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Activation of p70^{s6k} is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs

(Swiss 3T3 cells/*in vivo* $^{32}P_i$ labeling/two-dimensional mapping/phosphorylation sites)

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ABSTRACT **Partial amino acid sequences were obtained from 22 internal tryptic peptides of rat liver p70^{s6k} (M_r 70,000 ribosomal protein S6 kinase), 3 of which were found to contain phosphorylated residues. To determine whether these sites were associated with p70^{s6k} activation, the kinase was labeled to high specific activity with $^{32}P_i$ in Swiss mouse 3T3 cells. By sequential cleavage with CNBr and endoproteinase Lys-C followed by two-dimensional tryptic peptide analysis, it could be shown that all of the sites were located in a small endoproteinase Lys-C peptide of M_r 2400. Analysis of the p70^{s6k} protein sequence revealed a single candidate that could represent this peptide. Three tryptic peptides derived from the endoproteinase Lys-C fragment were chosen by a newly described computer program as the most likely candidates to contain the *in vivo* sites of phosphorylation. Synthetic peptides based on these sequences were phosphorylated either chemically or enzymatically and found to comigrate by two-dimensional thin-layer electrophoresis/chromatography with the four major *in vivo* labeled tryptic phosphopeptides. Three of the phosphorylation sites in these peptides were equivalent to those sequenced in the rat liver p70^{s6k}. In addition, all four sites display the motif Ser/Thr-Pro, typical of cell cycle-regulated sites, and are clustered in a putative autoinhibitory domain of the enzyme.**

Growth factors induce quiescent cells in culture to reenter the cell cycle, replicate their DNA, and divide through a complex array of biochemical responses (1). In most cases this process is initiated through specific ligand-activated receptor tyrosine kinases at the cell surface but is orchestrated intracellularly by a network of activated serine/threonine kinases (2). The mechanisms by which tyrosine kinases activate serine/threonine kinases are still unclear, with the possible exception of protein kinase C (2, 3). One of the many early obligatory responses elicited by growth factors is the activation and maintenance of high rates of protein synthesis, which is required for reentry and transit through the G_1 phase of the cell cycle (4, 5). This event is associated with increased phosphorylation of serine/threonine residues in a number of specific translational components, including 40S ribosomal protein S6 (6, 7). *In vitro* and *in vivo* studies have indicated that phosphorylation at five serine residues at the carboxyl terminus of S6 is required for the activation of protein synthesis (8–11).

The kinase mediating this event was first detected in extracts of quiescent cells stimulated to proliferate by serum or epidermal growth factor (12, 13). Purification of this S6 kinase activity eventually revealed an enzyme of M_r 70,000, referred to as p70^{s6k} (14, 15), which is highly specific for ribosomal protein S6 (14), biphasically activated (16, 17), and selectively inactivated by type 2A phosphatase (18). The p70^{s6k} phosphorylates four and possibly all five of the S6 sites observed *in vivo* (19), with recognition of the substrate absolutely dependent on the first and third arginine residues of a block of three at positions 231–233 (20). Sufficient amounts of p70^{s6k} were recently obtained from livers of cycloheximide-treated rats (21, 22) to allow protein sequencing and cloning of the enzyme (23, 24). These studies also revealed a second and less abundant isoform of the kinase (25, 26) named p85^{s6k} (26), which is derived from the same gene, appears to be nuclear-targeted, and is activated after mitogenic stimulation (26).

As a first step towards unraveling the upstream signaling pathways involved in p70^{s6k} activation, we set out to identify the sites of phosphorylation associated with activation of this kinase. Through a number of approaches, all of the sites were found to have a common motif and to be clustered within a 19-amino acid peptide located in a putative autoinhibitory domain (24). The role of this peptide sequence in serving as an autoinhibitor and the role of the kinases that may regulate the phosphorylation of these sites are discussed.

MATERIALS AND METHODS

Metabolic Labeling and Purification of p70^{s6k}. Swiss mouse 3T3 cells were grown, maintained, and labeled with $^{32}P_i$ as before (27), except that the volume of medium was reduced to 15 ml, and 65 μCi (1 μCi = 37 kBq) of $^{32}P_i$ was added per ml overnight. After stimulation of cells, the medium was removed and the cells were washed twice with 10 ml of ice-cold extraction buffer (120 mM NaCl/20 mM NaF/10 mM pyrophosphate/5 mM EGTA/1 mM EDTA/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride/30 mM 4-nitrophenyl phosphate/50 mM Tris·HCl, pH 8.0). Cells were scraped from the plate with a rubber policeman into 6 ml of extraction buffer made 1% with Nonidet P-40; after 20 strokes of a glass Dounce homogenizer, the homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was collected and recentrifuged at 460,000 × *g* for 30 min at 4°C. The final supernatant was divided into 300-μl aliquots, each being incubated for 2 hr at 4°C with 5 μl of p70^{s6k} M5 antiserum (28), after which 20 μl of 50% protein A-Sepharose was added and mixed for 30 min at 4°C. The protein A-Sepharose immune complex was pelleted for 5 min at 4°C at maximum speed in an Eppendorf centrifuge (model 5412). The resulting pellet was washed once in extraction buffer containing 1% Nonidet P-40 before loading on a 15% acrylamide/0.08% bisacrylamide/SDS gel and 30-min elec-

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Abbreviations: TLE/TLC, thin-layer electrophoresis/chromatography; >PhNCS, phenylthiohydantoin; DTT, dithiothreitol; endo Lys-C, endoproteinase Lys-C; 1-D or 2-D, 1 or 2 dimensional; p70^{s6k}, M_r 70,000 ribosomal protein S6 kinase.

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trophoresis. The kinase was localized by autoradiography, excised from the gel, and eluted from the gel piece in 0.1% SDS/100 mM NH_4HCO_3, pH 8.2, with a sample concentrator model 1750 (ISCO). To remove SDS, the kinase either extracted as described (29) or was dialyzed first against 0.5% Tween 20/100 mM NH_4HCO_3, pH 8.2, and second against 100 mM NH_4HCO_3 (pH 8.2).

$p70^{s6k}$ Cleavage and Peptide Separation. The $p70^{s6k}$ was cleaved with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (15 μg; Worthington), endoproteinase Lys-C (endo Lys-C; 15 μg; Wako Pure Chemical, Osaka), or CNBr (0.5 M; Fluka). Aliquots of the final digest were analyzed on a 15.5% acrylamide/1% bisacrylamide/Tricine/SDS gel (30). The products of the trypsin digest were made 0.1% with CF_3COOH and loaded onto a glass-lined C_{18} column (2 × 100 mm) (SGE) by using a totally inert HPLC system (19). Peptides were separated with a 0–70% gradient of acetonitrile in 0.1% CF_3COOH for 360 min and monitored at 214 nm. All peak fractions were dried in a Speed Vac concentrator. Nonsymmetrical peak fractions were resuspended in 30 mM sodium acetate (pH 5.7) and further purified on the same glass-lined C_{18} column with a 0–40% gradient of acetonitrile in 30 mM sodium acetate (pH 5.7) for 200 min. Tryptic phosphopeptides were resolved by two-dimensional (2-D) thin-layer electrophoresis/chromatography (TLE/TLC) as described (29). For phosphoamino acid analysis, radioactive spots were scraped from the plate, eluted with formic acid/acetonitrile, 1:1 (vol/vol), dried in a Speed Vac Concentrator, and analyzed as described (29).

Sequencing of $p70^{s6k}$ Peptides. Symmetrical peaks from both HPLC runs were sequenced in an Applied Biosystems 477A sequencer equipped with a miniaturized reaction cartridge; rapid-cycle chemistry and on-line analysis programs (31) were used. Phosphorylated serine and threonine were identified by the increase of their dithiothreitol (DTT)/phenylthiohydantoin (>PhNCS) addition products, >PhNCS-DTT-dehydroalanine and >PhNCS-DTT-dehydroaminobutyric acid, respectively (32).

***In Vitro* Phosphorylation of Synthetic Peptides.** Peptides for *in vitro* phosphorylation reactions were synthesized and sequenced as before (20) and then incubated at a final concentration of 500 μM with either purified $p42^{mapk}$ (33) in 10 mM Mops/0.2 mM DTT/2 mM $MgCl_2$/0.02% Triton X-100/200 μM [γ-^{32}P]ATP (specific activity, 2 μCi/nmol), pH 7.2, or with *Xenopus* $p34^{cdc2}$ precipitated with Sepharose-bound cyclin A in 20 mM Hepes/10 mM $MgCl_2$/5 mM 4-nitrophenylphosphate/0.4 mM DTT/200 μM [γ-^{32}P]ATP (specific activity 2.5 μCi/nmol), pH 7.4. Reactions were carried out for 30 min at 37°C in a final volume of 10 μl and terminated by adding 5 μl of glacial acetic acid. Peptides were resolved from [γ-^{32}P]ATP on 500-μl AG 501-X8 (Bio-Rad) self-packed columns, dried, and analyzed by one-dimensional TLE under the conditions described above (29). The chemically phosphorylated heptapeptide Thr-421 through Lys-427 was synthesized on a Milligen-9050 continuous-flow synthesizer by using an adapted software package and Kieselguhr-supported poly(dimethylacrylamide) as solid support (34).

RESULTS

Identification of Phosphorylated Peptides. To avoid loss of phosphorylated peptides during the sequencing of activated $p70^{s6k}$ from rat liver (23), tryptic peptides were resolved on an inert HPLC system with a narrow-bore glass-lined C_{18} column (Fig. 1). Of the peptides isolated, 15 were judged sufficiently pure for sequencing, whereas an additional 7 peptides had to be further purified. Sequencing was carried

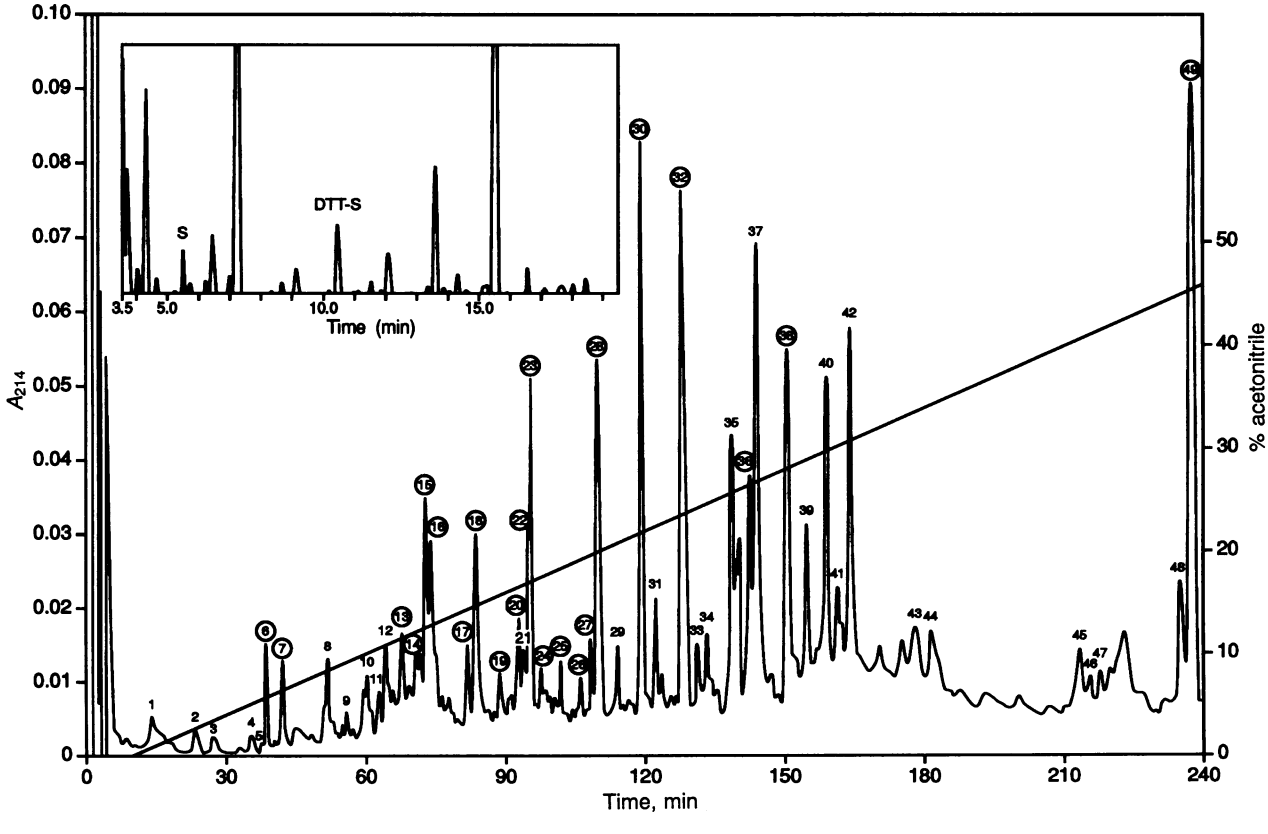

FIG. 1. Reversed-phase HPLC purification of $p70^{s6k}$ peptides. After trypsin digestion of ≈70 μg of kinase (23), the resulting peptides were separated on a C_{18} column as described in text. The peak peptides sequenced are circled. (*Inset*) Cycle three of the rapid-cycle sequencing of peak 7; S indicates serine and DTT-S indicates the >PhNCS-DTT breakdown product of phosphoserine (see text).

out with rapid-cycle chemistry (31). Under these conditions, the hydroxyl groups of serine and threonine are much more stable. However, the phosphate groups of phosphoserine and phosphothreonine are still readily eliminated, leading to the generation of >PhNCS-DTT-dehydroalanine and -dehydroaminobutyric acid, respectively (32). For example, using this approach, the ratio of >PhNCS-DTT-dehydroalanine to serine was found to be 5:1 in the third cycle of sequencing peptide 7, indicating that this site was heavily phosphorylated (Fig. 1 *Inset*). Of the 22 peptides sequenced, spanning 32% of the protein, 3 contained either phosphoserine or phosphothreonine. These included peptides 7 (Ile-Arg-*Ser*-Pro-Arg), 13 (Phe-Ile-Gly-*Ser*-Pro-Arg), and 19 (Phe-Ile-Gly-*Ser*-Pro-Arg-*Thr*), where the italic residue is phosphorylated. Interestingly, all four phosphorylated residues were clustered within a 13-amino acid stretch of the protein from Ile-409 through Thr-421 (23), which is part of a proposed autoinhibitory domain (24).

Localization of Phosphorylation Sites. To determine whether the phosphorylation sites above are involved in the activation of the enzyme, we labeled the kinase with $^{32}P_i$ after serum stimulation of quiescent Swiss 3T3 cells (35). The results above and studies with other proteins regulated by phosphorylation (36) suggested that these sites may be clustered in a small portion of the protein. To test this possibility, the kinase was immunoprecipitated from extracts of radioactively labeled cells, separated from IgGs by SDS/PAGE, electroeluted from the gel, and cleaved with CNBr. Analysis of the CNBr products revealed only a single radioactively labeled peptide of M_r 24,000 (Fig. 2*A*). Of the 18 potential CNBr peptides (23), only a fragment corresponding to Met-263–Pro-452 (molecular mass of 22,000 daltons) was large enough to fit this peptide. To further delineate the domain containing the phosphorylation sites, the CNBr fragment was digested with endo Lys-C, and the products were analyzed by Tricine/SDS/PAGE. Only two phosphopeptides, of M_r 2400 and M_r 3600, were detected (Fig. 2*B*). Preliminary analysis of these two peptides after trypsin digestion revealed that the larger peptide represented an incomplete digestion product of the smaller peptide (data not shown). This finding indicated that all of the phosphorylation sites resided within the M_r 2400 peptide.

Tryptic Peptide and Phosphoamino Acid Analysis. To ensure that all of the phosphorylation sites were present in the

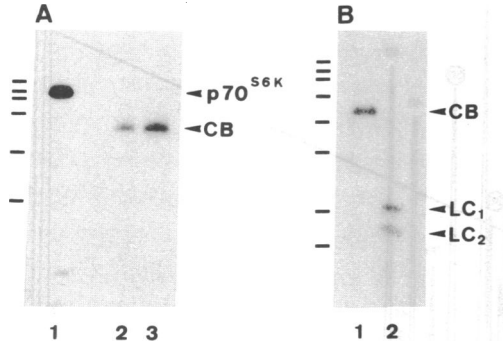

FIG. 2. Localization of p70^{s6k} phosphorylation sites. (*A*) ^{32}P-labeled p70^{s6k} (lane 1) was cleaved with either 0.5 M or 1 M CNBr (lanes 2 and 3, respectively). (*B*) CNBr cleavage product from lane 3 in *A* was electroeluted from the gel and incubated in either the absence (lane 1) or presence (lane 2) of endo Lys-C. Analysis in both cases was performed with Tricine/SDS/polyacrylamide gels. Molecular weight markers from top to bottom (to the left of lane 1) are phosphorylase b (98,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,000), and lysozyme (14,400). In *B*, the two additional lower molecular weight markers are synthetic peptides of 32 and 19 amino acid residues, respectively. CB, CNBr fragment; LC, endo Lys-C fragment.

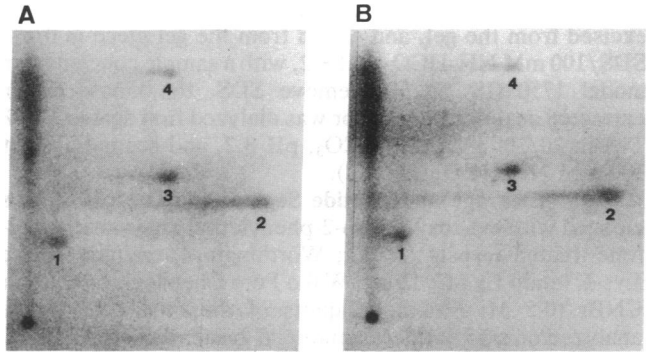

FIG. 3. Two-dimensional (2-D) tryptic phosphopeptide analysis of *in vivo* ^{32}P-labeled p70^{s6k}. p70^{s6k} was immunoprecipitated from extracts of ^{32}P-labeled cells stimulated for 1 hr with 10% fetal calf serum. The protein was digested with endo Lys-C, and the M_r 2400 peptide was resolved and electroeluted. The total endo Lys-C digest (*A*) or the M_r 2400 band (*B*) was digested with trypsin, and the resulting phosphopeptides were resolved by electrophoresis at pH 1.9 from left to right with the anode at the right in the first dimension and by ascending chromatography in the second dimension. The plates were autoradiographed for 3 weeks at −70°C.

M_r 2400 endo Lys-C peptide, either the total endo Lys-C-digested p70^{s6k} or the smaller peptide was cleaved with trypsin, and the products were examined by 2-D TLE/TLC (29). Both preparations yielded similar patterns, each having four major tryptic peptides (Fig. 3) and some minor spots probably representing incomplete digestion products. To determine the phosphoamino acid composition of the four peptides, each was scraped from the plate and analyzed as described. All four peptides contained phosphoserine while peptides 1 and 4 also contained phosphothreonine (Fig. 4). The relative ratio of phosphoserine to phosphothreonine in peptide 1 was 1:1 and in peptide 4 was 4:1. The amount of phosphothreonine in peptide 4 varied considerably; it was never more than 20% and in some cases was almost undetectable. The results are consistent with all of the sites of phosphorylation residing in a small peptide containing both serine and threonine residues, with phosphoserine more highly represented.

Identification of Phosphorylation Sites. From the results above and initial sequencing studies, a single peptide emerged as the most likely candidate to represent the phosphorylated endo Lys-C peptide. Ile-409–Lys-427 (Fig. 5). A recently designed computer program that predicts the mobility of tryptic phosphopeptides on 2-D TLE/TLC scored three peptides within this sequence as potential candidates for matching the *in vivo* tryptic phosphopeptides. These included peptides Ile-409–Arg-413, Phe-415–Arg-420, and

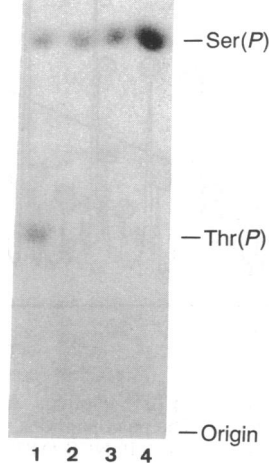

FIG. 4. Phosphoamino acid analysis of p70^{s6k} tryptic peptides. The four p70^{s6k} tryptic phosphopeptides (Fig. 3*A*) were scraped, eluted from the cellulose, and hydrolyzed for 1 hr in 6 M HCl at 100°C. Phosphoamino acids were separated on a thin-layer cellulose plate by 1-D electrophoresis at pH 1.9. Migration of phosphoserine [Ser(*P*)] and phosphothreonine [Thr(*P*)] standards is indicated. Lanes 1–4 correspond to phosphopeptides 1–4 of Fig. 3*A*. The plate was autoradiographed for 3 weeks at −70°C.

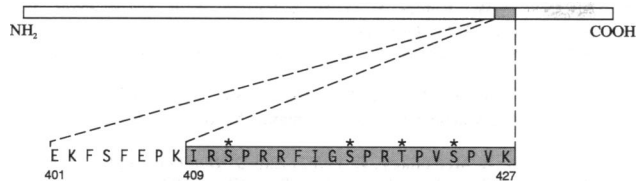

FIG. 5. Domain of the p70^{s6k} containing the sites of phosphorylation. The shaded area represents the endo Lys-C fragment Ile-409–Lys-427 (23), which contains the four sites of phosphorylation (denoted by asterisks).

Thr-421–Lys-427 (23), with the last peptide either singly or doubly phosphorylated. Peptides representing these sequences were synthesized and phosphorylated *in vitro* with either p34^{cdc2} or p42^{mapk}, both of which use *Ser*-Pro or *Thr*-Pro as recognition determinants (37–40). Since it was only possible to singly phosphorylate Thr-421–Lys-427 on serine with either kinase *in vitro*, the peptide was chemically phosphorylated on both residues (34). Mixing the *in vitro* and *in vivo* phosphorylated peptides together revealed that peptides 1–4 comigrated with Thr-421–Lys-427 (doubly phosphorylated), Ile-409–Arg-413, Thr-421–Lys-427 (singly phosphorylated), or Phe-415–Arg-420, respectively (Fig. 6 *A* and *C*). The only inconsistency was peptide 4, which *in vivo* was found to contain various amounts of phosphothreonine (Fig. 4). One possible explanation for the presence of phosphothreonine would be that peptide 4 represents a mixture of two peptides, one of which is Phe-415–Arg-420, and the other of which is closely related and contains phosphothreonine. Computer analysis suggested that Phe-415–Lys-427, resulting from incomplete trypsin cleavage of the parent endo Lys-C peptide and singly phosphorylated on serine or threonine, would migrate near Phe-415–Arg-420. Indeed, the two peptides, both singly phosphorylated, migrate in almost identical positions (Fig. 6*B*). Taken together, the results indicate that four major sites of phosphorylation are associated with activation of the p70^{s6k}, and that these sites all reside within the putative autoregulatory domain of the enzyme.

DISCUSSION

The results presented here suggest that all of the phosphorylation sites associated with p70^{s6k} activation reside within an endo Lys-C fragment of 19 amino acids, Ile-409–Lys-427. This conclusion is consistent with those sites identified by direct sequencing of activated p70^{s6k} from rat liver. The size of the endo Lys-C fragment, M_r 2400, closely fits the smaller of the two endo Lys-C phosphopeptides separated on Tricine/SDS/PAGE (Fig. 2*B*). The M_r 3600 endo Lys-C fragment, which contains all of the same tryptic phosphopeptides, probably represents an incomplete digestion product. From the size of this fragment, the most likely candidate would be a peptide beginning at Glu-401 and extending through Lys-427 (Fig. 5). Lys-402 may not serve as a good cleavage site for endo Lys-C, possibly due to the neighboring acidic residue Glu-403, as has been described for trypsin (29). Recently, we presented 2-D TLE/TLC tryptic maps of ^{32}P-labeled p70^{s6k} from epidermal growth factor-stimulated cells, which were much more complex than those found here (ref. 27 and Fig. 3). This difference is probably due to incomplete cleavage of p70^{s6k} by trypsin in the earlier study. Predigestion with endo Lys-C apparently eliminates "ragged ends," though in some preparations minor phosphopeptides still could be detected. Finally, note that there are four other Ser/Thr-Pro motifs within the kinase. Under the conditions used in these studies, they were never detected as being phosphorylated.

A frequent hallmark of regulatory phosphorylation is the clustering of phosphorylation sites (36). In addition, many monomeric kinases contain either substrate or pseudosubstrate autoinhibitory domains involved in their regulation (41). Recently, it was pointed out that p70^{s6k} may contain such a domain (24), Lys-400 through Gly-431 (23), which has 28% homology with the carboxyl terminus of S6 (24). When aligned with S6, Ser-418 of this domain aligns with the preferred site of S6 phosphorylation, Ser-236 (20). We recently questioned the role of this peptide as an autoinhibitory domain, since peptides spanning it do not serve as a substrate for the kinase (20, 42). Moreover, they only inhibit S6 phosphorylation with a K_i of 30 μM as compared with 0.5 μM for a peptide based on the S6 phosphorylation sites (20). However, here we show that all of the sites of phosphorylation of the p70^{s6k} reside in this domain, an expected structural feature if such a regulatory mechanism is being used.

It is clear that the activation of the p70^{s6k} is associated with multiple phosphorylation of the enzyme (35) through the sites described above and that removal of these phosphate groups

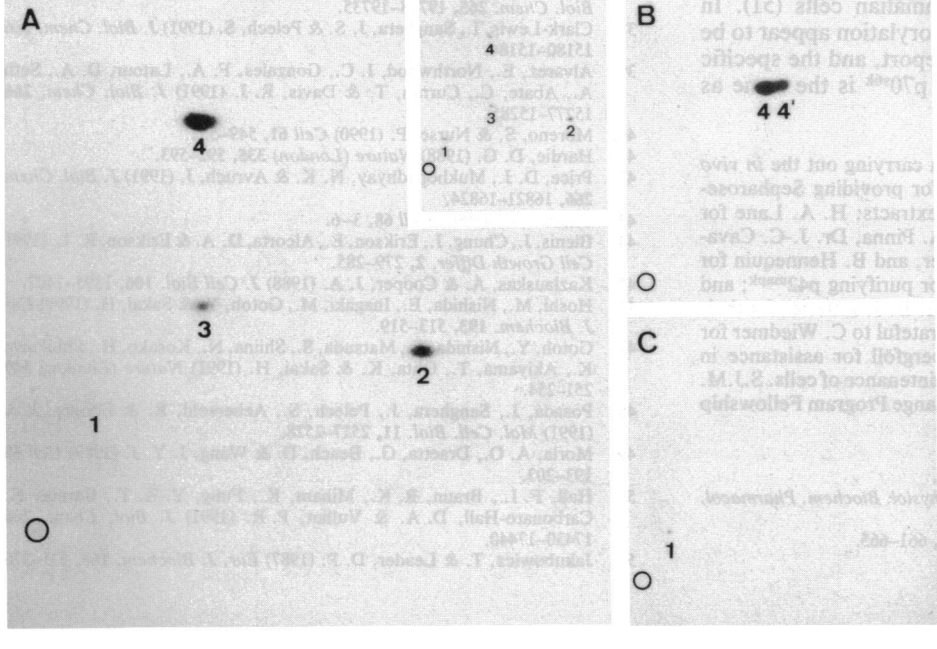

FIG. 6. Comparative analysis of tryptic phosphopeptide maps. Analysis of phosphopeptides was carried out as described in Fig. 3. (*A*) Approximately equal amounts of *in vivo* ^{32}P-labeled p70^{s6k} digested with trypsin and *in vitro* phosphorylated synthetic peptides were mixed. (*Inset*) *In vivo* ^{32}P-labeled p70^{s6k}. (*B*) 2-D migration of *in vitro* phosphorylated peptide Phe-415–Arg-420 (phosphopeptide 4) and *in vitro* singly phosphorylated peptide Phe-415–Lys-427 (phosphopeptide 4'). (*C*) Ninhydrin staining of the chemically phosphorylated peptide Thr-421–Lys-427. The thin-layer plates were autoradiographed for 1 week at −70°C.

leads to inactivation of the enzyme (18, 35). From this data it cannot be concluded that the causative event in $p70^{s6k}$ activation is phosphorylation; as discussed (5), other possibilities exist such as positive effector molecules. However, in either case, the phosphorylation sites described here play a key role in the $p70^{s6k}$ activation state, and knowledge of these sites offers a biochemical handle for searching for the $p70^{s6k}$ kinase. It would then be possible to determine whether phosphorylation of the four Ser/Thr-Pro sites is the causative event in $p70^{s6k}$ activation. To date such a kinase has not been identified (33, 43). Based on the peptide studies described here and the recognition motifs for $p42^{mapk}$ and $p34^{cdc2}$, either enzyme could be involved in modulating these sites. However, neither kinase effectively recognized $p70^{s6k}$ *in vitro*, and the amount of kinase used to carry out the peptide phosphorylation studies was much too high to justify as being physiologically relevant (*Materials and Methods*). Furthermore, a great deal of biological data have accumulated to suggest that neither enzyme is involved in the activation of $p70^{s6k}$ *in vivo*, including: (*i*) activation of $p70^{s6k}$ by insulin or cycloheximide without any effect on $p42^{mapk}$ activity (33, 43, 44); (*ii*) phorbol 12-myristate 13-acetate downregulation of protein kinase C in these same cells, attenuating platelet-derived growth factor-mediated activation of $p42^{mapk}$ (45, 46) with no effect on $p70^{s6k}$ activation (16); and (*iii*) progesterone induction of full $p70^{s6k}$ activation in stage VI *Xenopus* oocytes within 60 min of hormone treatment (28) hours before either $p34^{cdc2}$ or $p42^{mapk}$ is activated (47, 48). Furthermore, in the case of $p34^{cdc2}$, the enzyme is not activated during the G_0/G_1 transition (49), consistent with the fact that cyclin A is not detectable in cells during this time. However, note that a recent report showed that a proline-dependent protein kinase is activated during the G_0/G_1 transition and that this enzyme appears to be a $p34^{cdc2}$–cyclin A complex-like kinase (50). Because of this enzyme's substrate specificity and its point of activation in the cell cycle, it could be a potential candidate for the $p70^{s6k}$ kinase.

It also will be important to establish the relative role of each of the phosphorylation sites in the activation of $p70^{s6k}$; *in vitro*, such studies would be feasible if a source of $p70^{s6k}$ kinase were available. We recently have expressed $p70^{s6k}$ using a baculovirus construct. Surprisingly, the enzyme is activated without coinfection of possible upstream kinases (S. Kozma, E. McGlynn, C. Reinhard, S.F., and G.T., unpublished data). This is probably due to activation of the S6 kinase pathway in response to viral infection, as has been documented during infection of mammalian cells (51). In preliminary studies the sites of phosphorylation appear to be equivalent to those described in this report, and the specific activity of the baculovirus expressed $p70^{s6k}$ is the same as that described for the rat liver $p70^{s6k}$.

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