Mitogen-activated $p70^{s6k}$ signalling pathway

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ABSTRACT

$p70^{s6k}$ lies on a $p21^{ras}$-independent signalling pathway (1) and plays an important role in mitogenesis (2-5). Activation is associated with phosphorylation at multiple sites, four of which lie in an autoinhibitory region (6). The immunosuppressant rapamycin induces $p70^{s6k}$ inactivation through dephosphorylation of a second set of mitogen-induced sites (7). Here we identify these sites as T_{229}, T_{389}, and S_{404}. T_{229} resides in the "T loop" of the catalytic domain (8), an essential phosphorylation site in other kinases (9-11). However, $p70^{s6k}$ inactivation by rapamycin most closely parallels T_{389} dephosphorylation. Mutation of T_{389} to alanine ablates kinase activity, whereas mutation to glutamic acid confers constitutive kinase activity and rapamycin resistance, indicating an essential role for phosphorylation at this site. T_{389} resides in an unusual hydrophobic motif, not previously noted, between the catalytic and autoinhibitory domains. The importance of this site, and surrounding motif, is emphasised by its conservation in other kinases including homologues of $p70^{s6k}$ derived from such distantly related organisms as yeast and plant.

Serum-stimulation of quiescent Swiss 3T3 cells leads to an acute increase in the phosphorylation of seven $p70^{s6k}$ tryptic peptides, including three peptides termed b, c and d (Fig. 1A). The immunosuppressant rapamycin induces selective dephosphorylation of b, c and d, accompanied by kinase inactivation, indicating an essential role for phosphorylation at one or more of these sites (7). However, unlike the four known phosphorylation sites (Fig. 1A) residing in the autoinhibitory domain, initial attempts to identify the rapamycin sensitive sites employing a number of proteases together with a peptide mapping program (12) failed, as the proteolytic products did not migrate with the predicted mobility of phosphopeptides from $p70^{s6k}$.

Therefore, a strategy was developed to produce picomolar amounts of these peptides for direct analysis from a mutant $p70^{s6k}$ construct lacking the known sites of phosphorylation, $p70^{s6k}D_3E$ (7). Following transient transfection into human 293 cells, radioactively labelled phosphopeptides purified from the isolated $p70^{s6k}D_3E$ were found to migrate in the identical position as their respective counterparts from $p70^{s6k}$ of Swiss 3T3 cells (Fig. 1A and 1B). Phosphoamino acid analysis demonstrated that b contained phosphoserine whereas c and d contained phosphothreonine (Table 1). In the case of c, phosphate release at cycle 11 and the presence of phosphothreonine fit with this peptide being phosphorylated at T_{229} in the catalytic domain.

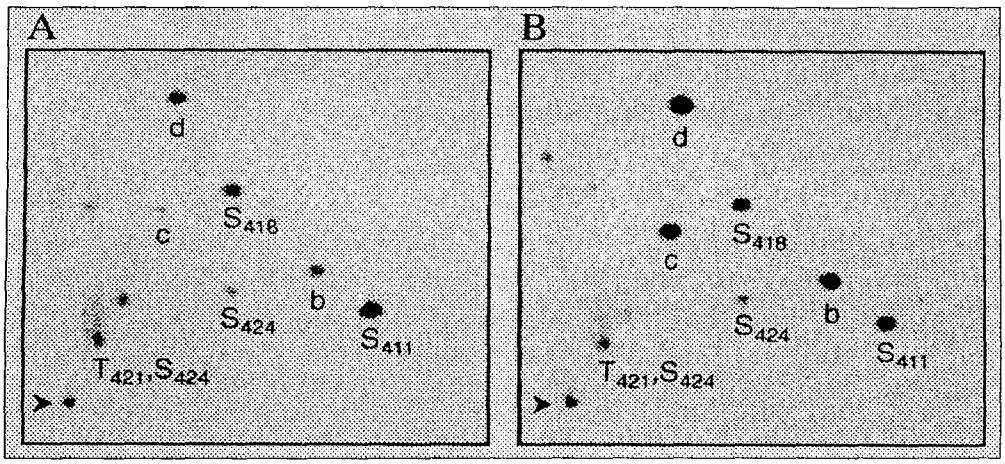

Fig. 1. Purification of phosphopeptides b, c and d. (A) $p70^{s6k}$ from $^{32}P_i$-labelled Swiss 3T3 cells stimulated with 10% FCS for 15 min was immunoprecipitated from cell extracts and subjected to two-dimensional tryptic phosphopeptide mapping. (B) His-tagged $p70^{s6k}D_3E$ was purified from $^{32}P_i$-labelled human 293 cells and digestedwith trypsin. Phosphopeptides b, c and d were purified by reversed phase chromatography followed by preparative two-dimensional thin layer electrophoresis/chromatography (2-D TLE/TLC), and 50 c.p.m. of each peptide fraction were mixed with 1000 c.p.m. of the [^{32}P]$p70^{s6k}$ trypsin digest used in (A). Previously identified phosphorylation sites from within the autoinhibitory domain are numbered according to the $p70^{s6k}$ sequence.

Chemical sequence and mass spectrometry confirmed this assignment and demonstrated that peptide c was singly phosphorylated (Table 1). Phosphate release, chemical sequencing and mass spectrometry identified peptides b and d as E_{401}-K_{408} and G_{387}-K_{400}, respectively, also singly phosphorylated at either S_{404} or T_{389}. (Table 1). Phosphopeptides c and d resulted from atypical trypsin cleavages whereas phosphopeptide b was an incomplete tryptic cleavage product. Thus, all three phosphopeptides were not limit tryptic peptides, explaining the failure of the mapping program to predict peptides matching their mobilities.

In contrast to the S/TP sites in the autoinhibitory domain, T_{229} is found in the "T loop" of the catalytic domain, whereas T_{389} and S_{404} reside in a linker region between the catalytic and autoinhibitory domains (Fig. 2). In a number of kinases, phosphorylation at sites in the "T loop" is essential for kinase activity (13), suggesting that the primary target of $p70^{s6k}$ inactivation by rapamycin is T_{229}. To examine this possibility, $p70_{s6k}$ activity was compared to the phosphorylation state of the enzyme in serum-stimulated Swiss 3T3 cells at times following rapamycin treatment. Within 4 to 5 min of the addition of 5nM rapamycin 50% of $p70^{s6k}$ activity is abolished with kinase activity reaching basal levels within 15 to 30 min. The loss of kinase activity is reflected as a progressive increase in the mobility of $p70^{s6k}$ on SDS-PAGE. Surprisingly, only a small decrease in the phosphorylation state of T_{229} was observed over the time course of rapamycin treatment despite complete loss of kinase activity. Indeed, inactivation of the kinase most closely paralleled dephosphorylation of T_{389}, followed by dephosphorylation of S_{404}. Thus, it appears that rapamycin inactivates $p70^{s6k}$ by inducing dephosphorylation of sites located outside the catalytic domain.

Table I. Characterization of purified phosphopeptides b, c and d

Phosphopeptide	Phosphoamino acid	Phosphate release	Mass	Sequence
b	P-Ser	Cycle 4	1091 (1092.9)	EKFS*FEPK
c	P-Thr	Cycle 11	2138 (2138.2)[a]	ESIHDGTVTHT*FCGTIEY
d	P-Thr	Cycle 3	1578 (1576.7)	GFT*YVAPSVLESVK

The residues identified by direct sequencing of each peptide are underlined and the phosphorylated residue is indicated by an asterisk.
[a]Phosphopeptide c contained cysteic acid due to performic acid oxidation of the protein prior to digestion.

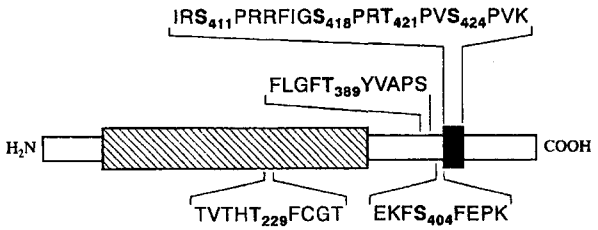

Fig. 2. Schematic representation of $p70^{s6k}$. Known sites of phosphorylation are shown in bold face with their surrounding sequence. The catalytic domain is hatched and the autoinhibitory domain is shown in black.

To assess their relative importance for kinase activation and in mediating rapamycin-induced kinase inactivation, each site was mutated to either a neutral or acidic residue. The mutated kinases were tagged with a myc-epitope, transiently transfected in human 293 cells, and the cells quiesced by serum deprivation for 24hrs. The extent of activation of the A_{404} and E_{404} mutants by serum was similar to that of the wild type enzyme. In contrast, the basal activity of these mutants was higher than the wild type $p70^{s6k}$ suggesting serine at this position contributes to maintaining the kinase in an inactive state.

In parallel studies mutations of T_{229} to either alanine or glutamic acid, abolished kinase activity, prohibiting further assessment of the importance of phosphorylation at this site. The activity of the E_{389} mutant in quiescent cells was significantly higher than wild type $p70^{s6k}$, though its ability to respond to serum was diminished. In contrast, no activity could be detected in the A_{389} mutant from either quiescent or serum-stimulated cells, further supporting T_{389} phosphorylation as essential for $p70^{s6k}$ activity. As the location of T_{389} suggested that it may function with the autoinhibitory domain in regulating $p70^{s6k}$ activity (Fig. 2), and $p70^{s6k}D_3E$ has higher basal kinase activity than $p70^{s6k}$, it was reasoned that the activity of the E_{389} mutant may be augmented if placed in a $p70^{s6k}D_3E$ background. This was found to be the case, with the basal activity of this mutant being higher than that of either $p70^{s6k}D_3E$ or $p70^{s6k}$-E_{389} and the serum-stimulated level being equal to that of the wild type construct. The results indicate that phosphorylation of T_{389} plays a critical role in regulating kinase activity.

That the E_{389} mutant was active offered the possibility of testing the role of this site in mediating rapamycin-induced $p70^{s6k}$ inactivation. Thus, serum-stimulated cells transiently expressing $p70^{s6k}$ or $p70^{s6k}D_3E$, as well as each construct containing the E_{389} mutation, were treated with 20nM rapamycin for 15 min. The results show that the two parent constructs rapidly lost activity whereas the E_{389} mutants were 50-70%

resistant to rapamycin. The E_{389} mutants still exhibited some rapamycin sensitivity, suggesting a secondary target of rapamycin-induced $p70^{s6k}$ inactivation. Indeed, preliminary data suggests this loss of activity correlates with dephosphorylation of T_{229}. Unexpectedly, however, longer treatment with rapamycin showed that after 1h the $p70^{s6k}D_3E$ mutant was still 35-40% resistant, a level of activity which did not decrease further after 2h rapamycin treatment. Under identical conditions, the $p70^{s6k}D_3E$ parent construct returned to basal levels within 15 min. Similar results were obtained with $p70^{s6k}$-E_{389}, though the extent of resistance was lower, approximately 21%. The results are consistent with T_{389} being the principle target of rapamycin action.

Though the results presented here demonstrate the importance of T_{389} phosphorylation, T_{229} and S_{404} may also play a role in regulating $p70^{s6k}$ activity. Earlier mutational analysis of the four S/TP sites lying in the autoinhibitory domain, combined with recent deletion studies (7, 14), suggest that this domain plays a critical role in regulating $p70^{s6k}$ activity as well. In contrast to the S/TP sites, the rapamycin sensitive sites are flanked by large hydrophobic residues (Fig. 2). The fact that the phosphorylation motifs displayed by these two sets of sites are quite distinct, as is their apparent mode of regulation, suggests that at least two upstream kinases are required for full $p70^{s6k}$ activation. The motif displayed by T_{389} is of particular interest as it is not only flanked by hydrophobic residues (Fig. 2), but lies in a long stretch of neutral uncharged residues, implying that regulation of $p70^{s6k}$ will involve a kinase of novel specificity. It is exciting to note that this previously unobserved motif, though outside the consensus catalytic domain (15), is conserved in other kinases including YPK1 (16) and in ATPK2 (17), putative $p70^{s6k}$ homologues from *Saccharomyces cerevisiae and Arabidopsis thaliana*. Indeed, the distance that this motif is situated downstream of the catalytic domain is also conserved, suggesting an important structural role in regulating the activity of these kinases. Obviously, the elucidation of this site should prove useful in characterising the molecules which regulate $p70^{s6k}$ activity via T_{389} phosphorylation, such as the recently reported FKBP12-rapamycin binding protein, FRAP (18).

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