

Multiple Independent Inputs Are Required for Activation of the p70 S6 Kinase

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Previous studies have shown that the noncatalytic carboxy-terminal tail of the p70 S6 kinase (amino acids 422 to 525) contains an autoinhibitory pseudosubstrate domain that is phosphorylated in situ during activation and in vitro by mitogen-activated protein kinases. The present study shows that a recombinant p70 deleted of the carboxy-terminal tail (p70 Δ CT104) nevertheless exhibits a basal and serum-stimulated 40S kinase activity and susceptibility to inhibition by wortmannin very similar to those of the parent, full-length p70 kinase. Carboxy-terminal deletion reduces the extent of maximal inhibition produced by rapamycin, from >95% in the full-length p70 to 60 to 80% in p70 Δ CT104, without altering the sensitivity to rapamycin inhibition (50% inhibitory concentration of 2 nM). Serum activation of p70 Δ CT104, as with the parent, full-length p70, is accompanied by an increase in ³²P content (about twofold) in situ and a slowing in electrophoretic mobility; both modifications are inhibited by pretreatment with wortmannin or rapamycin. ³²P-peptide maps of p70 Δ CT104 show multisite phosphorylation, and wortmannin and rapamycin appear to cause preferential dephosphorylation of the same subset of sites. Thus, it is likely that activation of the kinase requires phosphorylation of p70 at sites in addition to those previously identified in the carboxy-terminal tail. Evidence that the carboxy-terminal tail actually functions as a potent intramolecular inhibitor of kinase activity in situ is uncovered by deletion of a short acidic segment (amino acids 29 to 46) from the p70 amino-terminal noncatalytic region. Deletion of amino acids 29 to 46 causes a >95% inhibition of p70 activity despite continued phosphorylation of the carboxy-terminal tail in situ; additional deletion of the carboxy-terminal tail (yielding p70 Δ 29-46/ Δ CT104) increases activity 10-fold, to a level approaching that of p70 Δ CT104. Deletion of residues 29 to 46 also abolishes completely the sensitivity of p70 to inhibition by rapamycin but does not alter the susceptibility to activation by serum or inhibition by wortmannin. Although the mechanisms underlying the effects of the Δ 29-46 deletion are not known, they are not attributable to loss of the major in situ p70 phosphorylation site at Ser-40. Thus, activation of the p70 S6 kinase involves multiple, independent inputs directed at different domains of the p70 polypeptide. Disinhibition from the carboxy-terminal tail requires, in addition to its multisite phosphorylation, an activating input dependent on the presence of amino acids 29 to 46; this p70-activating input may be the same as that inhibited by rapamycin but is distinct from that arising from the wortmannin-inhibitable phosphatidylinositol 3-kinase. In addition, as exemplified by the rapamycin-resistant but mitogen- and wortmannin-sensitive p70 Δ 29-46/ Δ CT104 mutant, a further activating input, which probably involves site-specific phosphorylation in the segment between amino acids 46 to 421, is necessary.

Addition of insulin, polypeptide growth factors, and cytokines to a variety of cultured cells results in the phosphorylation of multiple serine residues on the 40S ribosomal protein S6, mediated by activation of S6 protein kinases (5). Two distinct subfamilies of insulin- and mitogen-stimulated protein kinases have been identified: the 85- to 90-kDa Rsk enzymes and the p70 S6 kinases (11). Rapamycin, an agent that inhibits the p70 S6 kinase but not Rsk, abolishes completely S6 phosphorylation in situ, indicating that the mitogen- and cytokine-stimulated S6 phosphorylation occurring in situ in mammalian cells is actually catalyzed by the p70 S6 kinase (4, 13). Although both the Rsk and p70 S6 kinases are usually activated in a coordinate fashion by mitogens, through a mitogen-stimulated Ser/Thr phosphorylation of the kinase polypeptide, the protein kinases that participate in the activation of the two S6 kinases

differ. The Rsk enzymes can be phosphorylated in vitro and activated by the p42 mitogen-activated protein (MAP) kinase, Erk-2 (18), whereas the p70 S6 kinase, although phosphorylated in vitro under certain conditions by p42 MAP kinase, is not activated thereby (12). Furthermore, rapamycin and, in many cells, wortmannin abolish the activation of p70 S6 kinase without altering the activity of Rsk or the p42 MAP kinases (3, 4, 13; unpublished data), providing additional evidence that there exist elements necessary for p70 S6 kinase activation that are not required for the activation of the Ras/Raf/Mek/Erk/Rsk pathway.

In attempting to understand the molecular basis for the mitogen activation of the p70 S6 kinase, we identified a 25-amino-acid segment in the noncatalytic carboxy-terminal tail (starting at residue K-423) whose amino acid sequence resembled (28% identity) that of its substrate, ribosomal S6, in the region surrounding the sites of S6 phosphorylation, and suggested that this segment of p70 S6 kinase functioned as an autoinhibitory pseudosubstrate domain (1). Activation of the enzyme was proposed to require the phosphorylation of multiple Ser/Thr residues within this pseudosubstrate segment,

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catalyzed by one or more insulin- and mitogen-activated protein kinases situated upstream. Considerable evidence now supports such a mechanism. A 37-amino-acid synthetic peptide whose sequence encompasses the putative p70 S6 kinase autoinhibitory pseudosubstrate domain (SKAIPS domain peptide) and a 32-amino-acid synthetic peptide corresponding to the homologous sequences in the S6 substrate itself were compared for the ability to interact with the p70 S6 kinase (14). Whereas the S6 peptide was avidly phosphorylated by the p70 S6 kinase, the SKAIPS peptide was not a substrate; nevertheless, both peptides inhibited p70-catalyzed phosphorylation of 40S subunits with an equal potency (50% inhibitory concentration of $\sim 20 \mu\text{M}$). Moreover, SKAIPS peptide inhibited p70 catalyzed phosphorylation of S6 peptide via a competitive mechanism, indicating that both peptides bound to a common site on the p70 S6 kinase, i.e., the substrate binding site. Ferrari et al. (6) reported that the majority of ^{32}P -p70 S6 kinase sites phosphorylated in ^{32}P -labelled, mitogen-stimulated cells are clustered within this SKAIPS domain at residues S-434, S-441, T-444, and S-447. Ferrari et al. (7) reported that mutation of all four of these amino acids to alanine reduced the activity of recombinant p70 by fivefold, whereas replacement by glutamic acid was slightly activating. Each of these S/T residues is followed directly by a proline, and using the synthetic SKAIPS peptide as a substrate, we showed that insulin activates an array of proline-directed SKAIPS peptide kinases that include Erk-1, Erk-2, a 150-kDa form of the cdc2 kinase, and some as yet unidentified proline-directed kinases (that probably include other Erk isoforms) (12). Erk-2 and MPF (*Xenopus* cyclin B-cdc2 complex) phosphorylate the intact p70 S6 kinase polypeptide in vitro at sites that comigrate on peptide mapping with those phosphorylated within the synthetic SKAIPS peptide by these two protein kinases; comigrating p70 ^{32}P -peptides are also generated in situ during mitogen stimulation of ^{32}P -labelled cells. Nevertheless, phosphorylation of recombinant or purified rat liver-derived p70 S6 kinase by Erk-2, MPF, or both in vitro did not activate p70 S6 kinase. This outcome led us to conclude that regulatory inputs in addition to that provided by phosphorylation of the autoinhibitory domain were necessary to the mitogen activation of the p70 S6 kinase.

In this study, we used mutagenesis and expression of recombinant p70 S6 kinase polypeptides to characterize the manner in which the SKAIPS domain participates in the mitogen regulation of the enzyme and to demonstrate the existence of mitogen-directed activating signals acting independently of the SKAIPS domain. In addition, we demonstrate that the susceptibility of p70 to inhibition in situ by rapamycin can be abolished by mutations in the p70 structure that leave intact the ability of the p70 to be activated by mitogens and inhibited by wortmannin, indicating that rapamycin inhibits p70 through effectors entirely distinct from those that mediate the actions of mitogens and wortmannin.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Sigma. Protein G-agarose and G418 were from Life Technologies, Inc. Restriction enzymes were obtained from New England Biolabs. [γ - ^{32}P]ATP, [α - ^{35}S]dATP, and $^{32}\text{P}_i$ were products of DuPont New England Nuclear. NIH 3T3, COS-7, and 293 human embryonal kidney cells were purchased from the American Type Culture Collection. DNA sequencing reagents were from United States Biochemical.

Construction of truncation mutant and site-specific mutant plasmids. A cDNA encoding rat p70 αI S6 kinase cloned into the *EcoRI* site of PMT2 (PMT2-p70 αI) (9) was modified by introduction of a nine-amino-acid epitope from the influenza virus hemagglutinin (HA) (8) at the amino terminus as follows. PMT2-p70 αI was digested with *ApaI*, and a pair of synthetic oligonucleotides were ligated into this site. The top strand, 5'-GAATTCGGCGCCGCACCATGTACCCATACGATGTTCCAGATTACGCTGGGCC-3', and bot-

tom strand, 5'-CAGCGTAATCTGGAACATCGTATGGGTACATGGTGGCGGCCGCGAATCC-3', encode, in the following order, *EcoRI* and *NoI* restriction sites, a consensus translation initiation sequence, an initiator methionine, the HA epitope YPYDVPDYA, and an *ApaI* site encoding the amino acids G and P, following which the p70 αI initiator methionine and sequences commence. This HA-tagged PMT2-p70 αI vector, referred to as full-length p70, was used as a basic template for construction of a set of amino-terminal and carboxy-terminal truncation mutants and site-specific mutants. p70- Δ 3-78 was generated by digestion of full-length p70 with *ApaI* and *SpeI* and ligation with a synthetic linker composed of a coding-strand oligonucleotide (5'-CAT GAG GCC TA-3') and a noncoding-strand oligonucleotide (5'-CTA GTA GGC CGC ATG GCC C-3'). The resultant p70 sequence encoded...R₂PTMV₇₉... and a diagnostic *StuI* restriction site. The p70 Δ 2-46 variant was produced by digestion of full-length p70 with *ApaI* and *SpeI* and ligation of an *ApaI*-*SpeI*-digested PCR fragment, prepared from a p70 template by using a forward primer (5'-GAT TAC GCT GGG CCC ATG GGG GGT CAG TTA AAT GAA AGC-3') encoding an *ApaI* site, Met-1, Gly-47, and succeeding p70 sequence and a reverse primer (5'-GTT CAC ACT AGT TTC TGA-3') encompassing the *SpeI* site. The p70 Δ 29-46 variant was created by a two-step PCR. In the first PCR, two overlapping fragments were prepared; fragment A was amplified with outer forward primer (5'-TTA CGC TGG GCC CAT GAG-3') covering an *ApaI* site and an inner reverse primer (5'-GGT ACC AAA CAC TCC TGC CAT GTC-3') beginning at amino acid residue Asp-23. Fragment B was amplified with inner forward primer (5'-GGT ACC GGG GGT CAG TTA AAT GAA-3') starting at the Gly-47 codon and outer reverse primer (5'-GTT CAC ACT AGT TTC TGA-3') covering the *SpeI* site at the 3' end. The inner forward and inner reverse primers are overlapping and contain a diagnostic *KpnI* site. Fragments A and B were annealed and used as a template for second-round PCR amplification with the outer forward and outer reverse primers. The resultant PCR product, encoding the sequence...F₂₈GTG₄₇..., was digested with *ApaI* and *SpeI* and subcloned into the *ApaI*-*SpeI*-digested p70 wild-type cDNA. The carboxy-terminal truncation mutant p70 Δ CT104 was constructed by introduction of an ochre stop codon after the Ser-421 codon by using a two-step PCR and cloning of the mutant PCR fragment as an *XhoI*-*BspMI* insert. The inner forward primer was (5'-CCA TCT GTA CTT GAA AGT TGA TAA GAA AAG-3'), and the inner reverse primer was 5'-CTT TTC TTA TCA ACT TTC AAG TAC AGA TGG-3'. The oligonucleotides 5'-ACA AGA AGC TCT GCA TCT GCT TAA AA-3' and 5'-TCC TCC ATC GAA AGC ATT CC-3' were used as outerprimers for PCR amplification to cover 5'-end *XhoI* and 3'-end *BspMI* sites, respectively. Construction of amino-terminal and carboxy-terminal double-truncation mutants was accomplished by excision of the DNA sequences encoding p70 residues Met-1 to Gly-165 from the Δ 3-78, Δ 2-46, and Δ 29-46 variants, using *ApaI* and *AgeI*, and subcloning the respective *ApaI*-*AgeI* fragments into a p70 Δ CT104 fragment from which the unmodified p70 amino terminus had been excised with *ApaI* and *AgeI*. All constructs were verified by double-stranded DNA sequence analysis.

Transient or stable expression of the p70 S6 kinase. NIH 3T3 and 293 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 100 U of streptomycin and penicillin per ml. For transient expression of p70 and its derivatives, approximately 10^6 293 cells were seeded and cultured on 10-cm-diameter tissue culture dishes for about 20 h prior to transfection. Ten or 20 μg of PMT2-based plasmids encoding wild-type or mutant p70 proteins was transfected per 100-mm-diameter dish, using the calcium phosphate coprecipitation method (17). Forty-eight hours posttransfection, the medium was replaced with DMEM containing 0.5% serum, and serum stimulation (10% for 15 min) was carried out 20 h thereafter. Rapamycin or wortmannin was added 15 min prior to serum stimulation. Stable transfectants were selected after cotransfection of full-length PMT2-p70 or PMT2-p70 Δ CT104 into NIH 3T3 cells and cultivation in the presence of G418 (400 $\mu\text{g}/\text{ml}$) for 2 weeks. Neomycin-resistant clones were purified by using a ring cloning method and screened further by immunoblotting for p70.

Immunoprecipitation. Cells were washed twice with ice-cold phosphate-buffered saline and scraped off in lysis buffer (10 mM morphinepropanesulfonic acid [MOPS], 5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 50 mM β -glycerophosphate, 10 mM MgCl_2 , 0.5% Triton X-100, 2 mM VO_4 , 2 mM leupeptin, 2 mM pepstatin, 1 mM diisopropylfluorophosphate, 1 mM phenylmethylsulfonyl fluoride [pH 7.5]). Supernatants were removed after centrifugation at $14,000 \times g$, and aliquots matched for protein content were incubated with the monoclonal anti-HA epitope antibody 12CA5 (1, 8) and protein G-agarose at 4°C for 3 h. Immunoprecipitate-agarose beads were washed three times with lysis buffer, once with 1 M NaCl in lysis buffer, and once with kinase reaction buffer. Under these transfection conditions, dilution of the extracts over a 10-fold range prior to 12CA5 immunoprecipitation did not alter the apparent specific activity of recombinant wild-type p70 S6 kinase. In some experiments, the endogenous p70 S6 kinase was immunoprecipitated with a polyclonal antiserum generated against a recombinant polypeptide encoding p70 amino acid residues 422 to 525.

S6 kinase assay. The assay for p70 S6 kinase activity was performed as described before (14, 15). ^{32}P -proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred from the gel onto an Immobilon membrane (Millipore Corp.). The membrane was exposed to X-ray film for the measurement of S6 kinase activity and also used for Western blot (immunoblot) analysis.

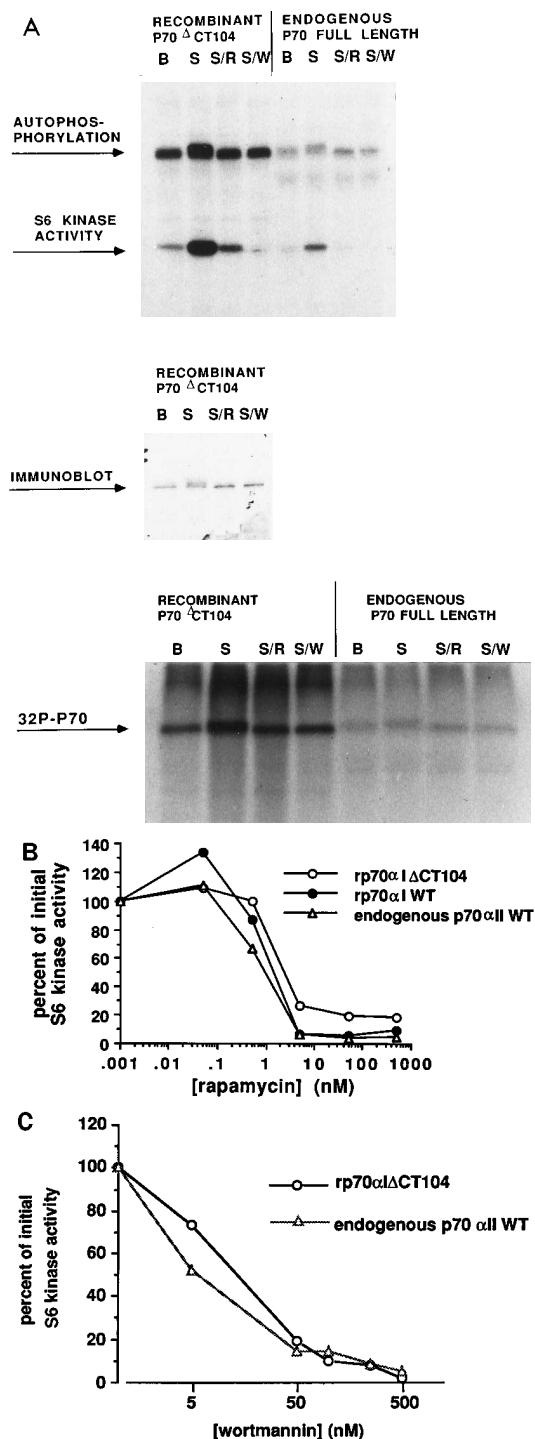


FIG. 1. Regulation of recombinant p70 Δ CT104 in comparison with full-length p70 polypeptides in NIH 3T3 stable transformants. (A) Regulation of recombinant p70 α I Δ CT104 and endogenous p70 α II S6 kinases in the same NIH 3T3 cell. Cells stably expressing p70 α I Δ CT104, maintained in DMEM supplemented with 10% fetal bovine serum, were labelled in the presence of 32 P_i (0.15 mCi/ml) for 6 h and then for an additional 18 h in DMEM containing 0.2% fetal bovine serum. The 32 P-labelled cells were stimulated by addition of DMEM (lanes B) or serum to 10% (lanes S). Cells were harvested 20 min after serum addition. Wortmannin (500 nM; lanes S/W) or rapamycin (50 nM; lanes S/R) was added 15 min prior to serum addition. Aliquots of the cell extracts containing equal amounts of protein were subject to immunoprecipitation with anti-HA antibody, to isolate the recombinant p70 Δ CT104, and then subjected to immunoprecipitation with a polyclonal antibody to a recombinant polypeptide encoding the carboxy-terminal 104 amino acids of p70, so as to selectively precipitate

Western blot analysis. The membranes were first blocked with 3% low-fat milk and then incubated with anti-p70 peptide (amino acids 337 to 352) antibody (9), washed, reacted with anti-rabbit immunoglobulin G coupled to peroxidase (Amersham), and developed with enhanced chemiluminescence reagents as instructed by the manufacturer.

RESULTS

The activation of endogenous p70 S6 kinase by serum or mitogens varies among cell lines and generally correlates with the degree to which cells can be brought to mitogenic quiescence by serum withdrawal. NIH 3T3 cells show a 5- to 10-fold activation upon serum readdition (Fig. 1), and 293 cells show a 2- to 4-fold activation, whereas COS-7 cells show no significant alteration in endogenous p70 kinase activity upon serum reduction (to 0.5%) or readdition to 10% (9, 13). We used the 293 cell line to examine the regulatory properties of mutant p70 polypeptide by transient expression, because the consistency of polypeptide expression was superior to that attained in NIH 3T3 cells. Recombinant full-length p70, both α I and α II, was modified by insertion of the nine-amino-acid epitope from influenza virus HA (8) immediately after the initiator methionine; this enabled all p70 variants to be recovered from cell extracts with equal efficiency by immunoprecipitation with the anti-HA monoclonal antibody 12CA5 without interference from the endogenous p70 polypeptide.

The full-length recombinant p70 polypeptide expressed transiently in 293 cells exhibits a two- to fourfold stimulation of 40S kinase activity when serum-deprived cells are exposed to 10% fetal calf serum for 20 min (Fig. 2, 3, and 5). A comparable degree of stimulation is elicited by platelet-derived growth factor, whereas epidermal growth factor and insulin at maximal concentrations give a slightly less potent activation (not shown). Rapamycin (at 10 nM), added 15 min prior to serum, inhibits recombinant wild-type p70 S6 kinase (at 15 min after serum readdition) by over 95% (Fig. 1, 3, and 4); addition of rapamycin after maximal serum activation has been achieved also produces rapid inhibition of p70 activity, down by 80% within 15 min (not shown). These responses are similar to those reported previously for endogenous p70 in several cell lines (4, 9, 14) as well as recombinant p70 expressed in COS-M7 cells (13). Finally, the responses of recombinant wild-type p70 α I and endogenous α II p70 kinase activities to serum and rapamycin were indistinguishable.

Effects of removal of the carboxy-terminal tail on the properties of p70 S6 kinase. Having characterized the regulation of recombinant full-length p70 polypeptide, we next examined a mutant p70 (p70 Δ CT104) whose entire carboxy-terminal non-catalytic tail, including the SKAIPS domain (residues 423 to 447), has been deleted by introduction of a stop codon after

endogenous, full-length p70. Washed immunoprecipitates were used for assay of 40S S6 kinase. The upper panel shows the 40S kinase assay; the S6 activities (picomoles of 32 P per minute) for lanes B, S, S/R, and S/W, respectively, were as follows: p70 Δ CT104, 13.1, 108, 22, and 5.3; endogenous p70, 2.1, 12.8, 4.0, and 3.4. The middle panel shows an immunoblot of the p70 Δ CT104 polypeptide. The lower panel shows the autoradiograph of the 12CA5 and anti-CT104 immunoprecipitates from 32 P-labelled cells; levels of 32 P recovered in the p70 Δ CT104 polypeptides were 1,788, 3,072, 2,408, and 1,798 cpm in lanes B, S, S/R, and S/W, respectively. (B) NIH 3T3 cell lines stably expressing either the full-length p70 α I or p70 α I Δ CT104 polypeptide were stimulated by serum as described above; rapamycin was added 15 min prior to serum. Inhibition of serum-stimulated activity of each recombinant and the endogenous p70 (in the Δ CT104 cells) is shown as a function of rapamycin concentration. WT, wild type. (C) NIH 3T3 cells stably expressing p70 Δ CT104 were stimulated with serum; wortmannin was added 15 min prior to serum. Inhibition of the serum-stimulated 40S kinase activity of the p70 Δ CT104 and endogenous p70 polypeptides by wortmannin is shown.

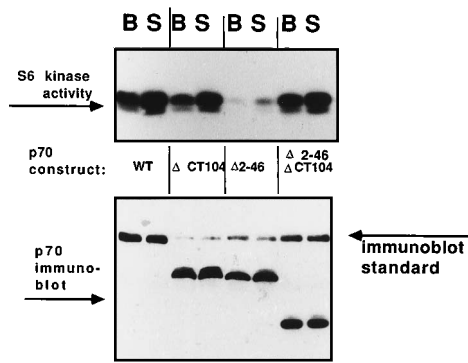


FIG. 2. Serum regulation of the 40S kinase activity of p70 variants during transient expression. cDNAs encoding full-length p70, p70 Δ 2-46, p70 Δ CT104, and p70 Δ 2-46/ Δ CT104 were transfected into 293 cells; the cells were placed in low serum 48 h later and after 20 h were stimulated by addition of DMEM (lanes B) or fetal calf serum to 10% (lanes S). After 15 min, cells were rinsed and disrupted, and the recombinant p70 polypeptides were immunoprecipitated with anti-HA monoclonal antibody 12CA5. The washed immunoprecipitates were assayed for 40S kinase activity as described in Materials and Methods. The upper panel displays 32 P incorporation into 40S S6, and the lower panel is an immunoblot of the recombinant p70 polypeptides in the assay using an antipeptide antibody to a catalytic domain sequence (9) found in all variants. The immunoblot standard is a recombinant p70 polypeptide run at increasing load in the same gel, used as an internal standard to normalize 40S kinase activity to polypeptide recovery (measured as units of protein) in those experiments in which variable recovery was encountered. The absolute values of 40S kinase (picomoles per minute per unit of protein) for lanes B and S, respectively, in this experiment were as follows: wild type (WT), 449 and 1,120; Δ CT104, 264 and 549; Δ 2-46, 29 and 71; Δ 2-46/ Δ CT104, 512 and 903. These results are representative of three experiments.

residue Ser-421. The p70 Δ CT104 polypeptide is expressed in 293 cells at levels similar to those of full-length p70 (Fig. 2 and 3). The 40S kinase activity of p70 Δ CT104 in serum-deprived cells is not appreciably different from that of the full-length recombinant enzyme, and p70 Δ CT104, like full-length p70, exhibits a two- to fourfold increase in activity after addition of 10% serum. The ability of rapamycin to inhibit the p70 Δ CT104 mutant has been altered modestly, however, in that concentrations of rapamycin that inhibit recombinant wild-type p70 by >95% give only 60 to 80% inhibition of p70 Δ CT104 kinase (Fig. 1 and 3).

The relatively normal behavior of the p70 Δ CT104 mutant was unexpected; it has been anticipated that deletion of the SKAIPS domain would produce an increase in the basal activity of the kinase and a consequent attenuation in the fold activation by serum. To verify the properties observed in 293 cells, lines of NIH 3T3 cells permanently expressing epitope-tagged full-length p70 and p70 Δ CT104 polypeptides were established. In the latter lines, the activity of the endogenous p70 (predominantly α II) and the recombinant p70 Δ CT104 can be evaluated independently in the same extracts: endogenous p70 is immunoprecipitated with a polyclonal antibody raised against the p70 carboxy-terminal 104-amino-acid tail, whereas the HA epitope-tagged p70 Δ CT104 polypeptide is immunoprecipitated selectively with 12CA5. (On SDS-PAGE, the mobilities of the recombinant p70 α I/ Δ CT104 polypeptide, stably expressed in NIH 3T3 cells, and the endogenous p70 α II polypeptide are nearly identical; this is coincidental and reflects the offsetting effects of carboxy-terminal truncation [which increases mobility] together with the 23-amino-acid amino-terminal extension [which slows mobility disproportionately because of a sequence containing six consecutive arginines {9}] present in the 525-amino-acid p70 α I that is lacking in the predominant endogenous p70 polypeptide, which is the

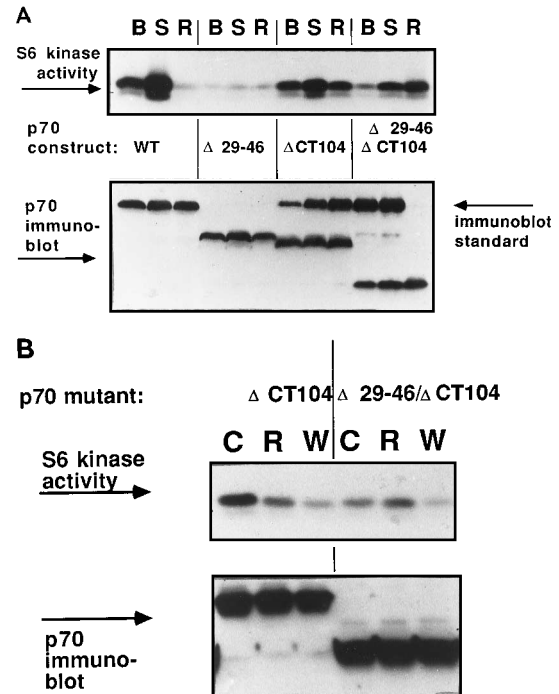


FIG. 3. Deletion of p70 residues 29 to 46 inhibits 40S kinase only if the p70 carboxy-terminal tail is present and greatly reduces rapamycin sensitivity. (A) The p70 variants indicated were expressed transiently in 293 cells as for Fig. 2. Serum-deprived cells were treated with basal medium (lanes B), 10% serum (lanes S), or rapamycin (10 nM) 15 min prior to serum addition (lanes R); extracts were prepared 20 min after serum addition; immunoprecipitation and the S6 kinase assay were performed as for Fig. 2. 40S kinase activities (picomoles per minute per unit of protein) for lanes B, S, and R, respectively, in this experiment were as follows: full-length (wild type [WT]), 1,780, 6,393, and 189; Δ 29-46, 86, 145, and 168; Δ CT104, 993, 2,887, and 932; Δ 29-46/ Δ CT104, 480, 1,333, and 1,253. These results were representative of three experiments. (B) Serum-replete 293 cells, transiently expressing p70 Δ CT104 or p70 Δ 29-46/ Δ CT104, were treated with a maximally inhibitory concentration of rapamycin (50 nM, lanes R), a submaximally inhibitory concentration of wortmannin (100 nM; lanes W), or carrier (lanes C) for 15 min prior to harvest, immunoprecipitation, and assay of S6 kinase activity. The S6 kinase activities (picomoles per minute per unit of protein) for lanes C, R, and W, respectively, were as follows: p70 Δ CT104, 444, 200, and 116; p70 Δ 29-46/ Δ CT104, 176, 220, and 91.

502-amino-acid α II isoform.) The behavior of p70 Δ CT104 in these permanent cell lines parallels closely the pattern seen after transient expression (Fig. 1). Both the endogenous full-length p70 and recombinant p70 Δ CT104 exhibit a relatively low activity in serum-deprived cells that is stimulated four- to eightfold within 15 min after addition of 10% serum. Rapamycin inhibits endogenous p70 (primarily p70 α II), recombinant full-length p70 α I, and recombinant p70 α I/ Δ CT104 with a 50% inhibitory concentration of \sim 2 nM; however, whereas the endogenous and recombinant p70 polypeptides with an intact carboxy-terminal tail are inhibited >95% by rapamycin, inhibition of p70 Δ CT104 plateaus at \sim 80% (Fig. 1B).

Wortmannin, a relatively selective inhibitor of phosphatidylinositol (PI) 3-kinase (3), inhibits p70 but not Rsk, Erk-1 or Erk-2 in several cell types. The impact of carboxy-terminal deletion on the ability of wortmannin to inhibit p70 S6 kinase was examined. Half-maximal and >95% inhibition of the 40S kinase activity of endogenous full length p70 and p70 Δ CT104, immunoprecipitated from the same extracts of serum-stimulated NIH 3T3 cells, is observed at comparable concentrations of wortmannin (about 10 and 500 nM, respectively; Fig. 1C). Thus, in contrast to rapamycin, whose inhibitory properties are

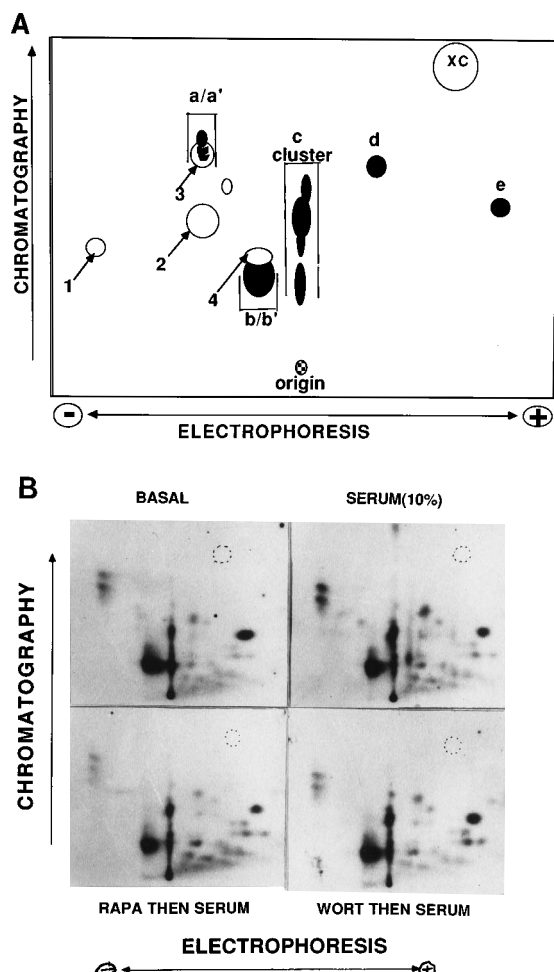


FIG. 4. ^{32}P -peptide maps of p70 S6 kinase. (A) Schematic representation of the ^{32}P -peptides visualized reproducibly in two-dimensional peptide maps of endogenous or recombinant p70 S6 kinase, ^{32}P labelled in NIH 3T3 cells, or during transient expression in 293 cells. The open symbols, which are numbered, are ^{32}P -peptides that are seen in full-length p70 but not in p70 Δ CT104 (see Fig. 4B) and correspond to peptides that contain the phosphorylation sites in the SKAIPS domain (6, 12). The filled symbols, which are given letter designations, are seen in maps of both full-length p70 and p70 Δ CT104. XC, xylene cyanol marker. (B) Autoradiographs of two-dimensional ^{32}P -peptide maps of digests prepared from the ^{32}P -labelled p70 Δ CT104 polypeptides shown in the bottom panel of Fig. 1A. Aliquots containing equal (1,000) ^{32}P cpm were subjected to thin-layer electrophoresis (TLE)/thin-layer chromatography and the plates were all exposed for 3 days. RAPA, rapamycin; WORT, wortmannin.

altered by carboxy-terminal truncation, deletion of the p70 carboxy-terminal tail does not alter the sensitivity to or extent of inhibition by wortmannin.

The finding that the p70 Δ CT104 mutant continues to show mitogen responsiveness, wortmannin inhibition, and partial inhibition by rapamycin establishes that crucial mitogen-directed inputs must occur into p70 domains outside of the putative autoinhibitory domain located in the carboxy-terminal tail; the further characterization of these inputs was undertaken.

The activation of p70 Δ CT104 by serum treatment of NIH 3T3 cells is accompanied by a slowing in the electrophoretic mobility of a portion of the p70 Δ CT104 polypeptides (Fig. 1A, middle panel), as has been shown to occur with full-length p70; inhibition of p70 Δ CT104 by pretreatment of cells with either rapamycin or wortmannin abolishes this upshift. The upshift of the full-length p70 that occurs upon activation has been shown

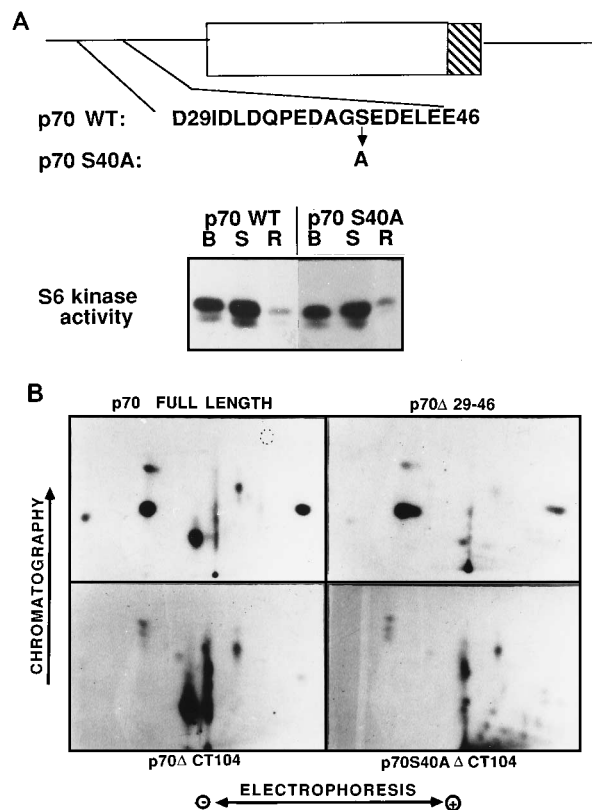


FIG. 5. p70 Ser-40, although a site of phosphorylation in situ, is not important for mitogen activation or sensitivity to rapamycin. (A) Mutation of Ser-40 does not alter the regulatory properties of p70 S6 kinase. The full-length (wild-type [WT]) p70 and full-length p70 Ser-40 \rightarrow Ala mutant were expressed transiently in 293 cells, and 40S kinase activity analyzed after immunoprecipitation with an anti-HA antibody. Cell treatments were as described in the legend to Fig. 3 and Materials and Methods. (B) ^{32}P -peptide maps of p70 variants ^{32}P labelled during transient expression in 293 cells. Full-length p70 and p70 Δ 29-46 were labelled with ^{32}P and 400 cpm of ^{32}P from each digest was exposed for 14 days; note in p70 Δ 29-46 the absence of spot b (Fig. 4A), whereas SKAIPS domain spots 1, 2, and 3 are clearly visualized. p70 Δ CT104 and S40Ap70 Δ CT104 were labelled with ^{32}P and 1,500 cpm of ^{32}P from each digest was exposed for 4 days; note in p70 Δ CT104 the absence of spots 1, 2, and 3 compared with full-length p70, whereas in S40Ap70 Δ CT104 spot b is lost selectively.

clearly to be due to phosphorylation of the p70 polypeptide; Fig. 1A (bottom panel) demonstrates that activation of p70 Δ CT104 is accompanied by an increased ^{32}P content (approximately twofold) that is largely prevented by rapamycin or wortmannin pretreatment. Thus, it is likely that p70 Δ CT104, despite the deletion of four major phosphorylation sites in the carboxy-terminal tail, is activated by phosphorylation of the p70 polypeptide. The relatively modest, 2-fold change in overall ^{32}P content in comparison with the 4- to 10-fold changes in 40S kinase activity between the basal, stimulated, and inhibited forms of the p70 Δ CT104 polypeptide suggests that the crucial sites of regulatory phosphorylation represent a subset of the total phosphorylation sites. The ^{32}P -labelled p70 Δ CT104 polypeptides shown in Fig. 1A were therefore subjected to proteolytic digestion, and aliquots containing equal ^{32}P cpm were analyzed by two-dimensional ^{32}P -peptide mapping so as to emphasize the relative abundance of ^{32}P -peptides. Multiple ^{32}P -peptides were reproducibly observed (Fig. 4B), as diagrammed in Fig. 4A. The most marked increase in relative ^{32}P content after serum stimulation is seen in spots a/a' and d, although the largest absolute increase in ^{32}P content is prob-

ably in the major site in spot b. Inhibition of the enzyme by pretreatment with wortmannin or rapamycin prior to serum addition was accompanied by rather consistent decreases in the relative ^{32}P content of spot a/a', especially in comparison with the basal pattern. Thus, activation of the p70 Δ CT104 by serum and its inhibition by wortmannin or rapamycin are accompanied by site-specific changes in ^{32}P content; the mechanisms underlying these altered phosphorylations and compelling evidence that the selective phosphorylation of spot a/a' regulates the activity of the p70 Δ CT104 polypeptide are not yet available.

A noncatalytic amino-terminal acidic (ATA) segment (residues 29 to 46) is required for release from inhibition by the carboxy-terminal tail. The failure of deletion of the carboxy-terminal tail to increase (i.e., disinhibit) the basal 40S kinase activity calls into question the premise that the carboxy-terminal segment actually contains an autoinhibitory domain; however, in view of the considerable data in support of a role for the SKAIPS segment located within the carboxy-terminal tail as an endogenous inhibitory domain, we sought to uncover evidence for such a function through further mutation.

The amino-terminal noncatalytic sequences of p70 contains a highly acidic segment from residues 29 to 46, wherein 10 of 18 amino acids are D or E and no basic residues are found. Deletion of this ATA domain either selectively (i.e., Δ 29-46) (Fig. 3) or from the first methionine (i.e., Δ 2-46) (Fig. 2) generates a p70 polypeptide whose level of expression is comparable to that of the full-length p70 and p70 Δ CT104 polypeptides but whose 40S kinase activity is reduced by 90 to 95%; despite the low activity, modest mitogen activation, ranging from 1.5- to 3.0-fold, is evident in both the Δ 2-46 (Fig. 2) and Δ 29-46 (Fig. 3) deletion mutants. Further truncation of the p70 amino terminus (Δ 3-78) yields a polypeptide whose expression after transient transfection is 10-fold lower than that of full-length p70 and p70 Δ CT104, precluding a reliable assessment; however, replacement of the p70 amino terminus down to residue 77 or 164 by the homologous segments from the rat Rsk-1 amino terminus (1, 10) yields chimeric polypeptides that are well expressed. Such Rsk-p70 chimeras also exhibit <5% of the 40S kinase activity of the wild-type p70 (or p70 Δ CT104) polypeptide (not shown). Although the low activity of these amino-terminal deletion mutants (through amino acid 46) or Rsk-p70 chimeras might be attributable to misfolding, the substantial expression achieved during transient expression suggests that an increased susceptibility to proteolysis in situ is not a feature of these mutants. Better evidence, however, that the loss of activity seen with these amino-terminal deletions is due to a functional inhibition of the enzyme rather than misfolding is the observation that introduction of the Δ CT104 carboxy-terminal deletion into the Δ 2-46 (Fig. 2) or Δ 29-46 (Fig. 3) background results in a 10-fold increase in 40S kinase activity with restoration of maximal activity and mitogen regulation to levels that approach those seen with the full-length and Δ CT104 p70 polypeptides (i.e., within ~50% of the specific activity). A similar 10-fold increase in activity is seen if the Δ CT104 deletion is introduced into the Rsk-p70 chimeras (not shown). Thus, the loss of activity engendered by the deletion of the p70 ATA domain (or its replacement by Rsk sequences) is largely if not entirely dependent on the presence of the carboxy-terminal tail; deletion of the ATA domain when introduced onto the Δ CT104 background does not greatly disturb (i.e., <2-fold change) the activity or mitogen regulation of the carboxy-terminally truncated p70 (Fig. 2 and 3). Conversely, the ability of the carboxy-terminal tail to act as an inhibitor is revealed only in the setting of the ATA deletion. Thus, the intact p70 amino-terminal noncatalytic sequences are appar-

ently necessary for release from the inhibitory effects of the carboxy-terminal tail. The simplest explanation of these results is that the ATA domain enables relief from the inhibition imposed by the SKAIPS domain, either by a direct interaction with the carboxy terminus or by enabling an input that is required for disinhibition. Consequently, deletion of the ATA domain prevents mitogen-induced disinhibition when the carboxy terminus is intact but has little or no impact when the carboxy terminus is deleted.

A noncatalytic amino-terminal segment (residues 29 to 46) is required for the susceptibility of p70 to inhibition by rapamycin but not wortmannin. Deletion of the 29-46 segment has a second effect on the regulatory properties of p70: the susceptibility of the enzyme to inhibition by rapamycin is abolished by this deletion (Fig. 3). This effect is evident when amino-terminal deletion is introduced on the full-length background; however, the very low maximal activity of those mutants (Δ 2-46 and Δ 29-46) tempers conclusions about the absence of further inhibition by rapamycin. The insensitivity of p70 Δ 29-46/ Δ CT104 (and p70 Δ 2-46/ Δ CT104; not shown) to rapamycin inhibition, however, is very clear-cut. In contrast to the loss of susceptibility to inhibition by rapamycin engendered by the Δ 29-46 deletion, no loss in susceptibility to inhibition by wortmannin is observed in the p70 Δ 29-46/ Δ CT104 variant compared with p70 Δ CT104; both variants as well as full-length p70 exhibit similar degrees of inhibition by submaximal (Fig. 3B) and maximal (not shown) concentrations wortmannin during transient expression. This disassociation in sensitivity to rapamycin and wortmannin exhibited by p70 Δ 29-46/ Δ CT104 demonstrates clearly that the rapamycin-sensitive input into p70 is entirely independent of the wortmannin-sensitive input, the latter presumably originating from the PI 3-kinase.

We next attempted to uncover the biochemical basis for the phenotypes induced by deletion of the ATA segment. A synthetic peptide corresponding to the p70 residues 29 to 46 added (up to 0.3 mM) to the p70 Δ 29-46 polypeptide immunoprecipitated from 293 cells failed to alter 40S kinase activity assayed in vitro (not shown). The 29-46 segment contains a single phosphorylatable residue, Ser-40, situated in a potential casein kinase 2 phosphorylation site. Replacement of this residue by Ala (Fig. 5A) or Glu (not shown) in the full-length p70 does not reproduce the inhibitory effects of deletion of the 29-46 segment or alter the sensitivity to or extent of inhibition by rapamycin. Deletion of the ATA segment does not appear to induce a failure in the phosphorylation of the carboxy terminus. After transient expression and ^{32}P labelling in intact 293 cells, overall levels of ^{32}P incorporation into full-length p70 and p70 Δ 29-46 polypeptides were comparable (within 2-fold), despite a 10- to 20-fold difference in S6 kinase activity. ^{32}P -peptide maps revealed proportionate ^{32}P labelling of the SKAIPS sites in both the full-length and Δ 29-46 p70 polypeptides (Fig. 5B). The latter, however, lacked the major ^{32}P -peptide designated spot b in Fig. 4A; this is due to the loss of Ser-40 in the Δ 29-46 deletion, as confirmed by ^{32}P labelling and mapping of the p70S40A Δ CT104 mutant, whose ^{32}P -peptide map also exhibits a selective loss of spot b compared with p70 Δ CT104 (Fig. 5B). As shown in Fig. 5A, replacement of Ser-40 by Ala (or Glu) does not reproduce the effects of deletion of the 29-46 segment or otherwise alter the regulatory properties of the enzyme.

Thus, the inhibition engendered by the 29-46 deletion occurs despite phosphorylation of the SKAIPS domain but is substantially, if not completely, reversed by deletion of the carboxy terminus. This observation together with earlier results (7, 14) implies that phosphorylation of the SKAIPS domain, although probably necessary, is not sufficient to disin-

hibit the catalytic domain; some further step that involves an input enabled by the ATA domain is needed.

The data presented indicate that two functions can be ascribed to the ATA domain: (i) it is required for mitogen-induced relief of the inhibition engendered by the SKAIPS domain, and (ii) the ATA domain is required in order for the enzyme to be susceptible to inhibition by rapamycin (but is not required for inhibition by wortmannin). However, the mechanism by which the ATA domain enables the disinhibition of the full-length p70 kinase and confers sensitivity to rapamycin inhibition is not known, nor is it known whether there exists a single common mechanism for these two functions of the ATA domain.

DISCUSSION

Mitogen activation of p70 requires an input independent of those directed at the pseudosubstrate autoinhibitory domain in the carboxy-terminal tail. The p70 S6 kinase deleted of its carboxy-terminal tail continues to exhibit a mitogen-stimulated, wortmannin-inhibitable activity very similar to that of the full-length enzyme, although the ability of rapamycin to suppress enzyme activity is reduced significantly by carboxy-terminal deletion. Mitogen activation of the p70 Δ CT104 polypeptide is accompanied by an upshift in electrophoretic mobility and a multisite phosphorylation that are inhibited by both wortmannin and rapamycin. Although serum probably stimulates phosphorylation at all sites, a preferential increase in 32 P incorporation occurs in a subset of 32 P-peptides (spots a/a' and d), and wortmannin and rapamycin inhibition appears to be accompanied by the preferential dephosphorylation of these same 32 P-peptides (i.e., spots a/a' and d). Little or none of the mitogen-stimulated phosphorylation of p70 Δ CT104 is attributable to autophosphorylation, inasmuch as the 32 P-peptide map of p70 Δ CT104, 32 P labelled in situ during transient expression, is not altered if K-123 in the ATP binding site is mutated to Met, thereby inactivating the p70 kinase activity. The identities of the protein kinases that act on p70 Δ CT104 are unknown, except for casein kinase 2. Ser-40, situated in a classical casein kinase 2 consensus site, is phosphorylated by casein kinase 2 in vitro, and peptide mapping generates a single major 32 P-peptide that comigrates with 32 P-peptide b. Conversion of Ser-40 to Ala selectively abolishes the generation of 32 P-spot b during 32 P labelling of p70 Δ CT104 in 293 cells (Fig. 4B), as well as the ability of casein kinase 2 to phosphorylate p70 Δ CT104 in vitro (not shown). The S40A mutation does not, however, appear to alter the mitogen activation of p70 (Fig. 4A) or its susceptibility to inhibition by rapamycin (or wortmannin). The identities of the sites of regulatory phosphorylation in p70 Δ CT104 as well as the identities of the kinases that act on these sites remain to be established. Nevertheless, we suggest that a site-specific phosphorylation of p70, somewhere between amino acids 46 and 421, is required for enzyme activation, in addition to and independent of the multiple phosphorylations within the carboxy-terminal SKAIPS domain, at residues 434, 441, 444, and 447.

A p70 amino-terminal noncatalytic domain, amino acids 29 to 46, is required for release from inhibition by the carboxy-terminal tail. An ATA segment, situated between residues 29 and 46, participates in a necessary way in the regulation of catalytic activity of the holoenzyme, in concert with the endogenous autoinhibitory pseudosubstrate (SKAIPS) domain located carboxy terminal to the catalytic domain. If the ATA segment is deleted or replaced by homologous Rsk sequences, the mutant p70 exhibits a severely inhibited kinase activity. The Δ 2-46 and Δ 29-46 deletion mutants both show a maximal ac-

tivity of only 1 to 5% of wild-type enzyme activity, although a modest mitogen responsiveness of this residual activity persists. A second deletion of the carboxy-terminal 104 amino acids increases specific activity 10-fold with nearly full mitogen responsiveness. This behavior illustrates the potency of the inhibitory effect of the carboxy-terminal tail and the ability of this segment to largely supersede the activating effects of others (non-SKAIPS) inputs when the ATA domain is unavailable. Unlike the carboxy-terminal tail, the ATA domain does not appear to directly influence, to a significant degree, the function of the catalytic domain, but acts largely through its functional interaction with the carboxy-terminal tail; thus, deletion of the ATA domain from a p70 polypeptide that lacks a carboxy-terminal tail has little effect on the basal or mitogen-stimulated kinase activity.

The mechanism by which the ATA domain modulates the inhibitory action of the SKAIPS domain is not known. Clearly, phosphorylation of Ser-40 is not important to the regulatory function of the ATA domain. The ATA domain does not appear to be necessary for phosphorylation of the SKAIPS domain; rather, the ATA segment appears to provide an input that enables release of the phosphorylated SKAIPS segment from the catalytic domain.

The ATA domain, residues 29 to 46, is required for p70 to be susceptible to inhibition by rapamycin but not wortmannin. The present studies have identified a second role for the ATA domain between residues 29 and 46, in that deletion of ATA domain abolishes nearly completely the ability of the p70 to be inhibited by treatment of cells with rapamycin without altering its susceptibility to inhibition by wortmannin. The mechanism of this phenomenon is unknown, just as the basis for rapamycin inhibition per se remains uncertain. If rapamycin acts to inhibit an upstream activator of p70, then a loss of rapamycin sensitivity would suggest that the ATA deletion creates a p70 polypeptide that no longer binds this activator or has been rendered insensitive to its activating input. Alternatively, if the rapamycin-FKBP complex activates a p70 inhibitor (e.g., a phosphatase or an inhibitory ligand), then the ATA deletion may render p70 unable to bind the inhibitor or insensitive to the inhibitory effects. The low activity of the p70 Δ 2-46 and Δ 29-46 mutants (when their carboxy-terminal tails are intact) is more consistent with the idea that the 29-46 segment is necessary for an activating input that is also the target of rapamycin inhibition. In this model, a rapamycin-sensitive activating input requires p70 amino acids 29 to 46, and deletion of these residues is the functional equivalent of the rapamycin-inhibited state. One implication of this model is that the maximal specific activity of p70 Δ 29-46/ Δ CT104 will be 20 to 40% that of the p70 Δ CT104 variant (i.e., the maximal activity of the rapamycin-inhibited p70 Δ CT104 polypeptide); activities measured during transient expression are usually consistent with this view but not sufficiently precise to provide strong support.

The rapamycin-sensitive input, however, must have a second component that does not involve the disinhibition from the carboxy-terminal tail, inasmuch as the p70 Δ CT104 mutant continues to be inhibitable by rapamycin, though unmistakably to a lesser extent than occurs with the full-length p70. Inhibition of p70 Δ CT104 by rapamycin is accompanied by a decrease in site-specific phosphorylation, and the sites most inhibited by rapamycin (spots a/a' and d) correspond to those that appear to be selectively dephosphorylated during wortmannin inhibition. Nevertheless, the disassociation of susceptibility to rapamycin and wortmannin that occurs with the 29-46 deletion establishes that the inputs inhibited by these two agents are entirely distinct. Inasmuch as the p70 Δ CT104 mutant, which is susceptible to inhibition by both rapamycin and wortmannin, and

p70 Δ 29-46/ Δ CT104, which is susceptible only to wortmannin, are comparably responsive to mitogen activation (Fig. 2 and 5), it appears that the second component of the rapamycin-sensitive input, as reflected in its action on p70 Δ CT104, synergizes with but is not absolutely necessary for the expression of the wortmannin-inhibitable, mitogen-responsive activating input.

In conclusion, the relatively modest effects of deletion of the p70 carboxy-terminal 104 amino acids (residues 422 to 525) that includes the putative pseudosubstrate autoinhibitory (SKAIPS) domain (residues 423 to 447) on the regulatory properties of the recombinant p70 polypeptide establish that in addition to the phosphorylation of the SKAIPS domain, regulatory inputs directed at other p70 domains are necessary to achieve activation of the p70 kinase. These additional inputs appear to include further site-specific regulatory phosphorylations; the identification of the crucial sites, the kinases that catalyze these modifications, and their upstream activating inputs is critically important to understanding the signal transduction pathways impinging on p70. The ability of wortmannin to cause a parallel and relatively selective inhibition of p70 S6 kinase and PI 3-kinase in several cell types (reference 7 and data not shown), together with the identification of the mammalian rapamycin target as a potential lipid kinase (2, 16), points to PI 3-kinase and perhaps other lipid kinases as candidate upstream regulators of the p70 S6 kinase. The creation of simplified versions of the p70 polypeptide, wherein the requirement for multiple, parallel regulatory inputs has been eliminated, may permit the identification of each of the necessary upstream elements, so as to ultimately enable reconstitution *in vitro* of the complex machinery required for activation of the full-length p70 polypeptide. The ultimate goal is the delineation of the pathways that couple each of these activating elements to the cell surface receptors.

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