

## PDK1 Homologs Activate the Pkc1–Mitogen-Activated Protein Kinase Pathway in Yeast

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**PDK1 (phosphoinositide-dependent kinase 1) is a mammalian growth factor-regulated serine/threonine kinase. Using a genetic selection based on a mutant form of the yeast MAP kinase kinase Ste7, we isolated a gene, *PKH2*, encoding a structurally and functionally conserved yeast homolog of PDK1. Yeast cells lacking both *PKH2* and *PKH1*, encoding another PDK1 homolog, were nonviable, indicating that Pkh1 and Pkh2 share an essential function. A temperature-sensitive mutant, *pkh1*<sup>D398G</sup> *pkh2*, was phenotypically similar to mutants defective in the Pkc1–mitogen-activated protein kinase (MAPK) pathway. Genetic epistasis analyses, the phosphorylation of Pkc1 by Pkh2 in vitro, and reduced Pkc1 activity in the *pkh1*<sup>D398G</sup> *pkh2* mutant indicate that Pkh functions upstream of Pkc1. The Pkh2 phosphorylation site in Pkc1 (Thr-983) is part of a conserved PDK1 target motif and essential for Pkc1 function. Thus, the yeast PDK1 homologs activate Pkc1 and the Pkc1-effector MAPK pathway.**

Mammalian PDK1 was first identified as a kinase that activates protein kinase B (PKB) (2, 39). PKB is a growth factor-regulated serine/threonine kinase that contains a pleckstrin homology (PH) domain at its amino-terminal end. Binding of phosphoinositide 3-OH kinase products to the PH domain results in translocation of PKB to the plasma membrane, where it is activated by phosphorylation. In response to growth factor treatment of cells, PKB becomes phosphorylated at two major sites, Thr-308 in the kinase domain and Ser-473 in the C-terminal tail (1). PDK1 phosphorylates Thr-308 in PKB, resulting in its activation. This PDK1-PKB cascade mediates the physiological effects of insulin and of several growth factors and stimuli. Furthermore, PKB plays a role in mediating the protective effects of survival factors against apoptosis (11, 20, 21). PDK1 has been shown to phosphorylate and activate a number of other protein kinases such as p70<sup>S6K</sup> (3, 34) and PKC isoforms (24).

Extracellular molecules that regulate cell proliferation and differentiation in eukaryotes exert their effects through pathways that transmit signals from the cell surface through the cytoplasm to the nucleus. Some of these signals are transmitted by protein kinase cascades that involve mitogen-activated protein kinases (MAPKs). MAPKs are activated by phosphorylation on both tyrosine and threonine residues, catalyzed by a family of dual-specificity MAPK kinases (MAPKKs). MAPKKs are in turn phosphorylated and activated by MAPKK kinases (MAPKKKs). This sequential activation of MAPKKK, MAPKK, and MAPK constitutes the MAPK module (16, 22, 35).

Elements of MAPK activation pathways have been conserved across evolution, as evidenced by the existence of MAPK pathways in organisms ranging from yeast to mammals. In *Saccharomyces cerevisiae*, several MAPK cascade modules

that mediate distinct responses to different extracellular or cell autonomous signals have been identified (4, 13, 15, 27). For example, the mating pheromone response pathway is activated by peptide pheromones and prepares cells for mating (23). The Pkc1-activated pathway, which includes the Mpk1 MAPK, responds to heat stress and hypotonic shock, and it controls both cell wall synthesis and organization of the actin cytoskeleton (14, 27). The high-osmolarity glycerol response pathway is activated by hypertonic stress (33).

The yeast MAPK cascade involved in mating comprises Ste11 (MAPKKK), Ste7 (MAPKK), and FUS3/KSS1 (MAPKs) (23). Ste11 is activated by a mechanism that probably involves Ste20 (a PAK-related protein kinase)-dependent phosphorylation. Phosphorylation activates Ste11, which consequently phosphorylates and activates Ste7. Ste11 phosphorylation of Ste7 appears to involve phosphorylation at two residues (Ser-359 and Thr-363) that are located in a region analogous to the phosphorylation lip of MAPKs. Once activated, Ste7 then activates Fus3 and Kss1 by phosphorylation at Thr and Tyr residues in the signature TEY motif within the respective Fus3 and Kss1 MAPK phosphorylation lips. Fus3 and Kss1 function redundantly in the transmission of pheromone-induced signals and induce the transcriptional activation of genes required for the process of mating.

We have recently identified a hyperactive mutant of Ste7, Ste7<sup>S368P</sup>, that is generated by mutation of serine to proline at position 368 (17, 45). Ste7<sup>S368P</sup> stimulates mating-specific transcriptional responses in the absence of mating pheromone. Although activity of Ste7<sup>S368P</sup> is higher than that of the wild-type enzyme in the absence of pheromone stimulation, its activity is still dependent on the presence of the upstream kinase Ste11. Pheromone stimulation causes a further increase in Ste7<sup>S368P</sup> activity to an amount equivalent to that of the fully activated Ste7 (45). These results suggest that Ste7<sup>S368P</sup> requires phosphorylation for full catalytic activity but that some fraction of Ste7<sup>S368P</sup> is present in the modified and active form even without pheromone stimulation. The proline substitution has one other interesting characteristic; it transforms Ste7<sup>S368P</sup>

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into a relatively nonspecific substrate for a variety of heterologous upstream MAPKKs. For example, an activated form of mammalian Raf (Raf $\Delta$ N), but not normal c-Raf-1, can activate Ste7<sup>S368P</sup> but not wild-type Ste7 (17). This relaxed specificity allowed us to identify other MAPKKs as activators of Ste7<sup>S368P</sup>. The genetic potential of this system has already been exploited in the identification of TAK1, a novel member of the MAPKKK family from mammalian cells, as a kinase that activates Ste7<sup>S368P</sup> in a manner homologous to Ste11 (44).

In this study, we have taken advantage of this genetic system to identify yeast genes that activate Ste7<sup>S368P</sup>. We isolated two yeast genes that were capable of activating Ste7<sup>S368P</sup> in a *ste11* $\Delta$  background when expressed from multicopy plasmids. The first gene was *BCK1*, which encodes the MAPKKK of the Pkc1-activated MAPK pathway (10, 25). The second gene, *PKH2*, encodes a protein kinase most similar to mammalian PDK1. We present evidence suggesting that yeast PDK1 homologs, Pkh1 and Pkh2, activate Pkc1 and the Pkc1-effector MAPK pathway. Furthermore, we isolated a multicopy suppressor of a *pkh1*<sup>D398G</sup> *pkh2* mutant. This gene, *PKH3*, encodes a third PDK1 homolog.

#### MATERIALS AND METHODS

**Strains and general methods.** *Escherichia coli* DH5 $\alpha$  was used for DNA manipulation; *E. coli* BL21 was used as the host for expression of heterologous proteins. Standard procedures were followed for yeast manipulations (18). The media used in this study included rich medium, synthetic complete medium (SC), synthetic minimal medium (SD), and sporulation medium. SC lacking amino acids or other nutrients (e.g., SC-Leu lacks leucine) were used to score auxotrophies and to select transformants. Recombinant DNA procedures were carried out as described by Sambrook et al. (37).

**Plasmids.** Plasmid YCpG22-PKH1 expresses *PKH1* under the control of the *GAL1* promoter. The N-terminal portion of the *PKH1* coding sequence was amplified by PCR using a 5' primer (5'-CTCGGATCCATGGGAAATAGGTC TTGACA-3', incorporating a *Bam*HI site) and a 3' primer (5'-ATCTCGTGC TGACATACCCGGGTCATCATTTGCCAGG-3'). A 190-bp *Bam*HI-*Spe*I fragment generated by PCR and a 2.1-kb *Spe*I-*Xho*I fragment containing the C-terminal portion of *PKH1* were inserted into the *Bam*HI-*Sal*I gap of YCpG22 harboring the *GAL1* promoter to generate YCpG22-PKH1 (*GAL1p-PKH1 TRP1*). Plasmids pKT10-GAL-HA2-PKH2 and pKT10-GAL-HA2-PKH2(KN) express influenza virus hemagglutinin epitope (HA)-tagged Pkh2 and Pkh2<sup>K208R</sup>, respectively, under the control of *GAL1* promoter. The N-terminal portion of the *PKH2* coding sequence was amplified by PCR using a 5' primer (5'-CTCGGATCCATGTATTTGATAAGGATAATTCC-3', incorporating a *Bam*HI site), and a 3' primer (5'-CGATAGTCTGTAAACTGTCAATTTAAA-3'). A 206-bp *Bam*HI-*Stu*I fragment generated by PCR and a 3-kb *Stu*I-*Sal*I fragment containing the C-terminal portion of Pkh2 and Pkh2<sup>K208R</sup> were inserted into the *Bam*HI-*Sal*I gap of pKT10-GAL-HA2, harboring the *GAL1* promoter and two copies of an HA tag, to generate pKT10-GAL-HA2-PKH2 and pKT10-GAL-HA2-PKH2(KN), respectively. Plasmid pKT10-PDK1 expresses human PDK1 under the control of the *TDH3* promoter. The 1.6-kb *Eco*RI fragment containing the *PDK1* cDNA (kindly provided by D. Stokoe and F. McCormick) was inserted into the *Eco*RI gap of pKT10 harboring the *TDH3* promoter, generating pKT10-PDK1. Plasmid pYS116 is a YEep-based *URA3* plasmid harboring the *lacZ* reporter gene containing LexA DNA binding sites in its promoter (31). Plasmid pYW71 is a YEep-based *TRP1* plasmid expressing LexA-Rlm1 $\Delta$ N (amino acids 222 to 676) fusion protein from the *ADH1* promoter (43). Plasmids pDL293 and pDL295 (kindly provided by D. Levin) express HA-tagged Pkc1 and Pkc1<sup>K853R</sup>, respectively, under the control of the *GAL1* promoter. Plasmids pE722 and pE738 are YCp-based *URA3* plasmids expressing wild-type Pkc1 and Pkc1<sup>T983A</sup>, respectively. The *pkc1*<sup>T983A</sup> mutant allele was generated by site-directed mutagenesis with PCR, and the mutation was confirmed by DNA sequencing.

**Construction of yeast strains containing deletion alleles of *PKH1* or *PKH2*.** The *pkh1* $\Delta$ ::*URA3* and *pkh2* $\Delta$ ::*URA3* disruption alleles were constructed by the one-step gene replacement method (36). After appropriate conversion of restriction sites, the 1.3-kb *Spe*I-*Bgl*II fragment of *PKH1* and the 0.85-kb *Stu*I-*Sna*BI fragment of *PKH2* were replaced with the 1.1-kb *Bam*HI fragment of *URA3*. The DNAs containing the entire *pkh1* $\Delta$ ::*URA3* and *pkh2* $\Delta$ ::*URA3* constructions were used to transform a diploid strain 15Du by selection for Ura<sup>+</sup>. Restriction mapping and Southern analysis of genomic DNAs from the resulting transformants were conducted to confirm that transplacement had occurred at each locus. The *pkh1* $\Delta$ ::*LEU2* and *pkh2* $\Delta$ ::*LEU2* strains were obtained by replacing *URA3* with *LEU2* in the *pkh1* $\Delta$ ::*URA3* and *pkh2* $\Delta$ ::*URA3* strains, respectively, with plasmid pUL2.

**Isolation of *pkh1* temperature-sensitive mutants.** Temperature-sensitive alleles of *pkh1* were created by the PCR mutagenesis method (29). A region of

*PKH1* (−543 to +2,636) was amplified under mutagenic PCR conditions (50 mM KCl, 10 mM Tris-HCl [pH 8.0]), 2 mM MgCl<sub>2</sub>, 2 mM each dATP, dCTP, dTTP, and dGTP]. After amplification, PCR products were digested and gel purified. The mutagenized PCR products were cotransformed into a strain of *pkh1* $\Delta$ ::*LEU2* *pkh2* $\Delta$ ::*LEU2* carrying YCpG33-PKH1 (*GAL1p-PKH1 URA3*) with gapped plasmids that contain homology to both ends (−543 to −7 and +2,328 to 2,636) of the mutagenized PCR product. The transformants were plated on YPGlu plates and incubated at 25°C. Then the colonies were replica stamped to YPGlu plates at 37°C for selecting temperature-sensitive (ts) colonies. Plasmids were recovered, rescued in *E. coli*, and reintroduced into yeast to confirm the phenotypes.

**Assessment of cell lysis.** Qualitative assessment of cell lysis in colonies was done by an alkaline phosphatase assay (6). Cells were spotted onto YPGlu plates, incubated at 25°C, and then shifted to 37°C. The plates were then overlaid with an alkaline phosphatase assay solution containing 0.05 M glycine hydrochloride (pH 9.5), 1% agar, and 10 mM chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate. Colonies which contained lysed cells stained blue, whereas intact colonies remained white.

**$\beta$ -Galactosidase assays.**  $\beta$ -Galactosidase assays were performed as described previously (18).

**Fluorescence microscopy.** Cells were grown to early logarithmic phase, fixed in formaldehyde, stained with tetramethyl rhodamine isocyanate (TRITC)-phalloidin to visualize the actin cytoskeleton, and observed by fluorescence and Nomarski microscopy as described previously (5, 38).

**Expression of Pkc1 in bacteria.** To express Pkc1 in *E. coli*, 1.3-kb *Sca*I-*Sph*I fragments (787 to 1151 amino acids) of Pkc1<sup>K853R</sup> and Pkc1<sup>K853R, T983A</sup> were inserted into the *Sma*I-*Sal*I gap of pGEX-5T-2 (Pharmacia Biotech) to produce pGEX-PKC1-K853R and pGEX-PKC1-K853R,T983A, respectively. A *pkc1*<sup>K853R</sup> mutant allele was kindly provided by D. Levin, and a *pkc1*<sup>K853R, T983A</sup> mutant was generated by site-directed mutagenesis with PCR. Glutathione *S*-transferase (GST)-Pkc1 fusion proteins were purified as previously described (43).

**Preparation of yeast extracts and immunoprecipitations.** Yeast cells were grown to an optical density (at 600 nm) of 0.5 to 1.0 and treated with 2.5% galactose for 1 h to induce the *GAL1* promoter. After treatment, yeast cultures were quickly chilled, and cells were collected by rapid centrifugation. The cell pellet was washed twice with Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and then suspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM NaF, 1 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mg of leupeptin per ml, 1 mg of pepstatin A per ml, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.4- to 0.6-mm diameter) was added to this suspension, and cells were broken by vigorous vortexing for 5 min at 4°C. The beads and cell debris were removed by centrifugation at 10,000  $\times$  g at 2°C, and the supernatant was further clarified by centrifugation at 100,000  $\times$  g at 2°C. Cell extracts (100 mg of protein) were incubated at 4°C for 2 h with 20  $\mu$ l of protein A-Sepharose beads (Sigma) containing covalently coupled mouse monoclonal HA.11 anti-HA immunoglobulin (Berkeley Antibody). Immune complexes were washed three times with lysis buffer and once with kinase buffer (100 mM Tris-HCl [pH 7.5], 50 mM MgCl<sub>2</sub>). Protein concentrations of cell extracts were measured with Bio-Rad protein determination reagent.

**In vitro phosphorylation assays.** Immunoprecipitated HA-Pkh2 or HA-Pkh2<sup>K208R</sup> was suspended in 20  $\mu$ l of kinase buffer with 70  $\mu$ g of GST-Pkc1 $\Delta$ N fusion proteins. The reaction was initiated by the addition of ATP to a final concentration of 0.1 mM along with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After incubation for 30 min at 30°C, the reaction was terminated by the addition of sodium dodecyl sulfate (SDS) sample buffer, and samples were boiled and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels. After electrophoresis, gels were stained with Coomassie brilliant blue R250 and washed in 45% isopropanol–10% acetic acid. Dried gels were subjected to autoradiography. Pkc1 kinase assays were performed as described previously (41). A synthetic peptide corresponding to the sequence surrounding Ser-939 of Bck1, a phosphorylation site for Pkc1, was used as the substrate in the Pkc1 kinase assays (41).

**Immunoblots.** Immunoprecipitated complexes were subjected to SDS-PAGE on 7.5% acrylamide gels followed by electroblotting onto Immobilon-P membranes (Millipore Corporation). Blots were blocked by incubation for 1 h at room temperature in TBS-M (TBS with 5% nonfat dry milk). Blots were then incubated with monoclonal antibody HA.11 diluted 1:1,000 in TBS-M for 20 h at 4°C. After three washes with TBS-M, blots were incubated for 2 h with peroxidase-linked secondary antibody (Calbiochem) diluted 1:2,500 in TBS-M. After four final washes with TBS-M, blots were developed using an enhanced chemiluminescence detection kit (Amersham).

## RESULTS

**Overexpression of *BCK1* or *PKH2* enhances Ste7<sup>S368P</sup> function.** The MAPK pathway mediating mating pheromone signal transduction is regulated by a cascade of protein kinases consisting of Ste11, Ste7, and Fus3/Kss1 (23). A terminal target of

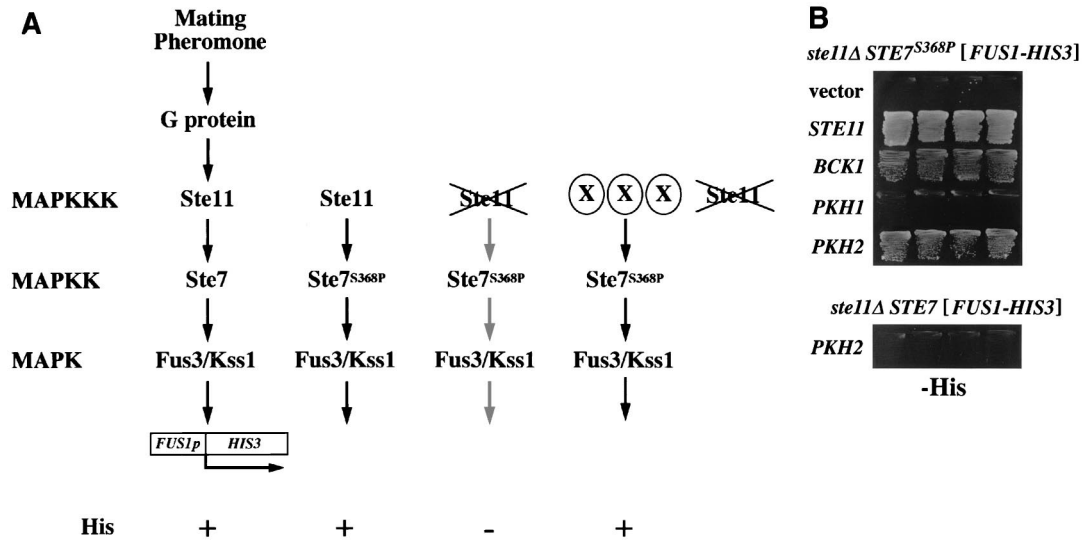


FIG. 1. Isolation of  $Ste7^{S368P}$  activators. (A) Model for the yeast pheromone-stimulated MAPK pathway. The pheromone-stimulated MAPK pathway induces transcription of mating-specific genes such as *FUS1*. The *FUS1p-HIS3* reporter gene consists of the *FUS1* upstream activation sequence joined to the *HIS3* open reading frame. This reporter allows signal activity in a *his3Δ FUS1p-HIS3* strain to be monitored by its ability to grow on medium lacking exogenous histidine (His phenotype). A *his3Δ ste11Δ FUS1p-HIS3 STE7<sup>S368P</sup>* strain has a His<sup>-</sup> phenotype because the activity of  $Ste7^{S368P}$  is still dependent on the presence of the upstream Ste11 MAPKKK (17). Presence of  $Ste7^{S368P}$  activators (shown as X) in this strain should confer a His<sup>+</sup> phenotype. (B) Suppression of *ste11Δ STE7<sup>S368P</sup>* by *BCK1* or *PKH2*. Strain SY1984-P (*his3Δ ste11Δ FUS1p-HIS3 STE7<sup>S368P</sup>*; top) (45) or SY1984 (*his3Δ ste11Δ FUS1p-HIS3 STE7*; bottom) (40) was transformed with the indicated plasmids, and each transformant was streaked onto SC-His plates and incubated at 30°C. Plasmids were YEplac195 (vector), YCp-STE11 (*STE11*), YE<sub>p</sub>-BCK1 (*BCK1*), YE<sub>p</sub>-PKH1 (*PKH1*), and YE<sub>p</sub>-PKH2 (*PKH2*).

this pathway is Ste12, a DNA-binding protein that recognizes the promoter regions of mating pheromone-inducible genes such as *FUS1* (12). Using a *his3Δ* yeast strain containing a *FUS1p-HIS3* fusion (40), one can readily assay activity of the mating pheromone signaling pathway by growth on media lacking histidine (His phenotype) (Fig. 1A). We previously isolated a strain containing a hyperactive mutation of *STE7*, *STE7<sup>S368P</sup>* (17, 45). The  $Ste7^{S368P}$  enzyme has higher kinase activity than wild type in the absence of pheromone stimulation but still requires modification by Ste11 for its activity. Therefore, *ste11Δ FUS1p-HIS3* cells containing *STE7<sup>S368P</sup>* exhibited a His<sup>-</sup> phenotype (Fig. 1). From a multicopy (YE<sub>p</sub>13) yeast genomic library we isolated two different clones that suppressed the His<sup>-</sup> phenotype (Fig. 1B). One contained the *BCK1* gene, which encodes the MAPKKK of the Pkc1-activated Mpk1 MAPK pathway (10, 25); the other contained the *PKH2* gene, which encodes a PDK1 homolog (Fig. 2). A multicopy plasmid carrying the *PKH2* gene (YE<sub>p</sub>-PKH2) failed to support *FUS1p-HIS3* expression in a *ste11Δ STE7<sup>+</sup>* background (Fig. 1B). This indicates that overexpression of *PKH2* can enhance the function of the  $Ste7^{S368P}$  variant but not of wild-type Ste7.

***PKH1* and *PKH2* encode protein kinases homologous to mammalian PDK1.** The *PKH2* gene encodes a 1,081-residue protein that shows most similarity to PDK1 from mammals (2, 39) (Fig. 2A). A GenBank database search revealed that *PKH2* shares homology with the *S. cerevisiae* gene *YDR490c*, also designated *PKH1*, which encodes a protein of 766 amino acids. The predicted protein kinase domains of these proteins share 73% amino acid identity (Fig. 2A), suggesting the possibility that Pkh1 and Pkh2 proteins functionally overlap. However, in contrast to *PKH2*, multicopy plasmids containing the *PKH1* gene failed to support *FUS1p-HIS3* expression in the *ste11Δ STE7<sup>S368P</sup>* strain (Fig. 1B).

To examine the phenotypic defect associated with loss of *PKH1* and/or *PKH2* function, we constructed yeast strains con-

taining deletion alleles of these genes (*pkh1Δ*, *pkh2Δ*, and *pkh1Δ pkh2Δ*) as described in Materials and Methods. Whereas the individual *pkhΔ* mutants were viable and grew normally at any temperature, the *pkh1Δ pkh2Δ* double mutant was not viable (Fig. 2B). To characterize the phenotype of the *pkh1Δ pkh2Δ* mutant cells, we constructed this strain carrying a plasmid expressing *PKH1* under the control of the *GAL1* promoter, YCpG22-*PKH1*, allowing *PKH1* to be induced or repressed on galactose- or glucose-containing medium, respectively. Haploid *pkh1Δ pkh2Δ* cells containing YCpG22-*PKH1* grew normally on galactose-containing medium but failed to grow on glucose-containing medium (Fig. 2B). Microscopic examination of these nongrowing cells revealed a high frequency of nonrefractile ghosts, suggesting that cell lysis had occurred. However, *pkh1Δ pkh2Δ* mutants containing YCpG22-*PKH1* were not rescued on glucose-containing medium supplemented with 1.2 M sorbitol (data not shown).

To test whether the structural similarity of mammalian PDK1 to Pkh1 and Pkh2 has any functional significance, we investigated whether expression of mammalian PDK1 would suppress the defect associated with loss of both *PKH1* and *PKH2*. We constructed a plasmid, pKT10-PDK1, that constitutively expresses PDK1 under the control of the yeast *TDH3* promoter (*TDH3p-PDK1*). This plasmid was transformed into the *pkh1Δ pkh2Δ* strain carrying YCpG22-*PKH1* (*GAL1p-PKH1*). On glucose-containing medium, where Pkh1 expression is repressed, expression of PDK1 allowed growth of *pkh1Δ pkh2Δ* cells (Fig. 2B). Similar results showing complementation of *pkh1Δ pkh2Δ* by mammalian PDK1 were obtained by Casamayor et al. while this study was in progress (7).

**Isolation of a ts *pkh1* mutation.** To isolate a ts mutant of *PKH1*, DNA encoding the entire *PKH1* gene was randomly mutagenized by PCR. The resulting amplification products were then introduced along with the *TRP1* marker into a *pkh1Δ pkh2Δ* strain which was maintained by the presence of YCpG33-*PKH1* (*GAL1p-PKH1 URA3*). Cells transformed



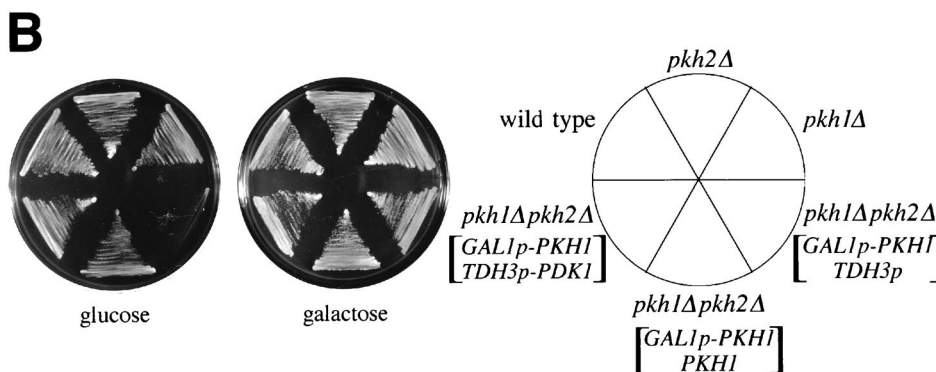
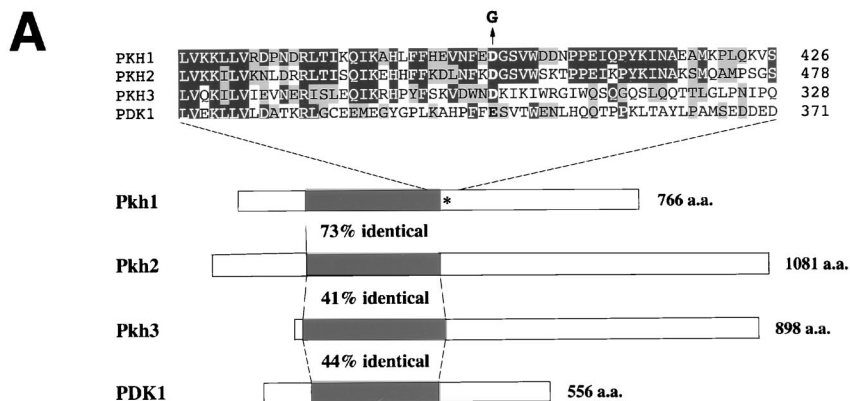


FIG. 2. Identification of yeast PDK1 homologs. (A) Schematic diagrams of the structures of PDK1, Pkh1, Pkh2, and Pkh3. Kinase domains are indicated by black boxes. The mutated residue in *pkh1<sup>D398G</sup>* is indicated by an asterisk. The amino acid (a.a.) residues in the region of the mutation site are aligned above the diagram. The Asp-to-Gly change in the *pkh1<sup>D398G</sup>* mutation is indicated as a G above the Pkh1 sequence. Amino acids which are identical or conserved are indicated by black or gray boxes, respectively. (B) Effect of the *pkh1Δ* and *pkh2Δ* mutations on cell growth. Yeast strains carrying the indicated plasmids were streaked onto YPGlu (glucose) medium and YPGal (galactose) medium and incubated at 30°C. Yeast strains were 15Dau (wild type), INA25-3B (*pkh1Δ::URA3*), INA28-1B (*pkh2Δ::URA3*), and INA38-6A (*pkh1Δ::URA3 pkh2Δ::URA3*). Plasmids were YCpG22-PKH1 (*GAL1p-PKH1*), pKT10 (*TDH3p*), YCplac22-PKH1 (*PKH1*), and pKT10-PDK1 (*TDH3p-PDK1*).

with a functional *PKH1* gene were selected by growth on glucose-containing medium at 25°C. The colonies were then replica plated onto glucose-containing medium and cultured at 37°C. Colonies that grew normally at 25°C but failed to grow at 37°C were subjected to further analysis. From a total of 5,000 transformants, 6 ts colonies were isolated, and their plasmid DNAs were retransformed into the parent strain. Among six candidate plasmids that conferred a ts growth defect, we characterized one *pkh1* allele (Fig. 3). This allele was found to harbor a single-base-pair mutation that resulted in an aspartate-to-glycine change at position 398 (Fig. 2A). Alignment of the Pkh1, Pkh2, and PDK1 protein sequences revealed that an acidic residue (Asp in Pkh1 and Pkh2 or Glu in PDK1) corresponding to position 398 in Pkh1 is conserved among these proteins (Fig. 2A). The ts phenotype was recessive, indicating that the phenotype was caused by a loss of *PKH1* function at 37°C. The mutant allele was named *pkh1<sup>D398G</sup>*. In strains wild type for *PKH2*, the *pkh1<sup>D398G</sup>* allele caused no growth defect at 37°C (data not shown).

When liquid cultures of exponentially growing *pkh1<sup>D398G</sup> pkh2Δ* cells were transferred from the permissive to the restrictive temperature, growth and viability rapidly decreased (Fig. 4A), and cell lysis and the accumulation of cell ghosts and debris were observed. As a further indication of cell lysis, the

*pkh1<sup>D398G</sup> pkh2Δ* mutant turned blue when cultured at the restrictive temperature on agar plates containing 5-bromo-4-chloro-3-indolylphosphate (Fig. 4B). Also, *pkh1<sup>D398G</sup> pkh2Δ* cells survived at 35°C in medium containing 1.2 M sorbitol

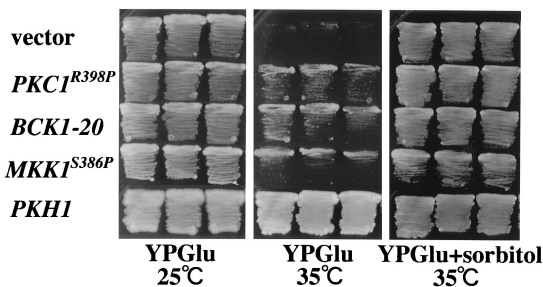


FIG. 3. A ts *pkh1* mutation. Transformants of the yeast strain INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ::LEU2*) carrying the indicated plasmids were streaked onto YPGlu medium and YPGlu medium supplemented with 1.2 M sorbitol and incubated at 25 or 35°C. Each patch represents an independent transformant. Plasmids were YCplac22 (vector), pRS316-PKC1(R398P) (*PKC1<sup>R398P</sup>*), pRS314-BCK1-20 (*BCK1-20*), YCplac22-MKK1(S386P) (*MKK1<sup>S386P</sup>*), and YCplac22-PKH1 (*PKH1*).

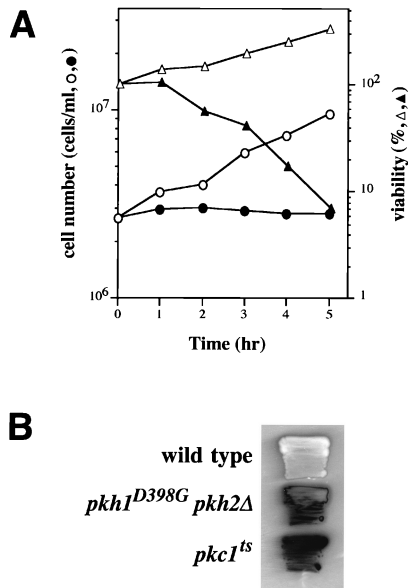


FIG. 4. Growth phenotypes of *pkh1<sup>D398G</sup> pkh2Δ* mutants. (A) Viability of *pkh1<sup>D398G</sup> pkh2Δ* mutants. INA106-3B cells (*pkh1<sup>D398G</sup> pkh2Δ::LEU2*) were grown in YPGlu medium at 25°C (open symbols) and shifted to 35°C (closed symbols). At the times indicated, cell number and viability were assayed. Cell number was determined by phase-contrast light microscopy with a hemacytometer. Percent viability was determined by comparing the colony number obtained after incubation for 48 h in YPGlu with that expected from the number of cells observed in the plated sample. (B) Cell lysis of *pkh1<sup>D398G</sup> pkh2Δ::LEU2* mutants. Yeast strains were patched onto YPGlu medium, incubated at 25°C for 1 day, and then shifted to 37°C for 1 day. The patches were assayed in situ for release of alkaline phosphatase as an indication of cell lysis. Yeast strains were 15Dau (wild type), INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ::LEU2*), and SYT11-12A (*pkc1<sup>ts</sup> stt1-1*).

(Fig. 3). This is characteristic of yeast mutants defective in cell wall integrity; i.e., lysis occurs in normal medium but not in high-osmolarity medium.

**Pkh1 and Pkh2 function in the Pkc1-MAPK pathway.** The cell lysis defect displayed by *pkh1<sup>D398G</sup> pkh2Δ* mutants was similar to that seen with mutants defective in the Pkc1-activated MAPK pathway, suggesting that Pkh1 and Pkh2 may function in this pathway. The Pkc1 pathway activates a MAPK cascade that consists of sequentially activated kinases: Bck1 (MAPKKK), the redundant Mkk1 and Mkk2 (MAPKKs), and Mpk1 (MAPK) (27). To test how loss of *PKH1* and *PKH2* affects the Pkc1-Mpk1 pathway, we examined the ability of *pkh1<sup>D398G</sup> pkh2Δ* mutants to activate a nuclear target of Mpk1, the Rlm1 transcription factor (42, 43). The extent of Mpk1 pathway activation can be monitored using a LexA-Rlm1ΔN fusion protein, which is phosphorylated by Mpk1, and a *LexA-lacZ* reporter gene, which is transcriptionally activated by LexA-Rlm1ΔN (43). Wild-type and *pkh1<sup>D398G</sup> pkh2Δ* cells expressing LexA-Rlm1ΔN were transformed with the *LexA-lacZ* plasmid, and transformants were tested for β-galactosidase activity (Fig. 5A). In contrast to the wild-type strain, LexA-Rlm1ΔN was unable to activate transcription of the reporter gene in a *pkh1<sup>D398G</sup> pkh2Δ* mutant. These results suggest that both Pkh1 and Pkh2 signal through the MAPK Mpk1.

The Pkc1-MAPK pathway is also required for organization of the actin cytoskeleton (14, 28). To investigate further whether Pkh1 or Pkh2 plays a role in the Pkc1-MAPK pathway, wild-type and *pkh1<sup>D398G</sup> pkh2Δ* mutant cells were grown at 24°C, shifted to 35°C, and processed for visualization of the actin cytoskeleton (Fig. 5B). Whereas wild-type cells displayed

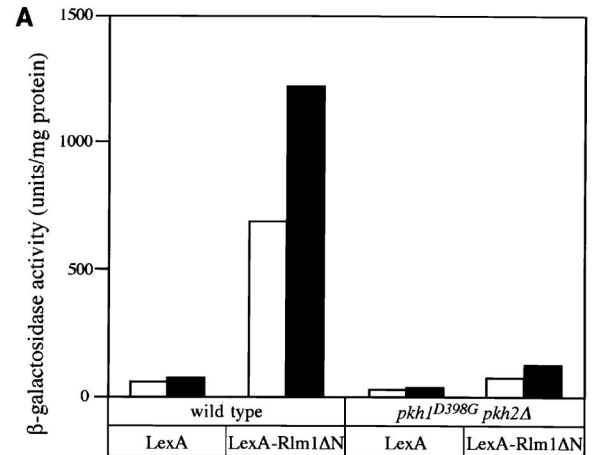


FIG. 5. Effect of Pkh on the Pkc1-MAPK pathway. (A) Effect of the *pkh1<sup>D398G</sup> pkh2Δ* mutation on Rlm1 transcriptional activity. Cells carrying the *LexA-lacZ* reporter gene and pBTM116 (LexA) or pYW71 (LexA-Rlm1ΔN) were grown at 23°C (open bars) and then shifted to 37°C for 30 min (solid bars) and assayed for β-galactosidase activity. The units shown are the average of two or three experiments. Yeast strains were 15Dau (wild type) and INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ*). (B) Effect of the *pkh1<sup>D398G</sup> pkh2Δ* mutation on actin organization. Cells were grown at 24°C, shifted to 35°C for 2.5 h, fixed, stained with TRITC-phalloidin, and observed by fluorescence (top) and Nomarski microscopy (bottom). Yeast strains were 15Dau (wild type) and INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ*). A field of mutant cells lacking lysed ghost cells was chosen.

the normal cell cycle-dependent polarized distribution of actin, *pkh1<sup>D398G</sup> pkh2Δ* cells were growth arrested at 35°C and exhibited random distribution of actin. Cells lacking only *PKH2* (*PKH1<sup>+</sup> pkh2Δ*) displayed a normal distribution of the actin cytoskeleton (data not shown). These results indicate that Pkh1 and Pkh2 are redundantly required for polarization of the actin cytoskeleton and further support a role for Pkh1 and Pkh2 in the Pkc1-MAPK pathway.

To determine the genetic relationship between the *PKH* genes and the Pkc1-MAPK pathway, we performed epistasis analyses. Mutant alleles encoding constitutively active Bck1 (*BCK1-20*) and Mkk1 (*MKK1<sup>S386P</sup>*) have been shown to suppress the phenotype of a *pkc1* deletion (25, 45). Expression of

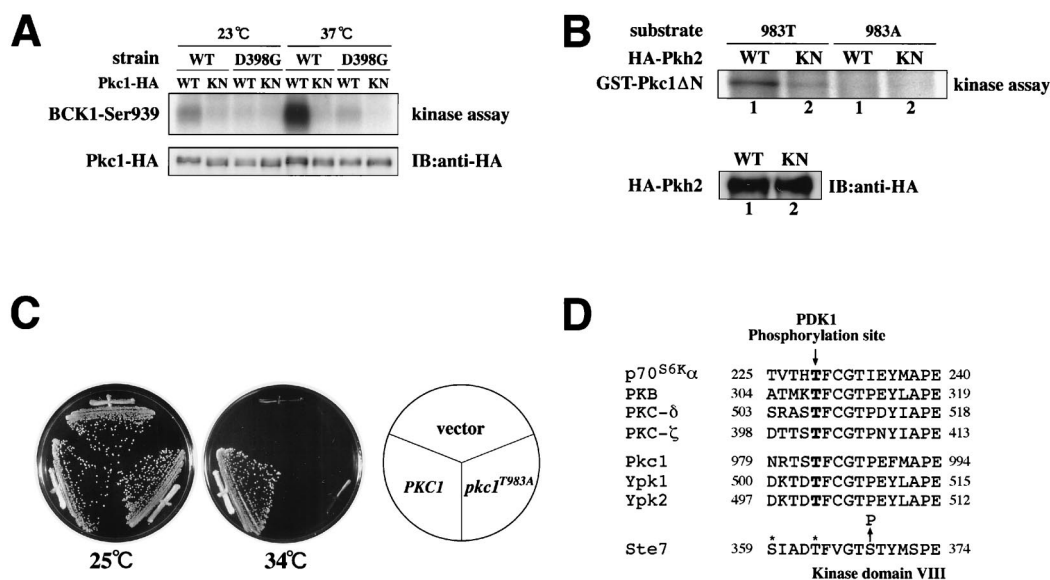


FIG. 6. Effect of Pkh on Pkc1. (A) Pkc1 kinase activity. Yeast strains 15Dau (wild type [WT]) and INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ::LEU2* [D398G]) were transformed with pDL293 [*GAL1p-PKC1-HA*] or pDL295 [*GAL1p-PKC1(K853R)-HA*]. Transformants were grown at 23°C, treated with galactose for 1 h to induce the *GAL1* promoter, and then shifted to 37°C for 1 h. Pkc1-HA (wild type [WT]) or Pkc1<sup>K853R</sup>-HA (KN) was immunoprecipitated from cell extracts. In vitro protein kinase assays were conducted with a synthetic peptide substrate corresponding to the sequence surrounding Ser-939 of Bck1, a phosphorylation site for Pkc1 (top). A parallel set of immune complexes was subjected to immunoblotting (IB) for detection of Pkc1-HA or Pkc1<sup>K853R</sup>-HA (bottom). (B) In vitro phosphorylation of Pkc1 by Pkh2. Yeast strain 15Dau (wild type) was transformed with pKT10-GAL-HA2-PKH2 (*GAL1p-HA-PKH2*) or pKT10-GAL-HA2-PKH2(K208R) [*GAL1p-HA-PKH2(K208R)*]. Transformants were grown at 30°C and treated with galactose for 1 h to induce the *GAL1* promoter. HA-Pkh2 (WT) or HA-Pkh2<sup>K208R</sup> (KN) was immunoprecipitated from cell extracts. In vitro protein kinase assays were conducted with GST-Pkc1ΔN(787-1151, K853R) (983T) and GST-Pkc1ΔN(787-1151, K853R, T983A) (983A) as substrates (top). A parallel set of immune complexes was subjected to immunodetection of HA-Pkh2 or HA-Pkh2<sup>K208R</sup> (KN) (bottom). (C) Mutation of Pkc1 phosphorylation site. Transformants of the yeast strain SYT11-12A (*pkc1<sup>ts</sup> stt1-1*) carrying the indicated plasmids were streaked onto YGlu medium and incubated at 25 or 34°C. Plasmids were YCplac33 (vector), pE722 (*PKC1*), and pE738 (*pkc1<sup>T983A</sup>*). (D) Alignment of amino acid sequences of the protein kinase subdomains VII and VIII. The amino acid sequences surrounding the residues equivalent to Thr-308 of PKB are highly conserved in these protein kinases. The positions of sites in Ste7 that are phosphorylated by Ste11 are indicated by asterisks, and the position of the substituted Ser residue in Ste7<sup>S368P</sup> is indicated by an arrow.

*BCK1-20* or *MKK1<sup>S386P</sup>* partially suppressed the temperature sensitivity of the *pkh1<sup>D398G</sup> pkh2Δ* mutant (Fig. 3). Furthermore, a constitutively active mutant of *PKC1*, *PKC1<sup>R398P</sup>* (30), also partially suppressed the growth defect of the *pkh1<sup>D398G</sup> pkh2Δ* mutant (Fig. 3). In contrast, overexpression of *PKH1* or *PKH2* failed to suppress the phenotype of a *ts pkc1* allele (*stt1-1*) (data not shown). These results suggest that Pkh1 and Pkh2 function upstream of Pkc1 in the Pkc1-MAPK pathway.

**Pkh phosphorylates and activates Pkc1.** The above results raise the possibility that Pkh proteins directly phosphorylate and activate Pkc1. To test this possibility, we assayed Pkc1 kinase activity in wild-type and *pkh1<sup>D398G</sup> pkh2Δ* mutant strains. Wild-type and mutant strains were transformed with a plasmid encoding Pkc1 tagged at its COOH terminus with HA (Pkc1-HA) and expressed under the control of the *GAL1* promoter (41). Transformants were grown at 23°C, treated with galactose for 1 h to induce the *GAL1* promoter, and then shifted to 37°C for 1 h. Pkc1-HA was immunoprecipitated from yeast extracts, and its protein kinase activity was measured by using a synthetic Bck1 peptide as a substrate (41). Compared to the wild-type strain, Pkc1 activity was significantly reduced in *pkh1<sup>D398G</sup> pkh2Δ* mutants even at the permissive temperature (Fig. 6A). Furthermore, the heat induction of Pkc1 kinase activity was severely compromised in the *pkh1<sup>D398G</sup> pkh2Δ* mutant relative to the wild type (Fig. 6A). These results indicate that Pkh proteins are required for Pkc1 activation, further supporting the results of the previous epistasis analyses.

To test whether Pkc1 is directly phosphorylated by Pkh in vitro, we developed an immune complex assay to measure Pkh2 protein kinase activity. HA-tagged wild-type Pkh2 (HA-Pkh2) was expressed under the control of the inducible *GAL1*

promoter. Expression of HA-Pkh2 was able to complement the growth defect of *pkh1Δ pkh2Δ* mutants, indicating that HA-Pkh2 is functional (data not shown). As a substrate, we used GST-Pkc1ΔN(787-1151; K853R), containing the inactivating K853R mutation, to eliminate background Pkc1 autophosphorylation. The epitope-tagged Pkh2 proteins were immunoprecipitated with an anti-HA antibody and incubated with [ $\gamma$ -<sup>32</sup>P]ATP and GST-Pkc1ΔN(787-1151; K853R). We found that Pkc1 was phosphorylated by immune complexes from cells expressing HA-Pkh2 (Fig. 6B). To rule out the possibility that this activity is due to another kinase that is tightly associated with HA-Pkh2 during the immunoprecipitation procedure, we also tested an HA-tagged kinase-inactive mutant (HA-Pkh2<sup>K208R</sup>) containing a lysine-to-arginine change in the kinase domain. Immunocomplexes from cells expressing HA-Pkh2<sup>K208R</sup> exhibited much lower kinase activity toward Pkc1, although the HA-Pkh2<sup>K208R</sup> protein was expressed at levels comparable to those for wild-type Pkh2 (Fig. 6B).

PDK1 activates PKB by phosphorylating a Thr residue in a conserved sequence motif located within the activation loop of its catalytic domain situated between conserved protein kinase subdomains VII and VIII (2, 39). This motif is also conserved in Pkc1 and in mammalian PKCs (Fig. 6D). PDK1 has been shown to phosphorylate sites within the activation loops of PKCξ and PKCδ (23). To test whether the conserved Thr-983 in the activation loop of Pkc1 is indeed the site of Pkh2 phosphorylation, we generated the GST fusion protein Pkc1ΔN(787-1151; K853R; T983A), in which the Thr residue at 983 was replaced with Ala. We found that this mutant protein was not phosphorylated by Pkh2 (Fig. 6B), indicating that Thr-983 is the site of Pkh2 phosphorylation.



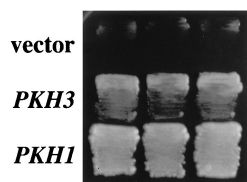


FIG. 7. Isolation of the *PKH3* gene. Transformants of the yeast strain INA106-3B (*pkh1<sup>D398G</sup> pkh2Δ::LEU2*) carrying the indicated plasmids were streaked onto YPGlu medium and incubated at 35°C. Each patch represents an independent transformant. Plasmids were YEp13 (vector), YEp-PKH3 (*PKH3*), and YCplac111-PKH1 (*PKH1*).

We next asked whether Thr-983 is important for Pkc1 function. We examined the ability of Pkc1<sup>T983A</sup> containing a mutation of Thr-983 to Ala to complement the growth defect of *pkc1<sup>ts</sup>* (*stt1-1*) mutants. Whereas wild-type Pkc1 complemented the *pkc1<sup>ts</sup>* defect, the T983A mutant did not (Fig. 6C), indicating that Thr-983 is required for Pkc1 function.

**Isolation of *PKH3* as a multicopy suppressor of *pkh1<sup>D398G</sup> pkh2*.** To identify additional components of the Pkh pathway, we isolated multicopy suppressors of the growth defect of a *pkh1<sup>D398G</sup> pkh2* mutant. A yeast genomic library cloned into the multicopy vector YEp13 was transformed into a *pkh1Δ pkh2Δ* strain that carried plasmid YCplac22-PKH1<sup>D398G</sup> (*pkh1<sup>D398G</sup> TRP1*). A total of 3,500 Leu<sup>+</sup> transformants were obtained and subsequently screened for the ability to grow at the restrictive temperature. Six transformants capable of forming colonies at 37°C were isolated (Fig. 7). The plasmids recovered from these yeast transformants were of two classes, based on restriction digest patterns. Sequence analysis revealed that three of these six plasmids contained the *PKH2* gene itself. The DNA sequences of the three remaining clones was determined, and all were found to contain a gene designated *YDR466w* by the Yeast Genome Project. The *YDR466w* gene encodes a protein of 898 amino acids and contains a protein kinase domain. The predicted protein has 40% sequence identity to Pkh1 and Pkh2 throughout its protein kinase domains (Fig. 2A). We therefore named this gene *PKH3*. Interestingly, *PKH3* restored growth only when coexpressed with *pkh1<sup>D398G</sup>*, even though *pkh1<sup>D398G</sup>* appears inactive at 37°C. This was shown by the observation that when *pkh1Δ pkh2Δ* strains carrying both YCplac22-PKH1<sup>D398G</sup> and YEp-PKH3 were cultured at 37°C, the YCplac22-PKH1<sup>D398G</sup> plasmid did not segregate away. Furthermore, *pkh1Δ pkh2Δ* cells transformed with both YEp-PKH3 and YCpG22-PKH1 (*GAL1p-PKH1 TRP1*) failed to grow in glucose-containing medium (data not shown). Thus, overexpression of *PKH3* does not compensate for disruption of both *PKH1* and *PKH2*, and the ts protein Pkh1<sup>D398G</sup> has some activity at the restrictive temperature that is required together with *PKH3* for viability.

We constructed strains containing a deletion allele of *PKH3* (*pkh3Δ*). These strains grew normally at any temperature. Furthermore, *pkh1Δ pkh3Δ* and *pkh2Δ pkh3Δ* double-mutant cells also grew normally at any temperature (data not shown), indicating that unlike Pkh1 and Pkh2, Pkh3 is dispensable for growth.

## DISCUSSION

We show that *S. cerevisiae* has two PDK1 homologs, Pkh1 and Pkh2. Single *pkh1Δ* and *pkh2Δ* mutants are viable, but the *pkh1Δ pkh2Δ* double mutant is nonviable, indicating that Pkh1 and Pkh2 share a role that is essential for cell growth. Expression of mammalian PDK1 suppresses the lethality of *pkh1Δ pkh2Δ* cells, demonstrating that Pkh1 and Pkh2 are function-

ally similar to PDK1. As many signal transduction pathways and mechanisms that regulate cell growth and proliferation are conserved between mammalian and yeast cells, it is not unexpected that PDK1 homologs are present in yeast. PDK1 activates PKB, PKCs and p70<sup>S6K</sup> by phosphorylating the Thr residue in a conserved sequence motif located within the activation loops of their catalytic domains (Fig. 6D). This conserved sequence motif is also found in the activation loop of the *S. cerevisiae* Pkc1 protein, raising the possibility that Pkc1 is a physiological substrate for Pkh1 and Pkh2.

In mammalian cells, PDK1 has been shown to phosphorylate PKC isoforms (24). However, the biological role of this phosphorylation in mammalian cells is not understood. Here we provide evidence indicating that Pkh1 and Pkh2 function in the Pkc1-MAPK pathway. First, temperature-sensitive *pkh1<sup>ts</sup> pkh2Δ* mutants display phenotypes similar to those of mutants defective in the Pkc1-MAPK pathway, notably osmoremedial cell lysis and loss of actin cytoskeleton polarity. Second, *pkh1<sup>ts</sup> pkh2Δ* mutants are defective in activation of the transcription factor Rlm1, whose activity is dependent on the Pkc1-MAPK pathway. Third, an activating mutation in *PKC1*, *BCK1*, or *MKK1* partially suppresses the growth defect of a *pkh1<sup>ts</sup> pkh2Δ* mutant. Fourth, Pkc1 activity is decreased in a *pkh1<sup>ts</sup> pkh2Δ* mutant. Finally, the Pkh2 protein phosphorylates Pkc1 in vitro at Thr-983; this residue is part of the conserved PDK1 target motif in the Pkc1 activation loop and is essential for Pkc1 function. Thus, Pkh1 and Pkh2 are in the Pkc1-MAPK pathway as activators of Pkc1.

Many protein kinases require phosphorylation within their activation loops to be fully activated. Phosphorylation within the activation loop is also important for protein kinases stringently regulated by allosteric effectors. This is exemplified by PKC, where activation of the Ca<sup>2+</sup>/diacylglycerol-dependent isotypes PKC $\alpha$  and PKC $\beta$  absolutely requires phosphorylation of respective activation loops (8, 32). PDK1 regulates multiple protein kinases, including PKB, p70<sup>S6K</sup>, and PKC isoforms (2, 3, 24, 34, 39). The specificity of PDK1 action on its downstream protein kinase targets could be determined by target-specific regulators. In *S. cerevisiae*, the GTP-bound form of Rho1 functions as an activator of Pkc1 (19, 30), raising the possibility that this Rho1-dependent activation of Pkc1 is controlled through Pkh1/Pkh2-dependent phosphorylation of the Pkc1 activation loop.

Several observations suggest that Pkc1 is not the only target of the Pkh kinases. *pkh1<sup>ts</sup> pkh2Δ* mutants resemble *pkc1* mutants in that they also exhibit defects in cell integrity resulting from aberrant cell wall construction. However, whereas cell lysis caused by loss of *PKC1* function is suppressed by osmotic stabilizing agents (26), the growth defect in *pkh1Δ pkh2Δ* mutants was not. Based on the observation that mammalian PDK1 activates PKB (2, 39), it is possible that the yeast PKB-like protein kinases, Ypk1 and Ypk2, which play an essential role in yeast cell growth (9), are also targets of Pkh. Consistent with this possibility, the sequence surrounding the site in PKB phosphorylated by PDK1 is also conserved in Ypk1 and Ypk2 (Fig. 6D). Furthermore, Casamayor et al. have recently shown that Pkh1 activates Ypk1 in vitro by phosphorylating the Thr-504 residue that corresponds to the site of PDK1 phosphorylation (7). Thus, Pkh1 and Pkh2 are likely to play a role in activating at least two types of protein kinases that are essential for cell growth, Pkc1 and Ypk1/Ypk2.

In this study, we isolated six different ts *pkh1* mutants and divided them into two classes. The growth defect of the first class, typified by *pkh1<sup>D398G</sup>*, was rescued by osmotic stabilization, whereas the growth defect of the second class (unpublished data) was not. This suggests that the second class of

mutants may be defective in activation of Ypk1/Ypk2. We thus attempted to rescue this class of mutants by overexpression of Ypk1 or Ypk2, but growth was not restored even in the presence of osmotic stabilizing agents (unpublished data). We suspect that activation of Ypk1 and Ypk2 may absolutely require phosphorylation by Pkh1 and Pkh2. If true, this hypothesis suggests a genetic approach to identify constitutive mutations in *YPK1* or *YPK2*, i.e., by isolating mutations that suppress the growth defect of a *pkh1<sup>ts</sup> pkh2Δ* strain. This work may elucidate the different modes by which mammalian PDK1 and PKB are regulated.

Overexpression of the *PKH2* gene, but not the *PKH1* gene, was shown to activate Ste7<sup>S368P</sup>, which suggests that Pkh2 basal kinase activity is higher than that of Pkh1. Consistent with this possibility, we could detect Pkh2 but not Pkh1 kinase activity when Pkc1 was used as a substrate (unpublished data). However, the *pkh2Δ* single mutant grows normally, indicating that endogenous Pkh1 activity is sufficient for growth. It is therefore likely that the activity of Pkh1 is tightly regulated. The fact that PDK1 contains a PH domain and can bind lipid vesicles containing phosphatidylinositol 3,4,5-trisphosphate or phosphatidylinositol 3,4-bisphosphate (39) may suggest that 3-phosphorylated lipids can activate PDK1 in some way. In contrast, Pkh1 and Pkh2 lack any obvious PH domain. It will be interesting to identify upstream activators of Pkh1. These analyses should further our understanding of the signal(s) that activates the Pkh-Pkc1 pathway.

The *STE7<sup>S368P</sup>* mutant, which activates mating response genes in the absence of pheromone, can be upregulated by overexpression of *PKH2*, whereas the wild-type *STE7* cannot. This suggests that Pkh2 phosphorylates and activates the Ste7<sup>S368P</sup> variant but not wild-type Ste7. The Ste7 protein contains a threonine residue in its activation loop, Thr-363, which is known to be the site of Ste11 phosphorylation. This residue is also analogous to a conserved Thr residue in the phosphorylation site of PDK1 (Fig. 6D). The residue in Ste7 that is mutated in Ste7<sup>S368P</sup>, i.e., Ser-368, lies within the activation loop between subdomains VII and VIII in proximity to the Thr-363 residue. Interestingly, all PDK1 target protein kinases except p70<sup>S6K</sup> have a proline residue at the site corresponding to Ser-368 of Ste7, which may be important for their interaction with PDK1, Pkh1, and/or Pkh2. Thus, the serine-to-proline change in Ste7<sup>S368P</sup> may convert Ste7 to a substrate of Pkh2, perhaps by altering the conformation of the activation loop in a way that makes the Thr-363 residue accessible to Pkh2.

We also identified a novel protein kinase, Pkh3, that functions as a multicopy suppressor of a *pkh1<sup>ts</sup> pkh2Δ* mutant and which exhibits homology to PDK1, Pkh1, and Pkh2. Growth of the *pkh3Δ* single mutant is normal and indistinguishable from that of wild-type cells. Also, *pkh1Δ pkh3Δ* and *pkh2Δ pkh3Δ* double mutants display no apparent phenotype. These results suggest that Pkh3 is able to phosphorylate the same essential target substrates as Pkh1 and Pkh2 when overexpressed but that Pkh1 and Pkh2 play a more important role in regulating cell growth under physiological conditions.

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