Regulation of the p70 S6 Kinase by Phosphorylation in Vivo
ANALYSIS USING SITE-SPECIFIC ANTI-PHOSPHOPEPTIDE ANTIBODIES*

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The p70 S6 kinase is activated by diverse stimuli through a multisite phosphorylation directed at three separate domains as follows: a cluster of (Ser/Thr) Pro sites in an autoinhibitory segment in the noncatalytic carboxyl-terminal tail; Thr-252 in the activation loop of the catalytic domain; and Ser-394 and Thr-412 in a segment immediately carboxyl-terminal to the catalytic domain. Phosphorylation of Thr-252 in vitro by the enzyme phosphatidylinositol 3-phosphate-dependent kinase-1 or mutation of Thr-412 → Glu has each been shown previously to engender some activation of the p70 S6 kinase, whereas both modifications together produce 20–30-fold more activity than either alone. We employed phospho-specific anti-peptide antibodies to examine the relative phosphorylation at several of these sites in wild type and various p70 mutants, in serum-deprived cells, and in response to activators and inhibitors of p70 S6 kinase activity.

Substantial phosphorylation of p70 Thr-252 and Ser-434 was present in serum-deprived cells, whereas Thr-412 and Thr-444/Ser-447 were essentially devoid of phospho-specific immunoreactivity. Activation of p70 by insulin was accompanied by a coordinate increase in phosphorylation at all sites examined, together with a slow decrease in mobility on SDS-PAGE of a portion of p70 polypeptides. Upon addition of rapamycin or wortmannin to insulin-treated cells, the decrease in activity of p70 was closely correlated with the disappearance of anti-Thr-412(P) immunoreactivity and the most slowly migrating portion of p70 polypeptides, whereas considerable phosphorylation at Ser-434 and Thr-252 persisted after the disappearance of 40 S kinase activity. The central role of Thr-412 phosphorylation in the regulation of kinase activity was further demonstrated by the close correlation of the effects of various deletions and point mutations on p70 activity and Thr-412 phosphorylation.

In conclusion, although p70 activity depends on a disinhibition from the carboxy-terminal tail and the simultaneous phosphorylation at both Thr-252 and Thr-412, p70 activity in vivo is most closely related to the state of phosphorylation at Thr-412.

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The p70 S6 kinase, an enzyme critical for cell cycle progression through G1, was among the first insulin/mitogen-activated protein (Ser/Thr) kinases to be identified, purified, and molecularly cloned. The enzyme was shown early on to be regulated by insulin/mitogen-stimulated (Ser/Thr) phosphorylation, and along with the kinases now known as Rsk provided the first evidence that insulin/mitogen signal transduction involved the recruitment of multiple, independently regulated cascades of protein (Ser/Thr) kinases (reviewed in Ref. 1). Nevertheless, whereas the in vitro activation of Rsk kinases by mitogen-activated protein kinase-catalyzed phosphorylation was accomplished early on (2), in vitro activation of p70 S6 kinase has proved much more difficult to reconstruct. During activation in vivo, p70 is phosphorylated at 10 or more sites by an array of independently regulated protein kinases. Enumerating the sites of phosphorylation, determining their individual functional roles in the process of p70 activation as well as the potential site-site interactions, has provided a formidable challenge. Work to date has identified four sets of phosphorylation sites on p70 that undergo insulin or mitogen-stimulated phosphorylation in situ, and whose mutation affects substantially p70 kinase activity. The first to be characterized were a cluster of 4–5 residues (Ser-434, Ser-441, Ser-447, Ser-452, and Thr-444) situated within an autoinhibitory pseudosubstrate segment in the p70 noncatalytic tail (3–6); conversion of 434, 441, 444, and 447 to Ala suppress mitogen-stimulated p70 activation, whereas conversion to Asp and Glu increases basal p70 activity (7). The phosphorylation of Thr-252 in the p70 activation loop within the catalytic domain is stimulated by serum and PI-3 kinase1 overexpression in situ, and whose mutation affects substantially p70 kinase activity. 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The abbreviations used are: PI-3 kinase, phosphatidylinositol 3-kinase; CHO-IR, Chinese hamster ovary-insulin receptor; HA, hemagglutinin; PDK, phosphatidylinositol 3-phosphate-dependent kinase; PAGE, polyacrylamide gel electrophoresis; PtdIns(3,4,5)P₃, phosphatidylinositol (3,4,5)-trisphosphate.
line and may also be a substrate for these kinases. Nevertheless, phosphorylation of PP2A-treated p70 in vitro with one or several proline-directed kinases does not restore any p70 S6 kinase activity (5).

Recently, the kinase PDK1 was shown to phosphorylate p70 in vitro selectively at Thr-252, with a resultant increase in p70 S6 kinase activity (11, 12). This finding together with the earlier demonstration of PI-3 kinase-stimulated, wortmannin-sensitive Thr-252 phosphorylation in vitro (8) establishes Thr-252 as a critical site of regulatory phosphorylation. Nevertheless, both the ability of p70 to be phosphorylated by PDK1 and the relative and absolute extent of p70 activation engendered by PDK1-catalyzed phosphorylation are greatly altered by a variety of p70 mutations (11). Thus conversion of the five clustered (Ser/Thr-Pro) phosphorylation sites in the p70 carboxyl-terminal tail to Ala strongly suppresses PDK1-catalyzed Thr-252 phosphorylation, whereas deletion of the entire carboxyl-terminal tail (to give p70 CT104) increases both the rate and extent of Thr-252 phosphorylation by PDK1 and also the degree of S6 kinase activation achieved at any level of Thr-252 phosphorylation. Deletion of p70 amino-terminal noncatalytic residues 2–46, a modification previously shown to greatly inhibit p70 activity, essentially abolishes the ability of p70 to be phosphorylated by PDK1 in vitro, whereas the additional deletion of the carboxyl-terminal tail (to give Δ2–46/ΔCT104), which restores p70 activity and mitogen responsiveness in vitro, also restores the ability of PDK1 to catalyze p70 Thr-252 phosphorylation in vitro. These results indicate that the p70 carboxyl-terminal tail exerts a strong influence on both the ability of PDK1 to catalyze the phosphorylation of Thr-252, as well as the extent of activation of S6 kinase activity resulting therefrom (11).

As regards the other sites of p70 phosphorylation, Thr-412 appears to play an especially important role, in that it influences both the ability of PDK1 to phosphorylate Thr-252 in vitro and the absolute extent of p70 activity that results from Thr-252 phosphorylation (11). Thus, conversion of Thr-412 in p70ΔCT104 to either Ala or Glu facilitates PDK1-catalyzed Thr-252 phosphorylation in vitro. Moreover, the functional interaction of Thr-252(P) and Thr-412 is the major, perhaps predominant, factor in determining p70 activity. Thus, whereas protein phosphatase 2A (PP2A)−treated p70ΔCT104 is essentially inactive, the selective phosphorylation of p70ΔCT104 at Thr-252 by PDK1 results in a 15-fold increase in 40 S kinase activity to a level comparable to that exhibited by PP2A−treated p70ΔCT104 (Thr-412 → Glu). This indicates that modification of either Thr-252 or Thr-412 singly is sufficient to confer some S6 kinase activity. This activity, however, represents no more than 3–5% of maximal S6 kinase activity, insomuch as PDK1 phosphorylation of PP2A−treated p70ΔCT104(T412E) increases its S6 kinase activity by a further 20–30-fold, to a level 240-fold greater than that exhibited by PP2A−treated (i.e., fully dephosphorylated) p70ΔCT104, demonstrating clearly the strong positive cooperativity between Thr-252 and Thr-412 in p70 activation (11). Thus, the phosphorylation state of the p70 carboxyl-terminal tail and Thr-412 both strongly influence the rate and extent of p70 Thr-252 phosphorylation catalyzed by PDK1 in vitro, as well as the absolute level of 40 S kinase activity engendered by a given extent of Thr-252 phosphorylation.

These data, derived for PDK1-catalyzed p70 phosphorylation in vitro, strongly suggest that any significant activation of S6 kinase in vivo requires the concurrent phosphorylation of both Thr-412 and Thr-252; the relevance of these in vitro data to p70 regulation in vivo as well as the determinants of Thr-252 and Thr-412 phosphorylation in vivo remain to be fully defined. To explore these questions we have employed anti-phosphopeptide antibodies developed toward several of the functionally important p70 phosphorylation sites as follows: Thr-412(P), Thr-252(P), Ser-434(P), and the cluster Ser-444(P)/Thr-447(P). These antibodies were used to examine the effects of insulin, recombinant PI-3 kinase, wortmannin, and rapamycin as well as the effects of p70 truncations and point mutations on site-specific p70 phosphorylation in vivo. We have attempted to correlate the changes in p70 S6 kinase activity with the site-specific phosphorylation, to verify whether the potent site-site interactions detected with in vitro phosphorylation obtain in vivo, and to identify which phosphorylation events determine S6 kinase activity during activation and deactivation in vivo.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium, fetal bovine serum, and chemicals were obtained from Sigma, Ham’s F-12 medium, LipojectAMINE, and protein G-agarose were purchased from Life Technologies, Inc. Restriction enzymes were products of New England Biolabs. [γ-32P]ATP, anti-rabbit Ig-linked horseradish peroxidase, and ECL were from Amersham Pharmacia Biotech.

Construction of p70 S6 Kinase Mutant Expression Vectors—A pMT2-based vector encoding an amino-terminal HA-tagged cognate proteins p70ΔCT104, p70Δ2–46, p70Δ2–46/ΔCT104, K123M, and T252A was described previously (13). Site-specific mutants p70T412A, T412E, Δ2–46/T412A, Δ2–46/T412E, T434A, T444A/S447A, S394A, and S394D were generated using a polymerase chain reaction-based method. The mutations were verified by DNA sequence and restriction analysis for newly introduced diagnostic restriction sites (11).

Transient Expression and Immunopurification of the p70 S6 Kinase—HEK293 cells and CHO-IR cells were maintained in Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 10% fetal bovine serum, respectively. HEK293 cells were transfected at 60% confluence in 100-mm dishes by the calcium phosphate method using 5 μg of plasmid DNA encoding wild type or variant p70. CHO-IR cells in 6-mm dishes were transfected with 1–2 μg of plasmid DNA using LipojectAMINE according to the manufacturer’s protocol (Life Technologies, Inc.). Twenty four hours post-transfection, cells were placed in serum-free medium for 16 h and then treated with insulin or 20% serum for the times indicated; in some experiments, rapamycin (50 nM) or wortmannin (100 nM) was added 10 min after insulin. Incubations were terminated by two rapid rinses with ice-cold phosphate buffered saline; the cells were then extracted with lysis buffer (13) and centrifuged at 12,000 g for 10 min. Aliquots of supernatants were matched for protein content, and HA p70 polypeptides were immunoprecipitated by addition of the monoclonal anti-HA epitope antibody 12CA5 and protein-G agarose at 4 °C for 3 h. The beads were washed three times with lysis buffer, once with 1 M NaCl in lysis buffer, and once with kinase reaction buffer. Endogenous p70 was immunoprecipitated using a rabbit polyclonal antisera generated against a recombinant fragment encoding p70 amino acids 422–525 (14).

S6 Kinase Assay—The immunocomplexes of the p70 S6 kinase were used to assay for p70 kinase activity using 40 S ribosomal subunit as substrate as described previously (13). The reaction mixtures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred a polyvinylidene difluoride membrane (Millipore Corp.), which was subjected to autoradiography for the detection of 32P-S6, and immunoblot analysis.

Anti-phosphopeptide Antibody Production—Phospho-specific antibodies directed against the various sites of p70 S6 kinase were produced by immunizing New Zealand White rabbits with the following synthetic phosphopeptides coupled to keyhole limpet hemocyanin: Thr-252(P) (HDGFTYHT*PGGGL), Thr-412(P) (NQQYPGLGPT*YVAPKKC), Ser-434(P) (EPKIR*SRFFGC), and Thr-444(P)/Ser-447(P) (SPRT*PV*PK). Enzyme-linked immunosorbent assay, using the phosphopeptide and corresponding nonphosphopeptide, was employed to identify the rabbits responding best. IgG was purified using protein A-Sepharose. Antibodies reactive with the nonphosphopeptide were removed by adsorption to a nonphosphopeptide (same sequence as above) immobilized affinity column. Antibodies that flowed through this column were next passed over a column of immobilized phosphopeptide; after washing, antibodies were eluted at low pH and dialyzed; these are subsequently referred to as “type 1” antibodies. The resulting antibodies were characterized by enzyme-linked immunosorbent assay against phospho- and nonphosphopeptides to determine the extent of phospho-specificity and subsequently by Western blotting to examine specificity.
against whole cell extracts. “Type 2” antibodies (seen only with the Thr-252(P) peptide immunogen) were those that bound to the affinity column that contained the nonphosphorylated peptide but showed little or no reactivity on immunoblot with a p70 Thr-252 → Ala mutant polypeptide (see “Results” and Fig. 1C).

Immunoblot Analysis of Phospho p70 Polypeptides—The electrophoresed p70 polypeptides were analyzed using a standard Western blot protocol. Briefly, the membrane was first blocked with 5% low-fat milk in TBST-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.2% Tween 20) at room temperature for 1 h, rinsed once with TBS-T, and probed with anti-phosphopeptide antibody at (0.02–0.05 μg/ml) in TBS-T for 1 h. Following three 20-min washes, the membranes were incubated with a horseradish peroxidase-labeled anti-IgG for 1 h at room temperature, followed by three 20-min washes with TBS-T. The membrane was wetted in ECL solution for 3 min, exposed to x-ray film, and developed.

RESULTS

The anti-p70 phosphopeptide antibodies were generated by immunization of female New Zealand White rabbits with synthetic phosphopeptide covalently coupled to keyhole limpet hemocyanin. The presence of phosphopeptide-specific immunoreactivity was detected by enzyme-linked immunoabsorbent assay using both the phosphorylated and nonphosphorylated peptides. After purification of IgG with protein A-agarose, the phosphopeptide-specific antibodies were purified by first passing the IgG over immobilized, nonphosphorylated peptide to remove antibodies reactive with nonphosphorylated epitopes; the nonadsorbed fraction was then passed over a column of immobilized phosphopeptide. After extensive washing, the retained immunoglobulins were eluted at low pH, rapidly neutralized, dialyzed, and concentrated. In this manner phosphopeptide-specific antibodies toward Thr-412(P), Thr-434(P), and the doubly phosphorylated peptide containing Thr-444(P)/Ser-447(P) were isolated.

The ability of each of these anti-p70 phosphopeptide antibodies to immunoblot recombinant p70 polypeptides was compared with an antibody prepared to a synthetic peptide corresponding to p70 amino acids 337–352, which contains no Ser or Thr residues (14). As is evident in Fig. 1A, this anti-p70 peptide antibody exhibits comparable reactivity with each of the recombinant wild type and mutant p70 polypeptides that is unaffected by insulin or serum treatment prior to extraction. The anti-p70 phosphopeptide antibodies react with all p70 variants except those wherein the Ser and/or Thr residue that was phosphorylated in the synthetic peptide antigen has been mutated to a nonphosphorylatable residue (Ala, Glu, or Asp). These features indicate that the anti-(Thr-412(P)), anti-(Thr-444(P)/Ser-447(P)), and anti-(Ser-434(P)) antibodies each are indeed specific for the peptide segment containing only a phosphorylated Ser and/or Thr. In addition, and to a varying extent, the immunoreactivity of each of these anti-p70 phosphopeptide antibodies is increased by insulin treatment prior to extraction.

As regards the anti-p70 Thr-252(P) antibodies, some sera (type 1) yielded anti-phosphopeptide antibodies with properties identical to those described above, i.e. nonadsorption to unphosphorylated peptide, adsorption to the phosphopeptide, and reactivity with wild type p70 but not with a p70 (Thr-252 → Ala) mutant polypeptide (Fig. 1B). These antibodies were, however, recovered in low yield, insufficient to complete the studies required. A second type of anti-(Thr-252(P)) antibody was found to adsorb to columns containing the immobilized nonphosphorylated peptide. These antibodies, when eluted from the peptide column, also showed highly selective reactivity with wild type p70 polypeptide over the p70 (Thr-252 → Ala), as well as a stimulus-dependent increase in immunoreactivity (Fig. 1C). It is not clear whether these type 2 antibodies are directly reactive with Thr-252(P) itself; conceivably, these antibodies may react with a conformationally dependent epitope on the activation loop that is generated as a consequence of Thr-252 phosphorylation but does not include the Thr-252(P) residue itself. This epitope must be highly dependent on Thr-252 phosphorylation inasmuch as the p70 (Thr-256 → Ala) mutant, which like the p70 (Thr-252 → Ala) mutant, is completely inactive, nevertheless exhibits an unimpaired insulin-stimulated increase in immunoreactivity with the type 2 anti-(Thr-252(P)) antibodies (Fig. 5B), a finding similar to that seen previously with 32P labeling in vivo (8). In practice, therefore, both types of anti-p70 (Thr-252(P)) antibodies provide a reliable monitor of the state of Thr-252 phosphorylation, even though the type 2 anti-p70 (Thr-252(P)) antibodies also have some reactivity with an epitope(s) present in the nonphosphorylated peptide segment.

Having defined the specificity of these antisera, we next examined the effects of serum (in 293 cells), insulin (in CHO-IR cells), or cotransfection with various candidate upstream activators on the 40 S kinase activity and site-specific phosphorylation of full-length, p70a1 polypeptides expressed transiently in 293 cells or CHO-IR cells. Insulin elicited a rapid 3–4-fold increase in 40 S kinase activity in CHO-IR cells, which attained 60% of maximal by the first time point examined, 2.5 min after insulin addition. All sites exhibited an increase in phosphorylation, with the largest relative increase observed with Thr-412 and Thr-444/Ser-447, primarily because these sites contain little or no detectable phosphate in serum-deprived cells (Fig. 2A). Thr-252 exhibits clear-cut basal phosphorylation, which nevertheless rapidly increases after insulin addition in parallel with the increased S6 kinase activity. Thus, even after the addition of submaximal concentrations of insulin, the increase in overall phosphorylation at all sites is comparably rapid, such that it cannot be reliably inferred from such data as to whether p70 phosphorylation occurs in an ordered sequence.

Given the relatively concerted phosphorylation in vivo of the wild type p70 after insulin addition, we attempted to evaluate the pattern of dephosphorylation that accompanies inhibition of p70 in vivo after addition of rapamycin or wortmannin, each added 10 min after the addition of insulin (Fig. 2B). Rapamycin led to the rapid inactivation of p70, with dephosphorylation evident at all sites, accompanied by a striking downshift, i.e. increase in p70 mobility on SDS-PAGE. The decrease in 40 S kinase activity, the disappearance most slowly migrating immunoreactive p70 polypeptide band (best seen in the anti-p70 (Thr-444(P)/Ser-447(P)) blot), and the disappearance of overall anti-(Thr-412(P)) immunoreactivity were each entirely congruent. Thr-252(P) immunoreactivity also diminished, but slightly more slowly, and did not disappear; this slower response was paralleled by the pattern seen with anti-(Ser-434(P)) immunoreactivity. The response to wortmannin, although somewhat slower, was also characterized by the concomitant disappearance of 40 S kinase activity, the most slowly migrating immunoreactive p70 polypeptide band, and total anti-p70 (Thr-412(P)) immunoreactivity, with somewhat slower and less pronounced dephosphorylation of Thr-252 and Ser-443.

These properties of recombinant p70 expressed in CHO-IR cells were compared with the behavior of the p70 polypeptide endogenous to these cells. Each of the anti-phosphopeptide antisera was sufficiently sensitive to dependably monitor the phosphorylation of the endogenous p70 except for the various anti-p70 (Thr-252(P)) antisera, whose sensitivity was at the limit of detection; visualization of p70 Thr-252(P) was therefore not achieved in every experiment. Given this limitation, insulin, after a brief lag, promoted the coordinate phosphorylation...
Regulation of p70 S6 Kinase Site-specific Phosphorylation

Fig. 1. Characterization of anti-p70 phosphopeptide antibodies. A, the specificity of various anti-p70 phosphopeptide antibodies. HEK-293 cells were transfected with plasmids encoding HA-tagged p70 wild type (WT), lanes 1 and 2) or HA p70α containing the point mutations Thr-412 → Ala (T412A, lanes 3 and 4); Thr-412 → Glu (T412E, lanes 5 and 6); Thr-252 → Ala (T252A, lanes 7 and 8); Lys-123 → Met (K123M, lanes 9 and 10); Ser-434 → Ala (S434A, lanes 11 and 12); and a double mutant, Thr-444 → Ala/Ser-447 → Ala (T444A,S447A, lanes 13 and 14). After 36 h, the cells were deprived of serum and 12 h later treated with medium (lanes 1, 3, 5, 7, 9, 11, and 13) or 10% serum (lanes 2, 4, 6, 8, 10, 12, and 14) for 20 min prior to harvest. Cell extracts matched for protein were immunoprecipitated with a monoclonal anti-HA antibody, 12CA5. Washed immunoprecipitates were assayed for S6 kinase activity (32P-S6) and immunoblot with the antibodies indicated. Type 2 anti-Thr-252(P) antibodies were used.32P cpm into S6, lane 1, 1250; lane 2, 3068; lane 3, 3461; lane 4, 4252; lane 5, 4163; lane 6, 4690; lane 7, 3600; lane 8, 1483; lane 9, 3590; lane 10, 3585; lane 11, 4453; lane 12, 3725; lane 13, 3527; lane 14, 3581. B, effect of rapamycin and wortmannin on the specificity of various anti-p70 phosphopeptide antibodies. CHO cells stably overexpressing human insulin receptor (CHO-IR cells) were transfected with pMT2 HA p70. Thirty six hours after the addition of insulin, rapamycin (200 nM, lanes 1, 3, 5) or insulin was added to a final concentration of 0.1 nM (lanes 2–7) or 10 nM (lanes 2–14). The time elapsed from addition of insulin to extraction is shown. Aliquots of extracts containing equal protein were subjected to immunoblot with the antibodies used. CAL27

Fig. 2. The relation of S6 kinase activity and site-specific phosphorylation of recombinant p70α1 in CHO-IR cells. A, time course of insulin-stimulated activation and site-specific phosphorylation of p70 S6 kinase. CHO cells stably overexpressing human insulin receptor (CHO-IR cells) were transfected with pMT2 HA p70. Thirty six hours after the cells were deprived of serum. After 12 h, carrier (lanes 1 and 8) or insulin was added to a final concentration of 0.1 nM (lanes 2–7) or 10 nM (lanes 2–14). The time elapsed from addition of insulin to extraction is shown. Aliquots of extracts containing equal protein were subjected to anti-HA immunoprecipitation followed by assay for S6 kinase (32P-S6) and immunoblot with the antibodies indicated. 32P cpm into S6, lane 1, 1250; lane 2, 3068; lane 3, 3461; lane 4, 4252; lane 5, 4163; lane 6, 4690; lane 7, 3600; lane 8, 1483; lane 9, 3590; lane 10, 3585; lane 11, 4453; lane 12, 3725; lane 13, 3527; lane 14, 3581. B, effect of rapamycin and wortmannin on the anti-HA immunoprecipitates were assayed for S6 kinase activity (32P-S6) and subjected to SDS-PAGE followed by electrophoretic transfer to a polyvinylidene difluoride membrane. Immunoblots using the following antisera were carried out. 1, anti-p70 peptide (amino acids 332–353); 2, anti-T412P, anti-p70 phosphopeptide (amino acids 405–416 Thr-412P); 3, anti-T444P/S447P, anti-p70 phosphopeptide (amino acids 441–452 Thr-444P/S447P); 4, anti-S434P, anti-p70 phosphopeptide (amino acids 429–440 anti-S434P). The actual 32P cpm incorporated into S6 in the kinase assay shown were: lane 1, 1022; lane 2, 7872; lane 3, 164; lane 4, 88; lane 5, 9393; lane 6, 14324; lane 7, 259; lane 8, 219; lane 9, 300; lane 10, 89; lane 11, 316; lane 12, 2930; lane 13, 2901; lane 14, 7168. C, the specificity of type 1 anti-p70 phosphopeptide (amino acids 244–257 Thr-252(P)) antibodies. HEK293 cells were transfected with either HA p70α1 wild type (lanes 1–4) or a mutant p70 (Thr-252 → Ala, lanes 5–8), singly (lanes 1, 2, 5, and 6) or together with a plasmid encoding a mutant, constitutively active p110 catalytic subunit of the PI-3 kinase (lanes 3, 4, 7, and 8). After 36 h, medium (lanes 1, 3, 5, and 7) or serum (to 10%, lanes 2, 4, 6, and 8) was added and the cells were harvested 20 min later. Anti-HA immunoprecipitates were assayed for S6 kinase (32P-S6) and subjected to SDS-PAGE followed by immunoblot with the antibodies used in A and, in addition, with type 1 anti-p70 phosphopeptide (Thr-252(P)) antibody. The actual 32P cpm incorporated into S6 in the kinase assay shown were as follows: lane 1, 2937; lane 2, 9089; lane 3, 7532; lane 4, 8079; lane 5, 519; lane 6, 168; lane 7, 94; lane 8, 46. C, the specificity of type 2 anti-p70 phosphopeptide (amino acids 244–257 Thr-252(P)) antibodies. HEK293 cells transiently expressing HA p70 wild type (lanes 1 and 2) or HA p70 Thr-252 → Ala (lanes 3 and 4) were treated with 10% serum and extracted, and anti-HA immunoprecipitates were prepared as described in A. These were subjected to SDS-PAGE followed by immunoblot with anti-p70 peptide (amino acids 332–353) antibody (panel 1) or type 2 anti-p70 phosphopeptide (amino acids 244–257 Thr-252(P)) antibodies (panel 2).
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stores insulin-stimulated 40 S kinase nearly completely, concomitant with the restoration of insulin-stimulated Thr-412 phosphorylation; Thr-252 phosphorylation, however, remains at the low level seen with either of the single deletions. These results suggest that the low activity accompanying the Δ2–46 deletion is attributable to a failure of Thr-412 (rather than Thr-252) phosphorylation. Support for this conclusion is provided by the ability of a Thr-412 → Glu mutation to overcome almost completely the inhibitory effect of Δ2–46, despite the continued presence of the carboxyl-terminal tail; conversion of Δ2–46 p70 (Thr-412) to Glu restores both 40 S kinase activity and Thr-252 phosphorylation (Fig. 4B). The Δ2–46/ΔCT104 double deletion mutant, although activated in vivo by insulin and inhibited by wortmannin, is almost completely resistant to inhibition by rapamycin (13). This correlates with the inability of rapamycin to reverse the mitogen-stimulated phosphorylation at Thr-412 in the Δ2–46/ΔCT104 variant, whereas wortmannin, presumably via inhibition of PI-3 kinase, causes the dephosphorylation of p70 Thr-412 and inactivation of Δ2–46/ΔCT104 (15). These findings indicate that the ability of rapamycin to inactivate wild type p70 through the dephosphorylation of p70 Thr-412 is not attributable to a rapamycin-induced inhibition of the PtdIns(3,4,5)P3-regulated kinases that act on Thr-412.

We next examined the effects of p70 point mutations on 40 S kinase activity and site-specific phosphorylation. Mutation of Lys-123 to methionine inactivates 40 S kinase activity completely but does not alter the pattern of basal or stimulated p70 phosphorylation, indicating that none of the insulin-stimulated p70 phosphorylations reflect (cis) autophosphorylation (Figs. 1A and 5F). As described above, Thr-256, a nonphosphorylated residue in the p70 activation loop, when converted to Ala, also inactivates the 40 S kinase activity without affecting p70 phosphorylation (Fig. 5F). In contrast, conversion of Thr-252 to Ala, in addition to abolishing the 40 S kinase activity, reduces the phosphorylation at Thr-412 to a great extent without affecting phosphorylation at sites within the carboxyl-terminal tail (Fig. 1A).

Reciprocally, mutation of Thr-412 to Ala abolishes 40 S kinase activity and Thr-252 phosphorylation (Fig. 5A) with little effect on Ser-434, Thr-444, or Ser-447 phosphorylation (Fig. 1A). Thus, whereas the truncation experiments indicate that the carboxyl-terminal tail influences strongly the phosphorylation at Thr-412 and Thr-252, inactivating mutations at either of these two residues, although each inhibiting phosphorylation at the other site, do not alter phosphorylation at the carboxyl-terminal sites. Mutation of Thr-412 to Glu results in a substantial activation of 40 S kinase activity in serum-deprived cells (Fig. 1A), an upshift in mobility on SDS-PAGE (although not as marked as with phosphorylation of Thr-412), and at least a partial restoration of the phosphorylation of Thr-252, as compared with its total absence in Thr-412 → Ala (Fig. 5A).

Although the degree of activation caused by Thr-412 → Glu mutation is somewhat more marked in the absence of the carboxyl-terminal tail (data not shown), the activity of both p70 (Thr-412 → Glu) and p70ΔCT104 (Thr-412 → Glu) can be further increased by insulin, an effect attributable, at least in part, to the insulin-stimulated phosphorylation of Thr-252 (e.g. Fig. 5A).

Recently p70 (Ser-394) was shown to be a site of mitogen-regulated phosphorylation, wherein mutation greatly diminished 40 S kinase activity (10). We confirm the inhibitory effect of Ser-394 mutation on 40 S kinase activity and find that mutation of Ser-394 to Ala or Asp greatly inhibits phosphorylation at Thr-412 (Fig. 5B). Neither removal of the carboxyl-terminal tail nor mutation of Thr-412 to Glu restores 40 S kinase activ-

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**Fig. 3.** The relation of site-specific phosphorylation of p70 S6 kinase endogenous to CHO-IR cells to S6 kinase activation and deactivation. A, time course of insulin activation. Serum-deprived CHO-IR cells were treated with carrier (lane 1) or insulin (10−7 M, lanes 2–7), and the cells were harvested thereafter at times indicated. The p70 polypeptide was immunoprecipitated with an antibody to the carboxyl-terminal tail (amino acids 422–525); the immunoprecipitates were assayed for S6 kinase activity (ΔP-P-S6) and immunoblotted with the antibodies indicated. B, site-specific phosphorylation of p70 endogenous to CHO-IR cells after addition of rapamycin or wortmannin to serum-depleted CHO-IR cells (Fig. 2A). The Δ2–46/ΔCT104 variant, whereas wortmannin, presumably via inhibition of PI-3 kinase, causes the dephosphorylation of p70 Thr-412 and inactivation of Δ2–46/ΔCT104 (15). These findings indicate that the ability of rapamycin to inactivate wild type p70 through the dephosphorylation of p70 Thr-412 is not attributable to a rapamycin-induced inhibition of the PtdIns(3,4,5)P3-regulated kinases that act on Thr-412.

Reciprocally, mutation of Thr-412 to Ala abolishes 40 S kinase activity and Thr-252 phosphorylation (Fig. 5A) with little effect on Ser-434, Thr-444, or Ser-447 phosphorylation (Fig. 1A). Thus, whereas the truncation experiments indicate that the carboxyl-terminal tail influences strongly the phosphorylation at Thr-412 and Thr-252, inactivating mutations at either of these two residues, although each inhibiting phosphorylation at the other site, do not alter phosphorylation at the carboxyl-terminal sites. Mutation of Thr-412 to Glu results in a substantial activation of 40 S kinase activity in serum-deprived cells (Fig. 1A), an upshift in mobility on SDS-PAGE (although not as marked as with phosphorylation of Thr-412), and at least a partial restoration of the phosphorylation of Thr-252, as compared with its total absence in Thr-412 → Ala (Fig. 5A).

Although the degree of activation caused by Thr-412 → Glu mutation is somewhat more marked in the absence of the carboxyl-terminal tail (data not shown), the activity of both p70 (Thr-412 → Glu) and p70ΔCT104 (Thr-412 → Glu) can be further increased by insulin, an effect attributable, at least in part, to the insulin-stimulated phosphorylation of Thr-252 (e.g. Fig. 5A).

Recently p70 (Ser-394) was shown to be a site of mitogen-regulated phosphorylation, wherein mutation greatly diminished 40 S kinase activity (10). We confirm the inhibitory effect of Ser-394 mutation on 40 S kinase activity and find that mutation of Ser-394 to Ala or Asp greatly inhibits phosphorylation at Thr-412 (Fig. 5B). Neither removal of the carboxyl-terminal tail nor mutation of Thr-412 to Glu restores 40 S kinase activ-
Regulation of p70 S6 Kinase Site-specific Phosphorylation

![Diagram](image)

**Fig. 4.** The effects of p70 S6 kinase amino- and carboxyl-terminal truncation on the S6 kinase activity and site-specific phosphorylation of p70. A, CHO-IR cells were transfected with HA-tagged p70 wild type (WT, lanes 1 and 2); p70 deleted of the carboxyl-terminal tail, amino acids 422–525 (ΔCT, lanes 3 and 4); p70 deleted of amino acids 2–46 (Δ2–46, lanes 5 and 6); and doubly deleted (Δ2–46/ΔCT). Twelve hours after removal of serum, the cells were treated with carrier (lanes 1, 3, 5, and 7) or insulin 10−7 M (lanes 2, 4, 6, and 8) and extracted 10 min later. Anti-HA immunoprecipitates were analyzed for S6 kinase activity ([32P]-S6) and by immunoblot using the antibodies indicated; type 2 anti-Thr-252P antibodies were used. [32P] cpm into S6: lane 1, 5953; lane 2, 19,517; lane 3, 3341; lane 4, 15,276; lane 5, 278; lane 6, 2570; lane 7, 3061; lane 8, 15,989. B, conversion of Thr-412 to Glu inhibits over the inhibition engendered by the Δ2–46 deletion. The experimental protocol was as in A. [32P] cpm into S6: lane 1, 3233; lane 2, 6937; lane 3, 427; lane 4, 1100; lane 5, 4057; lane 6, 5007.

**DISCUSSION**

The goal of these studies was to characterize, in an intact cell, the changes in phosphorylation of a cohort of sites on the p70 S6 kinase in response to agonists and inhibitors and to correlate those changes with p70 catalytic activity. This analysis was carried out through the use of anti-phosphopeptide antibodies, because the large number of data points examined would have rendered impractical such an analysis using conventional proteolysis and two-dimensional peptide mapping. Moreover, the residence of Thr-412 in a very hydrophobic domain has created difficulty in achieving reproducible tryptic/chymotryptic cleavage and recovery of this important site. Although the specificity of the antibodies employed has been clearly defined, the indirect nature of this analysis bears emphasis.

The sites chosen for study (Thr-252, Thr-412, Ser-434, Thr-444, and Ser-447) are a subset of those established by earlier work to be important in the regulation of p70 catalytic activity. Thus prior studies have shown that a cluster of sites in the p70 carboxyl-terminal tail (Ser-434, Ser-441, Ser-447, Ser-452, and Thr-444) (3–9), Thr-252 in the activation loop of the catalytic domain (8, 9, 11, 12, 16), and Ser-394 (10) and Thr-412 (9, 16), the latter two situated in a 65-amino acid segment immediately carboxyl-terminal to the catalytic domain, are each sites of insulin/mitogen-stimulated phosphorylation in vivo. Moreover, conversion of Thr-252, Thr-412, and Ser-394 to alanine each results in a >90% decrease in p70 activity, and the simultaneous conversion of amino acids 434, 441, 444, and 447 to alanine also suppresses the activation of p70 by over 80%. Furthermore, conversion of Thr-412 to Glu increases “basal” p70 activity, i.e., activity in serum-deprived cells; independently, the selective phosphorylation of Thr-252 in vitro by PDK1 also increases p70 activity (11, 12). When these two modifications are combined, p70 activity is 20–30-fold greater than with either modification singly (11). Thus each of the phosphorylations chosen for study is of established importance in p70 regulation. In the present study, we sought to understand how the phosphorylation of these sites is regulated in vivo and how each contributes to changes in p70 activity. A dominant conclusion derived from these results is that although the regulation of the p70 carboxyl-terminal tail and the phosphorylation of Thr-252 in the activation loop are each crucial to p70 activation, it is the changes in the phosphorylation of Thr-412 that corresponds most closely with the fluctuations of p70 activity in vivo (Fig. 6).

The cluster of phosphorylation sites in the p70 carboxy-terminal tail is situated in a pseudo-substrate autoinhibitory domain (3, 4), and it was inferred from the effects of mutation that multiple phosphorylation of these sites served to diminish the inhibitory potency of this segment by decreasing its ability to compete with the 40 S substrate for the catalytic domain (7). The present results uncover a separate, major mechanism of autoinhibition through the ability of the carboxyl-terminal tail to control access of upstream kinases to p70 phosphorylation sites crucial to activation, and in particular to Thr-412. The operation of the carboxyl-terminal tail as an inhibitor through this mechanism is revealed most clearly by deletion of p70 amino-terminal residues 2–46, which produces a profound inhibition of p70 that can be relieved by a further deletion of the carboxyl-terminal tail. Notably, the 2–46 deletion does not affect the phosphorylation of the carboxyl-terminal tail (at least at Ser-434, Thr-444, and Ser-447); however, the phosphorylation of Thr-412 is essentially abolished. Further deletion of the carboxyl-terminal tail restores Thr-412 phosphorylation and p70 activity. Alternatively, the need for Thr-412 phosphorylation can by bypassed by a Thr-412 → Glu mutation, which, like the carboxyl-terminal deletion, overcomes the inhibitory effect of the Δ2–46 deletion. The mechanism by which the p70 amino terminus controls the ability of the carboxyl-terminal tail to grant access to Thr-412 is unknown. The data in Fig. 5B and Ref. 10 indicate that the phosphorylation of Thr-412 is also dependent on the phosphorylation of Ser-394, another proline-directed site; it has not been determined as yet whether the Δ2–46 deletion alters Ser-394 phosphorylation.

Just as the p70 tail appears to control access to Thr-412, the phosphorylation of Thr-412 is one of the critical regulators of Thr-252 phosphorylation; mutation of the Thr-412 to Ala abolishes phosphorylation of Thr-252 (Fig. 5A). Inasmuch as Thr-252 is a site whose phosphorylation is known to be required for...
p70 activity, it could be argued that the effects of Thr-412 phosphorylation or p70 activity in vivo are achieved indirectly, through regulation of Thr-252. This cannot be entirely excluded until the effects of selective phosphorylation of Thr-412 has been determined. Nevertheless, it is clear that the activity of p70ΔCT104 (Thr-412 → Glu) is substantially greater than p70ΔCT104, even after both polypeptides have been extensively dephosphorylated in vitro with protein phosphatase 2A/PP2A (11). Moreover, the activity of PP2A-treated p70ΔCT104 (Thr-412 → Glu) is comparable to that of p70ΔCT104 selectively phosphorylated at Thr-252 by PDK1, and the finding that the modification of both sites concurrently results in potent synergistic activation strongly supports the conclusion that the phosphorylation of Thr-412 provides an important direct contribution to p70 activation in addition to its influence on the phosphorylation of Thr-252 in vivo (11). Moreover, it is clear that the phosphorylation of Thr-412 is in some manner dependent on Thr-252, as conversion of this amino acid to alanine greatly suppressed Thr-412 phosphorylation.

Given that the concurrent phosphorylation of Thr-252 and Thr-412 appears to be necessary for p70 activation, the question remains as to whether the phosphorylation at both sites is coordinately regulated in vivo under all circumstances. The data presented here, especially in Figs. 2 and 3, address this issue. They demonstrate that in serum-deprived cells, basal phosphorylation of the Thr-252 is detectable, whereas Thr-412 appears dephosphorylated. Consequently, to insulin addition, the phosphorylation at both these sites, as well as those in the carboxyl-terminal tail, increase concurrently with p70 activity. The coordinate phosphorylation of Thr-412 and Thr-252, however, is uncoupled in the presence of wortmannin and rapamycin. Both inhibitors cause dephosphorylation of p70 at all sites examined; however, the most rapid loss of phosphate occurs from Thr-412, followed closely by Thr-444/Ser-447, and the decrement in p70 kinase activity tracks directly with the loss of Thr-412(P) immunoreactivity. It is likely that the deactivation resulting from the selective Thr-412 dephosphorylation reflects the requirement for dual Thr-252/Thr-412 phosphorylation; however, this conclusion remains speculative in the absence of a selective inhibitor of Thr-252 phosphorylation. As to the mechanism underlying this rapid multisite dephosphorylation of p70, we infer that wortmannin, through the inhibition of PI-3 kinase, is deactivating the protein kinases responsible for p70 phosphorylation; direct evidence in support of this idea, however, is scant. The identity of the proline-directed kinases that act on p70 in vivo have not been unequivocally established; mitogen-activated protein kinases, stress-activated protein kinases, p38, and cdc2 can each phosphorylate some or all of these sites in vitro (5); the various Erks can be recruited through activation of PI-3 kinase in a cell- and stimulus-specific manner (17–19), and the actual effectors probably vary depending on the cell type and initiating stimulus, i.e. mitogens versus stress. PDK1, a candidate p70 Thr-252 kinase, appears to be constitutively active in vivo (21), and although it binds directly to PtdIns(3,4,5)P 3 in vitro, the lipid does not alter PDK1 catalytic activity. Thus wortmannin may inhibit Thr-252 phosphorylation either by deocalizing PDK1 from its site of action or by inhibiting the protein kinase acting on p70 (Thr-412). The p70 (Thr-412) kinase is probably activated by PtdIns(3,4,5)P 3 directly or indirectly, and the deactivation of Thr-412 phosphorylation may result in diminished phosphorylation or more rapid dephosphorylation of Thr-252.

The mechanism by which rapamycin promotes p70 Thr-412 dephosphorylation appears to be completely different than for wortmannin. Thus, whereas the S6 kinase activity and Thr-412 phosphorylation of the Δ2–46/ΔCT104 variant are both inhibited by wortmannin, they are resistant to rapamycin (15, 16). This indicates that rapamycin does not inhibit the Thr-412 kinase; instead, the rapamycin-induced p70 Thr-412 dephosphorylation probably reflects rapamycin activation of a protein phosphatase whose activity is normally restrained by the active mTOR kinase. This view contrasts with the conclusions of Burnett et al. (20) who found that immunoprecipitates of mTOR (RAFT-1) catalyze phosphorylation recombinant fragments of p70 directly at Thr-412. We find such in vitro p70 Thr-412 phosphorylation by mTOR to be trivial and probably irrelevant, inasmuch as the activity of p70 (41–364) expressed in Sf9 cells is completely inhibited by rapamycin.

A prominent feature of the p70 immunoblots is the presence of multiple, closely spaced immunoreactive polypeptide bands. This ladder-like array of p70 polypeptides on SDS-PAGE was first observed in isolates of purified rat liver p70 polypeptides (22) and was shown subsequently to reflect different degrees of
p70 phosphorylation (14). The progressively slower mobility is not due primarily to the increasing negative charge engendered by multiple phosphorylation but to an altered conformation caused by phosphorylations at specific sites, particularly Thr-252 and Thr-252, which have a disproportionate effect on p70 mobility, presumably reflecting the importance of phosphorylation at those sites in determining p70 structure.

The transiently expressed recombinant p70 polypeptide, in contrast to the endogenous p70 polypeptides, always exhibits a large predominance of rapidly migrating p70 bands, with only a small minority of bands exhibiting retarded mobility after insulin or serum treatment in vivo. This reflects the substantial overexpression of the recombinant p70 polypeptides, to an extent that far exceeds the capacity of the upstream activating kinases, so that only a minority of the recombinant p70 polypeptides undergo full phosphorylation (as reflected by maximal slowing of mobility on SDS-PAGE) and activation, even in response to maximal stimulation by insulin. Based on the relative elution of immunoreactive p70 polypeptides and 40 S kinase activity on Mono Q chromatography, as well as the effects of phosphatase (in vitro) (14) and rapamycin (in vivo) on p70 activity and mobility on SDS-PAGE (22, 23), it was concluded previously that only the most slowly migrating (presumably) fully phosphorylated p70 polypeptides possessed "any" 40 S kinase activity.

The immunostaining of p70 by each of the anti-phosphopeptide antibodies is biased toward the visualization of bands of slower mobility. This pattern is readily observed in the anti-(Thr-252(P)), anti-(Thr-444(P)/Ser-447(P)), and anti-(Thr-434(P)) immunoblots, and is most pronounced in the anti-(Thr-412(P)) blots, which in general show only a single band, corresponding in mobility to the most slowly migrating band visualized by the other antibodies. The predominant association of Thr-412(P) immunoreactivity with the most slowly migrating bands, which correspond to the catalytically "active" p70 polypeptides, can now be interpreted in the context of the very powerful positive cooperativity between Thr-412(P) and Thr-252(P) in conferring 40 S kinase activity on the p70 polypeptide. It is this doubly modified p70 polypeptide that probably corresponds to the catalytically active fraction, which is found exclusively among the most slowly migrating p70 polypeptides generated in vivo. The present results indicate that Thr-252 is phosphorylated on p70 polypeptides of every mobility, the majority of which lack substantial 40 S kinase activity (for example, especially as seen in serum-deprived cells or after wortmannin or rapamycin inhibition). Nearly all immunoreactive p70 (Thr-412(P)) polypeptides are found among the most slowly migrating p70 polypeptides, probably because the Thr-12 phosphorylation can itself cause the upshift. The fraction of these Thr-412(P) polypeptides that are also phosphorylated at Thr-252 cannot be ascertained; however, those that are doubly modified will be at least 20–30-fold more active than those phosphorylated at Thr-412 only. Serum-deprived cells contain a reservoir of Thr-252-phosphorylated p70 polypeptides, not yet phosphorylated at Thr-412; inasmuch as Thr-252 phosphorylation appears to require Thr-412 phosphorylation, the presence of p70 Thr-252(P) but not Thr-412(P) in serum-deprived cells must reflect a differential sensitivity of these two sites to phosphatase as suggested by Fig. 2B. The ability of insulin and serum to increase the phosphorylation of Thr-252 likely reflects, in part, a prior phosphorylation of Thr-412. These considerations, especially the very low abundance of

![Diagram of the insulin/mitogen activation of the p70 S6 kinase.](Image)
The phosphorylation of Thr-412(P) in serum-deprived or wortmannin/rapamycin-inhibited cells as compared with p70 Thr-252(P), indicate that it is the phosphorylation of Thr-412 that is the rate-limiting step in p70 activation. Presumably, it is also the last step in p70 activation (Fig. 6), although the concerted nature of the p70 multisite phosphorylation does not enable us to support this conclusion directly.

The identification of the p70 Thr-412 kinase remains one of the major unrealized goals necessary to complete the description of the apparatus interposed between PI-3 kinase and the p70 S6 kinase. The differences in the extent of phosphorylation at Thr-412 and Thr-252 in response to serum deprivation, rapamycin, wortmannin, as well as the ability to dissociate Thr-252 phosphorylation from Thr-412 phosphorylation in vivo through deletion of the carboxyl-terminal tail or conversion of Ser-394 to Ala all suggest that Thr-412 is phosphorylated in vivo by a different kinase than is Thr-252. This is further reinforced by the very marked selectivity of PDK1 for Thr-252 in vitro (11, 12), whereas Thr-252 and Thr-412 are phosphorylated with similar alacrity in vivo in response to insulin. A definitive answer requires the identification of a kinase specific for Thr-412, which is regulated by PI-3 kinase directly or indirectly, in a manner consistent with the patterns of insulin-stimulated p70 Thr-412 phosphorylation that occur in vivo, as described in this report.

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