Phosphorylation Sites in the Autoinhibitory Domain Participate in p70^65k Activation Loop Phosphorylation*

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Here we have employed p70^65k truncation and point mutants to elucidate the role played by the carboxy-terminal autoinhibitory domain S/TP phosphorylation sites in kinase activation. Earlier studies showed that truncation of the p70^65k amino terminus severely impairs kinase activation but that this effect was reversed by deleting the carboxyl terminus, which in parallel led to deregulation of Thr229 phosphorylation in the activation loop (Dennis, P. B., Pullen, N., Kozma, S. C., and Thomas, G. (1996) Mol. Cell. Biol. 16, 6242–6251). In this study, substitution of acidic residues for the four autoinhibitory domain S/TP sites mimics the carboxy-terminal deletion largely by rescuing kinase activation caused by the amino-terminal truncation. However, these mutations do not deregulate Thr229 phosphorylation, suggesting the involvement of another regulatory element in the intact kinase. This element appears to be Thr389 phosphorylation, because substitution of an acidic residue at this position in the p70^65k variant containing the S/TP mutations leads to a large increase in basal Thr229 phosphorylation and kinase activity. In contrast, an alanine substitution at Thr389 blocks both responses. Consistent with these data, we show that a mutant harboring the acidic S/TP and Thr389 substitutions is an excellent in vitro substrate for the newly identified Thr229 kinase, phosphoinositide-dependent kinase-1 (Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S., Hemmings, B. A., and Thomas, G. (1998) Science 279, 707–710), whereas phosphoinositide-dependent kinase-1 poorly utilizes the two p70^65k variants that have only one set of mutations. These findings indicate that phosphorylation of the S/TP sites, in cooperation with Thr389 phosphorylation, controls Thr229 phosphorylation through an intrasteric mechanism.

The concerted up-regulation of transcription and translation is required for a cell, responding to mitogenic stimuli, to grow and enter the cell cycle (1–3). Recent studies have implicated increased S6 phosphorylation in the selective translation of a subset of essential mRNAs containing an oligopyrimidine tract at their transcriptional start site (4, 5). This event is regulated by p70^65k/p85^65k (6), two mitogen-stimulated protein kinase isofoms that rely on multiple phosphorylation as a principal mechanism for activation (7). The p85^65k isoform is expressed from the same transcript as p70^65k through an alternative translational initiation start site, adding a 23-amino acid extension at the amino terminus and constitutively targeting it to the nucleus (8). Little is known about the role of p85^65k in the nucleus; however, S6 has been shown to reside in both the nucleoplasm, in a free form, and in the nucleolus, in preribosomal particles, where it is also phosphorylated in response to mitogens (9). In studies conducted to date, regulation of the nuclear isoform parallels that of p70^65k, so a concomitant role for p85^65k in mitogenesis is predicted (10, 11), perhaps involving transcription or processing of RNA (8).

The identification of intramolecular p70^65k regulatory elements, in the form of domains and phosphorylation sites, has increased our understanding of the mechanism by which the kinase autoregulates (7, 12, 13). To date, eight phosphorylation sites have been identified in the endogenous kinase (14, 15). In initial studies, Ser^411, Ser^418, Thr^421, and Ser^424, residing within a potential autoinhibitory domain at the carboxy terminus of the kinase (16, 17), were found to be principal sites of mitogen-induced phosphorylation (14). These sites are characterized by a proline in the +1 position and a hydrophobic residue in the −2 position. More recently, studies have led to the identification of Ser^371 (18) as a phosphorylation site that shares the same motif, and three additional sites, Thr^229, Thr^389, and Ser^404, which are flanked in the +1 and −1 positions by bulky aromatic amino acids (15). The phosphorylation of these latter sites in response to mitogens is blocked by treatment of cells with the immunosuppressant rapamycin or the fungal metabolite wortmannin (15, 19). Based on mutation studies, Thr^229, in the activation loop, as well as Ser^371 and Thr^389, in the linker region coupling the catalytic and autoinhibitory domains, appear to be critical for kinase activation (15, 18). The activation loop phosphorylation site, Thr^229 in p70^65k, is a common regulatory element found in many kinases (20, 21). In parallel studies, we have shown that the phosphorylation of this site is regulated by the newly described phosphoinositide-dependent protein kinase, PDK1 (22). Thr^229, Ser^371, and Thr^389, as well as the domains in which they reside, are strikingly conserved in most members of the AGC (protein kinases A, G, and C) family of Ser/Thr kinases (21).

In contrast to the domains containing Thr^229, Ser^371, and Thr^389 of the autoinhibitory domain, as well as the carboxy terminus of p70^65k, are conspicuously absent in the other members of the AGC family of Ser/Thr kinases (21). We have reported that mutation of the S/TP sites within the autoinhibitory domain, as well as Ser^404, to alanines or acidic residues modulates kinase activity (15, 19). However, others have

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1 Y. Chen, C. D. Hoemann, G. Thomas, and S. C. Kozma, submitted for publication.

2 The abbreviations used are: PDK1, phosphoinositide-dependent kinase-1; FCS, fetal calf serum.
claimed little to no effect of similar mutations on the activity of the kinase (23, 24). Indeed, the deletion of the p70 <sup>65k</sup> carboxyl terminus, containing the S/T and P sites, has little effect on either basal or mitogen-induced kinase activity (12, 13, 25), which has also been used to conclude that these sites are not involved in regulating kinase activation at either the G<sub>i</sub>/G<sub>i</sub> or M/G<sub>i</sub> transition state of the cell cycle (23, 24). Despite these observations, this domain is completely conserved in all the mammalian forms of p70 <sup>65k</sup> and is also present in the recently cloned <i>Drosophila</i> homolog, Dp70 <sup>65k</sup> (26, 27). More notably, a p70 <sup>65k</sup> amino-terminal truncation, which blocks kinase activation (12, 13, 25) and mitogen-induced Thr<sup>229</sup> and Thr<sup>389</sup> phosphorylation (13), is rescued by the same carboxyl-terminal deletion that removes the autoinhibitory domain (12, 13, 25). These observations suggest instead that the autoinhibitory domain, and possibly the phosphorylation sites residing within this domain, play a critical role in regulating p70 <sup>65k</sup> activity in the intact kinase through the modulation of Thr<sup>229</sup> and Thr<sup>389</sup> phosphorylation.

In this study, we utilized phosphorylation site and truncation mutations to elucidate the role of the autoinhibitory domain S/T and P sites in regulating mitogen-induced p70 <sup>65k</sup> activation. Next, we examined the nature of this event as it relates to the ability of the S/T sites, along with Thr<sup>389</sup>, to regulate Thr<sup>229</sup> phosphorylation in <i>vitro</i>. Finally, by employing PDK1 in <i>vitro</i>, we have determined that the mechanism by which these carboxyl-terminal phosphorylation sites control Thr<sup>229</sup> phosphorylation in <i>vitro</i> is synergistic and intrasteric.

**Experimental Procedures**

**Plasmid Construction and Mutagenesis—**Generation of the amino- and carboxyl-terminal truncation mutants of p70 <sup>65k</sup>, as well as point mutations at phosphorylation sites, was achieved using the Altered Site II Mutagenesis System (Promega), as described previously (15). All constructs were tagged with a myc epitope and placed immediately following the p70 <sup>65k</sup> or PDK1 initiator ATG codon (15, 22). Phosphorylation site mutants were placed in the appropriate background by BglII-PstI fragment exchanges. All constructs were subcloned into a cytosine-virus-driven expression vector after being verified by DNA sequencing.

**Cell Culture, Transfection, and Metabolic Labeling—**Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Twenty-four hours before transfection, cells were seeded at a density of 10<sup>6</sup> cells per 10-cm-diameter plate. The cells were then transiently transfected, using a modified calcium phosphate procedure (28, 29), with 1–5 μg of the appropriate construct. Total DNA transfected was kept at 10 μg for all experiments using the same reagent vector. After 12 h, the transfected cells were placed into serum-free Dulbecco's modfied Eagle's medium for an additional 24 h. Metabolic labeling was carried out in phosphate-free Dulbecco's modified Eagle's medium with 1–2 μCi <sup>32</sup>P ATP, per 5 ml of medium followed by extraction either with or without serum stimulation as described previously (30). Extracts were centrifuged at 12,000 × g for 5 min at 4 °C, and the supernatants were quickly frozen in liquid N <sub>2</sub> and stored at −70 °C.

**Immunoblotting, Kinase Assays, and Two-dimensional Phosphopeptide Mapping—**Protein concentrations were measured using the BioRad D/C protein assay. For Western blot analysis, 20 μg of extract protein were resolved by SDS-polyacrylamide gel electrophoresis before transfer onto an Immobilon P membrane (Millipore). Expression of the epitope-tagged proteins was detected by decorating the membrane with swine anti-rabbit tertiary antibody. Expression levels were determined by Western blotting as above, and total extracts containing myc-tagged p70 <sup>65k</sup> constructs harboring different mutations in indicated phosphorylation sites (Fig. 1). After serum deprivation, cells were extracted directly or stimulated with 10% FCS for 45 min before extraction. The ectopically expressed mutants were immunoprecipitated using the 9E10 antibody and assayed for kinase activity against 40S ribosomes as described under “Experimental Procedures.” Activities are shown as the percentage of serum-stimulated p70 <sup>65k</sup> activity when expressed at the same level. The error bars represent the standard error of three independent experiments.

**RESULTS**

**p70<sup>65k</sup>ΔN<sub>34</sub> Activation Is Rescued by Acidic Substitutions in the Carboxyl Terminus—**The inability of the p70 <sup>65k</sup> amino-
terminal truncation mutant, p70<sup>65k</sup>ΔN<sub>54</sub> (Fig. 1), to respond to mitogens can be largely rescued by removing the carboxyl terminus (12, 13, 25). To test whether mitogen-induced phosphorylation of the four autoinhibitory S/TP sites could mimic removal of the carboxyl terminus and rescue activity, acidic amino acids were substituted for these residues in p70<sup>65k</sup>ΔN<sub>54</sub>, and the activity of the newly generated variant, p70<sup>65k</sup>ΔN<sub>54</sub>zD3E (Fig. 1), was measured following transient expression in 293 cells. In contrast to the double truncation mutant (12, 13, 25), p70<sup>65k</sup>ΔN<sub>54</sub>zD3E displayed high basal activity (Fig. 2), resembling the elevated basal activity previously reported for the same acidic amino acid substitutions placed in wild-type p70<sup>65k</sup> (15, 19). Serum stimulation increased p70<sup>65k</sup>ΔN<sub>54</sub>zD3E activity to about 50% of the value obtained for the double truncation mutant and about 10-fold over that detected for the parent, p70<sup>65k</sup>ΔN<sub>54</sub> (Fig. 2). Because the basal activity of p70<sup>65k</sup>ΔN<sub>54</sub>zD3E can be further augmented by placing an acidic residue at Thr<sup>389</sup> (13, 15), the same mutation was placed in p70<sup>65k</sup>ΔN<sub>54</sub>D3E (ΔN<sub>54</sub>D3E-T389E), which further increased both basal and serum-stimulated activities (Fig. 2). Together, the results demonstrate that substitution of acidic amino acids for phosphorylation sites residing in the carboxyl terminus of p70<sup>65k</sup> largely rescues the activity of the amino-terminal truncation mutant.

**Deregulation of Thr<sup>229</sup> Phosphorylation**—Removal of the amino and carboxyl termini increases basal Thr<sup>229</sup> phosphorylation, whereas removal of the amino terminus alone suppresses Thr<sup>229</sup> phosphorylation (13). These findings suggest that access of the Thr<sup>229</sup> kinase is restricted by the p70<sup>65k</sup> carboxyl terminus, possibly through the phosphorylation state of the S/TP sites in the autoinhibitory domain. To examine this possibility, Thr<sup>229</sup> phosphorylation and kinase activity were first determined for p70<sup>65k</sup>D<sub>C104</sub>. In quiescent cells, p70<sup>65k</sup>D<sub>C104</sub> had high levels of Thr<sup>229</sup> phosphorylation but undetectable Thr<sup>389</sup> phosphorylation (Fig. 3B), consistent with results obtained for the double truncation mutant (13). Despite the high levels of Thr<sup>229</sup> phosphorylation, this variant has low basal kinase activity (Fig. 3A). Upon serum stimulation, Thr<sup>229</sup> phosphorylation increased approximately 2-fold, whereas Thr<sup>389</sup> phosphorylation was greatly enhanced (Fig. 3C), correlating with increased kinase activity (Fig. 3A). Therefore, truncation of the carboxyl terminus alone is sufficient to disrupt the regulation of Thr<sup>229</sup> phosphorylation in resting cells. To determine whether acidic mutations of the four S/TP sites in the
autoinhibitory domain could mimic this effect, a variant harboring these mutations, p70\textsuperscript{s6k}D\textsubscript{3E}, was transiently expressed in 293 cells. In quiescent cells, this mutant displayed elevated kinase activity, which could be further stimulated with serum (Fig. 4A). However, in contrast to the p70\textsuperscript{s6k}D\textsubscript{C104} truncation mutant, basal Thr\textsuperscript{229} phosphorylation in p70\textsuperscript{s6k}D\textsubscript{3E} was very low, although it could be stimulated with serum (Fig. 4, compare B and C). It should be noted that stronger exposures of the chromatogram depicted in Fig. 4B showed low levels of Thr\textsuperscript{389} phosphorylation (data not shown), reflecting the elevated basal kinase activity and rapamycin sensitivity of this construct (13). Thus, the acidic mutations alone are insufficient to increase basal Thr\textsuperscript{229} phosphorylation, indicating that another element is necessary for regulating Thr\textsuperscript{229} phosphorylation.

**Regulation of Thr\textsuperscript{229} Phosphorylation by Thr\textsuperscript{389} Phosphorylation**—An acidic residue substituted for Thr\textsuperscript{389} potentiates the ability of the S/TP mutations to rescue the p70\textsuperscript{s6k} amino-terminal truncation mutant (Fig. 2), and activation of p70\textsuperscript{s6k}D\textsubscript{3E} is paralleled by increased Thr\textsuperscript{389} phosphorylation (Fig. 4C). These findings suggest that Thr\textsuperscript{389} phosphorylation may be the additional element required to bring about Thr\textsuperscript{229} phosphorylation. To test this possibility, phosphopeptide analyses of p70\textsuperscript{s6k} were compared with those from p70\textsuperscript{s6k} variants harboring acidic mutations at Thr\textsuperscript{389} and in the S/TP sites. Phosphopeptide analysis of wild-type p70\textsuperscript{s6k} from quiescent cells revealed low levels of Thr\textsuperscript{229} phosphorylation, which were dramatically increased by serum stimulation (Fig. 5, compare B and C), as was activity (Fig. 5A). Substitution of a glutamate for Thr\textsuperscript{389} in the wild-type p70\textsuperscript{s6k} background raised basal kinase activity levels (Fig. 5A) and Thr\textsuperscript{229} phosphorylation (Fig. 5, compare B and D) approximately 2-fold over that of the wild-type enzyme. The corresponding mutation in the p70\textsuperscript{s6k}D\textsubscript{3E} variant led to a dramatic increase in basal Thr\textsuperscript{229} phosphorylation, reaching a value equivalent to the serum-stimulated wild-type kinase (Fig. 5, compare C with E). As previously shown (15), both constructs were further activated
by serum (Fig. 5A). In contrast, substitution of an alanine for Thr389 in either p70s6k or p70s6kD3E completely abolished kinase activity (Fig. 5A and Ref. 15) and Thr229 phosphorylation in response to serum (Fig. 6). These results support the hypothesis that in the wild-type kinase, phosphorylation of the autoinhibitory domain sites functions to up-regulate Thr229 phosphorylation by cooperating with Thr389 phosphorylation.

**Intrasteric Regulation of Thr229 Phosphorylation**—The results suggest that phosphorylation sites at the carboxyl terminus synergistically regulate Thr229 phosphorylation. However, the data do not address whether the observed synergy on Thr229 phosphorylation is through a single ordered intrasteric mechanism or is instead regulated in vivo through the interplay of multiple effector molecules. To obtain insight into this issue, advantage was taken of the recently described Thr229 kinase PDK1 (22, 32) and p70s6k mutants harboring the different acidic amino acid substitutions. When tested in vitro against the wild-type p70s6k (Fig. 7A), PDK1 only poorly phosphorylated the kinase (Fig. 7B) and had no effect on activity (data not shown and Ref. 22). Furthermore, even though p70s6kT389E was a slightly better substrate for PDK1 than p70s6kD3E, the response was only marginally enhanced for either variant over that obtained with wild-type p70s6k (Fig. 7B). This finding supports the hypothesis that neither of these...
individual sets of mutations is sufficient to allow PDK1 to access Thr\textsuperscript{229}. In contrast, substitution of both sets of acidic mutations resulted in synergistic phosphorylation of p70\textsuperscript{S6K}D\textsubscript{3}E-E389 by PDK1 (Fig. 7B), consistent with the observed effect of the combined mutations on Thr\textsuperscript{229} phosphorylation in vivo (Fig. 5F). This effect was abolished when an alanine was substituted for Thr\textsuperscript{389} in the wild-type p70\textsuperscript{S6K} and the p70\textsuperscript{S6K}D\textsubscript{3}E variant (Fig. 7B and data not shown). The data support the hypothesis that Thr\textsuperscript{229} phosphorylation is regulated by the collective efforts of the carboxyl-terminal phosphorylation sites, through an intrasteric mechanism, presumably by modulating a domain in p70\textsuperscript{S6K} that blocks access of PDK1 to the activation loop phosphorylation site.

**DISCUSSION**

Of the eight known p70\textsuperscript{S6K} phosphorylation sites, the first to be identified were the S/TP sites in the carboxyl-terminal autoinhibitory domain of the kinase (14). Although we reported that neutral and acidic mutations at these sites lower and raise basal kinase activity, respectively (15, 19), others found little to no effect of similar mutations (23, 24). This study emphasizes the importance of the autoinhibitory domain phosphorylation sites in p70\textsuperscript{S6K} activation. First, acidic amino acid substitutions at these sites largely rescue the activity of an amino-terminal truncation mutant, and second, these sites cooperate with an acidic mutation at Thr\textsuperscript{389} to synergistically regulate phosphorylation of the activation loop site, Thr\textsuperscript{229}. Although the p70\textsuperscript{S6K}ΔC\textsubscript{104} mutant is still regulated by mitogens, indicating that other elements are involved in p70\textsuperscript{S6K} activation, it does not exclude a role for the autoinhibitory domain S/TP in the activation of the intact kinase. Indeed, the synergistic effect conferred by the S/TP and Thr\textsuperscript{389} acidic mutations on Thr\textsuperscript{229} phosphorylation suggests a possible mechanism for the sensitive control of Thr\textsuperscript{229} phosphorylation, which is dependent on the stoichiometry of S/TP site phosphorylation in the autoinhibitory domain (see below). This is further supported by the observation that truncation of the carboxyl terminus largely deregulates Thr\textsuperscript{229} phosphorylation (Fig. 3).

The ability of the acidic mutations of the S/TP sites to rescue p70\textsuperscript{S6K}ΔN\textsubscript{54} activity was unexpected, because deletion of this domain did not significantly affect the serum-induced phosphorylation of these sites (15). However, this may be due to the degree of phosphorylation at the autoinhibitory domain S/TP sites in response to mitogen stimulation. Mitogen stimulation is hypothesized to first lead to an increase in S/TP site phosphorylation in the autoinhibitory domain, which functions together with the amino terminus to facilitate Thr\textsuperscript{389} phosphorylation (Fig. 8). In the absence of the amino terminus, the level of mitogen-induced S/TP site phosphorylation may not be sufficient to promote a net increase in Thr\textsuperscript{389} phosphorylation and subsequent Thr\textsuperscript{229} phosphorylation, attenuating kinase activation. However, substitution of an acidic amino acid at each of the S/TP sites would raise the overall of negative charge of this domain and overcome the effect of the amino-terminal truncation, triggering Thr\textsuperscript{389} phosphorylation. In support of this model, phosphopeptide maps show that Thr\textsuperscript{389} phosphorylation is rescued when acidic S/TP site mutations are placed in the p70\textsuperscript{S6K}ΔN\textsubscript{54} background. Thus, Thr\textsuperscript{389} phosphorylation would act as an intermediary step between autoinhibitory S/TP and activation loop site phosphorylation, which would be the final step in mitogen-induced p70\textsuperscript{S6K} activation (Fig. 8).

Although a number of candidate autoinhibitory domain S/TP kinases have been suggested, including cyclin-dependent kinase-1 and the mitogen-activated kinases p42\textsuperscript{MAPK}/p44\textsuperscript{MAPK} (33), their requirement has not been substantiated to date. Indeed, utilization of interfering mutants of p21\textsuperscript{ras} and p74\textsuperscript{raf}, as well as deletion mutants of the platelet-derived growth factor receptor, demonstrated that p42\textsuperscript{MAPK}/p44\textsuperscript{MAPK} were not

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3 P. B. Dennis and G. Thomas, unpublished results.
effectors of the p70s6k signaling pathway (11). It has been shown that over-expression of a kinase dead p70s6k blocks Thr229, Thr389, and Ser421 phosphorylation as well as strongly suppressing Ser411 and Thr421 phosphorylation in the autoinhibitory domain (5, 34), a pattern of inhibition resembling that induced by rapamycin (15). Furthermore, it was found that over-expression of kinase dead or wild-type p70s6k blocks the same sites of phosphorylation in the suppressor of protein synthesis initiation factor 4E, eIF4E-binding protein-1, as does rapamycin (34). These results suggest that overexpression of p70s6k might sequester a common upstream kinase that is also responsible for phosphorylating eIF4E-binding protein-1 (34). Consistent with this hypothesis, the phosphorylation sites in eIF4E-binding protein-1 also display S/TP motifs (35), and recently, it was shown that these sites are phosphorylated in vivo by the mammalian target of rapamycin (36). However, it is unlikely that mammalian target of rapamycin is the S/TP kinase for p70s6k, because rapamycin has no effect on serum-induced S/TP phosphorylation in the amino-terminal truncation mutant, p70s6kΔN54. Interest in the identity of the S/TP kinase has been further increased by the recent observation that Pin1, the conserved mitotic peptidyl-prolyl isomerase, binds to p70s6k, apparently through phosphorylated Ser411 (37).

The finding that in the carboxyl-terminal deletion mutant, p70s6kΔC104, kinase activity and Thr389 phosphorylation are tightly regulated suggests the existence of an additional regulatory element in p70s6k that is controlled by the phosphorylation of the S/TP and Thr389 sites. This element would function to modulate Thr229 phosphorylation and activity. An obvious candidate for such an element is the linker region, which couples the carboxyl and catalytic domains of p70s6k (Fig. 1). Previously, it was noted that many members of the AGC family of protein kinases (21) contain a site homologous to Thr389, as well as the conserved motif surrounding this site (15). In a more recent study, it was also pointed out that this conservation extends through the entire linker region (18). Within this region we identified a novel site, Ser371, the phosphorylation of which appears to be critical for kinase activation (18). The site equivalent to Ser371 has been identified as a major autophosphorylation site in protein kinase C βII (38, 39) and protein kinase C α (40), Thr641 and Thr638, respectively. In both cases, there is a proline in the +1 position and a hydrophobic residue in the −2 position, as with Ser421 (18). Furthermore, modeling studies with protein kinase C have suggested that the conserved linker region may interact with the amino terminus and that Thr641 is juxtaposed to the active site allowing autophosphorylation by an intramolecular reaction (41–43). Although Ser421 is not an autophosphorylation site in p70s6k (18), the modeling studies suggest that it could be strategically placed to modulate potential interactions between the amino terminus, the catalytic domain, and the autoinhibitory region. Indeed, mutation of this site to either an alanine or an aspartic acid blocked both Thr389 phosphorylation and kinase activation, but surprisingly, it did not affect Thr229 phosphorylation (18). This may indicate that mutations at Ser371 disrupt the normal function of the linker region in regulating Thr389 and Thr229 phosphorylation.

PKD1 has been identified as the common activation loop kinase for Thr229 phosphorylation in p70s6k (22) and Thr389 in protein kinase B (32). In a manner similar to the p70s6k S/TP and Thr389 phosphorylation sites, it has been shown that the access of PKD1 to the activation loop of protein kinase B is controlled by the binding of specific phosphatidylinositides to its PH domain (31, 32). Therefore, the two kinases are linked by a common, constitutively active upstream kinase that appears to catalyze the final event in activation. However, distinct internal regulatory elements control the differential activity of each kinase by regulating the access of PKD1 to the activation loop. This mechanism may provide an economical way for the two kinases to share a common upstream activator without sacrificing their ability to be independently regulated.

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REFERENCES
1. Brooks, R. F. (1977) Cell 12, 311–317
2. Hershey, J. W. B. (1991) Annu. Rev. Biochem. 60, 717–755
3. Karin, M., and Hunter, T. (1995) Curr. Biol. 5, 747–757
4. Jefferies, H. B. J., and Thomas, G. (1996) in Translational Control: Ribosomal Protein S6 Phosphorylation and Signal Transduction (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 389–409, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
5. Jefferies, H. B. J., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) EMBO J. 16, 3693–3704
6. Pearson, R. B., and Thomas, G. (1995) in Progress in Cell Cycle Research (Meijer, L., Guidet, S., and Tung, H. Y. L., eds) Vol. 1, pp. 21–32, Plenum Press, New York

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4 P. B. Dennis, N. Pullen, R. B. Pearson, S. C. Kozma, and G. Thomas, unpublished data.
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7. Pullen, N., and Thomas, G. (1997) FEBS Lett. 410, 78–82
8. Reinhard, C., Fernandez, A., Lamb, J. C., and Thomas, G. (1994) EMBO J. 13, 1557–1565
9. Franco, R., and Rosenfeld, M. G. (1990) J. Biol. Chem. 265, 4321–4325
10. Reinhard, C., Thomas, G., and Kozma, S. C. (1992) Proc. Natl. Acad. Sci. USA 89, 4052–4056
11. Reinhard, C., Pearson, R. B., Siegmann, M., Kozma, S. C., and Thomas, G. (1993) J. Biol. Chem. 268, 16091–16094
12. Weng, Q.-P., Andrabi, K., Kozlowski, M. T., Grove, J. R., and Avruch, J. (1995) Mol. Cell. Biol. 15, 2333–2340
13. Dennis, P. B., Pullen, N., Kozma, S. C., and Thomas, G. (1996) Mol. Cell. Biol. 16, 6242–6251
14. Ferrari, S., Bannwarth, W., Morley, S. J., Totty, N. F., and Thomas, G. (1992) Proc. Natl. Acad. Sci. USA 89, 7282–7285
15. Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H., and Thomas, G. (1995) EMBO J. 21, 5279–5287
16. Price, D. J., Mukhopadhyay, N. K., and Avruch, J. (1991) J. Biol. Chem. 266, 16281–16284
17. Flotow, H., and Thomas, G. (1992) J. Biol. Chem. 267, 3074–3078
18. Moser, B. A., Dennis, P. B., Pullen, N., Pearson, R. B., Williamson, N. A., Wettenhall, E. H., Kozma, S. C., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5648–5656
19. Han, J.-W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) J. Biol. Chem. 270, 21396–21403
20. Marshall, C. J. (1994) Nature 367, 686
21. Hanks, S. K., and Hunter, T. (1995) in The Protein Kinase Facts Book. Protein-Serine Kinases: The Eukaryotic Protein Kinase Superfamily (Hardie, G., and Hanks, S. K., eds) pp. 7–47, Academic Press, London
22. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S., Hemmings, B. A., and Thomas, G. (1998) J. Biol. Chem. 273, 963–971
23. Edeleanu, M. L., Kühne, C., Petritsch, C., and Ballou, L. M. (1996) J. Biol. Chem. 271, 963–971
24. Cheatham, L., Monfar, M., Chou, M. M., and Blenis, J. (1995) Proc. Natl. Acad. Sci. USA 92, 11696–11700
25. Stewart, M. J., Berry, C. O. A., Zilberman, F., Thomas, G., and Kozma, S. C. (1996) FEBS Lett. 4321–4325
26. Watson, K. L., Chou, M. M., Blenis, J., Gelbart, W. M., and Erickson, R. L. (1996) Proc. Natl. Acad. Sci. USA 93, 13684–13689
27. Okayama, H., and Chen, C. A. (1988) Biotechniques 6, 632–638
28. Jordan, M., Schnallhorn, A., and Wurm, F. M. (1997) Nucleic Acids Res. 24, 596–601
29. Ferrari, S., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
30. Alessi, D. R., Deak, M., Casamayor, A., Caubwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A., and Bownes, M. (1997) Curr. Biol. 7, 776–789
31. Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Pellech, S. L., Sanghera, J., and Avruch, J. (1992) J. Biol. Chem. 267, 3325–3335
32. Von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gnarra, A.-C., Sonenberg, N., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5426–5436
33. Fadden, P., Haystead, T. A. J., and Lawrence, J. C., Jr. (1997) J. Biol. Chem. 272, 10240–10247
34. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr., and Abraham, R. T. (1997) Science 277, 89–91
35. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J.-U., Xu, J., Kuo, J., Kirshner, M. W., Fischer, G., Cantley, L. C., and Lo, K. P. (1998) Science 278, 1957–1960
36. Flint, A. J., Paladini, R. D., and Koshland, D. E., Jr. (1990) Science 249, 408–411
37. Tsutakawa, S. E., Medzihradszky, K. F., Flint, A. J., Burlingame, A. L., and Koshland, D. E., Jr. (1995) J. Biol. Chem. 270, 26807–26812
38. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) Curr. Biol. 5, 1394–1403
39. Orr, J. W., and Newton, A. C. (1994) J. Biol. Chem. 269, 8383–8387
40. Newton, A. C. (1995) J. Biol. Chem. 270, 28485–28488
41. Borancin, F., and Parker, P. J. (1996) Curr. Biol. 6, 1114–1123