



Differential functional regulation of protein kinase C (PKC) orthologs in fission yeast

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The two PKC orthologs Pck1 and Pck2 in the fission yeast *Schizosaccharomyces pombe* operate in a redundant fashion to control essential functions, including morphogenesis and cell wall biosynthesis, as well as the activity of the cell integrity pathway and its core element, the MAPK Pmk1. We show here that, despite the strong structural similarity and functional redundancy of these two enzymes, the mechanisms regulating their maturation, activation, and stabilization have a remarkably distinct biological impact on both kinases. We found that, in contrast to Pck2, putative *in vivo* phosphorylation of Pck1 within the conserved activation loop, turn, and hydrophobic motifs is essential for Pck1 stability and biological functions. Constitutive Pck activation promoted dephosphorylation and destabilization of Pck2, whereas it enhanced Pck1 levels to interfere with proper downstream signaling to the cell integrity pathway via Pck2. Importantly, although catalytic activity was essential for Pck1 function, Pck2 remained partially functional independent of its catalytic activity. Our findings suggest that early divergence from a common ancestor in fission yeast involved important changes in the mechanisms regulating catalytic activation and stability of PKC family members to allow for flexible and dynamic control of downstream functions, including MAPK signaling.

The protein kinase C family of isozymes plays essential roles in signaling pathways controlling cell growth, proliferation, differentiation, and cell death (1, 2). Classical, novel, and atypical mammalian PKC isoforms share a basic structure with a variable N-terminal regulatory domain followed by a highly conserved C-terminal kinase domain, which contains three conserved phosphorylation sites critical for catalytic activity: the activation loop (AL),³ turn motif (TM), and hydrophobic motif (HM) (1, 2). AL phosphorylation is essential for the catalytic activation of most PKC isoforms and is carried out by 3-phosphoinositide-dependent kinase 1 (PDK-1) (1, 2). The mammalian

target of rapamycin (mTOR) or an autophosphorylation mechanism mediate TM and HM phosphorylation of most PKCs, although their requirement to regulate kinase activity varies among different family members (2). Given their essential biological roles, PKC isozymes are present throughout the eukaryotic lineage, from humans to simple organisms like yeasts (3). Indeed, the budding yeast *Saccharomyces cerevisiae* harbors a single and essential PKC ortholog named Pkc1, whose phosphorylation at Thr⁹⁸³ within AL by redundant PDKs Pkh1 and Pkh2 is indispensable for its catalytic and biological functions (4).

The fission yeast *Schizosaccharomyces pombe* has two non-essential PKC orthologs known as Pck1 and Pck2 (5, 6). Both kinases share extensive homology at their amino acid sequences, particularly within the catalytic domain (~70% identity within 180 amino acids; Fig. 1). Both Pck1 and Pck2, as *S. cerevisiae* Pkc1, retain the regulatory C1 and C2 domains found in mammalian PKCs but present an extended regulatory domain, including two polybasic coiled-coil HR1 domains that mediate binding and regulation by the GTP-bound Rho GTPase family members Rho1 and Rho2 (Fig. 1A) (7, 8). The HR1 domains in Pck1 and Pck1/Pck2 are closely related to those present in the mammalian Rho family-responsive protein kinase N kinases (PKNs) PKN1–3, a subfamily within the PKC family that binds and become regulated by Rho family members (9). Pck1 and Pck2 are short half-life proteins that contain N-terminal proline-, glutamic acid-, serine-, and threonine-rich (PEST) sequences, and their interaction with either GTP-Rho1 or GTP-Rho2 increases their stability (7, 8). Rho1 and Rho2 synergistically regulate, through Pck1 and Pck2, the biosynthesis of (1, 3) β -D-glucan and α -glucan, which are the two main cell wall polymers in fission yeast (7, 10). Pck1 and Pck2 share overlapping roles in cell viability and partially complement each other, and their simultaneous deletion elicits a synthetic lethal phenotype (7, 10).

Besides its role in controlling glucan synthesis, Pck2 is a major upstream activator of the cell integrity MAP kinase pathway (CIP). Its core component, MAP kinase Pmk1, becomes activated during growth and in response to adverse environmental conditions and regulates several processes, including cell separation, cell wall construction, and ionic homeostasis (5, 6, 11). Although the Rho2-Pck2 branch is essential for Pmk1 activation in response to hyper- and hypo-osmotic stress, both Rho1 and Rho2 target Pck2 to activate the CIP during vegeta-

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³ The abbreviations used are: AL, activation loop; TM, turn motif; HM, hydrophobic motif; mTOR, mammalian target of rapamycin; CIP, cell integrity pathway; PS, pseudosubstrate segment.

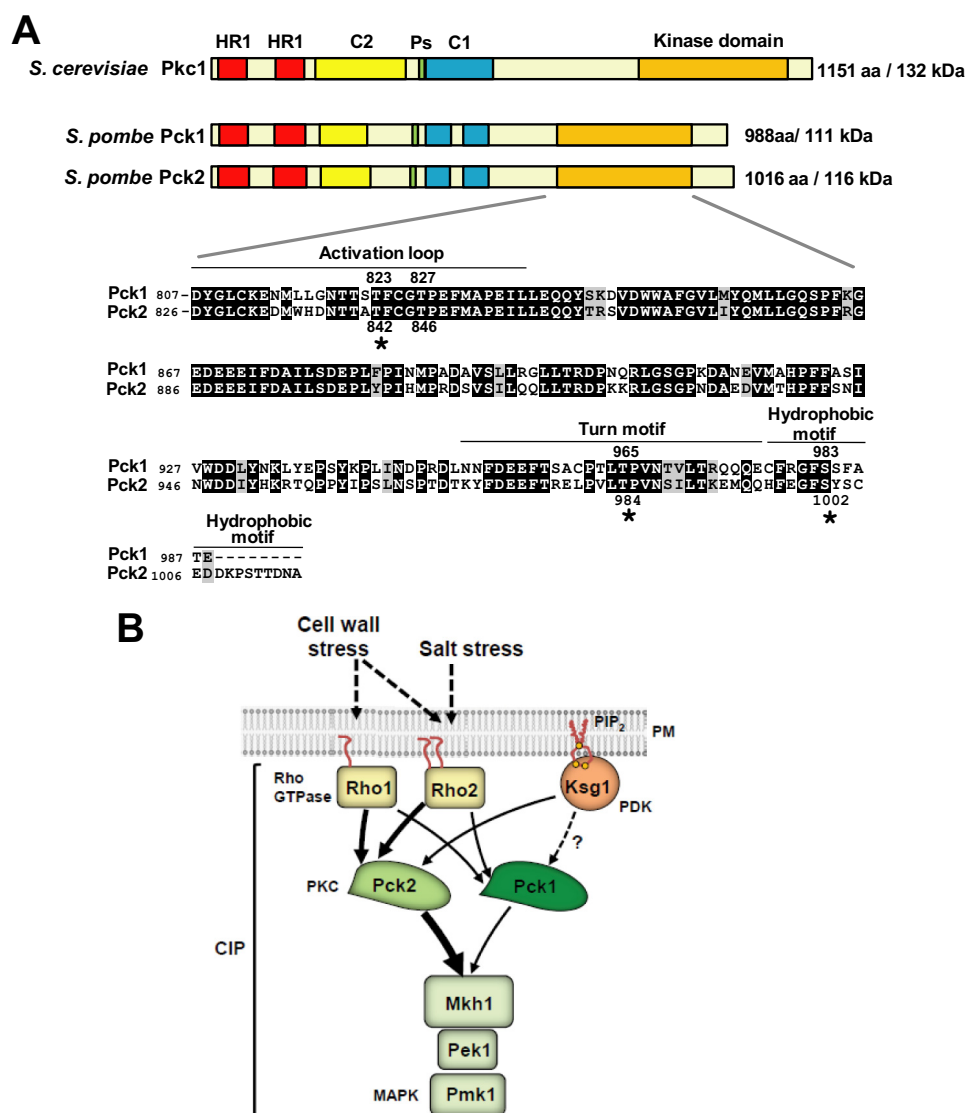


Figure 1. A, domain structure of the PKC orthologs Pck1 (*S. cerevisiae*) and Pck1 and Pck2 (*S. pombe*). HR1, putative rho-binding repeat; C2, putative Ca²⁺-binding motif; Ps, putative pseudosubstrate motif; C1, putative diacylglycerol binding motif. Amino acid (aa) sequence alignment of the Pck1 and Pck2 catalytic domains is also shown. Conserved canonical phosphorylated residues within the AL, TM, and HM are marked with asterisks. B, the cell integrity MAPK pathway in *S. pombe*. The thickness of the solid arrows denotes the relevance of each cascade component during downstream signaling to the MAPK module. For a detailed description of the pathway, see the text. PIP₂, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane.

tive growth and cell wall damage (12) (Fig. 1B). The PDK ortholog Ksg1 and an autophosphorylation mechanism are responsible for the *in vivo* phosphorylation of Pck2 at the conserved Thr⁸⁴² within the AL during vegetative growth and understress (13). These events, together with turn motif autophosphorylation at Thr⁹⁸⁴ and binding to Rho1 and/or Rho2, stabilize and render Pck2 competent to exert its biological functions, including activation of the CIP (13). In addition, we have discovered a novel mechanism involving the Akt ortholog Gad8 (a TORC2 target) and Psk1 (a TORC1 target) that promotes an increase in Pck2 protein levels to allow activation of the CIP in response to cell wall damage or glucose exhaustion (14, 15).

Initial observations suggested that Pck1 was a negative regulator of the CIP (16). However, later studies demonstrated that, instead, it plays a less prominent role than Pck2 as a positive regulator of Pmk1 activity during vegetative growth and cell wall stress (12, 16). Nevertheless, considering their shared func-

tions and strong structural similarity, it might be foreseen that the mechanisms regulating Pck1 function should be identical to those described for Pck2. Contrary to this prediction, in this work we show that, in fission yeast, the expansion of the PKC family from a single ancestor was accompanied by striking differences in the mechanisms regulating maturation, activation, and protein levels of both kinases. The early acquisition of differential regulatory activation and stabilization by Pck1 and Pck2 allows for fine-tuning of downstream MAPK signaling and regulation of cellular homeostasis in this simple organism.

Results

Pck1 is phosphorylated in vivo by Ksg1 within the AL at Thr⁸²³ and is more stable than Pck2

The C-terminal catalytic domains of Pck1 and Pck2 are strongly conserved (Fig. 1A). We showed previously that the

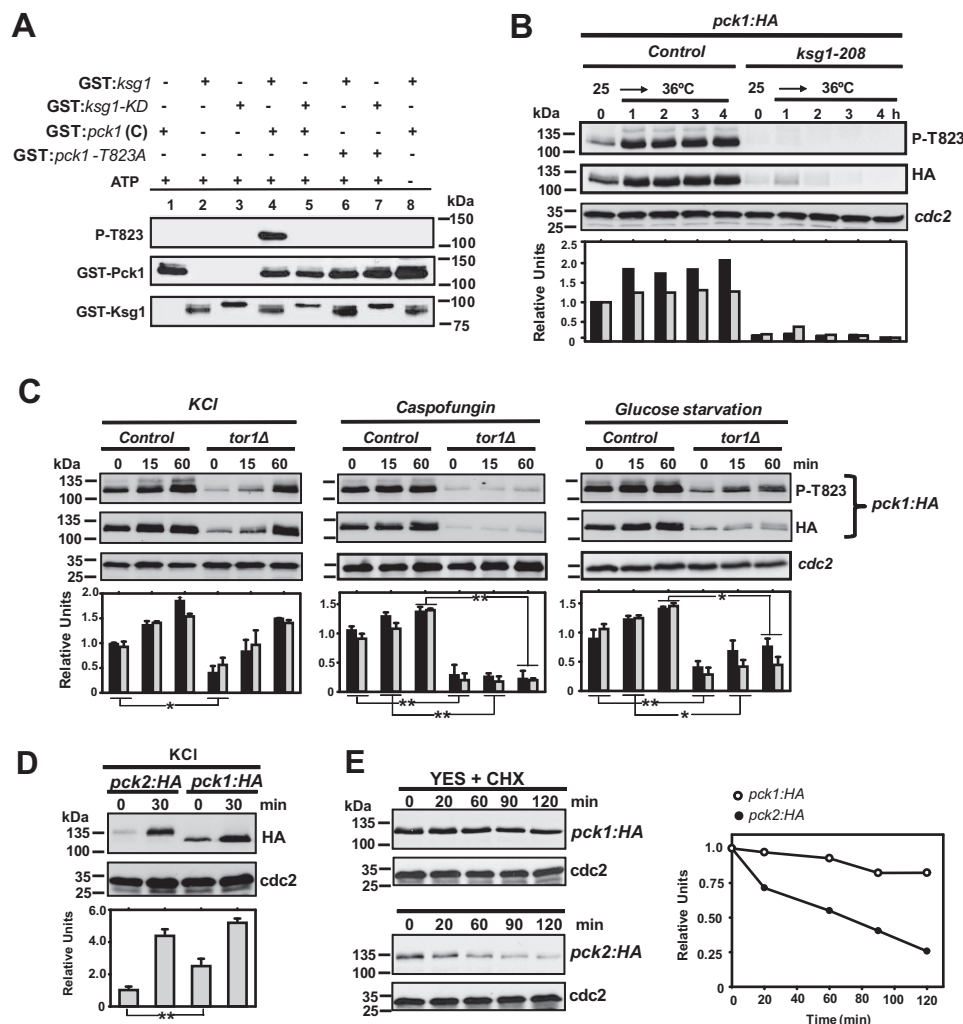


Figure 2. Pck1 is phosphorylated *in vivo* within the AL at Thr⁸²³ by Ksg1 and is more stable than the Pck2 isoform. *A*, GST:pck1 (control, C) and GST:pck1-T823A fusions were incubated at 30 °C for 1 h with either GST:ksg1-KD (kinase-dead mutant) or GST:ksg1 with or without ATP. Reaction mixtures were resolved and hybridized with anti-Thr(P)⁸²³ or anti-GST antibodies. Results representative of two independent experiments are shown. *B*, strains MM1578 (*pck1:HA*, Control) and MM1724 (*pck1:HA Ksg1-208*) were grown at 25 °C in YES medium and then incubated at 36 °C for the indicated times. Cell extracts were resolved by SDS-PAGE and hybridized with anti-Thr(P)⁸²³ and anti-HA antibodies. Relative units for total (gray columns) and Thr⁸²³ phosphorylated (black columns) Pck1 levels were estimated with respect to the internal control (*anti-cdc2 blot*) at each time point. Results representative of two independent experiments are shown. *C*, growing cultures of strains MM1578 (*pck1:HA*, Control) and MM1718 (*tor1Δ pck1:HA*) were treated with either 0.6 M KCl or 1 μg/ml caspofungin or shifted to the same medium without glucose and supplemented with 3% glycerol. Cell extracts were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸²³ and anti-HA antibodies. Relative units as mean ± S.D. (biological triplicates) for total (gray columns) and Thr(P)⁸²³ (black columns) Pck1 levels were estimated as above. * *p* < 0.05; ** *p* < 0.005; calculated by unpaired Student's *t* test. *D*, total (anti-HA) Pck1 and Pck2 levels were determined in growing cultures of control strains MM913 (*pck2:HA*) and MM1578 (*pck1:HA*) and after treatment with 0.6 M KCl for 30 min. Relative units as mean ± S.D. (biological triplicates) for total Pck1 and Pck2 levels were estimated with respect to the internal control (*anti-cdc2 blot*). ** *p* < 0.005; calculated by unpaired Student's *t* test. *E*, control strains MM913 and MM1578 were grown to early log phase and incubated with or without 100 μg/ml cycloheximide (CHX) for the indicated times. Cell extracts were hybridized with anti-HA antibodies. Relative units for total Pck1 and Pck2 levels were estimated with respect to the internal control (*anti-cdc2 blot*) at each time point. Results representative of two independent experiments are shown.

conserved Thr⁸⁴² located at the Pck2 AL is phosphorylated *in vivo* by the PDK1 ortholog Ksg1 (14). The equivalent threonine residue within Pck1 AL lies at position 823 (Fig. 1). Using a specific anti-Thr(P)⁸²³ antibody (see "Experimental Procedures"), we detected specific *in vivo* phosphorylation of Pck1 at this residue (supplemental Fig. S1). Incubation with anti-Thr(P)⁸²³ antibody revealed a strong phosphorylation signal by *in vitro* kinase assays performed with wild-type versions of Ksg1 and Pck1 (Fig. 2A, lane 4). This signal was totally absent when employing the kinase-dead version of Ksg1 (Fig. 2A, lane 5) or a T823A mutated version of Pck1 (Fig. 2A, lane 6), thus showing that Ksg1 phosphorylates the Pck1 AL at Thr⁸²³ *in vitro*. To explore the functional relationship between Ksg1 and Pck1 phos-

phorylation at Thr⁸²³ *in vivo*, we obtained control and *ksg1-208* strains expressing genomic Pck1-HA-tagged versions in a *pck1Δ* background. The *ksg1-208* allele shows normal growth at 25 °C and a thermosensitive phenotype above 34.5 °C. *ksg1-208* cells show marked morphology defects and sensitivity to staurosporine, a potent PKC inhibitor (17). In control cells, the levels of total (anti-HA antibody) and Thr(P)⁸²³ Pck1 were approximately 10 times higher than in *ksg1-208* cells either growing at the permissive temperature (25 °C) or incubated at 36 °C, which is a restrictive temperature for Ksg1 function (Fig. 2B). These results indicate that Ksg1 is also responsible for AL phosphorylation of Pck1 at Thr⁸²³ *in vivo*, and this event might regulate Pck1 stability (see below).

Pck2 protein levels increase in response to different stresses through a mechanism partially regulated by the TORC2 complex and its main component, Tor1 kinase (14, 15). We found that both total and Thr(P)⁸²³ Pck1 levels were ~2- or 3-fold lower in growing *tor1Δ* cells compared with control cells (Fig. 2C). Both Thr(P)⁸²³ and total Pck1 levels were augmented progressively in control cells subjected to saline stress (KCl) and cell wall stress with caspofungin (a specific β-glucan synthase inhibitor) or after starvation from glucose (Fig. 2C). Importantly, such a rise was strongly abrogated in *tor1Δ* cells during cell wall stress or glucose starvation but less evident in response to salt stress (Fig. 2C). Taken together, these findings suggest that TORC2 up-regulates Pck1 levels during growth and in response to specific stresses.

Total Pck1 protein levels were ~2- or 3-fold higher than those of Pck2 (Fig. 2D, *time 0*), confirming previous data on absolute proteome quantifications at the single-cell level (18). Remarkably, in contrast to Pck2, which is a relatively unstable protein with a short half-life (14), Pck1 protein levels decreased very slowly in growing cells treated with the protein synthesis inhibitor cycloheximide (Fig. 2E), implying that Pck1 is more stable than the Pck2 isoform.

Putative phosphorylation at conserved AL, TM, and HM residues differentially affects Pck1 and Pck2 levels and biological functions

In fission yeast, total Pck2 levels are very similar in control cells and in those expressing a non-phosphorylatable AL mutant (Pck2-*T842A*, Fig. 3A) (14). However, the observation that both total and Thr(P)⁸²³ Pck1 levels are quite low in *ksg1-208* cells suggests that Pck1 stability is dependent upon AL phosphorylation. Indeed, as shown in Fig. 3B, Pck1 levels were strongly reduced by ~95% in cells expressing the unphosphorylated AL mutant Pck1-*T823A*. Similar to Pck2 (14), bacterially purified or immunoprecipitated versions of wild-type and Pck1 mutants were not active either *in vitro* or *in vivo*, thus preventing direct biochemical confirmation of their kinase activity status. Instead, we tested the ability of genomic versions of wild-type or mutated alleles of Pck1 to suppress several known phenotypes of *pck1Δ* cells, including defective signaling to the CIP and growth sensitivity in the presence of caspofungin, Calcofluor white, and magnesium chloride (12, 16), as biological readouts to comparatively assess their function *in vivo*. The *T842A* mutation does not affect Pck2 signaling activity to the CIP or growth sensitivity in the presence of caspofungin (Fig. 3C) (14). On the contrary, compared with control cells, *pck1-T823A* cells displayed a strong or moderate growth sensitive phenotype in the presence of caspofungin, magnesium chloride, and Calcofluor white (Fig. 3C). Pck1-*T823A* was also synthetic lethal with the *pck2Δ* mutation (data not shown). Thus, *in vivo*, AL phosphorylation is a critical determinant for the stability and biological functions of Pck1, but not in the case of the Pck2 ortholog. We also noticed that the growth sensitivity of *pck1-T823A* cells to the above stressors was more pronounced than in *pck1Δ* cells (Fig. 3C). Although overexpression of wild-type Pck2 alters cell morphology and inhibits fission yeast growth because of hyperactivation of the CIP, overexpression of the *pck2-T842A* allele is not lethal (Fig. 3D) (19). Con-

trarily, overexpression of wild-type Pck1 is not lethal (12), whereas overexpression of the Pck1-*T823A* mutant allele induced a strong lytic phenotype and was deleterious for cell growth (Fig. 3D). Moreover, the maximal activation of the CIP core member MAPK Pmk1 in response to a salt stress, which is exclusively dependent upon Pck2 function (16), showed a modest but significant decrease in *pck1-T823A* cells compared with control cells (Fig. 3E). Collectively, these results suggest that Pck1 requires to be phosphorylated *in vivo* within the AL by Ksg1 to attain a stable and functional conformation and that failure to do so may negatively interfere with proper Pck2 signaling.

Together with the canonical AL site at Thr⁸⁴², Thr⁸⁴⁶ has been proposed to be phosphorylated *in vivo* and to play a role in Pck2 catalytic activation and biological functions (14). Both total and Thr(P)⁸²³ Pck1 levels were unaffected in cells expressing Pck1-*T827A* with a mutation in the amino acid residue equivalent to Pck2-*T846A* (Figs. 1A and 3B). However, this Pck1 mutant was as sensitive as *pck1Δ* cells to Calcofluor white and magnesium chloride but not to caspofungin (Fig. 3C). Conversely, mutation of Pck1 within the conserved phosphorylatable TM site (Pck1-*T965A*) decreased both total and Thr(P)⁸²³ kinase levels (Fig. 3B) and resulted in growth sensitivity only to caspofungin (Fig. 3F). Hence, *in vivo* putative phosphorylation of Pck1 at Thr⁸²⁷ (AL) and Thr⁹⁶⁵ (TM) may positively influence Pck1 biological functions in specific biological contexts. Mutation at the putative conserved phosphorylation site within Pck1 HM (Pck1-*S983A*) did not affect Pck1 stability, AL phosphorylation, or biological functions (Fig. 3, B and F). However, in sharp contrast with Pck2, the Pck1 mutant at both the TM and HM (Pck1-*T965A S983A*), which is equivalent to the Pck2-*T984A S1002A* mutant, showed very low protein levels (~80% decrease compared with control cells, Fig. 3B), and its sensitivity to different stresses was similar to that of *pck1Δ* cells (Fig. 3F). Taken as a whole, these observations reinforce the idea that, contrary to Pck2, *in vivo* phosphorylations of Pck1 within AL, TM and HM sites are essential for protein stability and biological functions.

Mutation at conserved pseudosubstrate motif has distinct effects on Pck1 and Pck2 stability and downstream signaling

PKCs, including PKCα, possess a pseudosubstrate segment (PS) that keeps the enzyme in a closed, autoinhibited conformation. This domain blocks the substrate-binding cavity and protects the priming AL, TM, and HM phosphosites within the catalytic domain from dephosphorylation (20). Deletion of the PS domain or a point mutation in the conserved alanine residue to a negatively charged glutamic acid renders the kinase constitutively active both *in vivo* and *in vitro* (21). Both Pck1 and Pck2 also harbor a PS domain with conserved alanine residues at positions 399 and 392, respectively (Fig. 4A). We found that total and Thr(P)⁸⁴² Pck2 levels were ~40% lower in the PS mutant (*pck2-A392E*) than in control cells (Fig. 4B). This finding was somehow expected because it is assumed that constitutive catalytic activation of PKCs elicits its subsequent dephosphorylation and degradation (22). Remarkably, Pmk1 phosphorylation was enhanced in growing *pck2-A392E* cells compared with control cells (Fig. 4C), and this feature was

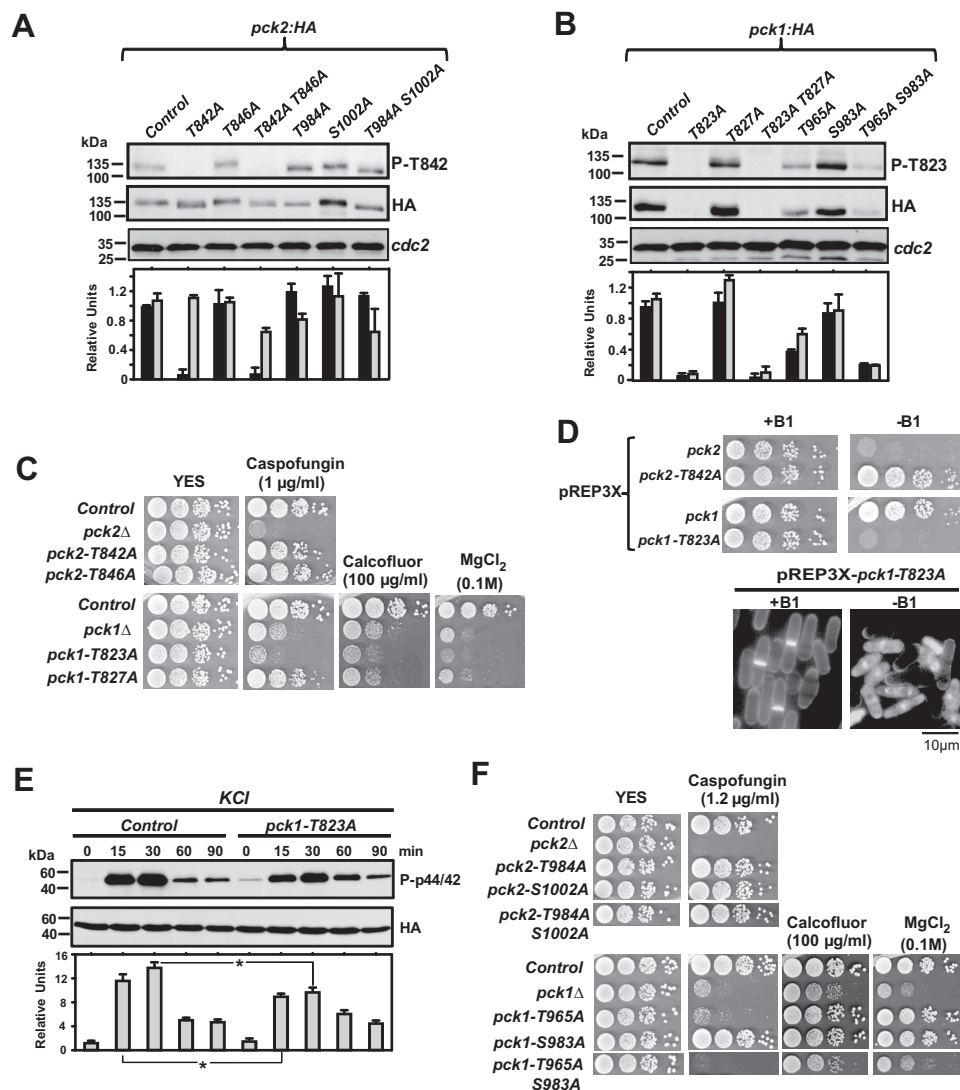


Figure 3. Putative *in vivo* phosphorylation within AL, TM, and HM sites is essential for protein stability and biological functions of Pck1 but not Pck2. A, both total (*anti-HA*) and Thr(P)⁸⁴²-phosphorylated (*anti-Thr(P)⁸⁴²* antibody) Pck2 levels were detected in strains expressing *pck2:HA* versions with the indicated mutations. Relative units as mean \pm S.D. (biological duplicates) for total (*gray columns*) and Thr(P)⁸⁴² (*black columns*) Pck2 levels were determined with respect to the internal control (*anti-cdc2 blot*) at each time point. B, total (*anti-HA*) and Thr(P)⁸²³ (*anti-Thr(P)⁸²³* antibody) Pck1 levels were detected in strains expressing *pck1:HA* versions with the indicated mutations. Relative units as mean \pm S.D. (biological duplicates) for total (*gray columns*) and Thr(P)⁸⁴² (*black columns*) Pck2 levels were determined with respect to the internal control as in A. C, serially diluted cells of strains with the indicated genotypes were spotted on YES plates supplemented with either 1 μ g/ml caspofungin, 100 μ g/ml Calcofluor white, or 0.1 M MgCl₂ and incubated for 3 days at 28 °C. Results representative of three independent experiments are shown. D, *top panel*, strains GB35 (*pck1::ura4⁺ pmk1:HA6H*) and GB3 (*pck2::kanR pmk1:HA6H*) were separately transformed with plasmids pREP3X-*pck1:HA* (control), pREP3X-*pck1-T823A:HA*, pREP3X-*pck2:HA* (control), and pREP3X-*pck2-T842A:HA*. Serially diluted cells from the above transformants were spotted on EMM2 plates with or without 5 mM thiamine (B1) and incubated for 4 days at 28 °C. Results representative of three independent experiments are shown. *Bottom panel*, the cell morphology of a transformant expressing pREP3X-*pck1-T823A:HA* plasmid growing in the presence or absence of thiamine (18 h) was analyzed by fluorescence microscopy after staining with Calcofluor white. E, strains MM1578 (*pck1:HA*, control) and MM1688 (*pck1-T823A:HA*), expressing a genomic *pmk1:HA6H* fusion, were grown in YES medium and treated with 0.6 M KCl. Aliquots were harvested at the times indicated, and activated/total Pmk1 was detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 activation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*) at each time point. *, $p < 0.05$; calculated by unpaired Student's *t* test. F, serially diluted cells of strains with the indicated genotypes were spotted on YES plates supplemented with either 1.2 μ g/ml caspofungin, 100 μ g/ml Calcofluor white, or 0.1 M MgCl₂ and incubated for 3 days at 28 °C. Results representative of three independent experiments are shown.

accompanied by increased growth sensitivity to magnesium chloride (Fig. 4D), which is a known phenotype associated with increased basal Pmk1 activity (23). The increase in total Pck2 abundance displayed by control cells when treated with a cell wall synthesis inhibitor (caspofungin) or KCl (salt stress) was reduced by ~50–55% in *pck2-A392E* cells (Fig. 4E). Pmk1 activation in the presence of caspofungin was partially defective in *pck2-A392E* cells (Fig. 4F), although they were not hypersensi-

tive to this compound (supplemental Fig. S2). In contrast, the response to salt stress was similar to control cells expressing *pck2⁺* (Fig. 4F). Taken together, these observations support the hypothesis that constitutive activation promotes dephosphorylation and destabilization of Pck2, resulting in increased basal Pmk1 activity and limited downstream signaling to the CIP under conditions that require *de novo* protein synthesis, like cell wall stress (13).

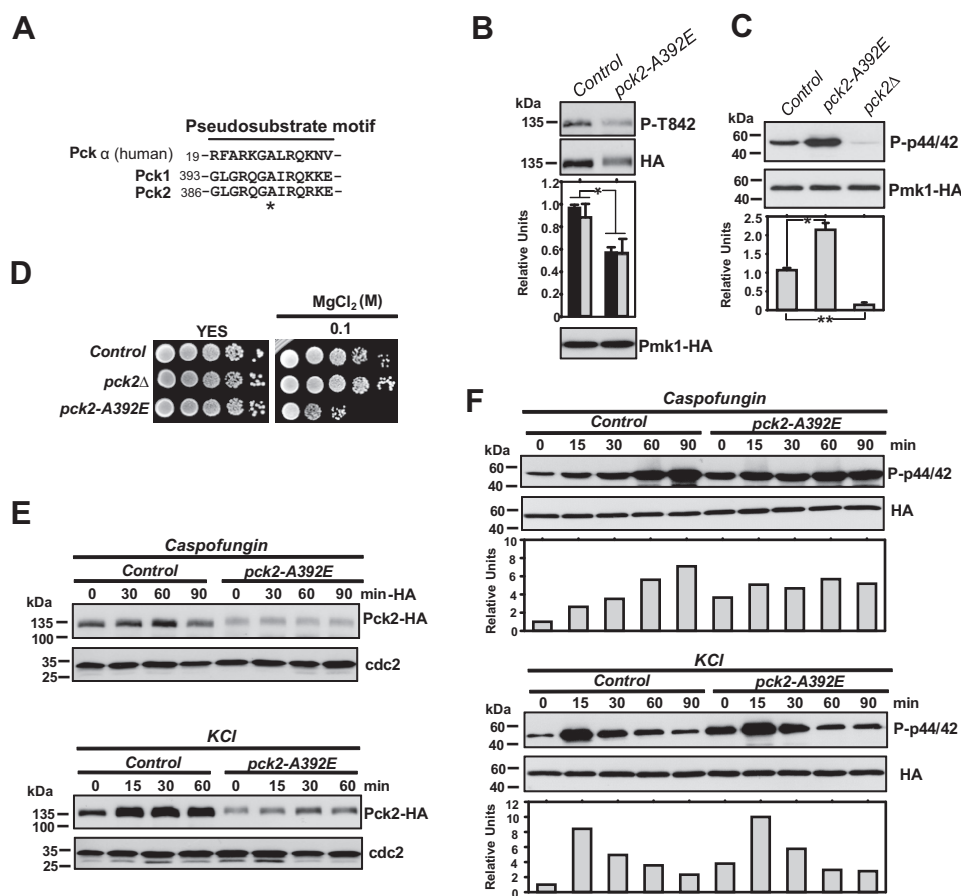


Figure 4. Constitutive activation promotes dephosphorylation and destabilization of Pck2 and altered downstream signaling to the CIP. *A*, amino acid sequence alignment of the pseudosubstrate motif present in human PKC α and fission yeast Pck1 and Pck2. The conserved alanine residue within the motif is marked with an asterisk. *B*, cell extracts from growing cultures of strains MM913 (*pck2::HA*, control) and BV813 (*pck2-A392E::HA*) were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸⁴² and anti-HA antibodies. Relative units as mean \pm S.D. (biological triplicates) for total (gray columns) and Thr(P)⁸⁴² (black columns) Pck2 levels were determined with respect to the internal control (anti-Pmk1-HA blot). *, $p < 0.05$; calculated by unpaired Student's t test. *C*, strains MM913 (*pck2::HA*, control), GB3 (*pck2 Δ*), and BV813 (*pck2-A392E::HA*) expressing a genomic *pmk1::HA6H* fusion were grown in YES medium, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 phosphorylation (anti-phospho-p44/42 blot) were determined with respect to the internal control (anti-HA blot). *, $p < 0.05$; **, $p < 0.005$; calculated by unpaired Student's t test. *D*, serially diluted cells of the strains described in *C* were spotted on YES plates supplemented with 0.1 M MgCl₂ and incubated for 3 days at 28 °C. Results representative of three independent experiments are shown. *E*, the growing strains described in *B* were treated with either 1 μ g/ml caspofungin or 0.6 M KCl. Total Pck2 levels were detected after incubation with anti-HA antibodies. Anti-cdc2 was used as a loading control. Results representative of two independent experiments are shown. *F*, the strains described in *B* were treated with either 1 μ g/ml caspofungin or 0.6 M KCl, and activated/total Pmk1 was detected as above. Relative units for Pmk1 phosphorylation (anti-phospho-p44/42 blot) were determined with respect to the internal control (anti-HA blot). Results representative of two independent experiments are shown.

Contrary to Pck2, the Pck1 PS mutant (*pck1-A399E*) showed total and Thr(P)⁸²³ levels that were approximately twice that of control cells expressing *pck1*⁺ (Fig. 5A). Interestingly, both total and Thr(P)⁸²³ Pck1 levels, which increase progressively in control cells in response to cell wall or salt stress, did not increase further in *pck1-A399E* cells under the same treatments but dropped slowly with longer incubation times (Fig. 5B). Pck2 plays a prominent role in the activation of the CIP during vegetative growth, whereas the Pck1 contribution to this response is minimal, as seen by the strong drop in basal Pmk1 phosphorylation detected in *pck2 Δ* cells compared with control cells (Fig. 5C) (16). However, *pck1-A399E* cells showed a marked increase in basal Pmk1 phosphorylation that was only partially attenuated in the absence of Pck2 (~50% reduction in *pck1-A399E pck2 Δ* cells versus ~80% in *pck2 Δ* cells expressing wild-type Pck1) (Fig. 5C). Growth sensitivity to magnesium chloride mirrored the basal Pmk1 phosphorylation levels displayed by these mutant strains (Fig. 5D). Interestingly, Pck2-mediated activa-

tion of Pmk1 in response to salt stress, which is independent of Pck1 function (16), was attenuated in *pck1-A399E* cells (Fig. 5E). Therefore, constitutive catalytic activation of Pck1 might interfere with proper downstream signaling of Pck2 to the MAPK Pmk1.

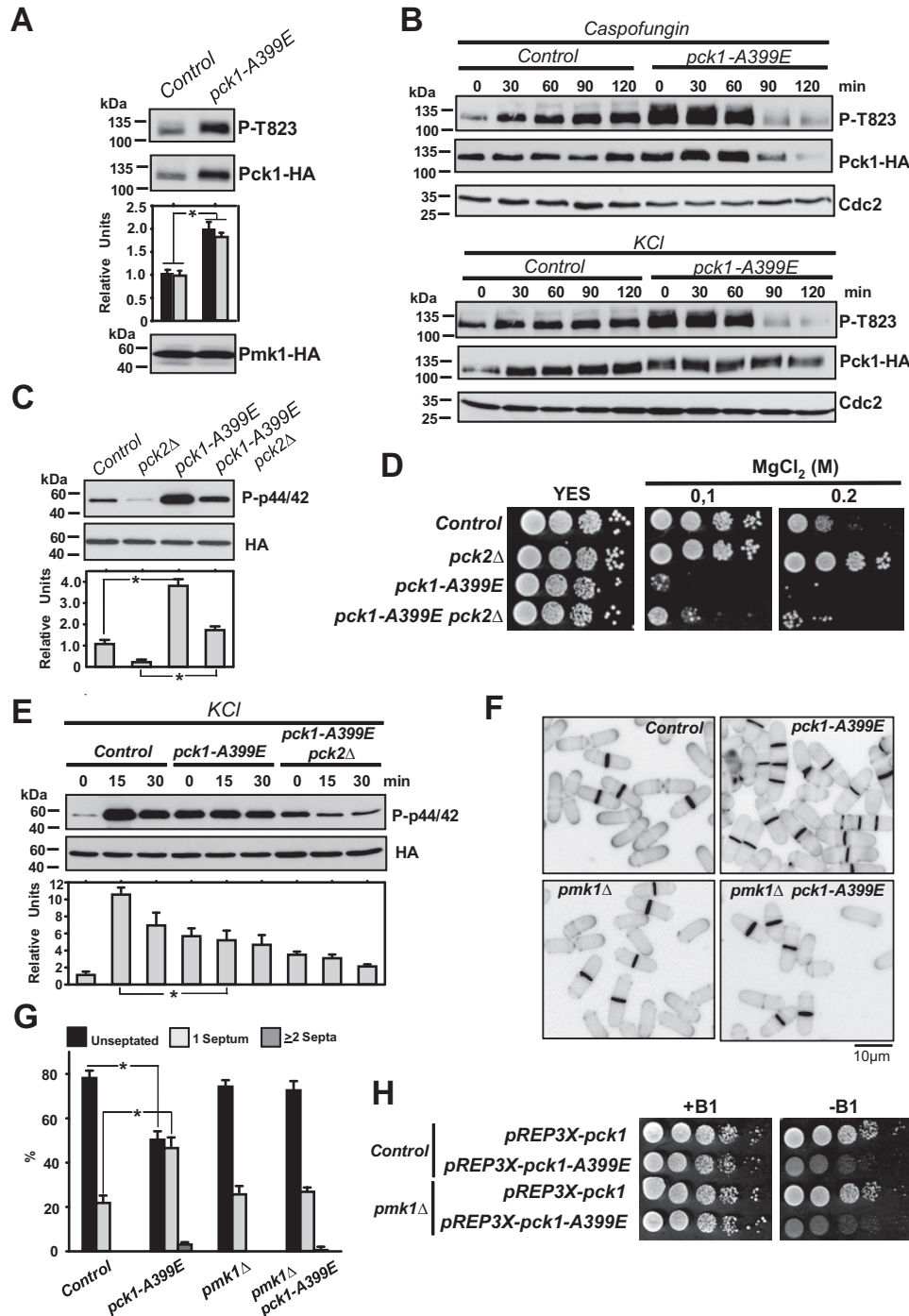
Exponentially growing fission yeast cultures show 20–25% of septated cells (Fig. 5, F and G, Control). Remarkably, *Pck1-A399E* cells showed strong cytokinesis defects with a notable increase in both septated and multiseptated cells (~50% and ~5% of the total cell number, respectively; Fig. 5, F and G). Constitutive activation of Pmk1 appears to be responsible for this defect because it was mostly suppressed by simultaneous deletion of Pmk1 (Fig. 5, F and G). However, despite the functional relationship between constitutive Pck1 activity and Pmk1 function, overexpression of Pck1-A399E was lethal in either control or *pmk1 Δ* cells (Fig. 5H), suggesting that Pck1 can modulate morphogenesis and/or cell growth through both Pmk1-dependent and -independent mechanisms.

Distinct regulation of PKC isoforms

Pck1 is a main *Rho1* effector during the control of cell growth and cell integrity signaling

The essential Rho GTPase *Rho1* is involved, together with *Rho2*, in the activation of the CIP during vegetative growth and in response to cell wall damage (12). Cells expressing the hypomorphic *Rho1* allele *rho1-596* show a thermosensitive phenotype and are hypersensitive to caspofungin (Fig. 6A) (24). Notably, both phenotypes were partially suppressed by the *Pck1-A399E* PS-mutated protein (Fig. 6A). The high percentage in septated and multiseptated cells present in the *Pck1-A399E* mutant (~50% and ~5%, respectively) was alleviated in

a *rho1-596 pck1-A399E* background (~27% and ~0.5% of septated and multiseptated cells, respectively; Fig. 6B). On the contrary, *rho1-596* thermosensitivity was aggravated by the simultaneous presence of the equivalent *Pck2-A392E* mutated protein (supplemental Fig. S2). The low total and Thr(P)⁸²³ *Pck1* levels present in *rho1-596* cells during growth and in response to stress (KCl) were partly restored in a *rho1-596 pck1-A399E* double mutant strain (Fig. 6C). The increase in basal *Pmk1* phosphorylation displayed by the *rho1-596* hypomorphic allele (24) and *pck1-A399E* cells (Fig. 5C) was significantly reduced in a *rho1-596 pck1-A399E* background (Fig.



6D). Moreover, the enhanced Pmk1 activity displayed by *pck1-A399E* cells was strongly alleviated in a *rho1-596 rho2Δ* double mutant compared with *rho2Δ* cells (Fig. 6E). Altogether, these results support the functional relevance of activated Pck1 as a main Rho1 effector during the control of cell growth and cell integrity signaling.

Catalytic activity is essential for Pck1 but not Pck2 function and promotes destabilization of both kinases

To gain more insight into how catalytic activation of Pck1 and Pck2 affects their stability and functions, we generated strains expressing catalytically inactive Pck1-*D789N* and Pck2-*D808N*, in which the conserved catalytic aspartate was substituted by asparagine, thus maintaining the integrity of the ATP binding pocket (Fig. 7A) (25). Mammalian PKCs carrying this mutation are constitutively phosphorylated (primed) within the AL, TM, and HM as they become protected from dephosphorylation (25). Compared with control cells, total and Thr(P)⁸⁴² Pck2 levels were detected in cells carrying *pck2-D808N* as shifted/slower migrating bands reminiscent of increased phosphorylation (Fig. 7B), and the total amount of either Pck2-*D808N* or Pck2-*A392E D808N* was approximately twice that of control cells. Importantly, the difference in Thr⁸⁴² phosphorylation was negligible in both *pck2-D808N* cells and in a *pck2-A392E D808N* double mutant (Fig. 7B), suggesting that intrinsic catalytic activity is responsible for destabilization triggered after activation of Pck2. These mutants also showed very low basal Pmk1 phosphorylation (Fig. 7C). Moreover, Pck2-*A392E D808N* cells failed to activate Pmk1 in response to salt stress, confirming that catalytic activation of Pck2 is essential for this response (Fig. 7D). Similar to the *pck2-D808N* mutant, total and Thr(P)⁸²³ Pck1 levels were present in *pck1-D789N* cells as multiple species with reduced electrophoretic mobility, and the total amount of either Pck1-*D789N* or Pck1-*A399E D789N* was approximately two times higher than in control cells (Fig. 7E). Notably, introduction of the *D789N* mutation in *pck1-A399E* suppressed both the increase in Pmk1 activity and the multiseptated phenotype shown by cells expressing the constitutively active PS mutant (Fig. 7, F and G). The negative effect of the *D789N* mutation was evident as the growth sensitivity to caspofungin of both *pck1-D789N* and *pck1-A399E D789N* cells was even higher than in *pck1Δ* cells

(Fig. 7H). However, contrary to *pck2Δ* cells, *pck2-D808N* and *pck2-A392E D808N* cells were moderately growth-resistant in the presence of this compound (Fig. 7I). Therefore, although Pck1 functions appear to be strictly dependent upon its catalytic activity, inactive Pck2 is biologically functional to a certain extent. Our results also indicate that intrinsic kinase activity promotes destabilization of both Pck1 and Pck2.

Discussion

The PKC orthologs Pck1 and Pck2 share an essential role to modulate cell growth and morphogenesis in fission yeast (6, 7). Taking into account their redundant functions and strong structural similarity in the regulatory and catalytic domains, it might be anticipated that the mechanisms responsible for catalytic activation and stabilization of both kinases should be identical. We found that, similar to Pck2 (14) and most PKC isoforms from higher eukaryotes (2, 26), the conserved Thr⁸²³ within the AL of Pck1 becomes phosphorylated *in vivo* by Ksg1, the fission yeast PDK ortholog. We also confirmed that, as in Pck2, TORC2 plays a major role to positively regulate Pck1 levels during growth and in response to most stresses. Notably, Pck1 up-regulation was clearly abrogated in *tor1Δ* cells during cell wall stress or glucose starvation but only slightly limited during salt stress. The nature of the above stresses is very different, and therefore it is likely that this specific treatment was not of enough strength for the effect to manifest in a clear fashion. In any case, despite the above similarities, we provide compelling evidence that regulation of catalytic activation and stabilization of Pck1 and Pck2 has a remarkably distinct biological impact on both kinases (Fig. 8).

The intracellular levels of most mammalian PKCs are strongly reduced in the absence of AL phosphorylation (2). For example, PKC α AL dephosphorylation decreases its sumoylation, which, in turn, promotes its ubiquitination and ultimately enhances its degradation via the ubiquitin-proteasome pathway (27). We observed that Ksg1-dependent *in vivo* AL phosphorylation is also a major mechanism controlling Pck1 stability and biological functions. In strong contrast, Pck2 protein levels, downstream signaling to the CIP, and biological functions are not significantly altered in the non-phosphorylatable AL mutant Pck2-*T842A* with respect to control cells (14). Moreover, although individual and/or combined mutations at

Figure 5. Constitutive catalytic activation stabilizes Pck1 and interferes with proper downstream signaling to the CIP triggered by Pck2. A, cell extracts from growing cultures of strains MM1578 (*pck1:HA*, control) and MM1746 (*pck1-A399E:HA*) were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸²³ and anti-HA antibodies. Relative units as mean \pm S.D. (biological triplicates) for total (gray columns) and Thr(P)⁸²³ (black columns) Pck1 levels were determined with respect to the internal control (*anti-Pmk1-HA blot*). *, $p < 0.05$; calculated by unpaired Student's *t* test. B, growing cultures of strains MM1578 (*pck1:HA*, control) and MM1746 (*pck1-A399E:HA*) were treated with either 1 μ g/ml caspofungin or 0.6 M KCl. Cell extracts were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸²³ and anti-HA antibodies. Anti-cdc2 was used as a loading control. Results representative of two independent experiments are shown. C, strains MM1578 (*pck1:HA*; control), MM2096 (*pck2Δ*), MM1746 (*pck1-A399E:HA*), and BV630 (*pck1-A399E:HA pck2Δ*), expressing genomic *pmk1:HA6H* fusions, were grown in YES medium, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 phosphorylation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*). *, $p < 0.05$; calculated by unpaired Student's *t* test. D, serially diluted cells of the strains described in C were spotted on YES plates supplemented with 0.1–0.2 M MgCl₂, and incubated for 3 days at 28 °C. Results representative of three independent experiments are shown. E, the strains described in C were grown in YES medium and treated with 0.6 M KCl. Aliquots were harvested at the times indicated, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 phosphorylation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*) at each time point. *, $p < 0.05$; calculated by unpaired Student's *t* test. F, the cell morphology of strains MM1578 (*pck1:HA*; control), MM1904 (*pmk1Δ*), MM1746 (*pck1-A399E:HA*), and MM2119 (*pck1-A399E:HA pmk1Δ*) was analyzed by fluorescence microscopy after staining with Calcofluor white. G, the percentage of unseptated/septated/multiseptated cells (as mean \pm S.D.; total number of cells \geq 400) in exponentially growing cultures of strains described in F was determined by fluorescence microscopy after cell staining with Calcofluor white. *, $p < 0.05$; calculated by unpaired Student's *t* test. H, control and *pmk1Δ* strains were separately transformed with plasmids pREP3X-*pck1:HA* (Control) and pREP3X-*pck1-A399E:HA*. Serially diluted cells from the above transformants were spotted on EMM2 plates with or without 5 mM thiamine (B1) and incubated for 4 days at 28 °C. Results representative of three independent experiments are shown.

Distinct regulation of PKC isoforms

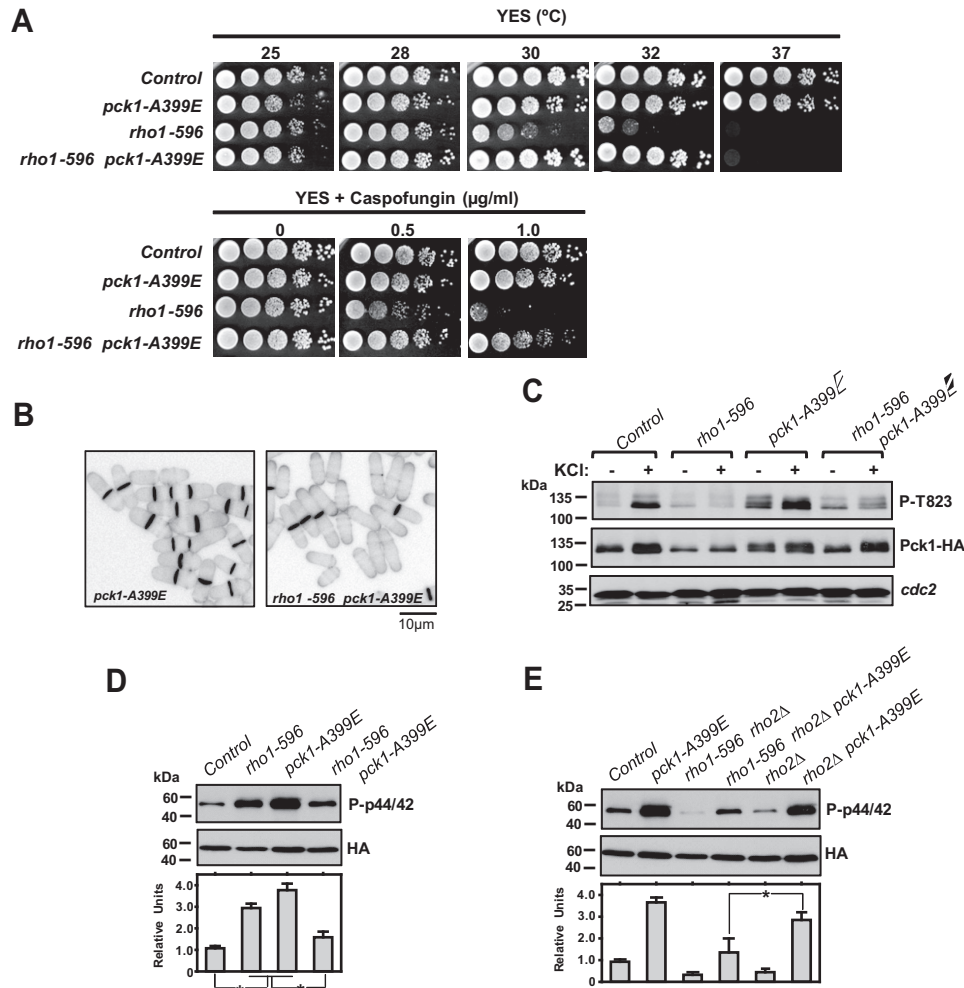


Figure 6. Pck1 is the main Rho1 effector during control of cell growth and cell integrity signaling. *A*, serially diluted cells of strains MM1578 (*pck1:HA*; Control), MM1746 (*pck1-A399E:HA*), MM2139 (*pck1:HA rho1-596*), and MM2143 (*pck1-A399E:HA rho1-596*), were spotted on YES plates and incubated for 3 days at 25, 28, 30, 32, and 37 °C (top panels) and YES plates supplemented with either 0.5 or 1 µg/ml caspofungin, and incubated for 3 days at 28 °C (bottom panels). Results representative of three independent experiments are shown. *B*, the cell morphology of strains MM1746 (*pck1-A399E:HA*) and MM2143 (*pck1-A399E:HA rho1-596*) growing in YES medium was analyzed by fluorescence microscopy after staining with Calcofluor white. *C*, growing cultures of strains described in *A* were treated with 0.6 M KCl for 60 min. Cell extracts were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸²³ and anti-HA antibodies. Anti-*cdc2* was used as a loading control. Results representative of two independent experiments are shown. *D*, the strains described in *A* and expressing a genomic Pmk1-HA6H fusion were grown in YES medium, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean ± S.D. (biological triplicates) for Pmk1 phosphorylation (anti-phospho-p44/42 blot) were determined with respect to the internal control (anti-HA blot). *, $p < 0.05$; calculated by unpaired Student's *t* test. *E*, strains MM1578 (*pck1:HA*, Control), MM1746 (*pck1-A399E:HA*), MM2140 (*pck1:HA rho1-596 rho2Δ*), MM2144 (*pck1-A399E:HA rho1-596 rho2Δ*), MM2135 (*pck1:HA rho2Δ*), and MM2183 (*pck1-A399E:HA rho2Δ*), expressing genomic *pmk1:HA6H* fusions, were grown in YES medium, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean ± S.D. (biological triplicates) for Pmk1 phosphorylation (anti-phospho-p44/42 blot) were determined with respect to the internal control (anti-HA blot). *, $p < 0.05$; calculated by unpaired Student's *t* test.

the putative *in vivo* phosphorylation TM and HM sites had an overall negative effect on Pck1 levels and function, this effect was not shown in the respective Pck2 mutant counterparts. Hence, fission yeast Pck1 resembles the majority of mammalian PKC family members, where maturation is dependent upon phosphorylation at conserved AL, TM, and HM sites within the catalytic domain, and converts newly synthesized kinases into a stable, degradation-resistant conformation (1). Our results also suggest that newly synthesized Pck1 is constitutively phosphorylated by PDK (Ksg1) at the AL residue (Thr⁸²³) and that failure to do so down-regulates Pck2 signaling to the CIP. Although presently unknown, this putative interference mechanism might be due to PDK trapping by the unphosphorylatable *pck1-T823A* mutant and the ensuing defect in Pck2 activation. Con-

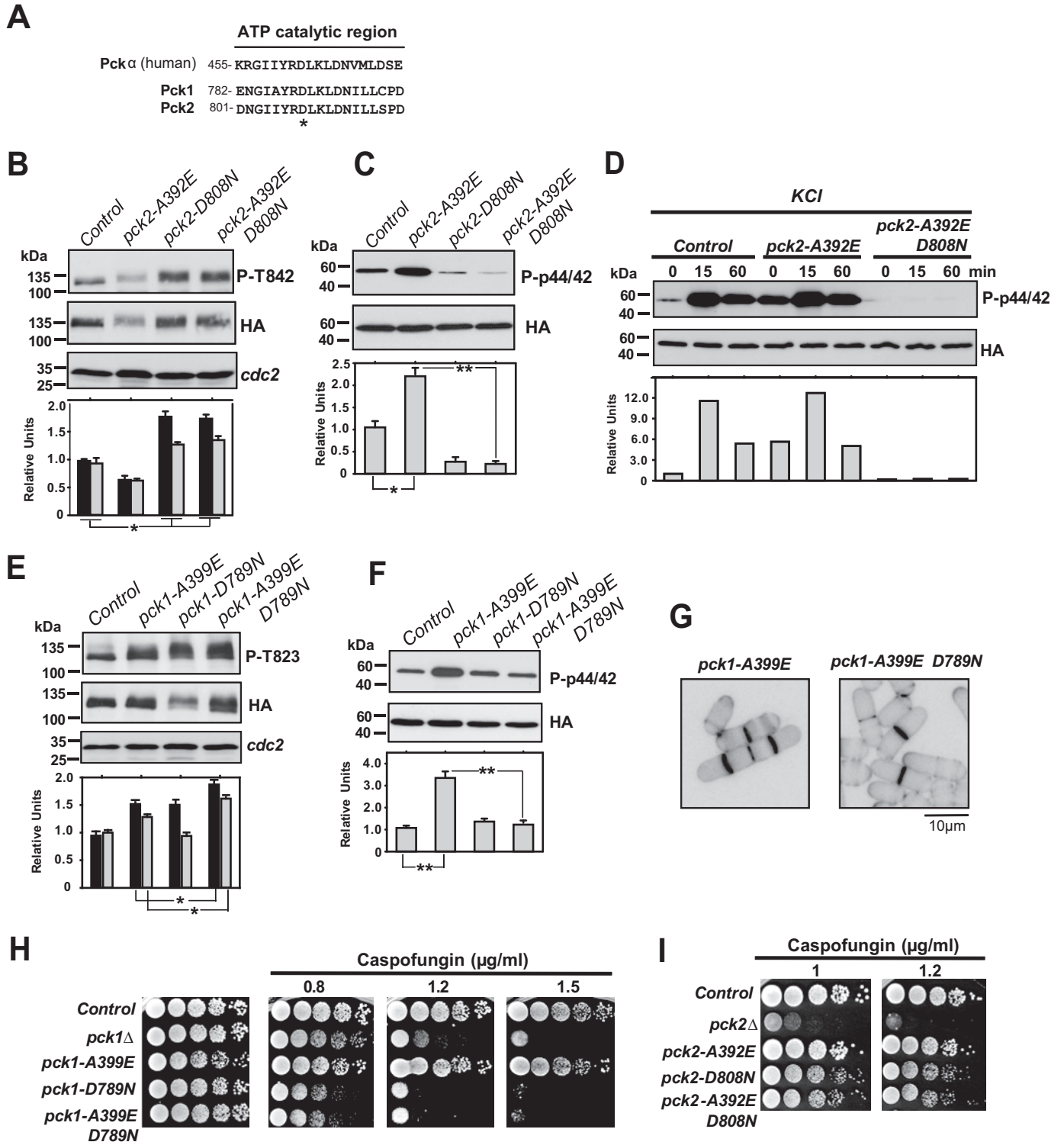
trarily, the fact that the *pck2-T842A* mutant is stable and functional strongly suggest that, in wild-type cells, Pck2 might exist as both AL-phosphorylated and -unphosphorylated isoforms (Fig. 8).

Mammalian PKCs bear a pseudosubstrate segment that blocks the substrate binding cavity and protects the priming AL, TM, and HM phosphosites within the catalytic domain from dephosphorylation and destabilization (1, 20). This model predicts that deletion or a point mutation of the PS domain constitutively activates the kinase and elicits its dephosphorylation and degradation, as shown for several PKC isoforms. Introduction of the PS mutation in Pck2 (*pck2-A392E* cells) increased its function as upstream activator of the CIP during vegetative growth but, at the same time, promoted the destabi-

lization of the kinase. The mechanism(s) responsible for Pck2 degradation might thus be similar to those present in mammalian PKCs. Remarkably, constitutive activation in the *pck1-A392E* PS mutant also enhanced its activity but, in contrast to Pck2, increased Pck1 phosphorylation and stability. Considering that the half-life of Pck1 is longer than that of Pck2, our observations depict a model where activated Pck2 might be

prone to rapid dephosphorylation and degradation whereas Pck1 is not (Fig. 8).

Nucleotide pocket occupation, but not intrinsic kinase activity, is necessary for PKC priming and maturation because kinase-inactive mutants that maintain the integrity of the ATP binding pocket are constitutively primed (25). Similarly, autophosphorylation does not compromise phosphorylation



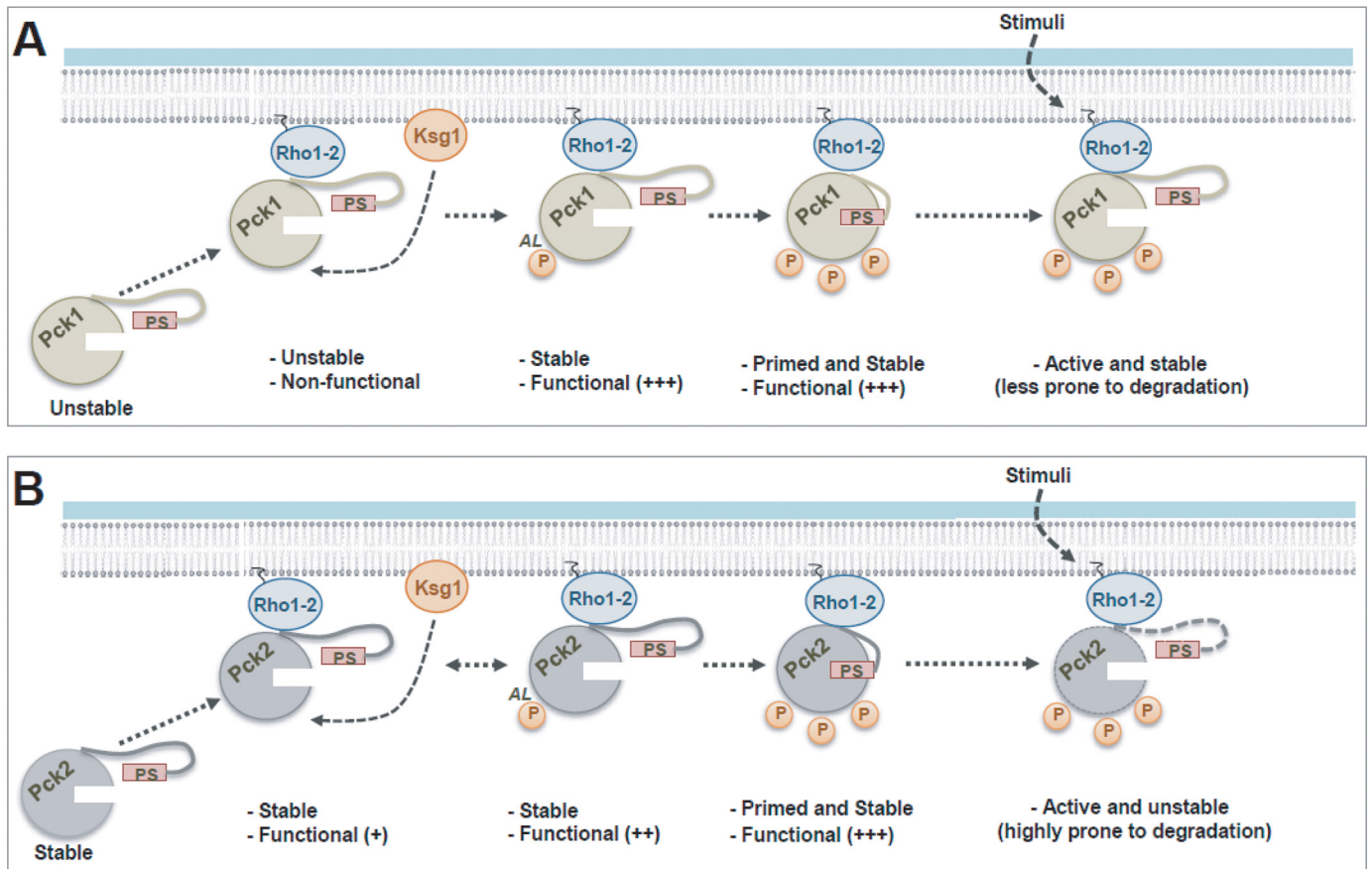


Figure 8. Proposed model for differential functional regulation of the fission yeast PKC orthologs Pck1 and Pck2. *A*, when synthesized, highly unstable Pck1 must be phosphorylated *in vivo* within the AL by Ksg1 (PDK) to promote kinase stabilization and biological functions. *B*, contrarily, Pck2 exists as a mixture of AL-phosphorylated and -unphosphorylated isoforms because AL-unphosphorylated Pck2 is stable and is partially functional (+) in the absence of kinase activity. Both kinases become fully phosphorylated within the catalytic domain to adopt a mature and primed conformation (+++); however, although activated Pck2 is prone to rapid degradation, active Pck1 remains stable and functional for longer times. Differential regulation of the stability and function of both kinase isoforms may allow for a graded and flexible control of their shared and specific downstream signaling functions.

and maturation of Pck1 and Pck2, as confirmed after introducing in both kinases the catalytic aspartate mutation (*D789N* and *D808N* mutants, respectively). Importantly, intrinsic catalytic activity is a determinant for destabilization triggered after constitutive activation of Pck2, as evidenced by the recovery in phosphorylated and total Pck2 levels by *pck2-A392E D808N* cells in comparison with the single pseudosubstrate

pck2-A392E mutant cells. In addition, the use of fully primed catalytic aspartate mutants described above allows us to formally demonstrate that, although catalytic activity is essential for Pck1 functions, Pck2 remains partially functional in the absence of intrinsic kinase activity. Our findings suggest that the existence of biological functions without kinase activity represents a common theme within the extended

Figure 7. Catalytic activity is essential for Pck1 function, whereas Pck2 remains partially functional in the absence of intrinsic kinase activity. *A*, amino acid sequence alignment of the ATP catalytic region present in human PKC α and fission yeast Pck1 and Pck2. The conserved catalytic aspartic acid residue within the motif is marked with an asterisk. *B*, cell extracts from growing cultures of strains MM913 (*pck2:HA*; control), BV813 (*pck2-A392E:HA*), BV623 (*pck2-D808N:HA*), and BV625 (*pck2-A392E D808N:HA*) were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸⁴² and anti-HA antibodies. Relative units as mean \pm S.D. (biological triplicates) for total (gray columns) and Thr(P)⁸⁴² (black columns) Pck2 levels were determined with respect to the internal control (*anti-cdc2 blot*). *, $p < 0.05$; calculated by unpaired Student's *t* test. *C*, the strains described in *B* and expressing a genomic *pmk1:HA6H* fusion were grown in YES medium, and activated/total Pmk1 were detected with anti-phospho-p44/42 and anti-HA antibodies, respectively. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 phosphorylation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*). *, $p < 0.05$; **, $p < 0.005$; calculated by unpaired Student's *t* test. *D*, the strains described in *B* were grown in YES medium and treated with 0.6 M KCl. Aliquots were harvested at the times indicated, and activated/total Pmk1 were as above. Relative units for Pmk1 phosphorylation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*) at each time point. Results representative of three independent experiments are shown. *E*, cell extracts from growing cultures of strains MM1578 (*pck1:HA*; Control), MM1746 (*pck1-A399E:HA*), BV627 (*pck1-D789N:HA*), and BV629 (*pck1-A399E D789N:HA*) were resolved by SDS-PAGE and hybridized separately with anti-Thr(P)⁸²³ and anti-HA antibodies. Relative units as mean \pm S.D. (biological triplicates) for total (gray columns) and Thr(P)⁸²³ (black columns) Pck1 levels were determined with respect to the internal control (*anti-cdc2 blot*). *, $p < 0.05$; calculated by unpaired Student's *t* test. *F*, the strains described in *E* and expressing a genomic *pmk1:HA6H* fusion were grown in YES medium, and activated/total Pmk1 were detected as above. Relative units as mean \pm S.D. (biological triplicates) for Pmk1 phosphorylation (*anti-phospho-p44/42 blot*) were determined with respect to the internal control (*anti-HA blot*). **, $p < 0.005$; calculated by unpaired Student's *t* test. *G*, the cell morphology of strains MM1746 (*pck1-A399E:HA*) and BV629 (*pck1-A399E D789N:HA*) was analyzed by fluorescence microscopy after staining with Calcofluor white. *H*, serially diluted cells of strains GB35 (*pck1 Δ*) plus those described in *F* were spotted on YES plates supplemented with 0.8–1.2–1.5 μ g/ml caspofungin and incubated for 3 days at 28 $^{\circ}$ C. Results representative of three independent experiments are shown. *I*, serially diluted cells of strains GB3 (*pck2 Δ*) plus those described in *B* were spotted on YES plates supplemented with 1–1.2 μ g/ml caspofungin and incubated for 3 days at 28 $^{\circ}$ C. Results representative of three independent experiments are shown.

PKC superfamily and might be attained early during evolution.

All the structural and regulatory elements that appear to be distributed among the members of the large mammalian PKC family are present in Pck1, the single and archetypal PKC enzyme present in *S. cerevisiae* (3). For the most part, this domain structure is similarly conserved in fission yeast Pck1 and Pck2 (Fig. 1A). Therefore, from an evolutionary perspective, these two functionally redundant kinases may represent a prime example in the expansion of the PKC superfamily from a common and single ancestor. Most importantly, our results strongly suggest that, in fission yeast, PKC duplication was accompanied by changes in the mechanisms that regulate catalytic activation and stability of the two kinase isoforms. The biological relevance of these distinct regulatory mechanisms is exemplified when MAPK Pmk1 activity is used as readout for Pck1 and Pck2-dependent downstream signaling. Strong Pmk1 activation in response to osmotic saline stress is a quick and transient event, does not require new protein synthesis, and is totally dependent on Pck2 catalytic activity (13, 14). Indeed, Pck2 appears to be perfectly suited to perform this role because, contrary to Pck1, constitutive activation elicits its dephosphorylation and degradation, thus decreasing the magnitude of the signal at long incubation times. However, Pmk1 activation under cell wall stress takes place in a progressive manner until reaching its maximum at long incubation times and depends on the enhanced synthesis and activity of both Pck1 and Pck2 (12, 14). In this situation, catalytic activation and induced stabilization of Pck1 might lead to a graded and robust downstream signaling to the MAPK module that increases with time. Differential regulation of Pck1 and Pck2 activation and/or stability may be important for the distinct roles of both kinases during cellular response to short- versus longer-term stress. We found that, compared with control cells, *pck2*Δ cells exhibited a defective growth recovery phenotype after being subjected to severe thermal stress (55 °C) during relatively short periods of time (supplemental Fig. S3). Notably, compared with control cells, we found that *pck2*Δ cells exhibited a defective growth recovery phenotype in response to heat shock, whereas cells expressing AL (*T842A*) and catalytically inactive (*D808N*) Pck2 alleles showed a fairly good growth recovery under the same conditions (supplemental Fig. S3). On the other hand, the equivalent Pck1 mutants showed a similar and very modest growth defect compared with control cells (supplemental Fig. S3). These results reinforce the suggestion that both catalytically active and inactive Pck2 isoforms might have a more prominent role than Pck1 in promoting cell survival in response to short-term stresses. At the same time, Pck1 and Pck2 activation status must be tightly coordinated *in vivo*, as indicated by the observation that cells expressing the up-regulated *pck1-A392E* allele hyperactivate the CIP constitutively in a Pck2-independent fashion and interfere with proper downstream signaling to the MAPK cascade by Pck2.

In higher eukaryotes, precise control of the amplitude of PKC signaling is essential for cellular homeostasis, and disruption of this control may lead to different pathophysiological states (28). Our results suggest that alternative regulation of the stability and PDK-mediated phosphorylation of both kinases emerged

as major factors to allow for a precise control of PKC signaling during the early diversification of this large and functionally relevant class of enzymes.

Experimental procedures

Strains, media, growth conditions, and gene disruption

The *S. pombe* strains used in this work (supplemental Table S1) were derived from control strain MI200 and express a genomic Pmk1-HA fusion (11). They were grown in rich (YES (0.5% yeast extract, 2% glucose)) or minimal (EMM2) medium with 2% glucose plus supplements (29). Transformants expressing pREP3X-based plasmids were grown in liquid EMM2 medium with thiamine (5 mg/liter) and either plated in solid medium with or without the vitamin or transferred to EMM2 lacking thiamine.

Gene fusion, site-directed mutagenesis, and expression plasmids

To construct the integrative plasmid pJK148-Pck1:HA, the *pck1*⁺ ORF plus regulatory sequences were amplified by PCR using fission yeast genomic DNA as a template and the 5'-oligonucleotide Pck1.XbaI-F (supplemental Table S2), which hybridizes 882–862 bp upstream of the *pck1*⁺ ATG start codon and contains a XbaI site, and the 3'-oligonucleotide Pck1HASmaI-R, which hybridizes at the 3' end of *pck1*⁺ ORF and incorporates a 64-nt sequence encoding one HA epitope (sequence GYPYDVPDYA) and a SmaI site. PCR fragments were digested with XbaI and SmaI and cloned into the integrative plasmid pJK148. The Pck1 ORF contains a NruI site that was deleted using the mutagenic 5'-oligonucleotide Pck1NruIX-F and the 3'-oligonucleotide Pck1NruIX-R with plasmid pJK148-Pck1:HA as a template. The mutagenized Pck1 sequence was digested with XbaI and SmaI and subcloned to generate pJK148-Pck1NruIΔ:HA. Integrative plasmids expressing HA-fused Pck1 mutants were obtained by site-directed mutagenesis PCR using plasmid pJK148-Pck1NruIΔ:HA as a template and the mutagenic oligonucleotide pairs described in supplemental Table S2. When confirmed, the mutagenized Pck1 sequences were subcloned into pJK148. The resulting integrative plasmids were digested at the unique NruI site within *leu1*⁺ and transformed into *pck1*Δ strain GB35 (supplemental Table S1). Transformants prototrophic for leucine were obtained, and the fusions were verified by both PCR and Western blot analysis. The integrative plasmids pJK148-Pck2.A392E:HA and pJK148-Pck2.D808N:HA were obtained by site-directed mutagenesis PCR using plasmid pJK148-Pck2NruIΔ:HA (14) as a template and the mutagenic oligonucleotide pairs described in supplemental Table S2. Mutant Pck1 overexpression constructs were obtained by site-directed mutagenesis PCR using plasmid pREP3X-*pck1*⁺ (7) as a template and the correspondent mutagenic oligonucleotide pairs (supplemental Table S2).

A GST-fused wild-type Pck1 construct was obtained by PCR employing a fission yeast cDNA library as a template and the oligonucleotides Pck1pKG-F (SmaI site) and Pck1pKG-R (XbaI site). The PCR product was then digested with SmaI and XbaI and cloned into plasmid pGEX-KG (30) to generate pGEX-KG-Pck1. Mutant Pck1 constructs were obtained by site-directed mutagenesis using pGEX-KG-Pck1 as a template and the cor-

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responding mutagenic oligonucleotide pairs (supplemental Table S2), digested with SmaI and XbaI, and cloned into pGEX-KG. Ksg1-GST fusion was obtained by PCR employing the oligonucleotide pair Ksg1pKG-F (SmaI site) and Ksg1pKG-F (NcoI site). A kinase-dead version of Ksg1 (K128R mutant) was obtained by site-directed mutagenesis with plasmid pGEX-KG-Ksg1 as a template (14) and the mutagenic oligonucleotides Ksg1K128R-F and Ksg1K128R-R (supplemental Table S2). Constructs were digested with SmaI and NcoI and cloned into pGEX-KG.

Kinase assays

In vitro kinase reactions were performed as described previously (31) with purified bacterially expressed GST-Ksg1 or GST-Ksg1-K128R (kinase-dead) as activating kinases and either wild-type or mutant GST-fused Pck1 constructs as substrates. GST-tagged fusions were detected with polyclonal goat anti-GST antibody conjugated to horseradish peroxidase (GE Healthcare) and the ECL system.

Stress treatments and detection of activated Pmk1

Log-phase cell cultures ($A_{600} = 0.5$) were supplemented with either KCl (Sigma), caspofungin (Sigma), or Calcofluor white (Sigma). In glucose starvation experiments, cells grown in YES medium with 7% glucose were resuspended in the same medium lacking glucose and osmotically equilibrated with 3% glycerol. Preparation of cell extracts, purification of HA-tagged Pmk1 with nickel-nitrilotriacetic acid-agarose beads (Qiagen), and SDS-PAGE was performed as described previously (11). Dual phosphorylation in Pmk1 was detected with rabbit polyclonal anti-phospho-p44/42 (Cell Signaling Technology), whereas total Pmk1 was detected with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals). Immunoreactive bands were revealed with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Sigma) and the ECL system (Amersham Biosciences-Pharmacia).

Detection of total and phosphorylated Pck1 and Pck2

Cell extracts were prepared using buffer IP (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM β -mercaptoethanol, 10% glycerol, 0.1 mM sodium orthovanadate, 1% Triton X-100, and protease inhibitors). Equal amounts of total protein were resolved in 6% SDS-PAGE gels and transferred to Hybond-ECL membranes. AL phosphorylation of Pck2 at Thr⁸⁴² was detected using a specific anti-Thr(P)⁸⁴² antibody as described previously (14). To detect AL phosphorylation of Pck1 at Thr⁸²³, an anti-phospho-polyclonal antibody was produced by immunization of rabbits with a synthetic phosphopeptide corresponding to residues surrounding Thr⁸²³ of Pck2 (GenScript). Total Pck2 and Pck1 were detected with mouse monoclonal anti-HA antibody. Anti-PSTAIR (anti-Cdc2, Sigma) was used as a loading control.

Quantification of Western blot experiments and reproducibility of results

Quantification of Western blot signals was performed using ImageJ (32). Briefly, bands plus background were selected or drawn as rectangles, and a profile plot was obtained for each

band (peaks). To minimize the background noise in the bands, each peak floating above the baseline of the corresponding profile plot was manually closed off using the straight-line tool. Finally, measurement of the closed peaks was performed with the wand tool. Relative units for Pmk1 activation were estimated by determining the signal ratio of the anti-phospho-p44/42 blot (activated Pmk1) with respect to the anti-HA blot (total Pmk1) at each time point. Relative units for phosphorylated and total Pck1/Pck2 levels were estimated by determining the signal ratio of either anti-phospho-P842 (AL-phosphorylated Pck2), anti-phospho-P823 (AL-phosphorylated Pck2), or anti-HA blot (total Pck2 or Pck1) with respect to the anti-cdc2 blot (internal control) at each time point. Unless otherwise stated, results shown correspond to experiments performed as biological triplicates. Mean relative units \pm S.D. and/or representative results are shown. The *p* values were analyzed by unpaired Student's *t* test.

Plate assay of stress sensitivity for growth

Decimal dilutions of *S. pombe* control and mutant strains were spotted per duplicate on YES solid medium or in the same medium supplemented with different concentrations of MgCl₂ (Sigma), FK506 (Alexis Biochemicals), Calcofluor white, or caspofungin. Plates were incubated at 28 °C for 3 days and then photographed. Results representative of three independent experiments are shown in the corresponding figures.

Fluorescence microscopy

Calcofluor white was employed for cell wall/septum staining as described previously (18). Images were taken on a Leica DM 4000B fluorescence microscope with a $\times 100$ objective and captured with a cooled Leica DC 300F camera and IM50 software. To determine the percentage of multiseptated cells, the number of septated cells was scored in each case ($n \geq 400$).

Author contributions—M. M. and J. C. conceived and designed the experiments. M. M., B. V. M., T. S., A. F., J. V. S., and E. G. G. performed the experiments. M. M., P. P., M. G., and J. C. analyzed the results and prepared the figures. J. C. wrote the main manuscript text with input from M. M., P. P., and M. G. All authors reviewed and approved the final version of the manuscript.

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