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A Pair of Functionally Redundant Yeast Genes (*PPZ1* and *PPZ2*) Encoding Type 1-Related Protein Phosphatases Function within the *PKC1*-Mediated Pathway

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The *PKC1* gene of *Saccharomyces cerevisiae* encodes a homolog of mammalian protein kinase C that is required for yeast cell growth. Loss of *PKC1* function results in cell lysis due to an inability to remodel the cell wall properly during growth. The *PKC1* gene has been proposed to regulate a bifurcated pathway, on one branch of which function four putative protein kinases that catalyze a linear cascade of protein phosphorylation culminating in the activation of the mitogen-activated protein kinase homolog, Mpk1p. Here we describe two genes whose overexpression suppress both an *mpk1Δ* mutation and a *pkc1Δ* mutation. One of these genes is identical to the previously identified *PPZ2* gene. The *PPZ2* gene is predicted to encode a type 1-related protein phosphatase and is functionally redundant with a closely related gene, designated *PPZ1*. Deletion of both *PPZ1* and *PPZ2* resulted in a temperature-dependent cell lysis defect similar to that observed for *bck1Δ*, *mkk1,2Δ*, or *mpk1Δ* mutants. However, *ppz1,2Δ mpk1Δ* triple mutants displayed a cell lysis defect at all temperatures. The additivity of the *ppz1,2Δ* defect with the *mpk1Δ* defect, combined with the results of genetic epistasis experiments, suggested either that the *PPZ1*- and *PPZ2*-encoded protein phosphatases function on a branch of the *PKC1*-mediated pathway different from that defined by the protein kinases or that they play an auxiliary role in the pathway. The other suppressor gene, designated *BCK2* (for bypass of C kinase), is predicted to encode a 92-kDa protein that is rich in serine and threonine residues. Genetic interactions between *BCK2* and other pathway components suggested that *BCK2* functions on a common pathway branch with *PPZ1* and *PPZ2*.

Members of the family of phospholipid-dependent, serine/threonine-specific protein kinases known collectively as protein kinase C (PKC) respond to extracellular signals that act through receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate (20). Diacylglycerol serves as a second messenger to activate PKC (29, 42, 43, 62), and inositol-1,4,5-triphosphate functions to mobilize Ca^{2+} from intracellular stores (4). Eight distinct subtypes of mammalian PKC have been reported (3, 28, 43), several of which (α, βI, βII, and γ) require Ca^{2+} for activity (29), whereas others (δ, ε, and ζ) do not (47, 49, 50).

Mammalian PKC is thought to play a pivotal role in the regulation of a host of cellular functions through its activation by growth factors and other agonists. These functions include cell growth and proliferation (26, 54, 56), release of various hormones (41, 46), and control of ion conductance channels (15, 38). Indirect evidence suggests that PKC induces the transcription of a wide array of genes, including the proto-oncogenes c-*myc*, c-*fos*, and c-*sis* (9, 11, 18, 27, 30), the human collagenase gene (2), the metallothionein II_A gene, and the simian virus 40 early genes (23). Several transcription factors have been implicated in this response, including components of the AP-1 complex, AP-2, AP-3, and NF-κB (4, 8, 22, 34).

Members of a family of enzymes called mitogen-activated protein (MAP) kinases have also been implicated in PKC-dependent signalling. These enzymes are thought to function as intermediaries between membrane-associated signalling molecules and the nucleus (53, 63). The p42 and p44 isoforms of MAP kinases are activated in response to a wide array of extracellular signals, including those that stimulate PKC activity (21). The intracellular locations of these enzymes—the cytoplasm and the nucleus (7)—make them excellent candidates for messengers from the membrane.

Although substantial progress has been made toward elucidating the pathways leading to PKC activation, the steps between this activation and subsequent nuclear events are only now beginning to emerge. To dissect this signalling pathway further, it is useful to study the role(s) of PKC in systems that are amenable to rigorous genetic analysis. The *PKC1* gene of *Saccharomyces cerevisiae* encodes a homolog of the Ca^{2+}-dependent subtypes of mammalian PKC that is essential for cell growth (37). Loss of *PKC1* function results in a cell lysis defect that is due to a deficiency in cell wall construction (35, 36, 51). The isolation of four genes that function within the *PKC1*-mediated signalling pathway has been reported previously. These genes, *BCK1* (33), *MKK1* and *MKK2* (24), and *MPK1* (32, 64), encode protein kinases that are proposed to catalyze a protein phosphorylation cascade culminating in the activation of the MAP kinase homolog Mpk1p. We have proposed that this protein kinase cascade functions on one branch of a bifurcated pathway mediated by Pkc1p (for reviews, see references 14 and 36). Here we report the isolation of two genes that bypass the requirement for *PKC1* and *MPK1* in a dosage-dependent fashion. One of these genes, designated *PPZ2*, and its redundant partner, *PPZ1*, are predicted to encode a pair of type 1-related protein phosphatases.

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
EG123	*MAT*α *leu2-3,112 ura3-52 trp1-1 his4 can-1*[r]	I. Herskowitz
1783	*MAT***a** EG123	I. Herskowitz
1788	*MAT***a**/*MAT*α isogenic diploid of EG123	I. Herskowitz
DL106	*MAT*α EG123 *pkc1*Δ::*LEU2* (YCp50[*PKC1*])	35
DL377	*MAT*α EG123 *pkc1*Δ::*LEU2*	This study
DL456	*MAT***a**/*MAT*α 1788 *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1*	32
DL737	*MAT***a**/*MAT*α 1788 *bck2*Δ::*TRP1*/*BCK2*	This study
DL750	*MAT***a**/*MAT*α 1788 *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1* (YCp50-*LEU2*[*MPK1*])	This study
DL761	*MAT***a** EG123 *bck2*Δ::*TRP1*	This study
DL762	*MAT***a** EG123 *bck2*Δ::*TRP1*	This study
DL763	*MAT***a**/*MAT*α 1788 *bck2*Δ::*TRP1*/*bck2*Δ::*TRP1*	This study
DL769	*MAT***a**/*MAT*α 1788 *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1* (YCp50-*LEU2*)	This study
DL776	*MAT***a**/*MAT*α 1788 *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1* (YEp352[*PPZ2*])	This study
DL786	*MAT***a** EG123 *ppz1*Δ::*URA3*	This study
DL787	*MAT*α EG123 *ppz1*Δ::*URA3*	This study
DL788	*MAT***a** EG123 *ppz2*Δ::*LEU2*	This study
DL789	*MAT*α EG123 *ppz2*Δ::*LEU2*	This study
DL790	*MAT***a**/*MAT*α 1788 *ppz1*Δ::*URA3*/*PPZ1 ppz2*Δ::*LEU2*/*PPZ2*	This study
DL791	*MAT***a** EG123 *ppz1*Δ::*URA3 ppz2*Δ::*LEU2*	This study
DL792	*MAT*α EG123 *ppz1*Δ::*URA3 ppz2*Δ::*LEU2*	This study
DL793	*MAT***a**/*MAT*α 1788 *ppz1*Δ::*URA3*/*ppz1*Δ::*URA3*	This study
DL794	*MAT***a**/*MAT*α 1788 *ppz2*Δ::*LEU2*/*ppz2*Δ::*LEU2*	This study
DL795	*MAT***a**/*MAT*α 1788 *ppz1*Δ::*URA3*/*ppz1*Δ::*URA3 ppz2*Δ::*LEU2*/*ppz2*Δ::*LEU2*	This study
DL797	*MAT***a**/*MAT*α 1788 *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1* (pACG-1)	This study
DL823	*MAT***a**/*MAT*α DL456 *ppz1*Δ::*URA3*/*ppz1*Δ::*URA3*	This study
DL824	*MAT***a**/*MAT*α DL456 *ppz2*Δ::*LEU2*/*ppz2*Δ::*LEU2*	This study
DL825	*MAT***a**/*MAT*α DL456 *ppz1*Δ::*URA3*/*ppz1*Δ::*URA3 ppz2*Δ::*LEU2*/*ppz2*Δ::*LEU2*	This study
DL827	*MAT***a**/*MAT*α DL456 *bck2*Δ::*TRP1*/*bck2*Δ::*TRP1*	This study
DL830	*MAT***a**/*MAT*α DL795 *bck2*Δ::*TRP1*/*bck2*Δ::*TRP1*	This study
DL831	*MAT*α EG123 *pkc1*Δ::*LEU2* (YEp352)	This study
DL832	*MAT*α EG123 *pkc1*Δ::*LEU2* (YEp352[*PPZ2*Δ*5′*])	This study
DL833	*MAT*α EG123 *pkc1*Δ::*LEU2* (YEp352[*PPZ2*])	This study
DL837	*MAT***a**/*MAT*α 1788 *ppz1*::*TRP1*/*ppz1*::*TRP1 ppz2*Δ::*LEU2*/*ppz2*Δ::*LEU2*	This study
DL843	*MAT*α EG123 *pck1*Δ::*LEU2* (YEp352[*BCK2*Δ*N*])	This study
DL844	*MAT*α EG123 *pkc1*Δ::*LEU2* (YEp352[*BCK2*])	This study
DL870	*MAT*α EG123 *pkc1*Δ::*LEU2 mpk1*Δ::*TRP1*	This study
DL873	*MAT*α EG123 *pkc1*Δ::*LEU2 ppz1*Δ::*URA3*	This study
DL876	*MAT*α EG123 *pkc1*Δ::*LEU2 bck2*Δ::*TRP1*	This study

MATERIALS AND METHODS

Strains, growth conditions, transformations, and nucleic acid manipulations. All yeast strains used in this study (Table 1) were derivatives of EG123 (*MAT*α *leu2-3,112 ura3-52 trp1-1 his4 can-1*[r]) (60). Yeast cultures were grown in YEP (1% yeast extract, 2% Bacto Peptone) supplemented with 2% glucose. Synthetic minimal medium (SD [59]) supplemented with the appropriate nutrients was used to select for plasmid maintenance and gene replacements. Yeast transformation was by the lithium acetate method (25). General genetic manipulation of yeast cells was carried out as described previously (59).

The initial genomic library screen (in the multicopy vector YEP24) was done by replicate plating transformants of a diploid *mpk1*Δ strain (DL456) grown at 30°C on SD medium supplemented with 1 M sorbitol to YEP-glucose at 37°C for 2 days. A diploid strain was used to prevent the accumulation of recessive suppressor mutations. Plasmids were rescued from colonies arising at the restrictive temperature. Full-length *PPZ2* was isolated from a genomic YCp50-*LEU2* library (provided by P. Hieter, Johns Hopkins University), using nick-translated *PPZ2* sequences as a hybridization probe, and an 11-kb *Kpn*I fragment bearing the entire gene was subcloned into the multicopy vector YEp352. Full-length *BCK2* was isolated from a genomic YEp13 library (provided by J. Thorner, University of Calif., Berkeley), and a 5.5-kb *Sph*I-*Xba*I fragment bearing the entire gene was subcloned into YEp352. Genomic yeast DNA and plasmids were isolated and then prepared for restriction endonuclease digestion and hybridization as described previously (33). Nick translation, hybridization, and DNA sequence analysis were also carried out as described previously (37).

Escherichia coli DH5α (19), HB101 (6), and TG1 (58) were used for the propagation of all plasmids and phage. Phage M13mp18 and M13mp19 (44) were used to generate single-stranded template DNA for sequence determination. *E. coli* cells were cultured in Luria broth or YT medium and transformed or were infected with M13 by standard methods (39).

Gene replacements. Deletion mutant alleles of *PPZ1*, *PPZ2*, and *BCK2* were constructed by the method of Rothstein (57). For construction of a deletion allele of *PPZ2*, a 5.5-kb *Sal*I (in vector)-*Xba*I fragment bearing the *PPZ2* gene (without 5′ regulatory sequences) was cloned into BluescriptII KS(−). After digestion of this plasmid with *Eco*RI to eliminate a 730-bp fragment corresponding to the predicted catalytic domain, the ends were made flush with Klenow fragment and dephosphorylated with calf intestinal alkaline phosphatase. An *Hpa*I fragment bearing the *LEU2* gene (from YEp13) was ligated into the blunt-end site of this

Bluescript construction. The resulting 3.2-kb *Sph*I fragment, bearing the *LEU2* gene flanked by *PPZ2* sequences (*ppz2*Δ::*LEU2*), was isolated and used for transformation of yeast strains to leucine prototrophy.

A deletion allele of *PPZ1* (*ppz1*Δ::*URA3*) was constructed previously by Posas et al. (55). The plasmid construction bearing this allele (pFP-2) was provided by J. Arino. For some experiments, an alternative selectable marker was preferred, so a *ppz1*::*TRP1* allele was constructed. An insertion allele of *PPZ1* (also provided by J. Arino) was constructed by first cloning a 1.9-kb *Eco*RI-*Xba*I fragment bearing the 3' half of the *PPZ1* gene (55) into Bluescript SK(+). A *Bam*HI-*Bgl*II fragment bearing the *TRP1* gene was cloned into the unique *Bgl*II site of this construction. The resulting 2.1-kb *Xho*I-*Sst*I fragment, bearing the *TRP1* gene flanked by *PPZ1* sequences (*ppz1*::*TRP1*), was isolated and used for transformations to tryptophan prototrophy.

A deletion of *BCK2* was constructed by first cloning a 3.5-kb *Sal*I (in vector)-*Xba*I fragment bearing the *BCK2* gene (without the 5' end of the gene) into YEp352. After digestion of this plasmid with *Bgl*II to eliminate a 1.1-kb fragment of coding sequence, the ends were made flush and dephosphorylated as described above. A *Sma*I fragment bearing the *TRP1* gene (from pUC18[*TRP1*]) was ligated into the blunt-end site of this YEp352 construction. Tandem ligation of two *TRP1*-containing fragments resulted in a 4.7-kb fragment bearing two *TRP1* genes flanked by *BCK2* sequences (*bck2*Δ::*TRP1*), which was isolated and used to transform diploid strain 1788. All gene replacements were confirmed by restriction and hybridization analysis.

Nucleotide sequence accession numbers. The GenBank accession numbers for *PPZ2* and *BCK2* are L10241 and L10242, respectively.

RESULTS

Isolation of dosage-dependent suppressors of an *mpk1* deletion mutant. To identify additional components of the *PKC1*-mediated pathway, we isolated dosage-dependent suppressors of an *mpk1* deletion mutant. A genomic yeast library, cloned in the multicopy shuttle vector YEp24 (contains *S. cerevisiae URA3*; provided by C. Guthrie, University of California), was used to transform a strain bearing the *mpk1*Δ::*TRP1* mutation (DL456 [32]) to uracil prototrophy, after which a screen for growth at the restrictive temperature was performed. Sixteen transformants from among 4,000 screened (approximately four genomic equivalents) were capable of growth at 37°C. The plasmids recovered from these yeast transformants were of eight classes, based on restriction digest patterns and genomic map positions (not shown), and were tentatively designated, genes 1 to 8. The *MPK1* gene was not represented among the cloned genes. Genes 1 to 3 were assessed to be the most effective at suppressing the *mpk1*Δ::*TRP1* defect. These clones also suppressed the temperature-dependent lysis defects associated with a *bck1*Δ mutation and *pkc1*^{ts} alleles (data not shown). Clones bearing gene 1 and clones bearing gene 2 were chosen for molecular and genetic analysis.

The plasmid containing gene 1 was subjected to deletion analysis (Fig. 1). Sequences to the left of the *Bam*HI site of this clone were necessary for suppression of *mpk1*Δ::*TRP1*. DNA sequence analysis of the left end of the clone revealed an open reading frame extending from the insert junction (at a *Sau*3A site; Fig. 2A) to 339 bp to the right of the *Bam*HI site. Because the 5' end of this gene was missing from the original clone, the full-length gene was isolated from a

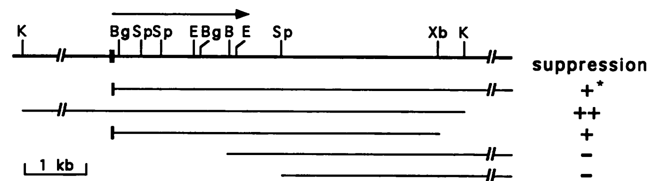

FIG. 1. Restriction and deletion maps of the *PPZ2* locus. Deletions within the genomic DNA carried in the multicopy vector YEp24 were generated by restriction endonuclease digestion. In some cases, fragments were subcloned into the multicopy vector YEp352. The ability (+) or inability (−) of the resulting plasmids to suppress the *mpk1*Δ::*TRP1* mutation is shown. The DNA fragments present in the plasmids are indicated. The original *PPZ2* isolate (*PPZ2*Δ*5'*) is marked with an asterisk, and the vertical bar demarcates the 5' end of this clone. The arrow indicates the direction and limits of the *PPZ2*-encoded open reading frame. Abbreviations for selected restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; Sp, *Sph*I; Xb, *Xba*I.

different genomic yeast library by molecular hybridization (see Materials and Methods). Although the original clone bearing gene 1 possesses the entire open reading frame, it carries only 14 bp 5' to the predicted translation initiation site. The lack of 5' regulatory sequences in the original isolate is consistent with the observation that it suppressed the *mpk1*Δ::*TRP1* defect less effectively than did the full-length gene (not shown). Moreover, full-length gene 1 (but not the original isolate) suppressed the unconditional cell lysis defect of a *pkc1*Δ mutant (Fig. 3A), further suggesting that this gene functions within the *PKC1*-mediated signalling pathway.

Gene 1 (*PPZ2*) encodes a protein phosphatase. The open reading frame encoded by gene 1 corresponds to a polypeptide with a predicted length of 710 amino acids (calculated molecular size of 78 kDa; Fig. 2A). This value assumes the use of the 5'-most methionine codon in the open reading frame. No consensus sequences for intron splicing (31) were found in the sequence 5' (150 bp) to the predicted translational initiation site. A consensus tripartite control sequence for transcription termination (65) starts 69 bp 3' to the translation termination site.

The predicted gene 1-encoded protein was compared with sequences in the GenBank and National Biomedical Research Foundation data bases (52) and found to possess sequence similarity to members of the type 1 protein phosphatase family through its C-terminal half. The gene 1 protein shares 62% sequence identity through its C-terminal 300 amino acids to the *S. cerevisiae* Dis2S1 protein (45), which is a type 1 protein phosphatase. It also shares 94% sequence identity through this region with the protein phosphatase encoded by *S. cerevisiae PPZ1* (Fig. 2B) (55). Partial sequence of a yeast gene closely related to *PPZ1*, designated *PPZ2*, has also been reported (12). The yeast *PPZ2* gene was isolated as a contaminant of a rabbit brain cDNA library by hybridization to a probe derived from rabbit protein phosphatase 1α. Gene 1 is identical to *PPZ2* over the 666 bp presented in a previous report (12) and probably represents the same locus. Gene 1 will henceforth be referred to as *PPZ2*. Ppz1p and Ppz2p share 63% overall sequence identity, suggesting that they may be functionally overlapping. A multicopy plasmid bearing the *PPZ1* gene (pACG-1 [55]; provided by J. Arino) suppressed the *mpk1*Δ::*TRP1* mutation poorly compared with *PPZ2* (Fig. 3B). Relative to known type 1 protein phosphatases, the predicted Ppz1 and

A

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                                                                                                                                                   *
-150 TCTCAGCATAGCATTAACATAGGTGCCTCAGAGTGACAAAGAGAGAAATAGTTAACTAGAATACGGTGCAAATTCAGAGAAAAAATATCAGATTCTATTCTATTTACCCCCCCTATAACTGCAGGATCGTACATTTGATCAGCAAGTACC
   1 ATGGGTAATTCCGGTTCGAAACAACATACCAAGCACAATAGCAAGAAGGATGACCACGACGGTGACAGGAAAAAGACACTAGATCTCCCACCACTGACAAAATCGGATACTACACATTCGCTGAAATCGTCAAGGTCGCTGAGGTCGCTG
     M  G  N  S  G  S  K  Q  H  T  K  H  N  S  K  K  D  D  H  D  G  D  R  K  K  T  L  D  L  P  P  L  T  K  S  D  T  T  H  S  L  K  S  S  R  S  L  R  S  L
                                                                                                                                                  50
 151 CGGTCGAAACGCTCAGAGGCGTCGTTGGCATCTAATGTTCAAGCTCAAACGCAACCCCTCTCCCGCAGATCGTCCACTCTGGGAAACGGCAATCGCAACCATAGGCGATCAAACAACGCCCCAATCACTCCTCCTAATAACCACTACCTA
     R  S  K  R  S  E  A  S  L  A  S  N  V  Q  A  Q  T  Q  P  L  S  R  R  S  S  T  L  G  N  G  N  R  N  H  R  R  S  N  N  A  P  I  T  P  P  N  N  H  Y  L
                                                                                                                                                 100
 301 ACCTCACATCCCAGTTCGTCAAGAAGACTCTCTTCCTCTTCGAGAAGGTCGAGCATGGGGAATAATAACAATTCAGAATTGCCCCCGTCAATGATCCAGATGGAGCCCAAATCACCCATCTTGAAAAATAGTACAAGCATGCATTCCACT
     T  S  H  P  S  S  S  R  R  L  S  S  S  S  R  R  S  S  M  G  N  N  N  N  S  E  L  P  P  S  M  I  Q  M  E  P  K  S  P  I  L  K  N  S  T  S  M  H  S  T
                                                                                                                                                 150
 451 AGCTCTTTCAACTCTTACGAAAATGCGTTGACTGATGATGACGACGATAGGGGGGATGATGGCGGCGAGAGCCCCTCAATGGCCAAAGTTACTCGCATCAACACTAGTTCTTCCGCAGATAGAGGCTCCAAGCGTACTCCTTTAAGACGT
     S  S  F  N  S  Y  E  N  A  L  T  D  D  D  D  D  R  G  D  D  G  G  E  S  P  S  M  A  K  V  T  R  I  N  T  S  S  S  A  D  K  G  S  K  R  T  P  L  R  R
                                                                                                                                                 200
 601 CATAATTCATTACAACCGGAAAAAGGTGTAACAGGATTTTCCTCGACTTCATCGAAATTACGCAGAAGATCAGACAATACTCTGCCAGCAACTTATCCTTTAAATGCAGAAGCAGGAGGGAATGGCAGCGACTATTTCTCAAACAGATCA
     H  N  S  L  Q  P  E  K  G  V  T  G  F  S  S  T  S  S  K  L  R  R  R  S  D  N  T  L  P  A  T  Y  P  L  N  A  E  A  G  G  N  G  S  D  Y  F  S  N  R  S
                                                                                                                                                 250
 751 AACTCGCATGCATCATCCAGGAAGTCGTCATTTGGCTCCACTGGGAATACAGCTTATAGCACACCACTGCATTCACCTGCGTTAAGAAAAATGAGCTCGAGGGACAACGACGATAGCGGAGACAACGTCAACGGTAGAGGCACCTCACCT
     N  S  H  A  S  S  R  K  S  S  F  G  S  T  G  N  T  A  Y  S  T  P  L  H  S  P  A  L  R  K  M  S  S  R  D  N  D  D  S  G  D  N  V  N  G  R  G  T  S  P
                                                                                                                                                 300
 901 ATCCCTAATCTAAACATAGACAAGCCTTCGCCGTCAGCGTCGTCAGCGTCAAAAAGAGAATATTTAAGTGCATATCCAACACTAGCTCACAGAGATTCATCATCTTCACTTAGTCCGCGGGGCAAAGGACAACGGTCTTCATCGTCTTCC
     I  P  N  L  N  I  D  K  P  C  P  S  A  S  S  S  A  S  K  R  E  Y  L  S  A  Y  P  T  L  A  H  R  D  S  S  S  S  L  S  P  R  G  K  G  Q  R  S  S  S  S
                                                                                                                                                 350
1051 AGTTCCAGTCAAAGAATATATGTTTCCCCACCATCTCCCACAGGTGATTTCGTACACGGGAGTTGTGCAGACGGTGATAATGGATCTAGGACTAATACTATGGTCGAAATGAAAAGGAAAAAACCAGTTCGTCCAGTGGACATAGATGAA
     S  S  S  Q  R  I  Y  V  S  P  P  S  P  T  G  D  F  V  H  G  S  C  A  D  G  D  N  G  S  R  T  N  T  M  V  E  M  K  R  K  K  P  V  R  P  V  D  I  D  E
                                                                                                                                                 400
1201 ATTATCCAGAGATTACTAGATGCCGGCTATGCCGCAAAGAGGACCAAGAATGTTTGCTTAAAGAATTCCGAGATTATTCAAATTTGCCATAAGGCTCGTGAATTATTCCTTGCCCAACCTGCTCTCTTAGAATTATCTCCCTCGGTAAAA
     I  I  Q  R  L  L  D  A  G  Y  A  A  K  R  T  K  N  V  C  L  K  N  S  E  I  I  Q  I  C  H  K  A  R  E  L  F  L  A  Q  P  A  L  L  E  L  S  P  S  V  K
                                                                                                                                                 450
1351 ATAGTGGGTGATGTTCACGGCCAATATGCAGATCTTTTGAGACTTTTTACCAAATGCGGTTTCCCCCCCATGGCAAATTACTTATTTTTAGGCGATTACGTAGATCGCGGTAAGCAGTCCCTGGAGACCATTTTACTATTACTATGCTAT
     I  V  G  D  V  H  G  Q  Y  A  D  L  L  R  L  F  T  K  C  G  F  P  P  M  A  N  Y  L  F  L  G  D  Y  V  D  R  G  K  Q  S  L  E  T  I  L  L  L  L  C  Y
                                                                                                                                                 500
1501 AAGATTAAATATCCTGAAAATTTCTTCCTCTTAAGAGGCAATCATGAATGTGCCAATGTTACAAGAGTCTACGGGTTTTATGATGAATGTAAACGACGTTGTAATATCAAGATTTGGAAAACCTTTGTTGACACGTTCAACACGCTACCG
     K  I  K  Y  P  E  N  F  F  L  L  R  G  N  H  E  C  A  N  V  T  R  V  Y  G  F  Y  D  E  C  K  R  R  C  N  I  K  I  W  K  T  F  V  D  T  F  N  T  L  P
                                                                                                                                                 550
1651 TTAGCAGCCATCGTCACAGGAAAAATATTTTGTGTTCATGGTGGACTATCACCTGTTCTAAATTCCATGGACGAAATTAGGCACGTTAGTAGGCCCACCGATGTACCCGACTTCGGCTTAATTAATGACCTTTTATGGTCGGATCCTACA
     L  A  A  I  V  T  G  K  I  F  C  V  H  G  G  L  S  P  V  L  N  S  M  D  E  I  R  H  V  S  R  P  T  D  V  P  D  F  G  L  I  N  D  L  L  W  S  D  P  T
                                                                                                                                                 600
1801 GATTCATCGAATGAATGGGAGGATAATGAGCGTGGAGTTAGTTTTTGTTACAATAAAGTGGCTATTAATAAATTTTTAAACAAATTCGGATTCGATTTAGTGTGTAGAGCACATATGGTGGTGGAAGATGGTTATGAATTCTTTAATGAC
     D  S  S  N  E  W  E  D  N  E  R  G  V  S  F  C  Y  N  K  V  A  I  N  K  F  L  N  K  F  G  F  D  L  V  C  R  A  H  M  V  V  E  D  G  Y  E  F  F  N  D
                                                                                                                                                 650
1951 AGAAGCTTAGTTACAGTGTTTTCGGCTCCCAACTATTGTGGTGAATTCGATAACTGGGGTGCTGTCATGACCGTTAGTGAAGGCCTACTCTGTTCTTTTGAGTTGTTGGACCCACTGGACAGTACCGCTTTGAAACAAGTGATGAAAAAA
     R  S  L  V  T  V  F  S  A  P  N  Y  C  G  E  F  D  N  W  G  A  V  M  T  V  S  E  G  L  L  C  S  F  E  L  L  D  P  L  D  S  T  A  L  K  Q  V  M  K  K
                                                                                                                                                 700
2101 GGCAGGCAAGAACGTAAATTAGCCAATCGCTGAAAAACTAGATATTAATGTTGCAGTTAATAAGCTCATTGAAAACTTTCTTTGGTTGTTCCTCTTCCTTCTTTTTTTCGCCGATAAAGTTGTCGTTATAGTTTCACTTATATCTTACGA
     G  R  Q  E  R  K  L  A  N  R  .

2251 GAATCTAAATAAGTAAATATTAATATTATTCAATTTCTCATTTCCTTTCCTCTGGGTTTTTTGAGAGTACGAGTTTTATTGCTCGCTTTTTGTATTTTCACTTTGCCGGCTTTTTCACTATTCCCTATTGTCTTAATAAAGTTCCTGCTT
2401 GGGCCCAGTAAACTTTTAAAATTTATGTGCTGAAAGAAAGTTGAAACAATCACAAGAGGAGAGCAGATTTGCCAAAAAAGAATCTGAGCTGTTTGTTCCTGTCTAATACTAAAAGATATAAATAAACAATATGTATACAAAAGAGTACTA
2551 CTGGTTTTCACAATATATGATAATAACAAGCACTTTGGTGCTCACCATTATATGGTCCATCTTACCATCATCGCTGGGTGAGGCTGCACCAAAGCAGTTTATCAACACGCTATTGGACATCTTCCCACAAAGAAGATGGATTATTACCTT
```

FIG. 2. (A) Nucleotide sequence and predicted amino acid sequence of the *PPZ2* gene. The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The asterisk indicates the *Sau*3A site representing the 5′ end of *PPZ2Δ5′*. The nucleotide sequence of *PPZ2* was determined for both strands. (B) Alignment of the predicted Ppz1p and Ppz2p sequences. Identical residues are boxed. The deduced amino acid sequence for Ppz1p has been published (55). Gaps were introduced as indicated by dashes.

Ppz2 proteins possess amino-terminal extensions of approximately 300 amino acids. These sequences are extremely rich in serine residues, 26% in PPZ1 and 22% in PPZ2. The significance of this amino acid composition is not clear. Similar amounts of *PPZ1*- and *PPZ2*-derived 2.7-kb mRNA were detected in proliferating diploid cells (data not shown).

Deletion of both *PPZ1* and *PPZ2* results in a temperature-dependent cell lysis defect. Loss of *PPZ1* function was reported to result in no apparent phenotypic defect (55). To examine the phenotypic defect associated with loss of *PPZ2* function, a deletion mutant of *PPZ2* was constructed in vitro. A 730-bp fragment of *PPZ2*, which includes the predicted catalytic domain, was replaced with the *S. cerevisiae LEU2* gene (see Materials and Methods). Anticipating that mutants defective in *PPZ2* would be viable, the deletion allele (*ppz2Δ::LEU2*) was transplaced into a haploid strain (EG123 [*MAT*α]) bearing multiple auxotrophic markers. Transformants were tested for possession of the *ppz2Δ::LEU2* allele by restriction and hybridization analysis (not shown). Haploids bearing the *ppz2Δ::LEU2* mutation grew normally on rich medium at 23, 30, and 37°C.

To test the possibility that *PPZ1* and *PPZ2* are functionally overlapping, a double mutant defective in both genes was constructed. First, a deletion mutant of *PPZ1* was constructed as described previously (55). The deletion allele (*ppz1Δ::URA3*) was transplaced into haploid strain 1783 (*MAT***a**). Transformants were tested for possession of the *ppz1Δ::URA3* allele by restriction and hybridization analysis (not shown). As reported previously, strains bearing the *ppz1Δ::URA3* allele grew normally on rich medium. Haploids bearing the *ppz1Δ::URA3* mutation were crossed to haploids bearing the *ppz2Δ::LEU2* mutation. The resulting diploids, heterozygous for both mutations, were induced to sporulate, and tetrads were dissected. Four spores from each tetrad gave rise to colonies at 23°C, but the Ura$^+$ Leu$^+$ segregants (bearing both *ppzΔ* mutations) grew very slowly at 37°C. A representative Ura$^+$ Leu$^+$ segregant is shown in Fig. 4A. Microscopic examination of these cells revealed a high frequency (approximately 50%) of nonrefractile ghosts at 37°C (not shown), suggesting that cell lysis was occurring at the high temperature.

Diploid cells homozygous for both *ppzΔ* mutations failed to grow at 37°C (Fig. 4) and were nearly all (>90%) nonrefractile. Although diploid cells lysed at the restrictive temperature, the dead cells had an elongated appearance similar to pseudohyphae (17). It is not clear why cells adopted this

```
B
PPZ1   1 MGNSSSKSSKKDSHSNSSSRNPRPQVSRTETSHSVKSAKSNKSSRSRRSLPSSSTTNTN-
PPZ2   1 MGNSGSKQHTKHNSKKDDHDGDRKKTLDLPPLTKSDTTHSLKSSRSLRSLRSKRSEASLA

PPZ1  60 SNVPDPSTPSKPNLEVNHQRHSSHTNRYHFPSSSHSHSNSQNELLTTPSSSSTKRPSTS-
PPZ2  61 SNVQAQTQPLSRRSSTLGNGNRNHRRSNNAPITPPNNHYLTSH----PSSS--RRLSSSS

PPZ1 119 RRSSYNTKAAADLPPSMIQMEPKSPILKTNNSSTHVSKHKSSYSSTYYENALTDDDND--
PPZ2 115 RRSSMGNNNNSELPPSMIQMEPKSPILKNSTSMHST----SSFNS--YENALTDDDDDRG

PPZ1 177 DKDNDISHTKRFSRSSNSRPSSIRSG-SVSRRKSDVTHEEPNNGSYSSNNQENYLVQALT
PPZ2 169 DDGGESPSMAKVTRINTSSSADKGSKRTPLRRHNSLQPEKGVTG-FSSTSSKLRRRSDNT

PPZ1 236 -------------------RSNSHASSLHSRKSSFGSDGNTAYSTPLNSPGLSKLT--
PPZ2 228 LPATYPLNAEAGGNGSDYFSNRSNSHASS---RKSSFGSTGNTAYSTPLHSPALRKMSSR

PPZ1 273 --DHSGEYFTSNSTSSLNHHSSRDIYPSKHISNDDDIENSSQLSNIHAS-----------
PPZ2 285 DNDDSGDNVNGRGTSPIPNLNIDKPCPSASSASKREYLSAYPTLAHRDSSSSLSPRGKGQ

PPZ1 320 ----------MENVNDKNNNITDSKKDPNEGFNDIMQSSGNKNAPKKFKKPIDIDETIQK
PPZ2 345 RSSSSSSSSQRIYVSPPSPTGDFVHGSCADGDNGSRTNTMVEMKRKKPVRPVDIDEIIQR

PPZ1 370 LLDAGYAAKRTKNVCLKNNEILQICIKAREIFLSQPSLLELSPPVKIVGDVHGQYGDLLR
PPZ2 405 LLDAGYAAKRTKNVCLKNSEIIQICHKARELFLAQPALLELSPSVKIVGDVHGQYADLLR

PPZ1 430 LFTKCGFPPSSNYLFLGDYVDRGKQSLETILLLFCYKIKYPENFFLLRGNHECANVTRVY
PPZ2 465 LFTKCGFPPMANYLFLGDYVDRGKQSLETILLLLCYKIKYPENFFLLRGNHECANVTRVY

PPZ1 490 GFYDECKRRCNIKIWKTFIDTFNTLPLAAIVAGKIFCVHGGLSPVLNSMDEIRHVVRPTD
PPZ2 525 GFYDECKRRCNIKIWKTFVDTFNTLPLAAIVTGKIFCVHGGLSPVLNSMDEIRHVSRPTD

PPZ1 550 VPDFGLINDLLWSDPTDSPNEWEDNERGVSYCYNKVAINKFLNKFGFDLVCRAHMVVEDG
PPZ2 585 VPDFGLINDLLWSDPTDSSNEWEDNERGVSFCYNKVAINKFLNKFGFDLVCRAHMVVEDG

PPZ1 610 YEFFNDRSLVTVFSAPNYCGEFDNWGAVMSVSEGLLCSFELLDPLDSAALKQVMKKGRQE
PPZ2 645 YEFFNDRSLVTVFSAPNYCGEFDNWGAVMTVSEGLLCSFELLDPLDSTALKQVMKKGRQE

PPZ1 670 RKLANQQQQMMETSITNDNESQQ
PPZ2 705 RKLANR
```

FIG. 2—*Continued.*

morphology only at the restrictive temperature, but this diploid-specific phenotype probably accounts for the greater severity of the lysis defect in the diploid *ppz1,2*Δ double mutants than in haploid mutants. The temperature sensitivity of the double mutants (of all mating types) was suppressed by incorporation of sorbitol to 1 M into the medium

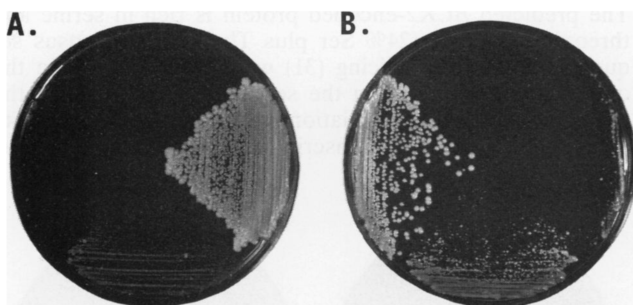

FIG. 3. Suppression of the *pkc1*Δ or *mpk1*Δ defect by *PPZ2*. (A) A *pkc1*Δ strain was transformed with plasmids in the presence of 1 M sorbitol. Representative transformants were streaked onto a YEP-glucose plate and incubated at 30°C for 4 days. Strains bear (clockwise from top) multicopy plasmid YEp352 (DL831), low-copy-number plasmid YCp50[*PKC1*] (DL106), YEp352[*PPZ2*] (DL833), or YEp352[*PPZ2*Δ*5'*] (DL832). The point of truncation in *PPZ2*Δ*5'* is indicated in Fig. 2A. (B) An *mpk1*Δ strain (DL456) was transformed with plasmids in the presence of 1 M sorbitol. Representative transformants were streaked onto a YEP-glucose plate and incubated at 37°C for 3 days. Strains bear (clockwise from top) low-copy-number plasmid YCp50-*LEU2* (DL769), multicopy plasmid pACG-1 (carrying *PPZ1*; strain DL797), multicopy plasmid YEp352[*PPZ2*] (DL776), or YCp50-*LEU2*[*MPK1*] (DL750).

for osmotic support (Fig. 4A). However, the elongated morphology of diploids at high temperature was retained in the presence of sorbitol. Diploids defective in either *PPZ1* or *PPZ2* alone grew normally at 37°C (Fig. 4B).

The temperature-dependent cell lysis defect displayed by the *ppz1,2*Δ mutant was similar to that observed for *mpk1*Δ (32), *mkk1,2*Δ (24), or *bck1*Δ (33) mutants except for the elongated morphology of the lysed cells. Epistatic interactions among the latter genes, all of which encode protein kinases, indicate that they function in a linear pathway, with *BCK1* acting first and *MPK1* acting last (24, 32). Two criteria were used to define this linear pathway. First, defects in one pathway component could be suppressed by overexpression or by mutational activation of pathway components that function downstream, but not upstream, of the defective component. Second, loss of function of multiple pathway components (e.g., *MPK1* and *BCK1*) resulted in a defect that was no more severe than that associated with loss of function of a single component. Because *PPZ2* was isolated as a dosage-dependent suppressor of an *mpk1*Δ mutation, we examined the possibility that the *PPZ1*- and *PPZ2*-encoded protein phosphatases function downstream of *MPK1* in the same linear pathway. We first tested the dosage-dependent ability of various *PKC1* pathway components to suppress the *ppz1,2*Δ-associated cell lysis defect. Maintenance of *MPK1*, *BCK1*, or *MKK1* in multiple copies weakly suppressed the *ppz1,2*Δ defect (data not shown), suggesting that *PPZ1* and *PPZ2* do not function downstream of *MPK1* in a linear, unbranched pathway. Overexpression of *PKC1* failed to suppress the double *ppz1,2*Δ defect.

We next examined the phenotypic effects resulting from loss of *PPZ1* and *PPZ2* function together with loss of *MPK1* function. Strains bearing an *mpk1*Δ mutation and a *ppz2*Δ mutation grew nearly as well as an *mpk1*Δ mutant at the temperature that is permissive for *mpk1*Δ mutants (23°C; Fig. 5). In contrast, *mpk1*Δ *ppz1*Δ double mutants grew very poorly at this temperature. The triple *mpk1*Δ *ppz1,2*Δ mutant was inviable. These defects were suppressed in the presence of 1 M sorbitol. The additivity of the *ppz1,2*Δ defect and the *mpk1*Δ defect closely approximated the *pkc1*Δ defect. These observations failed to establish an epistatic hierarchy between *PPZ1/2* and *MPK1* and therefore do not support a model in which the putative Ppz1 and Ppz2 protein phosphatases function downstream of Mpk1p in a linear, unbranched pathway.

Additivity of the *pkc1*Δ defect with the *ppz1*Δ defect. To determine whether the functions of *PPZ1* and *PPZ2* are entirely under the regulatory control of the *PKC1* gene, we examined the additivity of the *ppz1*Δ defect with the *pkc1*Δ defect. Although *pkc1*Δ mutants grow well at 30°C in the presence of 1 M sorbitol, they grow poorly in the presence of 0.5 M sorbitol at this temperature and do not grow appreciably if the sorbitol concentration is less than 0.5 M (not shown). Figure 6 shows that a *pkc1*Δ *mpk1*Δ double mutant grows as well as a *pkc1*Δ mutant under the semipermissive conditions established for the latter. The lack of additivity of the *mpk1*Δ and *pkc1*Δ defects is consistent with a model in which *MPK1* function is entirely under the control of the *PKC1* gene. In contrast, *pkc1*Δ *ppz1*Δ double mutants failed to grow under these conditions. The defect associated with this mutant was suppressed by 1 M sorbitol. The additivity of the *ppz1*Δ defect with the *pkc1*Δ defect suggests either that *PPZ1* and presumably *PPZ2* are not under the regulatory control of *PKC1* or that they are regulated by *PKC1* but retain residual function in a *pkc1*Δ mutant.

Molecular characterization of gene 2, *BCK2*. The plasmid

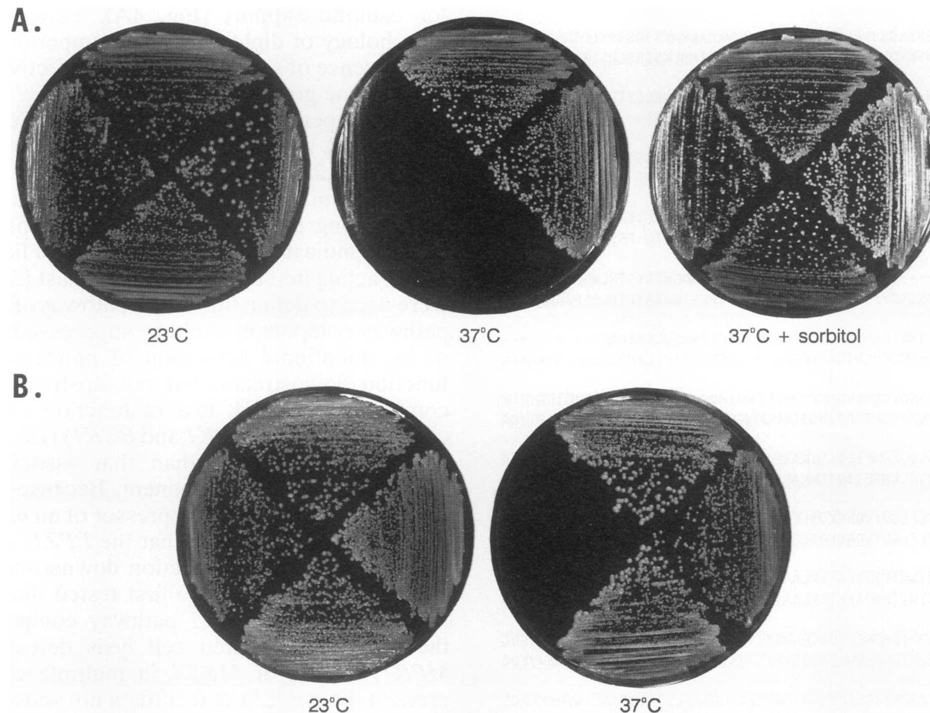

FIG. 4. Temperature-dependent cell lysis of *ppz1,2Δ* double mutants. (A) Haploid or diploid strains were streaked onto either YEP-glucose plates or YEP-glucose supplemented with 1 M sorbitol and incubated at the indicated temperature for 3 days. Sorbitol was added to 1 M where indicated to prevent cell lysis. Strains are (clockwise from top) wild-type haploid (1783), wild-type diploid (1788), *ppz1Δ ppz2Δ* diploid (DL795), and *ppz1Δ ppz2Δ* haploid (DL791). (B) Diploid strains defective in either *PPZ1* or *PPZ2* were streaked onto YEP-glucose plates as described above. Strains are (clockwise from top) wild type (1788), *ppz1Δ* (DL793), *ppz2Δ* (DL794), and *ppz1Δ ppz2Δ* (DL795).

containing gene 2 was subjected to deletion analysis (Fig. 7). Sequences on the left end of the clone (up to the leftmost *Hin*dIII site) were sufficient for suppression of *mpk1Δ::TRP1*. DNA sequence analysis of this region of the clone revealed an open reading frame extending from the insert junction (at a *Sau*3A site; Fig. 8) to approximately 500 bp before the *Hin*dIII site. Because the 5′ end of this gene was missing from the suppressing clone, we isolated the full-length gene from a different genomic yeast library by molecular hybridization (see Materials and Methods). The full-length gene 2, which was a more effective suppressor of *mpk1Δ::TRP1* than was the original isolate, also suppressed a *pkc1Δ* mutation (Fig. 9) and was therefore designated *BCK2* (for bypass of C kinase). The *BCK2* gene encodes a 2,553-bp open reading frame corresponding to a polypeptide with a predicted length of 851 amino acids (calculated molecular size of 94 kDa; Fig. 8). This value assumes the use of the 5′-most methionine codon in the open reading frame. The predicted *BCK2*-encoded protein is rich in serine and threonine residues (24% Ser plus Thr). No consensus sequences for intron splicing (31) were identified within the open reading frame or in the sequence 5′ (150 bp) to the predicted translational initiation site. A consensus tripartite control sequence for transcription termination (65) was

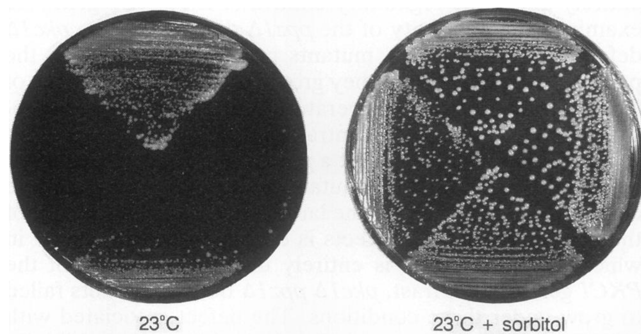

FIG. 5. The *mpk1Δ* and *ppz1,2Δ* defects are additive. Diploid strains were streaked onto either a YEP-glucose plate or a YEP-glucose plate supplemented with 1 M sorbitol and incubated at 23°C for 3 days. Strains are (clockwise from top) *mpk1Δ* (DL456), *mpk1Δ ppz1Δ* (DL823), *mpk1Δ ppz2Δ* (DL824), and *mpk1Δ ppz1Δ ppz2Δ* (DL825).

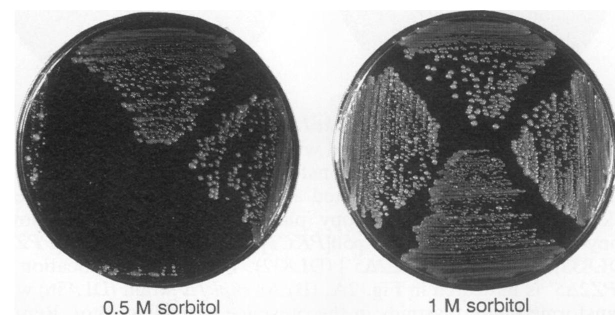

FIG. 6. The *ppz1Δ* defect and the *bck2Δ* defect are additive with the *pkc1Δ* defect. Haploid strains were streaked onto YEP-glucose plates with either 0.5 or 1 M sorbitol and incubated at 30°C for 3 days. Strains are (clockwise from top) *pkc1Δ* (DL377), *pkc1Δ mpk1Δ* (DL870), *pkc1Δ ppz1Δ* (DL873), and *pkc1Δ bck2Δ* (DL876).

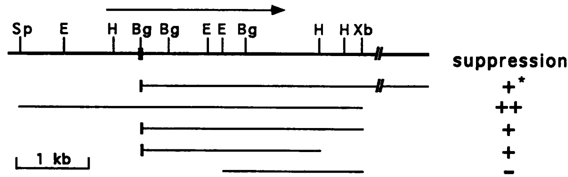

FIG. 7. Restriction and deletion maps of the *BCK2* locus. Deletions within the genomic DNA carried in the multicopy vector YEp24 were generated by restriction endonuclease digestion. In some cases, fragments were subcloned into the multicopy vector YEp352. The ability (+) or inability (−) of the resulting plasmids to suppress the *mpk1Δ::TRP1* mutation is shown. The DNA fragments present in the plasmids are indicated. The original *BCK2* isolate (*BCK2ΔN*) is marked with an asterisk,and the vertical bar demonstrates the 5′ end of this clone. The arrow indicates the direction and limits of the *BCK2*-encoded open reading frame. Abbreviations for selected restriction sites: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Sp, *Sph*I; Xb, *Xba*I.

identified starting 79 bp 3′ to the translation termination site. The predicted *BCK2*-encoded protein was not found to be similar to any proteins in the data bases. Apparently the amino-terminal third of the protein is not essential for suppressor function because these sequences were absent from the original *mpk1Δ*-suppressing isolate of *BCK2* (*BCK2ΔN*). Because both *BCK2ΔN* and the original isolate of *PPZ2* (*PPZ2Δ5′*), which was missing 5′ regulatory sequences, were cloned in the same orientation into the *Bam*HI site of YEp24, it seems likely that there exist sequences within the vector (proximal to the *Sph*I site in YEp24) that function as a transcriptional promoter in *S. cerevisiae*.

Deletion of *BCK2*. To examine the phenotypic defect associated with loss of *BCK2* function, a deletion mutant of *BCK2* was constructed in vitro. A 1.1-kb *Bgl*II fragment of the *BCK2* open reading frame was replaced with the *S. cerevisiae TRP1* gene (see Materials and Methods). This deletion allele (*bck2Δ::TRP1*) was transplaced into diploid strain 1788. Transformants were tested for possession of the

```
-150 TCAAATTATTGACTTTTTTTTCAGTGGATTTGTTGTTAATAAAGGAAACAATCAGATCAGCCATTTAGGGAAGTCTTTTAAGAAAGCTCAGAAGGAGATCTAAGACAAGAAGCAAGGCAGTCTTGATTTATCGCTCAAAACCAATAGCAC
   1 ATGCCGAAGCATTGTCACCACCATCGTTCCAGTTCGGTTAATTCCACAAAGAGTCGTTCTACGGAATCTACGAATAAATGGAAAATTCCGCACTATTATCGCAGATCCGCTAGCGGCAGCACACAAGCTTCGCCGGACAGGAACTCCTCA
     M P K H S H H H R S S S V N S T K S R S T E S T N K W K I P H Y Y R R S A S G S T Q A S P D R N S S
                                                                                                  50
 151 ACAGGCTCATGTAGCACTCCAGTATTACCTACTATGAATGTTATGTCTAGCCCGAAAAAAGTTTTGCTAGAGGATCCCAGAGACAACCATACTAAGGCGAAGAAGAGTAGTAGAAAGAAATCAGGTGAAATGGTCTTCGTCAATTATGCT
     T G S C S T P V L P T M N V M S S P K K V L L E D P R D N H T K A K K S S R K K S G E M V F V N Y A
                                                                                                 100
 301 GTACAGGACACGGCTAACGAAAATGATACTGACTTGCAAACCCAGCCGGTTTCTGTGCCAGCACCAAAGGCGAAATTAAAGAAGAAATCCTCCAAAAGGAGAATGCTGAAGATATTTGGATCGTCAAAGAACGAACACATAGAGGACATC
     V Q D T A N E N D T D L Q T Q P V S V P A P K A K L K K K S S K R R M L K I F G S S K N E H I E D I
                                                                            *                    150
 451 GTTGAAGAGCAACCAATGGTGCTTCAAATGGATTCAGAATCTAAACCCTTTTCGGGAACCCCTATCTCAGAAAGCGGAATCGACGCCTCGTCACTAACAACCAAAAGATCTTATAACTCGTTCTTAAAACACAATAGGCTAAACGGTAAA
     V E E Q P M V L Q M D S E S K P F S G T P I S E S G I D A S S L T T K R S Y N S F L K H N R L N G K
                                                                                                 200
 601 ACTCCGTTCTCTGGAAACTTGTCCTTTCCATCATTAAACATGATGGGTAACACTACTGATCTGCCTATTGACAATAACGATTTTTGTTCCGAGAAAGAAGTCGTTCCGAAATCAACTCATGATCCTTCACTAGCAAAACCGCCATCAAGG
     T P F S G N L S F P S L N M M G N T T D L P I D N N D F C S E K E V V P K S T H D P S L A K P P S R
                                                                                                 250
 751 TTCACTGAAAGCGAAACAAATTCTACTCCCAACTTATCAAGTATACCTCTCATGAACACAAAAAATACAAGATTGAAGTACAATAAAGTGGCGCCTCAGAGTTCAGACCGACAAAAGTCACAGGAAAGTGGGCTATATCATTCTACCGAA
     F T E S E T N P T P N L S S I P L M N T K N T R L K Y N K V A P Q S S D R Q K S Q E S G L Y H S T E
                                                                                                 300
 901 TCATTCAACTTCAAAGATCAAAACTACAGTAACAATAAATCCTCTTTAAGTCTGAACTCCGATTTAAGTACACCCCACTTTGCAAAGCATTCACCTGATTCACCAAGAACTTCAAGATCTTTCAACTGCGGAGACTCACAAAGTAAAGTT
     S F N F K D Q N Y S N N K S S L S L N F D L S T P H F A K H S P D S P R T S R S F N C G D S Q S K V
                                                                                                 350
1051 AAATTACCGGAAGAAAATGACGCTTCTATTGCATTTAGTAAGATGTTTACTAGAAAACGAGCCAACACTGGCGGGTCCACGTGTTCGCTAGCCTCACCCACTATTGCACAAACTATTCAGCAATCAAATATAAAAGTTAATAAATTGCCA
     K L P E E N D A S I A F S K M F T R K R A N T G G S T C S L A S P T I A Q T I Q Q S N I K V N K L P
                                                                                                 400
1201 ACTCAGCGAACCACTTCAGTTGGCTCATTATCATCCATGTCAAATCGTTATTCACCAATAAGAGTTGCATCGCCAGGAAGAGCAAGATCCGCAACTCGTGGGTCTTCCCTTTATAGATTATCCAGAGACCTTAATTCTTTACCAAGCGTC
     T Q R T T S V G S L S S M S N R Y S P I R V A C P G R A R S A T R G S S L Y R L F R D L N S L P S V
                                                                                                 450
1351 ACTGATCTACCAGAAATGGATAGTACAACCCCAGTTAACGAAATATTCTTGGATGGCCAACCACAGCATAAAAGTGGCAGTGTCAAAGGAGGGCATAGGAAGAAACAAGAATCTATCTCTGATGCTCAAAGAATTCAACATTCTAATTCG
     T D L P E M D S T T P V N E I F L D G Q P Q H K S G S V K G G H R K K Q E S I S D A Q R I Q H S N L
                                                                                                 500
1501 TACATTACAACACCTTCATCTTCTCTGGTGACTCCTCCTTACTACATGACAGGTTACACATTACCAAGTTCTGCATCTGCTTCTTCAACTCCAAATGTGCTTGAAACACACAATATGAACTTTGTTCCAAGTACCAGTACTGTTACAAGT
     Y I T T P S S S L V T P P Y Y M T G Y T L P S S A S A S S T R N V L E T H N M N F V P S T S T V T S
                                                                                                 550
1651 TATCGCCCATCTAGCAATTTTTCCTCATTTGACAAAGAATACAGTAACGAAAACGACGCTAGTGGGGAATTCTCTGCTTTCAATACTCCAATGGAGAATATACCGGCACTAAAAGGTATACCTAGATCCACGTTAGAAGAAAATGAAGAA
     Y R P S S N F F S F D K E Y S N E N D A S G E F S A F N T P M E N I P A L K G I P R S T L E E N E E
                                                                                                 600
1801 GAGGATGTCCTAGTACAAGATATTCCGAATACGGCACACTTCCAAAGAAGGGATATTATGGGGATGGATACTCACAGAAAGGATGACAGTTTAGATTTTAACTCTTTGATGCCACACGGTAGTACAACTAGTAGCAGCATCGTAGATTCT
     E D V L V Q D I P N T A H F Q R R D I M G M D T H R K D D S L D F N S L M P H G S T T S S S I V D S
                                                                                                 650
1951 GTTATGACGAACTCAATATCCACTACAACAAGCAACGCGACCGGAAACTACTTTCAAGATCAAGATAAGTATACATTGGTAAATACGGGATTGGGATTGAGTGATGCAAACCTCGATCATTTTATTAGATCTCAATGGAAACACGCTTCT
     V M T N S I S T T T S N A T G N Y F Q D Q D K Y T L V N T G L G L S D A N L D H F I R S Q W K H A S
                                                                                                 700
2101 CGATCAGAATCCAATAATAATACCGGAAATCGCGTTTCTTACAGTGGCTCAACACCAAACAATGTTGATACAACAAAGACTAATTTGCAAGTGTATACCGAGTTCGATTTTGAAAACCCAGAGTCGTTTTTCCATGAGCAATCGAAGCTA
     R S E S N N N T G N R V S Y S G S T P N N V D T T K T N L Q V Y T E F D F E N P E S F F H E Q S K L
                                                                                                 750
2251 CTAGGTGAGATGGGCCACAGCAATAACAACAGCAACAGCGCCATCAACATGAATGAACCCAAGTCTGCGGATACATACATTGGAAATATATCTCCGGATACTTCAGCAACTGTTTCATTAGGCGACCTGATGGGCTCAAACGTTTCAAAC
     L G E M G H S N N N S N S A I N M N E P K S A D T Y I G N I S P D T S A T V S L G D L M G S N V S N
                                                                                                 800
2401 AACAGTGAAAGAAATTTTTACGATGGTCATACTTGCGTTCCACAGTATCAGGCGAACTCATCTGTGGAAAACTCAAATAAACAAAATGCAGCACCAATTGCAAATAACGACATTGATAATAATTTACAGTCTTTTTATTTTGATAATAGC
     N S E R N F Y D G H T C V P Q Y Q A N S S V E N S N K Q N A A P I A N N D I D N N L Q S F Y F D N S
                                                                                                 850
2551 AACTAAAAAAGGAATGAAAAAAAAAAAAAAAAAGTTCAAAAATAGTAACAGATATCTTTCGAAAAAGTTAAAAACTGGTAAAAAATTTTGAATAGAGTATATAAAAACTAATAAATATGTTTAGTTATCATACACCATAGAATAACTACTA
     N .

2701 GAGATATTATTTCATTTTCGTTTTCATTTTTTTTTATTTTTTTTCTTTTCAATAAGGTAACAGAAAGTTATTTATGCATTCTTGTTCTACATGTCTTAAGTATACTAATAAGCTACTACAGTATTTTGGTAGTATGGCTTTTAAATGAGTTA
2851 TTAATTCTTGATCAGTCCCTGTAGGATTATCAAACTGCCGCCTAATCGCTTCGTTATTAATTTTACCCAGCCATGGACCTGGCTTCATTTGAAGTAGTTTTGCCATTTGTTTTCCGTCCACTATTGGTTTTAGTTCATGACAATTATTCA
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FIG. 8. Nucleotide sequence and predicted amino acid sequence of the *BCK2* gene. The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The asterisk indicates the *Sau*3A site representing the 5′ end of *BCK2ΔN*. The nucleotide sequence was determined for both strands.

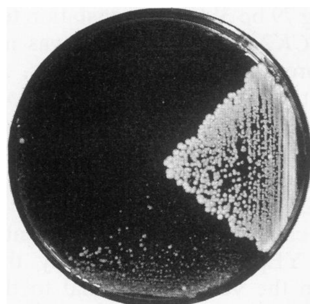

FIG. 9. Suppression of the *pkc1Δ* defect by *BCK2*. A *pkc1Δ* strain was transformed with plasmids in the presence of 1 M sorbitol. Representative transformants were streaked onto a YEP-glucose plate and incubated at 30°C for 4 days. Strains bear (clockwise from top) multicopy plasmid YEp352 (DL831), low-copy-number plasmid YCp50[*PKC1*] (DL106), YEp352[*BCK2*] (DL844), or YEp352[*BCK2ΔN*] (DL843). The point of truncation in *BCK2ΔN* is indicated in Fig. 8.

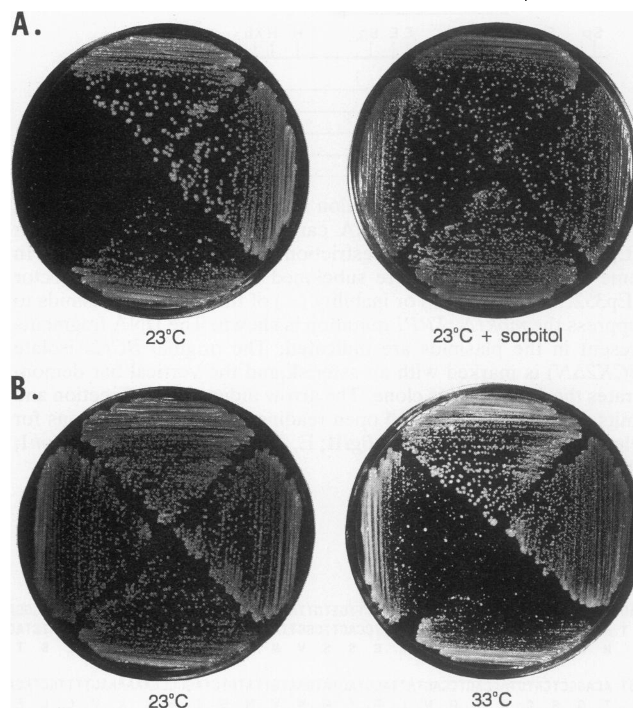

FIG. 10. The *bck2Δ* defect is additive with the *mpk1Δ* defect but not the *ppz1,2Δ* defect. (A) Diploid strains were streaked onto either a YEP-glucose plate or a YEP-glucose plate supplemented with 1 M sorbitol and incubated at 23°C for 3 days. Strains are (clockwise from top) wild type (1788), *bck2Δ* (DL763), *mpk1Δ* (DL456), and *mpk1Δ bck2Δ* (DL827). (B) Diploid strains were streaked onto YEP-glucose plates and incubated at the indicated temperature for 3 days. Strains are (clockwise from top) wild type (1788), *bck2Δ* (DL763), *ppz1Δ ppz2Δ* (DL795), and *ppz1Δ ppz2Δ bck2Δ* (DL830).

deletion allele by restriction and hybridization analysis (not shown). Two independently derived diploids, heterozygous at *BCK2* (*bck2Δ::TRP1/BCK2*), were induced to sporulate, and tetrads were dissected. Four spores from each tetrad gave rise to colonies. The *bck2Δ::TRP1* segregants grew normally at all temperatures ranging from 23 to 37°C. To identify yeast loci that are structurally related to (and potentially functionally overlapping with) *BCK2*, we used *BCK2*-derived DNA fragments to probe genomic yeast DNA under conditions of reduced stringency (see Materials and Methods). No additional hybridizing species were revealed (not shown).

To determine the point within the *PKC1*-mediated pathway at which *BCK2* functions, the additivity of the *bck2Δ::TRP1* mutation with other defects in the pathway was examined. A *bck2Δ mpk1Δ* double mutant grew extremely poorly without osmotic stabilizers at 23°C (Fig. 10A) and was inviable at temperatures above 23°C in the absence of osmotic support. This defect was considerably more severe than that associated with an *mpk1Δ* mutation alone, suggesting that *BCK2* does not function on the protein kinase branch of this pathway. Similar results were obtained when a *bck1Δ* mutation was used rather than an *mpk1Δ* mutation. In contrast, a *ppz1,2Δ bck2Δ* triple mutant displayed a defect that was no more severe than that of a *ppz1,2Δ* double mutant at the permissive (23°C) or semipermissive (33°C) temperature (Fig. 10B). The lack of additivity of the *bck2Δ* defect with the double *ppz1,2Δ* defect suggests that *BCK2* functions on the same branch of the pathway as these protein phosphatase-encoding genes do. Consistent with this observation, the *bck2Δ* defect displayed additivity with the *pkc1Δ* defect in a manner similar to that for the *ppz1Δ* defect (Fig. 6). Overexpression of *BCK2* failed to suppress the *ppz1,2Δ* defect, precluding definitive assignment of the order in which these pathway components function.

Mapping of the chromosomal locations of *PPZ1*, *PPZ2*, and *BCK2*. To determine the chromosomal map positions of *PPZ1*, *PPZ2*, and *BCK2*, probes derived from the coding sequences of these genes were hybridized to a set of λ clone and cosmid grid filters (provided by M. Olson, Washington University). The *PPZ1* probe hybridized with clones 4107 and 9571, which correspond to an overlapping region of chromosome XIII, approximately 60 to 70 kb centromere proximal to *SUP5* (48). Assignment of *PPZ1* to chromosome XIII is consistent with a previous report (55). The *PPZ2* probe hybridized with clone 5320, which corresponds to a region of chromosome IV approximately 40 to 70 kb centromere distal to *ADE8*. The *BCK2* probe hybridized with clones 7060, 7221, and 6197, which correspond to an overlapping region of chromosome V, approximately 10 to 20 kb centromere distal to *RAD4*. Approximately 450 bp of untranslated sequence at the 3′ end of the *BCK2* locus is nearly identical to sequences at the 3′ end of the *ts352* locus (1), indicating that these genes reside next to each other in opposite transcriptional orientation.

DISCUSSION

Isolation of novel components of the *PKC1*-mediated signalling pathway. We proposed previously that the PKC isozyme encoded by the *S. cerevisiae PKC1* gene regulates a bifurcated pathway. On one branch of this pathway, the putative Bck1p, Mkk1p, Mkk2p, and Mpk1p protein kinases function in a linear cascade (24, 32, 33). Yeast strains bearing deletions in any of the genes encoding these protein kinases display a temperature-dependent cell lysis defect (24, 32, 33), whereas a yeast strain bearing a deletion in the *PKC1* gene undergoes cell lysis at all temperatures (35, 51). Epistatic relationships among these genes indicate the following order of function: *BCK1* functions upstream of *MKK1* and *MKK2*, which function upstream of *MPK1*. The *MPK1* gene encodes a homolog of vertebrate MAP kinases (32), and the *MKK1* and *MKK2* genes encode functionally redundant

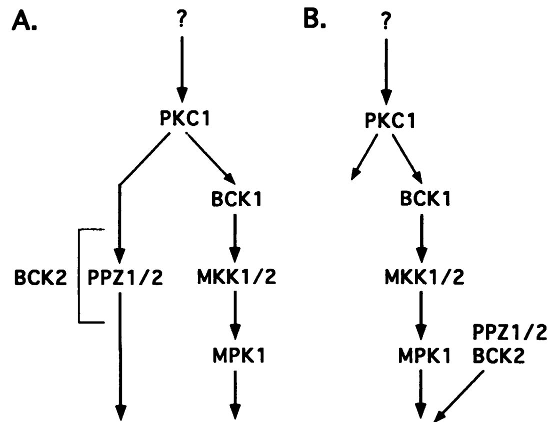

FIG. 11. Models for the interaction of *PPZ1*, *PPZ2*, and *BCK2* with the *PKC1*-mediated signal transduction pathway. Uncertainty regarding the order of function of *PPZ1*, *PPZ2*, and *BCK2* is indicated by the bracket.

homologs of vertebrate MAP kinase kinases (24). To identify additional components of the *PKC1*-mediated pathway, we used an *mpk1*Δ strain to isolate dosage-dependent suppressors of its conditional cell lysis defect. Eight suppressor genes were isolated. We presented in this study the molecular and genetic characterization of two of these genes, designated *PPZ2* and *BCK2*.

***PPZ1* and *PPZ2* encode functionally redundant protein phosphatases.** One of the *mpk1*Δ-suppressing genes was identical to the *PPZ2* gene on the basis of previously reported partial sequence of the latter (12). The *PPZ2* gene is predicted to encode a 78-kDa protein most closely related to type 1 protein phosphatases. The predicted *PPZ2*-encoded protein (Ppz2p) is also closely related to the predicted product of the *S. cerevisiae PPZ1* gene (55), sharing 94% sequence identity through its presumptive C-terminal catalytic domain and 63% identity overall with Ppz1p. The N-terminal halves of both Ppz1p and Ppz2p are extremely rich in serine residues (26 and 22%, respectively).

Deletion mutants of either *PPZ1* or *PPZ2* grew normally over a range of temperatures, but deletion mutants defective in both genes displayed a temperature-dependent cell lysis defect similar to that observed in mutants defective in *BCK1*, *MKK1* and *MKK2*, or *MPK1*. Any model in which *PPZ1* and *PPZ2* are proposed to function in a common pathway with *PKC1* must take into account three observations. (i) Because overexpression of *PPZ2* suppressed both the *pkc1*Δ defect and the *mpk1*Δ defect, *PPZ2* must not act at a point in the pathway before *PKC1* or *MPK1*. (ii) Although *PPZ2* was isolated by virtue of its dosage-dependent ability to suppress the *mpk1*Δ defect, overexpression of *BCK1*, *MKK1*, or *MPK1* also suppressed the *ppz1,2*Δ defect (although poorly), indicating a lack of epistatic hierarchy between the protein kinase-encoding genes and the protein phosphatase-encoding genes. (iii) The *ppz1*Δ and *ppz2*Δ defects were additive with the *mpk1*Δ defect and the *pkc1*Δ defect. Figure 11 outlines two models that are consistent with the observations described above. In the first model (Fig. 11A), *PKC1* regulates *PPZ1* and *PPZ2* on a branch of the pathway that is parallel to that mediated by the protein kinase cascade. The additivity of the *ppz1,2*Δ defect with the *mpk1*Δ defect (approximating the *pkc1*Δ defect) is consistent with loss of both branches of a pathway that is bifurcated below *PKC1*. However, the additivity of the *ppz1*Δ defect with the *pkc1*Δ defect requires that *PPZ1* and *PPZ2* retain partial function in a *pkc1*Δ mutant. Moreover, the dosage-dependent suppression of defects on one branch of the pathway by components on the other branch requires that both branches coordinately regulate interdependent processes associated with cell wall construction, such that increased activity of one branch compensates for reduced activity of the other branch. For these reasons, a second model is favored. In this model (Fig. 11B), *PPZ1* and *PPZ2* play an auxiliary role in the *PKC1*-mediated pathway. The putative protein phosphatases are not regulated by Pkc1p, but they contribute to the pathway at a point below or at the same level as *MPK1*. Additivity of the *ppz1*Δ defect with the *mpk1*Δ defect and the *pkc1*Δ defect would be expected if *MPK1*, *PPZ1*, and *PPZ2* coordinately regulate pathway components that function downstream of both. The reciprocal dosage-dependent suppression of the *mpk1*Δ and *ppz1,2*Δ defects by *PPZ1/2* and *MPK1*, respectively, is also consistent with coordinate regulation of downstream pathway components by these putative protein kinases and phosphatases. There are several examples of proteins whose activities are dependent on phosphorylation at one site and dephosphorylation of another site (13, 40). It should be noted in either model that because the *PPZ1* and *PPZ2* genes act cooperatively with the protein kinase-encoding genes, the putative protein phosphatases must not reverse the effects of the protein kinases.

***BCK2* encodes a serine/threonine-rich protein that functions on a common pathway branch with *PPZ1* and *PPZ2*.** Another of the *mpk1*Δ-suppressing genes encodes a predicted 94-kDa protein that is 24% serine plus threonine residues but is not closely related to any known protein. We designated this gene *BCK2* on the basis of its dosage-dependent ability to suppress a *pkc1*Δ defect. Although deletion of *BCK2* alone did not result in any apparent phenotypic defect, we propose that this gene functions on a common pathway branch with *PPZ1* and *PPZ2*. First, a *bck2*Δ mutation, in combination with a *bck1*Δ, *mpk1*Δ, or *pkc1*Δ mutation, resulted in a cell lysis defect that was considerably more severe than that observed for mutations in any of the protein kinase-encoding genes alone. This behavior was similar to the additivity observed for *ppz1*Δ and *ppz2*Δ defects with the *mpk1*Δ defect or the *pkc1*Δ defect. Second, a *bck2*Δ mutation, in combination with a *ppz1,2*Δ double mutation, resulted in a cell lysis defect that was no more severe than that observed for a *ppz1,2*Δ double mutant alone, consistent with one component being under the regulatory control of the other. The failure of *BCK2* overexpression to suppress the *ppz1,2*Δ defect, combined with the inability to do the reciprocal experiment, precluded assignment of the relative order of function of these pathway components.

The behavior of a *bck2*Δ mutant was similar to that observed previously for a *spa2*Δ mutant. The *SPA2* gene encodes a protein of unknown function (16) whose intracellular location is restricted to a small patch at the bud tip (61). Deletion of *SPA2* does not result in any growth defects, although subtle alterations in cell morphology have been reported. A synthetic lethal screen using a *spa2*Δ mutation revealed that this defect is lethal in combination with a *bck1* mutation (10). This finding suggests that *SPA2* may function within the *PKC1*-mediated pathway on a common branch with *PPZ1*, *PPZ2*, and *BCK2*.

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ADDENDUM IN PROOF

Posas et al. (F. Posas, A. Casamayor, and J. Arino, FEBS Lett. **318**:282–286, 1993) reported recently that *ppz1,2Δ* mutants are sensitive to growth inhibition by caffeine and that this defect is suppressed by 1 M sorbitol.

REFERENCES

1. **Aebi, M., G. Kirchner, J.-Y. Chen, U. Vijayraghavan, A. Jacobson, N. C. Martin, and J. Abelson.** 1990. Isolation of a temperature-sensitive mutant with an altered tRNA nucleotidyltransferase and cloning of the gene encoding tRNA nucleotidyltransferase in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **265**:16216–16220.
2. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729–739.
3. **Bacher, N., Y. Zisman, E. Berent, and E. Livneh.** 1991. Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. Mol. Cell. Biol. **11**:126–133.
4. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
5. **Berridge, M. J., and R. F. Irvine.** 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature (London) **312**:315–321.
6. **Boyer, H., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Chen, R.-H., C. Sarnecki, and J. Blenis.** 1992. Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases. Mol. Cell. Biol. **12**:915–927.
8. **Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockover, and M. Karin.** 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. Nature (London) **329**:648–651.
9. **Colamonici, O. R., J. B. Trepel, C. A. Vidal, and L. M. Neckers.** 1986. Phorbol ester induces c-*sis* gene transcription in stem cell line K-562. Mol. Cell. Biol. **6**:1847–1850.
10. **Costigan, C., S. Gehrung, and M. Snyder.** 1992. A synthetic lethal screen identifies SLK1, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. Mol. Cell. Biol. **12**:1162–1178.
11. **Coughlin, S. R., W. M. F. Lee, P. W. Williams, G. M. Giels, and L. T. Williams.** 1985. c-*myc* gene expression is stimulated by agents that activate protein kinase C and does not account for the mitogenic effect of PDGF. Cell **43**:243–251.
12. **Da Cruz e Silva, E. F., V. Hughes, P. McDonald, M. J. R. Stark, and T. T. W. Cohen.** 1991. Protein phosphatase $2B_w$ and protein phosphatase Z are *Saccharomyces cerevisiae* enzymes. Biochim. Biophys. Acta **1089**:269–272.
13. **Dent, P., A. Lavoinne, S. Nakielny, F. B. Caudwell, P. Watt, and P. Cohen.** 1990. The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature (London) **348**:302–308.
14. **Errede, B., and D. E. Levin.** 1993. A conserved kinase cascade for MAP kinase activation in yeast. Curr. Opin. Cell Biol. **5**:254–260.
15. **Farley, J., and S. Auerbach.** 1986. Protein kinase C activation induces conductance changes in *Hermissenda* photoreceptors like those seen in associative learning. Nature (London) **319**: 220–223.
16. **Gehrung, S., and M. Snyder.** 1990. The *SPA2* gene of *Saccharomyces cerevisiae* is important for pheromone-induced morphogenesis and efficient mating. J. Cell Biol. **111**:1451–1464.
17. **Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink.** 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and *RAS*. Cell **68**:1077–1090.
18. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–438.
19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
20. **Hokin, L. E.** 1985. Receptors and phosphoinositol-generated second messengers. Annu. Rev. Biochem. **54**:205–235.
21. **Hoshi, M., E. Nishida, and H. Sakai.** 1988. Activation of a Ca^{2+}-inhibitable protein kinase that phosphorylates microtubule-associated protein 2 in vitro by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. J. Biol. Chem. **263**:5396–5401.
22. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51**:251–260.
23. **Imbra, R. J., and M. Karin.** 1986. Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. Nature (London) **323**:555–558.
24. **Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto, and Y. Oshima.** 1993. *MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol. Cell. Biol. **13**:3076–3083.
25. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
26. **Kaibuchi, K., Y. Takai, and Y. Nishizuka.** 1985. Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. J. Biol. Chem. **260**:1366–1369.
27. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
28. **Kikkawa, U., A. Kishimoto, and Y. Nishizuka.** 1989. The protein kinase C family: heterogeneity and its implications. Annu. Rev. Biochem. **58**:31–44.
29. **Kishimoto, A., Y. Takai, T. Mori, U. Kikkawa, and Y. Nishizuka.** 1980. Activation of calcium and phospholipid dependent protein kinase by diacylglycerol: its possible relation to phosphatidyl inositol turnover. J. Biol. Chem. **255**:2273–2276.
30. **Kruijer, W., J. A. Cooper, T. Hunter, and I. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature (London) **312**: 711–720.
31. **Langford, C. J., and D. Gallwitz.** 1983. Evidence for an intron-contained sequence required for the splicing of yeast RNA polymerase II transcripts. Cell **33**:519–527.
32. **Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D. E. Levin.** 1993. A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. **13**:3067–3075.
33. **Lee, K. S., and D. E. Levin.** 1992. Dominant mutations in a gene encoding a putative protein knase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. Mol. Cell. Biol. **12**:172–182.
34. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) **325**:368–372.
35. **Levin, D. E., and E. Bartlett-Heubusch.** 1992. Mutants in the *S. cerevisiae PKC1* gene display a cell cycle-specific osmotic stability defect. J. Cell Biol. **116**:1221–1229.
36. **Levin, D. E., and B. Errede.** 1993. A multitude of MAP kinase activation pathways. J. NIH Res. **5**:49–52.
37. **Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner.** 1990. A candidate protein kinase C gene, *PKC1*, is required for the *S. cerevisiae* cell cycle. Cell **62**:213–224.
38. **Madison, D. V., R. C. Malenka, and R. A. Nicoll.** 1986. Phorbol esters block a voltage-sensitive chloride current in hippocampal

pyramidal cells. Nature (London) **321:**695–697.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Murray, A. W.** 1992. Creative blocks: cell-cycle checkpoints and feedback controls. Nature (London) **359:**599–604.
41. **Negro-Vilar, A., and E. G. Lapetina.** 1985. 1,2 didecanoylglycerol and phorbol 12, 13 dibutyrate enhance anterior pituitary hormone secretion *in vitro*. Endocrinology **117:**1559–1564.
42. **Nishizuka, Y.** 1986. Studies and perspectives of protein kinase C. Science **233:**305–312.
43. **Nishizuka, Y.** 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature (London) **334:**661–665.
44. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
45. **Ohkura, H., N. Kinoshita, S. Miyatani, T. Toda, and M. Yanagida.** 1989. The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. Cell **57:**997–1007.
46. **Ohmura, E., and H. G. Friesen.** 1985. 12-O-tetradecanoyl phorbol-13-acetate stimulates rat growth hormone (GH) release from different pathways from that of human pancreatic GH-releasing factor. Endocrinology **116:**728–733.
47. **Ohno, S., Y. Akita, Y. Konno, S. Imajoh, and K. Suzuki.** 1988. A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. Cell **53:**731–741.
48. **Olson, M., and L. Riles (Washington University).** Personal communication.
49. **Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, and Y. Nishizuka.** 1988. The structure, expression, and properties of additional members of the protein kinase C family. J. Biol. Chem. **263:**6927–6932.
50. **Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, and Y. Nishizuka.** 1989. Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc. Natl. Acad. Sci. USA **86:**3099–3103.
51. **Paravicini, G., M. Cooper, L. Friedii, D. J. Smith, J.-L. Carpentier, L. S. Klig, and M. A. Payton.** 1992. The osmotic integrity of the yeast cell requires a functional *PKC1* gene product. Mol. Cell. Biol. **12:**4896–4905.
52. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
53. **Pelech, S. L., and J. S. Sanghera.** 1992. Mitogen-activated protein kinases: versatile transducers for cell signalling. Trends Biochem. Sci. **17:**233–238.
54. **Persons, D. A., W. O. Wilkison, R. M. Bell, and O. J. Finn.** 1988. Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. Cell **52:**447–458.
55. **Posas, F., A. Casamayor, N. Moral, and J. Arino.** 1992. Molecular cloning and analysis of a yeast protein phosphatase with an unusual amino-terminal region. J. Biol. Chem. **267:**11734–11740.
56. **Rosengurt, E., A. Rodriquez-Pena, M. Coombs, and J. Sinnet-Smith.** 1984. Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: role of Ca^{+2}-sensitive phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. USA **81:**5748–5752.
57. **Rothstein, R.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
59. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
60. **Siliciano, P. G., and K. Tatchell.** 1984. Transcription and regulatory signals at the mating type locus in yeast. Cell **37:**969–978.
61. **Snyder, M.** 1989. The SPA2 protein of yeast localizes to sites of cell growth. J. Cell Biol. **108:**1419–1429.
62. **Takai, Y., A. Kishimoto, U. Kikkawa, T. Mori, and Y. Nishizuka.** 1979. Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. **91:**1218–1224.
63. **Thomas, G.** 1992. MAP kinase by any other name smells just as sweet. Cell **68:**3–6.
64. **Torres, L., H. Martin, M. I. Garcia-Saez, J. Arroyo, M. Molina, M. Sanchez, and C. Nombela.** 1991. A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae lyt2* mutants. Mol. Microbiol. **5:**2845–2854.
65. **Zaret, K. S., and F. Sherman.** 1982. DNA sequence required for efficient transcription termination in yeast. Cell **28:**563–573.