

# The Fission Yeast *pmk1*<sup>+</sup> Gene Encodes a Novel Mitogen-Activated Protein Kinase Homolog Which Regulates Cell Integrity and Functions Coordinately with the Protein Kinase C Pathway

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**We have isolated a gene, *pmk1*<sup>+</sup>, a third mitogen-activated protein kinase (MAPK) gene homolog from the fission yeast *Schizosaccharomyces pombe*. The predicted amino acid sequence shows the most homology (63 to 65% identity) to those of budding yeast *Saccharomyces* Mpk1 and *Candida* Mkc1. The Pmk1 protein contains phosphorylated tyrosines, and the level of tyrosine phosphorylation was increased in the *dsp1* mutant which lacks an attenuating phosphatase for Pmk1. The level of tyrosine phosphorylation appears constant during hypotonic or heat shock treatment. The cells with *pmk1* deleted ( $\Delta pmk1$ ) are viable but show various defective phenotypes, including cell wall weakness, abnormal cell shape, a cytokinesis defect, and altered sensitivities to cations, such as hypersensitivity to potassium and resistance to sodium. Consistent with a high degree of conservation of amino acid sequence, multicopy plasmids containing the *MPK1* gene rescued the defective phenotypes of the  $\Delta pmk1$  mutant. The frog *MAPK* gene also suppressed the *pmk1* disruptant. The results of genetic analysis indicated that Pmk1 lies on a novel MAPK pathway which does not overlap functionally with the other two MAPK pathways, the Spk1-dependent mating signal pathway and Sty1/Spc1/Phh1-dependent stress-sensing pathway. In *Saccharomyces cerevisiae*, Mpk1 is involved in cell wall integrity and functions downstream of the protein kinase C homolog. In contrast, in *S. pombe*, Pmk1 may not act in a linear manner with respect to fission yeast protein kinase C homologs. Interestingly, however, these two pathways are not independent; instead, they regulate cell integrity in a coordinate manner.**

Mitogen-activated protein kinases (MAPKs) are a group of protein kinases which execute a wide variety of roles in cellular signal transduction pathways (reviewed in references 7, 45, 59, and 85). The MAPK pathways constitute a cascade of protein kinases consisting of MAPK, a MAP kinase kinase (MAPKK), and a MAP kinase kinase kinase (MAPKKK). An ever-growing number of reports show the ubiquitous importance of the MAPK pathways in various biological phenomena (e.g., see references 20, 86, and 93). In particular, since the oncoprotein Ras was shown to act at least in part if not entirely (90) as an activator of another oncoprotein, Raf, which is a MAPKKK, much attention has been paid to both upstream and downstream elements which are linked to the MAPK pathways (45). The MAPK cascades are evolutionarily conserved; not only animal cells but also plants and unicellular organisms such as yeasts utilize these systems to monitor extracellular cues and respond to changes in these cues (57). Genetic and molecular analyses of the budding yeast *Saccharomyces cerevisiae* showed the existence of multiple parallel MAPK pathways, each of which consists of distinct members of protein kinases and responds to different extracellular signals (reviewed in references 1, 14, 27, and 41). Later it became clear that cells of other organisms, including fission yeast, plant, and animal cells, all

contain at least two or more MAPK cascades. In animal cells, these cascades include the classical p42/p44-extracellular signal-regulated kinase (ERK) pathway and the Jnk/stress-activated protein kinase (SAPK) and p38/Hog1 pathways.

In the fission yeast *Schizosaccharomyces pombe*, two distinct MAPK pathways have been identified so far. These pathways include the mating pheromone-responsive Spk1 MAPK pathway (21, 80) and the stress-sensing Sty1/Spc1/Phh1 MAPK pathway (12, 33, 50, 70, 72, 91). Spk1 is required for conjugation and meiosis, which take place in the presence of mating pheromones. We and others have shown that the *spk1*<sup>+</sup> gene complements the mating defect of a budding yeast *fus3* strain (*FUS3* encodes a MAPK homolog required for the mating signal transduction pathway [13]) and that MAPKs from frogs (X-MAPK) and rats (ERK2) are able to rescue the meiosis-defective phenotype of the  $\Delta spk1$  cells (21, 56). This result suggests that there is a functional conservation of the MAPK pathway between yeasts and animals. In line with this view, the second MAPK pathway, the Sty1/Spc1/Phh1 MAPK pathway, which is involved in cellular responses to various stress conditions, including high osmolarity, heat shock, and oxidative stress, has also been shown to be structurally and functionally conserved throughout evolution (5, 18, 24). Thus, human p38 and Jnk/SAPK kinases can substitute for the *hog1* mutant.

In *S. cerevisiae*, the third MAPK pathway, the Mpk1 MAPK pathway, has been isolated and characterized (reviewed in reference 14). This pathway consists of Bck1/Slk1 (MAPKKK) (9, 38), Mkk1 and Mkk2 (MAPKK) (29) and Mpk1/Slt2 (MAPK) (37, 46, 83). The Mpk1 MAPK pathway has been shown to regulate cell

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TABLE 1. Fission yeast strains used in this study

Strain	Relevant genotypes	Source or reference
HM123	<i>h<sup>-</sup> leu1</i>	Our stock
5A/1D	<i>h<sup>-</sup>/h<sup>+</sup> leu1/leu1 ura4/ura4 his2/+ ade6-M210/ade6-M216</i>	79
TP43	<i>h<sup>-</sup>/h<sup>+</sup> leu1/leu1 ura4/ura4 his2/+ ade6-M210/ade6-M216 pmk1::ura4<sup>+</sup>/+</i>	This study
TP319-13C	<i>h<sup>-</sup> leu1 ura4 pmk1::ura4<sup>+</sup></i>	This study
TP319-31A	<i>h<sup>-</sup> leu1 ura4 pmk1::ura4<sup>+</sup></i>	This study
TP114-9C	<i>h<sup>90</sup> leu1 ura4 spk1::ura4<sup>+</sup></i>	21
ED1011	<i>h<sup>-</sup> leu1 his1 wis1::his1<sup>+</sup></i>	Obtained from P. Fantès
TP355-7D	<i>h<sup>-</sup> leu1 pmk1::ura4<sup>+</sup> spk1::ura4<sup>+</sup> wis1::his1<sup>+</sup></i>	This study
TP47-2B	<i>h<sup>-</sup> leu1 pck2-8</i>	81
TP134-3B	<i>h<sup>-</sup> leu1 ura4 pck1::ura4<sup>+</sup></i>	81
TP170-2B	<i>h<sup>-</sup> leu1 pck2::LEU2</i>	81
TP385-1C	<i>h<sup>-</sup> leu1 ura4 pmk1::ura4<sup>+</sup> pck2::LEU2</i>	This study
KP264	<i>h<sup>-</sup> leu1 ura4 pmk1::ura4<sup>+</sup> dsp1::ura4<sup>+</sup></i>	This study
KP265	<i>h<sup>-</sup> leu1 ura4 dsp1::ura4<sup>+</sup></i>	This study

integrity, including cell wall construction and cell morphogenesis in response to stretching of the plasma membrane (14, 41). Genetic analyses have shown that this pathway is regulated by a protein kinase C (PKC) homolog, Pck1; defective phenotypes caused by mutations in these genes are similar, if not identical, and overexpression of or an activated mutation in genes encoding downstream kinases is capable of rescuing mutations in upstream kinase-encoding genes (40, 42). In addition, recently it was reported that the Pck1-mediated Mpk1 MAPK pathway is regulated by a ubiquitous small GTPase, Rho1 (44), which physically binds to the Pck1 molecules in a GTP-dependent manner (33, 60). These results may suggest that the third MAPK pathway is also evolutionarily conserved.

We and others have previously isolated and characterized fission yeast PKC-encoding genes, *pck1<sup>+</sup>* and *pck2<sup>+</sup>* (49, 81). *pck1<sup>+</sup>* and *pck2<sup>+</sup>* have overlapping roles in cell viability, and  $\Delta$ *pck2* cells have a defect in cell wall construction, like the budding yeast *pck1* mutant (71, 79, 81). However, downstream effectors of Pck1 and Pck2 remain to be established. Although components such as GTPases and protein kinases are conserved, the linkage between different effectors may not be. For example, in contrast to other organisms including *S. pombe*, budding yeast Ras1 and Ras2 proteins act independently of the MAPK pathway; instead, Ras primarily regulates adenyl cyclase (54, 82). Also, in contrast to the mammalian p38 and Jnk/SAPK and fission yeast Sty1/Spc1/Phh1 MAPK pathways, the budding yeast Hog1 kinase is activated by high osmolarity but not by other stress conditions (5, 12, 18, 72, 91).

In this study we have addressed the following two questions using *S. pombe* as a system. First, does an Mpk1-like MAPK exist in this yeast? Second, if it does, is a functional scheme on the Pck1-Mpk1 MAPK pathway established in *S. cerevisiae* conserved in *S. pombe*? Here we report the cloning of a third fission yeast MAPK-encoding gene, designated *pmk1<sup>+</sup>*. We show that *pmk1<sup>+</sup>* is a structural and functional homolog of budding yeast *MPK1*. In addition, our data suggest that, in spite of a high degree of conservation between budding and fission yeast MAPKs, the fission yeast PKC-like molecules may not directly regulate the Pmk1 MAPK pathway. Interestingly, however, these two pathways are not completely independent; cross-talk appears to exist between the Pmk1 and Pck pathways, two pathways which are involved in cell integrity. Two possible models are presented for a functional relationship between the Pmk1 MAPK and PKC pathways.

#### MATERIALS AND METHODS

**Strains and media.** *S. pombe* strains used in this study are listed in Table 1. Complete medium, YPD (1% yeast extract, 2% polypeptone, 2% dextrose),

modified synthetic EMM2 (53), and SPA and malt extract media for sporulation (23, 53) have been described elsewhere. Appropriate amino acids or bases (75  $\mu$ g/ml) were added if necessary. Plates contained 1.6% agar. Staurosporine (provided by H. Nakano, Kyowa Hakko Co.) was used as described previously (80).

**Genetic techniques and nomenclature.** The standard *S. pombe* genetic procedures of Gutz et al. (23) and Moreno et al. (53) were used. *S. pombe* cells were transformed by the lithium method (30). Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, *pmk1::ura4<sup>+</sup>*). In the text and figures, gene disruptions are abbreviated by the gene preceded by  $\Delta$  (for example,  $\Delta$ *pmk1*). Proteins are denoted by roman letters and only the first letter is capitalized (for example, Pmk1).

**Nucleic acid preparation and manipulation.** Standard molecular biology techniques were used as described previously (67). Enzymes were used as recommended by suppliers (Takara Shuzo Co., Toyobo Co., and New England Biolabs). Nonradioisotope labelling and chemiluminescence detection methods (digoxigenin [Boehringer Mannheim GmbH] and AMPPD [Tropix]) were used.

**Cloning of the *pmk1<sup>+</sup>* gene.** Degenerate oligonucleotides used for PCR were the 5' oligonucleotide 5'-TCAATCAGGATCCGGNGARGGNGCNTAYGG-3' (GS3) and the 3' oligonucleotide 5'-TAATCAGAATTCRAARTCRCA-3' (GS4) (N is G, A, T, or C; S is G, A, or T; R is G or A; Y is T or C). The underlined 5'-proximal sequence contains the *Bam*HI (GS3) or *Eco*RI (GS4) restriction site. GS3 and the reverse complement of GS4 correspond to highly conserved amino acid residues among the MAPK family members: Gly-Glu-Gly-Ala-Tyr-Gly (GS3) and Cys-Asp-Leu-Lys-Ile/Leu-Cys-Asp-Phe-Gly (GS4), respectively. An *S. pombe* cDNA library was used for PCR. Amplified fragments were subcloned into Bluescript (Stratagene), and the nucleotide sequence of the insert DNA was determined. One (pBS-m3) of the subclones contained a 400-bp fragment which encodes a potential novel MAPK. In order to obtain a genomic fragment, the 400-bp fragment was used as a probe to hybridize with a library of fission yeast genomic DNA constructed in a cosmid vector (52). The fragment hybridized with several overlapping cosmids located in contig 4 of the left arm of chromosome II. The most proximal known marker is *mei3*.

**Nucleotide sequence determination.** The dideoxy DNA sequencing method (68) was performed by using double-stranded plasmid DNA as templates (26). The 2,643-bp *Pst*I-*Spe*I fragment containing the entire *pmk1<sup>+</sup>* gene was completely sequenced.

**Gene disruption of *pmk1<sup>+</sup>*.** The 2.2-kb *Eco*RI fragment containing the *pmk1<sup>+</sup>* gene (Fig. 1A) was subcloned into pUC8 (87), yielding pM3-8A'. Then, a 1.8-kb *Sac*I fragment containing the *ura4<sup>+</sup>* gene (22) was inserted into the *Sac*I site located in the middle of the *pmk1<sup>+</sup>* gene (Fig. 1A), yielding *ppmk1::ura4<sup>+</sup>*. The 4.0-kb *pmk1::ura4<sup>+</sup>* fragment was used to transform the diploids (5A/1D [Table 1]). The disruption was verified by Southern hybridization of the *Ura<sup>+</sup>* heterozygous diploids and also *Ura<sup>+</sup>* haploid segregants.

**Construction and expression of the tagged *pmk1<sup>+</sup>* gene.** The tagged version of the *pmk1<sup>+</sup>* gene was constructed as follows. The *Nde*I site in the third intron of the *pmk1<sup>+</sup>* gene was destroyed by treatment with the Klenow enzyme. Then the entire *pmk1<sup>+</sup>* gene was amplified by PCR with two oligonucleotides; the 5' oligonucleotide is CCCCATATGGACCGTTCGACATCGTGTTC (pmk1-N) (the *Nde*I site is underlined), and the 3' oligonucleotide is AAAGCGGCCCGAGTTATGGCGATTATCATC (pmk1-C) (the *Not*I site is underlined). A PCR-amplified 1,736-bp fragment was cleaved with *Nde*I and *Not*I and subcloned into pREP41 (HA-6His) (provided by J. Millar) to yield pREP41-*pmk1<sup>+</sup>*-HA/6His, which expressed *pmk1<sup>+</sup>* under the control of a partially crippled version of the *nmt1* thiamine-repressible promoter (2) and which contains six His residues and two repeats of a peptide derived from the influenza virus hemagglutinin (HA peptide) (50). Tagging of 6His-HA in the C terminus did not interfere with the normal function of the Pmk1 protein, as pREP41-*pmk1<sup>+</sup>*-HA/6His, when intro-

duced into the  $\Delta pmk1$  mutant, suppressed the defective phenotypes (data not shown).

**Growth conditions and preparation of cell extracts.** Cells were grown at 30°C in rich YPD medium unless otherwise noted. For the heat shock experiment, exponentially growing cultures at 25°C were shifted to 36°C and aliquots were removed every 10 min for 60 min. Cold shock was performed by transferring the culture from 30 to 20°C. For hypotonic shock, yeast cells grown in minimal medium were collected and suspended in distilled water. Hypertonic shock was performed by transferring the culture grown in minimal medium to medium containing 1.2 M sorbitol. Cell extracts were prepared as described previously (47). When extracts were prepared from cells expressing the tagged  $pmk1^+$  gene, the procedures of Millar et al. (50) were followed with some modifications (75). Briefly, XB buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 8.0], 300 mM NaCl, 10 mM imidazole, and a cocktail of protease and phosphatase inhibitors) was used for disruption of cells with glass beads. The pH was checked at each step to maintain the pH above 7.5.

**Preparation of antisera and immunochemical assays.** To express a part of the Pmk1 protein in bacteria for antibody preparation, a 483-bp internal fragment of the  $pmk1^+$  gene which encodes internal peptides of 161 amino acid residues (methionine 107 to serine 286 [Fig. 1B]) was amplified by PCR with two oligonucleotides; the 5' oligonucleotide is GGCATATGGAGGCCGATTGAAT GCT (the *NdeI* site is underlined), and the 3' oligonucleotide is GGGGATCC AAGCTTCGAACATACTCCTGTGCA (the *BamHI* site is underlined). After cleavage with *NdeI* and *BamHI*, the amplified fragment was cloned into an expression vector, pAR3038 (65), which was also cut with *NdeI* and *BamHI*, yielding pT7- $pmk1$ . pT7- $pmk1$  was introduced in *Escherichia coli* [BL21(DE3)] (74) and the 20-kDa Pmk1 protein was gel purified and injected into rabbits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (35), and proteins were electrically transferred onto nitrocellulose filters (84). Anti-HA monoclonal antibody (12CA5) (58, 92) or anti-Pmk1 antibody was used to detect the Pmk1 protein. Antiphosphotyrosine antibodies (PY20 [ICN] and 4G10 [TCS Biologicals]) were used to detect tyrosine-phosphorylated Pmk1. Peroxidase-conjugated protein A or goat anti-mouse immunoglobulin G (Bio-Rad) and chemiluminescence (ECL system [Amersham]) were used to detect bound antibody.

**Expression of MAPK-encoding genes in the  $pmk1$  disruptant.** The 2,643-bp *PstI-SpeI* fragment containing the entire  $pmk1^+$  gene was subcloned into pSK248 (80), yielding pSK( $pmk1^+$ ). This plasmid was used as a multicopy plasmid containing the  $pmk1^+$  gene. pJD5 (provided by D. Hirata) or pART1-*X-MAPK* (21), containing the budding yeast *MPK1* gene or *Xenopus* MAPK gene, respectively, was used to transform the  $pmk1$  disruptant. *pwis1-1* (provided by P. Fantes) (88) and pADH-*spk1*<sup>+</sup> (80) were also used.

**Glucanase sensitivity.** The procedures described by Levin and Bishop (39) and Shiozaki and Russell (71) were followed. Briefly, exponentially growing cells (approximately 3 × 10<sup>6</sup>/ml) at 30°C were suspended at a concentration of 10<sup>7</sup> cells per ml.  $\beta$ -Glucanase (Zymolyase-20T [ICN]) was treated at a concentration of 100  $\mu$ g/ml at 29°C. Cell lysis was monitored by measuring the optical density at 600 nm.

**Nucleotide sequence accession number.** The nucleotide sequence data of the 2,643-bp *PstI-SpeI* fragment containing the entire  $pmk1^+$  gene reported in this study will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data-bases under accession number X98243.

## RESULTS

**Isolation of the  $pmk1^+$  gene and determination of the predicted amino acid sequence.** Using primers designed to amplify MAPK homologs with PCR, we isolated a DNA fragment which potentially encodes a novel MAPK from a fission yeast cDNA library. A genomic fragment which contains the entire gene (designated  $pmk1^+$  for *S. pombe* MAP kinase 1) was isolated from a fission yeast genomic library by using the PCR-amplified fragment as a probe (Materials and Methods) (Fig. 1A). The nucleotide sequence of a 2.6-kb *PstI-SpeI* fragment was determined (Fig. 1B). The  $pmk1^+$  gene encodes a protein of 422 amino acid residues and is interrupted by three introns. The three introns consist of 100, 270, and 82 bp and contain 5' GTAA/TG-----CTAA---T/CAG 3', which is a consensus sequence for fission yeast introns (63).

A comparison of the Pmk1 amino acid sequence with MAPK sequences is shown in Fig. 2. Pmk1 shows the most homology (63 to 65% identity) to two yeast MAPKs, *S. cerevisiae* Mpk1 (40) and *Candida albicans* Mkc1 (55). These two MAPKs have been shown to be structural and functional homologs; *MKC1* is capable of suppressing the *mpk1* mutant (55). Critical threonine and tyrosine residues which are known to be phosphory-

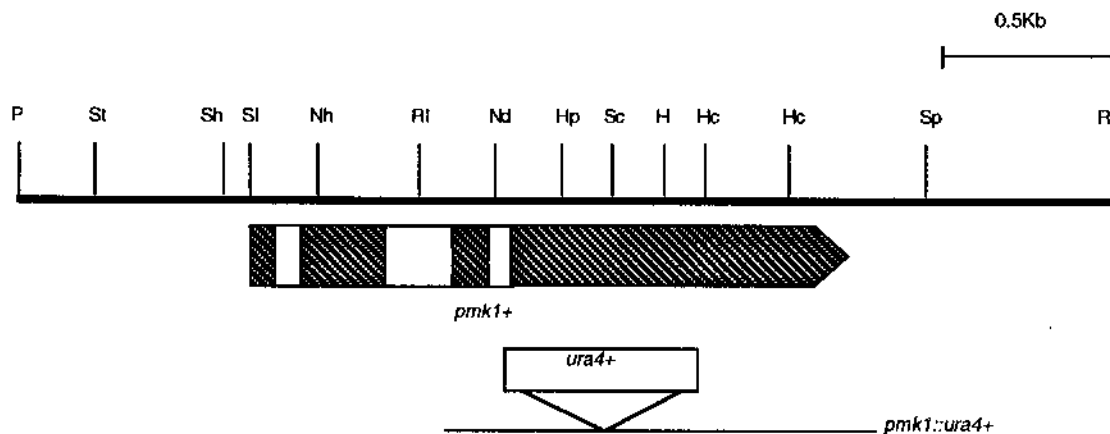
lated by upstream MAPKK are conserved in Pmk1 (indicated by arrowheads in Fig. 2). It should be noted that Pmk1 contains Thr-Glu-Tyr in this region, which is common to Erk1 but not to p38 or Jnk/SAPK (Fig. 2). The amino acid sequence around this region (between conserved kinase subdomains VII and VIII) (25) is again most homologous to that of Mpk1 and Mkc1 (Fig. 2). In contrast, Pmk1 is more distantly related to other MAPK family members (40 to 50%). For example, Pmk1 exhibits 50% identity to the fission yeast Spk1 (80) or budding yeast Fus3/Dac2 (13, 17) and Kss1 (10) (which are involved in mating pheromone signalling), 44% identity to budding yeast Hog1 (5) and fission yeast Sty1/Sp1/Phh1 (12, 34, 50, 70, 72, 91) (which are involved in responses to high osmolarity and various stress conditions), and 47% identity to metazoan Erk1 (4).

**Identification of the  $pmk1^+$  gene product.** To characterize the  $pmk1^+$  gene product biochemically, polyclonal antibody was prepared in rabbits with a fusion protein expressed in and purified from *E. coli* (see Materials and Methods). Immunoblotting was performed against total cell extracts prepared from wild-type or  $\Delta pmk1$  cells (see below) containing either vector plasmids or those carrying the  $pmk1^+$  gene. Two bands corresponding to approximately 48 and 50 kDa which are close to the predicted size (48 kDa) of the Pmk1 protein were detected in the wild type but not in the  $\Delta pmk1$  mutant (Fig. 3A, lanes 1 and 3). The intensities of these two bands greatly increased in extracts prepared from cells expressing the  $pmk1^+$  gene on a multicopy plasmid (lanes 2 and 4). Thus, the two bands are most probably derived from the Pmk1 protein. The ratio of the intensities of these two bands varies, depending on extract preparation. The precise natures of these two bands remain to be determined; either proteolytic cleavage or post-translational modification of the Pmk1 protein is possible (see below).

**The Pmk1 protein contains phosphorylated tyrosines.** In order to prove that Pmk1 is a MAPK, it is important to show that the Pmk1 protein is phosphorylated at a tyrosine residue. As the size (48 or 50 kDa) of the Pmk1 protein is close to that of immunoglobulin heavy chains, it hampered the detection of the Pmk1 protein by immunoprecipitation with anti-Pmk1 antibody or antiphosphotyrosine antibody. To overcome this difficulty, a tagged version of the Pmk1 protein (HA epitope and a six-histidine carboxy-terminal tail [pREP41- $pmk1^+$ -HA/6His]; see Materials and Methods for details) was constructed. By using this construct, the Pmk1 protein was purified in one step with Ni<sup>2+</sup>-nitrilotriacetic acid beads and analyzed by immunoblotting with either anti-Pmk1 antibody or anti-HA antibody. Similar to the pattern of the endogenous Pmk1 protein detected with anti-Pmk1 antibody (Fig. 3A), Pmk1-HA/6His protein was detected as two bands with anti-HA antibody (53 and 55 kDa) (Fig. 3B, lanes 1 and 2). When antiphosphotyrosine antibody was used, only the upper band was detected (Fig. 3B). This result suggests that the Pmk1 protein has phosphorylated tyrosines and that the lower band (53 kDa) may represent unphosphorylated Pmk1.

Independent genetic approaches which have been aimed at obtaining regulators of calcineurin (Ppb1, type 2B protein phosphatase) (97) have unexpectedly identified a gene, *dsp1*<sup>+</sup>, which encodes a member of the dual-specificity protein phosphatases or MAPK phosphatases (75). Subsequent genetic and biochemical analyses have demonstrated that Dsp1 acts through inactivation of Pmk1. These include the formation of a physical complex between Pmk1 and Dsp1 and suppression of the  $\Delta ppb1$  mutation either by overproduction of *dsp1*<sup>+</sup> or by deletion of the  $pmk1^+$  gene (75). We reasoned that in this mutant background ( $\Delta dsp1$ ), the level of the tyrosine-phospho-

**A**



**B**

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CTGCAGGTTCTGTGGAAACGAGGCCACTTTTTCATTCGCCCTTCTGGAAAGCCCTCAATGCGTTTATTTTGGAAATCCCAACAAATGTCCTGCTAGTTGGTGGTGAACCAAGCTCCGAGA -150
GTTCAAAACCTATCTTTAAATACATAAACAACCTCCTATCTACCAAAAAGTCTCTGGAACAACAGAATGTAGCCCAAGTCTTCGGGAGTGTCAAGGCTCTTCTATTCTTCAACACAAA -330
AGAACTCTACCTACCGAAAATGCTCTGACCCCATCGTTGACATGCGCTCTCTGTACCAACCGTACCGTATAGTCTTATTTGGTGTAGTCTGTGAATCTGCACTCTGCACTTAGTTTC
TTTGTAGTACTCTCATATATTTAATAGATTTCTATGATATCTCTTTAAATACCATAAATTCATCTGATAGTCTTCAAAATCTAAGCCGACTTCCATTTCAAATAGTGAATATAAAGGCATCGT -90
TAATCAGTACAAAGTAGTACTGTAGTTTTCACACATCCCTTTTGCCAAAACAGCTAAAATTCAGATGTCATGCTAATAGTCCGAAGSATGGACCGTGGACATCGTGTATACCGTGTGT 31
                                     *** M D R R H R V Y R F 11

TTAACCCAGAGATGTATGTGCGAGCCIAATTTTAAAGTATGAGATAFTTGGTAACTGCTGCATTTGCAAAAACATTTTGTTCCTTGACAGAAAATCGCATCTGCTTGAACCTTTTAGAAA 151
N Q E M Y V E P N F K 22

ACTAACATAGTTTAAAGTAGTGAAGGAGTTGGGTCAAGGCGCTTACCGCATTTGTTTGTGCGAGCAAGAAAAGTGGCTAGCAAGATCAAGAGCGCTGAGCCATAAAAAAAATACGAATGTC 271
V V K E L G Q G A Y G I V C A A R N V A S K D Q E A V A I K K I T N V 57

TTTTCTAAATCCATCTTACAAAAAGAGCTTTGAGAGAGATAAAAACGCGTATTCATTTTCGTAATCATCGTAAAGCTTAAAGTCTTTTATGCAAAAAGCGCTTCTGTGATCGATCTACTCAT 391
F S K S I L T K R A L R E I K L L I H F R N H R N 82

CATCACCCGGAGTATTAACGATGTGCATTTATGAAAATGCAAAATTTTATACAATGAAAAGTCTCTATGAGAAATGAGTTCTAATCATCTGAAAAGTATTTCTCTTATAAATCTTTGA 511
TATAATGTTTGGAAATCGGTTGATTAATAAATTTGAACTGCTCATTTGCTTTACATTTGCTAGATGATGATCTCTTCTTATTTTATAAACTTTAGTAAACATGAGATTACTTGCATTTAC 631
                                     I T C I Y 87

GATCTCGACATTATAAATCCATACAAATTTTAAATGAAGTTTACATTTACGAAAGAGTCTGATGTTTACCGGATGATCCACATAATGTTCTGTTTATGCAAGGAAATATTTCTATAAATAC 751
D L D I N P Y N F N E V Y I Y E E L 106

TTAATTAATATAAATGATGAGGCGGATTTGAATCTTATTAATAATCCGGTCAAGCCATTAACGGATGGGATTTTCAATCATTTTACAGATACTTTTGGTTTAAAGTATAT 871
M E A D L N A I E K S G Q P L T D A H F Q S F I Y Q I L C G L K Y I 140

TCATCTGCAACGTTATTCATCGTACTTAAAGCCAGTTAATCTTTTAAAGTAAAGCTGATTTGAGGCTGAAATTTGCGATTTTGGCTCCGCTGGGGATGTTCTGAAATCCAGAAGA 991
H S A N V I H R D L K P G N L L V N A D C E L K I C D F G L A R G C S E N F E E 180

AAATCTCGTTTAAATGACGAGTATGTGCAACGGCTGATACAGACTCCGGAATTTATGCTTTGGTTTCAGCAGCTATCATAAAGGATTTGATGTTTGGAGTCTGTGTGCAATTTTGGC 1111
N P G F M T E Y V A T R W Y R A P E I M L S F S S Y H X K G I D V W S V G C I L A 220

AGAGCTATTAGTGGGAACCCCTTTGTTTAAAGCAAAAGATTTTGTTCATCAGTTAAATTTAATTTTGCATCACTTGGCACCTGATGAGGAAACCCCTTCTCTCATATAAGCAGTTCGCG 1231
E L L G G T P L F K G K D F V H Q L N L I L H Q L G T P D E E T L S H I S S S R 260

TGCAACAGGATATCTGCAACCTTGGCAAGCCAAAGCACTATTCCTTTTGAACCAATTTTCTTAAAGCCAAATCTTTGGCTCTTGAAGCTGCTTAAAGCTCTTGGCTTTGCAACCCCAA 1351
A Q E Y V R S L P K Q R P I P P E T N F P K A N P L A L D L L A K L L I A F D P N 300

CAGAAGTATTTGGTGTGAGCATGCTTGAACATCCCTATTTAGCTGTTTGGCATGATCCCTCTGAGCAAGCTGTGTGATGCTGCTTTGATTTTCAGCTTTGAATACATTTGAAGATCC 1471
R R I S V D D A I E H P Y L A V W H D P S D B P V C D S V P D P S P E Y I E D A 340

AAATGAGTACCGCGTCTCATTTTGAATGAAAGTTTAAAGCTTCCGGTCAAGAAAGTCCGCAAGCGCTCTCATCAACAAACCCCAACAGTCAACATCCCGCAACAGCCAAACCGTTCCATC 1591
N E L R R V I L D E V L N F R Q K V R R R S H P T N P T V N I P Q P A Q T V P S 380

TAATGCAATGCTTCTTCAATGTTAGTACTCTTCAATCTCTCAAAACCTTAAACAAAAGCGCCATGATCAATCTTATAATGAAACTGCTCTATAGCCATAAGTCTGATGATAATCG 1711
N D N G S F N V S S S S S S Q T S N K K R H D H S Y N E T A A I D H K S D D N R 420

CCATAACTAATACATTTGCCAGCAGCTGTTTCAATGAAAGCTTTTCAAGCTTAACATTTGCAATACAAATCATCTATGCTGTTTCTCATTTTACATAAATAGCTATTCCTATCTTTTCAG 1831
H N * 422

CTTTGGTGGCTACTGTGGAATCTCTGTTTCTCTGTTTAAATTTGCATAAAAAATACTCATTTGCATTAGTCTCTCATTTACTGTTTGCATATAAATATATGCGCGCTTGGCTCCCTTTCTG 1951
CAGCTTTATAGTCTCAGAGCTGAAAATCTGTTATACATACATGCAATGCAATATACACCGCTACACAAACAGCTAGATATAAATAGAACAGCTAATTAATTAATAAATTAATTTACT 2071
AGT 2074
    
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FIG. 1. Restriction map, nucleotide sequence, and predicted amino acid sequences of the *pmk1+* gene and comparison of the amino acid sequences of Pmk1 and other MAPKs. (A) Restriction map of a genomic DNA sequence encompassing the *pmk1+* gene and construction of gene disruption (*pmk1::ura4+*) are shown. The *pmk1+*-encoding open reading frame is shown below the restriction map with the coding region in filled boxes and three introns in open boxes. The direction of transcription is from left to right. Restriction site abbreviations: H, *Hind*III; Hc, *Hinc*II; Hp, *Hpa*I; Nd, *Nde*I; Nh, *Nhe*I; P, *Pst*I; RI, *Eco*RI; Sc, *Sac*I; Sh, *Sph*I; Sl, *Sall*; Sp, *Spe*I; St, *Stu*I. (B) The nucleotide sequence of a 2,643-bp *Pst*I-*Spe*I fragment is shown together with the predicted amino acid sequence. The position of the initiator methionine is +1. The numbers to the right of the sequences are the nucleotide (upper) and amino acid (lower) positions. The termination codon (one asterisk) and the nearest in-frame termination codon in the 5' region (three asterisks) are indicated.

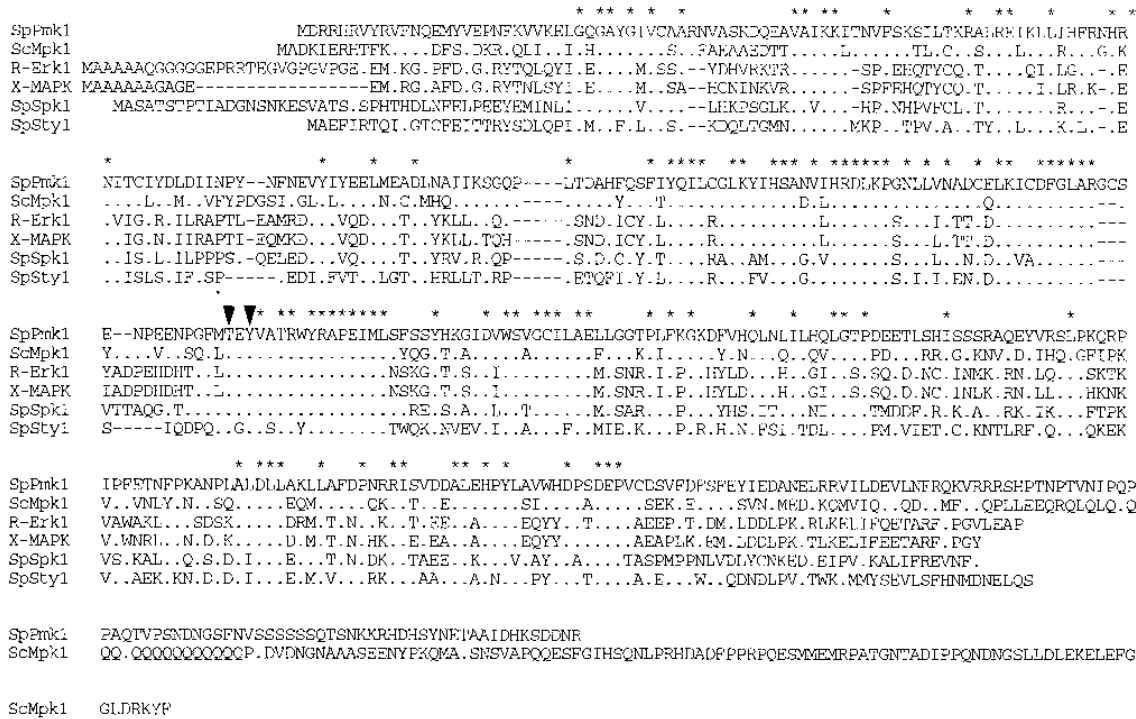


FIG. 2. Comparison of amino acid sequences of Pmk1 and other MAPKs. Identical amino acids (dots) and deletions (dashes) are indicated. The species each MAPK was derived from are abbreviated as follows: Sp, fission yeast; Sc, budding yeast; R, rat; X, frog. Amino acids which are conserved among all MAPKs (asterisks) and critical threonine and tyrosine residues (arrowheads) are indicated.

rylated Pmk1 protein would be increased because of the lack of attenuating phosphatases, and therefore, we tested the Pmk1-HA/6His protein in  $\Delta dsp1$  cells. When the purified Pmk1-HA/6His protein was immunoblotted with antiphosphotyrosine antibody, as expected, stronger signals were detected (compare lanes 2 and 7 in Fig. 3B) (75). This tyrosine-phosphorylated band was missing in cells which do not contain tagged Pmk1 (Fig. 3B, lanes 1 and 6, and Fig. 3C, lane 1). This result supports the notion that the 55-kDa band is indeed a tyrosine-phosphorylated form of Pmk1.

What signals or external conditions activate the Pmk1 MAPK? In *S. cerevisiae*, Mpk1 has been reported to be activated by various conditions including heat shock (32), hypotonic shock (11), and the addition of mating pheromone (98). As a first step to characterize activation mechanisms of Pmk1, the  $\Delta dsp1$  cells were treated with hypotonic or heat shock, and the level of tyrosine phosphorylation of the Pmk1 protein was examined by immunoblotting. No difference was found upon hypotonic shock (Fig. 3B, lanes 2 to 5 and 7 to 10). Likewise, no significant increase in tyrosine phosphorylation of the Pmk1 protein was apparent upon heat shock treatment (25 to 36°C) (Fig. 3C, lanes 2 to 5).

**The *pmk1*-deleted mutant is defective in cell shape and cytokinesis.** To construct *pmk1*-deleted cells, a one-step gene replacement method (66) was performed. The *ura4<sup>+</sup>* marker was inserted into the middle of the *pmk1<sup>+</sup>* coding region and used to transform the wild-type diploid strain (5A/1D [Table 1; also see Materials and Methods]). Subsequently, the heterozygous diploid was subjected to tetrad dissection. We obtained four viable spores, and the resulting colonies showed 2:2 segregation for the uracil auxotroph, indicating that the *pmk1<sup>+</sup>* gene is not essential for normal growth. *pmk1*-deleted ( $\Delta pmk1$ ) cells are fertile and can form colonies at any temperature

tested, including 20, 30, and 36°C. The generation time of the  $\Delta pmk1$  cells increased modestly (about 20%). Next we examined the cell morphology of the disruptant in various growth conditions. We found that the shape of the  $\Delta pmk1$  mutant cells is abnormal in the exponential growth phase. Wild-type cells are rod-shaped, and septum is formed in the middle of the cell (Fig. 4A). In contrast, unseptated mutant cells are often shorter and more rounded than wild-type cells (compare Fig. 4A and B). In addition, septa in the  $\Delta pmk1$  cells are often asymmetrically formed (5%), and sometimes (2%) multiseptated cells were observed (Fig. 4B). Abnormal cell morphology became more evident when the cells were grown in minimal medium; the reason for this is unknown. In addition, the percentage of septated cells in the exponentially growing  $\Delta pmk1$  culture in minimal medium is much higher (42%) than that of the wild type (14% [Table 2]), suggesting that the *pmk1<sup>+</sup>* gene is important for maintenance of cell morphology and completion of cytokinesis.

When wild-type fission yeast cells enter stationary phase, they cease division and arrest unseptated (53). We found that the percentage of septated cells of the  $\Delta pmk1$  mutant is still high in stationary phase (12% [Table 2]). Notably, we often observed abnormally multiseptated cells in the culture (5%) (Fig. 5A). The viability of the  $\Delta pmk1$  cells in the stationary phase is as high as that of the wild type, although recovery of the mutant from stationary phase appeared to take much longer than that of the wild type (Fig. 5B). If exponentially growing cells are plated, only a modest difference in colony size was observed (data not shown). Taken together, these results show that the *pmk1<sup>+</sup>* gene is required for maintenance of cell shape and execution of cell division in both the exponential-growth and stationary phases.

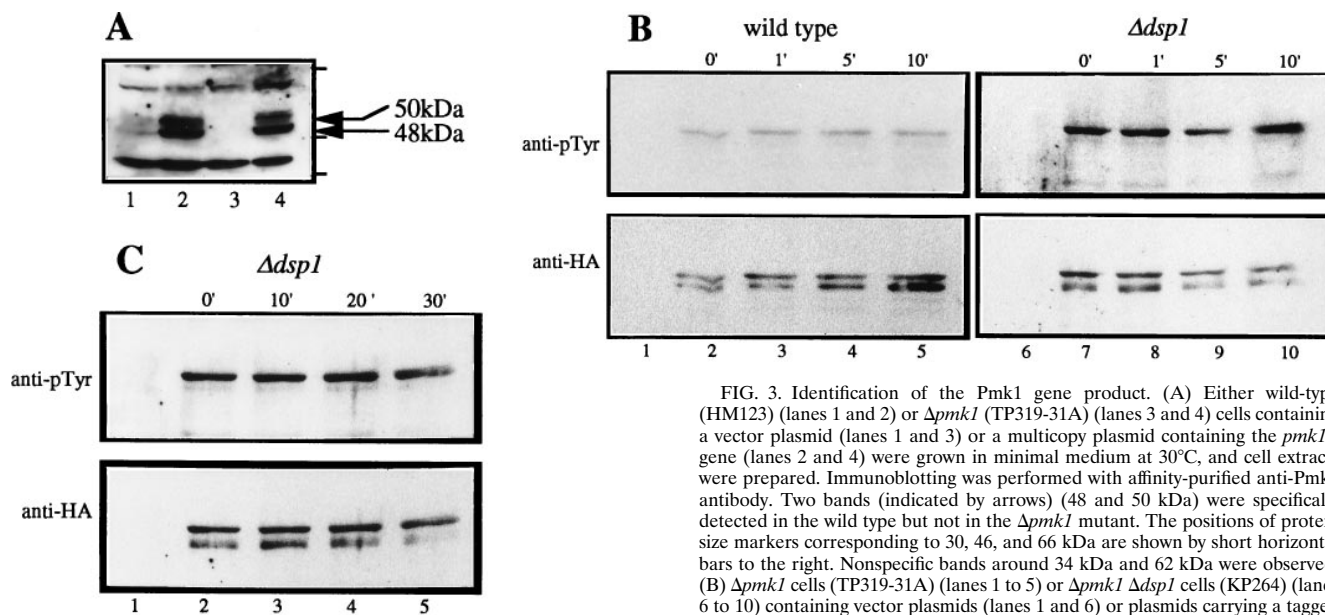


FIG. 3. Identification of the Pmk1 gene product. (A) Either wild-type (HM123) (lanes 1 and 2) or  $\Delta pmk1$  (TP319-31A) (lanes 3 and 4) cells containing a vector plasmid (lanes 1 and 3) or a multicopy plasmid containing the  $pmk1^+$  gene (lanes 2 and 4) were grown in minimal medium at 30°C, and cell extracts were prepared. Immunoblotting was performed with affinity-purified anti-Pmk1 antibody. Two bands (indicated by arrows) (48 and 50 kDa) were specifically detected in the wild type but not in the  $\Delta pmk1$  mutant. The positions of protein size markers corresponding to 30, 46, and 66 kDa are shown by short horizontal bars to the right. Nonspecific bands around 34 kDa and 62 kDa were observed. (B)  $\Delta pmk1$  cells (TP319-31A) (lanes 1 to 5) or  $\Delta pmk1 \Delta dsp1$  cells (KP264) (lanes 6 to 10) containing vector plasmids (lanes 1 and 6) or plasmids carrying a tagged version of the  $pmk1^+$  gene (pREP41- $pmk1^+$ -HA/6His) (lanes 2 to 5 and 7 to 10) were grown in minimal medium at 30°C. At time zero (lanes 2 and 7), the culture was washed, resuspended in distilled water, and incubated for 1 (lanes 1, 3, 6, and 8), 5 (lanes 4 and 9), and 10 (lanes 5 and 10) min. Cell extracts were affinity purified with  $Ni^{2+}$ -nitrilotriacetic acid beads and immunoblotted with either antiphosphotyrosine antibody (anti-pTyr) or anti-HA antibody. (C)  $\Delta pmk1 \Delta dsp1$  cells containing vector plasmids (lane 1) or plasmids carrying a tagged version of the  $pmk1^+$  gene (lanes 2 to 5) were grown in minimal medium at 25°C. The exponentially growing culture was shifted to 36°C, and aliquots were taken at 0 (lanes 1 and 2), 10 (lane 3), 20 (lane 4), and 30 (lane 5) min and processed for immunoblotting as described above for panel B.

**Three MAPK pathways act independently in fission yeast signal transduction pathways.** As mentioned previously, in *S. pombe* two other MAPK pathways are known: mating pheromone-responsive Spk1 MAPK (21, 57, 80) and stress-sensing Sty1/Spc1/Phh1 MAPK (12, 34, 50, 70, 72, 91). Cells of the  $\Delta spk1$  mutant are sterile, and those of the  $sty1/spc1/phh1$  mutant cannot proliferate in the presence of high osmolarity or fail to respond properly in various stress conditions. As described above, the  $\Delta pmk1$  cells are fertile and can form colonies under high osmolarity (data not shown), suggesting that Pmk1 constitutes a third independent MAPK pathway in *S. pombe*. In order to examine a functional relationship between these two MAPK pathways and Pmk1 pathways, double or triple mutants were constructed and phenotypic analysis was performed. It was found that any combination of the mutants in these MAPK pathways resulted in additive phenotypes. An example of the cell morphology of the  $\Delta pmk1 \Delta spk1 \Delta wis1$  triple mutant ( $wis1^+$  encodes a MAPKK for Sty1/Spc1/Phh1) (34, 50, 70) is shown in Fig. 4D. The single  $\Delta wis1$  mutant (Fig. 4C) showed elongated morphology due to a  $G_2$  delay (88), while the triple mutant showed, in addition to cell elongation, multiseptation and irregular cell shape, which was due to  $\Delta pmk1$  (the defect of  $\Delta spk1$  was not apparent in this vegetative condition) (80). Multicopy plasmids each containing MAPK ( $pmk1^+$  or  $spk1^+$ ) or MAPKK ( $wis1^+$ ) were introduced into each single mutant. No cross-suppression was observed. These results show that the Pmk1 protein participates in a third MAPK pathway which functions independently of the other two fission yeast MAPK pathways.

**The  $pmk1^+$  gene is required for cell wall integrity.** As described above, the predicted amino acid sequence of the  $pmk1^+$  gene shows the most homology to that of the budding yeast MPK1 gene. Previous studies of *S. cerevisiae* have shown that the Mpk1 MAPK pathway is involved in cell wall integrity and cell morphogenesis and acts downstream of the PKC-like protein Pkc1 (reviewed in references 1, 27, and 41). In *S. pombe*, which contains two PKC-like genes ( $pck1^+$  and  $pck2^+$ ) (81), the  $pck2$  mutant is also defective in cell wall integrity (71, 79). To examine whether  $pmk1^+$  is involved in cell wall integrity, the sensitivity to treatment of cell wall-digesting enzyme was examined. As a control, the  $pck2$  mutant was included. As

shown in Fig. 6A, we found that  $\Delta pmk1$  cells are hypersensitive to  $\beta$ -glucanase treatment and that sensitivity is even greater than that of the  $\Delta pck2$  mutant. This result shows that Pmk1 is involved in cell wall integrity.

**Budding yeast MPK1 and frog MAPK genes are capable of suppressing the  $\Delta pmk1$  mutant.** Next we asked whether MAPKs from other organisms are capable of rescuing the  $\Delta pmk1$  mutant. For this purpose, at first the budding yeast MPK1 gene, which encodes the most homologous MAPK, was tested. It was found that multicopy plasmids containing the MPK1 gene can complement efficiently the glucanase hypersensitivity of the  $\Delta pmk1$  mutant (Fig. 6B). Cells of the  $\Delta pmk1$  mutant containing the MPK1 gene also reverted to a normal rod shape, and no abnormally septated cells were observed (data not shown). Whether  $pmk1^+$  is capable of suppressing the  $mpk1$  mutant has not been tested, as a genomic sequence of the  $pmk1^+$  gene contains three introns (Fig. 1B) and fission yeast genes containing introns cannot function properly unless intronless genes are introduced in *S. cerevisiae* (3). In *S. cerevisiae*, the  $mpk1$  mutant was rescued by the expression of the frog MAPK gene (*X-MAPK*) (37). The *X-MAPK* gene connected under the fission yeast *adh* promoter was introduced into  $\Delta pmk1$ , and glucanase sensitivity was examined. It was found that frog MAPK also suppresses the hypersensitivity of  $\Delta pmk1$  cells (Fig. 6B). Suppression of *X-MAPK* appears weaker, as short and fat or multiseptated cells were still observed in transformants containing *X-MAPK* (data not shown).

**Fission yeast  $pmk1^+$  and budding yeast MPK1 may have a distinct role in cell wall integrity.** It has been shown that, in *S. cerevisiae*, defective phenotypes of the  $mpk1$  mutant are suppressed by the addition of osmotic stabilizers in the medium. To examine whether the  $\Delta pmk1$  disruptant is also rescued by

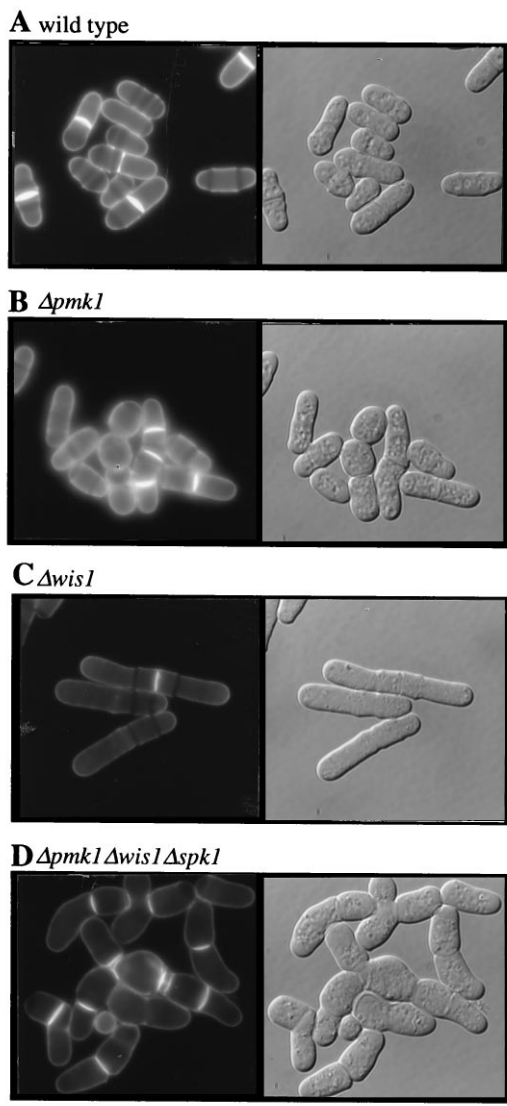


FIG. 4. Cell morphology of the  $\Delta pmk1$  disruptant. Cells grown in rich YPD medium were fixed with formaldehyde and stained with Calcofluor. Cell morphology was observed by either fluorescence microscopy (left) or differential-interference-contrast microscopy (right). The strains used are HM123 (wild type) (A), TP319-31A ( $\Delta pmk1$ ) (B), ED1011 ( $\Delta wis1$ ) (C), and TP355-7D ( $\Delta pmk1 \Delta spk1 \Delta wis1$ ) (D). See Table 1 for genotypes. Bar, 10  $\mu$ m.

high osmolarity, the  $\Delta pmk1$  mutant was grown in rich YPD medium containing 1.2 M sorbitol. No phenotypic suppression was found. Instead, that morphology became more abnormal in the presence of sorbitol, especially in the stationary-phase culture. Multiseptated cells were often found (approximately 5%) (right panel in Fig. 6C). Curiously, when these stationary cells were stained with Calcofluor (73), unusual accumulation of cell wall material was observed. In the wild type, this kind of abnormal accumulation of the cell wall material was never observed (left panel of Fig. 6C).

It has also been reported that budding yeast mutants defective in the Pkc1-Mpk1-MAPK pathway are supersensitive to caffeine (8, 9, 89). In contrast to the budding yeast *mpk1* mutants,  $\Delta pmk1$  mutants were not supersensitive to caffeine; either the  $\Delta pmk1$  or wild-type strain could form colonies on plates containing 10 mM caffeine but not 12.5 mM (data not

TABLE 2. Septation of cells in stationary phase<sup>a</sup>

Cell	Septated cells (%) at the following time (h):		
	5	24	48
Wild type	14	6	3
$\Delta pmk1$	42	17	12

<sup>a</sup> Wild-type (HM123) or  $\Delta pmk1$  (TP319-31A) cells were grown in minimal medium supplemented with leucine at a concentration of  $10^6$  cells per ml (0 h), and incubation was continued for 2 days. Aliquots were taken at 0, 5 (log phase), 24 (stationary phase), and 48 (stationary phase) h, and the percentages of septated cells were determined after the cells were stained with Calcofluor.

shown). This result might suggest that the molecular functions of the MAPK pathways between these two organisms in relation to cell wall integrity may not be the same (see Discussion).

**The *pmk1* and *pck2* mutations show a synergistic defect in cell wall integrity.** As described previously, the budding yeast Mpk1 MAPK pathway is regulated by Pkc1 (37). Mutants with mutations in this pathway show very similar, if not identical, phenotypes. To distinguish whether or not the Pmk1 MAPK and Pck kinases act in a single cascade, a double mutant  $\Delta pmk1 \Delta pck2$  strain was constructed (TP385-1C [Table 1]), and  $\beta$ -glucanase treatment was performed. It was found that this double mutant became even more sensitive to the treatment than each single mutant did (Fig. 6A). Half of the reduction in optical density occurred after only 10 min of incubation in the double disruptant, while it took 60 or 80 min in the case of a single  $\Delta pmk1$  or  $\Delta pck2$  disruptant, respectively. This result clearly shows that a defect in cell wall construction is not identical in  $\Delta pmk1$  and  $\Delta pck2$  mutants. It also suggests either that the Pmk1 MAPK regulates cell wall integrity independently of the Pck2 kinase or that Pck2 (and Pck1) has a dual role in cell wall integrity where one branch is regulated by the Pmk1 pathway (see Discussion).

*pck2*<sup>+</sup> mutants are supersensitive to staurosporine, a protein

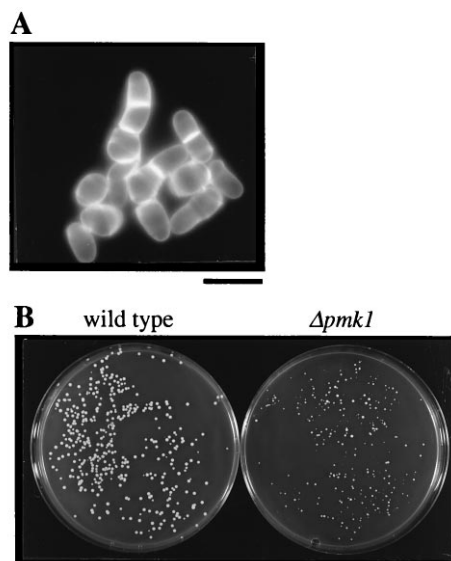


FIG. 5. Phenotypes of the  $\Delta pmk1$  mutants in stationary phase. (A)  $\Delta pmk1$  cells in stationary culture (24 h) are shown. Note that cell morphology is abnormal and multiseptated cells are observed. Bar, 10  $\mu$ m. (B) Stationary cultures (48 h) of wild-type and  $\Delta pmk1$  cells were spread on rich YPD plates after appropriate dilution ( $10^{-5}$ ) and incubated at 29°C for 3 days. The colonies of  $\Delta pmk1$  cells were smaller, although the numbers of viable cells were similar for these two strains.

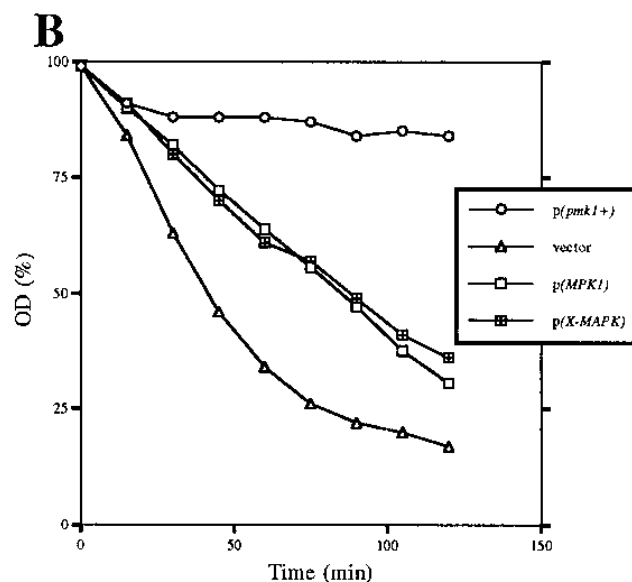
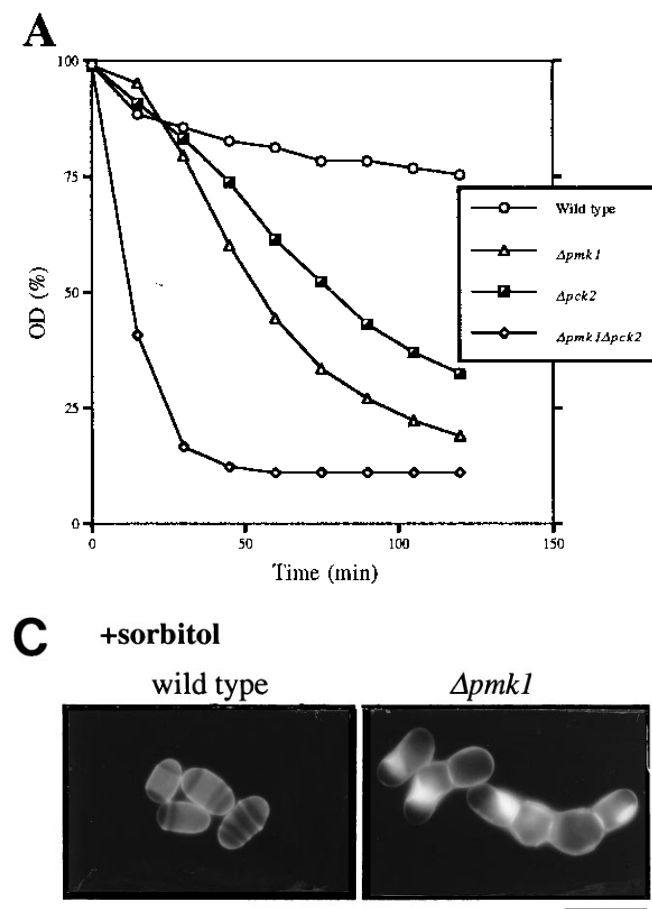


FIG. 6. Cell wall integrity in the  $\Delta pmk1$  mutant, suppression of the defect by other MAPKs, and cell morphology in the presence of sorbitol. (A) Cell wall digestion with  $\beta$ -glucanase (Zymolyase) was performed with wild-type (HM123),  $\Delta pmk1$  (TP319-31A),  $\Delta pck2$  (TP170-2B), and  $\Delta pmk1 \Delta pck2$  (TP385-1C) strains. The optical density (OD) at 0 min was set at 100%. (B) Cell wall digestion was performed with  $\Delta pmk1$  cells containing a vector or a multicopy plasmid carrying  $pmk1^+$ , budding yeast *MPK1*, or frog MAPK (*X-MAPK*). (C) Cell morphology and abnormal accumulation of cell wall materials in the  $\Delta pmk1$  mutant in the presence of 1.2 M sorbitol is shown. Wild-type (HM123) or  $\Delta pmk1$  (TP319-31A) cells were grown in rich medium containing 1.2 M sorbitol for 2 days and stained with Calcofluor. Bar, 10  $\mu$ m.

kinase inhibitor (76). The major target molecules of staurosporine in *S. pombe* are Pck1 and Pck2 (81). In *S. cerevisiae*, the *PKC1* gene was also identified as one of the staurosporine-supersensitive loci and mutants defective in genes involved in the Pck1 pathway were isolated in the same screening (95, 96). To test whether  $\Delta pmk1$  cells are also supersensitive to staurosporine, we have examined the staurosporine sensitivity of the  $\Delta pmk1$  mutant. No difference was found between the wild type and  $\Delta pmk1$  mutant (Table 3).

#### Suppression of $\Delta pmk1$ mutant by a high-dosage $pck1^+$ gene.

To examine more carefully a functional relationship between the Pmk1 and the Pck1 and Pck2 proteins in terms of cell wall integrity, multicopy plasmids containing each of these three genes were introduced into individual mutants and glucanase sensitivity was measured. As shown in Fig. 7,  $pck1^+$ -containing multicopy plasmids were capable of partially suppressing the  $\Delta pmk1$  mutant (Fig. 7A). In contrast,  $pmk1^+$ -containing multicopy plasmids had little effect on the glucanase hypersensitivity of the  $\Delta pck2$  mutant, although the kinetics of the digestion appeared slower than that of the  $\Delta pck2$  mutant containing only the vector (Fig. 7B). This result supports the notion that the Pmk1 and the Pck1 and Pck2 proteins act closely with each other in cell wall integrity; they are not functionally independent.

**Altered cation sensitivity in the  $\Delta pmk1$  mutant.** While looking for additional phenotypes of the  $\Delta pmk1$  disruptants, we found that the  $\Delta pmk1$  mutant shows altered sensitivity to monovalent cations such as sodium and potassium. As shown in Fig. 8,  $\Delta pmk1$  cells were capable of forming colonies on rich

YPD plates containing 1 M NaCl, while wild-type cells were not (compare Fig. 8A and B). In contrast to sodium resistance,  $\Delta pmk1$  cells were supersensitive to a high concentration of potassium. They could not form colonies on plates containing 1.4 M KCl (Fig. 8C). Sensitivities to other cations including calcium and magnesium were also examined and found to be indistinguishable from that of the wild type (Table 4). Cation sensitivity of the  $pck2$  mutant was also examined, and no difference was found between wild-type and  $pck2$  mutant cells (Table 4). Thus, the  $pmk1^+$  gene, not the  $pck2^+$  gene, is involved in the metabolism of sodium and potassium ions in an opposing manner.

TABLE 3. Staurosporine supersensitivity of the  $\Delta pmk1$  mutant<sup>a</sup>

Strain	Sensitivity <sup>b</sup> to staurosporine of strain carrying plasmid			
	Vector	$pck1^+$	$pck2^+$	$pmk1^+$
Wild type	+	+	+	+
$\Delta pmk1$	+	+	+	+
$\Delta pck1$	+	+	+	+
$pck2-8$	-	+	+	-

<sup>a</sup> Each transformant carrying various genes on multicopy plasmids was streaked on rich YPD plates with or without 2.0  $\mu$ g of staurosporine per ml and incubated at 29°C for 3 days. The strains used are as follows: HM123 (wild type), TP319-31A ( $\Delta pmk1$ ), TP134-3B ( $\Delta pck1$ ), and TP47-2B ( $pck2-8$ ) (see Table 1 for genotypes).

<sup>b</sup> Symbols: +, not sensitive; -, sensitive.



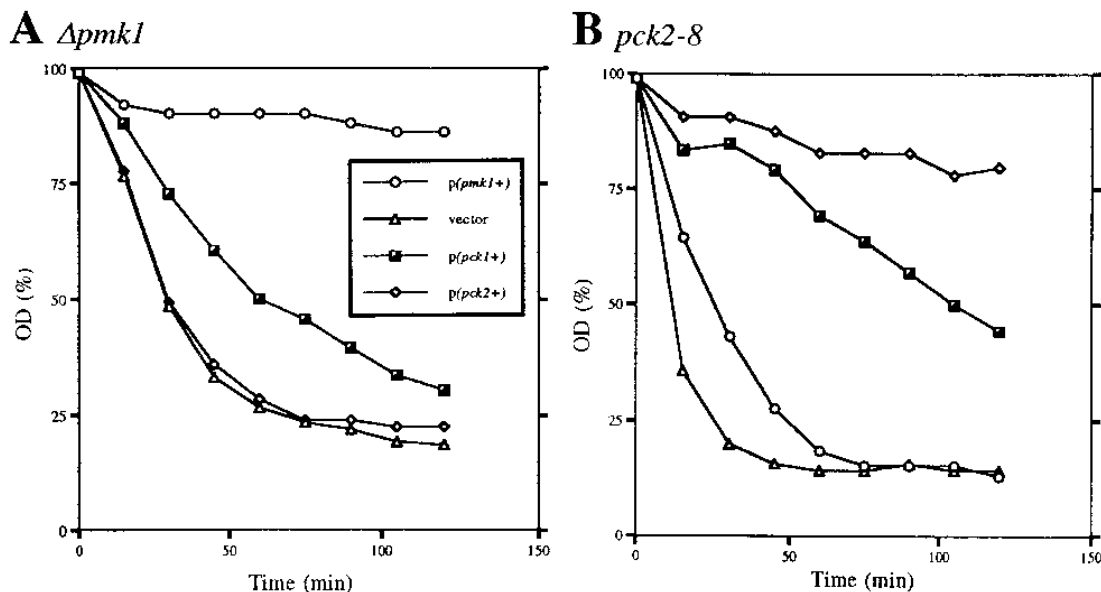


FIG. 7. Suppression of the defect in cell wall integrity of the  $\Delta pmk1$  mutant by a high-copy-number  $pck1^+$  gene. Cell wall digestion was performed with  $\Delta pmk1$  cells (TP319-31A) or  $pck2-8$  cells (TP47-2B) containing a vector or a multicopy plasmid carrying  $pmk1^+$ ,  $pck1^+$ , or  $pck2^+$ . OD, optical density.

## DISCUSSION

In this study we have isolated a third MAPK-encoding gene,  $pmk1^+$ , from *S. pombe*, identified the gene product, and characterized null phenotypes. We have shown that the Pmk1 pathway functions in parallel with two other MAPK pathways identified previously, the Spk1-dependent mating pheromone signalling and Sty1/Spc1/Phh1-dependent stress-sensing pathways (Fig. 9). The Pmk1-dependent pathway plays important roles in various aspects of cell integrity. These roles include cell wall architecture, cell shape control, cytokinesis in both exponential and stationary phases, and metabolism of ions.

**Three functionally distinct MAPK pathways in *S. pombe*.** It has become clear that multiple MAPK pathways exist in all eukaryotes. Analyses of *S. cerevisiae*, in which it appears that a total of five separate MAPK cascades operate in diverse signal transduction pathways, have also suggested the functional specificity and distinctness of each MAPK pathway except for certain mutant backgrounds (41, 94). In animal cells, these include Erk1/Erk2-, p38- and Jnk/SAPK-dependent pathways which respond to different external signals (7). In *Arabidopsis* plants, a family of MAPKs consisting of at least seven members has been identified and classified into three subfamilies on the basis of amino acid sequence comparison, although functional

heterogeneity of these MAPKs remains unknown (51). *S. cerevisiae* and *S. pombe* are known to have diverged long ago. It is often true that molecular pathways conserved between these two yeasts are ubiquitously conserved through evolution (e.g., see reference 61). The Pmk1 protein appears to be a genuine structural homolog of budding yeast Mpk1, as these two MAPKs show a high degree of amino acid identities with other MAPK members. Moreover, *MPK1* is capable of rescuing the defective phenotypes of  $\Delta pmk1$  cells. Although structural counterparts for  $pmk1^+/MPK1/MKC1$  have not been isolated from organisms other than yeasts, it is very possible that the Pmk1 MAPK is also conserved in higher eukaryotes.

We have shown that a frog MAPK (X-MAPK) is capable of suppressing the  $\Delta pmk1$  mutant. This result may be relevant because the budding yeast *mpk1* mutant is also suppressed by X-MAPK (37). However, it should be noted that X-MAPK is also capable of suppressing meiosis-defective phenotypes of  $\Delta spk1$  (21). The overall amino acid identities among Pmk1, Spk1, and X-MAPK are almost identical: 46% identity between Pmk1 and X-MAPK and 49% identity between Spk1 and X-MAPK. In this context, although a novel MAPK (Mmk2) from alfalfa plants, which shows 48% identity to budding yeast Mpk1, has been shown to specifically rescue the *mpk1* mutant (31), the demonstration of evolutionary conser-

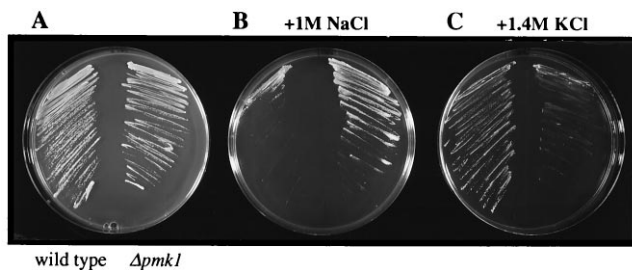


FIG. 8. Cation sensitivity of the  $\Delta pmk1$  mutant. Wild-type (HM123) or  $\Delta pmk1$  (TP319-31A) cells were streaked on a rich YPD plate (A) or a YPD plate containing 1 M NaCl (B) or 1.4 M KCl (C). Plates were incubated at 29°C for 3 (A) or 5 (B and C) days.

TABLE 4. Cation sensitivity of the  $\Delta pmk1$  mutant<sup>a</sup>

Strain	Sensitivity <sup>b</sup> of strain to the following cation concn (M):							
	NaCl		KCl		MgCl <sub>2</sub>		CaCl <sub>2</sub>	
	0.5	1.0	1.2	1.4	0.75	1.0	0.4	0.5
Wild type	+	-	+	+	+	-	+	-
$\Delta pmk1$	+	+	+	-	+	-	+	-
$\Delta pck2$	+	-	+	+	+	-	+	-

<sup>a</sup> Wild-type (HM123) or  $\Delta pmk1$  (TP319-31A) or  $\Delta pck2$  (TP170-2B) mutant *S. pombe* was streaked on YPD plates containing the indicated concentrations of cations and incubated at 29°C for 4 to 7 days.

<sup>b</sup> Symbols: +, not sensitive; -, sensitive.

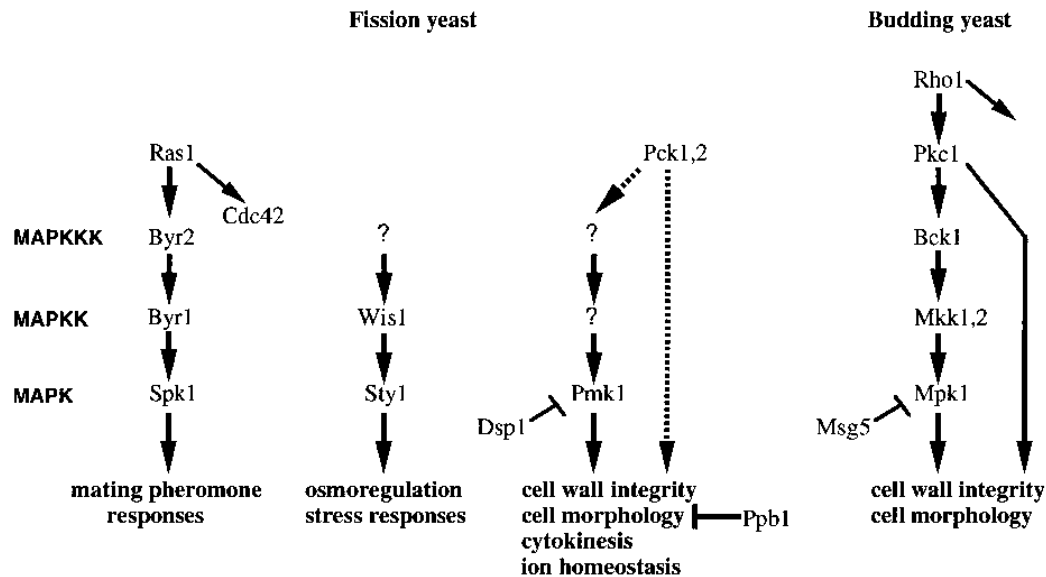


FIG. 9. MAPK pathways in *S. pombe*. Three fission yeast MAPK pathways are schematically depicted together with the budding yeast Mpk1 MAPK pathway. The Pmk1 MAPK pathway regulates diverse aspects of cell integrity including cell wall integrity, morphogenesis, cytokinesis, and ion homeostasis. Fission yeast PKC homologs (Pck1 and Pck2) regulate cell wall integrity either independently of the Pmk1 pathway (shown by the dotted line directly leading to the bottom) or in a bifurcated manner where one branch acts through the Pmk1 pathway (shown by the short dotted line leading to Pmk1). In *S. cerevisiae*, Pck1 acts upstream of the Mpk1 pathway and also appears to regulate cell wall integrity independent of the Mpk1 pathway (14). The Pck1-Mpk1 pathway is regulated by a small GTPase, Rho1 (60). Dsp1 is an attenuating phosphatase of the Pmk1 MAPK (this study and reference 75). The Msg5 phosphatase has been suggested to act negatively on Mpk1 (89). This and other studies have suggested that calcineurin (Ppb1) acts in an opposing manner to the Pmk1 MAPK pathway in *S. pombe* (75), while in *S. cerevisiae* it acts together with the Mpk1 MAPK pathway (19).

vation of the Pmk1/Mpk1/Mkc1 MAPK pathway must wait until structural homologs encoding novel mammalian MAPKs are isolated.

**Molecular pathways that interact with the Pmk1 MAPK pathway.** One of the most important results in this study is the demonstration of a functional linkage between the Pmk1 MAPK pathway and the PKC homologs Pck1 and Pck2. In animal cells, PKCs are known to regulate a wide variety of cellular events which are also regulated by the MAPK pathway (e.g., PC12 cell or T-cell activation) (16, 43, 45). Thus, a connection between PKCs and MAPK pathways might be a general scheme for all eukaryotes. How does the Pck1 and Pck2 pathway interact with the Pmk1 MAPK pathway? Two plausible models could be considered. In one model, as in *S. cerevisiae*, Pck1 and Pck2 act upstream of the Pmk1 pathway and regulate cell integrity (Fig. 9). In this model, however, Pck1 and Pck2 must have a bifurcated role in cell integrity where one of two branches acts through Pmk1, as the  $\Delta pck1\Delta pck2$  double mutation is lethal, while  $\Delta pmk1$  is not. Thus, Pck1 and Pck2 must have an essential role in which Pmk1 is not involved. It should be pointed out that in *S. cerevisiae* the Mpk1 pathway might not be the sole effector for Pck1, as a phenotypic difference exists between the *pck1* mutant and downstream MAPK component mutants, where loss of the Pck1 function results in a more severe defect than that of downstream molecules (14, 29, 36–38, 83).

The second model is that the Pmk1 and Pck1 and Pck2 pathways are functionally related but regulate independent aspects of cell integrity (Fig. 9). Synergistic phenotypes of cell wall weakness in the  $\Delta pmk1 \Delta pck2$  mutants might be consistent with this idea. Also, phenotypic differences between  $\Delta pmk1$  and  $\Delta pck2$  mutants, such as cation sensitivity and a defect in cytokinesis in the  $\Delta pmk1$  mutant and staurosporine sensitivity in the  $\Delta pck2$  mutant, could be explained in the second model. It is, nevertheless, still possible that the pres-

ence of the *pck1*<sup>+</sup> gene in  $\Delta pck2$  makes the interpretation difficult. Although *pck1*<sup>+</sup> appears to have a minor role in cell wall integrity and staurosporine sensitivity (81), the possibility that *pck1*<sup>+</sup> has a role in these processes in the absence of the *pck2*<sup>+</sup> gene cannot be excluded. At present, these two models are equally plausible.

We isolated the *dsp1*<sup>+</sup> gene as a high-dosage suppressor of  $\Delta ppb1$ , which is defective in the catalytic subunit of calcineurin (75, 95). Interestingly, not only overproduction of *dsp1*<sup>+</sup> but also deletion of the *pmk1*<sup>+</sup> gene is capable of suppressing  $\Delta ppb1$  mutants. Thus, the Pmk1-dependent MAPK pathway appears to act antagonistically with calcineurin. Further genetic as well as biochemical analyses have demonstrated that Dsp1 specifically inactivates Pmk1 (75). In line with these results, we were able to show increased tyrosine phosphorylation of the Pmk1 MAPK in the  $\Delta dsp1$  background. The Pmk1 pathway seems to act closely with Ca<sup>2+</sup> signalling pathways, in a parallel or dependent manner with respect to the PKC homologs Pck1 and Pck2 and in an opposing manner to calcineurin (Fig. 9). It would be interesting to see whether in higher organisms calcineurin and any of the MAPK pathways act in an opposing manner as they do in *S. pombe*. It should be noted that, in contrast to fission yeast calcineurin, budding yeast calcineurin has been proposed to perform independent but physiologically related functions with the Mpk1 pathway (19).

In terms of cell wall integrity, phenotypes of  $\Delta pmk1$  cells are similar to those with a deletion of the *kin1*<sup>+</sup> gene, which encodes a putative protein kinase (39). Like  $\Delta pmk1$  cells, the  $\Delta kin1$  cells are hypersensitive to treatment with cell wall-digesting enzymes, although the morphological defect seems to be more severe;  $\Delta kin1$  cells are almost round (39). The  $\Delta kin1$  cells are not supersensitive to staurosporine, suggesting that the *kin1*<sup>+</sup> gene is not involved in the Pck1- and Pck2-dependent cell morphogenetic pathway (77).

**Extracellular signals or conditions that activate the Pmk1 MAPK pathway.** Several important questions remain to be answered. These questions include which signals or extracellular conditions activate the Pmk1 pathway and upstream and downstream components of this pathway. In *S. cerevisiae*, protein kinase activity of the Mpk1 protein is induced by a variety of conditions, such as heat shock; addition of a cationic amphipath, chlorpromazine (32); mating pheromone (98); and hypotonic shock (11). In contrast, none of these conditions appears to increase the level of a tyrosine-phosphorylated form of the Pmk1 protein (this study and reference 77). Also, DNA-damaging agents do not activate the Pmk1 kinase, consistent with a normal response of  $\Delta pmk1$  cells upon UV irradiation (77). Further experiments including direct measurement of protein kinase activities of Pmk1 upon various treatments will be required to elucidate signals for the activation of the Pmk1 MAPK pathway.

Recently we have obtained genes which encode a putative MAPKK and MAPKKK for the Pmk1 kinase (77). Genetic and molecular analyses are in progress to clarify what is upstream of the Pmk1 MAPK pathway.

**Cellular roles of the Pmk1 MAPK pathway.** We have shown that Pmk1 is involved in cell wall integrity, as  $\Delta pmk1$  cells become hypersensitive to treatment with a cell wall-digesting enzyme. This defect is apparently similar to that observed in mutants which are defective in fission yeast Pck2 or in budding yeast Mpk1 MAPK pathway components (40, 71, 79). In *S. cerevisiae*, cell wall weakness of mutants in the pathway components appears to be due to the reduced  $\beta$ -glucan content in the cell wall (6, 48, 64, 69), and observations by electron microscopy supported this notion (40, 62). Ultrastructural analysis of the  $\Delta pmk1$  mutant by electron microscopy has failed to show apparent cell wall defects, while the  $\Delta pck2$  mutants have weaker cell wall structures (78). Thus, despite a similar supersensitivity to cell wall-digesting enzyme between  $\Delta pmk1$  and  $\Delta pck2$  or budding yeast *mpk1* mutants, the molecular mechanisms leading to cell wall weakness may not be identical. The cell wall is a complex structure where a number of factors are involved in maintenance of integrity (e.g., see reference 28). Biochemical analysis of cell wall composition or organization in  $\Delta pmk1$  cells would be required to clarify the defects of cell wall integrity in  $\Delta pmk1$  mutant cells.

In addition to the defect in cell wall integrity,  $\Delta pmk1$  cells show various pleiotropic phenotypes, including morphological defects, failure of cytokinesis, altered responses in stationary phase, hypersensitivity to potassium ions, and resistance to sodium ions. Ion homeostasis, e.g., sodium efflux and potassium uptake, is known to be mediated by P-type ATPases, channels, and transporters (15). Although it appears problematic that all of these pleiotropic phenotypes in  $\Delta pmk1$  cells are attributable to a single molecular pathway, it would be, nevertheless, intriguing to speculate that the Pmk1 MAPK regulates, for example, mechanosensitive ion channels which regulate membrane-cytoskeletal organization, ion homeostasis, and cytokinesis. Alternatively, the Pmk1 MAPK regulates diverse aspects of cell integrity through multiple downstream elements such as transcription factors, each of which is responsible for a subset of defective phenotypes. It would be interesting to examine whether transcription factors such as Nhp6A, Nhp6B, and Rlm1 (9, 89) which potentially act downstream of budding yeast Mpk1 MAPK are involved in the Pmk1 pathway. Further characterization of the Pmk1 pathway and dissection of other pathway components will provide a useful model to study fundamental functions of the MAPK pathways in higher eukaryotes.

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