Mitogen-activated protein kinase (MAPK) pathways are signal transduction mechanisms that regulate many cellular processes in eukaryotic organisms, from yeasts to mammals. The basic architecture of each functional cascade is composed of three sequentially acting protein kinases that become activated in response to triggering signals; the MAPK kinase kinases (MAPKKKs) phosphorylate and activate MAPK kinases (MAPKKs), which in turn phosphorylate and activate MAPKs (1, 2). Among other actions, the effector MAPKs control the activity of transcription factors either directly or indirectly. Thus, activation by specific stimuli of MAPK signal transduction pathways is accompanied by changes in gene expression that play a crucial adaptive role in the adjustment of cells to environmental conditions. In contrast to the six or more MAPK cascades present in budding yeast (3), three distinct MAPK signaling cascades have been so far identified in the fission yeast Schizosaccharomyces pombe. These include the mating pheromone-responsive MAPK pathway and the stress-activated protein kinase (SAPK) pathway, whose central elements are MAPKs Spk1 and Sty1/Spcl, respectively (4, 5). A third pathway, known as the cell integrity pathway, consists of a MAPK cascade composed by MAPKKK Mkh1 (6), MAPKK Pek1/Shk1 (7, 8), and the MAPK Pmk1/Spm1 (9, 10). Deletion of mkh1+ or pkm1+ causes hypersensitivity to B1,3-glucanases (6–10), growth inhibition in response to hyperosmotic stress or elevated temperatures, and morphological defects with cells displaying a multiseptate phenotype (6–10). These and other results suggest that this pathway is involved in cell wall construction, morphogenesis, cytoskeleton, and ion homeostasis in fission yeast (6–10).

Pmk1/Spm1 MAPK was independently characterized by two laboratories as a structural homolog of the budding yeast Mpk1/Slt2 MAPK (9, 10). Later work identified Mkh1 and Pek1 as the respective MAPKKK and MAPKK components of the pathway (6–8). In fact, yeast two-hybrid experiments indicate that Mkh1, Pek1, and Pmk1 physically interact to form a ternary complex (7). Pmk1 MAPK is a 48-kDa protein that can be dually phosphorylated by Pek1 at two conserved threonine and tyrosine amino acid residues in positions 186 and 188 (consensus sequence TEY) (8). Moreover, conclusive evidence indicates that inactive (unphosphorylated) Pek1 binds Pmk1 and acts as a potent inhibitor of Pmk1 signaling in the absence of a triggering stimulus (8). On the other hand, Pmp1 phosphatase is able to dephosphorylate Pmk1, suggesting that this phosphatase may negatively regulate the Pmk1 MAPK pathway (11).

The Pmk1 signal transduction pathway seems closely related to the Mpk1/Slt2 cell integrity pathway of the budding yeast Saccharomyces cerevisiae (3). However, whereas Mpk1/Slt2 is activated by phosphorylation in response to elevated temperatures, cell wall-damaging compounds, or oxidative agents (12, 13), S. pombe Pmk1 becomes activated only by high temperatures or sodium chloride (10). Besides, the MAPK activation domain in Pmk1 is homolog to that present in extracellular signal-regulated kinases 1 and 2 (ERK1/2) (p42/p44) of animal cells, which become strongly activated in response to growth factors, phorbol esters, and, to a lesser extent, cytokines or osmotic stress (14).
A common feature of the signaling pathways is the nuclear translocation of MAPKs as a critical step for their biological function at the transcriptional level. However, this process varies among different eukaryotic organisms. Whereas mammalian ERKs translocate from the cytoplasm to the cell nucleus upon stimulation by growth factors (14), budding yeast Slt2 constitutively switches between the nucleus and the cytoplasm (15). Similar to ERKs, fission yeast Sty1 is mainly cytoplasmic in unstressed cells but translocates into the cell nucleus upon phosphorylation (16).

The mechanisms responsible for the activation of the cell integrity MAPK cascade in *S. pombe* and the elements involved downstream from Pmk1 are poorly understood. Recent evidence supports a potential interaction between Pak2 and Mkh1 MAPKKK (17). Pak2/Shk2, a p21-activated kinase (PAK kinase), is an effector of the Cdc42 GTPase, which plays a key role in the establishment of cell polarity in fission yeast (18, 19). Pak2 associates with Mkh1 MAPKKK by a two-hybrid system, and overexpression of its catalytic domain is lethal in wild type cells of *S. pombe* and overexpression of its catalytic domain is lethal in wild type cells of *S. pombe*. Similar to ERKs, fission yeast Sty1 is mainly cytoplasmic in unstressed cells but translocates into the cell nucleus upon phosphorylation (16).

The mechanisms responsible for the activation of the cell integrity MAPK pathway and the subcellular location of its main components are lacking. In this work, we address some of the above questions to show that in *S. pombe* the Pmk1 MAPK pathway responds against a wide variety stresses, including the presence of cell wall-damaging compounds or oxidative conditions. We also demonstrate that Pmk1 MAPK may be found in the cytoplasm and nucleus, whereas Mkh1 MAPKKK, Pek1 MAPKK, and Pmp1 phosphatase are cytoplasmic proteins. Notably, Mkh1, Pek1, and Pmk1 locate at the septum during cell separation. In addition, our approach reveals that the stress-induced activation of the Pmk1 MAPK pathway is partially regulated by the SAPK pathway through Sty1 and that Cdc42 and Pak2 do not act as upstream elements in this cascade.

### EXPERIMENTAL PROCEDURES

**Strains, Plasmids, and Growth Conditions**—The *S. pombe* strains (Table 1) were routinely grown with shaking at 28 °C in YES or EMM2 medium (20) with 3% glucose. Culture media were supplemented with adenine, leucine, histidine, or uracil (100 mg/liter; Sigma), depending on the requirements for each particular strain. Transformation of yeast strains was performed by the lithium acetate method as described elsewhere (21). Plasmids pREP41X-HA6H-cdc42(T17N) and pREP41X-GST-cdc42 (22). Mutant strains were obtained by standard transformation procedures or by mating and selecting diploids in EMM2 medium without supplements. Spores were obtained in MEL medium (20), purified by glusulase treatment (21), and allowed to germinate in EMM2 plus the appropriate requirements. Correct construction of strains was verified by PCR and Southern and Western blot analyses (see below). In localization experiments with cdc25-22 thermosensitive mutant strains, the cells were grown in YES medium to an *A*$_{600}$ of 0.2 at 25 °C (permissive temperature), shifted to 37 °C for 3.5 h, and released from the growth arrest by transfer back to 25 °C.

**Gene Disruption and Epitope Tagging**—The pek1${}^+$, pmk1${}^+$, and pak2${}^-$ null mutants were obtained by entire deletion of the correspond-
ing coding sequences and their replacement with the KanMX6 cassette by a PCR-mediated strategy using plasmid pFA6a-kanMX6 as template (23). Primer sequences used in each case (80–100 bp in length) are available upon request. The Pmk1-HA6H-tagged strains MI200 and MI201 were obtained as follows. A 5’-truncated version of the pmk1+ ORF was amplified by PCR employing ACCACTCGAGATCGCATCTGGTGA as 5’-oligonucleotide (which hybridizes at positions +125 to +139 in the pmk1+ ORF and contains an XhoI site) and the 3’-oligonucleotide ACCATGGCCGGCGTTATGGCAGATTAC (which hybridizes at the 3’-end of the pmk1+ ORF and incorporates a NotI site placed immediately upstream of the TAA stop codon). Restriction sites in both oligonucleotides are italicized. PCR amplification employing the Expand high fidelity system (Roche Applied Science) generated a 1.6-kbp fragment that was cleaved with XhoI and NotI and cloned into integrative plasmid pIL-GFP to construct C-terminal fusions with the GFP tag and containing the pmk1+ ORF plus regulatory sequences was amplified by PCR employing the 5’-oligonucleotide PMKGF-5 (CCTTAGGATCTTATGGCAGATTAC, which hybridizes at positions 313–330 upstream of the pmk1+ ATG start codon and contains an XbaI site) and the 3’-oligonucleotide PMKGF-3 (CTTAGGATCTTATGGCAGATTATC, which hybridizes at the 3’-end of pmk1+ ORF and incorporates a BamHI site placed immediately upstream of the TAA stop codon). PCR amplification generated a 2.1-kbp fragment that was cleaved with XbaI and BamHI and cloned into integrative plasmid pIL-GFP to construct C-terminal fusions with the GFP tag and containing the S. pombe leu1+ gene as a selectable marker. The resulting plasmid (pIL-Pmk1-ura) was digested at the unique BstXI site within the pmk1+ coding region (position 1428), and the linear fragment was transformed into haploid strains MM1 and MM2 to target integration at the pmk1+ locus. Uracil prototrophs were selected, and the identification of strains MI200 and MI201 with one copy of pmk1-HA6H expressed from the genomic pmk1+ promoter was verified by Southern blot analysis and immunoblot of whole-cell extracts with anti-HA antibody (see below). To obtain strain MI301, which expresses a C-terminal tagged version of Pmk1 fused to green fluorescent protein (GFP), a DNA fragment with the pmk1+ ORF plus regulatory sequences was amplified by PCR employing the 5’-oligonucleotide PMKGF-5 (CCTTAGGATCTTATGGCAGATTTC, which hybridizes at positions 313–330 upstream of the pmk1+ ATG start codon and contains an XbaI site) and the 3’-oligonucleotide PMKGF-3 (CTTAGGATCTTATGGCAGATTATC, which hybridizes at the 3’-end of pmk1+ ORF and incorporates a BamHI site placed immediately upstream of the TAA stop codon). PCR amplification generated a 2.1-kbp fragment that was cleaved with XbaI and BamHI and cloned into integrative plasmid pIL-GFP to construct C-terminal fusions with the GFP tag and containing the S. pombe leu1+ gene as a selectable marker. The resulting plasmid (pIL-pmk1-GFP) was digested at the unique NruI site within leu1+, and the linear fragment was transformed into pkm1+–disrupted strain TP319-13c. leu1+ transformants were obtained, and the correct integration of the pkm1-GFP fusion plus regulatory sequences was verified as above. Additionally, pkm1+, pek1+, nkh1+, and pmp1+ were tagged in their chromosomal loci at their 3’-ends with GFP by employing plasmid pFA6a-GFP(S65T)-kanMX6 as template and the same PCR-mediated strategy used for gene disruption (23).

Stress Treatments—Most experiments to investigate Pmk1 activation under stress were performed using log phase cell cultures (A600 of 0.5) growing at 28°C in YES medium and the appropriate stress treatment. In glucose deprivation studies, cells were grown in YES medium with 7% glucose to an A600 of 0.5 (actual glucose concentration = 6%, determined by the glucose oxidase method), recovered by filtration, and resuspended in the same medium without glucose but osmotically equilibrated with 3% glycerol (25). Hypotonic treatment was achieved by growth of cells in YES medium plus 0.8 m sorbitol and transfer to the same medium without polyol. To monitor Pmk1 activation in strains bearing the orb2-34 allele, cultures were grown in YES medium to an A600 of 0.2, shifted to 37°C for 3 h, allowed to recover for 30 min at 28°C, and then subjected to the adequate treatment. In overexpression experiments, cells were first grown in EMM2 medium plus 10 µM thiamine, washed three times, and reincubated into fresh medium (with or without thiamine) for about 20 h at 28°C prior to the activating compound. At different times, 30 ml of culture were harvested by centrifugation at 4°C, the cells were washed with cold phosphate-buffered saline buffer, and the yeast pellets were immediately frozen in liquid nitrogen for analysis. Under these conditions, neither activation of Pmk1 nor the previously described Sty1 phosphorylation induced by centrifugation at room temperature was observed in unstressed cells (25).

Purification and Detection of Activated Pmk1-HA6H and Sty1-HA6H following Different Stresses—Cell homogenates were prepared under native conditions employing chilled acid-washed glass beads and lysis buffer (10% glycerol, 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, plus specific protease and phosphatase inhibitor mixtures for fungal and yeast extracts) (Sigma). The lysates were cleared by centrifugation at 13,000 rpm for 15 min, and HA6H-tagged Pmk1 or Sty1 was purified by using Ni2+-nitrilotriacetic acid-agarose beads (Qiagen Inc.) (21). The purified proteins were resolved in 10% SDS-PAGE gels, transferred to nitrocellulose filters (Amersham Biosciences), and incubated with either monoclonal mouse anti-HA (clone 12CA5; Roche Applied Science), polyclonal rabbit anti-phospho-p42/44 antibodies (Cell Signaling) (7, 10), or monoclonal mouse anti-phospho-p38 antibodies (Cell Signaling) (21). The immunoreactive bands were revealed with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma) and the Supersignal System (Pierce).

Co-immunoprecipitation Experiments—For immunoprecipitation of proteins interacting with Pmk1-HA6H, cell extracts were incubated for 12 h at 4°C with monoclonal anti-HA antibody (12CA5), and the immunocomplexes were adsorbed overnight at 4°C with protein A-agarose (Roche Applied Science). After extensive washing, the complexes were resolved by SDS-PAGE, transferred to nitrocellulose filters, and hybridized separately with anti-HA, anti-phospho-p44/42, or a monoclonal mouse anti-GFP antibody (clones 7.1 and 13.1; Roche Applied Science). The immunoreactive bands were revealed as indicated above.

Fluorescence Microscopy—Images were observed on a Leica DM 4000B fluorescence microscope with a 100× objective, captured with a cooled Leica DC 300F camera and IM50 software, and then imported into Adobe Photoshop 6.0 (Adobe Systems). Alternatively, fluorescence microscopy was performed on a Deltavision microscope containing a photometrics CH350L liquid-cooled CCD camera and an Olympus IX70 inverted microscope with ×100 objective equipped with a Delta-vision data collection system (Applied Precision, Issaquah, WA). To localize Pmk1-GFP, Pek1-GFP, Mkh1-GFP, and Pmp1-GFP fusions, cells grown in YES medium to an A600 of 0.2 were subjected to different stress treatments, and the cells of small aliquots (10 µl) were loaded onto poly-L-lysine-coated slides or fixed with formaldehyde as described (26). 4′,6-diamidino-2-phenylindole and Calcofluor white were employed for nuclear and cell wall/ septum staining, respectively (26).

Plate Assay of Stress Sensitivity for Growth—Wild type and mutant strains of S. pombe were grown in YES liquid medium to log phase. Appropriate dilutions were spotted in duplicate on YES solid medium containing 2% (w/v) bacto-agar (Difco) and supplemented with the indicated compounds. Incubation was either at 28°C for 3 days or at 37°C for 2 days.

RESULTS

Pmk1 Activation following Different Stresses—Exponentially growing cultures of strain MI200, which harbors a genomic copy of pkm1+ tagged with HA6H, were subjected to different environmental stimuli. The purified Pmk1-HA6H fusion was then assayed by Western blotting with a polyclonal anti-phospho-p42/44 antibody to detect phosphorylation/activation of Pmk1 protein at both Thr-186/Tyr-188 residues (7,
**FIGURE 1. Pmk1 MAPK is activated in response to a variety of stresses.** Wild type strain MI200 carrying a HA6H-tagged chromosomal version of pmk1 

10). Fig. 1A shows that the level of Pmk1 phosphorylation increased quickly upon salt-induced osmostress (NaCl or KCl), or after a shift to hypertonic medium (sorbitol). Also, Pmk1 became rapidly phosphorylated by CaCl₂, and the activation was more sustained than that induced by osmotic upshifts. Moreover, a delayed Pmk1 activation was evident after heat shock or by treatment with caffeine, sodium vanadate, calcofluor, diamide, DEM, or 8 mM paraquat (Fig. 1A). Treatment with different pro-oxidants, like diamide, DEM, t-BOOH, or paraquat, prompted as well a clear Pmk1 activation (Fig. 1E). These results indicate that Pmk1 is activated in S. pombe in response to multiple stress conditions and that the kinetics and degree of phosphorylation depend on the nature of the triggering stimulus.

Because Pmk1 was activated by a set of stresses similar to that inducing Sty1 phosphorylation, we determined cell viability under stress in the absence of Pmk1. As shown in Fig. 2, and confirming previous results (9, 10), Δpmk1 cells were hypertolerant to NaCl and hypersensitive to KCl, but they grew as wild type cells in medium containing high concentrations of sorbitol despite the fact that these compounds activated Pmk1 to a similar extent (Fig. 1). Also, cells lacking Pmk1 displayed lower growth at high temperature and in the presence of CaCl₂, sodium vanadate, calcofluor, diamide, DEM, t-BOOH, or paraquat, although they showed no growth defects in the presence of hydrogen peroxide or caffeine (Fig. 2). Hence, Pmk1 function plays an important role in maintaining S. pombe viability against most of the stressors that promote MAPK activation.
Pmk1 Activation in Fission Yeast

Osmotic Stabilization Does Not Inhibit the Stress-induced Activation of Pmk1—In S. cerevisiae, activation of Slt2 (the MAPK homolog to Pmk1) by heat shock, caffeine, sodium vanadate, or diamide is severely impaired in cells osmotically stabilized by preincubation with sorbitol (12, 13). The observations that caffeine alters multiple cellular targets (12, 13) has led to the hypothesis that these agents might trigger MAPK activation by perturbing cell wall architecture (12, 13). We thus analyzed the stress-induced activation of Pmk1 in S. pombe cultures growing in the presence or absence of 1 M sorbitol. Pregrowth of cells with or without the osmotic stabilizer did not significantly prevent Pmk1 activation by treatment with KCl, sodium vanadate, high temperature (40 °C), diamide, or caffeine (Fig. 3A). Similar results were obtained in cultures stimulated with NaCl, CaCl₂, calcofluor, hydrogen peroxide, DEM, t-BOOH, or paraquat. The plates were incubated at 28 or 37 °C (where indicated) for 2–3 days before being photographed.

Osmotic Stabilization Does Not Inhibit the Stress-induced Activation of Pmk1

FIGURE 2. Stress sensitivity analysis of Δpmk1 cells. Wild type (WT) control (MM1) and Δpmk1 (TP319-13c) strains were grown in YEP medium to an A₆₀₀ of 0.5. Samples containing 10⁴, 10⁵, and 10⁶ cells were spotted onto YEP plates supplemented with 0.2 M sodium chloride, 1 M potassium chloride, 1 M sorbitol, 100 mM calcium chloride, 5 mM sodium vanadate, 20 mM caffeine, 1 mg/ml calcofluor white, 1 mM hydrogen peroxide, 1.5 mM diamide, 0.75 mM DEM, 0.5 mM t-BOOH, or 0.5 mM paraquat. The plates were incubated for 2–3 days at 28 or 37 °C (where indicated) before being photographed.

FIGURE 3. The stress-induced activation of Pmk1 is not inhibited by osmotic stabilization. A, cultures of wild type (WT) strain MI200 (pmk1-HA6H) were grown in YEP medium with or without 1 M sorbitol to an A₆₀₀ of 0.5 and untreated (WT) or treated either with 0.6 M potassium chloride for 15 min or with 5 mM sodium vanadate, 4 mM diamide, 15 mM caffeine, or incubation at 40 °C for 90 min. Aliquots were harvested, and Pmk1-HA6H was purified by affinity chromatography. Activated Pmk1 was detected by immunoblotting with anti-phospho-p42/44 antibodies, and total Pmk1 was detected by immunoblotting with anti-HA antibody as loading control. B, wild type (WT1) and Δpmk1 (TP319-13c) strains were grown in YEP medium to an A₆₀₀ of 0.5. Samples with 10⁴, 10⁵, and 10⁶ cells were spotted onto YES + 1 M sorbitol plates containing either 0.2 M sodium chloride, 1 M potassium chloride, 100 mM calcium chloride, 5 mM sodium vanadate, 0.5 mM calcofluor white, 0.75 mM DEM, 0.5 mM t-BOOH, or 0.5 mM paraquat. The plates were incubated at 28 or 37 °C (where indicated) for 2–3 days before being photographed.

FIGURE 4. Basal and stress-induced activations of Pmk1 are channeled exclusively through Mkh1 MAPKKK and Pek1 MAPKK. A, co-immunoprecipitation analysis. Strains MI200 (pmk1-HA6H; lanes 1 and 2, negative controls), MI401 (pmk1-HA6H, pek1-GFP; lanes 3 and 4), MI403 (pmk1-HA6H, pek1-GFP, Δmkh1; lanes 5 and 6), MI400 (pmk1-HA6H, mkh1-GFP, lanes 7 and 8), and MI402 (pmk1-HA6H, mkh1-GFP, Δpek1; lanes 9 and 10) were grown in YEP medium to midlog phase and left untreated (odd numbered lanes), or supplemented with 0.6 M potassium chloride for 15 min (even numbered lanes). Cell extracts were immunoprecipitated (IP) with anti-HA antibody (12CA5), and the immunocomplexes were adsorbed with protein A-agarose. The complexes obtained were resolved by SDS-PAGE and hybridized separately with anti-HA, anti-phospho-p44/42, and anti-GFP antibodies. B, strains MI200 (pmk1-HA6H, control), MI202 (pmk1-HA6H, Δmkh1), and MI203 (pmk1-HA6H, Δpek1) were grown in YEP medium to an A₆₀₀ of 0.5. The cultures were then either left untreated (WT) or treated with 0.6 M potassium chloride, 0.5 M sodium chloride, 1 M sorbitol, or 50 mM calcium chloride for 15 min. Alternatively, they were incubated at 40 °C or treated with 5 mM sodium vanadate, 15 mM caffeine, 1 mg/ml calcofluor white, 4 mM diamide, 4 mM DEM, 4 mM t-BOOH, or 8 mM paraquat for 90 min. Treatment with 5 mM hydrogen peroxide was for 30 min. Aliquots were harvested, and Pmk1-HA6H was purified by affinity chromatography. The level of activated Pmk1 was detected by immunoblotting with anti-phospho-p42/44 antibodies and related to total Pmk1 detected by immunoblotting with anti-HA antibody.
Mkh1-GFP fusion was recovered by Pmk1-HA6H immunoprecipitation in growing or salt-stressed cells from double-tagged strain MI400 (Fig. 4A, lanes 7 and 8). However, whereas mkh1" deletion did not affect Pmk1-Pek1 association (Fig. 4A, lanes 5 and 6), we were unable to detect Mkh1-GFP after Pmk1 immunoprecipitation in strain MI402, which harbors a pek1" deletion (Fig. 4A, lanes 9 and 10). These data suggest that the association Mkh1-Pek1-Pmk1 is not affected in vivo by stress and that Pek1 mediates the interaction between Mkh1 and Pmk1.

To examine the functional organization of the Mkh1-Pek1-Pmk1 module and its relevance to Pmk1 activation under stress, we constructed strains MI202 and MI203, which express Pmk1-HA6H fusion in Δmkh1 or Δpek1 backgrounds, respectively. Both strains were grown to mid log phase and subjected to stressful conditions to promote maximal Pmk1 activation by treatment with KCl, NaCl, sodium vanadate, calcofluor, hydrogen peroxide, or high temperature (40 °C), as shown in Fig. 1. In all conditions tested, deletion of mkh1" and/or pek1" completely abolished Pmk1 activation and the basal level of activity observed in nonstressed control cells (Fig. 4B). These results confirm that the response to the stimuli analyzed is being funneled to Pmk1 through Mkh1-Pek1.

Subcellular Localization of the Pmk1 MAPK Cascade Components—To gain insight into the biological activity of each component within the signaling cascade, we investigated the subcellular localization of Mkh1, Pek1, and Pmk1 kinases. We employed strains MI303 and MI304, which express, respectively, genomic versions of Mkh1 and Pek1 fused to the GFP epitope at their C terminus. Both strains were grown to an A600 of 0.2 at 25 °C, shifted to 37 °C for 3.5 h, and then released from the growth arrest by transfer back to 25 °C. Different time points after the release of the arrest are shown. The solid and dotted arrows indicate the position of the mitotic spindle and septum, respectively.

Mkh1-GFP and Pek1-GFP are scarcely expressed proteins that were visualized throughout the cytoplasm in cells from asynchronous exponentially growing cultures and also as faint bands at the septum during cell separation (Fig. 5A). This localization pattern was unaffected by the phase of the cell cycle or the different stresses that induce activation of Pmk1 MAPK (not shown). Mkh1-GFP and Pek1-GFP are scarcely expressed proteins that were visualized throughout the cytoplasm in cells from asynchronous exponentially growing cultures and also as faint bands at the septum during cell separation (Fig. 5A). This localization pattern was unaffected by the phase of the cell cycle or the different stresses that induce activation of Pmk1 MAPK (not shown). Such a result is particularly intriguing in the case of Pek1, since the Wis1 MAPKK that induces activation of Pmk1 MAPK (not shown).

Pmk1 localization was investigated in a Δpmk1 mutant strain containing an integrated pmk1-GFP copy at the leu1-32 locus under the regulation of its own promoter (strain MI301). The morphology and growth properties of this strain are similar to those of the wild-type strain (not shown). Pmk1-GFP was mainly found at the nucleus and also in the cytoplasm along the mitotic cycle in exponentially growing cells both in vivo (Fig. 5, A and B) and after fixation with formaldehyde and double staining with calcofluor and 4',6-diamidino-2-phenylindole (not shown). Notably, the presence of the Pmk1-GFP fusion was evident in the mitotic spindle (Fig. 5B, c) and also in the septum as a fluorescent...
band overlapping the calcofluor white staining during cytokinesis (Fig. 5, A and B, c–e). A more accurate view of the space-temporal localization of Pmk1 along the cell cycle was obtained by introducing the Pmk1-GFP fusion into a cdc25-22 strain. Cells from this mutant (strain MI302) were grown at 25 °C to log phase, changed to 37 °C for 3.5 h to synchronize the cells in G2, and then returned to 25 °C. As shown in Fig. 5C, the nuclear and cytoplasmic localization of Pmk1 was confirmed along the cell cycle, with faintly visible long mitotic spindles and fluorescence localized to the septum from the initial formation steps until cell separation. Taking into account the defects in cell separation associated with pmk1Δ deletion (9, 10), this observation suggests that Pmk1 may be directly involved in the process of septum formation and/or cytokinesis in the fission yeast.

Finally, we focused on the subcellular localization of the dual specificity MAPK phosphatase Pmp1, which dephosphorylates and inactivates Pmk1 (11). In cells from strain MI305, expressing a genomic C-terminal fused version of Pmp1 tagged with GFP, this protein was visualized exclusively in the cytoplasm (Fig. 5A). Besides, this localization did not change in cultures subjected to treatments activating Pmk1 (not shown), indicating that Pmk1 inactivation by Pmp1 most probably occurs within the cytoplasm.

To determine a possible role of the MAPK cascade activation in Pmk1 localization, exponentially growing cultures of strain MI300 (pmk1-GFP) and MI310 (pmk1-GFP, ∆pak2 orb2-34) were observed by fluorescence microscopy. A, strains MI200 (pmk1-HA6H, wild type (WT)), MI205 (pmk1-HA6H, ∆pak2), and MI206 (pmk1-HA6H, ∆pak2 orb2-34) were grown in YES medium to midlog phase and subjected to stress treatments with 0.6 M potassium chloride, 0.5 M sodium chloride, 5 mM sodium vanadate, or 5 mM hydrogen peroxide. Aliquots were harvested at different times, and Pmk1-HA6H was purified by affinity chromatography. Activated Pmk1 was detected by immunoblotting with anti-phospho-p42/44 antibodies and normalized by the amount of total Pmk1 detected by immunoblotting with anti-HA antibody as an internal control. B, Pmk1 localization in pak mutants. Cells from log phase cultures of strains MI309 (pmk1-GFP, ∆pak2) and MI310 (pmk1-GFP, ∆pak2 orb2-34) were observed by fluorescence microscopy.

Role of Cdc42 and PAK Kinases in Pmk1 Activation under Stress—In S. pombe, the p21 activated kinases (PAK kinases) Pak1 and Pak2 are two effectors of the Cdc42 GTPase. Whereas pak1Δ is needed for polarized
considered the possibility that the wild type-like activation of Pmk1 in those related to sexual differentiation and mating (19). Therefore, we observed the activation of Pmk1 after treatment with NaCl, sodium vanadate, or hydrogen peroxide (strain MI205). As shown in Fig. 7A, the double mutant strain MI206, which harbors a pak2\textsuperscript{+} deletion in an orb2-34 (thermosensitive allele of pak1\textsuperscript{+}) background. This strain was grown to log phase at 28°C and then incubated at 37°C for 3 h to allow the expression of the orb2-34 thermosensitive phenotype. Except for an increased basal level of activation at zero time (due to the growth at 37°C) the \(\Delta\)pak2 orb2-34 double mutant exhibited the same profile of Pmk1 activation under stress as the \(\Delta\)pak2 single mutant (Fig. 7A). Moreover, Pmk1-GFP localized in \(\Delta\)pak2 and \(\Delta\)pak2 orb2-34 mutants as in wild type cells (Fig. 7B). From these results, we conclude that Pak1/Pak2 kinases do not appear to play a significant role on the stress-induced activation of Pmk1 in S. pombe.

To determine whether Pmk1 activity/activation was stress-regulated via Cdc42 through an as-yet-unidentified target, we transformed Pmk1-HA6H tagged strain MI200 with plasmids pREP41-HA6H-cdc42(G12V) and pREP41-GST-cdc42(T17N). These plasmids express, respectively, hyperactive and dominant negative alleles of cdc42\textsuperscript{+} fused to the HA or GST epitopes under the control of the medium strength thiamine repressible promoter (22). Cells from one transformant of each type were grown in minimal medium with or without thiamine and subjected to salt stress with KCl. Basal and salt-induced activation levels of Pmk1 were identical in cells growing in the presence or absence of thiamine and expressing HA6H-cdc42(G12V) or GST-cdc42(T17N) fusions (Fig. 7C). These results confirm that the Cdc42-Pak1/Pak2 cascade does not regulate the stress-induced activation of Pmk1 in S. pombe.

The SAPK Pathway Regulates Pmk1 Activation under Osmotic Stress—As indicated above, Pmk1 is activated by a range of stresses similar to that promoting activation of Sty1, which is the central element of the SAPK pathway in S. pombe (4, 33). We explored the possibility of cross-talk between both pathways in the strain MI204, which expresses the pmk1-HA6H fusion in a \(\Delta\)sty1 background, by monitoring Pmk1 activation under a wide array of treatments. Notably, deletion of sty1\textsuperscript{+} elicited an increased activation of Pmk1 by KCI (Fig. 8A) or sorbitol (not shown) that was maintained for a long time as compared with the response of control cells. This result suggests that Sty1 negatively regulates Pmk1 activity by osmostress. To determine whether this effect was dependent on \textit{de novo} protein synthesis, we examined KCI-induced Pmk1 activation in cultures from control strain MI200 pretreated with cycloheximide. In these conditions, Pmk1 phosphorylation was maintained for a long time (due to the growth at 37°C) the activation of Pmk1 was determined by immunoblotting with anti-phospho-p38 and anti-HA antibodies, respectively.

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growth and the sexual response, pak2\textsuperscript{+} is a nonessential gene, whose deletion does not confer any noticeable phenotype (18, 19, 31, 32). Earlier work has involved Pak2 kinase in the regulation of the activation of the Pmk1 pathway because Pak2, and not Pak1, associates with Mkh1 MAPKKK by two-hybrid analyses (17). Also, overexpression of the catalytic domain of Pak2 is lethal in wild type cells of the fission yeast but not in mutants disrupted in \textit{mkk1}\textsuperscript{+} or \textit{pmk1}\textsuperscript{+} (17). Hence, we investigated the stress-induced activation of Pmk1 in wild type and \(\Delta\)pak2 cells (strain MI205). As shown in Fig. 7A, the kinetics and intensity of activation of Pmk1 after treatment with NaCl, sodium vanadate, or hydrogen peroxide were virtually identical in both cases. Similar results were obtained after growth at 40°C or following treatment with \(\text{CaCl}_2\), calcifluor, diamide, DEM, \(t\)-BOOH, or parquat (not shown). The only effect of pak2\textsuperscript{+} disruption on Pmk1 activation was a maintained activation of the kinase after treatment with KCl (Fig. 7A). Pak2 kinase is somewhat redundant in function with Pak1, since pak2\textsuperscript{+} overexpression can rescue the morphological defects of \(\Delta\)pak1 mutants, although not those related to sexual differentiation and mating (19). Therefore, we considered the possibility that the wild type-like activation of Pmk1 in \(\Delta\)pak2 cells was due to redundant activity of Pak1 by using the double mutant strain MI206, which harbors a \(\text{pak2}^{+}\) deletion in an \textit{orb2-34} (thermosensitive allele of \textit{pak1}++) background. This strain was grown to log phase at 28°C and then incubated at 37°C for 3 h to allow the expression of the \textit{orb2-34} thermosensitive phenotype. Except for an increased basal level of activation at zero time (due to the growth at 37°C) the \(\Delta\)pak2 \textit{orb2-34} double mutant exhibited the same profile of Pmk1 activation under stress as the \(\Delta\)pak2 single mutant (Fig. 7A). Moreover, Pmk1-GFP localized in \(\Delta\)pak2 and \(\Delta\)pak2 \textit{orb2-34} mutants as in wild type cells (Fig. 7B). From these results, we conclude that Pak1/Pak2 kinases do not appear to play a significant role on the stress-induced activation of Pmk1 in S. pombe.

FIGURE 8. Sty1 activity regulates Pmk1 activation induced by osmostress. A, strains MI200 (\textit{pmk1-HA6H}, wild type (WT)), MI204 (\textit{pmk1-HA6H, \Delta sty1}), and MI207 (\textit{pmk1-HA6H}, \(\Delta\)aff1) were grown in YES medium to midlog phase and subjected to a stress treatment with 0.6\texttimes{}potassium chloride. Aliquots were harvested at the times indicated, and Pmk1-HA6H was purified by affinity chromatography. Activated and total Pmk1 was detected by immunoblotting with anti-phospho-p42/44 and anti-HA antibodies, respectively. B, effect of protein synthesis inhibition on the Pmk1 activation induced by salt. Cells from strain MI200 (\textit{pmk1-HA6H}, wild type) were treated for 60 min with 0.15 mg/ml cycloheximide. In these conditions, Pmk1 phosphorylation was maintained for a long time as compared with the response of control cells. This result suggests that Sty1 negatively regulates Pmk1 activity by osmostress. To determine whether this effect was dependent on \textit{de novo} protein synthesis, we examined KCI-induced Pmk1 activation in cultures from control strain MI200 pretreated with cycloheximide. In these conditions, Pmk1 phosphorylation was maintained for a long time (due to the growth at 37°C) the activation of Pmk1 was determined by immunoblotting with anti-phospho-p38 and anti-HA antibodies, respectively.

DISCUSSION

We have demonstrated that the sensitivity of the Pmk1 MAPK to environmental changes is greater than previously suspected. Pmk1 becomes activated by many treatments that trigger the activation response of Sty1, the central element of the SAPK pathway that orchestrates in S. pombe the induction of a wide number of relevant stress-
responsive genes through the bZIP transcription factor Atl1 (4, 28, 29, 33, 35). However, the respective patterns of activation are quite different. For instance, Pmk1 activations triggered by osmostress, high temperature, or oxidative conditions are slower than those observed in Sty1. Also, contrary to Sty1 (34), Pmk1 was activated by hypotonic stress but not by low doses of hydrogen peroxide or nitrogen withdrawal. Cells lacking Pmk1 lost viability under most stress treatments, although the effect was not as dramatic as when Sty1 was absent (4, 28, 29, 33) (our results). This supports that the Pmk1 MAPK pathway may reinforce the SAPK signaling pathway that controls survival and adaptation to sublethal stressing conditions. Nevertheless, this notion cannot be extended to all types of stress, because cells devoid of Pmk1 grew as control cells under osmostress induced by sorbitol and in the presence of caffeine or hydrogen peroxide. Hence, the exact function of Pmk1 under stress awaits further definition. In mammalian cells, activation of the ERK pathway can lead to antagonistic fates, and the duration of the activation specifies signal identity (36). However, in S. pombe, two distinct hyperosmotic stimuli (NaCl and KCl) can activate Pmk1 with comparable kinetics and magnitude, and the loss of Pmk1 renders cells hypersensitive to NaCl but hypersensitive to KCl. At present, there is no obvious explanation for this outcome, although a possible reason is that Pmk1 might regulate ion channels that control ion homeostasis (9).

The mechanisms controlling the stress-induced activation of Pmk1 in fission yeast differ from those modulating the activation of its ortholog Slt2 in budding yeast. The response to oxidative stresses is clearly distinct and, in contrast to Pmk1 (or to ERKs in mammalian ortholog Slt2 in budding yeast), Slt2 is not activated by osmostress (3). Moreover, preincubation of S. cerevisiae cells with sorbitol attenuates Slt2 activation induced by stress signals that are transduced to the cell integrity pathway through cell wall receptors MLT1, MID2, and/or WSC1–4, which detect surface structural changes (3, 12). Our data indicate that in S. pombe the activation of Pmk1 triggered by similar stresses is independent of the presence of stabilizer in the culture medium. Considering the different composition and structure of the respective cell walls (27), it seems possible that the stress-induced activation of the fission yeast cell integrity pathway may be transduced by alternative sensors.

Earlier studies provided evidence for the integration of Pmk1 activity within a phosphorylation cascade that is dependent on Mkh1 MAPKKK and Pek1 MAPKK (6–8). We present additional data to indicate that all stress signals activating Pmk1 are exclusively channeled through Mkh1 and Pek1. Moreover, our work confirms that the functional MAPK kinase module is composed by Mkh1, Pek1, and Pmk1 without additional components, with Mkh1 as the only MAPKKK able to activate Pek1 MAPKK, which in turn phosphorylates Pmk1 in response to diverse stressors. Also, co-immunoprecipitation studies support previous results from two-hybrid experiments suggesting that Pek1 associates in vivo with both Mkh1 and Pmk1, whereas Pmk1 and Mkh1 do not interact directly (7). Sugiyama et al. (8) reported that Pek1 regulates Pmk1 activity in a dual manner depending on its phosphorylation state, with the unphosphorylated form acting as a potent negative regulator of Pmk1 activation (8). Accordingly, the amount of active Pek1 associated with Pmk1 under stress should be comparatively lower than that bound under nonstressing conditions. However, we found that the amount of Pek1 and Mkh1 associated with Pmk1 did not change significantly during a salt stress (Fig. 6, lanes 3 and 4 and lanes 5 and 6). The reason for this result is unclear, but a likely explanation is that our immunoprecipitation analyses are not sensitive enough to detect subtle differences in the in vivo association between both kinases. This might be due in part to the use of strains expressing wild type levels of the corresponding protein fusions instead of overexpressing active or inactive forms of Pek1 as employed in previous studies (8).

Our results also show that the Cdc42 GTPase and two of its effectors, p21-activated kinases Pak2 and Pak1, do not play a significant role in either the basal level or the stress-induced activation response of Pmk1. Except for an altered activation kinetics under salt stress with KCl, neither pak2Δ deletion nor cdc42Δ (G12V) or cdc42Δ (T17N) overexpression had a conclusive effect on Pmk1 activation. Such observations were rather unexpected, since Pak2 associates with Mkh1 MAPKKK in a two-hybrid system (17), and overexpression of a dominant activated allele of cdc42 (G12V) or the catalytic domain of Pak2 is lethal in wild type cells but not in mutants disrupted in mkh1Δ or pmk1Δ (17). Moreover, Cdc42 and Pak1 functions have been shown to be essential for pheromone signaling in S. pombe (20, 21). In our experiments, Pmk1 localization was unaffected by deletion of pak1Δ and/or pak2Δ kinases. This apparent discrepancy could be explained if Pak2 hyperactivation should promote the activation of an as yet unidentified factor that also needs to be phosphorylated by Pmk1 to exert its biological function. In this scenario, both the Cdc42-Pak1/Pak2 cell polarity pathway and the Mkh1-Pek1-Pmk1 MAPK cascade might coordinate regulate morphogenesis and cell growth through at least one common target in fission yeast. Further studies are necessary to clarify this possibility.

Protein localization studies revealed some intriguing aspects of the dynamics and biological function of each component of the MAPK pathway. In control and stressed cells, Mkh1 and Pek1 localized at the cytoplasm and septum, whereas Pmk1 was found in both cytoplasm and nucleus as well as in the mitotic spindle and septum during cytokinesis. Most importantly, Pmk1 subcellular distribution was unaffected by stress or Mkh1/Pek1 deletion, implying that Pmk1 activation by the Mkh1-Pek1-Pmk1 cascade may take place at the cytoplasm or septum and that the active and inactive forms of this kinase are able to pass through the nuclear membrane. This is further supported by the observation that cells bearing a ts-allele of the exportin Crm1 show increased nuclear fluorescence for Pmk1-GFP as compared with control cells, whereas Pek1-GFP remained cytoplasmic.6 Similar findings were obtained by treatment with leptomycin B, a potent inhibitor of nuclear export (37). The combined evidence leads us to consider that Pmk1 (but not Pek1) is constitutively subjected to nuclear import and Crm1-mediated export. Thus, the pattern of Pmk1 targeting parallels that of Slt2 in budding yeast, which shuttles between nucleus and cytoplasm in a manner independent of the activation of the MAPK pathway (15). Although we have been unable to identify putative nuclear export signals in Pmk1, a search in the PROSITE data base reveals one "classical" and one bipartite-type putative nuclear localization signal at the C terminus of its amino acid sequence. The relevance of these import signals to Pmk1 nuclear localization awaits characterization by fluorescence microscopy of S. pombe strains expressing Pmk1-GFP versions mutated within the putative nuclear localization signals. On the other hand, phosphatase Pmp1 appears exclusively located at the cytoplasm in control and stressed cells, indicating that most likely Pmk1 deactivation takes place there. However, as discussed below, we cannot rule out the possibility that other phosphatases may also inactivate phosphorylated Pmk1. In any case, the localization of the components of the Pek1-Pmk1 pathway differs substantially from those of the Wis1-Sty1 cascade in S. pombe. Contrary to Pmk1, Sty1 localizes in the cytoplasm of unstressed cells but, similar to mammalian ERK MAPKs (14), translocates into the nucleus in response to a triggering stimulus (30). Wis1 MAPKKK has a

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MAPK-docking site and a nuclear export signal sequence in its N-terminal domain (30). The nuclear export signal sequence is essential not only for cytoplasmic localization of Wis1 but also for nuclear targeting of Sty1 under stress, and Wis1 translocates itself to the nucleus upon stress (30). Similar to Wis1, Pek1 shows a putative MAPK-docking motif at its N terminus (residues KKPVLNL at positions 3–9) resembling the MAPK-docking sites in human MEK1/MEK2 (38), which might be responsible for Pmk1 binding and activation. However, the absence of nuclear relocation of Pek1 under stress or in Crm1-ts mutants strongly suggest that, contrary to Wis1, this MAPKK remains exclusively at the cytoplasm without playing a role in the nuclear targeting of Pmk1.

An interesting finding is the location of Mkh1, Pek1, and Pmk1 in the cell division area. In S. pombe, cell separation is brought about by contraction of an actomyosin ring followed by deposition of a primary septum composed by linear β1,3-glucan, in a process coordinated by regulatory proteins of the septation initiation network (39). This is followed by the deposition of layers of the secondary septum (composed of β1.6-branched β1,3-glucan and β1,6-glucan) at both sides of the primary septum and by cleavage of the latter by glucanases that allow separation of the daughter cells (40). Previous microscope studies have described that a phenotypic feature associated with absence of Mkh1, Pek1, or Pmk1 is the appearance of multisepitate cells with thickened cell walls and prominent septa (9, 10). Our observation of Mkh1, Pek1, and Pmk1 at the septum during cell separation suggests a potential involvement of Pmk1 in the control of septum formation and/or its localized degradation. Accordingly, active Pmk1 might down-regulate septum formation by interacting with component(s) of the septation initiation network and the glucan synthases responsible for the building of the primary or secondary septa (i.e. Cps1/Bgs1) (41). Alternatively, Pmk1 might up-regulate the glucanases responsible for primary septum dissolution (i.e. Eng1 and Atn1) (42, 43).

As far as we are aware, this is the first study to clearly demonstrate the existence of cross-talk between Sty1 and Pmk1 MAPKs in S. pombe. The function of Sty1 is required for correct deactivation of Pmk1 in cells subjected to osmotic upshift by a mechanism dependent on the transcriptional activity of Atf1 and de novo protein synthesis. Since Pmk1 is currently the only phosphatase known to dephosphorylate and inactivate Pmk1, an interpretation for this control would be the up-regulation of Pmk1 mRNA levels by Sty1-Atf1 during osmotic stress. However, this possibility seems unlikely, since microarray analyses have shown that pmp1+ transcription does not significantly change in cells under osmotic treatment or by deletion of Sty1 and Atf1 (37). Moreover, stability of pmp1+ mRNA relies on the function of the RNA-binding protein Rnc1, whose activity is up-regulated by Pmk1-mediated phosphorylation (44). A more attractive possibility would be deactivation of phosphorylated Pmk1 by one or several phosphatases whose expression is up-regulated during osmostress by the stress-activated protein kinase pathway. The most probable candidates to play such a role are Pyp2 tyrosine-phosphatase and/or Pct1 threonine-phosphatase, whose corresponding genes, pyp2+ and pct1+, are expressed in a stress-induced fashion via Sty1-Atf1 through dual loops of negative feedback in the SAPK pathway (3). In fact, a similar scheme applies to budding yeast, where the MAPKs Fus3p (mating pheromone response), Kss1p (pseudohyphal development pathway), and Slk2 (Pmk1 ortholog) can be substrates for tyrosine phosphatases Ptp2 and Ptp3, which are the apparent counterparts of Pyp1 and Pyp2 in fission yeast (45, 46). Also, S. cerevisiae Pcc1, a type 2C Ser/Thr phosphatase homolog to Pct1 in fission yeast, is able to inactivate the MAPK Hog1 and probably Slt2 (47). Therefore, although not proven, it seems plausible that the SAPK-regulated MAPK phosphatases in fission yeast may not be strictly specific, and thus able to down-regulate both Pmk1 and Sty1 activities, depending on the nature of the stress stimulus.

In recent years, a considerable number of studies have focused on signaling pathways of yeast cells as model systems for basic signal transduction mechanisms, and the knowledge emerging makes now possible some comparative analyses. We show here that, contrary to structurally related MAPKs from other organisms, in S. pombe the Pmk1 MAPK is able to respond to multiple stressing conditions in a way similar, although not identical, to Sty1. The existence of only three MAPKs in fission yeast anticipates the need for extensive cross-talk modulation and the possibility that a same stress may trigger different MAPK cascades, a strategy quite different from the more stress-specific MAPK activation present in budding yeast, plant, or animal cells. Our results indicate that at least under osmotic stress, the response of Pmk1 is regulated by Sty1 function at a transcriptional level. Also, studies on protein localization draw a model in which Pmk1 distribution at the nucleus, cytoplasm, mitotic spindles, and septum is fully independent of the activation of the MAPK cascade, which is substantially different from the findings previously described for Sty1. Taken together, these results allow new progress in our understanding of the Mkh1-Pek1-Pmk1 MAPK cascade in fission yeast.

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