOR. To establish whether the activation of p70α in vitro requires the intrinsic kinase activity of mTOR, the recombinant wild-type and kinase-negative mutant of mTOR were employed for the assays (Fig. 3C). As in Fig. 3A, incubation of p70α with wild-type mTOR significantly increased the S6 kinase activity, whereas no activation is detected on incubation of p70α with kinase-negative mTOR.

These results clearly indicate that the kinase activity of p70α, which had been fully inactivated in vivo by the treatment of cells with rapamycin, was restored, at least in part, by the phosphorylation in vitro catalyzed by the kinase activity intrinsic to the mTOR catalytic domain.

PKD1-catalyzed phosphorylation of Thr-252 on the p70α activation loop is a critical and probably final step in the physiologic activation of p70α in vivo (7, 8, 21). The ability of PKD1 to phosphorylate Thr-252 is regulated primarily by the accessibility of the p70α activation loop to PKD1, which in turn is
Activation of p70α by mTOR in Vitro

Fig. 3. Activation of p70α by mTOR. A, HA-tagged wild-type p70α was immunoprecipitated with (+) or without (−) endogenous mTOR, and the immunoprecipitates were subjected to the two-step kinase assay as described under “Experimental Procedures.” The samples were analyzed by autoradiography (top) and immunoblot with the indicated antibodies (bottom). B, the activation of p70α by mTOR was carried out in the separate experiments, and 32P incorporated into S6 was quantified. 32P incorporated into S6 at the indicated times was expressed as a percentage of that at 45 min. Data are the mean ± S.D. of three experiments. C, FLAG-tagged wild-type p70α was immunoprecipitated without (−) or with either HA-tagged wild-type (W) or kinase negative mTOR. The immunoprecipitates were subjected to the two-step kinase assay as described in Fig. 4A and analyzed by autoradiography (top) and immunoblot with the indicated antibodies (bottom). 32P incorporated into S6 was quantified by BAS 2000 in arbitrary units: lane 1, 131; lane 2, 149; lane 3, 124; lane 4, 242; lane 5, 502; lane 6, 550; lane 7, 183; lane 8, 94.3; lane 9, 96.6.

Fig. 4. Synergistic activation of p70α by mTOR and PDK1. HA-tagged wild-type p70α that had been treated by 0.2 μM rapamycin (lanes 3–10) was immunoprecipitated with (+) or without (−) endogenous mTOR, and the immunoprecipitates were subjected to the three-step kinase assay as described under “Experimental Procedures.” In lanes 11–14, HA-tagged wild-type p70α was stimulated with 10% serum in vivo, and a different amount of HA-p70α (100, 80, 66.6, and 50% (lanes 11–14, respectively) of the amount of p70α used in lanes 3–10) was immunoprecipitated and subjected to the S6 kinase assay. The samples were analyzed by autoradiography (top) and immunoblot with the indicated antibodies (bottom). The 32P incorporated into S6 were quantified by BAS 2000 in arbitrary units: lane 1, 0; lane 2, 2.7; lane 3, 72; lane 4, 86; lane 5, 129; lane 6, 128; lane 7, 896; lane 8, 898; lane 9, 7607; lane 10, 10,800; lane 11, 6930; lane 12, 4960; lane 13, 4220; lane 14, 2720.
hardly activated p70a, presumably reflecting the relatively poor access of Thr-252 to PDK1 in full-length, inactive p70a as seen previously (7). In contrast, phosphorylation of p70a by PDK1 after a prior phosphorylation by mTOR increased the p70a activity by 10-fold over that engendered by mTOR alone, to a level roughly 70-fold greater than that generated by PDK1 acting alone. Moreover, the S6 kinase activity generated in vitro by the sequential action of mTOR and PDK1 is indistinguishable from that achieved in vivo by stimulation of cells with 10% serum (Fig. 4).

The present results demonstrate that mTOR can catalyze directly the phosphorylation and activation of p70a in vitro. In addition, mTOR can activate p70a in a synergistic manner with PDK1 in vitro, and it is likely that this occurs in vivo. Nevertheless, the nature of the physiologic inputs that control mTOR-catalyzed p70a phosphorylation and the relative contribution of mTOR-catalyzed p70a phosphorylation to overall p70a regulation in vivo are not clear. Pretreatment of 3T3-L1 cells with insulin has been reported to cause a modest (1.3–2-fold) increase in the ability of mTOR to catalyze eIF-4E BP1 phosphorylation in vitro. Moreover, cooperation with active protein kinase B may enhance mTOR kinase, although evidence for direct activation of mTOR by protein kinase B is lacking. On this basis, mTOR has been proposed to be an intermediate in the insulin/PI-3 kinase-dependent activation of p70a (22). To the contrary, the ability of the rapamycin-resistant p70a-Δ2–46/ΔCT104 mutant to undergo insulin-stimulated, wortmannin-inhibitable Thr-412 phosphorylation and activation in the presence of concentrations of rapamycin far in excess of those required for complete inhibition of endogenous wild-type p70a and mTOR indicates that insulin-responsive kinases exist that are capable of p70a (Thr-412) phosphorylation and activation, other than mTOR (9).

Thus, the contribution of mTOR to insulin-stimulated p70a Thr-412 phosphorylation in vivo is unsettled but may be minor. Another possible role for the mTOR kinase is as the mediator of the amino acid-stimulated phosphorylation and activation of p70a; as yet however, direct evidence supporting amino acid regulation of the mTOR kinase activity is lacking (9). A plausible synthesis for the operation of the mTOR kinase in vivo is that insulin- and amino acid-induced signals each converge independently in the regulation of mTOR, although insulin also controls an alternative, mTOR-independent set of p70a-kinases. In turn, mTOR controls p70a through direct phosphorylation, as well as through the negative regulation of a p70a phosphatase.

Gingras et al. (19) reported that mTOR phosphorylates eIF-4E BP1 in vitro primarily on Thr-37 and Thr-46; these phosphorylations do not themselves result in the release of eIF-4E but are required for the further phosphorylation on several carboxyl-terminal serum-sensitive sites, and these latter phosphorylations result in the release of eIF-4E. In the case of p70a, mTOR alone gives some activation, but by phosphorylating the Ser/Thr-Pro sites within the carboxyl-terminal 104 amino acids to improve access of PDK1 and by phosphorylating Thr-252, mTOR strongly promotes the ability of PDK1 to phosphorylate Thr-252. In both instances, mTOR phosphorylation acts primarily in a “priming” role rather than a sole activator.

An intriguing and unresolved aspect of mTOR function is the apparent ability of its single catalytic domain to catalyze phosphorylation of Ser/Thr-Pro sites, such as those on eIF-4E BP1 and p70a Thr-444/Ser-447, as well as Phe-Ser/Thr-Phe/Tyr sites, such as p70a Thr-412. Although these two kinds of mTOR kinase activity appear differentially sensitive to detergent, they are both inhibited by rapamycin/FKBP-12 in vitro and by mutations in the mTOR kinase domain. Assuming both activities are physiologically meaningful, such a breadth in substrate specificity is relatively unprecedented among the protein kinases. Finally, it should be noted that Phe-Ser/Thr-Phe/Tyr motifs homologous to p70a Thr-412 are found in many kinases of the AGC (protein kinase A, G, and C) subclass, and it is of interest to note the recent report that PKCδ activation and phosphorylation at Ser-662 (in the context Phe-Ser-Phe) is inhibitible by rapamycin (23). In conclusion, the present study demonstrates the in vitro activation of p70a by mTOR-catalyzed phosphorylation involving p70a Thr-412, a critical site conserved in the other AGC kinase subfamily members.

Acknowledgments—We are grateful to Dr. Y. Nishizuka for encouragement. We thank Dr. D. R. Alessi for providing cDNA of PDK1. We also thank Dr. U. Kikkawa for valuable advice and H. Miyamoto for technical assistance. The skillful secretarial assistance of M. Kusu is cordially acknowledged.

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Immunopurified Mammalian Target of Rapamycin Phosphorylates and Activates \textit{p70 S6 Kinase} \textit{\alpha} \textit{in Vitro}

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\textit{J. Biol. Chem.} 1999, 274:34493-34498.
doi: 10.1074/jbc.274.48.34493

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