Mutational analysis of ribosomal S6 kinase 2 shows differential regulation of its kinase activity from that of ribosomal S6 kinase 1

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Ribosomal S6 kinase 2 (S6K2) is a serine/threonine kinase identified as a homologue of p70 ribosomal S6 kinase 1 (S6K1). S6K1 and S6K2 show different cellular localization as well as divergent amino acid sequences in non-catalytic domains, suggesting that their cellular functions and/or regulation may not be identical. Many of the serine/threonine residues that become phosphorylated and contribute to S6K1 activation are conserved in S6K2. In this study we carry out mutational analyses of these serine/threonine residues on S6K2 in order to elucidate the mechanism of S6K2 regulation. We find that Thr-228 and Ser-370 are crucial for S6K2 activity, and the three proline-directed serines in the autoinhibitory domain, Ser-410, Ser-417 and Ser-423, play a role in S6K2 activity regulation in a mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK)-dependent manner. However, unlike S6K1, changing Thr-388 to glutamic acid in S6K2 renders the kinase fully active. This activity was resistant to the effects of rapamycin or wortmannin, indicating that mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K) regulate S6K2 activity via Thr-388. MEK-dependent phosphorylation of the autoinhibitory serines in S6K2 occurs prior to Thr-388 activation. Combining T388E and T228A mutations inhibited S6K2 activation, and a kinase-inactive phosphoinositide-dependent protein kinase (PDK1) diminished T388E activity, suggesting that the role of Thr-388 is to allow further phosphorylation of Thr-228 by PDK1. Thr-388 fails to become phosphorylated in Ser-370 mutants, suggesting that the role of Ser-370 phosphorylation may be to allow Thr-388 phosphorylation. Finally, using the rapamycin-resistant T388E mutant, we provide evidence that S6K2 can phosphorylate S6 *in vivo*.

Key words: mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase 1 (S6K1), phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent protein kinase (PDK1), ribosomal S6 kinase 2 (S6K2).

INTRODUCTION

Phosphorylation of the 40 S ribosomal protein S6 is one of the events that occur during cell proliferation in many different cell types under various mitogenic conditions. S6 phosphorylation results in up-regulation of translation of mRNA species that contain 5′-terminal oligopyrimidine tract, many of which encode proteins that are themselves involved in translation [1]. This in turn increases the cell's protein synthesis capability in preparation for cell proliferation. p70 Ribosomal S6 kinase 1 (S6K1), previously known as p70 S6 kinase, is a serine/threonine kinase responsible for S6 phosphorylation. The importance of S6K1 in cellular proliferation was underscored when it was discovered that a potent mitogenic inhibitor and immunosuppressant rapamycin might exert its function by inhibiting the activity of S6K1 [2–6]. However, rapamycin does not inhibit S6K1 directly; rather, rapamycin binds FK506-binding protein 12 (FKBP12), and this complex in turn binds and inhibits mammalian target of rapamycin (mTOR) [7]. mTOR plays a role as a nutrient sensor in cellular proliferation, and it controls not only S6K1 but also other proteins, including initiation factor 4E-binding protein [8–10]. Interestingly, cells derived from mice lacking S6K1 showed no defect in cellular proliferation and no defect in S6 phosphorylation *in vivo* [11]. These data suggest that there may exist additional kinase(s) that can substitute for S6K1. This has led to identification and cloning of ribosomal S6 kinase 2 (S6K2) [11–15].

S6K2 was cloned based on its nucleotide sequence homology to S6K1, and its cellular function and *in vivo* substrate(s) are not yet established. Initial studies on S6K2 showed that it can phosphorylate S6 *in vitro*, and that upstream kinases that have been shown to regulate the catalytic activity of S6K1, such as phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent protein kinase (PDK1), mTOR, Cdc42, Rac and protein kinase C ζ, may also play a role in S6K2 regulation [11–16]. The current hypothesis is that S6K1 and S6K2 may have common as well as distinct functions in cells. This is based on several lines of evidence. Although mouse embryonic fibroblasts from S6K1-knockout mice show normal proliferation and normal S6 phosphorylation *in vivo*, the mice had a small-body phenotype due to defects in cell size and growth regulation [11]. This suggests that S6K2 may be able to carry out some but not all of the functions of S6K1 in the absence of S6K1. Amino acid sequence comparison between the two kinases shows diverging sequences in non-catalytic regions, hinting at differential function and/or regulation. S6K1 has two isoforms that arise from alternative translational start sites that differ by 23 amino acids in the N-terminus (αI and αII) [17,18]. The nuclear localization signal for S6K1 resides in this 23 amino acid N-terminal portion, and therefore the αII isoform is found mainly in the cytoplasm whereas the longer αI isoform resides in the nucleus [19,20]. S6K2 also has two isoforms with alternative translational start sites (βI and βII), but the nuclear localization signal for S6K2 is in the C-terminal portion of the protein and therefore both isoforms

Abbreviations used: S6K2, ribosomal S6 kinase 2; S6K1, p70 ribosomal S6 kinase 1; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent protein kinase; MEK, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase; EGF, epidermal growth factor; HA, haemagglutinin; DTT, dithiothreitol; Ab, antibody.

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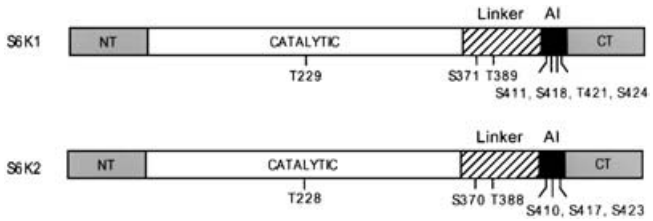

Figure 1 Sequence comparison of S6K1 and S6K2

The domains of the two kinases and the equivalent phosphorylation sites are shown. NT, N-terminal domain; AI, autoinhibitory domain; CT, C-terminal domain.

are found in the nucleus [12–15]. A recent study suggests that mTOR shuttling to and from the nucleus is important for S6K2 activation [21]. In addition, S6K2 contains a stretch of proline-rich sequences in the C-terminus that is not found in S6K1, a region that could potentially play a role in protein–protein interaction. These findings suggest that S6K2 may have a different subset of cellular substrate(s) and different function from that of S6K1, and/or that the catalytic activity of the two kinases may be regulated somewhat differently.

S6K1 is activated by a series of serine and threonine phosphorylation in various domains of the kinase [1]. S6K1 can be divided into five different domains; they are the N-terminal domain, catalytic domain, the linker region, autoinhibitory domain and the C-terminal domain (Figure 1). There are at least 11 different serine/threonine phosphorylation sites on S6K1 identified to date. Of these, seven have been demonstrated as important for S6K1 activity regulation, and sequential phosphorylation of these sites results in full activation of S6K1 [1]. The current model of S6K1 activation [1] is that the four proline-directed serine/threonine sites in the autoinhibitory domain (Ser-411, Ser-418, Thr-421 and Ser-424), so named because they are each followed immediately by a proline, become phosphorylated first. This allows access of upstream kinase(s) that phosphorylate Thr-389 and Ser-371 in an mTOR-dependent manner. A recent study suggests that NEK6/7 (NIMA-related kinases 6/7) may be responsible for Thr-389 phosphorylation [22]. Phosphorylation of these two sites is thought, in turn, to allow access by PDK1 that phosphorylates Thr-229 in the activation loop, thereby activating the kinase fully.

Of these seven major phosphorylation sites on S6K1, six of them are conserved in S6K2 (Figure 1). Of the four proline-directed sites in the autoinhibitory domain, three are conserved in human S6K2 (Ser-410, Ser-417 and Ser-423, equivalent to Ser-411, Ser-418 and Ser-424 in S6K1). The three other major phosphorylation sites, Thr-228, Thr-388 and Ser-370 (equivalent to Thr-229, Thr-389 and Ser-371 in S6K1) are also conserved in S6K2. We report that these six conserved residues are indeed important sites for S6K2 activation. We find that Thr-388 plays a major role in regulating S6K2, a greater role than the equivalent Thr-389 plays in S6K1 regulation, and that Thr-388 phosphorylation allows subsequent phosphorylation of Thr-228. The role of Ser-370 seems to be to allow phosphorylation of Thr-388. The mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK) pathway, which has been indicated to play a role in S6K2 activity regulation, does not play a direct role in Thr-388 activation. Our data show that, although S6K2 is regulated by mTOR and PI3K, as is the case with S6K1, the patterns of activity regulation for S6K1 and S6K2 are different. We also show evidence that S6K2 can be an *in vivo* kinase for the ribosomal protein S6.

EXPERIMENTAL

Mutagenesis

The template construct for all mutation was pCDNA3-HA-S6K2, which is the full-length βII S6K2 cDNA cloned into the pCDNA vector that contains haemagglutinin (HA) epitope tag sequences upstream of the multiple cloning site. Point mutations on target sites were made on pCDNA3-HA-S6K2 using Transformer (Clontech) or QuikChange (Stratagene) site-directed mutagenesis methods. Oligonucleotides designed to introduce the desired alanine, aspartic acid or glutamic acid mutations were used as mutagenic primers. Successful mutations were confirmed by DNA sequencing.

Cell culture and transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum, penicillin (250 units/ml), streptomycin (250 μg/ml) and L-glutamine (292 μg/ml). The cells (1×10^6 cells/60 mm plate) were transiently transfected with wild-type or mutant S6K2 constructs (12 μg of total DNA) using the calcium phosphate precipitation method for 6 h, followed by a PBS wash and recovery in the medium described above.

Cellular activation and immunoprecipitation

Post-transfection (24 h), the cells were synchronized in serum-free Dulbecco's modified Eagle's medium for 24 h. The following day, two experimental groups were prepared. The first group of cells transfected with wild-type and mutated S6K2 were activated by addition of fetal calf serum (20 %) for 20 min at 37 °C, whereas the second (control) group also expressing wild-type or mutated S6K2 remained inactivated at 37 °C. All cells were then washed with PBS and cell lysates were prepared by incubating cells in cell lysis buffer for 20 min [92 mM K_2HPO_4, 30 mM KH_2PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, pH 7.2, 0.5 % Igepal CA-630, 0.1 % Brij 35, 1 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 40 μg/ml PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin] followed by centrifugation at 14000 ***g*** for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody (Ab) and protein A–Sepharose beads (Amersham Biosciences). When inhibitors were used, the transfected cells were incubated with rapamycin (20 ng/ml), wortmannin (50 nM), U0126 (5 μM) or appropriate vehicle for 20 min prior to serum stimulation.

In vitro kinase assay

The *in vitro* kinase assay, also known as immune complex kinase assay, for S6 kinases has been described previously [16,23,24]. Briefly, HA immunoprecipitates above were washed once in buffer A (1 % Igepal CA-630, 0.5 % sodium deoxycholate, 100 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 40 μg/ml PMSF), once in buffer B (1 M NaCl, 0.1 % Igepal CA-630, 10 mM Tris, pH 7.2, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 40 μg/ml PMSF) and then once in buffer ST (150 mM NaCl, 50 mM Tris/HCl, pH 7.2, 50 mM Tris-base, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 40 μg/ml PMSF). The washed beads were incubated with kinase assay reaction mix {20 mM Hepes, 10 mM $MgCl_2$, 100 μg/ml BSA, 100 μM DTT, 50 μM unlabelled ATP, 10 μCi [γ-^{32}P]ATP

(ICN), 3 ng/μl protein kinase inhibitor and 1 μg GST-S6} at 30 °C for 15 min. The exogenous substrate for the reaction was GST-S6, which is a recombinant glutathione S-transferase fusion protein that contains the last 32 amino acids of S6, including the phosphorylated serine residues. The reaction mixture was then boiled for 3 min with sample buffer and separated via SDS/PAGE (12% gel). The gel was stained with Coomassie Brilliant Blue, dried and exposed to autoradiographic film to visualize the degree of S6 phosphorylation. The level of phosphate incorporation on to S6 was quantified using a Phosphorimager (BioRad).

Immunoblotting

Immunoblotting was performed by separating samples via SDS/PAGE (8% gel), transferring the proteins on to nitrocellulose membrane (Schleicher & Schuell) and incubating with appropriate primary Abs [anti-(phospho S6K1 Thr-389) Ab and anti-(phospho S6) Ab, Cell Signalling Technology; or anti-HA Ab], followed by horseradish peroxidase-conjugated Abs to the primary Ab species (Amersham Biosciences). The immunoblots were visualized by enhanced chemiluminescence (Pierce).

RESULTS

Thr-228 is crucial for S6K2 activity

Thr-229 on S6K1 is thought to be the last residue to become phosphorylated before full activation of S6K1 kinase activity [1]. Phosphorylation of the proline-directed serines/threonine in the autoinhibitory region followed by phosphorylation of Thr-389 and Ser-371 is thought to allow access of PDK1, a constitutively active kinase, to the Thr-229 site on S6K1 [25,26]. Mutation of Thr-229 on S6K1 to either alanine or aspartic acid inhibits S6K1 activation, indicating that this residue is crucial to S6K1 activity [27]. We mutated its equivalent site on S6K2, Thr-228, to either alanine, which removes the possibility of phosphorylation at this site, or glutamic acid, which has been shown to often mimic constitutive phosphorylation, in order to study the role of Thr-228 phosphorylation on S6K2 activity. The wild-type or T228A mutant were expressed in HEK-293 cells, and following serum-stimulation the expressed proteins were immunoprecipitated using anti-HA Ab and their kinase activities detected by *in vitro* kinase assay for S6 kinases [23,24]. Figure 2 shows that the T228A mutant showed a lack of inducible catalytic activity (Figure 2A). The T228E mutant also had basal-level catalytic activity when stimulated with serum (Figure 2C). This indicates that Thr-228 is indeed a crucial residue for S6K2 activation.

Ser-370 is an important residue for S6K2 activation

We assessed the role of Ser-370 in S6K2 activation. Ser-371 on S6K1 was identified as a rapamycin-sensitive site, and mutation of this residue to either alanine or aspartic acid inhibited S6K1 activation, highlighting the importance of this site [28]. A recent study suggested that Ser-371 phosphorylation regulates Thr-389 phosphorylation on S6K1 [29]. We mutated its equivalent site on S6K2, Ser-370, to either alanine or aspartic acid and assessed catalytic activity. Figures 3(A) and 3(C) show that changing this residue to either amino acid greatly diminished S6K2 activation by serum, suggesting that this residue is important in S6K2 regulation. S371A mutation of S6K1 has been shown to abolish Thr-389 phosphorylation, indicating that Thr-389 phosphorylation relies upon phosphorylation of Ser-371 [28,29]. In order to determine if Ser-370 of S6K2 similarly regulates Thr-388 phosphorylation, we made use of anti-(phospho S6K1 Thr-389) Ab. Since this Ab is a rabbit polyclonal Ab, and the sequences surrounding Thr-388 of S6K2 are similar to those around Thr-389 in S6K1, we tested to see if this Ab would cross-react with phosphorylated Thr-388 of S6K2. When we immunoprecipitated and immunoblotted wild-type S6K2, the anti-(phospho S6K1 Thr-389) Ab detected the phosphorylated Thr-388 site of S6K2 following serum stimulation (Figure 3E). Immunoblotting S370A or S370D with the same Ab showed that Thr-388 is not phosphorylated in Ser-370 mutant backgrounds, indicating that if Ser-370 activation is prevented by mutation, Thr-388 fails to become phosphorylated. This suggests that Ser-370 regulates phosphorylation of Thr-388 of S6K2.

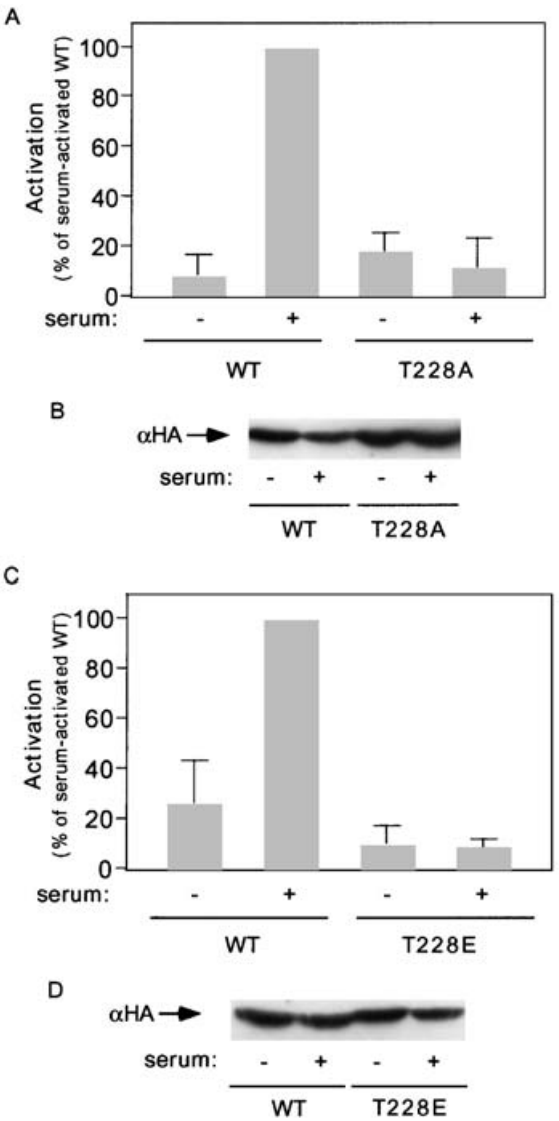

Figure 2 The role of Thr-228 in S6K2 activation

(**A**) HEK-293 cells that were transfected with HA-tagged wild-type S6K2 (WT) or T228A were stimulated with serum, lysed and anti-HA immunoprecipitation was carried out to isolate the transfected kinases. Wild-type or T228A catalytic activity was then determined via *in vitro* kinase assays in which recombinant S6 was used as exogenous substrate. The degree of radioactive phosphate incorporation on to S6 was quantified by Phosphorimager. The graph shown represents data from multiple experiments that were normalized by setting the serum-activated wild-type kinase activity level at 100%. (**B**) Cell lysates from the cells expressing wild-type or T228A were immunoblotted with anti-HA Ab to show equivalent protein expression. (**C**) Cells expressing wild-type or T228E were stimulated with serum and the kinase activity of wild-type or T228E was assayed as in (**A**). (**D**) Cell lysates from cells expressing wild-type or T228E were immunoblotted with anti-HA Ab to show equivalent protein expression.

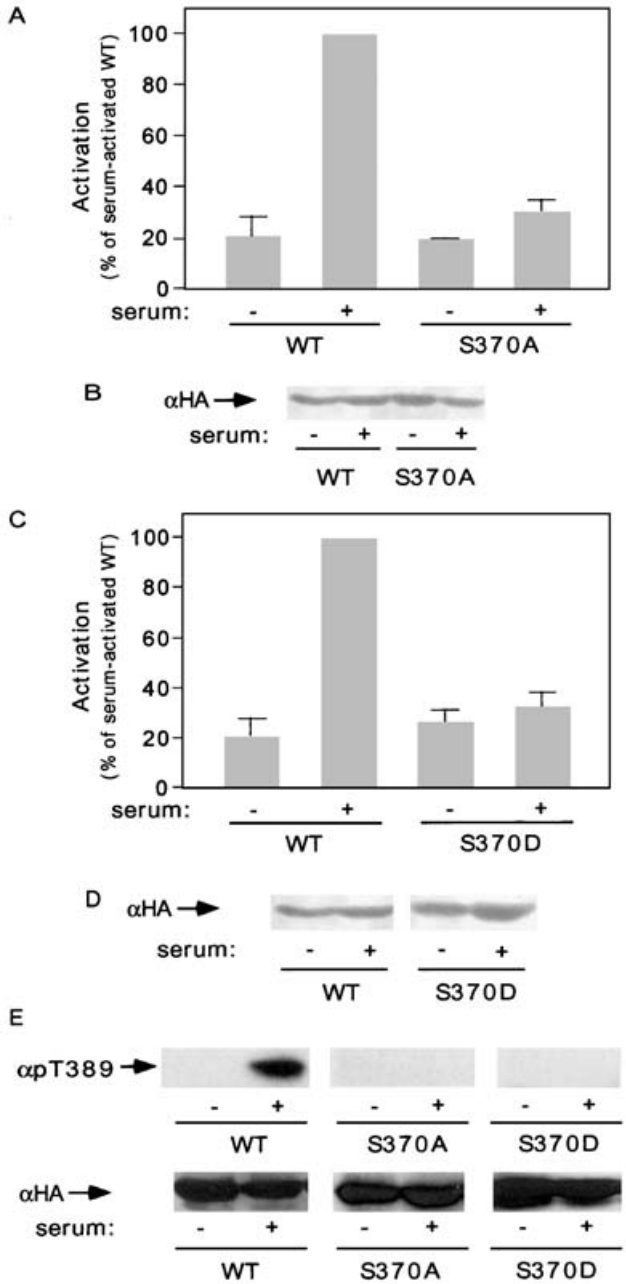

Figure 3 The role of Ser-370 in S6K2 activation

(**A**) Cells expressing wild-type (WT) or S370A were stimulated with serum and the kinase activities were assayed as in Figure 2(A). (**B**) Cell lysates from the cells expressing wild-type or S370A were immunoblotted with anti-HA Ab to show equivalent protein expression. (**C**) Cells expressing wild-type or S370D were stimulated with serum and the kinase activities were assayed as in Figure 2(A). (**D**) Cell lysates from cells expressing wild-type or S370D were immunoblotted with anti-HA Ab to show equivalent protein expression. (**E**) Cells that were transfected with wild-type, S370A or S370D were stimulated with serum and anti-HA Ab immunoprecipitates were separated by SDS/PAGE and immunoblotted using polyclonal anti-(phospho S6K1 Thr-389) Ab (top panel). Cell lysates were also immunoblotted with anti-HA Ab to show protein-expression levels (bottom panel).

Autoinhibitory domain mutant shows slightly higher activity

We next assessed the importance of the three proline-directed serine residues in the autoinhibitory domain, Ser-410, Ser-417 and Ser-423. We generated point mutations of these three residues to aspartic acid to mimic phosphorylation of these sites. The

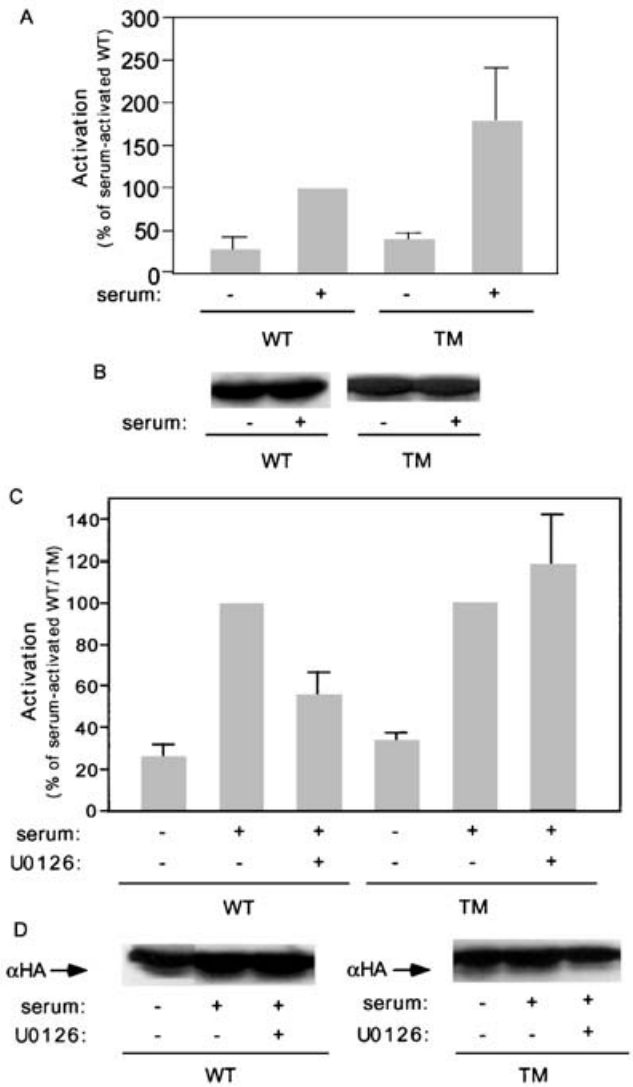

Figure 4 The role of proline-directed serines in S6K2 activation

(**A**) Cells expressing wild-type (WT) or TM (triple mutation) mutant S6K2 were stimulated with serum and the kinase activities were assayed as in Figure 2(A). (**B**) Cell lysates from the cells expressing wild-type or TM were immunoblotted with anti-HA Ab to show equivalent protein expression. (**C**) Cells that were transfected with wild-type or TM mutant were treated with U0126 MEK inhibitor prior to serum stimulation and *in vitro* kinase assays were performed as in Figure 2(A). U0126 itself did not have an effect on the unstimulated wild-type or TM S6K2 activity (results not shown). The graph represents data from multiple experiments that were normalized by setting the serum-activated wild-type (for wild-type samples) or TM (for TM samples) kinase activity level as 100 %. (**D**) Cell lysates from the cells expressing wild-type or TM for (**C**) were immunoblotted with anti-HA Ab to show equivalent protein expression.

mutation of the equivalent residues in S6K1 shows slightly higher basal activity of the kinase, although it was still activated further by serum stimulation [30]. This mutant, termed TM for triple mutation, showed enhanced activity over that of wild-type S6K2 when stimulated by serum (Figure 4A). Martin et al. [31] have reported that their similar S6K2 mutant (termed D3) showed slightly higher basal activity that is further enhanced by addition of epidermal growth factor (EGF) or insulin [31]. These data suggest that phosphorylation of the serines in the autoinhibitory domain potentiates partial activation of S6K2 that is further enhanced by additional phosphorylation at additional sites by other kinases. Martin et al. [31] also showed that their D3 mutant was resistant to the effects of MEK inhibitor U0126 when activated by EGF,

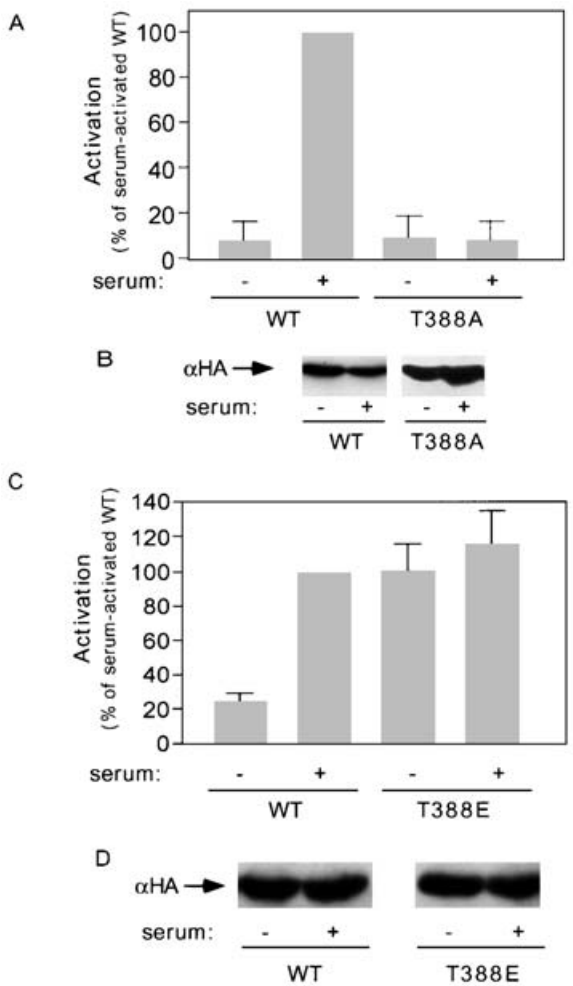

Figure 5 The role of Thr-388 in S6K2 activation

(**A**) Cells expressing wild-type (WT) or T388A were stimulated with serum and the kinase activities were assayed as in Figure 2(A). (**B**) Cell lysates from the cells expressing wild-type or T388A were immunoblotted with anti-HA Ab to show protein-expression levels. (**C**) Cells expressing wild-type or T388E were stimulated with serum and their kinase activities were assayed as in Figure 2(A). (**D**) Cell lysates from cells expressing wild-type or T388E were immunoblotted with anti-HA Ab to show equivalent protein expression.

but not insulin. Our data show that U0126 inhibits the activation of wild-type S6K2, but serum activation of the TM mutant was resistant to U0126 treatment (Figure 4C), indicating that activation of autoinhibitory serines in S6K2 by serum is also in part mediated by the MEK pathway.

Thr-388 plays a key role in S6K2 activation

We next assessed the role of Thr-388, the residue equivalent to Thr-389 in S6K1. This is one of the major sites that are dephosphorylated when cells are treated with rapamycin, and wortmannin has been also shown to affect phosphorylation of this site [27,32]. It is thought that this may be the site where mTOR and PI3K signalling pathways converge to regulate S6K1 activity. We substituted Thr-388 on S6K2 with either alanine or glutamic acid. T388A mutation inhibits activation of S6K2 upon serum stimulation, which is similar to what was seen when similar mutation was introduced to S6K1 (Figure 5A) [27]. However, T388E mutation showed a surprising difference between S6K1 and S6K2. When Thr-388 was mutated to glutamic acid, this alone was sufficient to raise the basal activity level to that of serum-stimulated wild-type S6K2 (Figure 5C). This high basal activity of T388E was unchanged or modestly enhanced following serum stimulation. This was unexpected since T389E mutation of S6K1 raises the basal activity only slightly and exhibits decreased serum-stimulated catalytic activity level [27]. Only when the serine and threonine mutations in the autoinhibitory domain are combined with the T389E mutation does one begin to see enhanced basal activity of S6K1 [27]. Our data suggest that Thr-388 phosphorylation plays a greater role in activation of S6K2 than seen in S6K1, and that phosphorylation at this residue may be sufficient to allow full activation of S6K2.

We next tested to see if mTOR- and PI3K-dependent activation of S6K2 is mediated by Thr-388. When cells transfected with T388E were treated with rapamycin or wortmannin, T388E did not lose its catalytic activity (Figure 6A), indicating that mutation of this site now rendered the kinase independent of mTOR and PI3K. Martin et al. [31] have shown that the MEK pathway plays a role in the C-terminal autoinhibition mechanism of S6K2. We tested to see whether the MEK pathway plays a role in Thr-388-mediated activation of S6K2. Figure 6(C) shows that T388E is resistant to U0126, a MEK inhibitor, indicating that the input of the MEK pathway on S6K2 activation occurs prior to Thr-388 phosphorylation.

The role of Thr-388 is to allow phosphorylation of Thr-228

It has been shown in S6K1 that phosphorylation of the C-terminal serine/threonine sites in the autoinhibitory domain results in phosphorylation of Ser-371 and Thr-389, and that once all of these sites are phosphorylated, constitutively active PDK1 can access Thr-229 and phosphorylate it, resulting in full activation of S6K1 [1,25,26]. We have shown previously that PDK1 plays a role in activation of S6K2 [12]. We set out to assess whether full activation seen with T388E in the absence of serum indicates that Thr-388 phosphorylation alone is sufficient for activation of the kinase regardless of the status of Thr-228 phosphorylation, or that the role of Thr-388 phosphorylation is to allow subsequent Thr-228 phosphorylation by constitutively active PDK1. To this end, we combined the T388E mutation with the T228A mutation. This combinatory mutant did not have the high basal activity that was seen in T388E, and it showed reduced serum-stimulated activity (Figure 7A). This suggests that T388E phosphorylation alone is not the mechanism by which S6K2 is activated. Rather, Thr-388 phosphorylation seems to be leading to phosphorylation of Thr-228, and this subsequent phosphorylation may be causing full activation of S6K2. Further supporting this model, co-transfection of kinase-inactive PDK1 diminished basal and stimulated activities of T388E, indicating that the effects of PDK1 are downstream of Thr-388 phosphorylation (Figure 7C).

S6K2 can mediate S6 phosphorylation *in vivo*

Since S6K2 was identified based on its nucleotide sequence homology to S6K1, the cellular function and *in vivo* substrate for S6K2 remain to be determined. S6K2 can phosphorylate the ribosomal protein S6 *in vitro*, and S6K2 expression is up-regulated in S6K1-null mice [11]. However, there is no direct evidence that S6K2 is a kinase for S6 *in vivo*; indeed, S6K2 can also phosphorylate a number of other substrates used routinely in *in vitro* kinase assays, such as histone H2B (results not shown). Taking advantage of the fact that rapamycin blocks S6 phosphorylation (and therefore inhibits all S6 kinases, identities

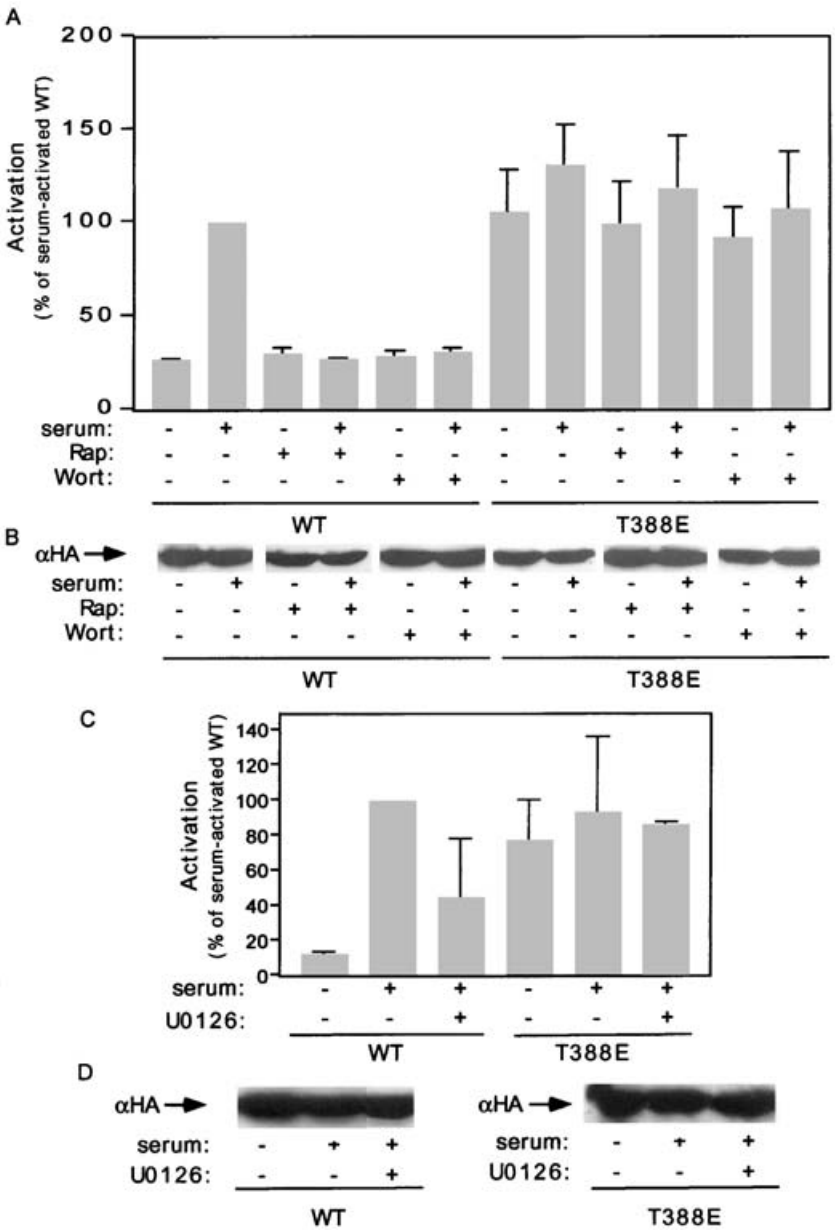

Figure 6 The effects of inhibitors on Thr-388 activity

(**A**) Cells that were transfected with wild-type (WT) or T388E were treated with rapamycin (Rap) or wortmannin (Wort) prior to serum stimulation and *in vitro* kinase assays were performed as in Figure 2(A). (**B**) Cell lysates from (**A**) were immunoblotted with anti-HA Ab to show equivalent protein expression. (**C**) Cells that were transfected with wild-type or T388E were treated with U0126 MEK inhibitor prior to serum stimulation and *in vitro* kinase assays were performed as in Figure 2(A). The graph represents data from multiple experiments that were normalized by setting the serum-activated wild-type kinase activity level to 100 %. (**D**) Cell lysates from (**C**) were immunoblotted with anti-HA Ab to show equivalent protein expression.

known and unknown), and that T388E is a constitutively active mutant that is rapamycin-resistant, we set out to test whether S6K2 could act as a kinase for S6 *in vivo*. Cells were transfected with wild-type S6K2 or T388E, and they were treated with rapamycin to inhibit all endogenous S6 kinase(s). We tested to see if endogenous S6 is still phosphorylated in the presence of T388E and rapamycin. The only kinase able to phosphorylate S6 in the presence of rapamycin would be T388E if it can function as an *in vivo* kinase for S6. The phosphorylation status of the endogenous S6 was assayed using anti-(phospho S6) Ab immunoblots. Figure 8 shows that S6 in cells that express wild-type was phosphorylated following serum stimulation, and that S6 phosphorylation was inhibited completely when these cells were treated with rapamycin. The low amount of S6 phosphorylation that is seen in the absence of serum and rapamycin (Figure 8, first lane) is due to overexpression of transfected wild-type S6K2, which increases the overall S6K2 kinase activity in cells. The T388E-transfected cells showed a similar S6 phosphorylation pattern in the absence of rapamycin; the low amount of S6 phosphorylation was enhanced markedly when the cells were stimulated with serum. However, S6 phosphorylation was still seen in the T388E-expressing cells when the cells were treated with rapamycin, indicating that T388E could act as a kinase for endogenous S6 *in vivo*. This phosphorylation was independent of serum stimulation of rapamycin-treated cells, which correlates with constitutive activity of T388E in the absence of serum

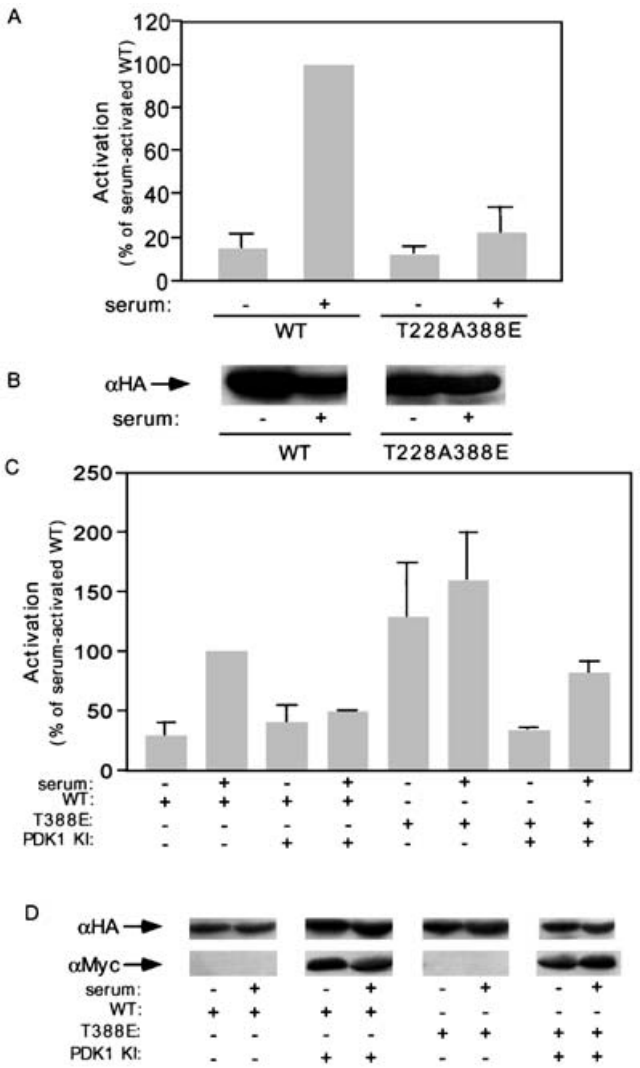

Figure 7 The role of Thr-388 is to allow phosphorylation of Thr-228

(**A**) Cells expressing wild-type (WT) or T228A388E double mutant were stimulated with serum and the kinase activities were assayed as in Figure 2(A). (**B**) Cell lysates from (**A**) were immunoblotted with anti-HA Ab to show protein-expression levels. (**C**) Cells that were transfected with wild-type or T388E, with or without kinase-inactive PDK1 (PDK1 KI), were stimulated with serum and *in vitro* kinase assays were performed as in Figure 2(A). (**D**) Cell lysates in (**C**) were immunoblotted with anti-HA Ab (for wild-type and T388E; top panels) or anti-Myc (for PDK1 KI; bottom panels) to show equivalent protein expression.

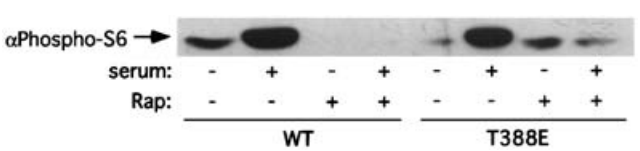

Figure 8 T388E can mediate endogenous S6 phosphorylation in cells

Cells were transiently transfected with wild-type (WT) or T388E, treated with or without rapamycin (Rap), stimulated with or without serum and total cell lysates were immunoblotted with anti-(phospho S6) Ab. Anti-HA immunoblot showed equivalent protein expression (results not shown).

activation. The comparatively low level of S6 phosphorylation in rapamycin-treated T388E cells, as compared with that of serum-stimulated S6 phosphorylation level, is due to the transfection efficiency of T388E being less than 100 %. Our data suggest that S6K2 can phosphorylate S6 in cells.

DISCUSSION

S6K1 activation involves multi-step phosphorylation events by upstream kinases that result in conformational changes that allow full activation of the kinase. The current model [1] is that the proline-directed serines and threonine in the autoinhibitory domain of the kinase are phosphorylated first. This phosphorylation then allows conformational changes that result in phosphorylation of Thr-371 and Thr-389, which then allows access to Thr-229 by constitutively active PDK1, thereby fully activating S6K1. Our study yields insight into the steps involved in activation of an S6K1 homologue, S6K2.

Our data suggest that the equivalent residues that are conserved in S6K2 and shown to be important for S6K1 also play a role in S6K2 activation. Thus Thr-228, Ser-370, the proline-directed autoinhibitory serines, and Thr-388 all play a role in activation of S6K2. Martin et al. [31] showed that the proline-directed serines in the autoinhibitory domain are phosphorylated in a MEK pathway-dependent manner in EGF-mediated cellular activation, thereby potentiating S6K2 for further activation. Truncation of the C-terminal portion of S6K2 resulted in higher basal as well as mitogen-activated kinase activity, and this was mostly attributed to the proline-directed serines in the autoinhibitory domain [31]. We found similar results when HEK-293 cells were transfected with our TM mutant and activated by serum. We have extended the findings of Martin et al. [31] by showing that relief of repression of S6K2 by autoinhibitory serines occurs prior to Thr-388 phosphorylation since the T388E mutant was insensitive to the effects of the MEK inhibitor.

We were surprised to find that changing Thr-388 to glutamic acid was sufficient to fully activate S6K2. The equivalent mutation, T389E, in S6K1 shows diminished serum activation of S6K1 catalytic activity. Only when a combination of T389E with acidic mutations of the proline-directed autoinhibitory serines and threonine was made did it result in higher activity that could be activated further by mitogen stimulation [27]. This suggests that Thr-388 phosphorylation plays a greater role in the release of S6K2 activity repression upon mitogenic activation. Martin et al. [31] have shown that the proline-directed serines in the autoinhibitory domain exert a bigger role in regulating S6K2 activity than the same sites do for S6K1. This is not incompatible with our data on T388E, whose phosphorylation seems sufficient to release the repression of S6K2 to the point of Thr-228 activation. We believe that the autoinhibitory serines in S6K2 play a greater role in leading to the eventual phosphorylation of Thr-388 than the equivalent sites on S6K1 do for Thr-389. The role of the autoinhibitory serines may be to lead to conformational changes that allow Thr-388 phosphorylation. T388E would therefore bypass involvement of phosphorylation of the autoinhibitory serines. Immunoblots using phospho-specific S6 Abs show that both wild-type and T388E have the same substrate specificity among the phosphorylated serines on S6 (results not shown).

T388E was resistant to the effects of rapamycin or wortmannin, suggesting that mTOR and PI3K exert their regulatory role through phosphorylation of Thr-388. There may be other rapamycin- and wortmannin-sensitive sites on S6K2, but their regulation must be prior to activation of Thr-388 (and their role to contribute to activation of Thr-388) since T388E could bypass the effects of rapamycin and wortmannin. Another possibility is that Thr-388 is the only rapamycin- and wortmannin-sensitive phosphorylation site. Given the similarities between S6K1 and S6K2 sequences this seems unlikely since S6K1 Ser-371 has

shown to be also sensitive to these drugs. A plausible model involves sequential phosphorylation of Ser-370 and Thr-388, and the role of Ser-370 may indeed be to allow phosphorylation of Thr-388. T388E would therefore bypass the effects of rapamycin or wortmannin on Ser-370 on S6K2 activation. Saitoh et al. [29] suggest that in S6K1 Ser-371 and Thr-389 are regulated by the mTOR pathway, and that the role of Ser-371 seems to be to regulate phosphorylation of Thr-389. Our data are consistent with this in that, in S6K2, the role of Ser-370 is to allow Thr-388 phosphorylation. When Ser-370 activation is prevented by point mutation Thr-388 phosphorylation is also abolished. Minami et al. [33] have shown that S370G mutation in the other S6K2 isoform (the longer βI isoform) still retains 50% of activity, and they concluded that Ser-370 plays a minor role in S6K2 activation. Our data show that Ser-370 still plays an important role in S6K2 βII isoform activation. The discrepancy between the two studies may be due to the difference in the isoforms the two groups used; indeed, the βI and βII isoforms have been shown to reside in distinct fractions of the nucleus, indicating that they may have differential function and/or regulation [12]. It should be also pointed out that Minami et al. [33] reported that their S6K2 βI shows relative insensitivity to rapamycin, which is different from all other reports to date on S6K2 for reasons that are as yet unclear but which may also be a factor in the discrepancies.

Although the T388E mutant shows constitutive activity, our data indicate that phosphorylation of Thr-388 alone is not sufficient for S6K2 activation; rather, the role of Thr-388 seems to be to allow subsequent phosphorylation of Thr-228. This is supported by analysis of a combinatorial mutant, T228A388E, which restored low basal and serum-stimulated activity seen in T228A. Moreover, kinase-inactive PDK1 was still able to inhibit T388E activity, suggesting that phosphorylation of Thr-228 by PDK1 is still necessary to activate S6K2.

We used the T388E mutant to see if S6 phosphorylation can be carried out by S6K2 *in vivo*. Although the S6K1-null mice showed enhanced expression of S6K2 and therefore provided circumstantial evidence that S6K2 may act as another S6 kinase [11], to date there is no direct evidence that S6K2 can function as an *in vivo* S6 kinase. Our data show that S6K2 can indeed mediate phosphorylation of S6 in cells. This is not to suggest that S6 is the only substrate for S6K2. S6K1 and S6K2 may have some shared as well as some distinct substrates. The localizations of S6K1 and S6K2 are different in cells [15,19,20]. Since most S6 resides in the cytoplasm as a part of the 40 S ribosome, it seems unlikely that the main role of S6K2, which is found in the nucleus, is to participate in phosphorylation of ribosomal S6 and therefore in translational control. However, there is a nuclear pool of free S6 whose function remains unknown at this time [34], and S6K2 may play a role in its phosphorylation. Further research needs to be carried out to address these questions.

Our data present a clearer picture of S6K2 activation. The data support a model in which S6K2 activity is repressed by dephosphorylation of the proline-directed autoinhibitory serines. Phosphorylation of these serines, carried out by MEK-dependent signalling pathways, leads to phosphorylation of Ser-370, which allows phosphorylation of Thr-388, an event regulated by mTOR- and PI3K-dependent pathways. Phosphorylation of Thr-228 by PDK1 then follows, leading to full activation of S6K2. S6K2 can serve as an *in vivo* kinase for S6, although the full range of S6K2 substrates and its function remain to be seen.

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