Ribosomal S6 Kinase 2 Inhibition by a Potent C-terminal Repressor Domain Is Relieved by Mitogen-activated Protein-Extracellular Signal-regulated Kinase Kinase-regulated Phosphorylation*

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Ribosomal S6 kinase 2 (S6K2) is a recently identified serine/threonine protein kinase that phosphorylates the 40 S ribosomal protein S6 in vitro. S6K2 is highly homologous to S6K1 in the core kinase and linker regulatory domains but differs from S6K1 in the N- and C-terminal regions and is differently localized primarily to the nucleus because of a C-terminal nuclear localization signal unique to S6K2. We have recently demonstrated that S6K2 is regulated similarly to S6K1 by the mammalian target of rapamycin pathway and by multiple P38-K pathway effectors in vivo. However, deletion of the C-terminal domain of S6K2 enhances kinase activity, whereas analogous deletion of S6K1 is inhibitory. Here, we characterize the S6K2 C-terminal motifs that confer this differential regulation. We demonstrate that the inhibitory effects of the S6K2 C-terminal domain are only partly attributable to the nuclear localization signal but that three C-terminal proline-directed potential mitogen-activated protein kinase phosphorylation sites are critical mediators of this inhibitory effect. Site-specific mutation of these sites to alanine completely desensitizes S6K2 to activating inputs, whereas mutation to aspartic acid to mimic phosphorylation results in an activated enzyme which is hypersensitive to activating inputs. Pretreatment of cells with the mitogen-activated protein-extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 inhibited S6K2 activation to a greater extent than S6K1. Furthermore, S6K2 mutants with C-terminal deletion or acidic phosphorylation site mutations displayed greatly reduced U0126 sensitivity. Thus, MEK-dependent inputs to C-terminal phosphorylation sites appear to be essential for relief of S6K2 inhibition but less critical for activation of S6K1. These data suggest a mechanism by which weak P38-K agonists can regulate S6 phosphorylation and selective translation in the presence of mitogen-activated protein kinase signaling.

S6 phosphorylation is a conserved mitogenic response that regulates translation of 5′-terminal oligopyrimidine tract-containing mRNAs encoding components of the protein synthetic machinery. This critical translational response that regulates ribosome biosynthesis is mediated by the ubiquitously expressed serine/threonine protein kinase ribosomal S6 kinase 1 (S6K1)1 (1, 2). Drosophila and mice lacking S6K1 exhibit a small animal phenotype, implicating this kinase in regulation of cell size (3, 4). However, S6 phosphorylation and 5′-terminal oligopyrimidine tract-containing mRNA translation appear normal in cells derived from mice lacking S6K1, suggesting a compensatory mechanism for these functions. S6K2, a mitogen-responsive S6K1 homolog, has recently been identified by our lab and others as a candidate for the compensatory S6 kinase (4–7).

Despite a high degree of sequence homology between S6K1 and S6K2 overall, it is likely that their physiological functions do not overlap entirely. The small size phenotype persists in the S6K1-deleted mice despite the presence of S6K2 (4). Both isoforms (β1 and βII) of S6K2 appear to be localized primarily to the nucleus because of a C-terminal nuclear localization sequence (6, 7). In contrast, the 70-kDa αI S6K1 isoform is predominantly cytosolic, but the 85-kDa αII isoform, which contains an N-terminal nuclear localization sequence, is nuclear (8). Finally, there are regions in the N- and C-terminal domains of S6K2 that lack homology to S6K1 (5).

S6K2 shares a conserved four domain organization with S6K1. Both contain a highly conserved core catalytic kinase domain and linker regulatory region. Interaction between regulatory N-terminal acidic and C-terminal basic domains is thought to maintain S6K1 in an inactive conformation in unstimulated cells. In this inactive state, a pseudosubstrate region in the C terminus may occlude the catalytic site. Phosphorylation of C-terminal proline-directed motifs is thought to disrupt the autoinhibitory interaction of the N- and C-terminal domains, exposing other critical regulatory sites in the linker and catalytic regions (9, 10). Three of the four C-terminal proline-directed sites, as well as other mitogen-stimulated regulatory phosphorylation sites identified in S6K1, are conserved in S6K2, suggesting that these related kinases may share similar activation mechanisms. Several studies have implicated MEK-dependent signals in regulation of the S6K1 C-
terminal proline-directed sites (11–13), which conform to the consensus motif for ERK phosphorylation sites (14).

We have recently determined that S6K1 and S6K2 are regulated similarly by effectors of the PI3-K pathway, including Cdc42, Rac, protein kinase Cζ, and PDK1 (15). Both kinases are also activated by overexpression of Akt/protein kinase B (7). Inhibition of S6K1 and S6K2 activity by rapamycin treatment of cells implicates them as effectors of the nutrient-sensitive mammalian target of rapamycin pathway (6). Despite these similarities, we recently reported a major difference in the role of the C-terminal domain in S6K2 regulation. Deletion of the C-terminal domain of S6K2 enhances basal kinase activity and renders S6K2 hypersensitive to activation by growth factors and PI3-K-regulated effectors (15). In contrast, deletion of the analogous region of S6K1 inhibits kinase activity and does not result in the dramatic potentiation of activation seen with S6K2. Here, we explore the roles of the unique features of the C-terminal region of S6K2 to address the mechanism of S6K2 regulation. We find that nuclear localization contributes to the lesser activity of S6K2 but is not the primary inhibitory influence on its activity. We report that the proline-directed phosphorylation sites are essential for activation of full-length S6K2 but not S6K1 and that these sites confer greater sensitivity of S6K2 to MEK-inhibition than S6K1.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Eukaryotic expression vectors encoding rat p70 S6K1 (HA-S6K1/pRK7) or human p54 S6K2 βRI (HA-S6K2/pcDNA3) under the control of the cytomegalovirus promoter have been described (6). Alignments of human S6K1 and S6K2 isoforms identifying primary sequence homology, domain junctions, and phosphorylation sites have been published (5, 15). HA-S6K plasmids were mutagenized using the Quik-Change polymerase chain reaction-based method (Stratagene). To generate HA-S6K2-ΔCT, a stop codon was introduced at amino acid 399. HA-S6K1-ΔCT and HA-S6K1-ΔSE mutants have been described (9). Plasmids encoding GST-Cdc42 mutants (16) and Myc-PDK1/pcDNA3 (17) have been described elsewhere.

Cell Culture and Transfection—HEK293E cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.2% fetal calf serum, and 200 ng/ml gentamycin. Cells were seeded at 4–6 × 10⁴ cells/ml into 100-mm dishes 2–3 days prior to calcium phosphate transfection with 2 µg of total DNA. Cells were washed with phosphate-buffered saline after 18–20 h and starved in serum-free Dulbecco’s modified Eagle’s medium for 24 h prior to 30 min of stimulation with 100 nM EGF. Cells were lysed as described, and protein expression levels were assayed by immunoblotting with anti-HA or -Myc antibodies. After Western blotting, lysates were normalized for HA-S6K2 expression levels prior to immune complex kinase assay. Activity of HA-S6K2 constructs is indicated in the top panel. An anti-HA Western blot of the immunoprecipitated kinase assay samples and an anti-Myc Western blot of whole cell lysate are shown in the bottom panel. Data are representative of three experiments.

RESULTS

The PI3-K and mammalian target of rapamycin pathways contribute to the mitogen-dependent activation of S6K1 and S6K2. These kinases are highly homologous overall but diverge in the N- and C-terminal domains. Because deletion of the C-terminal domain dramatically potentiates S6K2 activation (15), whereas truncation of S6K1 is inhibitory (9, 10), we sought to determine which S6K2 C-terminal motifs may mediate the inhibitory effect on the kinase.

Disruption of the Putative Nuclear Localization Signal Modestly Enhances S6K2 Activation by PI3-K Effectors—Because the C-terminal domain of S6K2 contains several features distinct from S6K1, we isolated the effects of these differences by mutagenesis. Two unique features of the S6K2 C terminus are the presence of a polyproline-rich domain and a nuclear localization sequence. Previous analysis of the polyproline-rich domain suggests that its deletion does not affect S6K2 regulation (5). However, mutation of a single amino acid in the putative nuclear localization signal within the context of full-length HA-S6K2 (K474M) results in cytoplasmic localization of the kinase in transfected HEK293 cells by immunolocalization (7). We assayed the ability of cotransfected Myc-PDK1 to activate the putative nuclear localization sequence point mutant HA-S6K2-K474M in HEK293 cells. This point mutant was activated to a greater extent than wild type HA-S6K2 by Myc-PDK1 (Fig. 1). However, this activity was intermediate.

FIG. 1. PDK1 regulation of HA-S6K2 wild type, K474M, and ΔCT. HEK293 cells were transfected with 1.0 µg of HA-S6K2 wild type (wt) or K474M or 2.0 µg of ΔCT in the pcDNA3 vector and 0.5 or 1.0 µg Myc-PDK1/pcDNA3 as indicated. Transfected cells were quiesced in serum-free medium for 24 h prior to 30 min of stimulation with 100 nM insulin. Cells were lysed as described, and protein expression levels were assayed by immunoblotting with anti-HA or -Myc antibodies. After Western blotting, lysates were normalized for HA-S6K2 expression levels prior to immune complex kinase assay. Activity of HA-S6K2 constructs is indicated in the top panel. An anti-HA Western blot of the immunoprecipitated kinase assay samples and an anti-Myc Western blot of whole cell lysate are shown in the bottom panel. Data are representative of three experiments.
Ser417, and Ser423) are conserved in S6K2. To determine the contribution of these putative phosphorylation sites to regulation of S6K2, we mutated these sites to aspartic acid (HA-S6K2-D3) to mimic phosphorylation or to alanine (HA-S6K2-A3) to prevent phosphorylation. We find that these sites play a major role in activation of S6K2, because HA-S6K2-D3 exhibits elevated basal and EGF- or insulin-stimulated activity similar to that of HA-S6K2-ΔCT in HEK293 cells (Fig. 2), whereas substitution of two of these sites to alanine (S417A, S423A) results in an inactive HA-S6K2-A2 mutant (Fig. 2A). Similarly, when all three sites are mutated to alanine, the HA-S6K2-A3 cannot be activated by EGF (see Fig. 7) or insulin stimulation (data not shown). These findings indicate that these C-terminal phosphorylation sites are essential for activation of the full-length kinase. This is surprising because mutation of the corresponding sites in S6K1 to alanine (A4) reduces kinase activity 5-fold, but the kinase retains mitogen-responsive activity (see Fig. 7) (19). Like HA-S6K2-ΔCT (15), HA-S6K2-D3 is hypersensitive to activation by insulin, PDK1, or Cdc42V12 (Fig. 3). Notably, the analogous acidic mutant of S6K1, HA-S6K1-D3E (S411D, S418D, T421E, and S424D), demonstrates elevated basal and insulin-stimulated activity but is not hypersensitive to Cdc42V12 or PDK1 (data not shown). In multiple experiments, HA-S6K2-ΔCT and -D3 mutants exhibit comparable activities, suggesting that these three sites may largely account for the inhibitory effect conferred by the intact C-terminal domain.

The Ser-Pro motifs in S6K2 conform to a consensus motif for ERK substrates (14), and S6K1 has been shown to be modestly inhibited by pharmacological inhibition of MEK activity (11, 12). To determine whether S6K2 activation requires this pathway, HEK293 cells transfected with HA-S6K2 or HA-S6K1 were treated with varying doses of the MEK inhibitor U0126 prior to stimulation with EGF. EGF-stimulated HA-S6K1 activity was modestly inhibited (0–30% inhibition, n = 4) by maximal doses (5–10 μM) of U0126 in multiple experiments (Fig. 4A; see also Fig. 7). By contrast, EGF-stimulation of HA-S6K2 was potently inhibited by U0126 in a dose-dependent manner, with 50–90% inhibition by maximal doses of U0126 in multiple experiments (Fig. 4); see also Figs. 6 and 7). HA-S6K2 is also potently inhibited by U0126 in U2OS cells (Fig. 4B). The greater sensitivity of S6K2 relative to S6K1 was also noted in assays of the endogenous kinases in HEK293 (93% inhibition of S6K2, 45% inhibition of S6K1; Fig. 5A) and U2OS cells (Fig. 5B), suggesting that MEK-regulated kinases may be more important for activation of S6K2 than S6K1.

To identify potential targets of the MEK pathway in S6K2, we assessed the effects of U0126 on the HA-S6K2 C-terminal...
The indicated dose of U0126 or Me2SO vehicle. Cells were then stimulated in serum-free medium for 24 h and then pretreated for 30 min with the absence (100%) or presence of the indicated doses of U0126.

The percentage of maximal EGF-stimulated HA-S6K activity in the S6K2 expression levels were confirmed by immunoblotting. Equal medium with 10% fetal bovine serum, cells were quiesced for 24 h in D

A conspicuous feature unique to the C terminus of S6K2 is the nuclear localization signal. Disruption of this basic sequence by a single point mutation (K474M) confers predominantly cytosolic expression to HA-S6K2-K474M as assayed by immunofluorescence (7). We demonstrate here that this mutant is more sensitive to activation by insulin or cotransfected PI3-K effectors. However, cytosolic localization alone does not account for the dramatic potentiation of the S6K2-DCT mutant, because HA-S6K2-DCT is significantly more active than HA-S6K2-K474M. Activation of wild type S6K2 by cytosolic proteins such as Cdc42 and PDK1 suggests that the kinase may exit the nucleus during the course of its activation. It is likely that cytosolic localization of the K474M mutant facilitates interaction with cytosolic S6K2 activators. However, as the nuclear localization sequence is rich in basic residues, it is also possible that disruption of the basic sequence itself may lessen the inhibitory potential of the C terminus.

The S6K2 proline-directed phosphorylation sites appear to play an essential role in relieving S6K2 autoinhibition. In fact, acidic substitution of these sites (S6K2-D3) results in a mutant that is regulated very similarly to S6K2-DCT, suggesting that phosphorylation of these sites is sufficient to disrupt C-terminal mediated inhibitory intermolecular or intramolecular interactions. The current model of S6K1 activation based on structure function analyses postulates that an interaction between basic residues in the C-terminal pseudosubstrate domain and an acidic region in the N terminus contributes to kinase autoinhibition, which is disrupted by mitogen-stimulated phosphorylation of the C-terminal proline-directed sites. The acidic residues are conserved between the S6K1 and S6K2 N termini, suggesting that this mechanism may also apply to S6K2. However, the kinases diverge completely between the end of the acidic region and beginning of the conserved catalytic domain. It is possible that the divergent N- and C-terminal regions of S6K2 may form a stronger autoinhibitory intramolecular interaction than occurs in S6K1, which may account for the diminished specific activity of S6K2 relative to

![Figure 4](image-url)

**FIG. 4.** HA-S6K3 is more sensitive than HA-S6K1 to inhibition by U0126. A, dose-dependent inhibition of HA-S6K2, but not HA-S6K1, by U0126 in HEK293 cells. HEK293 cells were transfected with 1.0 µg of HA-S6K2/pCNA3 or 0.5 µg of HA-S6K1/pRK7. Cells were quiescent in serum-free medium for 24 h and then pretreated for 30 min with the indicated dose of U0126 or Me2SO vehicle. Cells were then stimulated with 50 ng/ml EGF for 30 min and lysed as in Fig. 2. The top panel indicates the percentage of maximal EGF-stimulated HA-S6K activity in the absence (100%) or presence of the indicated doses of U0126. Western blots for anti-HA or anti-phospho-ERK1/2 (both quiescent and EGF-stimulated) are shown in the bottom panel. Data are representative of four experiments. B, dose-dependent inhibition of HA-S6K2 by U0126 in U2OS cells. U2OS cells were transfected with 2.0 µg of HA-S6K2/pCNA3 using LipofectAMINE. After overnight recovery in medium with 10% fetal bovine serum, cells were quiescent in serum-free media prior to treatment with the indicated doses of U0126 and 50 ng/ml EGF as above. Lysates were normalized for total protein content following Bradford assay (Bio-Rad reagent), and equal HA-S6K2 expression levels were confirmed by immunoblotting. Equal amounts of protein were subjected to immune complex kinase assay. The percentage of maximal EGF-stimulated HA-S6K2 activity in the absence (100%) or presence of the indicated doses of U0126 is presented. Data are representative of two experiments.

We have employed EGF in these studies because it is an agonist for both S6K and ERK activation. However, at physiological levels of insulin receptor, insulin is a potent activator of S6K1 and S6K2 but a poor agonist for ERKs (11). In contrast to EGF, we find that insulin-stimulated HA-S6K2 activity is insensitive to U0126 (Fig. 8), suggesting that MEK-independent signals relieve S6K2 autoinhibition in response to insulin. Thus, EGF and insulin activation of S6K2 may employ distinct signal transduction mechanisms.

**DISCUSSION**

Studies in mice lacking S6K1 reveal that S6K1 and S6K2 may mediate both common and nonredundant functions in vivo. S6K2 possesses unique regions of primary structure, as well as a primarily nuclear subcellular localization distinct from S6K1. Understanding the regulation of these related kinases may provide insight into the physiological functions unique to each kinase. We have recently demonstrated similarities and notable differences in the regulation of S6K1 and S6K2 by PI3-K pathway effectors. Of particular interest is the observation that the C-terminal domain of S6K2 mediates a potent inhibitory influence on kinase activity; deletion of the C terminus of S6K2 activates the kinase, whereas analogous deletion of S6K1 is inhibitory (15). In the present study, we dissect the roles of the distinct features of the S6K2 C terminus and conclude that the proline-directed phosphorylation sites are MEK-regulated and contribute to the major regulatory influence of this domain on S6K2 kinase activity.
S6K1. It is interesting to note that only three of the four mitogen-stimulated S6K1 proline-directed sites are conserved in human S6K2. It is attractive to speculate that the lesser mitogen-stimulated net negative charge because of three sites, in the context of a more basic C terminus (because of the basic nuclear localization signal) may render S6K2 more sensitive to autoinhibition via N- and C-terminal charge-dependent interaction. The enhanced sensitivity of S6K2-D3 to activation by PDK1 or Cdc42V12 relative to the corresponding S6K1-D3E mutant further supports a stronger influence of these C-terminal proline-directed sites in regulation of S6K2 than S6K1.

Our data suggest that different kinases may phosphorylate the C-terminal proline-directed sites in response to different upstream signals and that MEK-dependent kinases play a more critical role in regulation of S6K2 than S6K1. We demonstrate that S6K2 is more sensitive than S6K1 to MEK inhibition by U0126. Further, it is likely that the C-terminal Ser-Pro motifs may be targets of MEK-dependent kinases, because S6K2-D3 and S6K2-ΔCT are less sensitive to U0126 inhibition than wild type S6K2. Residual MEK dependence could potentially be mediated by the proline-directed S370 site, because the analogous S6K1 S371 site, which is phosphorylated by an unknown kinase, is essential for S6K1 activity (20). Interestingly, the residual EGF-stimulated activity of the HA-S6K1-A4 mutant is insensitive to U0126, suggesting that the C-terminal proline-directed motifs may be the major targets of the MEK pathway in S6K1 but that additional MEK-dependent inputs may regulate S6K2.

The kinases that regulate the C-terminal sites in S6K1 and S6K2 in vivo are not known, but ERK1/2 or cdc2 (M-phase
promoting factor) can phosphorylate the S6K1 C terminus in vitro (21). Previous studies have indicated that Ras and Raf are neither necessary nor sufficient for S6K1 activation by mitogens (22). However, other data suggest a role for MEK-dependent signals in S6K1 activation. Inducible Raf, although less potent than serum stimulation, was reported to activate S6K1 in an ERK-independent manner (13). Scott and Lawrence (11) have shown that ERK1/2 are not involved in insulin-stimulated phosphorylation of S6K1 or of the rapamycin-sensitive translational regulator 4E-BP1/PHAS-I (eIF-4E binding protein 1/phosphorylated heat- and acid-stable protein regulated by insulin) but that the MEK inhibitor PD098059 blunts insulin stimulation of these events. A recent study also implicates a role for basal MEK activity, independent of ERK1/2, for phosphorylation of S6K1, 4E-BP1/PHAS-I and stimulation of protein synthesis in response to insulin or phorbol ester (12). Based on our data, we hypothesize that in the presence of a strong PI3-K signal, MEK-independent kinases may be able to phosphorylate the S6K2 C terminus, whereas growth factors that are weaker PI3-K agonists may require MEK-dependent signals to stimulate C-terminal phosphorylation. Alternatively, insulin and EGF may activate other distinct pathways that influence the S6K2 C terminus.

**FIG. 6.** Activating C-terminal HA-S6K2 mutations reduce sensitivity to U0126. HEK293 cells were transfected with 1.0 μg of the indicated HA-S6K2 plasmid. Cells were serum-starved for 24 h and then pretreated with Me2SO or 5 μM U0126 for 30 min prior to 30 min of stimulation with 50 ng/ml EGF. Cells were lysed and subjected to immune complex kinase assay. Equivalent amounts of protein were expressed as assessed by anti-HA Western blotting. The maximal EGF-stimulated activity in the absence of U0126 for each construct was normalized to 100%. Data averaged from four experiments are reported as the percentages of maximal activity and S.E. of EGF-stimulated activity after U0126 treatment.

**FIG. 7.** Alanine mutation of the C-terminal proline-directed motifs results in greater inhibition of HA-S6K2 than HA-S6K1. HEK293 cells were transfected with 1.0 μg of HA-S6K2 wild type (wt) or 1.25 μg of A3 constructs in pcDNA3 or 0.5 μg of HA-S6K1 wild type or A4 mutant in pRK7. Cells were starved and stimulated with 50 ng/ml EGF as above and lysed for analysis by Western blotting and kinase assay. Activity of anti-HA immunoprecipitates is shown in the top panel. The anti-HA and anti-phospho-ERK1/2 Western blots are shown in the bottom panel. Data are representative of at least two experiments.

**FIG. 8.** Insulin-stimulated HA-S6K2 activity is insensitive to U0126. HEK293 cells were transfected with 1 μg of HA-S6K2/pcDNA3. Cells were quiesced and pretreated with 5 μM U0126 as above, prior to 30 min of stimulation with 100 nM insulin. Cells were lysed, and equal protein expression levels were verified by anti-HA Western blot prior to immune complex kinase assay. Data are presented as fold activation of HA-S6K2, mean and S.D., n = 2.
Deletion of the S6K1 gene by homologous recombination suggests that S6K1 and S6K2 may serve both distinct and common functions. Because S6 phosphorylation and 5′-terminal oligopyrimidine tract-containing mRNA translation are preserved in MEFs from S6K1-deficient mice (4), it is likely that both S6K1 and S6K2 may mediate these functions. The small size phenotype of these mice, however, suggests that S6K1 may have functions for which S6K2 cannot entirely compensate (4). The primarily nuclear localization of both S6K2 isoforms also suggests that S6K2 may have targets discrete from those of the cytosolic p70 S6K1 isoform. We have demonstrated similarities in the pathways that regulate S6K1 and S6K2, as well as notable differences, particularly in regulation of the C-terminal domain. Differential regulation by MEK signaling, along with discrete subcellular localization, may confer specificity toward potentially unique substrates of these S6 kinases.

Acknowledgments—We thank members of the Blenis laboratory and John Hwa for critical reading of this manuscript.

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