Characterization of Phosphatidylinositol 3-Kinase-dependent Phosphorylation of the Hydrophobic Motif Site Thr\(^{389}\) in p70 S6 Kinase 1

Phosphorylation of the highly conserved hydrophobic motif site in AGC kinases is necessary for phosphotransferase activity. Phosphorylation of this motif (FLGFT389Y) in p70 S6 kinase (S6K1) is both rapamycin- and wortmannin-sensitive, suggesting a role for both mammalian target of rapamycin- and phosphatidylinositol 3-kinase-dependent pathways. We report here that co-expression of phosphoinositide-dependent kinase-1 (PDK1) and the phosphatidylinositol 3-kinase-regulated atypical protein kinase C\(_{\gamma}\) cooperate to increase both phosphorylation of the hydrophobic motif site Thr\(^{389}\), as well as the activation loop site Thr\(^{229}\). Interestingly, although PDK1 alone can promote an increase in Thr\(^{389}\) phosphorylation in both wild type S6K1 and a kinase-inactive mutant of S6K1, the cooperative effect between PDK1 and protein kinase C\(_{\gamma}\) required S6K1 activity. Furthermore, Akt, another phosphatidylinositol 3-kinase effector and regulator of S6K1, also increased Thr\(^{389}\) phosphorylation in a S6K1 activity-dependent manner. Consistent with this, epidermal growth factor-induced Thr\(^{389}\) phosphorylation in wild type S6K1 persisted for up to 120 min, whereas kinase-inactive mutants of S6K1 displayed only a reduced and transient increase in Thr\(^{389}\) phosphorylation. We conclude that S6K1 activity is required for maximal Thr\(^{389}\) phosphorylation by mitogens and by multiple phosphatidylinositol 3-kinase-dependent inputs including PDK1, PKC\(_{\gamma}\), and Akt, and we propose that autophosphorylation is an important regulatory mechanism for phosphorylation of the hydrophobic motif Thr\(^{389}\) site in S6K1.

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p70 S6 kinase 1 (S6K1\(^{1}\); aII isofrom) is an important regulator of both cell proliferation and cell growth (cell size/cell mass) (1–4). S6K1 is activated by a wide array of growth factors, and its activity is also dependent on sufficient nutrient availability (5, 6). Mitogen-induced activation of S6K1 requires multiple inputs including phosphatidylinositol 3-kinase-dependent and -independent mechanisms (7–9). The mammalian target of rapamycin (mTOR; also known as FRAP or RAPT) acts as a nutrient sensor and plays a major role in nutrient-dependent signaling to S6K1. mTOR also contributes to mitogen-dependent activation of S6K1 (5, 10, 11).

S6K1 is a member of the AGC (cAMP- and cGMP-dependent kinases and protein kinase C) superfamily of kinases. AGC kinases share high sequence homology in their catalytic domains (12). This domain includes a highly conserved region in the activation loop (or T loop) of the kinase that contains a phosphorylation site within the consensus motif TFGCT (Thr\(^{229}\) in S6K1, aII isofrom; Fig. 1). A second conserved site resides within a hydrophobic motif, FXFPTY (Thr\(^{389}\) in S6K1; see Fig. 1) and is ~160 amino acids downstream of the activation loop site in AGC kinases. Phosphorylation of both these sites in AGC kinases is critical for phosphotransferase activity. PDK1, also a member of the AGC family of kinases, has been unequivocally identified as the in vivo activation loop kinase for S6K1 and many other AGC kinases, including Akt, RSK, and several PKC isoforms (13–19). Phosphorylation of the hydrophobic motif site in S6K1 (Thr\(^{389}\), Akt (Ser\(^{473}\)), and some novel PKCs is mitogen-regulated and wortmannin-sensitive, implicating a PI3K-dependent pathway in its regulation (20). It has therefore been coined the “PDK2” site, in anticipation of a second lipid-regulated kinase that specifically phosphorylates this site. However, the existence of a PDK2 enzyme that regulates all AGC kinases remains controversial. It has been reported that, in addition to the S6K1 activation loop (Thr\(^{229}\)), the S6K1 activation loop phosphorylation both in vivo and in vitro (21). PDK1 –/– ES cells do not display Thr\(^{389}\) phosphorylation or activation of S6K1 in response to insulin-like growth factor-1, supporting a role for PDK1 in regulating phosphorylation of both the activation loop site and Thr\(^{389}\) (18).

More recently, the NIMA family kinases NEK6/7 have been identified as regulators of the hydrophobic motif Thr\(^{389}\) site, and possibly other sites, in S6K1 (22). NEK6 activity is modestly activated by insulin, and its high basal activity is only partially inhibited (30%) by the PI3K inhibitors, wortmannin or LY294002, whereas stimulation of Thr\(^{389}\) phosphorylation and activation of S6K1 is more strongly inhibited by these drugs (22, 23). It is therefore likely that other mitogen-induced, PI3K-dependent mechanisms, perhaps in addition to NEK6/7, are involved in the regulation of Thr\(^{389}\) phosphorylation. Consistent with the rapamycin sensitivity of Thr\(^{389}\) phosphorylation, mTOR has also been shown to phosphorylate Thr\(^{389}\) in vitro (24, 25). However, mTOR cannot solely account for
mitogen-dependent increases in Thr\textsuperscript{389} phosphorylation. A truncation mutant of S6K1 whose activity is rapamycin-insensitive and is not regulated by mTOR retains mitogen-stimulated Thr\textsuperscript{389} phosphorylation and sensitivity to wortmannin (23, 26, 27). Finally, autophosphorylation has also been described as a regulatory mechanism for the phosphorylation of the hydrophobic motif site in some AGC kinases including Ser\textsuperscript{473} in Akt (28) and Ser\textsuperscript{606} in PKC\textbeta II (29).

Previous work has shown that the PI3K-regulated atypical PKC\textgamma and \lambda participate in the activation of S6K1 (30, 31). PKC\textgamma cooperates with PDK1 to activate S6K1 and its homolog, S6K2 (30, 32). Akt, another target of PI3K, has also been identified as a regulator of S6K1 (33). The mechanisms whereby these effectors of the PI3K pathway regulate S6K1 still remain unknown.

In the present study, we have sought to further characterize the PI3K-dependent mechanisms involved in the phosphorylation of the hydrophobic motif Thr\textsuperscript{389} in S6K1. We report that PKC\textgamma and PDK1 cooperate to enhance phosphorylation of both the hydrophobic motif Thr\textsuperscript{389} and the activation loop Thr\textsuperscript{229} sites in S6K1. Interestingly, whereas PDK1 alone could significantly increase Thr\textsuperscript{389} phosphorylation, independent of S6K1 kinase activity, the cooperative effect of PKC\textgamma and PDK1 on Thr\textsuperscript{389} phosphorylation and Akt-dependent Thr\textsuperscript{389} phosphorylation requires S6K1 activity. We also observe that S6K1 activity is required for maximal and sustained mitogen-dependent phosphorylation of Thr\textsuperscript{389}. Furthermore, we find that binding of PDK1 to S6K1 does not require phosphorylation of the hydrophobic motif site, which has been described as a PDK1 docking site (34). We propose that mitogen- and PI3K-regulated Thr\textsuperscript{389} phosphorylation is dependent on phosphorylation of the activation loop Thr\textsuperscript{229} and stimulation of S6K1 activity and requires an autophosphorylation mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Extract Preparation**—HEK-293 and NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum or 10% calf serum, respectively, and were transfected using LipofectAMINE (Invitrogen). The cells were starved in serum-free Dulbecco’s modified Eagle’s medium for 16 or 24 h prior to harvesting, and the lysates were prepared at 24 or 48 h post-transfection, as previously reported (30).

**Immunoblotting**—Whole cell lysate (10% of total extract) or washed immunoprecipitates were resolved by 7.5 or 12% SDS-PAGE, and Western blots were performed as previously reported (30).

**Immunoprecipitations and Immune Complex Assays**—For evaluation of Thr\textsuperscript{389}, Thr\textsuperscript{229}, and Thr\textsuperscript{421}/Ser\textsuperscript{424} phosphorylation and immune complex kinase assays, the lysates were prepared as described above, and HA-S6K1 was immunoprecipitated (33% of total cell extract) with an anti-HA antibody. The immunoprecipitates were stringently washed once in each 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, 40 mg/ml phenylmethylsulfonyl fluoride, 10 \mu g/ml leupeptin, 5 \mu g/ml pepstatin, pH 7.2), Buffer B (same as Buffer A except for 0.1% Nonidet P-40, and 1 mM NaCl), and ST (50 mM Tris-HCl, 5 mM Tris base, 150 mM NaCl). For Western blotting, the washed immunoprecipitates were resolved by 7.5% or 12% SDS-PAGE and immunoblotted as described above with the appropriate antibody.

For co-immunoprecipitation experiments, the lysates (33% of total cell extract) were immunoprecipitated with an anti-Myc antibody and washed twice with phosphate-buffered saline containing 1% Nonidet...
P-40 and once in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4). The proteins were resolved and immunoblotted as described above. S6K1 immune complex kinase assays were carried out as described previously using a GST fusion of the last 32 amino acids of the 40 S ribosomal protein S6 (GST-S6) as a substrate (30). For autophosphorylation assays, GST-S6 was excluded from the kinase reaction mixture.

cDNA Constructs—HA-S6K1 WT (wild type), HA-S6K1-K100R (Lys100→Arg), and HA-S6K1-ΔNT/CT (N- and C-terminal deletion mutant) were described previously (26) and subcloned into the mammalian expression vector pRK7. HA-S6K1-T389E (Thr389→Glu) were generated using the Stratagene QuikChange site-directed mutagenesis kit. Myr-PKCζ/ΔH9256-FLAG, FLAG-PKCζ/ΔH9256-T410A (Thr410→Ala, activation loop site mutant), and Myc-PDK1 were described previously (16, 30). Myr-Akt was obtained from P. Tsichlis.

RESULTS

Mitogen-induced phosphorylation of the hydrophobic motif residue Thr389 in S6K1 correlates with S6K1 activity and is sensitive to both rapamycin and wortmannin (23, 35). In this study, we set out to further understand how PI3K effectors regulate Thr389 phosphorylation. We have previously demonstrated that the atypical PKCζ interacts with and participates in the activation of S6K1 (30). Because phosphorylation of Thr389 and PKCζ are both regulated by PI3K, we hypothesized that PKCζ may directly or indirectly regulate Thr389 phosphorylation. To verify this, we asked whether a kinase-inactive mutant of PKCζ could interfere with EGF-dependent Thr389 phosphorylation. We co-expressed, in HEK-293 cells, FLAG-PKCζ/ΔH9256-T410A (rendered inactive by mutation of the activation loop threonine residue to an alanine residue), with HA-S6K1. Fig. 2 shows that a dose-dependent, inhibitory effect of PKCζ-T410A on EGF-dependent S6K1 activation correlated with a decrease in Thr389 phosphorylation, indicating that PKCζ participates in mitogen-dependent Thr389 phosphorylation.

Because PDK1 can increase Thr389 phosphorylation (21) and PKCζ cooperates with PDK1 to increase basal S6K1 activity (30), we wanted to determine whether PKCζ could influence the ability of PDK1 to regulate Thr389 phosphorylation. In co-expression experiments (Fig. 3a, top panel), a constitutively active and membrane-targeted PKCζ mutant (Myr-PKCζ/ΔH9256-FLAG) (30) alone increased Thr389 phosphorylation in S6K1 almost 3-fold over the basal level of phosphorylation observed in unstimulated HEK-293 cells (lanes 1 and 4). As previously
reported (21), PDK1 alone caused a 13-fold increase in Thr<sup>389</sup> phosphorylation (lane 3), similar to the level of Thr<sup>389</sup> phosphorylation achieved by EGF. Thr<sup>389</sup> phosphorylation was increased almost 33-fold over basal when S6K1 was co-expressed with both Myr-PKC<sub>C</sub>/H9256 and PDK1 (lane 5). Surprisingly, Myr-PKC<sub>C</sub>/H9256 also enhanced the ability of PDK1 to phosphorylate the activation loop site, Thr<sup>229</sup> (middle panel, lane 5 compared with lane 3). Myr-PKC<sub>C</sub>/H9256 alone also increased Thr<sup>229</sup> phosphorylation, although to a lesser extent than PDK1 (lanes 4 and 3, respectively). These data suggest that PKC<sub>C</sub>/H9256 and PDK1 cooperate to increase phosphorylation of both the hydrophobic motif, Thr<sup>389</sup>, and the activation loop, Thr<sup>229</sup>.

To distinguish between PI3K- and mTOR-dependent mechanisms regulating S6K1 phosphorylation, we utilized a truncation mutant of S6K1, ΔNT/CT, lacking both its N and C termini. This mutant is rapamycin-insensitive with respect to activity and phosphorylation and is not regulated by mTOR (26, 27). As seen in Fig. 3b, PDK1 and PKC<sub>C</sub> alone increased Thr<sup>229</sup> phosphorylation in the ΔNT/CT mutant, similarly to S6K1-WT. PKC<sub>C</sub> and PDK1 can therefore regulate phosphorylation of Thr<sup>229</sup> and Thr<sup>389</sup> independently of mTOR.

PKC<sub>C</sub> has been hypothesized to directly phosphorylate the novel PKCδ at its hydrophobic motif site (36). However, we have been unable to detect a specific phosphorylation of S6K1 at Thr<sup>389</sup>, or at any other site, in vitro by PKC<sub>C</sub> (data not shown). It is therefore likely that PKC<sub>C</sub> does not regulate phosphorylation of S6K1 directly. This led us to consider other mechanisms for the cooperative effect of PKC<sub>C</sub> with PDK1. The equivalent hydrophobic motif site in Akt and some PKCs has been described as an autophosphorylation site (28, 29). We therefore hypothesized that S6K1 also autophosphorylates at this site and that PKC<sub>C</sub> is stimulating autophosphorylation at Thr<sup>389</sup> as a result of enhancing S6K1 activity (30). To verify this possibility, we co-expressed Myr-PKC<sub>C</sub> and PDK1 with a kinase-inactive mutant of S6K1, HA-S6K1-K100R (described under “Experimental Procedures”). We observed that PDK1 alone was able to increase Thr<sup>389</sup> phosphorylation in the kinase-inactive mutant of S6K1, although to a lesser extent than that observed with HA-S6K1-WT (Fig. 4a, lane 7 versus lane 3). This is consistent with the ability of PDK1 to directly phosphorylate Thr<sup>389</sup> (21), as well as to cooperate with S6K1 activity in the phosphorylation of this site. Moreover, Myr-PKC<sub>C</sub> was only able to enhance Thr<sup>389</sup> phosphorylation in the catalytically competent S6K1 (Fig. 4a, compare lanes 8 and 7 with lanes 4 and 3), suggesting that PKC<sub>C</sub> cooperates with PDK1 to enhance autophosphorylation at Thr<sup>389</sup>. As expected, Thr<sup>229</sup> phosphorylation induced by PDK1 and PDK1 together with PKC<sub>C</sub> is not dependent on S6K1 activity (Fig. 4b, middle panel). We then asked whether S6K1 activity was required for Thr<sup>389</sup> phosphorylation mediated by other PI3K-dependent inputs such as Akt. Unlike PDK1, Akt does not directly phosphorylate S6K1, but it has been reported to increase both Thr<sup>229</sup> and Thr<sup>389</sup> phosphorylation (data not shown and Ref. 37). As shown in Fig. 4b, both PDK1 and an activated mutant of Akt (Myr-Akt), individually, increased basal Thr<sup>389</sup> phosphorylation of WT-S6K1 (Fig. 4b, lanes 2 and 3, respectively versus lane 1). Interestingly, Akt-induced Thr<sup>389</sup> phosphorylation was completely absent in
Regulation of the Hydrophobic Motif Site in S6K1

Fig. 5. EGF-dependent phosphorylation of the hydrophobic motif site Thr389, but not the C-terminal proline-directed Thr389/Ser424, is transient and reduced in the inactive HA-S6K1-K100R mutant. HEK-293 cells were transfected with HA-S6K1 WT or the K100R mutant, starved for 16 h in serum-free medium, and stimulated with EGF (50 ng/ml) for the indicated times. α and β, anti-phospho-Thr389 and anti-phospho-Thr389/Ser424 blots evaluating immunoprecipitated (IP) HA-S6K1 protein and anti-HA blots comparing expression levels of HA-S6K1 in whole cell lysate are shown. b, S6 kinase activity was evaluated in anti-HA immunoprecipitates as described under “Experimental Procedures.”

S6K1-K100R (Fig. 4b, lane 6 versus lane 3), suggesting that Akt is solely stimulating autophosphorylation of S6K1 at Thr389. Taken together, these data invoke autophosphorylation as a relevant mechanism for phosphorylation of Thr389 by PI3K-dependent inputs.

Activation of S6K1 by various mitogens is largely dependent on PI3K-dependent mechanisms. We therefore wanted to determine to what extent mitogen-induced phosphorylation of Thr389 is dependent on S6K1 activity. It has already been reported that a kinase-inactive mutant of S6K1 (K100M,K123M according to the authors’ nomenclature) is still phosphorylated in vivo at Thr389 in mitogen-stimulated HEK-293 cells (23), suggesting that autophosphorylation is an unlikely mechanism. In those reports, Thr389 phosphorylation was only evaluated at one time point (20 min) after stimulation with insulin. We performed a kinetic analysis comparing Thr389 phosphorylation of HA-S6K1-WT and the kinase-inactive HA-S6K1-K100R mutant, in HEK-293 cells stimulated with EGF. We found that EGF induced a sustained increase in the phosphorylation of Thr389 in HA-S6K1-WT that was still detectable up to 120 min post-stimulation with EGF (Figs. 5a-c) and correlated with a sustained increase in S6K1 activity (Fig. 5b). Interestingly, we consistently observed that the increase in phosphorylation of Thr389 in the kinases-inactive S6K1-K100R mutant was transient and was significantly reduced after the first 10–20 min of stimulation (Fig. 5). We have performed this experiment several times (n = 10) and have found that the transient increase in Thr389 phosphorylation in S6K1-K100R is 60–80% of that found in S6K1-WT at the early time points, whereas at later time points, Thr389 phosphorylation was barely increased above basal levels. As expected, EGF-dependent phosphorylation of the C-terminal, proline-directed sites was not reduced in S6K1-K100R (Fig. 5a, middle panel). To confirm that the transient and reduced phosphorylation of Thr389 in S6K1-K100R was not cell type-specific, we performed the same experiment in NIH-3T3 fibroblasts. As seen in HEK-293 cells, Thr389 phosphorylation in the K100R mutant was only detectable at early time points (10 min) of stimulation with EGF (Fig. 6), whereas it persisted for at least 60 min in S6K1-WT.

We considered the possibility that the reduction in Thr389 phosphorylation in S6K1-K100R is due to increased sensitivity of this site to phosphatases compared with S6K1-WT or that S6K1 activity is required for the inhibition of a phosphatase. Protein phosphatase (PP) 2A has been reported to interact with S6K1 and has been implicated in the regulation of S6K1 phosphorylation by mTOR (38, 39). We speculated that PP2A might be responsible for dephosphorylation of Thr389. We tested the effect of a known PP2A and PP1C inhibitor, okadaic acid, on the phosphorylation state of Thr389 in both wild type and the K100R mutant of S6K1. For this experiment, we used the NIH-3T3 cell line because HEK-293 cells no longer adhere to tissue culture dishes in the presence of okadaic acid (our observation). As seen in Fig. 6 (compare right panels to left panels), okadaic acid enhanced EGF-induced Thr389 phosphorylation in S6K1-WT and enhanced both basal and transiently increased Thr389 phosphorylation in S6K1-K100R. This observation confirms that phosphatases inhibited by okadaic acid (including PP2A) are indeed regulating Thr389 phosphorylation. However, okadaic acid did not prolong the kinetics of Thr389 phosphorylation in the K100R mutant. This result suggests that the transient phosphorylation observed in this mutant is not a result of increased sensitivity of this mutant to phosphatases. It is likely that a positive input dependent on S6K1 activity, such as autophosphorylation, is required for sustained Thr389 phosphorylation.

We also evaluated phosphorylation of Thr389 in a different kinase-inactive S6K1 mutant, S6K1-T229A, where the activation loop Thr229 site is mutated to an alanine residue. Like the K100R mutant, this mutant is devoid of phosphotransferase activity toward S6 (data not shown). It has been previously reported that Thr389 phosphorylation in the S6K1-T229A mutant was not significantly increased in response to insulin-like growth factor 1 or upon co-transfection of this mutant with an
activated mutant of PI3K (21, 23). As seen in Fig. 7, mitogen-dependent and basal Thr 389 phosphorylation in the T229A mutant was even more dramatically reduced compared with the K100R mutant (Fig. 5). A slight increase in Thr 389 phosphorylation was observed in the T229A mutant at the 20-min time point in response to EGF. We have consistently seen that Thr389 phosphorylation in the T229A mutant, at early time points, is only about 20% of the phosphorylation observed with WT-S6K1. These data suggest that phosphorylation of the activation loop site by PDK1 is required for phosphorylation of the hydrophobic motif Thr389, which is consistent with the lack of Thr389 phosphorylation in PDK1/H11002/H11002 ES cells (18).

To confirm that S6K1 can autophosphorylate, we immunoprecipitated HA-S6K1-WT, K100R, or the T229A mutant from HEK-293 cells and performed in vitro kinase assays in the absence of exogenous substrate for S6K1. We found that EGF increases the incorporation of \(^{32}\)P label for WT-S6K1, indicative of autophosphorylation (Fig. 8). This was not observed with the kinase-inactive mutants of S6K1, ruling out the possibility that a co-immunoprecipitating mitogen-regulated kinase phosphorylates S6K1.

It has been proposed that phosphorylation of the hydrophobic motif Thr389 creates a docking site for PDK1 and thus primes S6K1 for phosphorylation of the activation loop Thr229 by PDK1 (13, 34, 40). This model implies that Thr389 phosphorylation occurs prior to and independently of Thr229 phosphorylation. However, this is not consistent with a lack of Thr389 phosphorylation in the PDK1−/− ES cells. We therefore wanted to examine whether Thr389 phosphorylation is required for binding of PDK1 to S6K1. For this purpose, we performed co-immunoprecipitation experiments with PDK1 and different mutants of S6K1 that exhibit different levels of Thr389 phosphorylation. We co-expressed, in HEK-293 cells, HA-S6K1-WT or mutants of S6K1 (described under “Experimental Procedures”) together with Myc-PDK1 or expressed S6K1-WT alone as a control. We evaluated the extent of S6K1 binding to PDK1 by monitoring S6K1 co-immunoprecipitating with PDK1 in anti-Myc immunoprecipitates. The background caused by non-specific binding of HA-S6K1 to protein G-Sepharose beads was detected in Myc immunoprecipitates from cells expressing HA-S6K1-WT but not Myc-PDK1. Thr389 phosphorylation of S6K1 was evaluated in anti-HA immunoprecipitates from lysates. We observed that the S6K1-T389E mutant, where an acidic residue mimics phosphorylation at this site, has a higher affinity for PDK1 than does S6K1-WT (21, 41) (Fig. 9). We also noted that mutants of S6K1 (including the S371A and T229A mutants) that exhibit very little or no Thr389 phosphorylation (second panel) in the presence or absence of EGF were able to co-immunoprecipitate with PDK1. As a control, co-immunoprecipitating S6K1 was not detected in cells not expressing Myc-PDK1. It is important to note here that the S371A mutant, which is kinase-inactive (42) and exhibits no detectable increase in Thr389 phosphorylation, is still phosphorylated upon Thr229, albeit at a reduced rate (our data not shown and Ref. 23). These observations suggest that although Thr389 phosphorylation increases the affinity of PDK1 binding to S6K1, it is not a prerequisite (41). Therefore, PDK1 binding and Thr229 phosphorylation can probably occur prior to and in the absence of mitogen-stimulated Thr389 phosphorylation.
DISCUSSION

The discovery of PDK1 as the activation loop site kinase for many of the AGC kinases was a significant step toward understanding how PI3K regulates other AGC kinases such as S6K1, Akt, and PKCs. Since then, much effort has focused on the identification of a PDK2 enzyme that phosphorylates the critical hydrophobic motif site of these kinases. Phosphorylation of this site in the AGC kinases is predicted to be necessary for a stable, active conformation of the kinase (43, 44). It has already been shown that in the conventional PKC isoforms (PKCβII) and Akt, this site is modulated by autophosphorylation (28, 29). In the present study, we present data supporting an important role for autophosphorylation in the regulation of the hydrophobic motif site of yet another AGC kinase, S6K1. In the case of S6K1, however, evidence for additional mechanisms for regulating phosphorylation of this site has also been presented (10, 21, 22). In the present study, we observe that the optimal, prolonged Thr<sup>389</sup> phosphorylation in the wild type enzyme, in response to EGF (Figs. 5–7), is dependent on S6K1 activity because kinase-inactive K100R and T229A S6K1 mutants exhibit only a reduced and transient increase in Thr<sup>389</sup> phosphorylation after mitogen treatment of cells. It is unlikely that the kinase-inactive S6K1 mutant is “misfolded” and therefore not recognizable by the PDK2 enzyme because co-expression of PDK1 with the K100R mutant does result in a significant increase in Thr<sup>389</sup> phosphorylation (Fig. 4). Furthermore, similar mitogen-dependent phosphorylation kinetics of the proline-directed sites within the C-terminal pseudosubstrate domain of

![Fig. 8. EGF stimulates autophosphorylation of S6K1.](image)

![Fig. 9. Phosphorylation of Thr<sup>389</sup> is not a prerequisite for interaction between S6K1 and PDK1.](image)
PI3K-regulated kinases proposed as upstream regulators of S6K1 include PDK1, the atypical PKCs ζ and λ, and Akt. PKD1 has been unequivocally described as the activation loop site (Thr240) kinase. PKD1 has also been shown to directly phosphorylate Thr389. However, the primary target of PKD1 is Thr389 because it phosphorylates Thr389 much less efficiently in vitro (21). It is likely that co-expression of PKD1 with S6K1 increases the efficiency of phosphorylation of Thr389 by PKD1 in vivo, and this accounts for the PKD1-dependent increase in Thr389 phosphorylation in kinase-inactive S6K1 (Fig. 4). Moreover, our data strongly suggest that Thr389 phosphorylation mediated by PI3K-dependent inputs including PKD1, PKCζ, and Akt requires S6K1 activity. Because EGFP-induced Thr389 phosphorylation is also largely dependent on S6K1 activity, we propose that the majority of the observed mitogen- and PI3K-dependent Thr389 phosphorylation is probably due to autophosphorylation, which is dependent on Thr249 phosphorylation and S6K1 activity. This model is consistent with the lack of mitogen- and PI3K-dependent Thr389 phosphorylation (Fig. 7 and data not shown) in S6K1-T229A (activation loop site mutant) and with the absence of Thr389 phosphorylation in PDK1-null ES cells.

Recent evidence suggests that mTOR may also respond to mitogenic signals, in addition to nutrient-dependent signals (11). The initial transient mitogen-dependent Thr389 phosphorylation that is revealed in the K100R mutant is both rapamycin- and wortmannin-sensitive (data not shown) and therefore may be achieved by mTOR and/or PKD1.

An unexpected yet very interesting observation that we have made here is the positive effect that PKCζ exerts on Thr249 phosphorylation. This is reminiscent of the stimulatory effect of the PDK1-interacting fragment (PIF) peptide on the phosphorylation of Akt by PKD1 at its activation loop and hydrophobic motif sites (45). PIF includes the hydrophobic motif consensus sequence of protein kinase C-related kinase 2 (PRDF-D-Y) that is analogous to that of PKCζ (FEFG-E-Y). It was initially speculated that the binding of PIF to PKD1 changes the specificity of PKD1 so that it is able to phosphorylate a site within a different consensus sequence, allowing it to directly phosphorylate the hydrophobic motif site of Akt, Ser473 (45). However, it was subsequently noted that Ser473 phosphorylation was increased by PKD1 and PIF only in active Akt recombinant protein and not in a kinase-inactive Akt mutant (44). Bondi et al. (44) have proposed that the bulky hydrophobic phenylalanine residues in the hydrophobic motif of PIF mediate its interaction with a hydrophobic pocket in the kinase domain of PKD1 and thus stabilize an active conformation of PKD1. Because the hydrophobic motif of PKCζ closely resembles that of protein kinase C-related kinase 2 (PIF), we speculate that PKCζ probably binds directly to the hydrophobic pocket of PKD1 and stabilizes an active conformation of PKD1. Consistent with this, PKCζ and PKD1 have been shown to co-immunoprecipitate (16, 30, 46). Furthermore, Fig. 3A shows that co-expression of Myr-PKCζ with PKD1 induces a mobility shift in PKD1, which is indicative of an increase in phosphorylation of PKD1. This could be the result of autophosphorylation of PKD1 caused by an increase in PKD1 activity (47). In support of this hypothesis, the hydrophobic motif site in RSK, also an AGC kinase, was defined as a docking site for PKD1. RSK binding to PKD1 was shown to stimulate autophosphorylation of PKD1 (48). Because our present and previous observations (Fig. 2 and Ref. 30) suggest that PKCζ activity is required for its cooperative effect with PKD1, we cannot rule out the possibility that PKCζ is phosphorylating PKD1 and thus regulating its activity (inducing a positive feedback loop). Alternatively, the interaction between PKCζ and PKD1 could be modulated by and require PKCζ activity. At this point we cannot exclude either of these possibilities, and the potential modulation of PDK1 activity by complex formation with other AGC kinases such as PKCζ is an important question that needs to be addressed.

We have focused here on PI3K-dependent mechanisms regulating Thr389 phosphorylation. However, it is clear that mTOR also plays a critical role in Thr389 phosphorylation and S6K1 activation via the conserved TOS motif in the N termini of S6Ks (10). Based on the data presented here, it is likely that various combinations of inputs from autophosphorylation, mTOR, PKD1, and NEK6/7 can contribute to Thr389 phosphorylation by specific stimuli in specific cell types. Because NEK6/7 activity is only partially wortmannin-sensitive (22), it will be interesting to determine whether these kinases participate in PI3K-independent responses previously shown to contribute to S6K1 activation (7–9). In any case, the requirement for autophosphorylation and additional mechanisms for regulating S6 kinase hydrophobic motif phosphorylation is consistent with the activation of S6K1 by a variety of mitogenic (PI3K-dependent and -independent), metabolic, and stress-inducing agonists (5).

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