Characterization of a Conserved C-terminal Motif (RSPRR) in Ribosomal Protein S6 Kinase 1 Required for Its Mammalian Target of Rapamycin-dependent Regulation*

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The mammalian target of rapamycin, mTOR, is a Ser/Thr kinase that promotes cell growth and proliferation by activating ribosomal protein S6 kinase 1 (S6K1). We previously identified a conserved TOR signaling (TOS) motif in the N terminus of S6K1 that is required for its mTOR-dependent activation. Furthermore, our data suggested that the TOS motif suppresses an inhibitory function associated with the C terminus of S6K1. Here, we have characterized the mTOR-regulated inhibitory region within the C terminus. We have identified a conserved C-terminal “RSPRR” sequence that is responsible for an mTOR-dependent suppression of S6K1 activation. Deletion or mutations within this RSPRR motif partially rescue the kinase activity of the S6K1 TOS motif mutant (S6K1-F5A), and this rescued activity is rapamycin resistant. Furthermore, we have shown that the RSPRR motif significantly suppresses S6K1 phosphorylation at two phosphorylation sites (Thr-389 and Thr-229) that are crucial for S6K1 activation. Importantly, introducing both the Thr-389 phosphomimetic and RSPRR motif mutations into the catalytically inactive S6K1 mutant S6K1-F5A completely rescues its activity and renders it fully rapamycin resistant. These data show that the N-terminal TOS motif suppresses an inhibitory function mediated by the C-terminal RSPRR motif. We propose that the RSPRR motif interacts with a negative regulator of S6K1 that is normally suppressed by mTOR.

The mammalian target of rapamycin, mTOR, is a conserved Ser/Thr kinase that integrates signals from nutrients (such as amino acids), energy sufficiency, and growth factors to coordinate the regulation of cell growth and cell cycle progression (reviewed in Refs. 1 and 2). The best-characterized function of mTOR is the regulation of translation initiation via the ribosomal protein S6 kinases 1 and 2 (S6K1 and S6K2) and the translational inhibitors, the eukaryotic initiation factor 4E-binding proteins (4E-BP1/2/3) (3, 4). Studies in Drosophila and mouse revealed that the S6 kinases play a critical role in the regulation of cell growth and development (1, 2). Consistent with the S6K knock-out experiments, RNA interference-mediated reduction of S6K1 expression reduces cell size, whereas overexpression of S6K1 increases cell size (5, 6).

Mitogen- and mTOR-dependent pathways coordinately regulate the activity of S6K1 by phosphorylation of at least 8 Ser/Thr residues (reviewed in Ref. 4). The phosphoinositide 3-kinase pathway is a major mitogen-dependent contributor of S6K1 activation, which is mediated by a variety of effectors, including Akt/protein kinase B, 3-phosphinositide-dependent kinase 1, protein kinase Cζ, and the small G proteins Cdc42 and Rac1 (reviewed in Ref. 4). Direct phosphorylation of S6K1 at Thr-229 by 3-phosphinositide-dependent kinase 1 is a crucial step in S6K1 activation. mTOR-dependent signaling is absolutely required for S6K1 activation. For instance, treatment of cells with the specific mTOR inhibitor, rapamycin, causes rapid dephosphorylation of S6K1 at several sites, namely Thr-389, Thr-229 and Ser-404 (7, 8), and leads to S6K1 inactivation even when mitogen-activated S6K1 regulators remain unaffected. Thr-389, a phosphorylation site situated within a hydrophobic amino acid motif in the linker region, is the most rapamycin-sensitive site, and its phosphorylation is crucial for S6K1 phosphotransferase activity.

We previously identified a conserved TOR signaling (TOS) motif in the N terminus of S6K1 and in the C terminus of 4E-BP1 that is required for mTOR-dependent S6K1 and 4E-BP1 phosphorylation (9, 10). This TOS motif facilitates the association of S6K1 and 4E-BP1 to the mTOR regulator raptor (regulatory associated protein of mTOR) and their efficient in vitro phosphorylation by the mTOR-raptor complex (10, 11, 12). We showed that a single point mutation in the TOS motif (S6K1-F5A) prevented the mTOR signaling input to S6K1 (9). Mimicking Thr-389 phosphorylation by mutating this site to an acidic residue (S6K1-F5A-E389) partially rescued the activity of this inactive, mTOR-insensitive mutant (S6K1-F5A). A combination of the Thr-389 phosphomimetic mutant with a C-terminal deletion (F5A-E389ΔCT401) rescued the inhibitory effect of the TOS motif mutant and rendered it rapamycin resistant (9). These data indicated that the TOS motif mediates mTOR-dependent S6K1 activation by at least two mechanisms: direct phosphorylation of the hydrophobic motif site Thr-389 and suppression of an inhibitory activity associated with the C terminus of S6K1 (9).
Here, we have identified a conserved sequence in the C terminus of S6K1 (RSPRR) that mediates an inhibitory effect on enzyme activity. Deletion of or mutation within the conserved C-terminal RSPRR motif partially rescued a catalytically inactive TOS motif mutant (S6K1-F5A) and rendered S6K1 partially rapamycin resistant. Furthermore, a combination of an RSPRR motif mutation and mimicking of Thr-389 phosphorylation completely rescued the activity of S6K1-F5A and generated a mutant of S6K1 that was completely rapamycin resistant. We propose that the RSPRR motif interacts with a negative regulator of S6K1 that is suppressed by mTOR.

MATERIALS AND METHODS

Plasmids and Mutagenesis—The HA-S6K1 (wild-type, HA-S6K1-ΔCT401, HA-S6K1-F5A, HA-S6K1-F5A-ΔCT401, and HA-S6K1-F5A-E389ΔCT401) constructs were generated as previously described (9). Point mutations were introduced using the QuikChange PCR method (Stratagene). C-terminal truncation S6K1 mutants were generated by introducing a premature stop codon into the coding sequence of S6K1.

Antibodies—Anti-HA antibodies were kindly provided by M. Chou (University of Pennsylvania, Philadelphia). The anti-S6K1-phospho-Thr-389 and -Thr-229 antibodies were obtained from Cell Signaling Inc. (Beverly, MA) and R&D Systems (Minneapolis, MN), respectively. The Ser-371 antibody was described previously (9).

Cell Culture, Transfection, and Western Blotting—HEK293E cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (vol/vol) heat-inactivated fetal bovine serum, 20 units/ml penicillin, and 200 ng/ml streptomycin. Ca3PO4 transfections were performed as previously described (13). After treatment, cells were harvested and Western blot analysis was carried out as before (13).

Immune Complex Kinase Assay—For immunoprecipitation studies of HA-tagged S6K1, cell extracts were immunoprecipitated with anti-HA antibodies bound to protein A-Sepharose (Amersham Biosciences) for 3 h. Immunoprecipitates were washed as described previously (13). S6K1 kinase activity was determined in vitro by using recombinant GST-S6 (32 C-terminal amino acids of ribosomal protein S6) as a substrate as described previously (13). Quantification of incorporation of the [32P] label was quantified by phosphorimaging (Bio-Rad) with ImageQuant software.

RESULTS

Characterization of the C-terminal Inhibitory Region of S6K1—Deletion of the S6K1 C terminus (S6K1-ΔCT401) or the S6K2 C terminus (S6K2-ΔCT401) renders these kinases partially rapamycin resistant (Refs. 14 and 15, and data not shown), suggesting an mTOR-regulated, inhibitory activity associated with the C terminus (discussed in Schalm and Blenis, Ref. 9). To identify the region in the C terminus responsible for this inhibitory effect, we generated a series of progressively shorter C-terminal truncation mutants (see Fig. 1A) and assayed their kinase activities under starved, insulin-stimulated, or rapamycin-treated conditions (Fig. 1B). In contrast to the partial rapamycin-resistant S6K1-ΔCT401 mutant, C-terminal truncation mutants at amino acids 417 (S6K1-ΔCT417) and 422 (S6K1-ΔCT422) were completely sensitive to rapamycin (Fig. 1B). These results suggest that the region between amino acids 401 and 417 possesses an mTOR-antagonized inhibitory activity. In the presence of rapamycin, this inhibitory function is no longer suppressed and S6K1 activation is therefore prevented.

Phosphorylation of Ser-404 Does Not Regulate the Inhibitory Function of the C Terminus—This 16-amino acid region within the C terminus contains two interesting features: a rapamycin-sensitive phosphorylation site, Ser-404, and a highly conserved 5-amino acid motif (RSPRR) (see Fig. 1A). A second partially conserved SPR motif (amino acids 417–420, Fig. 1A) seemed to be dispensable for this inhibitory effect, as its presence (S6K1-ΔCT422) or absence (S6K1-ΔCT417) did not affect the rapamycin sensitivity of S6K1 (see Fig. 1B).

To determine whether Ser-404 phosphorylation contributed to the inhibitory property of the C terminus, Ser-404 was mutated to Asp to mimic phosphorylation or to Ala to prevent phosphorylation. When compared with wild-type protein, mutation of Ser-404 to Asp or Ala did not alter the rapamycin sensitivity of S6K1 (Fig. 2), making it unlikely that Ser-404 phosphorylation mediates the inhibitory function.

FIG. 1. Characterization of the C-terminal inhibitory effect of S6K1. A, C-terminal sequence alignment of S6Ks. Identical (black box) and conserved (shadowed box) amino acids are highlighted. Truncation mutants are indicated. Conserved RSPRR and SPR sequences are boxed. The C-terminal sequences were aligned using the CLUSTAL W algorithm. B, HEK293 cells were transfected with HA-S6K1-ΔCT417 (ΔCT417), HA-S6K1-ΔCT422 (ΔCT422), HA-S6K1 wild-type (WT), or HA-S6K2-ΔCT401 (ΔCT401). Transfected cells were serum starved, pretreated with rapamycin (Rap) (20 ng/ml) for 30 min, and stimulated with 100 nM insulin (Ins) for 30 min, and lysed as described under “Materials and Methods.” Protein expression levels were assayed by immunoblotting with anti-HA-antibodies, and kinase assays were performed as described.
Ser-404 phosphorylation does not regulate the inhibitory function of the C terminus. HEK293 cells were transfected with HA-S6K1 wild-type (WT), HA-S6K1-S404A (S404A), or HA-S6K1-S404D (S404D). Transfected cells were serum starved, pretreated with rapamycin (Rap) (20 ng/ml) for 30 min, and lysed as described under “Materials and Methods.” Protein expression levels were assayed by immunoblotting with α-HA antibodies, and kinase assays were performed as described.

C-terminal Deletions from Amino Acids 401 or 409 Rescue the Kinase Activity of the S6K1 TOS Motif Mutant S6K1-F5A—The very weak insulin-stimulated activity of the S6K1 TOS motif mutant S6K1-F5A was partially restored (to 5–15% of wild-type S6K1) upon deletion of the C terminus (S6K1-F5A-ΔCT401) (Fig. 3A) (9). To identify the inhibitory region within the C terminus, we examined which of the C-terminal truncation mutants rescued the kinase activity of S6K1-F5A (Fig. 3A). Deletion of the extreme 92 C-terminal amino acids (ΔCT409) partially rescued the inhibitory effect of the TOS motif mutant, similar to that of F5A-ΔCT401. The rescued kinase activity of S6K1-F5A-ΔCT409 was completely insensitive to rapamycin, as observed for F5A-ΔCT401. In contrast, deletion of the extreme 83 C-terminal amino acids (F5A-ΔCT417) did not rescue the inhibitory effect of the TOS motif mutant S6K1-F5A. These data indicate that the amino acid sequence between residues 409 and 417 mediates the mTOR-regulated inhibitory effect on S6K1 kinase activity. It is possible that the C-terminal region between residues 409 and 417 inhibits phosphorylation of S6K1 at an mTOR-regulated site. Two rapamycin-sensitive phosphorylation sites important for S6K1 activation (Thr-229 and Thr-389) were phosphorylated in F5A-ΔCT417, a mutant possessing the conserved RSPRR motif (Fig. 3C). These data suggest that the S6K1 C-terminal region between residues 409 and 417 is required for the mTOR-dependent phosphorylation of Thr-229 and Thr-389. Thr-389 phosphorylation was insulin stimulated but rapamycin resistant in F5A-ΔCT401 and F5A-ΔCT409 (Fig. 3C), indicating that Thr-389 phosphorylation in these mutants was regulated by an insulin-dependent, but mTOR-independent (rapamycin-insensitive), input. Similarly, Thr-229 phosphorylation in F5A-ΔCT401 and F5A-ΔCT409 was partially rescued (when compared with F5A-S6K1) and was rapamycin resistant (Fig. 3C). However, Thr-229 phosphorylation in these mutants was already basally high and not further increased upon insulin stimulation. Thus, the C-terminal sequence between residues 409 and 417 appears to mediate an inhibitory effect on the basal phosphorylation of Thr-229. In contrast, the rapamycin-resistant phosphorylation site Ser-371 (9) was phosphorylated in F5A-ΔCT401 and F5A-ΔCT409 and to a lesser extent in F5A-ΔCT417. As reported before (9), phosphorylation of Ser-371 was not increased upon insulin stimulation and not affected by rapamycin treatment under our conditions. Thus, the RSPRR motif-mediated inhibition of S6K1 kinase seems to be mainly the result of suppression of Thr-229 and Thr-389 phosphorylation.

Mimicking Thr-389 Phosphorylation within the F5A-ΔCT (409) Mutant Renders S6K1 Highly Active and Rapamycin Resistant—As shown previously, mimicking Thr-389 phosphorylation by mutating this residue to a glutamic acid weakly rescued the insulin-stimulated activity of the S6K1-F5A. In contrast, a combination of mimicking Thr-389 phosphorylation and deletion of the C terminus (S6K1-F5A-E389ΔCT401) completely overcame the inhibitory effect of the F5A mutation and rendered S6K1 constitutively active and rapamycin resistant (Fig. 4) (9). To extend our characterization of the C terminus, we determined whether C-terminal truncations from residue 409 or residue 417 rescued S6K1 phosphotransferase activity in the context of the S6K1-F5A-E389 mutant. Indeed, truncation of the C terminus from amino acid 409 in the F5A-E389 mutant (referred to as S6K1-F5A-E389ΔCT401) completely rescued the kinase activity of S6K1-F5A in a manner similar to that of S6K1-F5A-E389ΔCT401. In contrast, truncation of the C terminus from amino acid 417 failed to rescue the kinase activity of S6K1-F5A-E389 (Fig. 4). Thus, in addition to Thr-389 phosphorylation, deletion of a negative regulatory region between amino acids 409–417 is needed to rescue the inhibitory effect caused by mutation of the TOS motif (F5A). Both S6K1-F5A-E389ΔCT401 and S6K1-F5A-E389ΔCT409 have a high basal Thr-229 phosphorylation, which is resistant to rapamycin and not further stimulated by insulin. Interestingly, phosphorylation of Thr-229 in these two S6K1 truncation mutants was significantly higher than that observed with wild-type S6K1 or S6K1-F5A-E389. Thus, the C-terminal S6K1 region between amino acids 409 and 417 is important for
modulating Thr-229 phosphorylation, which is at least partially independent of Thr-389 phosphorylation.

Alanine Point Mutations within the Conserved RSPRR Motif Rescue the Activity of S6K1 TOS Motif Mutant S6K1-F5A—To determine whether the RSPRR motif mediates the mTOR-modulated inhibitory function of the S6K1 C terminus, we asked whether mutations within this motif rescued the activity of S6K1-F5A analogous to the C-terminal deletion (S6K1-ΔCT401). Mutating either the three arginines or all the residues within the RSPRR motif to alanines (referred as 3A or 5A, respectively) partially rescued the inhibitory effect of the TOS motif mutant (Fig. 5). In contrast to F5A-ΔCT401 or F5A-ΔCT409 (Fig. 3B), however, the rescued activity of this mutant was only partially rapamycin resistant. We also noted that the insulin-stimulated activity of S6K1-F5A-3A and S6K1-F5A-5A was higher than that observed with F5A-ΔCT401 and F5A-ΔCT409 (Fig. 5). The alanine mutations of the RSPRR in the context of the TOS motif mutant increased the insulin-stimulated phosphorylation of Thr-229 and Thr-389 over that observed in the S6K1-F5A mutant, indicating that the RSPRR motif is important for the regulation of Thr-229 and Thr-389 phosphorylation. Furthermore, under non-stimulated conditions Thr-229 phosphorylation was high in S6K1-F5A-3A and S6K1-F5A-5A, consistent with the notion that the RSPRR motif affects basal phosphorylation of Thr-229. The insulin-stimulated phosphorylation of Thr-389 in S6K1-F5A-3A and S6K1-F5A-5A was still rapamycin sensitive. These results suggest that other regions within the C terminus may also be important for regulating insulin-stimulated and rapamycin-sensitive Thr-389 phosphorylation.

Mimicking Thr-389 Phosphorylation Renders the F5A-3A Mutant Fully Rapamycin Resistant with High Specific Activity—Finally, we wanted to determine whether mimicking Thr-389 phosphorylation rendered the RSPRR motif mutant S6K1-F5A-3A (referred to as S6K1-F5A-E389–3A) constitutively active and fully rapamycin resistant (Fig. 6). Indeed, S6K1-F5A-E389–3A had a high basal kinase activity that was not further stimulated by insulin and was completely rapamycin resistant, making it indistinguishable from the S6K1-F5A-E389ΔCT401 mutant. Moreover, phosphorylation of Thr-229 in S6K1-F5A-E389–3A was also completely rapamycin insensitive. These data suggest that the RSPRR motif, in the context of phosphorylated Thr-389, also directly contributes to the regulation of Thr-229 phosphorylation. Unlike Thr-389 phosphorylation, which may be directly phosphorylated by mTOR as well as indirectly regulated via the RSPRR motif, regulation of Thr-229 is not likely to be directly phosphorylated by mTOR (4).

DISCUSSION

The mechanism of how mTOR signaling regulates multisite S6K1 phosphorylation and activation is poorly understood. Recently, we identified a conserved TOS motif in the N terminus of S6K1 (9) that is crucial for S6K1 binding to and regulation by the mTOR-raptor complex (10, 11, 12). Mutating the S6K1 TOS motif (F5A) dramatically reduced S6K1 kinase activity and phosphorylation at Thr-389 and Thr-229 (9). However, mimicking Thr-389 phosphorylation by mutating this site to an acidic residue only partially rescued the activity of the TOS motif mutant S6K1-F5A (9), indicating that Thr-389 phosphorylation was not the only TOS motif-mediated input for S6K1 activation. Only a combination of mimicking Thr-389 phosphorylation and deletion of the C-terminal domain (F5A-E389ΔCT) completely overcame the inhibitory effect of the F5A mutation and rendered this S6K1 mutant fully active and rapamycin resistant (9). In contrast to deletion of the C terminus, mimicking C-terminal phosphorylation was not sufficient to bring the activity of S6K1-F5A-E389 up to wild-type levels (9).
data suggested that the TOS motif suppressed an inhibitory activity associated with the C terminus that was not due to C-terminal phosphorylation.

Here, we have characterized this inhibitory function of the S6K1 C terminus. We show that a C-terminal deletion at amino acid 409, but not amino acid 417, rendered S6K1 partially rapamycin resistant and rescued the activity of the catalytically inactive TOS motif mutant (S6K1-F5A) (Figs. 1A and 3A). Thus, the region between amino acids 409 and 417 within the C terminus mediates an mTOR-dependent inhibitory function. Interestingly, this S6K1 C-terminal region contains an RSPRR motif, which is the only conserved sequence within the C terminus of all known S6 kinases (Fig. 1A). We show that mutating residues within this RSPRR motif to alanines partially rescued the kinase activity of S6K1-F5A (Fig. 5). Moreover, deletion or mutation of the RSPRR motif in combination with mimicking Thr-389 phosphorylation completely rescued the inhibitory effect of the TOS motif mutant (Fig. 6).

How does the RSPRR motif affect S6K1 activation? The three phosphorylation sites, Thr-229, Thr-389, and Ser-404, still present in the S6K1 C-terminal truncation mutants are known to be rapamycin sensitive (7, 8). Conflicting data exist about the rapamycin sensitivity of a fourth site, Ser-371 (16, 17). We did not detect rapamycin sensitivity of this site in our cell systems (Fig. 3C) (9). Phosphorylation of Ser-404 does not appear to be important for the regulatory function of the RSPRR motif, as mutating Ser-404 to Asp to mimic phosphorylation did not affect the activity or rapamycin sensitivity of S6K1 (Fig. 2) (7, 14).

Phosphorylation of Thr-389 is influenced by the RSPRR motif, as insulin-stimulated Thr-389 phosphorylation was increased in the S6K1 TOS motif mutants (S6K1-F5A) lacking an intact RSPRR motif (S6K1-F5A-3A, S6K1-F5A-5A, S6K1-F5A-ΔCT401, and S6K1-F5A-ΔCT409 (Fig. 2B) (4), but not in the S6K1 TOS motif mutants with an intact RSPRR motif (S6K1-F5A and S6K1-F5A-ΔCT417). Thus, the RSPRR motif prevents Thr-389 phosphorylation in the TOS motif mutant (S6K1-F5A). In addition to the effect of RSPRR mutations on S6K1 Thr-389 phosphorylation, basal phosphorylation of Thr-229 is also significantly elevated and rapamycin resistant in these S6K1 mutants (Fig. 5A) (6). Consistently, deletion of the S6K1 C terminus has been reported to cause high basal Thr-229 phosphorylation (18). 3-Phosphoinositide-dependent kinase 1 is known to phosphorylate Thr-229, but its activity is insensitive to rapamycin. Our data suggest that the RSPRR motif negatively modulates Thr-229 phosphorylation. It is possible that this negative regulation may involve a phosphatase. Several lines of evidence suggest that a PP2A (protein phosphatase 2A)-like phosphatase mediates mTOR-dependent regulation of S6K1 and 4E-BP1. In Saccharomyces cerevisiae, TOR regulation of several substrates involves PP2A-like phosphatases. Furthermore, PP2A has been shown to form a complex with S6K1 (19) and to bind to wild-type S6K1, but not to the rapamycin-resistant N- and C-terminal truncated form of S6K1 (20). It is likely that a PP2A-like phosphatase binds to the C terminus, possibly to the RSPRR motif. To date we have been unable to reproducibly detect PP2A association with S6K1, but ongoing studies are aimed at addressing this possibility.

Summary—We have identified a highly conserved motif (RSPRR) within the C terminus of S6K1 that mediates an inhibitory effect on mTOR-dependent S6K1 activation. Without a positive mTOR input, the RSPRR motif suppresses S6K1 phosphorylation at Thr-389 and Thr-229. We anticipate that future work will reveal the identity of the RSPRR-associated negative regulator. Importantly, the full-length rapamycin-resistant S6K1 mutant (S6K1-F5A-E389–3A) described in this study will be a useful tool to study rapamycin-sensitive signaling through S6K1. For instance, defining S6K1 function in cultured cells is problematic as overexpression of S6K1 with an intact TOS motif inhibits mTOR-raptor signaling to its other TOS motif-containing targets (e.g. 4E-BP1). As a result, overexpression of S6 kinases indirectly interferes with eukaryotic initiation factor 4E function, complicating any interpretation of data using this experimental approach (21).

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