The Fission Yeast pmk1+ Gene Encodes a Novel Mitogen-Activated Protein Kinase Homolog Which Regulates Cell Integrity and Functions Coordinate with the Protein Kinase C Pathway

TAKASHI TODA,1,2* SUSHEELA DHUT,1 GIULIO SUPERTI-FURGA,3 YUKIKO GOTOH,4 EISUKE NISHIDA,4 REIKO SUGIURA,5 AND TAKAYOSHI KUNO5

Cell Regulation Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom;2 European Molecular Biology Laboratory, 69012 Heidelberg, Germany;3 and Department of Biophysics, Faculty of Science,2 and Department of Genetics and Molecular Biology, Institute of Virus Research,4 Kyoto University, Sakyo-ku, Kyoto 606-01, and Department of Pharmacology, School of Medicine, Kobe University, Chuo-ku, Kobe 650,5 Japan

Received 14 June 1996/Returned for modification 11 July 1996/Accepted 9 September 1996

We have isolated a gene, pmk1+, 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 Mkl. The Pmk1 protein contains phosphorylated tyrosines, and the level of tyrosine phosphorylation is increased in the dpk1 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 (Δpmk1) are viable but show various defective phenotypes, including cell wall weakness, abnormal cell shape, 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 Δpmk1 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 oncprotein Ras was shown to act at least in part if not entirely (90) as an activator of another oncprotein, 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+ 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 Δspk1 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/Sik1 (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
We and others have previously isolated and characterized fission yeast PKC-encoding genes, pck1\(^+\) and pck2\(^+\) (49, 81). pck1\(^+\) and pck2\(^+\) have overlapping roles in cell viability, and Δpck2 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 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 (26). The budding yeast Hog1 kinase is activated by high osmolarity (79). Jnk/SAPK and fission yeast Sty1/Spc1/Phh1 MAPK pathways, instead, RKı primarily regulates adenyl cyclase (80).

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 it 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\(^+\). We show that pmk1\(^+\) 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, 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 Pck pathways.

**MATTERIALS 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 μg/ml) were added if necessary. Plates contained 1% 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 Gautz 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\(^+\)). In the text and figures, gene disruptions are abbreviated by the gene preceded by Δ (for example, Δpmk1). 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 Bio-labs). Nonradioisotope labeling and chemiluminescence detection methods (digoxigenin [Boehringer Mannheim GmbH] and AMPPD [Tropix]) were used.

**Cloning of the pmk1\(^+\) gene.** Degenerate oligonucleotides used for PCR were the 5′ oligonucleotide 5′-TCAATACGAGGATCGGAGGAGGGAGTATATGGTCA-3′ (GS3) and the 3′ oligonucleotide 5′-TAATCTGAGGAATCCRAARTCRCASACCAAAATTTRCAGGCATA-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 BamHI (GS3) or EcoRI (GS4) restriction site. GS3 and the reverse complement of GS4 correspond to highly conserved amino acid residues among the MAPK family members: Gly-Val-Gly-Ala-Tyr-Gly (GS3) and Cys-Asp-Leu-Lys-Ile-Leu-Cys-Asp-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 mej.

**Nucleotide sequence determination.** The dideoxy DNA sequencing method (68) was performed by using double-stranded plasmid DNA as templates (26). The 2,643-bp Par-SpeI fragment containing the entire pmk1\(^+\) gene was completely sequenced.

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

**Construction and expression of the tagged pmk1\(^+\) gene.** The tagged version of the pmk1\(^+\) gene was constructed as follows. The NdeI site in the third intron of the pmk1\(^+\) gene was destroyed by treatment with the Klenow enzyme. Then the entire pmk1\(^+\) gene was amplified by PCR with two oligonucleotides; the 5′ oligonucleotide is CCCCTATGGACGGCTGACATCTGTTT (pmk1-N) (the NdeI site is underlined), and the 3′ oligonucleotide is AAAAGGCGCCGC AGTTATGCGGATTATCATC (pmk1-C) (the NdeI site is underlined). A PCR-amplified 1,736-bp fragment was cloned with NdeI and NotI and subcloned into pREP41 (HA-His) (provided by J. Millar) to yield pREP41-pmk1\(^+\)-HA-His, which expressed pmk1\(^+\) 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 His-His in the C terminus did not interfere with the normal function of the Pmk1 protein, as pREP41-pmk1\(^+\)-HA-His, when intro...
duced into the \textit{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. Hypotonic 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 \textit{pmk1}+ gene, the procedures of Millar et al. (50) were followed with some modifications (75).

Briefly, XB buffer (50 mM NaH$_2$PO$_4$ [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 antiseras and immunoblot analysis.** To express a part of the \textit{Pmk1} protein in bacteria for antibody preparation, a 483-bp internal fragment of the \textit{pmk1}+ gene which encodes internal peptides of 161 amino acid residues (methionine 107 to serine 254 [Fig. 1B]) was amplified by PCR with two oligonucleotides: the 5’ oligonucleotide is GGCGATATGAGCCGCTTGGATA GT (the NdeI site is underlined), and the 3’ oligonucleotide is GGGGATCC AAGGATGCGAATACTCCTGTCGA (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 20kDa 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 electrochemically 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 [Amer sham]) were used to detect bound antibody.

**Expression of MAPK-encoding genes in the \textit{pmk1} disruptant.** The 2.643-bp PstI-SpeI fragment containing the entire \textit{pmk1}+ gene was subcloned into pSK248 (80), yielding pSK(pmk1+)1. This plasmid was used as a multicopy plasmid containing the \textit{pmk1}+ gene. pJD5 (provided by D. Hirata) or pART1-X-MAPK (21), containing the budding yeast \textit{MPK1} gene or \textit{Xenopus} MAPK gene, respectively, was used to transform the \textit{pmk1} disruptant. pwis1-1 (provided by P. Fantie) (88) and pADH1-pmk1+ (80) were also used.

**Glucanase sensitivity.** The procedures described by Levin and Bishop (59) and Shiozaki and Russell (71) were followed. Briefly, exponentially growing cells (approximately \(3 \times 10^8\) cells/ml) at 30°C were suspended at a concentration of 10°C cells per ml. β-Glucanase (Zymolyase-20T [ICN]) was treated at a concentration of 100 μg/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 \textit{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 \textit{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 \textit{pmk1}+ for \textit{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 \textit{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’. This is a consensus sequence for fission yeast introns (63).

A comparison of the \textit{Pmk1} amino acid sequence with MAPK sequences is shown in Fig. 2. \textit{Pmk1} shows the most homology (63 to 65% identity) to two yeast MAPKs, \textit{S. cerevisiae} Mpk1 (40) and \textit{Candida albicans} Mkc1 (55). These two MAPKs have been shown to be structural and functional homologs; \textit{Mkc1} is capable of suppressing the \textit{mpk1} mutant (55). Critical threonine and tyrosine residues which are known to be phosphorylated by upstream MAPKK are conserved in \textit{Pmk1} (indicated by arrowheads in Fig. 2). It should be noted that \textit{Pmk1} contains Thr-Glu-Tyr in this region, which is common to \textit{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, \textit{Pmk1} is more distantly related to other MAPK family members (40 to 50%). For example, \textit{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/Spc1/Phl1 (12, 34, 50, 70, 72, 91) (which are involved in responses to high osmolarity and various stress conditions), and 47% identity to metazoan \textit{Erk1} (4).

**Identification of the \textit{pmk1}+ gene product.** To characterize the \textit{pmk1}+ gene product biochemically, polyclonal antibody was prepared in rabbits with a fusion protein expressed in and purified from \textit{E. coli} (see Materials and Methods). Immunoblotting was performed against total cell extracts prepared from wild-type or \textit{Δpmk1} cells (see below) containing either vector plasmids or those carrying the \textit{pmk1}+ gene. Two bands corresponding to approximately 48 and 50 kDa which are close to the predicted size (48 kDa) of the \textit{Pmk1} protein were detected in the wild type but not in the \textit{Δpmk1} mutant (Fig. 3A, lanes 1 and 3). The intensities of these two bands greatly increased in extracts prepared from cells expressing the \textit{pmk1}+ gene on a multicopy plasmid (lanes 2 and 4). Thus, the two bands are most probably derived from the \textit{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 \textit{Pmk1} protein is possible (see below).

The \textit{Pmk1} protein contains phosphorylated tyrosines. In order to prove that \textit{Pmk1} is a MAPK, it is important to show that the \textit{Pmk1} protein is phosphorylated at a tyrosine residue. As the size (48 or 50 kDa) of the \textit{Pmk1} protein is close to that of immunoglobulin heavy chains, it hampered the detection of the \textit{Pmk1} protein by immunoprecipitation with anti-\textit{Pmk1} antibody or antiphosphotyrosine antibody. To overcome this difficulty, a tagged version of the \textit{Pmk1} protein (HA epitope and a six-histidine carboxy-terminal tail [\textit{PREP41-pmk1}+ -HA/6His]; see Materials and Methods for details) was constructed. By using this construct, the \textit{Pmk1} protein was purified in one step with \textit{Ni}²⁺-nitrilotriacetic acid beads and analyzed by immunoblotting with either anti-\textit{Pmk1} antibody or anti-HA antibody. Similar to the pattern of the endogenous \textit{Pmk1} protein detected with anti-\textit{Pmk1} antibody (Fig. 3A), \textit{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 \textit{Pmk1} protein has phosphorylated tyrosines and that the lower band (53 kDa) may represent unphosphorylated \textit{Pmk1}.

Independent genetic approaches which have been aimed at obtaining regulators of calcineurin (Ppb1, type 2B protein phosphatase) (97) have unexpectedly identified a gene, \textit{dsp1}+ (75), which encodes a member of the dual-specificity protein phosphatases or MAPK phosphatases (75). Subsequent genetic and biochemical analyses have demonstrated that \textit{Dsp1} acts through inactivation of \textit{Pmk1}. These include the formation of a physical complex between \textit{Pmk1} and \textit{Dsp1} and suppression of the \textit{Δdsp1} mutation either by overproduction of \textit{ dsp1}+ or by deletion of the \textit{pmk1}+ gene (75). We reasoned that in this mutant background (\textit{Δdsp1}), the level of the tyrosine-phospho-
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, HindIII; Hc, HincII; Hp, HpaI; Nc, NcoI; Nh, NheI; P, PstI; RI, EcoRI; Sc, SacI; Sh, SphI; Sl, SalI; Sp, SpeI; St, SmaI. (B) The nucleotide sequence of a 2,643-bp PstI-SpeI 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.
rylated Pmk1 protein would be increased because of the lack of attenuating phosphatases, and therefore, we tested the Pmk1-HA/6His protein in Δdsp1 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 thenotion 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 Δdsp1 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 pmk1Δ-deleted mutant is defective in cell shape and cytokinesis. To construct pmk1Δ-deleted cells, a one-step gene replacement method (66) was performed. The uaa4Δ marker was inserted into the middle of the pmk1Δ 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 pmk1Δ gene is not essential for normal growth. pmk1Δ-deleted (Δpmk1) cells are fertile and can form colonies at any temperature tested, including 20, 30, and 36°C. The generation time of the Δpmk1 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 Δpmk1 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 Δpmk1 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 Δpmk1 culture in minimal medium is much higher (42%) than that of the wild type (14% [Table 2]), suggesting that the pmk1Δ 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 Δpmk1 mutant is still high in stationary phase (12% [Table 2]). Notably, we often observed abnormal multiseptated cells in the culture (5%). The viability of the Δpmk1 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 pmk1Δ gene is required for maintenance of cell shape and execution of cell division in both the exponential-growth and stationary phases.
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 Δspk1 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 predicted amino acid sequence of the pmk1 gene is shown in Fig. 4D. The single Δpmk1 mutant (wis1) showed elongated morphology due to a G2 delay (88), while the triple mutant showed, in addition to cell elongation, multiseptation and irregular cell shape, which was due to the defect of Δspk1 (the defect of Δspk1 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 Δpmk1 cells are hypersensitive to β-glucanase treatment and that sensitivity is even greater than that of the Δpck2 mutant. This result shows that Pmk1 is involved in cell wall integrity.

Budding yeast MPK1 and frog MAPK genes are capable of suppressing the Δpmk1 mutant. Next we asked whether MAPKs from other organisms are capable of rescuing the Δpmk1 mutant. For this purpose, at first the budding yeast MPK1 and frog MAPK genes were expressed in Δpmk1 mutant cells (Fig. 6B). Cell growth was tested. It was found that multicopy plasmids containing the MPK1 gene can complement efficiently the glucanase hypersensitivity of the Δpmk1 mutant (Fig. 6B). Cells of the Δpmk1 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 Δpmk1, and glucanase sensitivity was examined. It was found that frog MAPK also suppresses the hypersensitivity of Δpmk1 cells (Fig. 6B). Suppression of X-MAPK appears weaker, as short and fat or multiseptated cells were 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 Δpmk1 disruptant is also rescued by...
high osmolarity, the \( \Delta \text{pmk1} \) 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 Pck1-Mpk1-MAPK pathway are supersensitive to caffeine (8, 9, 89). In contrast to the budding yeast \( mpk1 \) mutants, \( \Delta \text{pmk1} \) mutants were not supersensitive to caffeine; either the \( \Delta \text{pmk1} \) or wild-type strain could form colonies on plates containing 10 mM caffeine but not 12.5 mM (data not 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 \( \Delta \text{pmk1} \) and \( \Delta \text{pck2} \) mutations show a synergistic defect in cell wall integrity.** As described previously, the budding yeast Mpk1 MAPK pathway is regulated by Pck1 (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 \( \Delta \text{pmk1} \Delta \text{pck2} \) 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 \( \Delta \text{pmk1} \) or \( \Delta \text{pck2} \) disruptant, respectively. This result clearly shows that a defect in cell wall construction is not identical in \( \Delta \text{pmk1} \) and \( \Delta \text{pck2} \) 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).

\( \Delta \text{pck2}^+ \) mutants are supersensitive to staurosporine, a protein

### Table 2. Septation of cells in stationary phase

<table>
<thead>
<tr>
<th>Cell</th>
<th>Septated cells (%) at the following time (h):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>14 6 3</td>
</tr>
<tr>
<td>( \Delta \text{pmk1} )</td>
<td>42 17 12</td>
</tr>
</tbody>
</table>

\( ^a \) Wild-type (HM123) or \( \Delta \text{pmk1} \) (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.
kinase inhibitor. The major target molecules of staurosporine in S. pombe are Pck1 and Pck2. In S. cerevisiae, the PKC1 gene was also identified as one of the staurosporine-supersensitive loci and mutants defective in genes involved in the Pkc1 pathway were isolated in the same screening. To test whether Dpmk1 cells are also supersensitive to staurosporine, we have examined the staurosporine sensitivity of the Dpmk1 mutant. No difference was found between the wild type and Dpmk1 mutant (Table 3).

Suppression of Dpmk1 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 Dpmk1 mutant. In contrast, pmk1+ -containing multicopy plasmids had little effect on the glucanase hypersensitivity of the Dpck2 mutant, although the kinetics of the digestion appeared slower than that of the Dpck2 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 Dpmk1 mutant. While looking for additional phenotypes of the Dpmk1 disruptants, we found that the Dpmk1 mutant shows altered sensitivity to monovalent cations such as sodium and potassium. As shown in Fig. 8, Dpmk1 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, Dpmk1 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 Dpmk1 mutant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sensitivity to staurosporine of strain carrying plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>Dpmk1</td>
<td>+</td>
</tr>
<tr>
<td>Dpck1</td>
<td>+</td>
</tr>
<tr>
<td>Dpck2-8</td>
<td>–</td>
</tr>
</tbody>
</table>

*Each transformant carrying various genes on multicopy plasmids was streaked on rich YPD plates with or without 2.0 μg of staurosporine per ml and incubated at 29°C for 3 days. The strains used are as follows: HM123 (wild type), TP319-31A (Dpmk1), TP153-5B (Dpck1), and TP47-2B (pck2-8) (see Table 1 for genotypes). Symbols: +, not sensitive; –, sensitive.
DISCUSSION

In this study we have isolated a third MAPK-encoding gene, pmk11, 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 Δpmk1 cells. Although structural counterparts for pmk11/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 Δpmk1 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 Δspk1 (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. 7. Suppression of the defect in cell wall integrity of the Δpmk1 mutant by a high-copy-number pck11 gene. Cell wall digestion was performed with Δpmk1 cells (TP319-31A) or pck2-8 cells (TP47-2B) containing a vector or a multicopy plasmid carrying pck11, pck11, or pck21. OD, optical density.](http://mcb.asm.org/)

![FIG. 8. Cation sensitivity of the Δpmk1 mutant. Wild-type (HM123) or Δpmk1 (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.](http://mcb.asm.org/)

<table>
<thead>
<tr>
<th>Strain</th>
<th>NaCl</th>
<th>KCl</th>
<th>MgCl2</th>
<th>CaCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δpmk1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δspk2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Wild-type (HM123) or Δpmk1 (TP319-31A) or Δspk2 (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.

* Symbols: +, not sensitive; -, sensitive.
Second model. It is, nevertheless, still possible that the presence of the pck1+ gene in Δpck2 makes the interpretation difficult. Although pck1+ appears to have a minor role in cell wall integrity and staurosporine sensitivity (81), the possibility that pck1+ has a role in these processes in the absence of the pck2+ gene cannot be excluded. At present, these two models are equally plausible.

We isolated the dsp1+ gene as a high-dosage suppressor of Δpck1, which is defective in the catalytic subunit of calcineurin (75, 95). Interestingly, not only overproduction of dsp1+ but also deletion of the pck1+ gene is capable of suppressing Δpck1 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 Δdsp1 background. The Pmk1 pathway seems to act closely with Ca2+ 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 Δpck1 cells are similar to those with a deletion of the kin1+ gene, which encodes a putative protein kinase (39). Like Δpck1 cells, the skin1 cells are hypersensitive to treatment with cell wall-digesting enzymes, although the morphological defect seems to be more severe; Δskin1 cells are almost round (39). The skin1 cells are not supersensitive to staurosporine, suggesting that the kin1+ 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 Δpmk1 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, Δpmk1 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 β-glucan content in the cell wall (6, 48, 64, 69), and observations by electron microscopy supported this notion (40, 62). Ultrastructural analysis of the Δpmk1 mutant by electron microscopy has failed to show apparent cell wall defects, while the Δpck2 mutants have weaker cell wall structures (78). Thus, despite a similar super-sensitivity to cell wall-digesting enzyme between Δpmk1 and Δpck2 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 Δpmk1 cells would be required to clarify the defects of cell wall integrity in Δpmk1 mutant cells.

In addition to defect in cell wall integrity, Δpmk1 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 Δpmk1 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 Rim1 (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.

**ACKNOWLEDGMENTS**

We thank Peter Fantes, Dai Hirata, and Jonathan Millar for plasmids and strains, Hirofumi Nakano for staurosporine, and Takahisa Hachiya for the preparation of anti-Pmk1 antibody. We also thank Jonathan Cooper for communicating unpublished results. Mitsuhiro Yangida for encouragement, Dai Hirata for constructive discussion, David Levin for comments on the manuscript, Shusuke Kuge for guidance PCR-based mutagenesis, and Rich Treisman for critical reading of the manuscript and useful comments. We thank Dallas Young for agreeing to change the name of the gene (previously called pmk1) also encoding a putative MAPKK which his group isolated, to avoid confusing nomenclature for these two genes.

This work was in part supported by grants from Ministry of Education, Science, and Culture of Japan and from Kyowa Hakko Co.

**REFERENCES**

A NOVEL MAP KINASE PATHWAY IN S. POMBE


