MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5648–5655 Vol. 17, No. 9
0270-7306/97/$04.00+0

Dual Requirement for a Newly Identified Phosphorylation Site in p70^{s6k}

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Received 7 April 1997/Returned for modification 16 May 1997/Accepted 12 June 1997

The activation of p70^{s6k} is associated with multiple phosphorylations at two sets of sites. The first set, S_{411}, S_{418}, T_{421}, and S_{424}, reside within the autoinhibitory domain, and each contains a hydrophobic residue at −2 and a proline at +1. The second set of sites, T_{229} (in the catalytic domain) and T_{389} and S_{404} (in the linker region), are rapamycin sensitive and flanked by bulky aromatic residues. Here we describe the identification and mutational analysis of three new phosphorylation sites, T_{367}, S_{371}, and T_{447}, all of which have a recognition motif similar to that of the first set of sites. A mutation of T_{367} or T_{447} to either alanine or glutamic acid had no apparent effect on p70^{s6k} activity, whereas similar mutations of S_{371} abolished kinase activity. Of these three sites and their surrounding motifs, only S_{371} is conserved in p70^{s6k} homologs from *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Saccharomyces cerevisiae*, as well as many members of the protein kinase C family. Serum stimulation increased S_{371} phosphorylation; unlike the situation for specific members of the protein kinase C family, where the homologous site is regulated by autophosphorylation, S_{371} phosphorylation is regulated by an external mechanism. Phosphopeptide analysis of S_{371} mutants further revealed that the loss of activity in these variants was paralleled by a block in serum-induced T_{389} phosphorylation, a phosphorylation site previously shown to be essential for kinase activity. Nevertheless, the substitution of an acidic residue at T_{389}, which mimics phosphorylation at this site, did not rescue mutant p70^{s6k} activity, indicating that S_{371} phosphorylation plays an independent role in regulating intrinsic kinase activity.

The ability of quiescent cells to transit G_0/G_1 in response to mitogens or of cycling cells to exit metaphase and initiate a new cell cycle is tightly associated with the activation of a number of specific protein kinases (5, 11, 15). Among the kinases affected are the p70^{s6k} and p85^{s6k} isoforms, which are differentially targeted to the cytoplasm and nucleus, respectively (reviewed in references 5 and 15). The 40S ribosomal protein S6 is the target of p70^{s6k} and p85^{s6k} in both compartments of the cell (reviewed in reference 43). Microinjection of p70^{s6k} and p85^{s6k} inhibitory antibodies or treatment of cells with the immunosuppressant rapamycin, which selectively blocks p70^{s6k} and p85^{s6k} activation, inhibits the ability of cells to progress through G_1 (7, 26, 27, 39, 41). Consistent with these findings, rapamycin in parallel suppresses the selective translational upregulation of an essential family of mRNA transcripts which contain a polypyrimidine tract at the transcriptional start site, 5′TOP (23, 43a). This family of mRNA transcripts has been shown to encode for a number of components of the translational apparatus, including ribosomal proteins and elongation factors (30). Recent studies from this laboratory have demonstrated that the effects of rapamycin on these transcripts are elicited through inhibition of p70^{s6k} activation, presumably by blocking S6 phosphorylation (22). Translational inhibition of these transcripts would suppress upregulation of components of the protein synthetic apparatus and thus cell growth (33).

Activation of p70^{s6k}, and presumably p85^{s6k}, has been demonstrated to be regulated by phosphorylation at two distinct sets of sites (reviewed in reference 40). The first set of phosphorylation sites described are clustered within a small region of the molecule which has been characterized as a potential autoinhibitory domain (17). All these sites, S_{411}, S_{418}, T_{421}, and S_{424}, contain a proline at the +1 position and a hydrophobic residue at the −2 position (13). Substitutions of alanines at these four sites severely impair the ability of the kinase to be activated by mitogens, whereas substitutions of acidic residues at these positions raise basal kinase activity (18, 37). Although the recognition motif described closely fits that described for the mitogen-activated protein (MAP) kinase family of proteins (8) and MAP kinase phosphorylates some of these residues in vitro (32), the results with different growth factors (1), specific inhibitors (45), growth factor receptor docking site mutants (6, 31), and dominant interfering signalling components (31) have discounted a role for MAP kinase in modulating p70^{s6k} and p85^{s6k} activation. The second set of phosphorylation sites have recently been identified as T_{229} in the activation loop (37, 46) and T_{389} and S_{404} (37) in the linker region which couples the catalytic and autoinhibitory domains. Unlike the sites in the autoinhibitory domain, these sites are flanked by large bulky aromatic residues at both the +1 and −1 positions. Phosphorylations of these sites, as well as S_{411}, are selectively inhibited by rapamycin (18, 37). The direct target of rapamycin in a gain-of-function inhibitory complex with FKBP12 was initially identified as TOR1/TOR2 in yeast (20), followed by identification of the mammalian homolog FRAP (3). TOR1/TOR2 and FRAP have homologies with lipid and protein kinases, but substrates still await identification (3, 21). The conversion of the principal site of rapamycin-induced p70^{s6k} dephosphorylation and inactivation, T_{389}, to an acidic residue confers rapamycin resistance on a p70^{s6k} variant, p70$^{s6k}E_{389}$ (37). More recent studies have further demonstrated that acute phosphorylation of this site, together with hyperphosphorylation of the serine/threonine-proline (S/T-P) sites, acts in concert with spe-

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TABLE 1. Characterization of purified phosphopeptides A, Q, and N

Phosphopeptide	Phosphoamino acid(s)[c]	Phosphate release	Molecular mass (Da)[b]	Sequence[a]
A	P-T	Cycle 11	4,151 (4,172.43)	GASASTANPQT*PVEYP-//-PLPIR
Q	P-T, P-S	Cycles 5 and 9	2,832 (2,831.89)	FTRQT*PVDS*PDDSTLSESANQVFL
N	P-S		2,322 (2,320.47)	QTPVDS*PDDSTLSESANQVFL

[a] The residues identified by direct sequencing of each peptide are underlined, and phosphorylated residues are indicated by asterisks.
[b] Parenthetical data are predicted molecular masses.
[c] P-T, phosphothreonine; P-S, phosphoserine.

cific domains in the molecule (4, 47) to bring about kinase activation (9).

For the identification of rapamycin-sensitive sites, large amounts of the corresponding phosphopeptides were isolated from a histidine-tagged variant of the kinase (37). During these studies, three additional phosphopeptides, two of which contain S/T-P sites not previously noted in the kinase, were isolated. Here we describe the identification of these sites, as well as the site present in the third phosphopeptide, and an assessment of their functional importance through mutational analysis. Next, we determined whether one of these sites, S_{371}, whose mutation had a severe effect on kinase activity, is phosphorylated in the endogenous kinase and whether it serves as a site for autophosphorylation, as previously described for other members of the second-messenger family of serine/threonine kinases, such as protein kinase C (PKC) (34). Finally, we determined whether S_{371} phosphorylation increases in response to serum and whether mutating this site affects kinase function through blocking phosphorylation at this site or by affecting phosphorylation of a secondary site.

MATERIALS AND METHODS

Plasmid constructs and mutagenesis. With the exception of His-tagged p70^{s6k} (37) (see Table 1), the p70^{s6k} constructs used in this study were tagged with the myc 9E10 epitope at the amino terminus of the p70^{s6k} isoform as previously described (31). Site-directed mutagenesis was carried out by using the Altered Site II mutagenesis system (Promega) with either wild-type p70^{s6k}, p70$^{s6k}\Delta C_{104}$ (9), or p70^{s6k} D3E (37) as the template. The glutathione *S*-transferase (GST)–p70^{s6k} and GST-p70$^{s6k}Q_{100}$ constructs will be described elsewhere. The mutations were confirmed by sequencing, and all constructs were subcloned in a cytomagalovirus promoter-driven expression vector.

Cell cultures, transfections, and metabolic labeling. Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and seeded at 10^6 cells per 10-cm-diameter plate for 24 h prior to transfections. Transient transfections were performed overnight by a modified calcium phosphate procedure (35) with either between 1 and 10 μg of plasmid DNA or (for in vivo labeling) between 0.5 and 5 μg of plasmid DNA, complemented with vector DNA to 10 or 5 μg of total DNA. After 12 h, cells were washed twice in serum-free Dulbecco's modified Eagle's medium and incubated for an additional 24 h prior to 1-h serum stimulation and subsequent extraction. For in vivo labeling, 1 mCi of $^{32}P_i$ per 5 ml was added after the 24-hour starvation period and cells were incubated for an additional 7 h prior to serum stimulation and extraction. Except where indicated, 20 nM rapamycin or the vehicle alone and 0.1% ethanol were added 15 min prior to serum stimulation.

Immunoprecipitation and kinase assay. Kinase assays were performed directly (24) or after immunoprecipitation with the 9E10 antibody as previously described (31).

Purification of phosphopeptides. The expression, purification, and digestion of His-tagged p70^{s6k} (see Table 1) were carried out as previously described (37). Endogenous p70^{s6k} or transiently expressed p70$^{s6k}\Delta C_{104}$ or p70$^{s6k}\Delta C_{104}T_{371}$ was purified from total cell extracts of in vivo- and $^{32}P_i$-labeled 293 cells with either M5 (38) or 9E10 antiserum (12) as previously described (28, 31). The immunoprecipitated kinase was washed and digested with a mixture of chymotrypsin-trypsin (9), and the resulting phosphopeptides were resolved by reversed-phase high-pressure liquid chromatography (HPLC) on a glass-lined C_{18} column (2 by 100 mm; SGE). Fractionated peptides were collected and separated by two-dimensional, thin-layer electrophoresis–chromatography (TLE-TLC), and the resultant chromatograms were visualized and quantitated where stated by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Characterization of phosphopeptides A, Q, and N. The amino acid and cycle at which ^{32}P-labeled phosphate was released were determined by automated solid-phase sequence analysis as described previously (37). The molecular masses of phosphopeptides were determined with a matrix-assisted laser-induced time-of-flight mass spectrometer (MAT LASERMAT; Finnigan) and a matrix of α-cyano-4-hydroxycinnamic acid. The experimental molecular mass values given are the means of 10 independent 50-shot-averaged spectra. The experimentally determined and predicted molecular mass values, including those for the peptide sample which gave rise to a weak and therefore less accurate signal, were within the experimental error.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis. Two-dimensional chymotryptic-tryptic phosphopeptide analysis and one-dimensional phosphoamino acid analysis were performed as previously described (13).

RESULTS

Identification of three new S/T-P sites. During large-scale expression experiments aimed at identifying the rapamycin-sensitive phosphorylation sites of p70^{s6k} (37), three additional phosphopeptides, termed A, Q, and N, were purified (Table 1). Chemical sequencing, phosphate release, phosphoamino acid analysis, and mass spectrometry identified phosphopeptide A as G_{437} to R_{476}, singly phosphorylated at T_{447} (see Materials and Methods) (Fig. 1; Table 1), whereas phosphopeptide Q was identified as F_{363} to L_{386}, doubly phosphorylated at resi-

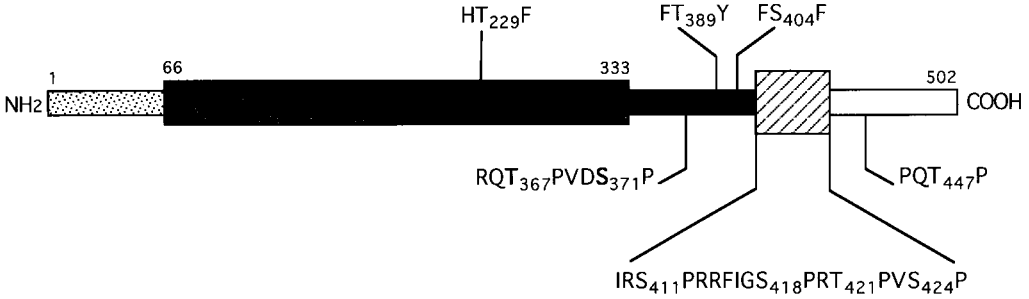

FIG. 1. Schematic representation of p70^{s6k}. Phosphorylation sites are shown with corresponding amino acid positions and surrounding sequences indicated. The sites analyzed here are in bold. Each p70^{s6k} domain is indicated as follows: the amino terminus is dotted, the catalytic domain is grey, the linker domain is black, the autoinhibitory domain is hatched, and the carboxy terminus is white.

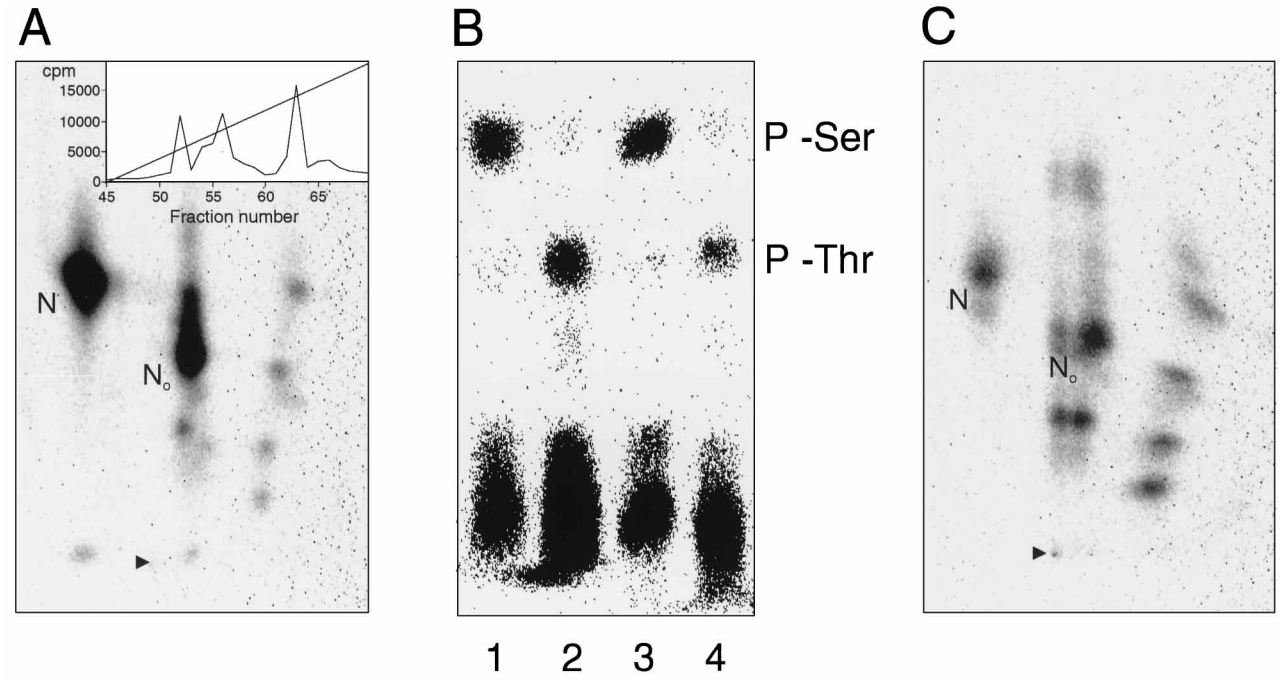

FIG. 2. Two-dimensional TLE-TLC phosphopeptide analysis of $p70^{s6k}\Delta C_{104}$ (A) and $p70^{s6k}\Delta C_{104}T_{371}$ (C) mutants. $p70^{s6k}\Delta C_{104}$ and $p70^{s6k}\Delta C_{104}T_{371}$, transiently expressed in human 293 cells, were immunoprecipitated, digested with chymotrypsin-trypsin, and purified by HPLC, and fractions 55 to 57 were analyzed by two-dimensional TLE-TLC as described in Materials and Methods. In panel A, 10,000 cpm was applied; in panel C, 3,000 cpm was applied. The origins (arrowheads) and positions of phosphopeptides N and N_0 are indicated. The HPLC elution profile of radiolabeled $p70^{s6k}\Delta C_{104}$ is shown in the inset of panel A. The dotted line indicates the acetonitrile gradient (15 to 23%). (B) Phosphoamino acid analysis of N peptides (lanes 1 and 2) and N_0 peptides (lanes 3 and 4) obtained from $p70^{s6k}\Delta C_{104}$ (lanes 1 and 3) and $p70^{s6k}\Delta C_{104}T_{371}$ (lanes 2 and 4). P-Ser and P-Thr, phosphoserine and phosphothreonine, respectively.

dues T_{367} and S_{371} (Table 1). Phosphorylation at T_{367} in the Q phosphopeptide most likely inhibits cleavage by trypsin at R_{365}, leading to the recovery of the larger peptide, as previously reported for other trypsin cleavage sites in $p70^{s6k}$ (37). Although the N peptide was blocked at the amino terminus, mass spectrometry and phosphoamino acid analysis identified it as Q_{366} to L_{386}, containing a single phosphoserine (Table 1). Under mild-acid conditions, Q_{366} probably undergoes ring closure, forming the amino-terminal-blocked pyroglutamyl peptide (see below). The obvious candidate site for phosphorylation in the N phosphopeptide was S_{371}, though three other potential sites of phosphorylation exist (Table 1).

To determine whether S_{371} is the site of phosphorylation, it was mutated to a threonine in the $p70^{s6k}\Delta C_{104}$ mutant (9) and ectopically expressed in 293 cells in parallel with the parent construct. The $p70^{s6k}\Delta C_{104}$ mutant was chosen because it lacks the last 104 amino acids of the kinase and thus six phosphorylation sites. HPLC resolution of the chymotryptic-tryptic digest obtained from the $^{32}P_i$-labeled parent construct revealed a major peak of radioactivity that emerged between fractions 55 and 57 (Fig. 2A, inset), where the N and Q peptides had been previously found to elute (37). An analysis by two-dimensional TLE-TLC revealed two major phosphopeptides in this fraction, the N peptide and a second peptide, termed N_0 (Fig. 2A). Although the N_0 peptide was assumed to be the earlier described Q phosphopeptide (37) (Table 1), phosphoamino acid analysis revealed mostly phosphoserine, with only trace amounts of phosphothreonine detected, probably due to contaminating phosphopeptides (Fig. 2B, lane 3). The migration of the N_0 peptide by TLE-TLC and its coelution by HPLC with the N peptide suggested that it represented the N peptide with a free amino terminus. Consistent with this and the hypothesis that S_{371} is the site of phosphorylation in the N peptide, phosphoamino acid analysis of both peptides from the T_{371} mutant (Fig. 2C) revealed that in each case, phosphothreonine was observed instead of phosphoserine (Fig. 2B; compare lanes 1 and 3 with lanes 2 and 4). The T_{371} mutant incorporated less ^{32}P in vivo than did the parent construct, as reflected in proportionally lower levels of the N and N_0 phosphopeptides (Fig. 2C). Collectively, the results demonstrate the existence of three additional $p70^{s6k}$ phosphorylation sites, which exhibit surrounding amino acid motifs similar to those in the autoinhibitory domain (Fig. 1).

S_{371}, a critical phosphorylation site. To obtain insight into the importance of the three new phosphorylation sites for kinase function, each residue was changed singly to either an alanine or an acidic residue, glutamate for threonine and aspartate for serine, and placed in a myc-epitope-tagged, wild-type $p70^{s6k}$ construct (31). Extracts from either quiescent or serum-stimulated 293 cells, transiently expressing each mutant, were immunoprecipitated and assayed for S6 kinase activity with 40S ribosomes as the substrate. The basal activity of each mutant was comparable to that of the wild-type kinase (Fig. 3), and the activities of T_{367} and T_{447} mutants in response to serum were similar to that of wild-type $p70^{s6k}$ (Fig. 3). In contrast, the substitution of an alanine or aspartic acid for S_{371} nearly abolished serum-induced kinase activation (Fig. 3), in a manner comparable with that reported for similar amino acid substitutions at T_{229} in the activation loop (37, 46). In all cases, the expression levels of transfected kinase mutants were equivalent (data not shown). Although these observations do not eliminate a role for T_{367} or T_{447} phosphorylation in regulating kinase activity, they do suggest a critical function for S_{371} phosphorylation in regulating $p70^{s6k}$ activity. To ensure that

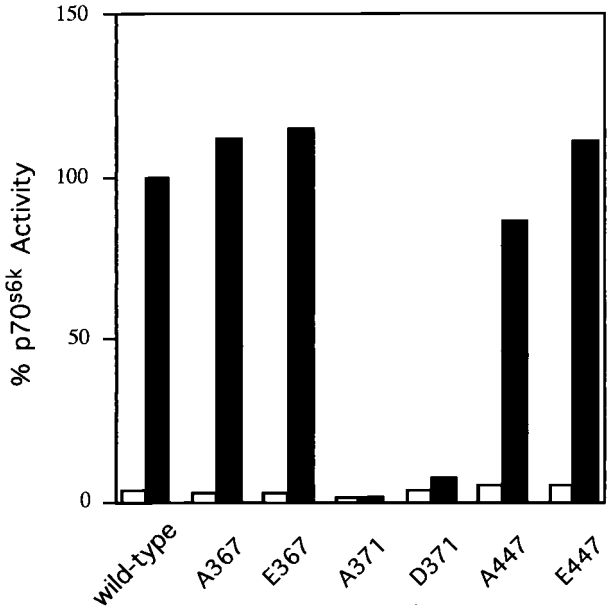

FIG. 3. Effects of T_{367}, S_{371}, and T_{447} point mutations on p70^{s6k} activity. The activity of each mutant, transiently expressed in 293 cells, was determined by an immunocomplex assay before (open bars) and after (black bars) serum stimulation (see Materials and Methods). Activities were normalized for the level of expressed kinase and are representative of the results obtained from three independent experiments.

phosphorylated S_{371} is present in the endogenous kinase and is not due to ectopic expression, p70^{s6k} was isolated from serum-stimulated 293 cells and the N and N_0 phosphopeptides were purified as described above (Fig. 2). An analysis of fractions 55 to 57 revealed two phosphopeptides that migrated to positions identical to those of the N and N_0 phosphopeptides (Fig. 4A). Consistent with this finding, the N phosphopeptide contained only phosphoserine, as did the predicted homolog of the N_0 peptide (Fig. 4B). Furthermore, incubation of the N_0 peptide from the endogenous kinase with acetic acid and formic acid led to generation of the N peptide (Fig. 4C, lane 2), whereas such treatment had no effect on the N peptide. In contrast to quiescent 293 cells, the addition of serum led to a marked increase in the level of S_{371} phosphorylation (Fig. 5). Although this site was not as acutely upregulated as the rapamycin-sensitive sites T_{229}, T_{389}, S_{404}, and S_{411} were, the relative increase induced by serum was approximately threefold, as measured with ImageQuant software (see Materials and Methods). Together, the results indicate that S_{371} is a critical site of endogenous p70^{s6k} phosphorylation and that its phosphorylation state increases after serum stimulation.

S_{371} is regulated by an upstream kinase. It has been demonstrated that the PKC β_{II} isotype, a member of the second-messenger family of serine/threonine kinases (34), is phosphorylated at T_{500}, T_{641}, and S_{660} (25, 44), positions equivalent to T_{229}, S_{371}, and T_{389} in p70^{s6k}. Furthermore, T_{641}, the p70^{s6k} S_{371} homologous site, has been reported to be a major site of autophosphorylation (44). These observations raised the possibility that S_{371} phosphorylation is mediated by autophosphorylation. The strategy employed to examine this hypothesis was to first generate a kinase-dead form of p70^{s6k} by mutating the functionally critical lysine in subdomain II, K_{100}, to a glutamine, thereby attenuating intramolecular autophosphorylation. This construct, p70^{s6k}Q$_{100}$, was tagged with GST at the carboxy terminus so that it could be readily isolated. Next, 293 cells overexpressing this construct were pretreated with rapamycin prior to the addition of $^{32}P_i$ and serum in order to eliminate intermolecular autophosphorylation by endogenous p70^{s6k}. Compared with wild-type GST-p70^{s6k}, the kinase-dead GST-p70^{s6k}Q$_{100}$ variant exhibited no S6 kinase activity (Fig. 6A; compare lanes 1 and 2). Although endogenous p70^{s6k} activity was measured from serum-stimulated cells expressing GST-p70^{s6k}Q$_{100}$ (Fig. 6A, lane 3), this activity was totally abolished by rapamycin treatment (Fig. 6A, lane 4). In contrast to two-dimensional phosphopeptide maps of GST-p70^{s6k}Q$_{100}$ from untreated cells (Fig. 6B), equivalent maps from cells treated with rapamycin revealed either the absence or severe reduction of the macrolide-sensitive phosphorylation sites T_{229}, T_{389}, S_{404}, and S_{411} (Fig. 6C). Nevertheless, such treatment had no apparent effect on the phosphorylation of S_{371}. Similar results were obtained for the wild-type parent construct (data not shown). It should be noted that in comparison to

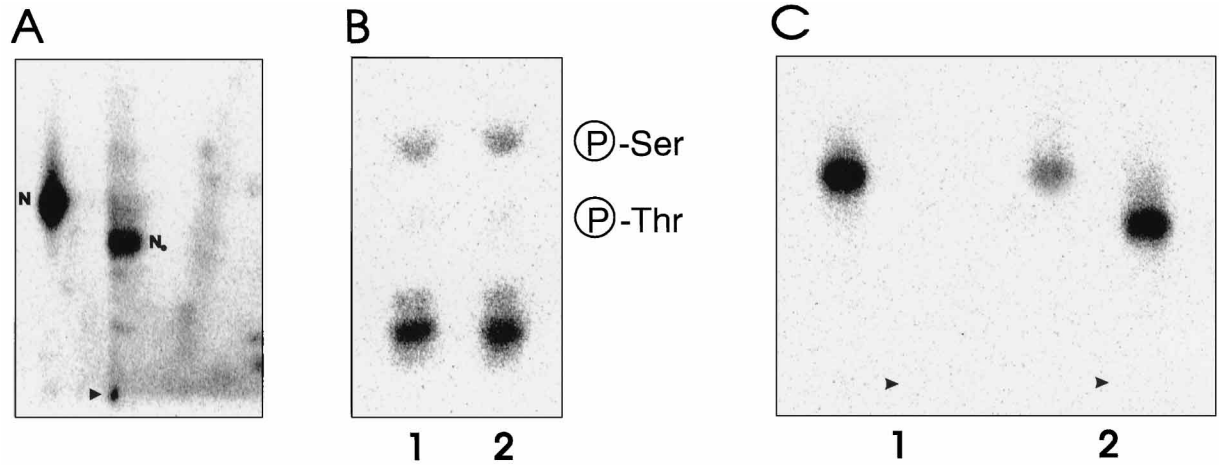

FIG. 4. Characterization of N and N_0 peptides from endogenous p70^{s6k}. (A) Endogenous p70^{s6k} from in vivo-labeled 293 cells was immunoprecipitated, digested with chymotrypsin-trypsin, and purified by HPLC, and fractions 55 to 57 were analyzed by two-dimensional TLE-TLC as described in Materials and Methods. An arrowhead indicates the origin. (B) Phosphoamino acid analysis of the N (lane 1) and N_0 (lane 2) peptides from panel A. P-Ser and P-Thr, phosphoserine and phosphothreonine, respectively. (C) Two-dimensional tryptic-chymotryptic phosphopeptide mapping of the N (lane 1) and the N_0 (lane 2) peptides from panel A after treatment with acetic acid and formic acid (see Materials and Methods). Arrowheads indicate origins.

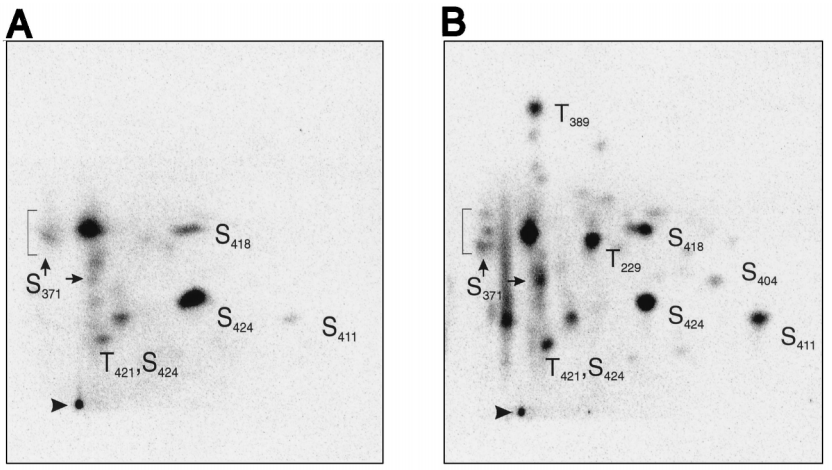

FIG. 5. Upregulation of S_{371} in serum-stimulated cells. Endogenous $p70^{s6k}$ two-dimensional tryptic-chymotryptic phosphopeptide maps from quiescent (A) and serum-stimulated (B) 293 cells are shown. Arrows and brackets indicate the positions of phosphorylated S_{371}. Arrowheads indicate origins.

phosphopeptide maps of endogenous $p70^{s6k}$ (Fig. 5B), the rapamycin-sensitive sites T_{229}, T_{389}, and S_{404} in transiently expressed $p70^{s6k}$ were less represented (Fig. 6B), as previously noted (9). Recent studies suggest that this is due to the fact that the signalling mechanism which regulates phosphorylation at these sites is limiting (22) (unpublished data). Together, these data support a model in which S_{371} phosphorylation is mediated by a rapamycin-resistant $p70^{s6k}$ kinase rather than through intra- or intermolecular autophosphorylation.

Dual role for S_{371} phosphorylation. Recent studies have suggested that mitogen-induced increases in the phosphorylation of specific $p70^{s6k}$ sites may be interdependent (reviewed in references 9 and 40). The loss of activity in S_{371} mutants (Fig. 3) might be due to inhibition of phosphorylation at a second site rather than prevention of phosphorylation at the primary residue. Indeed, it has been suggested that the S_{371} homologous site in PKC regulates phosphorylation at a site equivalent to that of T_{389} (25). To test this possibility, epitope-tagged wild-type $p70^{s6k}$ and $p70^{s6k}D_{371}$ were expressed in serum-stimulated cells and analyzed by two-dimensional TLE-TLC phosphopeptide mapping. Compared with wild-type $p70^{s6k}$, the $p70^{s6k}D_{371}$ mutant lacks the two peptides containing phosphorylated S_{371} (Fig. 7). However, just as striking is the complete absence of the T_{389} phosphopeptide (Fig. 7B). In contrast to T_{389}, the remaining sites of phosphorylation associated with $p70^{s6k}$ activation, including T_{229} in the activation loop, S_{404} in the linker region, and S_{411}, S_{418}, T_{421}, and S_{424}, were not significantly affected (Fig. 7B). These findings suggest that S_{371} mutants block $p70^{s6k}$ activation by suppressing T_{389} phosphorylation. If the only role of S_{371} phosphorylation is to regulate T_{389} phosphorylation, the effects of the alanine and aspartic acid mutations at this site should be rescued by placing an acidic residue at T_{389}. When this mutation is expressed in the $p70^{s6k}D_3E$ background, it has high basal kinase activity and can be activated by serum to the same extent as the wild-type kinase is (37). The results of such an experiment demonstrate that the activity of neither mutant was rescued during expression in the background of the $p70^{s6k}D_3E$-E_{389} variant (Fig. 8). Collectively, these results indicate that in addition to controlling T_{389} phosphorylation, S_{371} phosphorylation plays an independent role in determining $p70^{s6k}$ activity.

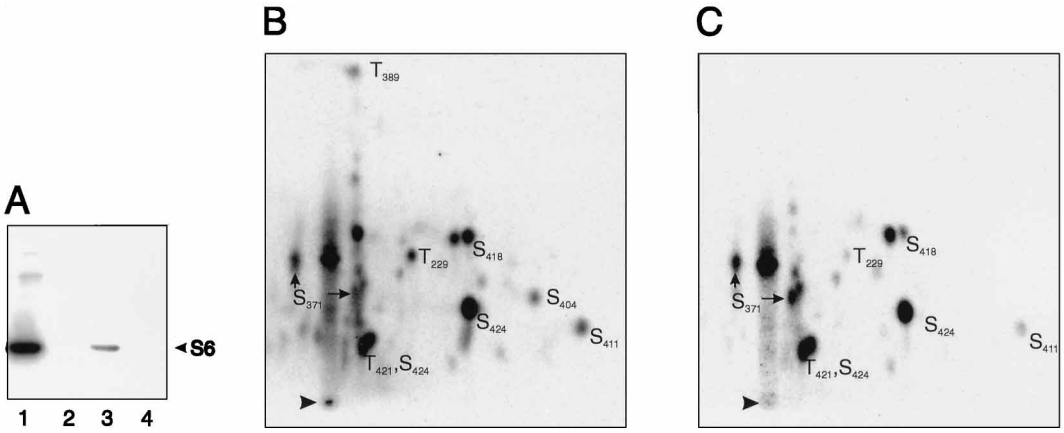

FIG. 6. Activity and phosphopeptide analysis of a kinase-dead $p70^{s6k}$, $p70^{s6k}Q_{100}$. (A) S6 kinase activities from serum-stimulated cell extracts expressing either GST-$p70^{s6k}Q_{100}$ (lanes 2 to 4) or wild-type GST-$p70^{s6k}$ (lane 1) after affinity purification (lanes 1 and 2) and from total cell extracts of cultures treated with the vehicle alone (lane 3) or the vehicle and rapamycin (lane 4) (see Materials and Methods) are shown. (B and C) Two-dimensional tryptic-chymotryptic phosphopeptide maps of affinity-purified GST-$p70^{s6k}Q_{100}$ from 293 cells treated with the vehicle alone and with the vehicle plus rapamycin, respectively. Arrowheads indicate origins.

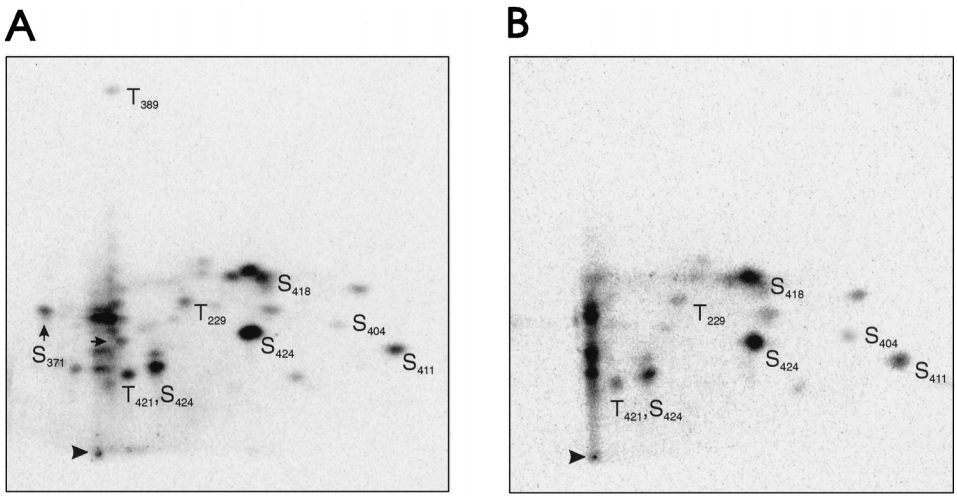

FIG. 7. Phosphopeptide analysis of the p70^{s6k}D$_{371}$ mutant. Wild-type p70^{s6k} (A) and the p70^{s6k}D$_{371}$ mutant (B) were compared after two-dimensional tryptic-chymotryptic phosphopeptide mapping as described in Materials and Methods. Arrowheads indicate origins.

DISCUSSION

Initial studies aimed at identifying the sites of phosphorylation associated with p70^{s6k} activation led to discovery of four clustered residues within the putative autoinhibitory domain (13). As four discrete bands were readily detected by electrophoretic mobility shifts of the kinase after mitogenic stimulation (7, 18, 37), it was reasoned that the four identified sites were responsible for kinase activation. Although conversion of these residues to acidic amino acids raised basal kinase activity (18), activation was further augmented by the addition of mitogens, a response which was blocked by rapamycin pretreatment (14). Furthermore, the acidic mutations had little effect on the electrophoretic mobility of the kinase (14), collectively indicating that additional phosphorylation sites which displayed rapamycin sensitivity were implicated in kinase activation. These sites were subsequently identified as T$_{229}$ in the activation loop and T$_{389}$ and S$_{404}$ in the linker region that couples the catalytic and autoinhibitory domains of the kinase (37). Mutations of T$_{229}$ and T$_{389}$ to alanine abolished kinase activity, suggesting that these sites are essential in regulating this response (37). In the case of T$_{389}$, this was further substantiated by the finding that conversion of this residue to an acidic amino acid conferred rapamycin resistance on the kinase (9, 37). The data presented here suggest that in addition to T$_{229}$ and T$_{389}$ phosphorylation, S$_{371}$ phosphorylation plays a critical role in regulating kinase activity.

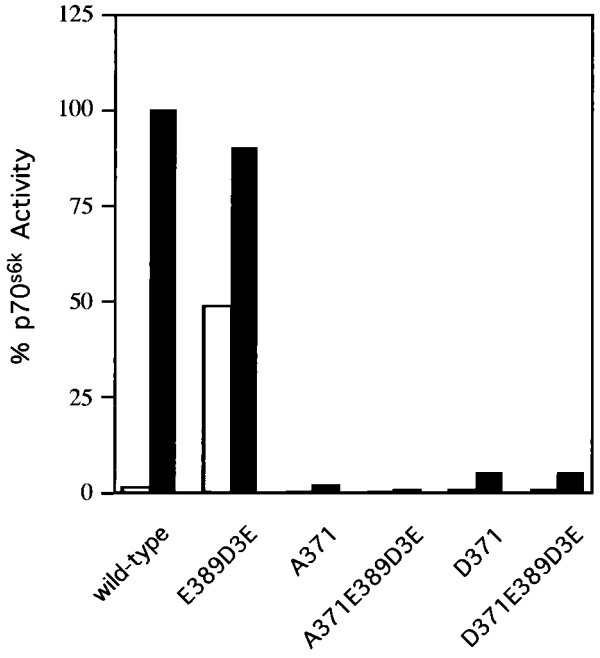

FIG. 8. Effect of expressing S$_{371}$ mutants in the p70^{s6k}D$_3$E-E$_{389}$ background. The activity of each mutant, transiently expressed in human 293 cells, was determined by an immunocomplex assay before (open bars) and after (black bars) serum stimulation (see Materials and Methods). Activities were normalized for the level of expressed kinase and are representative of the results obtained from three independent experiments.

In a recent analysis of various p70^{s6k} mutants, peptides migrating to positions equivalent to those of the N and N$_0$ peptides were designated as being doubly phosphorylated at T$_{367}$ and S$_{371}$ (9). This designation was based on the R_f values of the initially identified Q phosphopeptide. However, the data presented here demonstrate that the N peptides are singly phosphorylated at S$_{371}$. This observation prompted an analysis of the phosphoamino acid contents of all phosphopeptides migrating near the origin by using both the whole kinase and the double-truncation mutant p70$^{s6k}\Delta$N$_{54}\Delta$C$_{104}$ (9). Unexpectedly, no phosphopeptides were found to contain similar proportions of phosphoserine and phosphothreonine. Possibly these differences were due to the fact that the initial analysis (Table 1) utilized a different construct and the resulting material did not undergo the same digestion protocol as that used in this study (see Materials and Methods).

Recently, we noted that many members of the second-messenger family of serine/threonine kinases contain not only T$_{389}$ but also the conserved motif surrounding this site (37) (Fig. 9). Furthermore, the distance from this site to the site in the activation loop, T$_{229}$, is also conserved, suggesting an intrinsic interaction between these residues which is modulated by phosphorylation (37). In the case of p70^{s6k}, recent studies have shown that T$_{389}$ mutants modulate phosphorylation of T$_{229}$, implying a regulatory link between these two sites (unpublished data). Closer inspection of the complete linker region

FIG. 9. Conservation of the domain carboxy terminal to the catalytic core in p70^{s6k} homologs and PKC. Shown is an alignment of the p70^{s6k} sequence from the end of the catalytic domain until amino acid residue 396 and the corresponding sequences from the p70^{s6k} homologs of *D. melanogaster* (Dp70^{s6k}) (42), *A. thaliana* (ATK2) (49), and *S. cerevisiae* (YPK1) (29) as well as from PKC α and PKC β_{II} (34). The black background indicates consensus sequence, and the grey background indicates similarity to the consensus sequence. The percentage of sequence identity to the consensus is indicated at the extreme right of each sequence. The conserved motifs surrounding S_{371} and T_{389} are underlined.

shows that not only the sequence motif surrounding T_{389} but the whole domain is highly conserved (Fig. 9). Indeed, as with T_{389}, S_{371} and its surrounding motif appear to be conserved in all p70^{s6k} homologs, including those from *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Saccharomyces cerevisiae*, as well as in PKC α and β_{II} (Fig. 9). Interestingly, this is not the case for T_{367} and T_{447}, suggesting that they play a role which is unique to the mammalian p70^{s6k}. Although it has been suggested that the homologous S_{371} site is autophosphorylated in members of the PKC family, the data presented in Fig. 6 strongly imply that S_{371} is phosphorylated by a p70^{s6k} kinase, not by an intra- or intermolecular autophosphorylation mechanism. In fact, S_{371} also displays a hydrophobic residue at the −2 position and a proline at the +1 position, equivalent to the S/T-P sites in the autoinhibitory domain (Fig. 1). Interestingly, we found that when the autoinhibitory domain sites were changed to acidic residues, they facilitated phosphorylation of T_{389} (unpublished data). The fact that a mutation of S_{371} to either an acidic or neutral residue suppresses phosphorylation at T_{389} fits a model in which phosphorylation at all five residues by the same p70^{s6k} S/T-P kinase regulates the access of a distinct kinase to T_{389} and subsequent phosphorylation at T_{229}. Consistent with such a model, the substitution of an alanine for either T_{229} or T_{389} or rapamycin treatment, which blocks their phosphorylation, had no effect on S_{371} phosphorylation (Fig. 6 and data not shown). Thus, one role of phosphorylated S_{371} is to participate in regulating phosphorylation at a secondary site that is involved in acute p70^{s6k} activation.

Similar to the effects observed by mutation of T_{229} in the T loop of p70^{s6k} (37), both acidic and neutral substitutions at S_{371} severely attenuated the activity of the kinase (Fig. 3). Furthermore, the inhibitory effects of S_{371} mutants cannot be rescued by placing either mutant in the background of the p70$^{s6k}D_3E$-E_{389} variant (Fig. 8). Mutations at T_{367} had little or no effect on kinase activity, although this site is proximal to S_{371} (Fig. 3). This is in contrast to mutations at residues surrounding T_{229}, which resulted in inactivation of the kinase, although none has been reported to be phosphorylated (46). Together, these observations suggest that S_{371} is precisely positioned not only for a role in regulating T_{389} phosphorylation but possibly for a more central role in regulating kinase activity. The importance of this site is further underscored by the observation that the homologous site in other kinases of the second-messenger family is spatially conserved in relation to the catalytic domain (19).

As pointed out earlier, the site equivalent to S_{371} has been identified as a major autophosphorylation site in PKC β_{II} (44) and more recently in PKC α (25), T_{641} and T_{638}, respectively. In both cases, there is a proline at the +1 position and a hydrophobic residue at the −2 position (Fig. 9). Although this motif does not represent a PKC recognition motif, modelling studies have suggested that it is juxtaposed to the active site, allowing autophosphorylation by an intramolecular reaction (36). This intramolecular mode of regulation is consistent with in vitro phosphorylation studies (16). Initially, it was demonstrated that the mutation of this site to an alanine in PKC β_I, T_{642}, led to the abolishment of kinase activity and loss of the slower-migrating phosphorylated form of the kinase (48). Similarly selective removal of phosphate from this site in PKC β_{II} with phosphatase 1 led to the production of an inactive kinase (25). By employing this approach, it was further argued that transphosphorylation of T_{500} in the activation loop generates an active form of the kinase which autophosphorylates at T_{641}, equivalent to S_{371} in p70^{s6k}, followed by autophosphorylation at S_{660}, equivalent to T_{389} in p70^{s6k}. Surprisingly, selective dephosphorylation of T_{500} and S_{660} by phosphatase 2A did not ablate PKC β_{II} activity (25). However, the importance of this same residue in PKC α is not so marked (2). In PKC, the phosphorylation of these sites is involved in the maturation of the kinase (2, 10, 48). In contrast, acute or hyperphosphorylation of the homologous sites in p70^{s6k} is associated with activation of the mature enzyme in response to mitogens. The emerging paradigm is that many of the residues and motifs implicated in the activation of this family of kinases are the same but that the regulatory mechanisms employed in their utilization can vary dramatically among members of the family.

ACKNOWLEDGMENTS

We thank C. Hamilton for typing the manuscript and F. Zilbermann for assistance with tissue culture. We thank M. Andjelkovic and T. Myles for critically reading the manuscript. We also thank all the members of the laboratory for their discussions and support during these studies.

B.A.M. is a recipient of a predoctoral fellowship from Novartis, Basel, Switzerland. P.B.D. and N.P. are recipients of long-term postdoctoral fellowships from the Human Frontier Science Program Organization. These studies were supported in part by grants from the National Health and Medical Research Council (R.E.H.W.) and the Human Frontier Science Program Organization (G.T.).

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