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# The Principal Rapamycin-Sensitive p70$^{s6k}$ Phosphorylation Sites, T-229 and T-389, Are Differentially Regulated by Rapamycin-Insensitive Kinase Kinases

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**Mitogen-induced activation of p70$^{s6k}$ is associated with the phosphorylation of specific sites which are negatively affected by the immunosuppressant rapamycin, the fungal metabolite wortmannin, and the methylxanthine SQ20006. Recent reports have focused on the role of the amino terminus of the p85$^{s6k}$ isoform in mediating kinase activity, with the observation that amino-terminal truncation mutants are activated in the presence of rapamycin while retaining their sensitivity to wortmannin. Here we show that the effects of previously described amino- and carboxy-terminal truncations on kinase activity are ultimately reflected in the phosphorylation state of the enzyme. Mutation of the principal rapamycin-targeted phosphorylation site, T-389, to an acidic residue generates a form of the kinase which is as resistant to wortmannin or SQ20006 as it is to rapamycin, consistent with the previous observation that T-389 was a common target of all three inhibitors. Truncation of the first 54 residues of the amino terminus blocks the serum-induced phosphorylation of three rapamycin-sensitive sites, T-229 in the activation loop and T-389 and S-404 in the linker region. This correlates with a severe reduction in the ability of the kinase to be activated by serum. However, loss of mitogen activation conferred by the removal of the amino terminus is reversed by additional truncation of the carboxy-terminal domain, with the resulting mutant demonstrating phosphorylation of the remaining two rapamycin-sensitive sites, T-229 and T-389. In this double-truncation mutant, phosphorylation of T-229 occurs in the basal state, whereas mitogen stimulation is required to induce acute upregulation of T-389 phosphorylation. The phosphorylation of both sites proceeds unimpaired in the presence of rapamycin, indicating that the kinases responsible for the phosphorylation of these sites are not inhibited by the macrolide. In contrast, activation of the double-truncation mutant is blocked in the presence of wortmannin or SQ20006, and these agents completely block the phosphorylation of T-389 while having only a marginal effect on T-229 phosphorylation. When the T-389 site is mutated to an acidic residue in the double-truncation background, the activation of the resulting mutant is insensitive to the wortmannin and SQ20006 block, but interestingly, the mutant is activated to a significantly greater level than a control in the presence of rapamycin. These data are consistent with the hypothesis that T-389 is the principal regulatory phosphorylation site, which, in combination with hyperphosphorylation of the autoinhibitory domain S/TP sites, is acutely regulated by external effectors, whereas T-229 phosphorylation is regulated primarily by internal mechanisms.**

p70$^{s6k}$ and p85$^{s6k}$, which appear to play a key role in $G_1$ progression, are encoded by a common gene and are generated from the same transcript through alternative translation initiation start sites (22). The two isoforms differ by a 23-amino-acid extension at the amino terminus of p85$^{s6k}$ which constitutively targets p85$^{s6k}$ to the nucleus, whereas p70$^{s6k}$ appears to be exclusively cytoplasmic (44). Although studies comparing their modes of activation are limited, the available data suggest that both isoforms are regulated in a coordinated fashion (10, 14, 38, 45). Such a finding is consistent with the fact that the major target in both compartments of the cell appears to be the 40S ribosomal protein S6 (46). While the role of this event in the nucleus is unclear, recent studies have implicated increased S6 phosphorylation in the cytoplasm, with the translational upregulation of a family of mRNAs which contain a polypyrimidine tract at their 5′ transcriptional start sites (32). As a class, these mRNAs represent 0.1% of the mRNA complexity in cells, but in abundance they can embody as much as 30% of cellular mRNA (37). With a few possible exceptions, these mRNAs have been found to encode ribosomal proteins and elongation factors of the translational apparatus, gene products whose increased expression is essential for cell growth and development (37).

In contrast to S6, little is known concerning the kinases which regulate p70$^{s6k}$/p85$^{s6k}$ phosphorylation and activation. This stems largely from the failure to reconstitute p70$^{s6k}$/p85$^{s6k}$ activation in vitro (1, 39). It is now becoming evident that this problem is due to the fact that full activation of p70$^{s6k}$/p85$^{s6k}$ is a complex event which requires multiple interdependent phosphorylation events (19, 20, 25, 41). A number of indirect approaches have been applied in an attempt to identify upstream regulatory components of the signalling pathway leading to kinase activation. One such approach has led to the finding that the fungal metabolite wortmannin, which blocks phosphatidylinositol 3-OH kinase (PI3K), also blocks mitogen- but not tetradecanoyl phorbol acetate-induced p70$^{s6k}$/p85$^{s6k}$ activation (13). Although the role of PI3K in triggering this pathway (28, 38) as well as the specificity of wortmannin for PI3K (15, 30) has been questioned, wortmannin clearly blocks a key element in p70$^{s6k}$ activation. More recently, it has been shown that the *Akt* kinase is also wortmannin sensitive (9, 24), is activated in vitro by phosphatidylinositols (24), and, in cotransfection studies, can selectively activate the p70$^{s6k}$ isoform (9). The immunosuppressant rapamycin, like wortmannin, also

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blocks p70$^{s6k}$/p85$^{s6k}$ activation (14, 21, 35, 42). The target of rapamycin is a high-molecular-weight protein termed TOR in *Saccharomyces cerevisiae* (34) or FRAP in mammals (7). It has structural homology to phosphatidylinositol kinases and protein kinases (30), although no allied kinase activity has been yet assigned to FRAP (8). Mutants of FRAP which fail to bind the rapamycin-FKBP12 gain-of-function inhibitory complex protect p70$^{s6k}$ from inactivation (8). This has led to the hypothesis that FRAP is an upstream component of the p70$^{s6k}$ signalling pathway (8). Unlike wortmannin, rapamycin blocks p70$^{s6k}$ activation induced by either mitogens or TPA (14) and, in addition, has no effect on the activation of PI3K or *Akt* (9, 24). These findings led to a recent model depicting the order of events leading to p70$^{s6k}$ activation (5, 17, 18), such that mitogen stimulation triggers the activation first of PI3K, then of *Akt*, and then, further downstream, of FRAP, the target of rapamycin.

An alternative approach to identify immediate upstream components in the pathway has been to unravel the complex pattern of mitogen-induced phosphorylation associated with p70$^{s6k}$/p85$^{s6k}$ activation (20, 41). These studies have focused largely on the cytoplasmic p70$^{s6k}$ isoform. This has led to the finding that mitogen-induced p70$^{s6k}$ activation is associated with the phosphorylation of at least two distinct sets of sites. Members of the first set are flanked by a proline in the +1 position, are rapamycin resistant (except for S-411), and appear to modulate kinase activity (11, 25). In contrast, members of the second set of sites, i.e., T-229, T-389, and S-404, are flanked by large aromatic residues and are rapamycin sensitive, and T-229 and T-389 are essential for kinase activity (41). Furthermore when T-389, the principal target of rapamycin-induced p70$^{s6k}$ dephosphorylation and inactivation, is converted to an acidic residue, this confers rapamycin resistance on p70$^{s6k}$ (41). Although not previously noted, the importance of this site and its surrounding domain to kinase function is emphasized by their presence in a large number of protein kinases of the second messenger family (41). The relative importance of the second site, T-229, residing in the highly conserved activation loop, has been more difficult to assess because similar mutations abrogate kinase activity (41, 49). Consistent with the wortmannin target lying upstream of FRAP, selective dephosphorylation of the rapamycin-sensitive sites is also induced by wortmannin, as well as by the methylxanthine inhibitor SQ20006, which, like wortmannin and rapamycin, selectively blocks p70$^{s6k}$/p85$^{s6k}$ activation (25). In seeming conflict with this model, however, is the recent finding that deletion of either the complete amino terminus or a short sequence within this domain of the nuclear p85$^{s6k}$ isoform, together with the removal of the regulatory carboxy terminus, generates a form of the kinase which is rapamycin resistant but still inactivated by wortmannin (50). This finding raises a number of issues. It suggests that wortmannin, unlike rapamycin, may induce p70$^{s6k}$ inactivation through dephosphorylation of a site distinct from T-389 or possibly that in the truncated form of the kinase, rapamycin does not induce p70$^{s6k}$ inactivation because it can no longer provoke T-389 dephosphorylation. Finally, it brings into question the ordering of specific signalling components, implying that wortmannin does not block p70$^{s6k}$ activation by acting on an upstream activator of FRAP.

To assess the relative importance of T-389 phosphorylation in inhibitor-induced p70$^{s6k}$ inactivation, we set out to determine whether mutation of this site confers wortmannin and SQ20006 resistance to the kinase, as it does for rapamycin. Next, we generated either single- or double-truncation constructs of p70$^{s6k}$ harboring specific point mutations and tested their sensitivities to all three inhibitory agents. Finally, we have examined the sites of phosphorylation in a number of these mutants to determine whether differential effects of the inhibitors on patterns of p70$^{s6k}$ activity can be attributed to the phosphorylation state of T-389 or other distinct phosphorylation sites.

## MATERIALS AND METHODS

**Plasmid construction.** All p70$^{s6k}$ constructs used in this study were tagged by the insertion of the myc 9E10 epitope immediately following the p70$^{s6k}$ isoform initiator ATG codon, as described previously (38). The tagged p70$^{s6k}$D$_3$E and p70$^{s6k}$D$_3$E-E$_{389}$ constructs have been described elsewhere (41). Generation of all truncation mutants and site-directed mutagenesis were performed with the Altered Site II Mutagenesis System (Promega) according to the manufacturer's instructions. The p70$^{s6k}\Delta$N$_{54}$ truncation was made by looping out the intervening sequence from the 3′ end of the myc tag to S-55. Similarly p70$^{s6k}\Delta$C$_{104}$ was constructed by deletion of the 3′ sequence preceding K-400 and insertion of a stop codon upstream of a *Pst*I cloning site. Chimeras containing both truncations and specific mutations were constructed by replacing *Xba*I-*Bgl*II fragments from appropriate vectors. All constructs were placed in a cytomegalovirus-driven expression vector and confirmed by DNA sequencing.

**Cell culture, transfection, and $^{32}PO_4$ labelling.** Human embryonic kidney 293 cells were maintained in 10% fetal calf serum–Dulbecco modified Eagle medium as described previously (38) and seeded at $10^6$ cells per 10-cm-diameter plate 24 h prior to transfection. Transient transfection was performed overnight by using a modified calcium phosphate procedure with between 1 and 10 μg of plasmid DNA (41). The next day, cells were washed twice and then made quiescent for 24 h with Dulbecco modified Eagle medium or, for cells to be used for $^{32}$P labelling, Dulbecco modified Eagle medium lacking phosphate. The cells were then incubated with $^{32}PO_4$ (1 to 2 mCi/5 ml) for a further 7 h and then treated with the appropriate inhibitor in the absence or presence of serum. After removal of medium and two washes in ice-cold phosphate-buffered saline, cells were harvested in extraction buffer A (41). The cells were Dounce homogenized on ice and centrifuged at 12,000 × *g* for 5 min at 4°C, and the supernatants were immediately frozen in liquid $N_2$ and stored at −70°C.

**Immunoblotting and kinase assay.** The protein concentration in samples was determined with the Bio-Rad D/C protein assay. Samples (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to Immobilon P (Millipore). Construct expression was quantified by Western blotting (immunoblotting) with monoclonal antibody 9E10 (41) and $^{125}$I-labelled sheep anti-mouse immunoglobulin G (Amersham) or, for samples from in vivo labelling, by incubating with monoclonal antibody 9E10, rabbit anti-mouse immunoglobulin G (Sigma), and finally fluorescein isothiocyanate-labelled swine anti-rabbit immunoglobulin G (DAKO). Storage PhosphorImagery and fluorimetry (Molecular Dynamics) were used to normalize the data obtained from kinase assays as a function of construct expression. S6 kinase assays were performed following immunoprecipitation as described previously (41).

**Two-dimensional phosphopeptide mapping.** Analysis of phosphopeptides was performed as previously reported (41) with the following modifications. After electroelution and performic oxidation, samples were digested with a 1:250 mixture of chymotrypsin-trypsin (three 5-μg portions over 36 h). This modified digestion procedure generated a single migrating phosphopeptide incorporating T-389, which migrated further in thin-layer chromatography than previously reported (25, 41). Similarly, T-229 migrates within a peptide in a slightly different position. Confirmation of T-389 and T-229 phosphorylation was carried out by amino acid replacement (16). It is noteworthy that the T-389 and T-229 sites were originally identified in phosphopeptides generated by atypical tryptic digestion (41). Use of the protocol described above largely eliminates the differences in the patterns obtained with different batches of trypsin.

## RESULTS

**Effects of wortmannin and SQ20006 on the rapamycin-resistant p70$^{s6k}$D$_3$E-E$_{389}$ mutant.** To determine whether wortmannin-induced p70$^{s6k}$ inactivation occurs through dephosphorylation of a site separate from T-389, the mutant form of the kinase, termed p70$^{s6k}$D$_3$E-E$_{389}$ (41), was transiently transfected in human embryonic kidney 293 cells, and the S6 kinase activity in cells before or after serum stimulation was assessed for its sensitivity to wortmannin as well as rapamycin and SQ20006. In this mutant, the E-389 mutation was placed in the p70$^{s6k}$D$_3$E background (Fig. 1), since substitution of acidic residues for the S/TP sites in the autoinhibitory domain confers higher specific activity to the kinase harboring the E-389 mutation than the wild-type background does (41). However, it should be noted that similar results were obtained with the

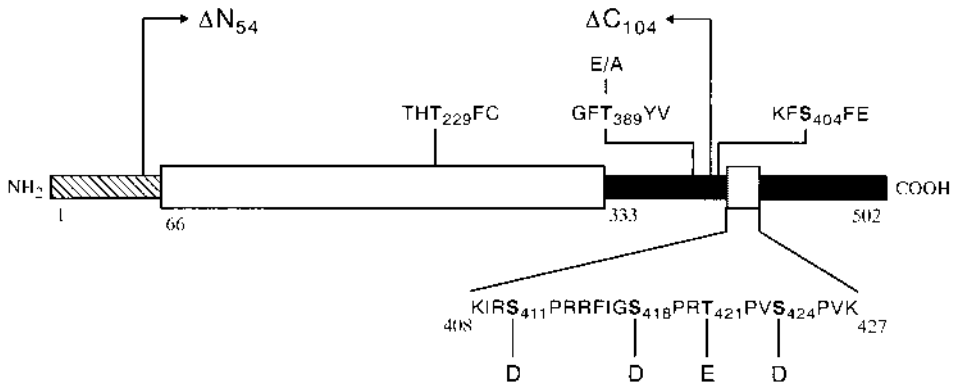


FIG. 1. Schematic representation of $p70^{s6k}$ phosphorylation sites and points of truncation. The model is drawn to scale and indicates the locations of the N-terminal domain (hatched area), the catalytic domain (open area), and the autoinhibitory domain (shaded area). The rapamycin-sensitive phosphorylation sites are indicated above the bars, and the autoinhibitory domain phosphorylation sites are indicated below the bars. Amino acid substitutions for the phosphorylation site mutants are shown next to each site. Starting points for both N- and C-terminal truncations are indicated with arrows.

E-389 mutation in the wild-type $p70^{s6k}$ background (unpublished data). As noted previously (41), in cells arrested by serum deprivation, both $p70^{s6k}D_3E$ and $p70^{s6k}D_3E$-$E_{389}$ show elevated basal activity compared with the wild-type enzyme (Fig. 2A, compare bars 1 and 2 with inset). However, $p70^{s6k}D_3E$-$E_{389}$ was qualitatively as resistant to wortmannin- or SQ20006-induced inactivation as it was to rapamycin, regardless of whether the inhibitors were added after (Fig. 2A, compare bars 6 and 8 with bar 4) or before (Fig. 2B, compare bars 4 and 6 with bar 2) serum stimulation. However, following either regimen, all three agents lowered the activity of $p70^{s6k}D_3E$ to below basal levels, although some resistance to wortmannin was noted (Fig. 2A, bars 3, 5, and 7, and 2B, bars 1, 3, and 5). The data are consistent with T-389 dephosphor-

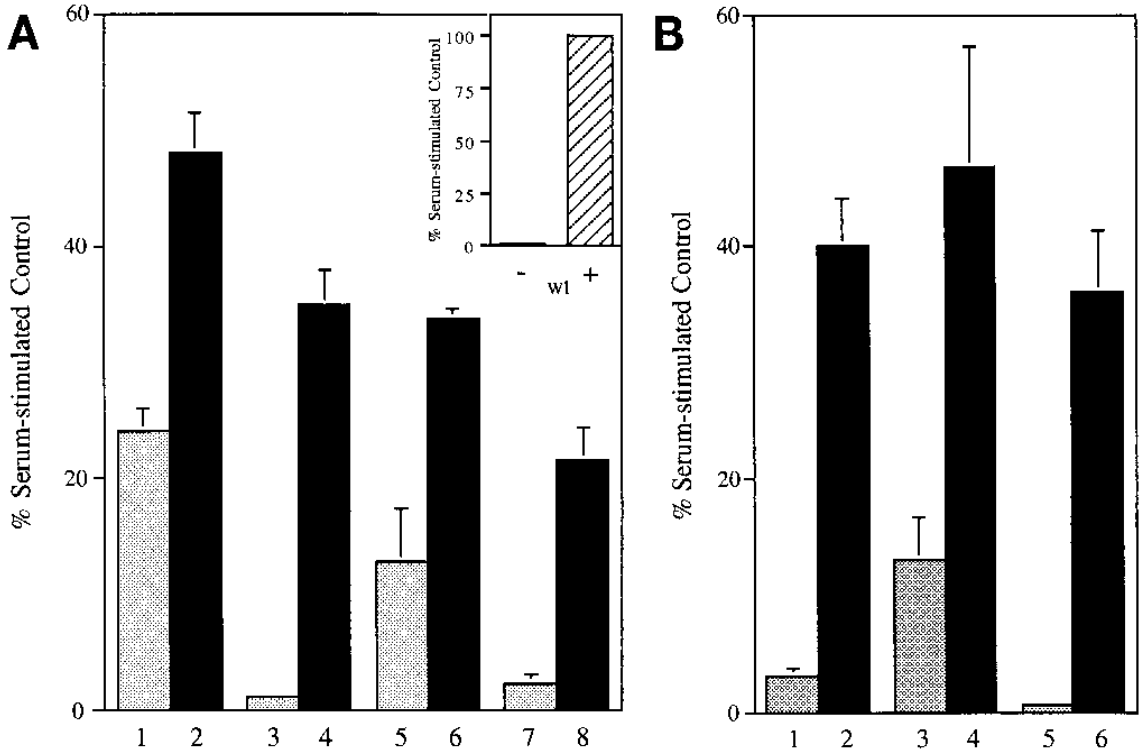


FIG. 2. Insensitivity of the $p70^{s6k}D_3E$-$E_{389}$ phosphorylation site mutant to rapamycin, wortmannin, and SQ20006. (A) Cells were transiently transfected with either $p70^{s6k}D_3E$ (shaded bars) or $p70^{s6k}D_3E$-$E_{389}$ (solid bars) and made quiescent as described in Materials and Methods. Cell lysates were prepared directly from serum-deprived cells (bars 1 and 2) or following treatment with 10% serum for 45 min before treatment with 20 nM rapamycin (bars 3 and 4), 250 nM wortmannin (bars 5 and 6), or 1.2 mM SQ20006 (bars 7 and 8) for 2 h. The activities of ectopically expressed wild-type (wt) $p70^{s6k}$ from quiescent (−) and serum-stimulated (+) cells are presented in the inset for comparison. (B) Cells were transfected and serum-starved as described for panel A but instead were treated with rapamycin (15 min) (bars 1 and 2), wortmannin (30 min) (bars 3 and 4), or SQ20006 (30 min) (bars 5 and 6) prior to stimulation with 10% serum for 45 min. Cells were extracted, and following immunoprecipitation, each lysate was assayed for S6 kinase activity as described in Materials and Methods. The results are presented as the average percent activity of each construct compared with the measured activity of that construct isolated from serum-stimulated cells. Error bars indicate the standard error from three independent experiments.

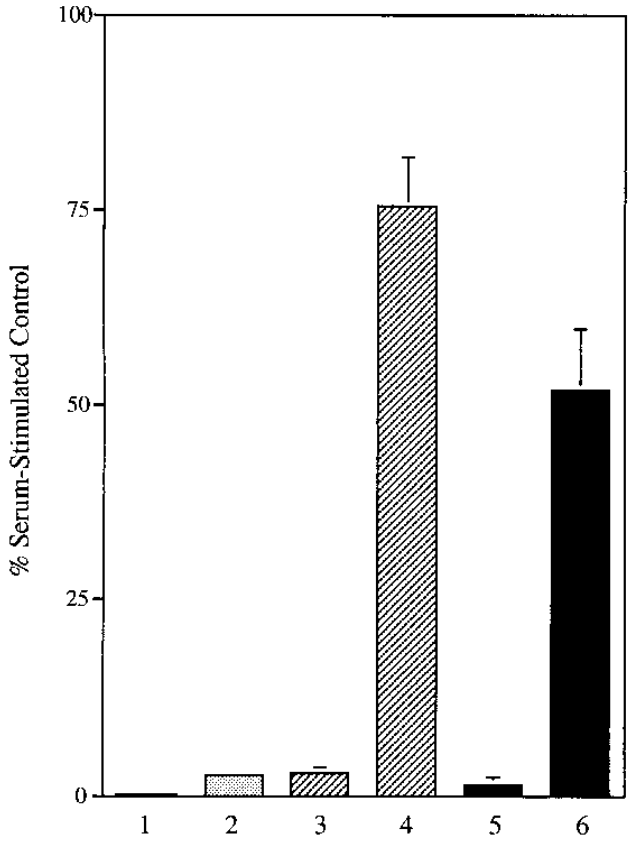


FIG. 3. Specific activities of truncation mutants. Cells were transfected with p70$^{s6k}\Delta C_{104}$ (hatched bars), p70$^{s6k}\Delta N_{54}$ (shaded bars), or p70$^{s6k}\Delta N_{54}\Delta C_{104}$ (solid bars). After transfection, cells were treated as described for Fig. 2. Serum-starved cells were either extracted directly (bars 1, 3, and 5) or stimulated with 10% serum prior to extraction (bars 2, 4, and 6). Extracts expressing equivalent levels of each mutant were assayed for S6 kinase activity, and the results are expressed as described for Fig. 2.

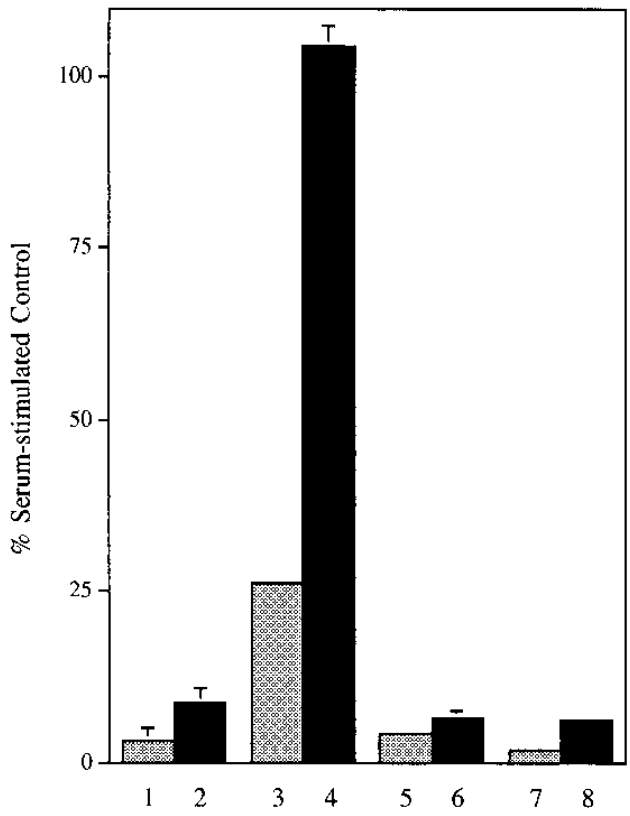


FIG. 4. Sensitivity of p70$^{s6k}$ truncation mutants to rapamycin, wortmannin, and SQ20006. Cells were transfected with myc-p70$^{s6k}\Delta C_{104}$ (shaded bars) or myc-p70$^{s6k}\Delta N_{54}\Delta C_{104}$ (solid bars). Serum-starved cells were extracted immediately (lanes 1 and 2) or stimulated with serum (lanes 3 to 8) as described for Fig. 2. Prior to stimulation, cells were treated with rapamycin (bars 3 and 4), wortmannin (bars 5 and 6), or SQ20006 (bars 7 and 8) as described for Fig. 2B. Results are expressed as described for Fig. 2, except that each value is adjusted to that for the corresponding mutant in the presence of serum alone.

ylation being the principal mediator of p70$^{s6k}$ inactivation by all three inhibitors.

**Effects of rapamycin, wortmannin, and SQ20006 on activities of p70$^{s6k}$ truncation mutants.** The data described above are consistent with wortmannin- and SQ20006-induced p70$^{s6k}$ inactivation being mediated through dephosphorylation of T-389, as shown for rapamycin (41). However, the doubly truncated p85$^{s6k}$ isoform is inactivated by wortmannin treatment but not by rapamycin treatment (50). Thus, it may be that in this mutant rapamycin is unable to induce T-389 dephosphorylation and subsequent kinase inactivation or that wortmannin brings about the inactivation of this form of the kinase through some as-yet-unknown mechanism. To distinguish between these possibilities, three mutants similar to those described for p85$^{s6k}$, each containing a myc epitope tag at the amino terminus, were constructed: (i) p70$^{s6k}\Delta N_{54}$, lacking the first 54 amino acids; (ii) p70$^{s6k}\Delta C_{104}$, lacking the last 104 amino acids; and (iii) p70$^{s6k}\Delta N_{54}\Delta C_{104}$, lacking both domains (Fig. 1). Each construct was transiently expressed in 293 cells, and its activity following serum stimulation was assessed. The results demonstrate that p70$^{s6k}\Delta C_{104}$ and p70$^{s6k}\Delta N_{54}\Delta C_{104}$ are activated to approximately 75 and 50%, respectively, of the value obtained for p70$^{s6k}$ (Fig. 3, bars 4 and 6, respectively), whereas serum has little effect on p70$^{s6k}\Delta N_{54}$ (Fig. 3, bar 2). Furthermore, pretreatment with wortmannin or SQ20006 of cells transiently expressing either p70$^{s6k}\Delta C_{104}$ or p70$^{s6k}\Delta N_{54}\Delta C_{104}$ blocks activation of both forms of the kinase (Fig. 4, compare bars 5 and 7 with bars 6 and 8, respectively). Although p70$^{s6k}\Delta C_{104}$ displayed a low level of rapamycin insensitivity, p70$^{s6k}\Delta N_{54}\Delta C_{104}$ exhibited total rapamycin resistance (Fig. 4, bars 3 and 4, respectively). The findings in Fig. 3 and 4 are similar to those obtained for the p85$^{s6k}$ nuclear isoform (50), supporting the hypothesis that the nuclear and cytoplasmic forms of the kinase are regulated in equivalent manners. However, it should be noted that p70$^{s6k}\Delta N_{54}\Delta C_{104}$ isolated from serum-stimulated cells exhibited slightly greater activity in the presence of rapamycin (Fig. 4, bar 4) (see below). Although not previously commented on, similar results were obtained by Weng et al. for the p85$^{s6k}$ isoform (50) and more recently were obtained with p70$^{s6k}$ (11). Thus, removal of the amino and carboxy termini of p70$^{s6k}$ generates a form of the kinase which is still regulated by mitogens and is inactivated by wortmannin and SQ20006 but which displays rapamycin resistance.

**Phosphopeptide maps of p70$^{s6k}\Delta N_{54}$.** The amino-terminal truncation of the first 54 amino acids of p70$^{s6k}$ severely reduced the ability of the kinase to be activated by serum (Fig. 3, bar 2), an effect which is largely reversed by also truncating the carboxy terminus (Fig. 3, bar 6). On the basis of the putative role of phosphorylation in mediating p70$^{s6k}$ activation, the absence of the amino terminus may prevent an upstream kinase from phosphorylating key sites involved in p70$^{s6k}$ activation. To resolve this issue, two-dimensional thin-layer chromatogra-

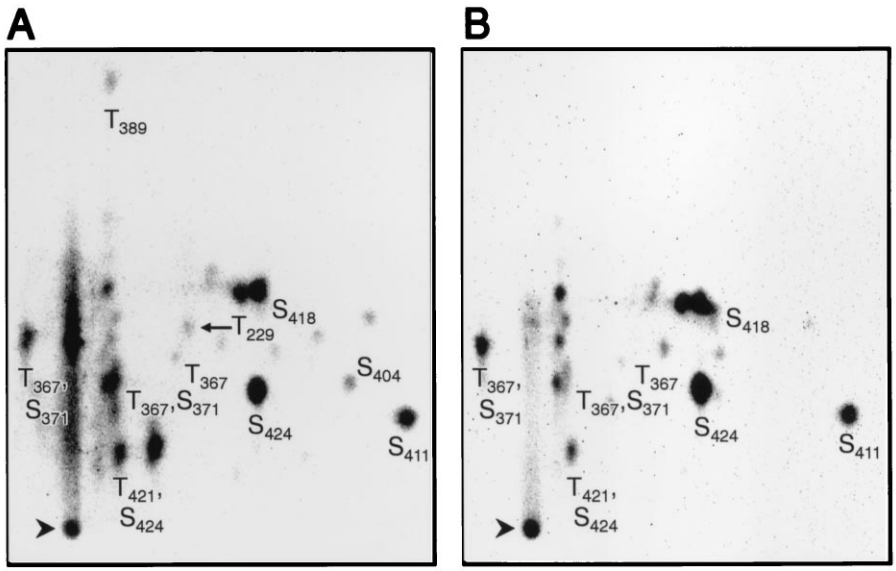


FIG. 5. Phosphopeptide analysis of $p70^{s6k}$ and $p70^{s6k}\Delta N_{54}$ from serum-stimulated cells. Cells were transfected with the myc-$p70^{s6k}$ construct (A) or the myc-$p70^{s6k}\Delta N_{54}$ mutant (B), serum starved, and labelled with $^{32}PO_4$ as described in Materials and Methods. After 45 min of stimulation with 10% serum and extraction, the epitope-tagged constructs were immunoprecipitated with the 9E10 antibody, digested, and analyzed by two-dimensional thin-layer electrophoresis–thin-layer chromatography as previously described (41) according to the protocol of Boyle et al. (6). The origin (arrowhead) and the positions of phosphopeptides containing identified phosphorylation sites are indicated.

phy–thin-layer electrophoresis tryptic [$^{32}$P]phosphopeptide maps derived from either $p70^{s6k}$ or $p70^{s6k}\Delta N_{54}$ following serum stimulation of cells transiently expressing the two constructs were compared (Fig. 5). Because of a modified kinase digestion protocol (see Materials and Methods), the phosphopeptides containing T-229 and T-389 migrate in positions different from those previously reported (41). Comparison of the $p70^{s6k}$ and $p70^{s6k}\Delta N_{54}$ maps showed no obvious differences in the phosphorylation statuses of the known S/TP sites, including T-367 and S-371 (41). However, all three of the rapamycin-sensitive phosphorylated sites, T-229, T-389, and S-404, are clearly absent in $p70^{s6k}\Delta N_{54}$ compared with $p70^{s6k}$ (Fig. 5). Since phosphorylation of T-229 and T-389, in contrast to that of S-404, appears to be essential for kinase activity (41), the lack of $p70^{s6k}\Delta N_{54}$ activity in response to serum is consistent with the absence of detectable phosphate at these sites.

**Phosphopeptide maps of $p70^{s6k}\Delta N_{54}\Delta C_{104}$.** In contrast to the case for the amino-terminal truncation mutant, removal of both termini generated a form of the kinase, $p70^{s6k}\Delta N_{54}\Delta C_{104}$, which was activated by mitogen stimulation (Fig. 3, compare bars 5 and 6). This mutant lacks six of the sites which show increased phosphorylation upon serum stimulation (Fig. 1), although T-389 and T-229 are present. To determine whether phosphorylation of T-229 and T-389 was rescued in the $p70^{s6k}\Delta N_{54}\Delta C_{104}$ mutant and whether this phosphorylation correlated with the ability of this form of the kinase to be activated, phosphopeptide maps of $p70^{s6k}\Delta N_{54}\Delta C_{104}$ from serum-deprived or serum-stimulated cells were analyzed. The maps from serum-deprived cells revealed high basal phosphorylation of T-367, S-371, and, unexpectedly, T-229 (Fig. 6A). Despite the high level of phosphorylation at T-229 in serum-deprived cells, the activity of the mutant under these conditions is low (Fig. 3, bar 2). Addition of serum led to a twofold increase in the amount of phosphate incorporated into $p70^{s6k}\Delta N_{54}\Delta C_{104}$ (data not shown). Qualitatively, the pattern of phosphorylation was quite similar to that detected in serum-deprived cells, indicating that the increased phosphate was largely incorporated into the same phosphorylation sites (Fig. 6B). However, the results clearly show that in addition to these sites, T-389 phosphorylation is dramatically upregulated following serum stimulation (Fig. 6B). The increase in T-389 phosphorylation, in contrast to that of T-229, correlated well with kinase activation (Fig. 3 and 6B) and is compatible with phosphorylation at this site being responsible for triggering the acute upregulation of $p70^{s6k}$ activity following mitogenic stimulation.

**Phosphopeptide mapping of $p70^{s6k}\Delta N_{54}\Delta C_{104}$ from cells treated with rapamycin, wortmannin, or SQ20006.** To determine whether the differential inhibitory effect of rapamycin versus wortmannin and SQ20006 on $p70^{s6k}\Delta N_{54}\Delta C_{104}$ activity (Fig. 4) correlated with the phosphorylation status of T-229 and T-389 (Fig. 5A and 6B, respectively), phosphopeptide maps of the double-truncation mutant from cells pretreated with each of the inhibitors prior to serum stimulation were examined (Fig. 7). The results show that in rapamycin-treated cells, there is no loss of phosphate from any of the four sites of phosphorylation (compare Fig. 7A with Fig. 6B). In contrast to the case for rapamycin, in either wortmannin- or SQ20006-pretreated cells, no phosphate was detected at T-389 in the mutant kinase, whereas there was no detectable loss of phosphate from T-229 (Fig. 7B and C, respectively). As with T-229, no change in the phosphorylation state of T-367 or S-371 was observed with wortmannin or SQ20006, indicating that the upstream kinase responsible for the phosphorylation of all three sites is not blocked by any of the three inhibitors. Taken together, these results are consistent with a model in which rapamycin is unable to obstruct the phosphorylation of T-389 in the double-truncation mutant, thus protecting $p70^{s6k}$ from inactivation by the bacterial macrolide. The results also imply that T-389 is the principal target of wortmannin- and SQ20006-induced $p70^{s6k}\Delta N_{54}\Delta C_{104}$ inactivation. Indeed, mutation of this site to acidic residues in $p70^{s6k}\Delta N_{54}\Delta C_{104}$ confers wortmannin and SQ20006 resistance on the doubly truncated kinase (Fig. 8, bars 6 and 7) to a level similar to that detected in $p70^{s6k}D_3E\text{-}E_{389}$ (Fig. 2B) (41). Surprisingly, rapamycin greatly

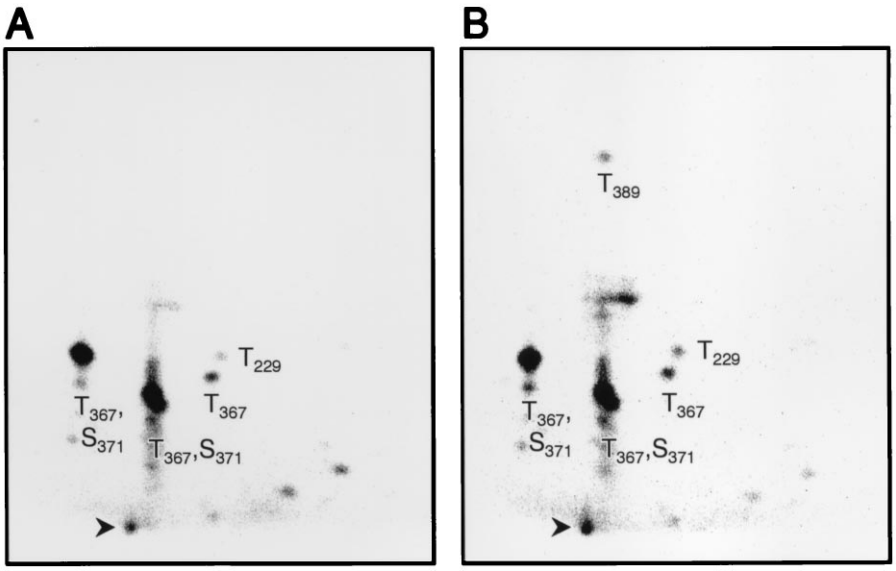


FIG. 6. Phosphopeptide analysis of p70$^{s6k}\Delta N_{54}\Delta C_{104}$ from serum-starved and stimulated cells. Cells transfected with myc-p70$^{s6k}\Delta N_{54}\Delta C_{104}$ were serum starved and labelled with $^{32}PO_4$ as described in Materials and Methods. The cells were then extracted either immediately (A) or after 45 min of serum stimulation (B). Two-dimensional tryptic phosphopeptide analysis was carried out as described for Fig. 5.

enhances the activity of this form of the kinase (Fig. 8, bars 4 and 5). In this context, it should be noted that the ability to activate the double-truncation mutant harboring the E-389 mutation is less than that of the parent construct, similar to the previous observation made for p70$^{s6k}$-$E_{389}$ (41). As expected, substitution of an alanine residue for T-389 in this construct abolished kinase activity (Fig. 8, bars 1 and 2), as previously observed for either p70$^{s6k}$ or p70$^{s6k}D_3E$ (41). The findings with rapamycin argue that removal of the amino terminus may alter but does not abrogate the ability of the macrolide to influence p70$^{s6k}$ activity.

## DISCUSSION

A tentative outline of the pathway describing p70$^{s6k}$ activation initially emerged from studies employing specific inhibitory agents combined with dominant-negative signalling molecules and growth factor receptor mutants (17). The envisaged pathway was triggered by the recruitment of PI3K to the mitogen-activated receptor, which led to the production of phosphatidylinositol phosphates followed by the sequential activation of FRAP and p70$^{s6k}$ through as-yet-unidentified signalling components. More recently, the model was refined to include

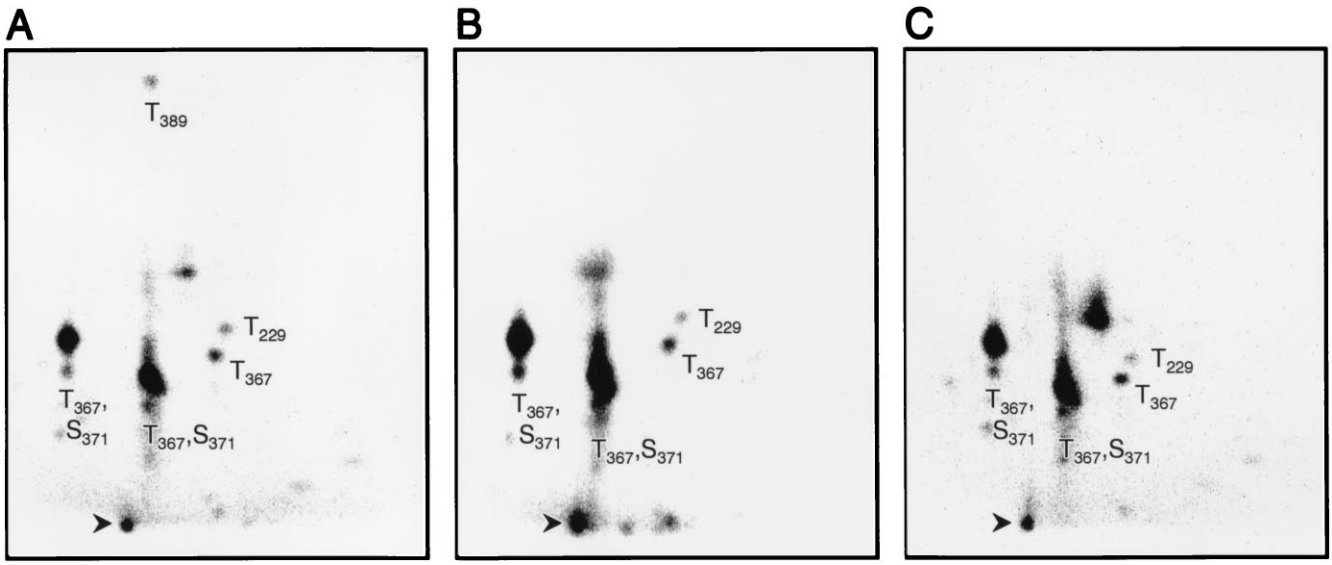


FIG. 7. Phosphopeptide analysis of p70$^{s6k}\Delta N_{54}\Delta C_{104}$ from rapamycin-, wortmannin-, and SQ20006-treated cells. Cells transfected with the myc-p70$^{s6k}\Delta N_{54}\Delta C_{104}$ mutant were serum starved and labelled with $^{32}PO_4$ as described for Fig. 5. Treatment with rapamycin (A), wortmannin (B), and SQ20006 (C) was carried out prior to serum stimulation as described for Fig. 2B.

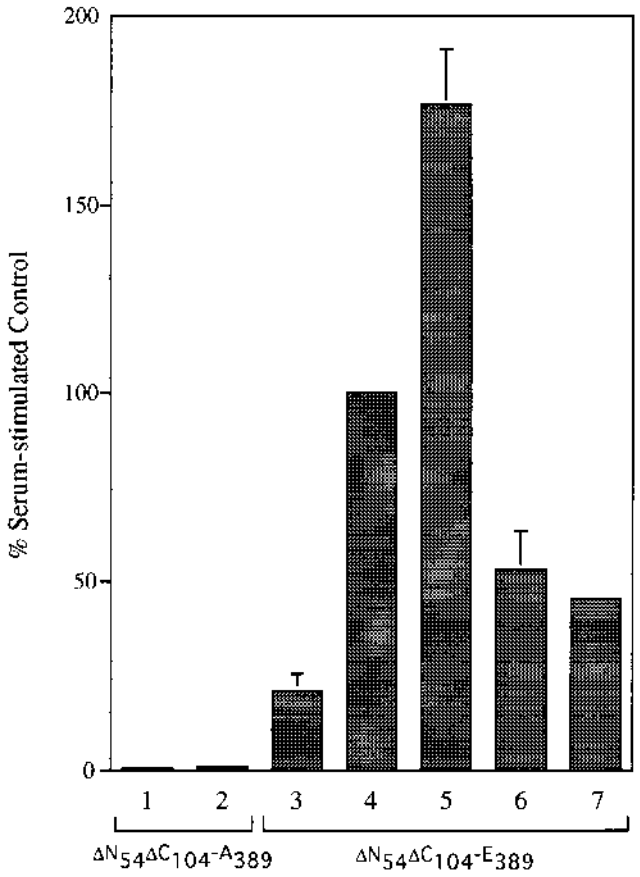


FIG. 8. Insensitivity of $p70^{s6k}\Delta N_{54}\Delta C_{104}$-$E_{389}$ to the inhibitor-induced block in kinase activation. Cells were transfected either with myc-$p70^{s6k}\Delta N_{54}\Delta C_{104}$-$A_{389}$ (bars 1 and 2) or with myc-$p70^{s6k}\Delta N_{54}\Delta C_{104}$-$E_{389}$ (bars 3 to 7). S6 kinase activity was determined for serum-starved cells either extracted immediately (bars 1 and 3), stimulated with serum (bars 2 and 4), or pretreated with rapamycin (bar 5), wortmannin (bar 6), or SQ20006 (bar 7) and then serum stimulated. Treatments and assays were performed as described for Fig. 2B.

the proto-oncogene kinase *Akt*, which was placed as an immediate target of PI3K, located at a distal upstream point in the pathway from FRAP (5, 18). In conflict with this model was the observation that platelet-derived growth factor treatment of porcine aortic endothelial cells stably transfected with the Y-740→F (Y740F) single point mutant of the platelet-derived growth factor receptor, the major PI3K binding site (33), did not impair $p70^{s6k}$ activation (38). In parallel, platelet-derived growth factor in these same cells failed to recruit PI3K to the activated receptor (9, 38) and to bring about *Akt* activation (9). Furthermore, in later studies, the $p70^{s6k}$ activity of the Y740F single point mutant was found to be wortmannin sensitive (unpublished data). Indeed, it has been subsequently shown that $p70^{s6k}$ activation in response to insulin in CHO cells overexpressing both the human insulin receptor and a dominant-negative form of the p85 regulatory subunit of PI3K was unimpaired (28). Despite this finding, insulin treatment of these cells failed to induce recruitment of PI3K to the receptor (28), raise phosphatidylinositol 3,4,5-phosphate levels, and increase glucose uptake (27). This $p70^{s6k}$ activity was also shown to exhibit wortmannin sensitivity (28), supporting the hypothesis that this agent has other targets distinct from PI3K (15, 30). Although the ordering of the pathway relied largely on the differential inhibitory effects of wortmannin and rapamycin (17), it was consistent with each of these inhibitory agents inducing the inactivation of $p70^{s6k}$ through the dephosphorylation of the same specific subset of sites (25). However, the validity of this approach in ordering the pathway was also put into question by the observation that a nuclear $p85^{s6k}$ mutant construct lacking a portion of the amino terminus (either residues 2 to 46 or 29 to 46) and the last 104 residues of the carboxy terminus displayed rapamycin resistance while retaining its sensitivity to wortmannin (50). Despite the fact that wortmannin and rapamycin apparently interfere with $p70^{s6k}$ inactivation through distinct mechanisms (see below), the data presented here are consistent with our earlier view that both agents, as well as SQ20006, inactivate $p70^{s6k}$ principally through preventing phosphorylation of T-389.

The low basal activity of the amino-terminally truncated $p85^{s6k}$ mutants in quiescent cells and their resistance to rapamycin led to the hypothesis that this domain serves as the target for a rapamycin-sensitive activating input (50). Moreover, it was reported (36) that removal of a further 31 amino acids from the amino terminus of $p85^{s6k}$ conferred constitutive activity on the kinase. This finding led to the conclusion that an autoinhibitory domain resided immediately adjacent to the rapamycin-sensitive domain and that the suppressive effect of the autoinhibitory domain was nullified upon the binding of a mitogen-induced positive effector to the rapamycin-sensitive sequence. This mutant is identical to $p70^{s6k}\Delta N_{54}$ described here (Fig. 1), except for the epitope tag. Here a myc epitope tag of 10 residues was employed (38), whereas Mahalingam and Templeton used a 13-amino-acid tag derived from the middle T antigen of polyomavirus (36). In the immune complex $p70^{s6k}$ assay, the myc-tagged $p70^{s6k}\Delta N_{54}$ construct has no detectable basal activity and is stimulated by serum to less than 3% of the activity of the wild-type kinase (Fig. 3). The fact that Mahalingam and Templeton (36) eluted the kinase from the immune complex prior to assaying for activity raised the possibility that the discrepancy between these two sets of findings could be attributed to the myc antibody inhibiting the constitutively active $p70^{s6k}\Delta N_{54}$ kinase in the immune complex assay. However, $p70^{s6k}\Delta N_{54}\Delta C_{104}$ activation is not inhibited in the same assay (Fig. 3), and the absence of serum-induced $p70^{s6k}\Delta N_{54}$ activation is paralleled by the absence of phosphorylation at T-229 and T-389 (Fig. 5), responses which appear to be essential for kinase activation (41, 49). Alternatively, it could be argued that the myc epitope tag mimics the putative amino-terminal autoinhibitory domain. Since the myc sequence is only 10 residues in length and has no homology to the 33 amino acids of the putative autoinhibitory domain, it seems unlikely that it could serve a similar purpose in the truncated $p70^{s6k}$. Furthermore, placement of this same tag in the $p70^{s6k}\Delta N_{54}\Delta C_{104}$ construct does not block kinase activation (Fig. 4). Instead, it may be that the constitutive activity observed previously (36) is conferred by the middle T antigen epitope tag. It would be of interest to know whether this form of the kinase is constitutively phosphorylated at T-229 and T-389.

The principal target of rapamycin-induced $p70^{s6k}$ inactivation is T-389 (41). In response to mitogenic stimulation, this site becomes phosphorylated in the $p70^{s6k}\Delta N_{54}\Delta C_{104}$ mutant (Fig. 6B), despite the absence of the amino terminus. The obvious interpretation of this finding is that FRAP is not an upstream signalling component which mediates the activity of the T-389 kinase. Instead, this result raises the real possibility that the rapamycin-FKBP12 complex enhances the activity of a negative effector, e.g., a phosphatase, which requires the amino terminus to exert its inhibitory effect. However, it is not possible to rule out a deficiency in an intrinsic function of the

amino and carboxy termini that is initiated by a downstream positive effector of FRAP, which is abrogated by removal of the amino terminus. Initially, the involvement of a negative effector was not favored, since removal of the amino terminus did not liberate p70$^{s6k}$ activity (50). However, it is evident that even in the absence of the amino terminus, mitogen-induced T-389 phosphorylation is required to bring about kinase activation (Fig. 6). Since the carboxy-terminally truncated p85$^{s6k}$ was still rapamycin sensitive, it also had been reasoned that the hypothetical rapamycin-sensitive input contained a second component (50). Moreover, the sensitivity of this mutant to rapamycin was paralleled by a quantitatively small reduction in the amount of phosphate incorporated into two phosphopeptides, and these same two phosphopeptides are argued to be inhibited by wortmannin (50). This led those authors (50) to suggest that the second component of the rapamycin-sensitive input synergizes with the wortmannin-inhibitable mitogen-activating input but is not necessary for the latter's effect. Nonetheless, both of these phosphopeptides actually were quite resistant to either rapamycin or wortmannin treatment (50), similar to phosphorylated T-229 in the doubly truncated p70$^{s6k}$ described here (Fig. 7A and B), and were highly labelled in serum-deprived cells. The identity of one of the peptides is unknown, whereas by mutational analysis the other was argued to contain T-229 (49). Given that T-229 is highly phosphorylated in mitogen-deprived cells when the carboxy tail is removed (Fig. 6A) (reference 50 and unpublished data) and that its phosphorylation increases little in response to subsequent mitogen stimulation, it seems highly unlikely that the kinase which controls phosphorylation at T-229 is significantly regulated by mitogens. Besides, on the basis of previous studies (25, 41) and those presented here (Fig. 7), neither T-229 nor the second phosphopeptide detected previously (50) would appear to be the principal target of either rapamycin or wortmannin action. Indeed, this function would appear to be engendered in T-389, whose activity is acutely upregulated by mitogens (25, 41) and is highly sensitive to all three inhibitory agents (25).

The results described above and those depicted in Fig. 6 point to the importance of T-389 phosphorylation in triggering p70$^{s6k}$ activation. Nonetheless, either removal of the carboxy terminus (Fig. 3) or substitution of acidic residues for the S/TP sites in the autoinhibitory domain is sufficient to bring about activation of the amino-terminal truncation mutant (unpublished data). This region of the kinase was designated a potential autoinhibitory domain (3) on the basis of its similarity in sequence to the domain surrounding the S6 phosphorylation sites (2). Synthetic peptides covering this region of the molecule inhibit the kinase in vitro in the low micromolar range (23, 43), and increased phosphorylation of five residues residing in this sequence, including S-404, S-411, S-418, T-421, and S-424, is associated with p70$^{s6k}$ activation (20, 25). Conversion of the last four of these sites to neutral residues, in a construct termed p70$^{s6k}$A$_4$, has been shown to diminish basal kinase activity and to suppress the ability of p70$^{s6k}$ to be activated (25). Substitution of acidic residues in these positions raises basal kinase activity, although mitogen stimulation does not raise the activity to a level higher than that observed with the wild-type kinase (25, 41). Thus, phosphorylation of these sites was predicted to disrupt the putative autoinhibitory sequence, freeing the active site and allowing the kinase to access the substrate (20). This hypothesis is consistent with the positive effects on p70$^{s6k}\Delta$N$_{54}$ activity of either removing the carboxy terminus or substituting acidic residues for the four S/TP sites. However, as mentioned above, the p70$^{s6k}$D$_3$E mutant has high basal kinase activity (Fig. 2A, bar 1) (25, 41), whereas p70$^{s6k}\Delta$C$_{104}$ has basal kinase activity equivalent to that of the wild-type kinase (Fig. 3, bar 3). In addition, the carboxy-truncation mutant cannot be activated to the same extent as the p70$^{s6k}$D$_3$E mutant (25, 41) (Fig. 3). These findings indicate that the role of this domain is not confined to that of a negative effector but that in the phosphorylated state it participates in bringing about maximal kinase activation.

The finding that rapamycin can stimulate activity of the p70$^{s6k}\Delta$N$_{54}\Delta$C$_{104}$-E$_{389}$ mutant (Fig. 8) and, to a lesser extent, of the parent p70$^{s6k}\Delta$N$_{54}\Delta$C$_{104}$ construct (Fig. 4) is not immediately reconcilable with a model in which the rapamycin-FKBP12 complex stimulates a negative input acting through the amino terminus. One simple explanation, nevertheless, would be that addition of rapamycin to mitogen-stimulated cells activates the upstream pathway leading to p70$^{s6k}$ activation, as part of a stress or SOS response (29). In such a model, addition of rapamycin would block p70$^{s6k}$ activation and thus the selective upregulation of 5′ polypyrimidine tract-encoded mRNAs. The translational upregulation of these mRNAs is essential for the production of nascent ribosomes and thus for cell growth. In such a scenario the cell would respond to rapamycin treatment by activating the upstream macrolide-sensitive signalling pathways. This effort would fail, since the downstream rapamycin block would be dominant. However, the double-truncation mutants, which are no longer sensitive to the rapamycin interfering input, would have the potential to become superactivated. Since the mutant harboring the E-389 mutation is partially crippled in its ability to be activated (Fig. 8) (41), the effect of the superactivated pathway on this construct might be expected to be greater. A precedent for such a model has already been described. Blenis and colleagues (4) have demonstrated a dose-dependent activation of p70$^{s6k}$ by cycloheximide, a potent inhibitor of protein synthesis, which directly paralleles the ability of the drug to inhibit translation. Since cycloheximide acts at a step further downstream in the initiation pathway (47), p70$^{s6k}$ activation cannot overcome this response. Consistent with this model, cycloheximide at submaximal protein synthesis-inhibitory concentrations induces the selective upregulation of polypyrimidine tract mRNAs (35a). A similar case can be made for virally infected cells, in which host translation is shut down and p70$^{s6k}$ is activated (31).

The activation of p70$^{s6k}$ is obviously a complex event requiring a precise interplay between specific domains and multiple phosphorylation sites. By employing a number of complementary strategies, 10 unique phosphorylation sites have been identified (20, 41), all of which are apparently phosphorylated in the endogenous enzyme (references 20 and 41 and unpublished data). In addition to these sites, Weng et al. (50), using a mutational approach have identified S-40 (S-17 in p70$^{s6k}$) as a phosphorylation site in p85$^{s6k}$. With the exception of S-17 and T-229, all of the identified sites are in the carboxy end of the molecule. On the basis of a number of criteria, we have found that the phosphorylation of 3 of the 10 sites identified, T-229, S-371, and T-389, appear to be critical for kinase function (reference 41 and unpublished data). Parallel studies also favor a model in which phosphorylation of T-389, in concert with the carboxy tail, controls the ability of an upstream kinase to access and phosphorylate T-229 (unpublished data). Mutation of the remaining phosphorylation sites, such as those in the autoinhibitory domain, including S-404, S-411, S-418, T-421, and S-424, suggests that their phosphorylation is involved in bringing about full kinase activation rather than playing an absolute role in this event (25, 41). The emerging model of p70$^{s6k}$ activation is that hyperphosphorylation of sites residing in the autoinhibitory domain, combined with acute phosphorylation of T-389, in the conserved linker region, induces a conformational change which disrupts an interaction between

the carboxy and amino termini, thus exposing the activation loop and allowing phosphorylation of T-229 and activation of $p70^{s6k}$. Independent of these ordered steps, each of these responses is envisaged to contribute to full kinase activation, which is compatible with the observation that total enzymatic activity decreases as a function of increasing deletions (Fig. 3) (50). In contrast to that of rapamycin, the inhibitory effect of wortmannin and SQ20006 would be through interfering with the activation of the T-389 kinase, either directly or through blocking the activation of an upstream kinase. In conclusion, this model highlights the importance of identifying the kinases which regulate $p70^{s6k}$ activity.

$p70^{s6k}$ is a member of the second messenger family of serine-threonine protein kinases (26). Other members of this family include cyclic AMP-dependent protein kinase, protein kinase C, and *Akt*/protein kinase B. The activity of these enzymes is known to be regulated by effector molecules or second messengers, with which phosphorylation at the activation loop appears to play a maintenance function, in contrast to the case for the mitogen-activated kinases. Indeed, the recent finding that the small GTPases rac1 and cdc42 can interact with $p70^{s6k}$ and alter its activity may identify such an effector molecule (12). The identification of T-389 and the domain surrounding this sequence should provide a powerful tool to unravel the events leading to $p70^{s6k}$ activation. Downstream, it will be essential to establish whether the impact of $p70^{s6k}$ activation on translation is delivered solely through S6 phosphorylation or whether other translational components, such as 4E-BP1 (40), are also targeted (48).

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P.B.D. and N.P. contributed equally to this study.

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