Regulation of Ribosomal S6 Kinase 2 by Effectors of the Phosphoinositide 3-Kinase Pathway*

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Kathleen A. Martin‡‡, Stefanie S. Schalm‡‡, Celeste Richardson‡, Angela Romaneli‡‡, Kristen L. Keon‡, and John Blenis‡‡††

From the ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 and the ‡Freie Universitat Berlin, Institut fur Biochemie, Thielallee 63, 14195 Berlin, Germany

Ribosomal S6 kinase (S6K1), through phosphorylation of the 40 S ribosomal protein S6 and regulation of 5′-terminal oligopyrimidine tract mRNAs, is an important regulator of cellular translational capacity. S6K1 has also been implicated in regulation of cell size. We have recently identified S6K2, a homolog of S6K1, which phosphorylates S6 in vitro and is regulated by the phosphatidylinositide 3-kinase (PI3-K) and mammalian target of rapamycin pathways in vivo. Here, we characterize S6K2 regulation by PI3-K signaling intermediates and compare its regulation to that of S6K1. We report that S6K2 is activated similarly to S6K1 by the PI3-K effectors phosphoinositide-dependent kinase 1, Cdc42, Rac, and protein kinase Cζ but that S6K2 is more sensitive to basal activation by myristoylated protein kinase Cζ than is S6K1. The C-terminal sequence of S6K2 is divergent from that of S6K1. We find that the S6K2 C terminus plays a greater role in S6K2 regulation than does the S6K1 C terminus by functioning as a potent inhibitor of activation by various agonists. Removal of the S6K2 C terminus results in an enzyme that is hypersensitive to agonist-dependent activation. These data suggest that S6K1 and S6K2 are similarly activated by PI3-K effectors but that sequences unique to S6K2 contribute to stronger inhibition of its kinase activity. Understanding the regulation of the two S6K homologs may provide insight into the physiological roles of these kinases.

The 70-kDa ribosomal S6 kinase 1 (S6K1) is a ubiquitously expressed serine/threonine protein kinase that phosphorylates the 40 S ribosomal protein S6 in response to mitogen stimulation (1). S6 phosphorylation up-regulates translation of mRNAs with 5′-terminal oligopyrimidine tracts, many of which encode ribosomal proteins and translation elongation factors (2). S6K1 activation thus up-regulates ribosome biosynthesis and enhances the translational capacity of the cell.

Deletion of S6K1 in Drosophila and mice has implicated S6K1 in regulation of cell size. The Drosophila knockout has a high incidence of embryonic lethality, but surviving flies exhibit a marked reduction in size that is cell autonomous (3). Mice lacking S6K1 through targeted disruption also exhibit a small animal phenotype (4). Interestingly, S6 phosphorylation and 5′-terminal oligopyrimidine tract mRNA translation were found to be normal in fibroblasts derived from mice lacking S6K1, suggesting a compensatory mechanism for these S6K1 functions. Our lab and others have recently identified S6K2, a homolog of S6K1 that phosphorylates S6 in vitro (4–7). Elevated S6K2 mRNA levels have been reported in the S6K1 knockout mice (4). Drosophila are thought to express only S6K1, which may account for the more severe S6K1 knockout phenotype in flies. S6K2 is a good candidate kinase that may supply some but not all of the functions of S6K1 in the knockout mouse, because the small animal phenotype persists despite the presence of S6K2, S6 phosphorylation, and 5′-terminal oligopyrimidine tract mRNA translation.

There are two isoforms of both S6K1 and S6K2 derived from alternative splicing at the N terminus. The p70S6K1α isoform is cytosolic, whereas p85 S6K1α is nuclear (8). In contrast, both isoforms of S6K2 (p54 S6K2βI, p60 S6K2βII) (5, 6) are primarily nuclear, because of the presence of a C-terminal putative nuclear localization signal sequence (NLS) (7) not found in S6K1. Point mutation of the putative NLS (K474M) results in cytosolic immunolocalization of S6K2βII (7). The S6K2 βI and βII isoforms may reside in distinct nuclear compartments based on subcellular fractionation studies (6).

S6K1 and S6K2 are highly homologous overall, with the greatest sequence homology in the kinase domain and adjacent regulatory linker domain. A schematic diagram of S6K1 and S6K2 outlining regions of homology, features unique to S6K2, and regulatory phosphorylation sites is provided in Fig. 1. Seven of eight mitogen-stimulated regulatory phosphorylation sites identified in S6K1 are conserved in human S6K2. There are interesting differences in S6K2 primary structure that may confer differential regulation and functions to this kinase. There are regions of sequence divergence between S6K1 and S6K2 in the N- and C-terminal domains. In addition to the nuclear localization signal, the C terminus of S6K2 also contains a proline-rich domain not found in S6K1.

In both S6K1 and S6K2, the conserved core catalytic and linker domains are flanked by regulatory N- and C-terminal domains. For S6K1, it is thought that interaction between
N-terminal acidic residues and C-terminal basic residues inhibits the kinase by allowing a C-terminal pseudosubstrate region to occlude the kinase domain. Mitogen-stimulated phosphorylation of the C-terminal Ser/Thr-Pro motifs is believed to disrupt these interactions, relieving autoinhibition of S6K1 and exposing other regulatory sites, including the major rapamycin-sensitive site, T389, and the catalytic activation loop site, Thr229 (9, 10). The PI3 kinase (PI3-K) and mTOR signaling pathways mediate multiple mitogen-stimulated phosphorylation events that lead to S6K1 activation. Consistent with the important roles of these pathways in S6K1 regulation, S6K1 activation is inhibited by pharmacological inhibitors of these pathways (11, 12). The immunosuppressant rapamycin, an inhibitor of mTOR, leads to rapid and complete dephosphorylation and inactivation of S6K1 (11). The role of mTOR in S6K1 activation may be suppression of an S6K1 phosphatase (13). There is also evidence suggesting that mTOR may directly phosphorylate S6K1 (14). S6K2 activation is also sensitive to rapamycin, and the analogous rapamycin-sensitive mitogen-stimulated S6K1 phosphorylation sites are conserved in S6K2 (Thr229, Ser410, Ser417, and Ser423) (4–7).

Multiple PI3-K effectors provide distinct inputs to S6K1 activation. Phosphoinositide-dependent kinase 1 (PDK1) is a constitutively active kinase whose access to many substrates is disrupted by preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18).

Preliminary studies using pharmacological inhibitors suggest that, like S6K1, S6K2 is regulated by the PI3-K and mTOR signaling pathways (4–7). We have shown that a constitutively active PI3-K p110 subunit activates S6K2 and that S6K2 is activated by PDK1 (6). Others have found that S6K2, like S6K1, can be regulated by the PI3-K effector Akt/PKB (7). However, further characterization of the signaling intermediates that regulate S6K2 has not yet been addressed. Given the differences in subcellular localization and primary sequence and the lack of complete functional redundancy between these homologs in the S6K1 knockout mice (4), we aimed to examine in detail the regulation of S6K2. Here, we report that although S6K2 is regulated similarly to S6K1 by PI3-K effectors, the C terminus of S6K2 exerts a more potent inhibitory effect on the kinase.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Mutagenesis**—Eukaryotic expression vectors encoding rat p70 S6K1 cII (HA-S6K1/pRK7) or human p54 S6K2 βII (HA-S6K2/pcDNA3) under the control of the cytomegalovirus promoter have been described (6). A schematic alignment of rat S6K1 cII and human S6K2 βII isoforms identifying domain junctions and phosphorylation sites is conserved in S6K2 (Thr229, Ser410, Ser417, and Ser423) (4–7).

Multiple PI3-K effectors provide distinct inputs to S6K1 activation. Phosphoinositide-dependent kinase 1 (PDK1) is a constitutively active kinase whose access to many substrates is disrupted by preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18). Our lab has also demonstrated the existence of preassembled complexes of PI3-K-regulated signaling molecules (18).
blotted using α-HA, α-GST (Santa Cruz), α-PKCζ (22), or α-Myc (22), and horseradish peroxidase-conjugated secondary antibody, and detected with enhanced chemiluminescence reagents.

**Immune Complex Kinase Assay**—One-third of total lysate was immunoprecipitated using α-HA antibody and protein A-Sepharose. Alternatively, volumes of lysate assayed were normalized to reflect S6K expression levels determined by Western blotting. Immunoprecipitates were washed with 1 ml each of buffer A (10 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg/ml leupeptin, and 5 μg/ml pepstatin, pH 7.2), buffer B (buffer A except with 0.1% Nonidet P-40 and 1M NaCl), and ST buffer (50 mM Tris-HCl, 5 mM Tris-base, 150 mM NaCl, pH 7.2). Kinase activity toward a recombinant GST-S6 peptide (32 final amino acids of ribosomal S6) in washed immunoprecipitates was assayed in a reaction containing 20 mM HEPES, 10 mM MgCl₂, 50 μM ATP unlabeled, 5 μCi of [γ-32P]ATP (PerkinElmer Life Sciences), 3 ng/μl PKI, pH 7.2, for 12 min at 30 °C. Reactions were subjected to 12% SDS-polyacrylamide gel electrophoresis, and the amount of 32P incorporated into GST-S6 was assessed by autoradiography and quantitated by phosphorimaging (Bio-Rad).

**RESULTS**

S6K2, like S6K1, is regulated by the PI3-K pathway. S6K2 is activated by constitutively active p110 PI3-K and inhibited when cells are treated with wortmannin (6). Wortmannin-sen-

tive phosphorylation sites found in S6K1 are conserved in S6K2 and S6K1. Multiple PI3-K pathway effectors have been implicated in S6K1 activation. Given the differences in primary structure and subcellular localization between S6K2 and S6K1, we sought to determine whether S6K2 is regulated by the same downstream PI3-K effectors known to regulate S6K1 and to investigate the roles of divergent S6K2 C-terminal sequences.

**Rho Family G Proteins Regulate S6K2**—Evidence suggests that PI3-K regulates the Rho family G proteins Cdc42 and Rac through activation of their guanine nucleotide exchange factors, such as Dbl and Vav (23). We have previously shown that Cdc42 and Rac regulate S6K1; cotransfection of GTPase-deficient constitutively active point mutants of Cdc42 and Rac (G12V) activates HA-S6K1, and dominant negative point mutants (T17N) with high GDP affinity (which sequester guanine nucleotide exchange factors) antagonize growth factor activation (20). Similarly, transient cotransfection of HEK293 cells with HA-S6K2 and GST-Cdc42V12 or GST-RacV12 enhanced basal (2–5-fold) and insulin-stimulated (2–3-fold) activity as measured in immune complex kinase assays (Fig. 2A). Consistent with a role for Rho family G proteins in
regulation of S6K2, cotransfection of HA-S6K2 with the dominant negative GST-Cdc42N17 mutant inhibits insulin stimulation of HA-S6K2, as well as HA-S6K1, activity (Fig. 2B). PDK1 activates S6K1 by phosphorylating Thr\(^{229}\) in the catalytic activation loop (15, 16). It is likely that Cdc42 contributes an S6K-activating function distinct from that of PDK1, because cotransfection of submaximally activating levels of Myc-PDK1 and GST-Cdc42V12 cooperatively activate S6K2. We demonstrate here for the first time that this is also the case with S6K1 (Fig. 3).

The mechanism by which Cdc42 activates S6K1 is not yet known, but Cdc42V12 and S6K1 coimmunoprecipitate and a mutation that prevents Cdc42 isoprenylation (C189S) fails to activate S6K1 (20). This suggests that membrane targeting of Cdc42 may be required. An attractive hypothesis is that association with Cdc42 may transiently target S6K1 to a cellular membrane in the course of its activation. This membrane targeting may be important for access to other membrane-associated S6K1 activators such as PDK1 and PKC\(_z\). Because S6K2 is thought to be primarily nuclear, it is notable that the cytosolic proteins Cdc42 and Rac can regulate this kinase. We determined that isoprenylation of GST-Cdc42V12 is required for this effect, because GST-Cdc42V12,C189S fails to activate HA-S6K2 when cotransfected (Fig. 4). These data suggest that despite localization primarily to the nucleus, S6K2 is regulated by cytosolic, isoprenylated low molecular weight G proteins. We hypothesize therefore that S6K2 may shuttle in and out of the nucleus and target to a membrane during the course of its activation.

**Atypical PKC\(_z\) Regulates S6K2—**

S6K1 associates with and is regulated by the atypical PKC\(_z\) (18). PKC\(_z\) is activated by binding PI3-K-derived phosphatidylinositol 3,4,5-trisphosphate and by interaction with and phosphorylation by PDK1 (22, 24). Although PKC\(_z\) is not sufficient to activate S6K1 under basal conditions, when coexpressed with PDK1, a strong S6K1 activation is observed (18). Coimmunoprecipitation of S6K1, PDK1, and PKC\(_z\) suggests their participation in a PI3-K-regulated signaling complex (18). To address whether PKC\(_z\) can regulate S6K2 in vivo, we cotransfected a constitutively active FLAG-tagged, myristoylated PKC\(_z\) (myr-PKC\(_z\)) construct with HA-S6K2, which resulted in basal and insulin-stimulated HA-S6K2 activation (Fig. 5A). There was a notable difference between S6K1 and S6K2, because HA-S6K1 was activated only modestly (up to 2-fold) by cotransfection of myr-PKC\(_z\) in quiescent cells (Fig. 5A), whereas HA-S6K2 was activated 5–30-fold under basal conditions (Fig. 5A), suggesting that S6K2 may be more sensitive to regulation by atypical PKCs. Further supporting a role for PKC\(_z\) in activation of HA-S6K2 was the observation that insulin-stimulated activity was inhibited by cotransfection of the dominant negative FLAG-PKC\(_z\)K281W (PKC\(_z\)K/W) (Fig. 5B), as is the case with S6K1 (18). In addition, HA-S6K2 is cooperatively activated by combined cotransfection of submaximally activating levels of myr-PKC\(_z\) and PDK1 (Fig. 6). Under these conditions,
the constitutively active myr-PKC\(\zeta\) is not further activated by PDK1 (22), but the modest overexpression of both activators results in synergistic activation of S6K2. Although both S6K1 and S6K2 are inhibited by dominant negative PKC\(\zeta\), only S6K2 basal activity is substantially activated by myr-PKC\(\zeta\) alone. These data provide the first evidence that S6K1 and S6K2 can be differentially regulated.

**C-terminal Truncation Potentiates S6K2 Activation—**Structure-function mutational analyses have provided important insight into regulation and activation of S6K1 (9, 10). We employed this approach to further study the regulation of S6K2. Because the C-terminal domain is a region of divergence between S6K1 and S6K2, we sought to determine the effect of deletion of this domain on regulation of S6K2. Amino acids 399–482 encoding the pseudosubstrate and proline-rich regions, as well as the NLS, were deleted from HA-S6K2 (Fig. 1), and the activity of the resulting mutant (HA-S6K2-D\(\text{CT}\)) was assayed in transfected HEK293 cells. Surprisingly, deletion of the C terminus resulted in enhanced basal and insulin-stimulated activity (Figs. 7 and 8). By contrast, the analogous truncation mutant of S6K1 is mitogen-regulated but is less active than the full-length kinase and is not more sensitive to insulin (9, 10) and (Fig. 8). The S6K2 C-terminal truncation did not significantly alter sensitivity of the kinase to wortmannin (Fig. 7B), suggesting that essential inputs from the PI3-K pathway are primarily integrated in regions upstream of amino acid 399.

Another marked difference was that basal and insulin-stimulated HA-S6K2-D\(\text{CT}\) activity is also more sensitive to activation by Cdc42V12 than HA-S6K2 wild type (Fig. 8). As with PDK1 activation, Cdc42V12 stimulation of the S6K2 C-terminal mutant is stronger than of the analogous S6K1 mutant under both starved and stimulated conditions (Fig. 8). These data demonstrate that the presence of the intact C-terminal domain exerts a more potent inhibitory influence on S6K2 than on S6K1, because deletion of the domain greatly facilitates S6K2 activation by insulin or PI3-K effectors.

There is a striking difference (10–25-fold) in the specific

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**Fig. 5. Atypical PKC\(\zeta\) regulates HA-S6K2.** A, myristoylated PKC\(\zeta\) activates HA-S6K2. HEK293 cells were transfected with 1.0 \(\mu\)g of HA-S6K2/pcDNA3 or 0.5 \(\mu\)g of HA-S6K1/prk7 and 1.0 \(\mu\)g of FLAG-myr-PKC\(\zeta\)/pCMV6. Cells were quiesced, insulin-stimulated, and lysed as in Fig. 2. HA-S6K activity is presented in the top panel. Anti-HA and anti-PKC\(\zeta\) Western blots are shown in the bottom panel. Data are representative of three experiments. B, HA-S6K2 activation is inhibited by dominant negative PKC\(\zeta\). HEK293 cells were transfected with 1.0 \(\mu\)g of HA-S6K2/pcDNA3 and 4.0 \(\mu\)g of FLAG-PKC\(\zeta\)K281W/pCMV as indicated. Cells were quiesced, insulin-stimulated, and lysed as in Fig. 2. HA-S6K2 activity is presented in the top panel. Anti-HA and anti-PKC\(\zeta\), Western blots are shown in the bottom panel. Data are representative of at least two experiments.
activity of S6K1 and S6K2. In our immune complex kinase assays, with equivalent protein expression levels, S6K2 is a significantly less active kinase toward GST-S6 or histone H2B substrates (Figs. 2B, 3, 5A, and 8 and Ref. 6). However, the insulin-stimulated specific activity of HA-S6K2-DCT upon co-transfection with Myc-PDK1 or Cdc42V12 was similar to the specific activity of wild type HA-S6K1 (Figs. 7A and 8). These data show that the intrinsic specific activity of the S6K2 kinase domain is not less than that of S6K1 but that S6K2 kinase activity is subject to repression \textit{in vivo}, mediated by the C-terminal domain. Thus, a second major difference between these closely related S6 kinases is the role of the C terminus in the activation process.

**DISCUSSION**

\textit{In vivo} studies suggest that S6K1 and S6K2 may serve both distinct and overlapping physiological functions, because it appears that S6K2 can only partly compensate in cells lacking S6K1 (4). The primarily nuclear localization of S6K2 (7) also suggests that S6K2 may serve unique functions. Given the likely functional differences, as well as regions of divergence in primary sequence, it is important to understand the upstream signaling pathways and intermediates that regulate each S6 kinase to coordinate these physiological responses. Our studies of the recently identified S6K1 homolog, S6K2, reveal similarities as well as differences in the regulation of these related kinases. Both are activated by common effectors of the PI3-K pathway, including Akt (7), Cdc42, Rac, PKC\(\zeta\), and PDK1 (this report). In addition, we demonstrate that regions of sequence divergence, particularly in the C terminus, dramatically influence the specific activity and growth factor activation of S6K2.

The PI3-K pathway is critical to activation of S6 kinases, because PI3-K inhibition with wortmannin or LY294002 potently inhibits activation of both S6K1 and S6K2 (4–7). Here, we show that PI3-K effectors known to participate in S6K1 activation also activate S6K2. In addition, Akt overexpression activates both S6K1 and S6K2 (7). Because the various PI3-K effectors examined here exhibit distinct patterns of cytosolic, membrane, and nuclear localization, it is surprising that all were implicated in S6K2 activation. Because there is evidence that Akt and PKC\(\zeta\) can translocate to the nucleus in stimulated cells (25, 26), it is reasonable to hypothesize that these kinases may regulate S6K2, which is primarily nuclear. However, Cdc42, Rac, and PDK1, thought to be cytosolic and associated with membranes (21, 27), are potent activators of S6K2. It is likely that this regulation is not merely due to overexpression of these proteins, because inhibition of endogenous Cdc42 or PDK1 by expression of dominant negative mutants inhibits S6K2 (this report and Ref. 6). We therefore hypothesize that S6K2 may shuttle between the nucleus and the cytosol during the course of its activation. We and others do not detect a stable

![Fig. 6. PKC\(\zeta\) and PDK1 cooperatively activate HA-S6K2.](image)

**Fig. 6.** PKC\(\zeta\) and PDK1 cooperatively activate HA-S6K2. HEK293 cells were transfected with 1.0 \(\mu\)g of HA-S6K2/pcDNA3 and 1.0 \(\mu\)g of FLAG-myr-PKC\(\zeta\)/pCMV6 and/or 0.5 \(\mu\)g of Myc-PDK1/pcDNA3 as indicated. Cells were quiesced, insulin-stimulated, and lysed as in Fig. 2. HA-S6K2 activity is presented in the top panel. Anti-HA, -PKC\(\zeta\), and -Myc Western blots are shown in the bottom panel. Data are representative of three experiments.

![Fig. 7. C-terminal truncation potentiates HA-S6K2 activation.](image)

**Fig. 7.** C-terminal truncation potentiates HA-S6K2 activation. A, enhanced basal and PDK1 or insulin-stimulated activity of HA-S6K2-DCT. HEK293 cells were transfected with 1.0 \(\mu\)g of HA-S6K2 wild type (wt) or 2 \(\mu\)g of HA-S6K2-DCT (DCT) in the pcDNA3 vector and 1.0 \(\mu\)g of Myc-PDK1/pcDNA3 as indicated. Cells were serum-starved, insulin-stimulated, and lysed as in Fig. 2. Lysates were normalized for HA-S6K2 expression levels after Western blotting, and kinase activity was assayed. Data are representative of at least two experiments. B, HA-S6K2 wild type and HA-S6K2-DCT are sensitive to wortmannin. HEK293 cells were transfected with 1.0 \(\mu\)g of HA-S6K2 wild type (wt) or 2 \(\mu\)g of HA-S6K2-DCT in the pcDNA3 vector. Cells were serum-starved for 24 h and then pretreated with 100 nm wortmannin or vehicle for 30 min prior to a 30-min stimulation with 100 nm insulin. Cells were lysed and subjected to immunoblotting and kinase assay as in Fig. 2. Activity and anti-HA Western blots are shown. Data are representative of two experiments.
change in subcellular localization in HA-S6K2 by immunofluorescence in quiescent versus growth factor-stimulated cells (data not shown and Ref. 7), suggesting that any cytosolic translocation may be rapid and transient and/or involve levels of protein below the range of detection of this method. Such nuclear/cytosolic shuttling models have been suggested for other signaling proteins and kinases including Ste5, MEK, and extracellular signal-regulated kinase (28, 29). Furthermore, our data suggest that S6K2 may transiently associate with a cytosolic membrane during the activation process, as a preylation-deficient Cdc42 mutant does not associate with membranes fails to activate S6K2. Additionally, all of the PI3-K effectors implicated in S6K2 regulation can associate with cellular membranes in growth factor-stimulated cells (23).

One difference we have identified in PI3-K-mediated regulation of full-length S6Ks is that S6K2 is more sensitive to activation by PKCζ than is S6K1. Because the mechanism by which PKCζ activates S6K1 is not yet understood, why the effect may be stronger on S6K2 is uncertain. The ability of PKCζ to translocate to the nucleus suggests a potential mechanism (26). We have previously shown that PKCζ activates S6K1 lacking the C-terminal MEK-dependent sites (18). Similarly, HA-S6K2-D3, in which C-terminal MEK-dependent sites are replaced with aspartic acid residues, is activated by PKCζ (data not shown). These data suggest that PKCζ-mediated extracellular signal-regulated kinase activation (30–32) is not a likely mechanism for preferential HA-S6K2 versus S6K1 regulation. In addition, we do not detect activation of extracellular signal-regulated kinase 1/2 by transfection of myr-PKCζ in our experiments (data not shown and Ref. 18).

We report here a significant difference in the role of the C-terminal domain in regulation of S6K1 and S6K2. Both contain a pseudosubstrate region with high homology to S6. Autokinase of S6K1 by this pseudosubstrate domain is thought to be relieved by phosphorylation of four proline-directed sites (Ser41, Ser42, Ser442, and Ser444) within this region. Deletion of the C-terminal domain of S6K1 results in a kinase with activity slightly lower than that of full-length S6K1, which is still sensitive to activation by mitogens and inhibition by wortmannin (9, 10). By contrast, the analogous C-terminal truncation of S6K2 is also sensitive to wortmannin but confers elevated basal activity and hypersensitivity to mitogens or PI3-K effectors. Thus, the greatly reduced specific activity of S6K2 relative to S6K1 appears to be mediated in part by the S6K2 C terminus, because HA-S6K2-ΔCT can be activated by PDK1 and growth factors to levels similar to S6K1 activity.

The C termini of S6K1 and S6K2 are highly homologous (73% identity) in the pseudosubstrate region. However, there is only 25% sequence identity between the kinases from the end of this region to the extreme C terminus (S6K2 βII amino acids 429–482) (5). It is likely that sequences within this divergent region account for the particularly strong inhibition of S6K2. Of note, this region of S6K2 encodes a polyproline-rich domain not found in S6K1. This domain could potentially interact with SH3 domain containing proteins that may confer unique regulation to S6K2. However, Gout et al. (5) cite unpublished data indicating that selective deletion of this proline-rich domain does not alter activity of S6K2. It is not known whether the effects of coexpressed PDK1 or other PI3-K effectors might be greater on this mutant, as is the case for S6K2-ΔCT.

One hypothesis for the enhanced activity of the S6K2-ΔCT mutant is that deletion of the C-terminal NLS and consequent cytosolic localization facilitates S6K2 activation. Immunofluorescence studies indicate that this mutant is in fact localized to the cytosol (data not shown). We have recently examined the roles of the unique S6K2 C-terminal features in an accompanying study (33). We report that disruption of the unique S6K2 C-terminal NLS by point mutation potentiates S6K2 activation but is not responsible for the dramatic effects observed upon truncation of the entire C-terminal domain. Instead, we find that MEK-dependent regulation of three C-terminal proline-directed phosphorylation sites is the critical regulatory influence on this domain.

PI3-K-derived lipid messengers mediate signals affecting the critical processes of cell growth and size, proliferation, and survival. We demonstrate here that despite distinct patterns of subcellular localization and divergences in primary sequence, S6K1 and S6K2 are regulated similarly by effectors of the PI3-K pathway. However, we also identify differences in activity and regulation of these related kinases, such as reduced specific activity of S6K2 compared with S6K1, greater S6K2 sensitivity to atypical PKCζ, and a differential C-terminal domain regulatory mechanism. The phenotype of the mice lacking S6K1 through homologous recombination suggests that S6K1 and S6K2 share common as well as nonredundant functions. Our regulatory data further support the possibility that these related kinases may regulate both common and distinct cellular substrates and functions. Differential response to down-
stream effectors of common pathways, along with discrete subcellular localization, may confer specificity toward potentially unique substrates of these S6 kinases.

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REFERENCES

1. Dufner, A., and Thomas, G. (1999) Exp. Cell Res. 253, 100–109
2. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) EMBO J. 16, 3693–3704
3. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999) Science 283, 2126–2129
4. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S. C. (1998) EMBO J. 17, 6649–6659
5. Gout, I., Minami, T., Hara, K., Tsujishita, Y., Filonenko, V., Waterfield, M. D., and Yonezawa, K. (1998) J. Biol. Chem. 273, 30061–30064
6. Lee-Fruman, K. K., Kuo, C. J., Lippincott, J., Terada, N., and Blenis, J. (1999) Oncogene 18, 5108–5114
7. Koh, H., Jee, K., Lee, B., Kim, J., Kim, D., Yun, Y. H., Kim, J. W., Choi, H. S., and Chung, J. (1999) Oncogene 18, 5115–5119
8. Reinhard, C., Thomas, G., and Kozma, S. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4052–4056
9. Cheatham, L., Monfort, M., Chou, M. M., and Blenis, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11696–11700
10. Weng, Q., Andrahi, K., Kozlowski, M. T., Grove, J. R., and Avruch, J. (1995) Mol. Cell. Biol. 15, 2333–2340
11. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) Cell 69, 1227–1236
12. Chung, J., Grammer, T., Lemon, K., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
13. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4438–4442
14. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., and Sabatini, D. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1432–1437
15. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Merrise, N., and Avruch, J. (1998) Curr. Biol. 8, 69–81
16. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1999) Science 279, 707–710
17. Balendran, A., Currie, R., Armstrong, C. G., Avruch, J., and Alessi, D. R. (1999) J. Biol. Chem. 274, 37400–37406
18. Romanelli, A., Martin, K. A., Toker, A., and Blenis, J. (1999) Mol. Cell. Biol. 19, 2921–2928
19. Akimoto, K., Nakaya, M., Yamanaka, T., Tanaka, J., Matsuda, S., Weng, Q. P., Avruch, J., and Ohno, S. (1998) Biochem. J. 335, 417–424
20. Chou, M. K., and Blenis, J. (1996) Cell 85, 573–583
21. Coghill, M. P., Chou, M. M., and Carpenter, C. L. (2000) Mol. Cell. Biol. 20, 2880–2889
22. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C.-S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) Curr. Biol. 8, 1069–1077
23. Leevers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) Curr. Opin. Cell Biol. 11, 219–225
24. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2042–2045
25. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 31515–31524
26. Mizukami, Y., Hirata, T., and Yoshida, K. (1997) FEBS Lett. 401, 247–251
27. Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Dowes, C. P., Cohen, P., Alessi, D. R., and Lucocq, J. (1999) Biochem. J. 347, 575–583
28. Mahanty, S. K., Wang, Y., Farley, F. W., and Elion, E. A. (1999) Cell 98, 501–512
29. Adachi, M., Fukuda, M., and Nishida, E. (2000) J. Cell Biol. 148, 849–856
30. Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sanchez, P., Sanz, L., and Moscat, J. (1995) EMBO J. 14, 6157–6163
31. van Dijk, M. C., Hilkmann, H., and van Blitterswijk, W. J. (1997) Biochem. J. 325, 303–307
32. Schonwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, P. J. (1998) Mol. Cell. Biol. 18, 790–798
33. Martin, K. A., Schalm, S. S., Romanelli, A., Keon, K. L., and Blenis, J. (2001) J. Biol. Chem. 276, 7882–7888